

Metal Binding to Nsp1, a SARS-CoV-2 protein

Thesis by
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In Partial Fulfillment of the Requirements for
the Degree of
Doctor of Philosophy

The Caltech logo, featuring the word "Caltech" in a bold, orange, sans-serif font, centered within a light orange rectangular background.

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To my family

“We are the sum of those who have supported us.” -Unknown

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ABSTRACT

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to unprecedented global health and socioeconomic challenges since its emergence in late 2019. Widespread vaccination efforts have lessened the severity of infections, however, the continuous evolution of the virus highlights the need for novel antiviral treatments. A critical aspect of SARS-CoV-2 pathogenesis is its ability to suppress host immune responses, allowing efficient viral replication and persistence. Among the 16 nonstructural proteins encoded by the viral ORF1ab polyprotein, nonstructural protein 1 (Nsp1) plays a pivotal role in immune evasion by inhibiting type I interferon (IFN-I) responses and shutting down host protein translation. Nsp1 achieves this by promoting host mRNA degradation, blocking nuclear export of transcripts, and binding to the 40S ribosomal subunit, where its intrinsically disordered C-terminal region adopts a helix–turn–helix structure that blocks the mRNA entry tunnel. Even at low micromolar concentrations, Nsp1 effectively halts host translation, making it a key virulence factor and an attractive antiviral drug target.

However, the lack of a stable folded structure within the C-terminal domain presents a significant challenge for conventional structure-based drug discovery. This work investigates an alternative strategy by leveraging the coordination chemistry of transition metals to target the disordered regions of Nsp1. Specifically, copper(II) and cobalt(III) complexes were explored for their ability to bind to histidine residues, particularly H165, which is essential for Nsp1's interaction with the ribosome.

A combination of biophysical techniques, including fluorescence spectroscopy, electron paramagnetic resonance (EPR), and ^{59}Co nuclear magnetic resonance (NMR), alongside computational modeling, was employed to characterize metal coordination to Nsp1-derived peptides and the full-length protein. Cu(II) was found to coordinate through both histidine side chains and backbone amide nitrogens within the disordered C-terminal region, exhibiting pH-dependent binding modes. Co(II) complexes, upon controlled oxidation to Co(III), formed substitution-inert complexes, effectively “locking” the metal ion in place and stabilizing interactions with Nsp1. These studies revealed multi-site binding behavior and provided insights into the kinetics of metal-protein interactions with Nsp1.

Additionally, *in vitro* translation assays demonstrated that while certain metal complexes could modulate translation processes, their ability to selectively inhibit Nsp1 function remains complex, highlighting both the potential and challenges of metal-based therapeutic approaches. This research establishes a foundation for targeting intrinsically disordered viral proteins using coordination chemistry, offering a novel perspective for antiviral drug development beyond traditional small molecule inhibitors. The insights gained from this work contribute to a deeper understanding of Nsp1's biochemical properties and open new avenues for combating SARS-CoV-2.

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M.M. performed research, contributed new reagents/analytic tools, analyzed data, and co-wrote the paper.

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Chapter 1

INTRODUCTION

COVID-19 Pandemic: A Global Challenge and Scientific Response

As of March 2025, COVID-19 has resulted in approximately 676 million cases and 6.8 million deaths worldwide.¹ The disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). While the peak of the pandemic has passed, the virus continues to circulate due to ongoing transmission and the emergence of mutations that enhance its ability to spread.² Although vaccines have been developed to prevent infection, understanding the virus remains essential for advancing therapeutic strategies and better preparing us for future pandemics.

In December 2019, several cases of pneumonia of unknown origin were reported in Wuhan, Hubei province, China. Most of these patients were linked to the Huanan Seafood Wholesale Market. SARS-CoV-2 was later identified as the causative agent of these pneumonia cases.^{3,4} Phylogenetic analysis revealed a distinct lineage with Bat-SARS, confirming a zoonotic origin.^{5,6} Bats are the second most numerous mammals after rodents and have been known as sources of coronaviruses and other zoonotic viruses.⁷

Initially, scientists believed the disease was transmitted solely from animals to humans and only between symptomatic individuals. However, cases in Germany revealed that human transmission could also occur from asymptomatic carriers.^{8,9} By April 2020, the disease had spread to over 200 countries, with 1.4 million confirmed cases and more than 100,000 deaths. The pandemic prompted global authorities to impose travel restrictions, limit large gatherings, and advise people to stay home unless absolutely necessary.

Worldwide leadership began to institute control measures to try and keep the pandemic from spreading. Travel advisories and flight bans to and from infected countries were put into place. Quarantine measures were established and traveler screenings were added as part of travel measures. Healthcare specialist emphasized the importance of social distancing, hand washing and wearing face masks.¹⁰

From the onset of the COVID-19 pandemic, the world turned to scientists for solutions, and vaccination quickly emerged as the most effective and safe strategy to control the virus's spread.¹¹ To accelerate vaccine development, researchers leveraged preclinical data from SARS-CoV and MERS-CoV vaccine studies.¹²⁻¹⁵ As a result, seven different types of COVID-19 vaccines were

developed. Inactivated vaccines involve culturing the virus *in vitro* and inactivating it with chemical agents, allowing the entire virus to act as an immunogen that induces a broad antibody response.^{16,17} Live attenuated vaccines use a weakened form of the virus, obtained through reverse genetics or adaptation, which retains key amino acid sequences to generate a strong immune response, including mucosal immunity that protects the upper respiratory tract.¹¹ Viral vector vaccines utilize engineered, replication-attenuated viruses that carry genetic material of viral proteins, inducing a robust Th1 cell response, as seen in the Johnson & Johnson vaccine.^{18,19} Protein subunit vaccines rely on cell expression systems to produce viral proteins or peptides that stimulate a targeted Th1 response.²⁰ DNA vaccines encode viral antigens within recombinant plasmids, triggering the production of neutralizing antibodies.¹⁷ mRNA vaccines, such as the Pfizer-BioNTech and Moderna vaccines, encapsulate mRNA to instruct cells to produce viral proteins or peptides, stimulating a strong Th1 and B-cell response while promoting long-lived plasma and memory cells.^{21,22} Finally, virus-like particle (VLP) vaccines are composed of non-infectious particles that contain viral structural proteins and polypeptides, which facilitate antigen loading and induce neutralizing antibodies against immune epitopes.¹⁷

In the United States, the FDA granted emergency use authorization for the Pfizer-BioNTech and Moderna mRNA vaccines in December 2020, followed by the Janssen/Johnson & Johnson viral vector vaccine in 2021. It was not until 2022 that the Moderna and Pfizer-BioNTech vaccines received full FDA approval. Widespread vaccination and other public health measures caused COVID-19 cases to decline, hospitalizations to decrease, and the world slowly began adjusting to a new normal, one reminiscent of life before the pandemic, yet undeniably changed.

The COVID-19 pandemic has had a profound impact on global health and daily life. The peak of the crisis has passed, however, the virus continues to evolve requiring continuous monitoring and research. The rapid development of vaccines played a crucial role in mitigating the severity of the disease and preventing further loss of life. However, the emergence of new variants highlights the importance of research into viral transmission, immunity, and treatments. As the world adapts to a post-pandemic reality, research and public health efforts remain essential to controlling outbreaks and preparing for future pandemics. The lessons learned from COVID-19 will serve as a foundation for more effective responses to emerging infectious diseases.

SARS-CoV-2 Taxonomy

Coronaviruses are a large group of viruses classified under the order *Nidovirales*, suborder *Cornidovirineae*, and family *Coronaviridae*. The *Coronaviridae* family is divided into two subfamilies: *Letovirinae* and *Orthocoronavirinae*. The latter is further categorized into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The coronavirus responsible for the COVID-19 pandemic belongs to the *Betacoronavirus* genus and the *Sarbecovirus* subgenus. A taxonomy diagram illustrating these classifications is shown in Figure 1.

The name “coronavirus” is derived from the Latin word *corona*, meaning “crown,” referring to the virus’s crown-like appearance under an electron microscope due to spike proteins protruding from its envelope. Additionally, the term *Nido*, meaning “nest” in Latin, this refers to the virus’s ability to generate a nested set of subgenomic mRNAs.^{23–25}

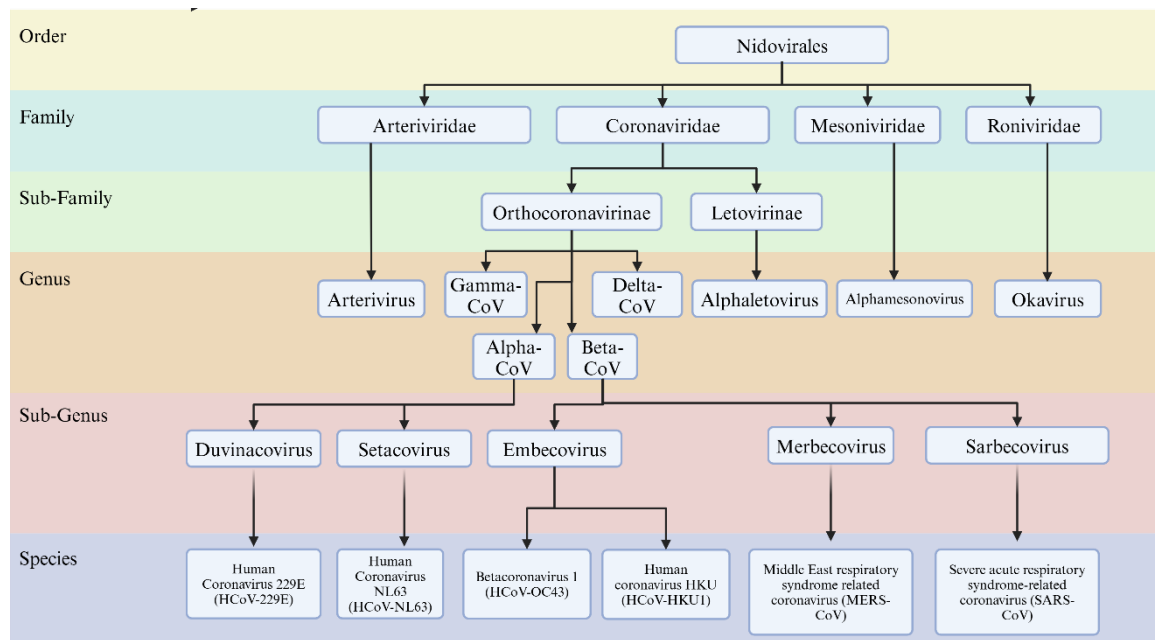


Figure 1.2 Classification of *Coronaviridae* according to the International Committee of Taxonomy of Viruses. This figure was created with BioRender.com.

History of Coronaviruses

Coronaviruses have been evolving for over a thousand years.²⁶ The first coronaviruses were isolated from chickens in 1937 with the discovery of infectious bronchitis virus (IBV) and from mice in 1949 with murine hepatitis virus (MHV).^{27,28} Human coronaviruses were first characterized in the 1960s from respiratory tract infections.²⁹

As its name suggests, SARS-CoV-2 is the second identified strain of SARS-CoV. The first strain, SARS-CoV, caused the 2002–2004 SARS outbreak, which originated in Guangdong Province, China. The virus spread to 29 countries, resulting in 8,422 reported cases and 916 deaths.³⁰ The disease presented as atypical pneumonia with symptoms including fever, headache, and respiratory distress, which in severe cases led to respiratory failure.³¹ Genome sequencing linked SARS-CoV to a virus found in Himalayan palm civets, suggesting they served as an intermediate host, with bats as the natural reservoir.^{32,33}

Another significant coronavirus within this family is Middle East respiratory syndrome coronavirus (MERS-CoV), which caused the 2012 MERS outbreak. Symptoms ranged from mild upper respiratory illness to severe pneumonia and multi-organ failure.³⁴ Both the SARS and MERS outbreaks led to severe human diseases.³⁵ Most of these cases were reported in Saudi Arabia.³⁶ The genome sequence for MERS-CoV closely resembles bat coronaviruses HKU4 and HKU5.³⁷

In addition to these highly pathogenic coronaviruses, there are four other known human coronaviruses: HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1. These typically cause milder symptoms resembling the common cold. These strains are also depicted in Figure 1.1.³⁸

SARS-CoV-2 Biology

SARS-CoV-2 shares approximately 82% sequence identity with SARS-CoV based on whole genome alignment.^{6,9} These alignments revealed 380 amino acid substitutions between the two viruses. Of these, 348 occur in nonstructural proteins (including *ORF1ab*, *3a*, *3b*, *7a*, *7b*, *9b*, and *ORF14*), 27 are found in the spike protein, and 5 in the nucleocapsid protein.³⁹

SARS-CoV-2 has a ~30 kb genome, which is one of the largest known RNA genomes.³⁹ the genome contains 14 open reading frames (ORFs), preceded by transcriptional regulatory sequences. The two main transcriptional subunits are ORF1a and ORF1b, these two encode polyprotein 1a (PP1a) and polyprotein 1ab (PP1ab). PP1ab makes Nsp1-16 which are responsible for making the complex replicase machinery.^{40,41} At the 3' end the viral genome encodes four structural proteins: the spike protein (S), the envelope protein (E), and the nucleocapsid protein (N).^{4,40,42} Throughout the structural genes, there are also nine ORFs for accessory factors.⁴³ The genome can be seen depicted in Figure 1.2.

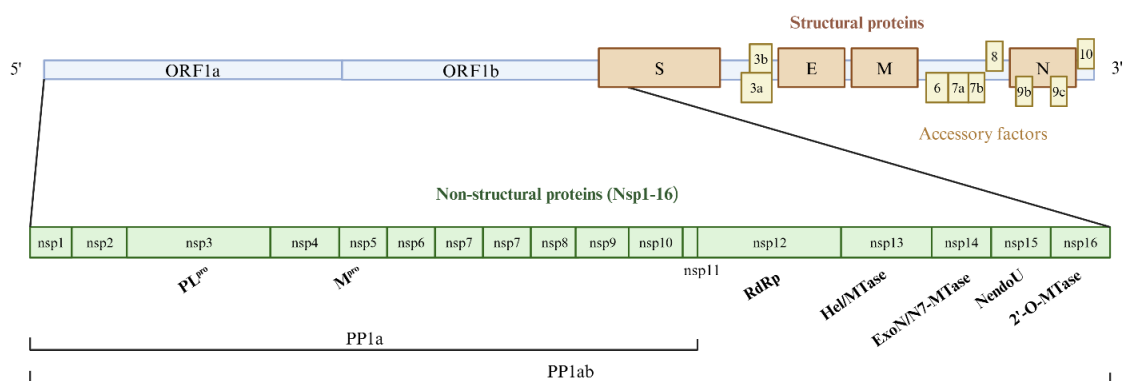


Figure 1.2 The genome of SARS-CoV-2 can be seen as organized by individual ORFs. This figure was created with BioRender.com.

Viral entry, attachment to the host cell membrane, and fusion are mediated by the spike (S) glycoprotein. The S protein assembles as a homotrimer and is inserted into the viral membrane, giving the virus its characteristic crown-like appearance. Upon entry into the host cell, the S protein is cleaved by proprotein convertases.^{44,45} It is then further cleaved in the Golgi apparatus by furin into the S1 and S2 subunits. The S1 subunit binds to angiotensin-converting enzyme 2 (ACE2), while the S2 subunit anchors the S protein to the membrane, facilitating membrane fusion.⁴⁴

In normal physiology, ACE2 is responsible for converting angiotensin I to angiotensin II. However, when SARS-CoV-2 binds to ACE2, this receptor is downregulated, contributing to disease severity by disrupting the renin-angiotensin-aldosterone system. This disruption plays a critical role in the development of lethal lung failure.⁴⁶

After viral entry through membrane proteins, the virion is internalized via endocytosis, and fusion occurs following spike cleavage. This process leads to the release of the genomic RNA (gRNA).^{44,47} The capped and polyadenylated gRNA is used for translation of ORF1a and ORF1b, resulting in the synthesis of two large polyproteins, pp1a and pp1b. These polyproteins are proteolytically cleaved into 16 nonstructural proteins (nsps) by the papain-like protease (PL^{pro}) in nsp3 and the main protease (M^{pro}) in nsp5.^{48,49} Nsp1 is rapidly released into the cytoplasm, where it interferes with host translation and the innate immune response.^{50–52} Nsp2 through nsp16 establish the viral replication and transcription complexes.

Coronaviruses induce extensive reorganization of host intracellular membranes to create replication organelles.⁵³ The endoplasmic reticulum (ER) is remodeled into a network of double-membrane vesicles (DMVs) attached to the ER via thin double-membrane connectors derived from collapsed ER tubules.⁵⁴ Nsp3, nsp4, and nsp6 contain multiple transmembrane domains that contribute to the formation of the replication organelles.^{55,56} Coexpression of nsp3 on the outer membrane and nsp4 on the inner membrane facilitates the formation of double-membrane vesicles.^{56,57} Nsp6 plays a key role in establishing connections between the double-membrane vesicles and the ER, serving as a spatial organizer for these vesicle clusters.⁵⁵

With the exception of nsp1, the nonstructural proteins assemble into a replication and transcription complex that facilitates viral RNA synthesis.⁵⁸ The holoenzyme responsible for RNA synthesis consists of nsp12, the nsp7-nsp8 heterodimer, and an additional nsp8. Nsp12 contains the RNA-dependent RNA polymerase (RdRp) domain, while nsp7 and nsp8 act as co-factors to stabilize the RdRp.^{59,60} Nsp8 also serves as a scaffold for viral proteins, aiding in RNA unwinding and proofreading, and is involved in RNA capping.⁵⁸ Nsp13, the viral helicase, is responsible for unwinding the highly structured viral genome.^{61,62}

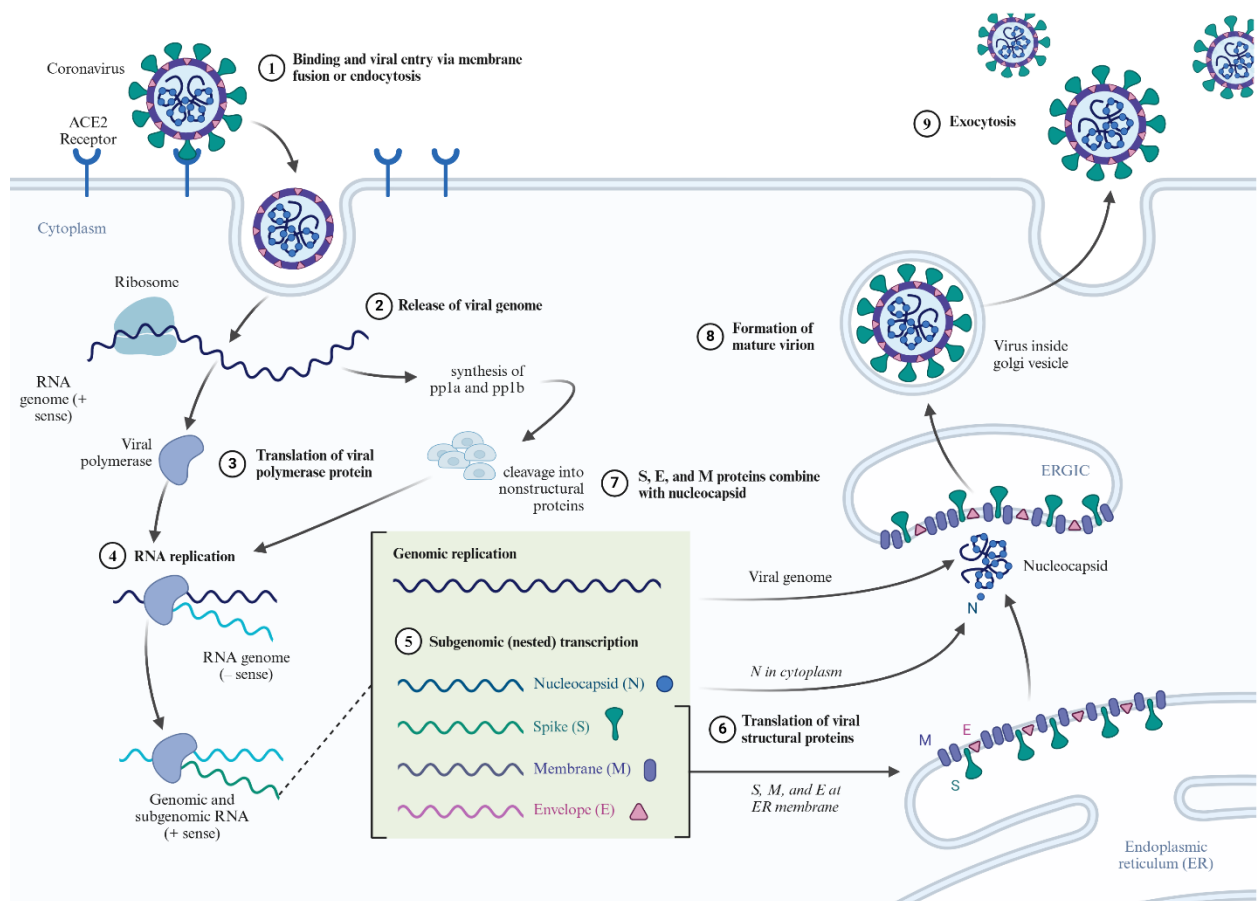


Figure 1.3 The viral biology and process of replication in SARS-CoV-2. This figure was created with BioRender.com.

The RNA-dependent RNA polymerase (RdRp) in SARS-CoV-2 is one of the fastest viral RdRps, however, this speed comes at the cost of low accuracy, which may benefit the virus by allowing it to better adapt to selective pressures.⁶³ Due to this low fidelity, the virus employs proofreading mechanisms to enhance replication and maintain genomic stability.⁶⁴ Proofreading is carried out by the N-terminal 3' to 5' exoribonuclease (ExoN) domain within nsp14. Nsp14 binds to nsp10, forming the RNA proofreading machinery that removes misincorporated nucleotides.^{65,66}

After the replication and transcription complex (RTC) is assembled, the viral gRNA serves as a template for both replication and transcription. The RTC synthesizes a full negative-sense copy of the viral genome, which then acts as a template for the positive-sense gRNA. The newly synthesized genomes are used to generate additional nonstructural proteins (nsps) and RTCs, or

they become part of new virions.⁶⁷ Viral translation occurs through a discontinuous process, producing a set of nested 3' and 5' co-terminal subgenomic RNAs (sgRNAs).⁶⁸ During negative-strand RNA synthesis, the RTC interrupts transcription upon encountering transcription regulatory sequences (TRS). Upon reaching a TRS, transcription stops and is reinitiated at the TRS next to the leader sequence. When reinitiation occurs, a negative-strand copy of the leader sequence is added to the developing RNA, completing the synthesis of the sgRNA.^{40,69,70} These sgRNA are used as templates to transcribe the positive sensed sgRNAs that are then used for the translation of structural and accessory proteins.⁷¹

Virion packaging begins with the assembly of the N protein, synthesized in the cytosol, and the viral gRNA into ribonucleoproteins (RNPs). The N protein selectively binds the viral gRNA, excluding sgRNA and cellular RNA, to form the ribonucleoprotein complex. This complex then interacts with the M protein, which ensures proper packaging into the virus particle. Meanwhile, the M, E, and S proteins are synthesized in the endoplasmic reticulum (ER).⁷² The RNP must locate the M/S/E protein clusters for packaging into new virions. The N protein binds to the cytoplasmic tail of the M protein, this promotes the packaging of viral RNPs and potentially stimulating M oligomerization, which aids in particle formation.⁷³ This process occurs in the ER-Golgi intermediate compartment (ERGIC), where N, S, and M proteins accumulate.⁷⁴ Newly formed single-membrane vesicles bud off from the ERGIC.^{72,75} The precise mechanism by which SARS-CoV-2 and other coronaviruses bud remains unclear, as it does not follow typical viral budding pathways. It is suggested that the E protein may play a role, as it is highly conserved and is responsible for budding in other viruses.^{76–78} Studies have reported that the E protein induces asymmetry between the two leaflets of the lipid bilayer, which favors vesicle formation.⁷⁹ However, the full mechanism of budding is still under investigation.

The egress of SARS-CoV-2 follows a lysosome-dependent pathway.⁸⁰ The exact mechanism by which SARS-CoV-2 particles reach the lysosomes remains unclear, however, it is known that the viral particles are transported to the lysosomes after their formation in the ERGIC.⁸¹ ORF3a plays a role in promoting the movement of lysosomes towards the periphery of the cytoplasm, where they are eventually released.^{82,83}

SARS-CoV-2 Immune Evasion Mechanisms

A key factor contributing to the persistence of SARS-CoV-2 is its ability to evade and suppress the host immune response through a range of strategies. These include shielding viral replication intermediates, mimicking host molecular structures, disrupting antiviral signaling pathways, and suppressing interferon responses.^{54,84–95} These mechanisms allow the virus to establish infection, replicate efficiently, and delay immune detection, contributing to disease severity.

Host Immune Response

Upon viral entry, SARS-CoV-2 is detected by host pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), which sense viral RNA and initiate innate immune signaling.^{96,97} TLR2, TLR3, TLR7, TLR8, and cytosolic sensors such as MDA5 and LGP2 play pivotal roles in recognizing viral components, leading to the production of pro-inflammatory cytokines (such as TNF, IL-6, IFN- γ) and type I/III interferons (IFN-I, IFN-III).^{98–100} MDA5, in particular, serves as the primary sensor of SARS-CoV-2 RNA, detecting long double-stranded RNA intermediates generated during replication.^{100,101}

Activation of these pathways triggers downstream signaling via mitochondrial antiviral signaling proteins (MAVS), promoting phosphorylation and nuclear translocation of IRF3 and IRF7, which induce interferon expression.^{102,103} Secreted interferons engage JAK/STAT pathways, via IFNAR or IFNLR receptors, culminating in the formation of the ISGF3 complex and activation of interferon-stimulated genes (ISGs).^{104,105} These ISGs coordinate a broad antiviral response by inhibiting viral entry, replication, transcription, and assembly, forming a critical defense against SARS-CoV-2.

In parallel, SARS-CoV-2 activates additional host defenses such as the cGAS-STING pathway through mitochondrial damage, and the NLRP3 inflammasome via viral RNA and nucleocapsid protein recognition, contributing to inflammatory responses and, in severe cases, cytokine storms.^{106–113}

To overcome these defenses, SARS-CoV-2 employs a comprehensive immune evasion strategy. Viral proteins, including nonstructural proteins (NSP1, NSP3, NSP5, NSP12–15), structural proteins (M, N), and accessory proteins (ORF3a, ORF6, ORF7a/b, ORF8, ORF9b) target

nearly every stage of innate immune signaling.^{90,91,94,95,114} These proteins inhibit PRR signaling by blocking IRF3 phosphorylation, degrading MAVS and TBK1, and disrupting nuclear transport through interactions with importins and nucleoporins.^{115–118} Additionally, SARS-CoV-2 impairs downstream IFN signaling by degrading IFN receptors (such as Nsp14-mediated IFNAR1 degradation), preventing STAT1/STAT2 phosphorylation, and inhibiting ISGF3 nuclear translocation. Accessory proteins like ORF6, ORF7a, and N further interfere with JAK/STAT signaling and ISG expression.^{88,94,114}

This multilayered suppression of antiviral responses enables SARS-CoV-2 to establish infection before the immune response can be activated. This then contributes to viral persistence, immune dysregulation, and disease severity. The redundancy of these evasion mechanisms underscores the virus's evolutionary adaptation to circumvent innate immunity and highlights the challenges in developing therapeutics that can restore or enhance host antiviral defenses.

Avoiding Host Detection

Viruses manage to survive and evolve by avoiding detection, similarly SARS-CoV-2 has done the same. Typically, virus derived RNA can be detected via its missing modifications, inclusion of virus-distinguishing elements, or the presence of extensive secondary structures.¹¹⁹ Virus-DNA can be identified by its location in the cell or by aspects of its sequence that might not be present within the host.¹²⁰ These unique characteristics are what is called pathogen-associated molecular patterns (PAMPs). The detection of PAMPs in cells is either a direct process or aided by autophagy.¹²¹ This is why avoiding viral detection is crucial for the virus' survival.

To avoid host detection the viral genomic RNA conforms to the rules of host translation. Coronaviruses modify the 5'-triphosphate(ppA) of their genomic RNA and sgRNA via capping and methylation which not only allows for ribosomal loading but also for evading host detection. The capping process occurs as four sequential steps. The first is the removal of γ -phosphate from pppA to form the 5'diphosphate (ppA). Then, guanylyltransferase creates the cap core structure by transferring guanine monophosphate to the ppA. This is followed by the formation of the core structure by methylation of the guanine at position N7 by N7-methyltransferase. As the last part of this process, 2'-O-methyltransferase methylates the ribose at the 2'-O position of the first transcribed nucleotide, making the final capped structure.¹²²

The result of this masking mechanism in the viruses results in a late detection by the host immune response. As viral RNA synthesis continues dsRNA intermediates trigger host immune response. This is because both genomic RNA and sgRNA need to proceed through negative strand intermediates. Therefore, the virus has evolved mechanism that decrease the early detection of dsRNA by minimizing its accumulation and hiding it from the host. Nsp15 is responsible for minimizing the negative-stranded RNA and dsRNA through its endonuclease activity, which targets the negative-sense transcripts.^{123,124} At the same time, the virus induces the assembly of double-membrane vesicles (DMVs) that compartmentalize the viral replication away from cellular sensors.^{125,126} These replication organelles (ROs) diminish the host capacity to recognize the presence of a viral infection by exploiting autophagy biology.^{127,128} The assembly of RO is mediated by Nsp3 and Nsp4 with Nsp6 forming a tether to the ER that allows for the flow of lipids.⁵⁵ The time it takes to build the levels of Nsp3, Nsp4, Nsp6 and Nsp15 is slow enough that at even at low multiplicity the optimal infection has already been achieved.

SARS-CoV-2 employs a multifaceted immune evasion strategy, combining molecular mimicry, RNA shielding, and active suppression of host antiviral pathways. These mechanisms allow the virus to delay immune detection, suppress interferon responses, and manipulate host cellular machinery, which all contribute to its high infectivity and pathogenicity. Among the numerous viral proteins involved, Nsp1 plays a particularly critical role as a key immune evasion factor. By shutting down host protein synthesis and interfering with innate immune signaling, Nsp1 serves as a central player in the virus's ability to suppress host defenses. Given its pivotal function and conserved nature, Nsp1 has emerged as a compelling target for therapeutic intervention.

Nsp1

An important part of viral strategy is what is called “host shut off,” this is the process in which the virus impairs the translation of mRNA. In doing so it helps redirect translation resources and it blocks the ability of infected cell to respond to the virus.¹²⁹ SARS-CoV-2 leads to shut off of host protein synthesis and Nsp1 is one of the central factors in coronaviruses that inhibit host cellular mRNA translation.⁵²

Upon SARS-CoV-2 infection, the overlapping reading frames ORF1ab are translated from the positive-sense genomic RNA to form a polypeptide that is then autoproteolyzed into 16 non structural proteins (nsps).¹³⁰ The first of these proteins is Nsp1.

Nsp1 uses a multipronged strategy to shut down host protein synthesis.¹³¹ Nsp1 binds to the 40S ribosomal subunit, in doing so its C-terminal domain blocks the mRNA entry channel by adopting a helix turn helix shape, this then leads to reduction in translation.^{50,51,132,133} Through this process, it promotes cellular mRNA degradation. Nsp1 also interacts with export factor NXF1 to inhibit the nuclear export of cellular transcripts.¹³⁴

The interaction of binding to the 40S ribosomal subunit is crucial for translation inhibition. The first C-terminal helix (residues 153-160) interact with ribosomal proteins uS5 and uS3, through a few of the hydrophobic side chains including Y154, F157 and W161. The two helices that form the helix-turn-helix shape that disrupt the mRNA entry channel are connected by a short loop that contains K164 and H165. K164 and H165 establish stacking interactions with the helix of h18 of the 18S rRNA through U607 and U630 as well as backbone binding. K164 and H165 are important to the virus as without them it can no longer promote mRNA degradation, it no longer inhibits translation, and it stops inducing type 1 IFN expression.¹³⁵ The second helix (residues 166-178) interact with the phosphate backbone of h18 through the conserved arginines R171 and R175 and also with ribosomal protein eS30. Nsp1 also sterically occludes the entrance of the 43S pre-initiation complex and the non-translating 80S ribosomes. This indicates that Nsp1 interacts with multiple different ribosomal states.⁵¹

The process of inhibiting nuclear mRNA export through NXF1 is done through the N-terminal of Nsp1 (residues 1-129) and is independent of the C-terminal domain that blocks the ribosome. By directly binding to NXF1 it disrupts the interactions with mRNA export adaptors and the nuclear pore complex. Nsp1 is visible in the cytoplasm and near the nuclear pore complexes, which indicate that it could be shuttling between the nucleus and the cytoplasm.¹³⁴

It is clear that the C-terminal region of Nsp1 is an important player for controlling cellular response to viral infections. It would make an excellent target for combatting the virus as it plays a crucial role in shutting down the host immune response. Drug design for Nsp1 would need to

prevent binding to the ribosome to allow cellular defense systems the ability to respond to the virus.

Intrinsically Disordered Proteins

The C-terminal of Nsp1 that is responsible for binding to the 40S ribosome is considered an intrinsically disordered protein region (IDPR). Therefore, the following section will give insights into and characterize what it means for a protein to be disordered or have intrinsically disordered regions.

There are two paradigms regarding the structure and function of proteins. The first is the well-known ‘structure-function paradigm,’ this paradigm states that the native structure of a protein under physiological conditions is what is needed for the protein to function. The second is the ‘disorder-function paradigm,’ this paradigm is based on proteins that perform cellular functions without attaining a stable structure under physiological conditions.¹³⁶

Intrinsically disordered proteins (IDPs) fall into the ‘disorder-function paradigm,’ they are proteins that cannot attain a single stable three-dimensional structure under physiological conditions, instead they are constantly changing and are in various conformational states. These types of proteins are highly abundant in the proteome.

Intrinsically disordered proteins are characterized by their biased amino acid composition, low sequence complexity, low proportion of bulky hydrophobic residues and high proportions of hydrophilic amino acids. These proteins do not fold spontaneously into stable, defined three dimensional structures. They are dynamically disordered and fluctuate between a range of conformations.^{137,138}

The propensity of a protein to fold or not fold is contained within the amino acid sequence of the protein. The number, charge, and relative positioning of charged residues is an important determinant of the properties of IDRs. A large reason for the unfolded structures of IDPs is their strong electrostatic repulsion due to a higher net charge and a lack of driving force due to low average hydrophobicity.¹³⁹ Residues such as alanine, arginine, glycine, glutamic acid, glutamine, lysine, proline and serine occur more often in IDPs/IDPRs.^{140–142} Aromatic residues allow for intramolecular interactions driven by their side chain π - π interactions, cation- π

interactions, and methyl- π interactions.^{143–145} Aliphatic residues drive intramolecular interactions through the hydrophobic effect and desolvation.

Upon the interaction of IDPs/IDPRs with their specific binding partner, the protein/region will adopt a disorder to order conformational transition known as ‘induced folding.’^{137,138,141,146} These disordered proteins can also bind to multiple partners and take on different conformations.^{147,148} The process in which these disordered proteins fold upon binding with their targets is a process called coupled folding and binding.^{149,150} There are studies that suggest that there are pre-formed secondary structural elements in the conformational ensemble that favor the binding process.¹⁵¹ Not all intrinsically disordered proteins undergo folding to perform their biological function, some remain disordered even after binding to their targets, forming ‘fuzzy’ complexes.^{152–155}

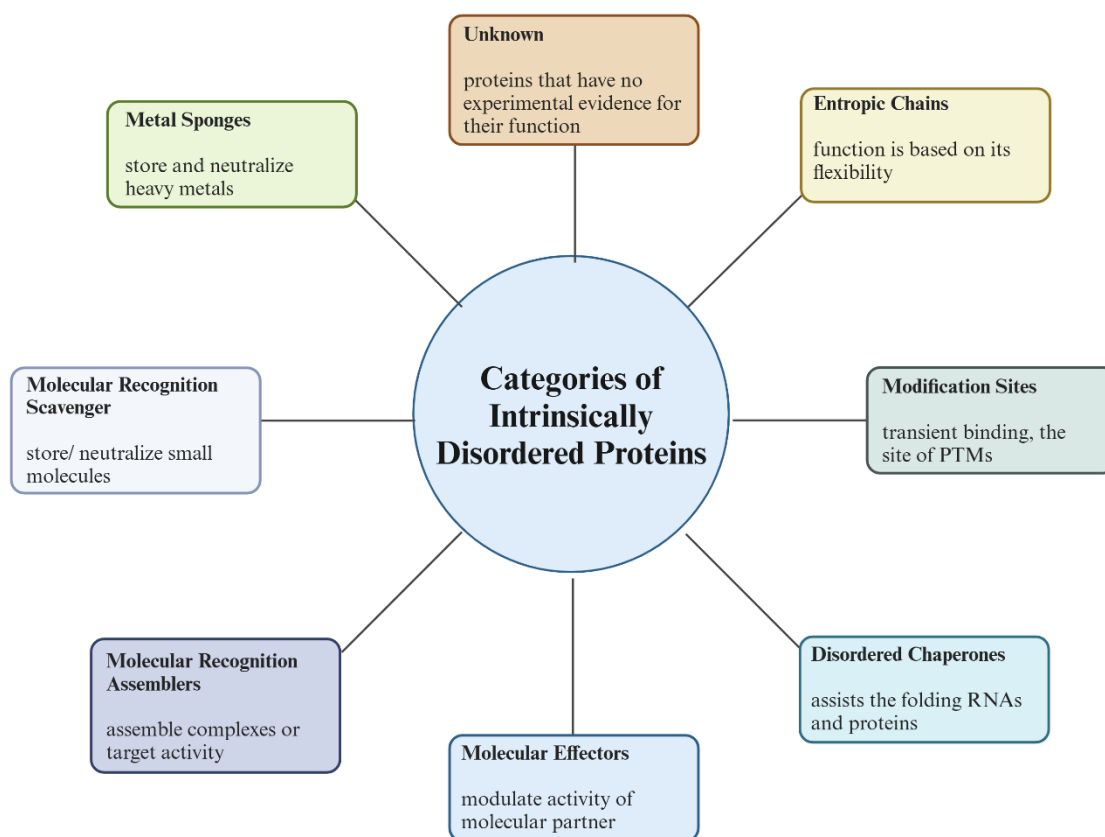


Figure 1.4 The 8 main categories of intrinsically disorder proteins.
Figure was created with BioRender.com.

IDPs and IDPRs have been classified into 8 different categories based on their biological function: entropic chains, modifications sites, disordered chaperones, molecular effectors, molecular recognition assemblers, molecular recognition scavengers, metal sponges and those of unknown class (Figure 1.4).¹⁵⁶ Entropic chains never adopt a structural function while they perform their biological function, this would include things like flexible linkers and spacers.^{157,158} Modification sites refer to IDPs/IDPRs that facilitate the electrostatic interactions with the lipid head groups in the membranes, which allow for fine tuning of the signaling pathways. These tend to have transient binding and are the sites of post translational modifications.¹⁵⁹ Disordered chaperones are proteins that help RNA and other proteins attain their functional folded state.^{160–162} Molecular recognition effectors bind permanently to other proteins and modify their actions.¹⁶³ Molecular assemblers interact with multiple binding partners in order to form higher-order protein complexes.¹⁵⁵ Molecular effectors store and neutralize small ligands. Metal sponges are IDPs/IDPRs that can store and neutralize heavy metals. The unknown category refers to IDPs/IDPRs who do not have an established functional role.¹⁵⁶ Understanding these functional classifications is essential, as they highlight how intrinsic disorder equips proteins with unique capabilities absent in structured proteins. The flexibility inherent to IDPs allows them to engage in transient, promiscuous, or highly specific interactions, often acting as central hubs in cellular signaling pathways. This dynamic nature enables rapid responses to environmental changes, regulation through post-translational modifications, and the ability to bind multiple partners via conformational adaptability.

Viruses exploit intrinsically disordered regions to enhance pathogenicity. Many viral proteins leverage disorder to modulate host processes, evade immune surveillance, and hijack cellular machinery. The disordered regions often mimic host motifs, facilitating interactions with key regulatory proteins while remaining elusive to immune detection.¹⁶⁴

Despite their biological importance, targeting IDPs poses significant challenges due to their lack of stable structure. The intrinsically disordered nature of Nsp1's C-terminal region presents both a challenge and an opportunity for therapeutic development. While its lack of a stable structure under physiological conditions complicates traditional structure-based drug design, the conformational plasticity of this region allows for dynamic and potentially selective interactions with small molecules, peptides, or metal complexes. The ability of IDPs and IDPRs to undergo

induced folding or remain in ‘fuzzy’ complexes upon binding provides a unique avenue for modulating their function. Given the critical role of Nsp1 in immune suppression and its reliance on a disordered region to bind the ribosome, targeting this domain represents a promising and underexplored antiviral strategy.

Problem Statement and Research Approach

SARS-CoV-2 continues to pose a global health challenge, not only due to its rapid transmission and mutation rates but also because of its immune evasion mechanisms. Key to these mechanisms is nonstructural protein 1 (Nsp1), a key virulence factor responsible for shutting down host protein synthesis and impairing innate immune responses. Despite its critical role in viral pathogenicity, Nsp1 has received comparatively less attention as a therapeutic target than structural proteins such as the spike (S) glycoprotein.

A major feature that complicates traditional drug design efforts against Nsp1 is the intrinsically disordered nature of its C-terminal region. This disordered segment is essential for binding to the 40S ribosomal subunit, where it adopts a helix–turn–helix conformation upon interaction, effectively blocking host mRNA translation. The dynamic and flexible characteristics of intrinsically disordered protein regions (IDPRs) present both challenges and unique opportunities in drug discovery. Unlike well-structured proteins, IDPRs lack stable binding pockets in their unbound state, making conventional structure-based drug design approaches less effective. However, their conformational flexibility allows for diverse modes of interaction, particularly with small molecules or metal complexes capable of inducing or stabilizing specific conformations.

Given Nsp1's pivotal role in immune suppression and its highly conserved sequence across SARS-CoV-2 variants, it represents a compelling yet underexplored target for antiviral intervention. Targeting the C-terminal IDPR of Nsp1 offers the potential to disrupt its interaction with the ribosome, thereby restoring host translation and immune function.

The research presented in this thesis focuses on leveraging metal coordination chemistry, specifically using copper(II) and cobalt(II/III) complexes, as a novel strategy to modulate the structure and function of Nsp1. Metal complexes offer a versatile platform for interacting with

disordered regions due to their ability to form stable coordination bonds with amino acid side chains, particularly histidine residues. By exploiting these properties, this work aims to investigate whether metal-based compounds can effectively bind to Nsp1, alter its conformation, and impair its ability to inhibit host translation.

References

- (1) Dong, E.; Du, H.; Gardner, L. An Interactive Web-Based Dashboard to Track COVID-19 in Real Time. *The Lancet Infectious Diseases* **2020**, *20* (5), 533–534. [https://doi.org/10.1016/S1473-3099\(20\)30120-1](https://doi.org/10.1016/S1473-3099(20)30120-1).
- (2) Van Egeren, D.; Novokhodko, A.; Stoddard, M.; Tran, U.; Zetter, B.; Rogers, M.; Pentelute, B. L.; Carlson, J. M.; Hixon, M.; Joseph-McCarthy, D.; Chakravarty, A. Risk of Rapid Evolutionary Escape from Biomedical Interventions Targeting SARS-CoV-2 Spike Protein. *PLoS One* **2021**, *16* (4), e0250780. <https://doi.org/10.1371/journal.pone.0250780>.
- (3) Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; Cheng, Z.; Yu, T.; Xia, J.; Wei, Y.; Wu, W.; Xie, X.; Yin, W.; Li, H.; Liu, M.; Xiao, Y.; Gao, H.; Guo, L.; Xie, J.; Wang, G.; Jiang, R.; Gao, Z.; Jin, Q.; Wang, J.; Cao, B. Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. *The Lancet* **2020**, *395* (10223), 497–506. [https://doi.org/10.1016/S0140-6736\(20\)30183-5](https://doi.org/10.1016/S0140-6736(20)30183-5).
- (4) Zhou, P.; Yang, X.-L.; Wang, X.-G.; Hu, B.; Zhang, L.; Zhang, W.; Si, H.-R.; Zhu, Y.; Li, B.; Huang, C.-L.; Chen, H.-D.; Chen, J.; Luo, Y.; Guo, H.; Jiang, R.-D.; Liu, M.-Q.; Chen, Y.; Shen, X.-R.; Wang, X.; Zheng, X.-S.; Zhao, K.; Chen, Q.-J.; Deng, F.; Liu, L.-L.; Yan, B.; Zhan, F.-X.; Wang, Y.-Y.; Xiao, G.-F.; Shi, Z.-L. A Pneumonia Outbreak Associated with a New Coronavirus of Probable Bat Origin. *Nature* **2020**, *579* (7798), 270–273. <https://doi.org/10.1038/s41586-020-2012-7>.
- (5) Chan, J. F.-W.; Yuan, S.; Kok, K.-H.; To, K. K.-W.; Chu, H.; Yang, J.; Xing, F.; Liu, J.; Yip, C. C.-Y.; Poon, R. W.-S.; Tsoi, H.-W.; Lo, S. K.-F.; Chan, K.-H.; Poon, V. K.-M.; Chan, W.-M.; Ip, J. D.; Cai, J.-P.; Cheng, V. C.-C.; Chen, H.; Hui, C. K.-M.; Yuen, K.-Y. A Familial Cluster of Pneumonia Associated with the 2019 Novel Coronavirus Indicating Person-to-Person Transmission: A Study of a Family Cluster. *The Lancet* **2020**, *395* (10223), 514–523. [https://doi.org/10.1016/S0140-6736\(20\)30154-9](https://doi.org/10.1016/S0140-6736(20)30154-9).

- (6)Paraskevis, D.; Kostaki, E. G.; Magiorkinis, G.; Panayiotakopoulos, G.; Sourvinos, G.; Tsiodras, S. Full-Genome Evolutionary Analysis of the Novel Corona Virus (2019-nCoV) Rejects the Hypothesis of Emergence as a Result of a Recent Recombination Event. *Infect Genet Evol.* **2020**, *79*, 104212. <https://doi.org/10.1016/j.meegid.2020.104212>.
- (7)Luis, A. D.; Hayman, D. T. S.; O'Shea, T. J.; Cryan, P. M.; Gilbert, A. T.; Pulliam, J. R. C.; Mills, J. N.; Timonin, M. E.; Willis, C. K. R.; Cunningham, A. A.; Fooks, A. R.; Rupprecht, C. E.; Wood, J. L. N.; Webb, C. T. A Comparison of Bats and Rodents as Reservoirs of Zoonotic Viruses: Are Bats Special? *Proc. R. Soc. B.* **2013**, *280* (1756), 20122753. <https://doi.org/10.1098/rspb.2012.2753>.
- (8)Rothe, C.; Schunk, M.; Sothmann, P.; Bretzel, G.; Froeschl, G.; Wallrauch, C.; Zimmer, T.; Thiel, V.; Janke, C.; Guggemos, W.; Seilmaier, M.; Drosten, C.; Vollmar, P.; Zwirgmaier, K.; Zange, S.; Wölfel, R.; Hoelscher, M. Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany. *N Engl J Med* **2020**, *382* (10), 970–971. <https://doi.org/10.1056/NEJMc2001468>.
- (9)Chan, J. F.-W.; Kok, K.-H.; Zhu, Z.; Chu, H.; To, K. K.-W.; Yuan, S.; Yuen, K.-Y. Genomic Characterization of the 2019 Novel Human-Pathogenic Coronavirus Isolated from a Patient with Atypical Pneumonia after Visiting Wuhan. *Emerg Microbes Infect* **2020**, *9* (1), 221–236. <https://doi.org/10.1080/22221751.2020.1719902>.
- (10)Cowling, B. J.; Leung, G. M. Epidemiological Research Priorities for Public Health Control of the Ongoing Global Novel Coronavirus (2019-nCoV) Outbreak. *Eurosurveillance* **2020**, *25* (6). <https://doi.org/10.2807/1560-7917.ES.2020.25.6.2000110>.
- (11)Krammer, F. SARS-CoV-2 Vaccines in Development. *Nature* **2020**, *586* (7830), 516–527. <https://doi.org/10.1038/s41586-020-2798-3>.
- (12)Zhou, Y.; Jiang, S.; Du, L. Prospects for a MERS-CoV Spike Vaccine. *Expert Rev Vaccines* **2018**, *17* (8), 677–686. <https://doi.org/10.1080/14760584.2018.1506702>.
- (13)Mubarak, A.; Alturaiki, W.; Hemida, M. G. Middle East Respiratory Syndrome Coronavirus (MERS-CoV): Infection, Immunological Response, and Vaccine Development. *J Immunol Res* **2019**, *2019*, 6491738. <https://doi.org/10.1155/2019/6491738>.
- (14)Modjarrad, K. MERS-CoV Vaccine Candidates in Development: The Current Landscape. *Vaccine* **2016**, *34* (26), 2982–2987. <https://doi.org/10.1016/j.vaccine.2016.03.104>.

- (15) Su, S.; Du, L.; Jiang, S. Learning from the Past: Development of Safe and Effective COVID-19 Vaccines. *Nat Rev Microbiol* **2021**, *19* (3), 211–219. <https://doi.org/10.1038/s41579-020-00462-y>.
- (16) Creech, C. B.; Walker, S. C.; Samuels, R. J. SARS-CoV-2 Vaccines. *JAMA* **2021**, *325* (13), 1318–1320. <https://doi.org/10.1001/jama.2021.3199>.
- (17) Dai, L.; Gao, G. F. Viral Targets for Vaccines against COVID-19. *Nat Rev Immunol* **2021**, *21* (2), 73–82. <https://doi.org/10.1038/s41577-020-00480-0>.
- (18) Folegatti, P. M.; Ewer, K. J.; Aley, P. K.; Angus, B.; Becker, S.; Belij-Rammerstorfer, S.; Bellamy, D.; Bibi, S.; Bittaye, M.; Clutterbuck, E. A.; Dold, C.; Faust, S. N.; Finn, A.; Flaxman, A. L.; Hallis, B.; Heath, P.; Jenkin, D.; Lazarus, R.; Makinson, R.; Minassian, A. M.; Pollock, K. M.; Ramasamy, M.; Robinson, H.; Snape, M.; Tarrant, R.; Voysey, M.; Green, C.; Douglas, A. D.; Hill, A. V. S.; Lambe, T.; Gilbert, S. C.; Pollard, A. J.; Oxford COVID Vaccine Trial Group. Safety and Immunogenicity of the ChAdOx1 nCoV-19 Vaccine against SARS-CoV-2: A Preliminary Report of a Phase 1/2, Single-Blind, Randomised Controlled Trial. *Lancet* **2020**, *396* (10249), 467–478. [https://doi.org/10.1016/S0140-6736\(20\)31604-4](https://doi.org/10.1016/S0140-6736(20)31604-4).
- (19) Stephenson, K. E.; Le Gars, M.; Sadoff, J.; de Groot, A. M.; Heerwegh, D.; Truyers, C.; Atyeo, C.; Loos, C.; Chandrashekar, A.; McMahan, K.; Tostanoski, L. H.; Yu, J.; Gebre, M. S.; Jacob-Dolan, C.; Li, Z.; Patel, S.; Peter, L.; Liu, J.; Borducchi, E. N.; Nkolola, J. P.; Souza, M.; Tan, C. S.; Zash, R.; Julg, B.; Nathavitharana, R. R.; Shapiro, R. L.; Azim, A. A.; Alonso, C. D.; Jaegle, K.; Ansel, J. L.; Kanjilal, D. G.; Guiney, C. J.; Bradshaw, C.; Tyler, A.; Makoni, T.; Yanosick, K. E.; Seaman, M. S.; Lauffenburger, D. A.; Alter, G.; Struyf, F.; Douoguih, M.; Van Hoof, J.; Schuitemaker, H.; Barouch, D. H. Immunogenicity of the Ad26.COV2.S Vaccine for COVID-19. *JAMA* **2021**, *325* (15), 1535–1544. <https://doi.org/10.1001/jama.2021.3645>.
- (20) Legros, V.; Denolly, S.; Vogrig, M.; Boson, B.; Siret, E.; Rigail, J.; Pillet, S.; Grattard, F.; Gonzalo, S.; Verhoeven, P.; Allatif, O.; Berthelot, P.; Pélissier, C.; Thiery, G.; Botelho-Nevers, E.; Millet, G.; Morel, J.; Paul, S.; Walzer, T.; Cosset, F.-L.; Bourlet, T.; Pozzetto, B. A Longitudinal Study of SARS-CoV-2-Infected Patients Reveals a High Correlation between Neutralizing Antibodies and COVID-19 Severity. *Cell Mol Immunol* **2021**, *18* (2), 318–327. <https://doi.org/10.1038/s41423-020-00588-2>.
- (21) Turner, J. S.; O'Halloran, J. A.; Kalaidina, E.; Kim, W.; Schmitz, A. J.; Zhou, J. Q.; Lei, T.; Thapa, M.; Chen, R. E.; Case, J. B.; Amanat, F.; Rauseo, A. M.; Haile, A.; Xie, X.; Klebert, M. K.; Suessen, T.; Middleton, W. D.; Shi, P.-Y.; Krammer, F.; Teefey, S. A.; Diamond, M. S.; Presti,

- R. M.; Ellebedy, A. H. SARS-CoV-2 mRNA Vaccines Induce Persistent Human Germinal Centre Responses. *Nature* **2021**, *596* (7870), 109–113. <https://doi.org/10.1038/s41586-021-03738-2>.
- (22) Goel, R. R.; Apostolidis, S. A.; Painter, M. M.; Mathew, D.; Pattekar, A.; Kuthuru, O.; Gouma, S.; Hicks, P.; Meng, W.; Rosenfeld, A. M.; Dysinger, S.; Lundgreen, K. A.; Kuri-Cervantes, L.; Adamski, S.; Hicks, A.; Korte, S.; Oldridge, D. A.; Baxter, A. E.; Giles, J. R.; Weirick, M. E.; McAllister, C. M.; Dougherty, J.; Long, S.; D'Andrea, K.; Hamilton, J. T.; Betts, M. R.; Luning Prak, E. T.; Bates, P.; Hensley, S. E.; Greenplate, A. R.; Wherry, E. J. Distinct Antibody and Memory B Cell Responses in SARS-CoV-2 Naïve and Recovered Individuals Following mRNA Vaccination. *Sci Immunol* **2021**, *6* (58), eabi6950. <https://doi.org/10.1126/sciimmunol.abi6950>.
- (23) Knipe, D. M.; Howley, P. *Fields Virology*; Wolters Kluwer: Philadelphia, 2015.
- (24) Tyrrell, D. A. J.; Almeida, J. D.; Cunningham, C. H.; Dowdle, W. R.; Hofstad, M. S.; McIntosh, K.; Tajima, M.; Zakstelskaya, L. Ya.; Easterday, B. C.; Kapikian, A.; Bingham, R. W. Coronaviridae. *Intervirology* **1975**, *5* (1–2), 76–82. <https://doi.org/10.1159/000149883>.
- (25) Helmy, Y. A.; Fawzy, M.; Elasad, A.; Sobieh, A.; Kenney, S. P.; Shehata, A. A. The COVID-19 Pandemic: A Comprehensive Review of Taxonomy, Genetics, Epidemiology, Diagnosis, Treatment, and Control. *J Clin Med* **2020**, *9* (4), 1225. <https://doi.org/10.3390/jcm9041225>.
- (26) Forni, D.; Cagliani, R.; Clerici, M.; Sironi, M. Molecular Evolution of Human Coronavirus Genomes. *Trends in Microbiology* **2017**, *25* (1), 35–48. <https://doi.org/10.1016/j.tim.2016.09.001>.
- (27) F.R. Beaudette; C.B. Hudson. Cultivation of the Virus of Infectious Bronchitis. *J Am Vet Med Assoc* **1937**, *90*, 51–60.
- (28) Cheever, F. S.; Daniels, J. B.; Pappenheimer, A. M.; Bailey, O. T. A Murine Virus (JHM) Causing Disseminated Encephalomyelitis with Extensive Destruction of Myelin. *J Exp Med* **1949**, *90* (3), 181–194. <https://doi.org/10.1084/jem.90.3.181>.
- (29) Kahn, J. S.; McIntosh, K. History and Recent Advances in Coronavirus Discovery. *Pediatr Infect Dis J* **2005**, *24* (11), S223–S227. <https://doi.org/10.1097/01.inf.0000188166.17324.60>.
- (30) Cherry, J. D.; Krogstad, P. SARS: The First Pandemic of the 21st Century. *Pediatr Res* **2004**, *56* (1), 1–5. <https://doi.org/10.1203/01.PDR.0000129184.87042.FC>.

- (31)Graham, R. L.; Donaldson, E. F.; Baric, R. S. A Decade after SARS: Strategies for Controlling Emerging Coronaviruses. *Nat Rev Microbiol* **2013**, *11* (12), 836–848. <https://doi.org/10.1038/nrmicro3143>.
- (32)Guan, Y.; Zheng, B. J.; He, Y. Q.; Liu, X. L.; Zhuang, Z. X.; Cheung, C. L.; Luo, S. W.; Li, P. H.; Zhang, L. J.; Guan, Y. J.; Butt, K. M.; Wong, K. L.; Chan, K. W.; Lim, W.; Shortridge, K. F.; Yuen, K. Y.; Peiris, J. S. M.; Poon, L. L. M. Isolation and Characterization of Viruses Related to the SARS Coronavirus from Animals in Southern China. *Science* **2003**, *302* (5643), 276–278. <https://doi.org/10.1126/science.1087139>.
- (33)Cui, J.; Li, F.; Shi, Z.-L. Origin and Evolution of Pathogenic Coronaviruses. *Nat Rev Microbiol* **2019**, *17* (3), 181–192. <https://doi.org/10.1038/s41579-018-0118-9>.
- (34)Omran, A. S.; Shalhoub, S. Middle East Respiratory Syndrome Coronavirus (MERS-CoV): What Lessons Can We Learn? *J Hosp Infect* **2015**, *91* (3), 188–196. <https://doi.org/10.1016/j.jhin.2015.08.002>.
- (35)Dudas, G.; Carvalho, L. M.; Rambaut, A.; Bedford, T. MERS-CoV Spillover at the Camel-Human Interface. *Elife* **2018**, *7*, e31257. <https://doi.org/10.7554/eLife.31257>.
- (36)Zaki, A. M.; Van Boheemen, S.; Bestebroer, T. M.; Osterhaus, A. D. M. E.; Fouchier, R. A. M. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *N Engl J Med* **2012**, *367* (19), 1814–1820. <https://doi.org/10.1056/NEJMoa1211721>.
- (37)Lau, S. K. P.; Li, K. S. M.; Tsang, A. K. L.; Lam, C. S. F.; Ahmed, S.; Chen, H.; Chan, K.-H.; Woo, P. C. Y.; Yuen, K.-Y. Genetic Characterization of Betacoronavirus Lineage C Viruses in Bats Reveals Marked Sequence Divergence in the Spike Protein of Pipistrellus Bat Coronavirus HKU5 in Japanese Pipistrelle: Implications for the Origin of the Novel Middle East Respiratory Syndrome Coronavirus. *J Virol* **2013**, *87* (15), 8638–8650. <https://doi.org/10.1128/JVI.01055-13>.
- (38)Corman, V. M.; Muth, D.; Niemeyer, D.; Drosten, C. Hosts and Sources of Endemic Human Coronaviruses. *Adv Virus Res* **2018**, *100*, 163–188. <https://doi.org/10.1016/bs.aivir.2018.01.001>.
- (39)Wu, A.; Peng, Y.; Huang, B.; Ding, X.; Wang, X.; Niu, P.; Meng, J.; Zhu, Z.; Zhang, Z.; Wang, J.; Sheng, J.; Quan, L.; Xia, Z.; Tan, W.; Cheng, G.; Jiang, T. Genome Composition and Divergence of the Novel Coronavirus (2019-nCoV) Originating in China. *Cell Host & Microbe* **2020**, *27* (3), 325–328. <https://doi.org/10.1016/j.chom.2020.02.001>.

- (40) Kim, D.; Lee, J.-Y.; Yang, J.-S.; Kim, J. W.; Kim, V. N.; Chang, H. The Architecture of SARS-CoV-2 Transcriptome. *Cell* **2020**, *181* (4), 914–921.e10. <https://doi.org/10.1016/j.cell.2020.04.011>.
- (41) Gorbalenya, A. E.; Enjuanes, L.; Ziebuhr, J.; Snijder, E. J. Nidovirales: Evolving the Largest RNA Virus Genome. *Virus Res* **2006**, *117* (1), 17–37. <https://doi.org/10.1016/j.virusres.2006.01.017>.
- (42) Jungreis, I.; Sealfon, R.; Kellis, M. SARS-CoV-2 Gene Content and COVID-19 Mutation Impact by Comparing 44 Sarbecovirus Genomes. *Res Sq* October 1, 2020. <https://doi.org/10.21203/rs.3.rs-80345/v1>.
- (43) Wu, F.; Zhao, S.; Yu, B.; Chen, Y.-M.; Wang, W.; Song, Z.-G.; Hu, Y.; Tao, Z.-W.; Tian, J.-H.; Pei, Y.-Y.; Yuan, M.-L.; Zhang, Y.-L.; Dai, F.-H.; Liu, Y.; Wang, Q.-M.; Zheng, J.-J.; Xu, L.; Holmes, E. C.; Zhang, Y.-Z. A New Coronavirus Associated with Human Respiratory Disease in China. *Nature* **2020**, *579* (7798), 265–269. <https://doi.org/10.1038/s41586-020-2008-3>.
- (44) Hoffmann, M.; Kleine-Weber, H.; Pöhlmann, S. A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell* **2020**, *78* (4), 779–784.e5. <https://doi.org/10.1016/j.molcel.2020.04.022>.
- (45) Shang, J.; Wan, Y.; Luo, C.; Ye, G.; Geng, Q.; Auerbach, A.; Li, F. Cell Entry Mechanisms of SARS-CoV-2. *Proc Natl Acad Sci USA* **2020**, *117* (21), 11727–11734. <https://doi.org/10.1073/pnas.2003138117>.
- (46) Kuba, K.; Imai, Y.; Rao, S.; Gao, H.; Guo, F.; Guan, B.; Huan, Y.; Yang, P.; Zhang, Y.; Deng, W.; Bao, L.; Zhang, B.; Liu, G.; Wang, Z.; Chappell, M.; Liu, Y.; Zheng, D.; Leibbrandt, A.; Wada, T.; Slutsky, A. S.; Liu, D.; Qin, C.; Jiang, C.; Penninger, J. M. A Crucial Role of Angiotensin Converting Enzyme 2 (ACE2) in SARS Coronavirus-Induced Lung Injury. *Nat Med* **2005**, *11* (8), 875–879. <https://doi.org/10.1038/nm1267>.
- (47) Zhao, M.-M.; Yang, W.-L.; Yang, F.-Y.; Zhang, L.; Huang, W.-J.; Hou, W.; Fan, C.-F.; Jin, R.-H.; Feng, Y.-M.; Wang, Y.-C.; Yang, J.-K. Cathepsin L Plays a Key Role in SARS-CoV-2 Infection in Humans and Humanized Mice and Is a Promising Target for New Drug Development. *Signal Transduct Target Ther* **2021**, *6* (1), 134. <https://doi.org/10.1038/s41392-021-00558-8>.
- (48) Osipiuk, J.; Azizi, S.-A.; Dvorkin, S.; Endres, M.; Jedrzejczak, R.; Jones, K. A.; Kang, S.; Kathayat, R. S.; Kim, Y.; Lisnyak, V. G.; Maki, S. L.; Nicolaescu, V.; Taylor, C. A.; Tesar, C.; Zhang, Y.-A.; Zhou, Z.; Randall, G.; Michalska, K.; Snyder, S. A.; Dickinson, B. C.; Joachimiak,

A. Structure of Papain-like Protease from SARS-CoV-2 and Its Complexes with Non-Covalent Inhibitors. *Nat Commun* **2021**, *12* (1), 743. <https://doi.org/10.1038/s41467-021-21060-3>.

(49) Jin, Z.; Du, X.; Xu, Y.; Deng, Y.; Liu, M.; Zhao, Y.; Zhang, B.; Li, X.; Zhang, L.; Peng, C.; Duan, Y.; Yu, J.; Wang, L.; Yang, K.; Liu, F.; Jiang, R.; Yang, X.; You, T.; Liu, X.; Yang, X.; Bai, F.; Liu, H.; Liu, X.; Guddat, L. W.; Xu, W.; Xiao, G.; Qin, C.; Shi, Z.; Jiang, H.; Rao, Z.; Yang, H. Structure of Mpro from SARS-CoV-2 and Discovery of Its Inhibitors. *Nature* **2020**, *582* (7811), 289–293. <https://doi.org/10.1038/s41586-020-2223-y>.

(50) Thoms, M.; Buschauer, R.; Ameisemeier, M.; Koepke, L.; Denk, T.; Hirschenberger, M.; Kratzat, H.; Hayn, M.; Mackens-Kiani, T.; Cheng, J.; Straub, J. H.; Stürzel, C. M.; Fröhlich, T.; Berninghausen, O.; Becker, T.; Kirchhoff, F.; Sparrer, K. M. J.; Beckmann, R. Structural Basis for Translational Shutdown and Immune Evasion by the Nsp1 Protein of SARS-CoV-2. *Science* **2020**, *369* (6508), 1249–1255. <https://doi.org/10.1126/science.abc8665>.

(51) Schubert, K.; Karousis, E. D.; Jomaa, A.; Scaiola, A.; Echeverria, B.; Gurzeler, L.-A.; Leibundgut, M.; Thiel, V.; Mühlemann, O.; Ban, N. SARS-CoV-2 Nsp1 Binds the Ribosomal mRNA Channel to Inhibit Translation. *Nat Struct Mol Biol* **2020**, *27* (10), 959–966. <https://doi.org/10.1038/s41594-020-0511-8>.

(52) Fisher, T.; Gluck, A.; Narayanan, K.; Kuroda, M.; Nachshon, A.; Hsu, J. C.; Halfmann, P. J.; Yahalom-Ronen, Y.; Tamir, H.; Finkel, Y.; Schwartz, M.; Weiss, S.; Tseng, C.-T. K.; Israely, T.; Paran, N.; Kawaoka, Y.; Makino, S.; Stern-Ginossar, N. Parsing the Role of NSP1 in SARS-CoV-2 Infection. *Cell Rep* **2022**, *39* (11), 110954. <https://doi.org/10.1016/j.celrep.2022.110954>.

(53) Cortese, M.; Lee, J.-Y.; Cerikan, B.; Neufeldt, C. J.; Oorschot, V. M. J.; Köhrer, S.; Hennies, J.; Schieber, N. L.; Ronchi, P.; Mizzon, G.; Romero-Brey, I.; Santarella-Mellwig, R.; Schorb, M.; Boermel, M.; Mocaer, K.; Beckwith, M. S.; Templin, R. M.; Gross, V.; Pape, C.; Tischer, C.; Frankish, J.; Horvat, N. K.; Laketa, V.; Stanifer, M.; Boulant, S.; Ruggieri, A.; Chatel-Chaix, L.; Schwab, Y.; Bartenschlager, R. Integrative Imaging Reveals SARS-CoV-2-Induced Reshaping of Subcellular Morphologies. *Cell Host Microbe* **2020**, *28* (6), 853–866.e5. <https://doi.org/10.1016/j.chom.2020.11.003>.

(54) Wolff, G.; Melia, C. E.; Snijder, E. J.; Bárcena, M. Double-Membrane Vesicles as Platforms for Viral Replication. *Trends Microbiol* **2020**, *28* (12), 1022–1033. <https://doi.org/10.1016/j.tim.2020.05.009>.

(55) Ricciardi, S.; Guarino, A. M.; Giaquinto, L.; Polishchuk, E. V.; Santoro, M.; Di Tullio, G.; Wilson, C.; Panariello, F.; Soares, V. C.; Dias, S. S. G.; Santos, J. C.; Souza, T. M. L.; Fusco, G.;

- Viscardi, M.; Brandi, S.; Bozza, P. T.; Polishchuk, R. S.; Venditti, R.; De Matteis, M. A. The Role of NSP6 in the Biogenesis of the SARS-CoV-2 Replication Organelle. *Nature* **2022**, *606* (7915), 761–768. <https://doi.org/10.1038/s41586-022-04835-6>.
- (56) Twu, W.-I.; Lee, J.-Y.; Kim, H.; Prasad, V.; Cerikan, B.; Haselmann, U.; Tabata, K.; Bartenschlager, R. Contribution of Autophagy Machinery Factors to HCV and SARS-CoV-2 Replication Organelle Formation. *Cell Rep* **2021**, *37* (8), 110049. <https://doi.org/10.1016/j.celrep.2021.110049>.
- (57) Tabata, K.; Prasad, V.; Paul, D.; Lee, J.-Y.; Pham, M.-T.; Twu, W.-I.; Neufeldt, C. J.; Cortese, M.; Cerikan, B.; Stahl, Y.; Joecks, S.; Tran, C. S.; Lüchtenborg, C.; V'kovski, P.; Hörmann, K.; Müller, A. C.; Zitzmann, C.; Haselmann, U.; Beneke, J.; Kaderali, L.; Erfle, H.; Thiel, V.; Lohmann, V.; Superti-Furga, G.; Brügger, B.; Bartenschlager, R. Convergent Use of Phosphatidic Acid for Hepatitis C Virus and SARS-CoV-2 Replication Organelle Formation. *Nat Commun* **2021**, *12* (1), 7276. <https://doi.org/10.1038/s41467-021-27511-1>.
- (58) Malone, B.; Urakova, N.; Snijder, E. J.; Campbell, E. A. Structures and Functions of Coronavirus Replication–Transcription Complexes and Their Relevance for SARS-CoV-2 Drug Design. *Nat Rev Mol Cell Biol* **2022**, *23* (1), 21–39. <https://doi.org/10.1038/s41580-021-00432-z>.
- (59) Hillen, H. S.; Kokic, G.; Farnung, L.; Dienemann, C.; Tegunov, D.; Cramer, P. Structure of Replicating SARS-CoV-2 Polymerase. *Nature* **2020**, *584* (7819), 154–156. <https://doi.org/10.1038/s41586-020-2368-8>.
- (60) Gao, Y.; Yan, L.; Huang, Y.; Liu, F.; Zhao, Y.; Cao, L.; Wang, T.; Sun, Q.; Ming, Z.; Zhang, L.; Ge, J.; Zheng, L.; Zhang, Y.; Wang, H.; Zhu, Y.; Zhu, C.; Hu, T.; Hua, T.; Zhang, B.; Yang, X.; Li, J.; Yang, H.; Liu, Z.; Xu, W.; Guddat, L. W.; Wang, Q.; Lou, Z.; Rao, Z. Structure of the RNA-Dependent RNA Polymerase from COVID-19 Virus. *Science* **2020**, *368* (6492), 779–782. <https://doi.org/10.1126/science.abb7498>.
- (61) Snijder, E. J.; Decroly, E.; Ziebuhr, J. The Nonstructural Proteins Directing Coronavirus RNA Synthesis and Processing. *Adv Virus Res* **2016**, *96*, 59–126. <https://doi.org/10.1016/bs.aivir.2016.08.008>.
- (62) Mickolajczyk, K. J.; Shelton, P. M. M.; Grasso, M.; Cao, X.; Warrington, S. E.; Aher, A.; Liu, S.; Kapoor, T. M. Force-Dependent Stimulation of RNA Unwinding by SARS-CoV-2 Nsp13 Helicase. *Biophys J* **2021**, *120* (6), 1020–1030. <https://doi.org/10.1016/j.bpj.2020.11.2276>.

- (63) Shannon, A.; Selisko, B.; Le, N.-T.-T.; Huchting, J.; Touret, F.; Piorkowski, G.; Fattorini, V.; Ferron, F.; Decroly, E.; Meier, C.; Coutard, B.; Peersen, O.; Canard, B. Rapid Incorporation of Favipiravir by the Fast and Permissive Viral RNA Polymerase Complex Results in SARS-CoV-2 Lethal Mutagenesis. *Nat Commun* **2020**, *11* (1), 4682. <https://doi.org/10.1038/s41467-020-18463-z>.
- (64) Smith, E. C.; Blanc, H.; Surdel, M. C.; Vignuzzi, M.; Denison, M. R. Coronaviruses Lacking Exoribonuclease Activity Are Susceptible to Lethal Mutagenesis: Evidence for Proofreading and Potential Therapeutics. *PLoS Pathog* **2013**, *9* (8), e1003565. <https://doi.org/10.1371/journal.ppat.1003565>.
- (65) Lin, S.; Chen, H.; Chen, Z.; Yang, F.; Ye, F.; Zheng, Y.; Yang, J.; Lin, X.; Sun, H.; Wang, L.; Wen, A.; Dong, H.; Xiao, Q.; Deng, D.; Cao, Y.; Lu, G. Crystal Structure of SARS-CoV-2 Nsp10 Bound to Nsp14-ExoN Domain Reveals an Exoribonuclease with Both Structural and Functional Integrity. *Nucleic Acids Res* **2021**, *49* (9), 5382–5392. <https://doi.org/10.1093/nar/gkab320>.
- (66) Liu, C.; Shi, W.; Becker, S. T.; Schatz, D. G.; Liu, B.; Yang, Y. Structural Basis of Mismatch Recognition by a SARS-CoV-2 Proofreading Enzyme. *Science* **2021**, *373* (6559), 1142–1146. <https://doi.org/10.1126/science.abi9310>.
- (67) V'kovski, P.; Kratzel, A.; Steiner, S.; Stalder, H.; Thiel, V. Coronavirus Biology and Replication: Implications for SARS-CoV-2. *Nat Rev Microbiol* **2021**, *19* (3), 155–170. <https://doi.org/10.1038/s41579-020-00468-6>.
- (68) Sawicki, S. G.; Sawicki, D. L. Coronaviruses Use Discontinuous Extension for Synthesis of Subgenome-Length Negative Strands. *Adv Exp Med Biol* **1995**, *380*, 499–506. https://doi.org/10.1007/978-1-4615-1899-0_79.
- (69) Sola, I.; Almazán, F.; Zúñiga, S.; Enjuanes, L. Continuous and Discontinuous RNA Synthesis in Coronaviruses. *Annu Rev Virol* **2015**, *2* (1), 265–288. <https://doi.org/10.1146/annurev-virology-100114-055218>.
- (70) Di, H.; McIntyre, A. A.; Brinton, M. A. New Insights about the Regulation of Nidovirus Subgenomic mRNA Synthesis. *Virology* **2018**, *517*, 38–43. <https://doi.org/10.1016/j.virol.2018.01.026>.
- (71) Steiner, S.; Kratzel, A.; Barut, G. T.; Lang, R. M.; Aguiar Moreira, E.; Thomann, L.; Kelly, J. N.; Thiel, V. SARS-CoV-2 Biology and Host Interactions. *Nat Rev Microbiol* **2024**, *22* (4), 206–225. <https://doi.org/10.1038/s41579-023-01003-z>.

- (72)Mendonça, L.; Howe, A.; Gilchrist, J. B.; Sheng, Y.; Sun, D.; Knight, M. L.; Zanetti-Domingues, L. C.; Bateman, B.; Krebs, A.-S.; Chen, L.; Radecke, J.; Li, V. D.; Ni, T.; Kounatidis, I.; Koronfel, M. A.; Szykiewicz, M.; Harkiolaki, M.; Martin-Fernandez, M. L.; James, W.; Zhang, P. Correlative Multi-Scale Cryo-Imaging Unveils SARS-CoV-2 Assembly and Egress. *Nat Commun* **2021**, *12* (1), 4629. <https://doi.org/10.1038/s41467-021-24887-y>.
- (73)Lu, S.; Ye, Q.; Singh, D.; Cao, Y.; Diedrich, J. K.; Yates, J. R.; Villa, E.; Cleveland, D. W.; Corbett, K. D. The SARS-CoV-2 Nucleocapsid Phosphoprotein Forms Mutually Exclusive Condensates with RNA and the Membrane-Associated M Protein. *Nat Commun* **2021**, *12* (1), 502. <https://doi.org/10.1038/s41467-020-20768-y>.
- (74)Scherer, K. M.; Mascheroni, L.; Carnell, G. W.; Wunderlich, L. C. S.; Makarchuk, S.; Brockhoff, M.; Mela, I.; Fernandez-Villegas, A.; Barysevich, M.; Stewart, H.; Suau Sans, M.; George, C. L.; Lamb, J. R.; Kaminski-Schierle, G. S.; Heeney, J. L.; Kaminski, C. F. SARS-CoV-2 Nucleocapsid Protein Adheres to Replication Organelles before Viral Assembly at the Golgi/ERGIC and Lysosome-Mediated Egress. *Sci Adv* **2022**, *8* (1), eabl4895. <https://doi.org/10.1126/sciadv.abl4895>.
- (75)Murigneux, E.; Softic, L.; Aubé, C.; Grandi, C.; Judith, D.; Bruce, J.; Le Gall, M.; Guillonnet, F.; Schmitt, A.; Parissi, V.; Berlioz-Torrent, C.; Meertens, L.; Hansen, M. M. K.; Gallois-Montbrun, S. Proteomic Analysis of SARS-CoV-2 Particles Unveils a Key Role of G3BP Proteins in Viral Assembly. *Nat Commun* **2024**, *15* (1), 640. <https://doi.org/10.1038/s41467-024-44958-0>.
- (76)Miura, K.; Suzuki, Y.; Ishida, K.; Arakawa, M.; Wu, H.; Fujioka, Y.; Emi, A.; Maeda, K.; Hamajima, R.; Nakano, T.; Tenno, T.; Hiroaki, H.; Morita, E. Distinct Motifs in the E Protein Are Required for SARS-CoV-2 Virus Particle Formation and Lysosomal Deacidification in Host Cells. *J Virol* **2023**, *97* (10), e00426-23. <https://doi.org/10.1128/jvi.00426-23>.
- (77)Alsaadi, E. A. J.; Neuman, B. W.; Jones, I. M. Identification of a Membrane Binding Peptide in the Envelope Protein of MHV Coronavirus. *Viruses* **2020**, *12* (9), 1054. <https://doi.org/10.3390/v12091054>.
- (78)Maeda, J.; Maeda, A.; Makino, S. Release of Coronavirus E Protein in Membrane Vesicles from Virus-Infected Cells and E Protein-Expressing Cells. *Virology* **1999**, *263* (2), 265–272. <https://doi.org/10.1006/viro.1999.9955>.

- (79)Wölk, C.; Shen, C.; Hause, G.; Surya, W.; Torres, J.; Harvey, R. D.; Bello, G. Membrane Condensation and Curvature Induced by SARS-CoV-2 Envelope Protein. *Langmuir* **2024**, *40* (5), 2646–2655. <https://doi.org/10.1021/acs.langmuir.3c03079>.
- (80)Ghosh, S.; Dellibovi-Ragheb, T. A.; Kerviel, A.; Pak, E.; Qiu, Q.; Fisher, M.; Takvorian, P. M.; Bleck, C.; Hsu, V. W.; Fehr, A. R.; Perlman, S.; Achar, S. R.; Straus, M. R.; Whittaker, G. R.; De Haan, C. A. M.; Kehrl, J.; Altan-Bonnet, G.; Altan-Bonnet, N. β -Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. *Cell* **2020**, *183* (6), 1520–1535.e14. <https://doi.org/10.1016/j.cell.2020.10.039>.
- (81)Katiyar, H.; Arduini, A.; Li, Y.; Liang, C. SARS-CoV-2 Assembly: Gaining Infectivity and Beyond. *Viruses* **2024**, *16* (11), 1648. <https://doi.org/10.3390/v16111648>.
- (82)Pu, J.; Schindler, C.; Jia, R.; Jarnik, M.; Backlund, P.; Bonifacino, J. S. BORC, a Multisubunit Complex That Regulates Lysosome Positioning. *Dev Cell* **2015**, *33* (2), 176–188. <https://doi.org/10.1016/j.devcel.2015.02.011>.
- (83)Chen, D.; Zheng, Q.; Sun, L.; Ji, M.; Li, Y.; Deng, H.; Zhang, H. ORF3a of SARS-CoV-2 Promotes Lysosomal Exocytosis-Mediated Viral Egress. *Dev Cell* **2021**, *56* (23), 3250–3263.e5. <https://doi.org/10.1016/j.devcel.2021.10.006>.
- (84)Bouayad, A. Innate Immune Evasion by SARS-CoV -2: Comparison with SARS-CoV. *Rev Med Virol* **2020**, *30* (6), 1–9. <https://doi.org/10.1002/rmv.2135>.
- (85)Walker, A. P.; Fan, H.; Keown, J. R.; Knight, M. L.; Grimes, J. M.; Fodor, E. The SARS-CoV-2 RNA Polymerase Is a Viral RNA Capping Enzyme. *Nucleic Acids Res* **2021**, *49* (22), 13019–13030. <https://doi.org/10.1093/nar/gkab1160>.
- (86)Park, G. J.; Osinski, A.; Hernandez, G.; Eitson, J. L.; Majumdar, A.; Tonelli, M.; Henzler-Wildman, K.; Pawłowski, K.; Chen, Z.; Li, Y.; Schoggins, J. W.; Tagliabracci, V. S. The Mechanism of RNA Capping by SARS-CoV-2. *Nature* **2022**, *609* (7928), 793–800. <https://doi.org/10.1038/s41586-022-05185-z>.
- (87)Banerjee, A. K.; Blanco, M. R.; Bruce, E. A.; Honson, D. D.; Chen, L. M.; Chow, A.; Bhat, P.; Ollikainen, N.; Quinodoz, S. A.; Loney, C.; Thai, J.; Miller, Z. D.; Lin, A. E.; Schmidt, M. M.; Stewart, D. G.; Goldfarb, D.; De Lorenzo, G.; Rihn, S. J.; Voorhees, R. M.; Botten, J. W.; Majumdar, D.; Guttman, M. SARS-CoV-2 Disrupts Splicing, Translation, and Protein Trafficking to Suppress Host Defenses. *Cell* **2020**, *183* (5), 1325–1339.e21. <https://doi.org/10.1016/j.cell.2020.10.004>.

- (88) Hayn, M.; Hirschenberger, M.; Koepke, L.; Nchioua, R.; Straub, J. H.; Klute, S.; Hunszinger, V.; Zech, F.; Prelli Bozzo, C.; Aftab, W.; Christensen, M. H.; Conzelmann, C.; Müller, J. A.; Srinivasachar Badarinarayan, S.; Stürzel, C. M.; Forne, I.; Stenger, S.; Conzelmann, K.-K.; Münch, J.; Schmidt, F. I.; Sauter, D.; Imhof, A.; Kirchhoff, F.; Sparrer, K. M. J. Systematic Functional Analysis of SARS-CoV-2 Proteins Uncovers Viral Innate Immune Antagonists and Remaining Vulnerabilities. *Cell Rep* **2021**, *35* (7), 109126. <https://doi.org/10.1016/j.celrep.2021.109126>.
- (89) Li, Z.-L.; Zhang, H.-L.; Huang, Y.; Huang, J.-H.; Sun, P.; Zhou, N.-N.; Chen, Y.-H.; Mai, J.; Wang, Y.; Yu, Y.; Zhou, L.-H.; Li, X.; Yang, D.; Peng, X.-D.; Feng, G.-K.; Tang, J.; Zhu, X.-F.; Deng, R. Autophagy Deficiency Promotes Triple-Negative Breast Cancer Resistance to T Cell-Mediated Cytotoxicity by Blocking Tenascin-C Degradation. *Nat Commun* **2020**, *11* (1), 3806. <https://doi.org/10.1038/s41467-020-17395-y>.
- (90) Li, J.-Y.; Liao, C.-H.; Wang, Q.; Tan, Y.-J.; Luo, R.; Qiu, Y.; Ge, X.-Y. The ORF6, ORF8 and Nucleocapsid Proteins of SARS-CoV-2 Inhibit Type I Interferon Signaling Pathway. *Virus Research* **2020**, *286*, 198074. <https://doi.org/10.1016/j.virusres.2020.198074>.
- (91) Shemesh, M.; Aktepe, T. E.; Deearin, J. M.; McAuley, J. L.; Audsley, M. D.; David, C. T.; Purcell, D. F. J.; Urin, V.; Hartmann, R.; Moseley, G. W.; Mackenzie, J. M.; Schreiber, G.; Harari, D. SARS-CoV-2 Suppresses IFN β Production Mediated by NSP1, 5, 6, 15, ORF6 and ORF7b but Does Not Suppress the Effects of Added Interferon. *PLoS Pathog* **2021**, *17* (8), e1009800. <https://doi.org/10.1371/journal.ppat.1009800>.
- (92) Stukalov, A.; Girault, V.; Grass, V.; Karayel, O.; Bergant, V.; Urban, C.; Haas, D. A.; Huang, Y.; Oubraham, L.; Wang, A.; Hamad, M. S.; Piras, A.; Hansen, F. M.; Tanzer, M. C.; Paron, I.; Zinzula, L.; Engleitner, T.; Reinecke, M.; Lavacca, T. M.; Ehmann, R.; Wölfel, R.; Jores, J.; Kuster, B.; Protzer, U.; Rad, R.; Ziebuhr, J.; Thiel, V.; Scaturro, P.; Mann, M.; Pichlmair, A. Multilevel Proteomics Reveals Host Perturbations by SARS-CoV-2 and SARS-CoV. *Nature* **2021**, *594* (7862), 246–252. <https://doi.org/10.1038/s41586-021-03493-4>.
- (93) Vazquez, C.; Swanson, S. E.; Negatu, S. G.; Dittmar, M.; Miller, J.; Ramage, H. R.; Cherry, S.; Jurado, K. A. SARS-CoV-2 Viral Proteins NSP1 and NSP13 Inhibit Interferon Activation through Distinct Mechanisms. *PLoS One* **2021**, *16* (6), e0253089. <https://doi.org/10.1371/journal.pone.0253089>.

- (94)Xia, H.; Cao, Z.; Xie, X.; Zhang, X.; Chen, J. Y.-C.; Wang, H.; Menachery, V. D.; Rajsbaum, R.; Shi, P.-Y. Evasion of Type I Interferon by SARS-CoV-2. *Cell Rep* **2020**, *33* (1), 108234. <https://doi.org/10.1016/j.celrep.2020.108234>.
- (95)Yuen, C.-K.; Lam, J.-Y.; Wong, W.-M.; Mak, L.-F.; Wang, X.; Chu, H.; Cai, J.-P.; Jin, D.-Y.; To, K. K.-W.; Chan, J. F.-W.; Yuen, K.-Y.; Kok, K.-H. SARS-CoV-2 Nsp13, Nsp14, Nsp15 and Orf6 Function as Potent Interferon Antagonists. *Emerg Microbes Infect* **2020**, *9* (1), 1418–1428. <https://doi.org/10.1080/22221751.2020.1780953>.
- (96)Zheng, M.; Karki, R.; Williams, E. P.; Yang, D.; Fitzpatrick, E.; Vogel, P.; Jonsson, C. B.; Kanneganti, T.-D. TLR2 Senses the SARS-CoV-2 Envelope Protein to Produce Inflammatory Cytokines. *Nat Immunol* **2021**, *22* (7), 829–838. <https://doi.org/10.1038/s41590-021-00937-x>.
- (97)Planès, R.; Bert, J.-B.; Tairi, S.; BenMohamed, L.; Bahraoui, E. SARS-CoV-2 Envelope (E) Protein Binds and Activates TLR2 Pathway: A Novel Molecular Target for COVID-19 Interventions. *Viruses* **2022**, *14* (5), 999. <https://doi.org/10.3390/v14050999>.
- (98)Rebendenne, A.; Valadão, A. L. C.; Tauziet, M.; Maarifi, G.; Bonaventure, B.; McKellar, J.; Planès, R.; Nisole, S.; Arnaud-Arnould, M.; Moncorgé, O.; Goujon, C. SARS-CoV-2 Triggers an MDA-5-Dependent Interferon Response Which Is Unable to Control Replication in Lung Epithelial Cells. *J Virol* **2021**, *95* (8), e02415-20, JVI.02415-20. <https://doi.org/10.1128/JVI.02415-20>.
- (99)Yang, D.-M.; Geng, T.-T.; Harrison, A. G.; Wang, P.-H. Differential Roles of RIG-I like Receptors in SARS-CoV-2 Infection. *Military Med Res* **2021**, *8* (1), 49. <https://doi.org/10.1186/s40779-021-00340-5>.
- (100)Yin, X.; Riva, L.; Pu, Y.; Martin-Sancho, L.; Kanamune, J.; Yamamoto, Y.; Sakai, K.; Gotoh, S.; Miorin, L.; De Jesus, P. D.; Yang, C.-C.; Herbert, K. M.; Yoh, S.; Hultquist, J. F.; García-Sastre, A.; Chanda, S. K. MDA5 Governs the Innate Immune Response to SARS-CoV-2 in Lung Epithelial Cells. *Cell Rep* **2021**, *34* (2), 108628. <https://doi.org/10.1016/j.celrep.2020.108628>.
- (101)Sampaio, N. G.; Chauveau, L.; Hertzog, J.; Bridgeman, A.; Fowler, G.; Moonen, J. P.; Dupont, M.; Russell, R. A.; Noerenberg, M.; Rehwinkel, J. The RNA Sensor MDA5 Detects SARS-CoV-2 Infection. *Sci Rep* **2021**, *11* (1), 13638. <https://doi.org/10.1038/s41598-021-92940-3>.

- (102) Anaegoudari, A.; Mollaei, H. R.; Arababadi, M. K.; Nosratabadi, R. Severe Acute Respiratory Syndrome Coronavirus 2: The Role of the Main Components of the Innate Immune System. *Inflammation* **2021**, *44* (6), 2151–2169. <https://doi.org/10.1007/s10753-021-01519-7>.
- (103) Kasuga, Y.; Zhu, B.; Jang, K.-J.; Yoo, J.-S. Innate Immune Sensing of Coronavirus and Viral Evasion Strategies. *Exp Mol Med* **2021**, *53* (5), 723–736. <https://doi.org/10.1038/s12276-021-00602-1>.
- (104) Schindler, C.; Levy, D. E.; Decker, T. JAK-STAT Signaling: From Interferons to Cytokines. *Journal of Biological Chemistry* **2007**, *282* (28), 20059–20063. <https://doi.org/10.1074/jbc.R700016200>.
- (105) Schoggins, J. W. Interferon-Stimulated Genes: What Do They All Do? *Annu. Rev. Virol.* **2019**, *6* (1), 567–584. <https://doi.org/10.1146/annurev-virology-092818-015756>.
- (106) Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z. J. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. *Science* **2013**, *339* (6121), 786–791. <https://doi.org/10.1126/science.1232458>.
- (107) Schoggins, J. W.; MacDuff, D. A.; Imanaka, N.; Gainey, M. D.; Shrestha, B.; Eitson, J. L.; Mar, K. B.; Richardson, R. B.; Ratushny, A. V.; Litvak, V.; Dabelic, R.; Manicassamy, B.; Aitchison, J. D.; Aderem, A.; Elliott, R. M.; García-Sastre, A.; Racaniello, V.; Snijder, E. J.; Yokoyama, W. M.; Diamond, M. S.; Virgin, H. W.; Rice, C. M. Pan-Viral Specificity of IFN-Induced Genes Reveals New Roles for cGAS in Innate Immunity. *Nature* **2014**, *505* (7485), 691–695. <https://doi.org/10.1038/nature12862>.
- (108) Rui, Y.; Su, J.; Shen, S.; Hu, Y.; Huang, D.; Zheng, W.; Lou, M.; Shi, Y.; Wang, M.; Chen, S.; Zhao, N.; Dong, Q.; Cai, Y.; Xu, R.; Zheng, S.; Yu, X.-F. Unique and Complementary Suppression of cGAS-STING and RNA Sensing- Triggered Innate Immune Responses by SARS-CoV-2 Proteins. *Signal Transduct Target Ther* **2021**, *6* (1), 123. <https://doi.org/10.1038/s41392-021-00515-5>.
- (109) Christgen, S.; Kanneganti, T.-D. Inflammasomes and the Fine Line between Defense and Disease. *Curr Opin Immunol* **2020**, *62*, 39–44. <https://doi.org/10.1016/j.coi.2019.11.007>.
- (110) Kelley, N.; Jeltema, D.; Duan, Y.; He, Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *IJMS* **2019**, *20* (13), 3328. <https://doi.org/10.3390/ijms20133328>.
- (111) Campbell, G. R.; To, R. K.; Hanna, J.; Spector, S. A. SARS-CoV-2, SARS-CoV-1, and HIV-1 Derived ssRNA Sequences Activate the NLRP3 Inflammasome in Human Macrophages

through a Non-Classical Pathway. *iScience* **2021**, *24* (4), 102295. <https://doi.org/10.1016/j.isci.2021.102295>.

(112)Pan, P.; Shen, M.; Yu, Z.; Ge, W.; Chen, K.; Tian, M.; Xiao, F.; Wang, Z.; Wang, J.; Jia, Y.; Wang, W.; Wan, P.; Zhang, J.; Chen, W.; Lei, Z.; Chen, X.; Luo, Z.; Zhang, Q.; Xu, M.; Li, G.; Li, Y.; Wu, J. SARS-CoV-2 N Protein Promotes NLRP3 Inflammasome Activation to Induce Hyperinflammation. *Nat Commun* **2021**, *12* (1), 4664. <https://doi.org/10.1038/s41467-021-25015-6>.

(113)Xu, H.; Akinyemi, I. A.; Chitre, S. A.; Loeb, J. C.; Lednicky, J. A.; McIntosh, M. T.; Bhaduri-McIntosh, S. SARS-CoV-2 Viroporin Encoded by ORF3a Triggers the NLRP3 Inflammatory Pathway. *Virology* **2022**, *568*, 13–22. <https://doi.org/10.1016/j.virol.2022.01.003>.

(114)Lei, X.; Dong, X.; Ma, R.; Wang, W.; Xiao, X.; Tian, Z.; Wang, C.; Wang, Y.; Li, L.; Ren, L.; Guo, F.; Zhao, Z.; Zhou, Z.; Xiang, Z.; Wang, J. Activation and Evasion of Type I Interferon Responses by SARS-CoV-2. *Nat Commun* **2020**, *11* (1), 3810. <https://doi.org/10.1038/s41467-020-17665-9>.

(115)Li, A.; Zhao, K.; Zhang, B.; Hua, R.; Fang, Y.; Jiang, W.; Zhang, J.; Hui, L.; Zheng, Y.; Li, Y.; Zhu, C.; Wang, P.-H.; Peng, K.; Xia, Y. SARS-CoV-2 NSP12 Protein Is Not an Interferon- β Antagonist. *J Virol* **2021**, *95* (17), e00747-21. <https://doi.org/10.1128/JVI.00747-21>.

(116)Zheng, Y.; Zhuang, M.-W.; Han, L.; Zhang, J.; Nan, M.-L.; Zhan, P.; Kang, D.; Liu, X.; Gao, C.; Wang, P.-H. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Membrane (M) Protein Inhibits Type I and III Interferon Production by Targeting RIG-I/MDA-5 Signaling. *Sig Transduct Target Ther* **2020**, *5* (1), 299. <https://doi.org/10.1038/s41392-020-00438-7>.

(117)Sui, L.; Zhao, Y.; Wang, W.; Wu, P.; Wang, Z.; Yu, Y.; Hou, Z.; Tan, G.; Liu, Q. SARS-CoV-2 Membrane Protein Inhibits Type I Interferon Production Through Ubiquitin-Mediated Degradation of TBK1. *Front. Immunol.* **2021**, *12*, 662989. <https://doi.org/10.3389/fimmu.2021.662989>.

(118)Loo, Y.-M.; Gale, M. Immune Signaling by RIG-I-like Receptors. *Immunity* **2011**, *34* (5), 680–692. <https://doi.org/10.1016/j.immuni.2011.05.003>.

(119)Kawai, T.; Akira, S. The Roles of TLRs, RLRs and NLRs in Pathogen Recognition. *Int Immunol* **2009**, *21* (4), 317–337. <https://doi.org/10.1093/intimm/dxp017>.

(120)Ma, Z.; Ni, G.; Damania, B. Innate Sensing of DNA Virus Genomes. *Annu Rev Virol* **2018**, *5* (1), 341–362. <https://doi.org/10.1146/annurev-virology-092917-043244>.

- (121) Mogensen, T. H. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clin Microbiol Rev* **2009**, *22* (2), 240–273, Table of Contents. <https://doi.org/10.1128/CMR.00046-08>.
- (122) Yan, L.; Ge, J.; Zheng, L.; Zhang, Y.; Gao, Y.; Wang, T.; Huang, Y.; Yang, Y.; Gao, S.; Li, M.; Liu, Z.; Wang, H.; Li, Y.; Chen, Y.; Guddat, L. W.; Wang, Q.; Rao, Z.; Lou, Z. Cryo-EM Structure of an Extended SARS-CoV-2 Replication and Transcription Complex Reveals an Intermediate State in Cap Synthesis. *Cell* **2021**, *184* (1), 184–193.e10. <https://doi.org/10.1016/j.cell.2020.11.016>.
- (123) Frazier, M. N.; Dillard, L. B.; Krahn, J. M.; Perera, L.; Williams, J. G.; Wilson, I. M.; Stewart, Z. D.; Pillon, M. C.; Deterding, L. J.; Borgnia, M. J.; Stanley, R. E. Characterization of SARS2 Nsp15 Nuclease Activity Reveals It's Mad about U. *Nucleic Acids Res* **2021**, *49* (17), 10136–10149. <https://doi.org/10.1093/nar/gkab719>.
- (124) Hackbart, M.; Deng, X.; Baker, S. C. Coronavirus Endoribonuclease Targets Viral Polyuridine Sequences to Evade Activating Host Sensors. *Proc Natl Acad Sci U S A* **2020**, *117* (14), 8094–8103. <https://doi.org/10.1073/pnas.1921485117>.
- (125) Versteeg, G. A.; Bredenbeek, P. J.; van den Worm, S. H. E.; Spaan, W. J. M. Group 2 Coronaviruses Prevent Immediate Early Interferon Induction by Protection of Viral RNA from Host Cell Recognition. *Virology* **2007**, *361* (1), 18–26. <https://doi.org/10.1016/j.virol.2007.01.020>.
- (126) Zhou, H.; Perlman, S. Mouse Hepatitis Virus Does Not Induce Beta Interferon Synthesis and Does Not Inhibit Its Induction by Double-Stranded RNA. *J Virol* **2007**, *81* (2), 568–574. <https://doi.org/10.1128/JVI.01512-06>.
- (127) Chen, D.; Zhang, H. Autophagy in Severe Acute Respiratory Syndrome Coronavirus 2 Infection. *Curr Opin Physiol* **2022**, *29*, 100596. <https://doi.org/10.1016/j.cophys.2022.100596>.
- (128) Levine, B.; Mizushima, N.; Virgin, H. W. Autophagy in Immunity and Inflammation. *Nature* **2011**, *469* (7330), 323–335. <https://doi.org/10.1038/nature09782>.
- (129) Stern-Ginossar, N.; Thompson, S. R.; Mathews, M. B.; Mohr, I. Translational Control in Virus-Infected Cells. *Cold Spring Harb Perspect Biol* **2019**, *11* (3), a033001. <https://doi.org/10.1101/cshperspect.a033001>.
- (130) Finkel, Y.; Mizrahi, O.; Nachshon, A.; Weingarten-Gabbay, S.; Morgenstern, D.; Yahalom-Ronen, Y.; Tamir, H.; Achdout, H.; Stein, D.; Israeli, O.; Beth-Din, A.; Melamed, S.; Weiss, S.;

- Israely, T.; Paran, N.; Schwartz, M.; Stern-Ginossar, N. The Coding Capacity of SARS-CoV-2. *Nature* **2021**, *589* (7840), 125–130. <https://doi.org/10.1038/s41586-020-2739-1>.
- (131) Finkel, Y.; Gluck, A.; Nachshon, A.; Winkler, R.; Fisher, T.; Rozman, B.; Mizrahi, O.; Lubelsky, Y.; Zuckerman, B.; Slobodin, B.; Yahalom-Ronen, Y.; Tamir, H.; Ulitsky, I.; Israely, T.; Paran, N.; Schwartz, M.; Stern-Ginossar, N. SARS-CoV-2 Uses a Multipronged Strategy to Impede Host Protein Synthesis. *Nature* **2021**, *594* (7862), 240–245. <https://doi.org/10.1038/s41586-021-03610-3>.
- (132) Lapointe, C. P.; Grosely, R.; Johnson, A. G.; Wang, J.; Fernández, I. S.; Puglisi, J. D. Dynamic Competition between SARS-CoV-2 NSP1 and mRNA on the Human Ribosome Inhibits Translation Initiation. *Proc Natl Acad Sci USA* **2021**, *118* (6), e2017715118. <https://doi.org/10.1073/pnas.2017715118>.
- (133) Yuan, S.; Balaji, S.; Lomakin, I. B.; Xiong, Y. Coronavirus Nsp1: Immune Response Suppression and Protein Expression Inhibition. *Front Microbiol* **2021**, *12*, 752214. <https://doi.org/10.3389/fmicb.2021.752214>.
- (134) Zhang, K.; Miorin, L.; Makio, T.; Dehghan, I.; Gao, S.; Xie, Y.; Zhong, H.; Esparza, M.; Kehrer, T.; Kumar, A.; Hobman, T. C.; Ptak, C.; Gao, B.; Minna, J. D.; Chen, Z.; García-Sastre, A.; Ren, Y.; Wozniak, R. W.; Fontoura, B. M. A. Nsp1 Protein of SARS-CoV-2 Disrupts the mRNA Export Machinery to Inhibit Host Gene Expression. *Sci Adv* **2021**, *7* (6), eabe7386. <https://doi.org/10.1126/sciadv.abe7386>.
- (135) Narayanan, K.; Huang, C.; Lokugamage, K.; Kamitani, W.; Ikegami, T.; Tseng, C.-T. K.; Makino, S. Severe Acute Respiratory Syndrome Coronavirus Nsp1 Suppresses Host Gene Expression, Including That of Type I Interferon, in Infected Cells. *J Virol* **2008**, *82* (9), 4471–4479. <https://doi.org/10.1128/JVI.02472-07>.
- (136) Pinet, L.; Assrir, N.; Van Heijenoort, C. Expanding the Disorder-Function Paradigm in the C-Terminal Tails of Erbbs. *Biomolecules* **2021**, *11* (11), 1690. <https://doi.org/10.3390/biom11111690>.
- (137) Wright, P. E.; Dyson, H. J. Intrinsically Unstructured Proteins: Re-Assessing the Protein Structure-Function Paradigm. *J Mol Biol* **1999**, *293* (2), 321–331. <https://doi.org/10.1006/jmbi.1999.3110>.
- (138) Dyson, H. J.; Wright, P. E. Intrinsically Unstructured Proteins and Their Functions. *Nat Rev Mol Cell Biol* **2005**, *6* (3), 197–208. <https://doi.org/10.1038/nrm1589>.

- (139)Uversky, V. N. What Does It Mean to Be Natively Unfolded? *European Journal of Biochemistry* **2002**, *269* (1), 2–12. <https://doi.org/10.1046/j.0014-2956.2001.02649.x>.
- (140)Dunker, A. K.; Lawson, J. D.; Brown, C. J.; Williams, R. M.; Romero, P.; Oh, J. S.; Oldfield, C. J.; Campen, A. M.; Ratliff, C. M.; Hipps, K. W.; Ausio, J.; Nissen, M. S.; Reeves, R.; Kang, C.; Kissinger, C. R.; Bailey, R. W.; Griswold, M. D.; Chiu, W.; Garner, E. C.; Obradovic, Z. Intrinsically Disordered Protein. *J Mol Graph Model* **2001**, *19* (1), 26–59. [https://doi.org/10.1016/S1093-3263\(00\)00138-8](https://doi.org/10.1016/S1093-3263(00)00138-8).
- (141)Radivojac, P.; Iakoucheva, L. M.; Oldfield, C. J.; Obradovic, Z.; Uversky, V. N.; Dunker, A. K. Intrinsic Disorder and Functional Proteomics. *Biophys J* **2007**, *92* (5), 1439–1456. <https://doi.org/10.1529/biophysj.106.094045>.
- (142)Williams, R. M.; Obradovic, Z.; Mathura, V.; Braun, W.; Garner, E. C.; Young, J.; Takayama, S.; Brown, C. J.; Dunker, A. K. The Protein Non-folding Problem: Amino Acid Determinants of Intrinsic Order and Disorder. In *Biocomputing 2001*; WORLD SCIENTIFIC: Mauna Lani, Hawaii, 2000; pp 89–100. https://doi.org/10.1142/9789814447362_0010.
- (143)Beveridge, R.; Migas, L. G.; Das, R. K.; Pappu, R. V.; Kriwacki, R. W.; Barran, P. E. Ion Mobility Mass Spectrometry Uncovers the Impact of the Patterning of Oppositely Charged Residues on the Conformational Distributions of Intrinsically Disordered Proteins. *J Am Chem Soc* **2019**, *141* (12), 4908–4918. <https://doi.org/10.1021/jacs.8b13483>.
- (144)Martin, E. W.; Holehouse, A. S.; Peran, I.; Farag, M.; Incicco, J. J.; Bremer, A.; Grace, C. R.; Soranno, A.; Pappu, R. V.; Mittag, T. Valence and Patterning of Aromatic Residues Determine the Phase Behavior of Prion-like Domains. *Science* **2020**, *367* (6478), 694–699. <https://doi.org/10.1126/science.aaw8653>.
- (145)Martin, E. W.; Holehouse, A. S.; Grace, C. R.; Hughes, A.; Pappu, R. V.; Mittag, T. Sequence Determinants of the Conformational Properties of an Intrinsically Disordered Protein Prior to and upon Multisite Phosphorylation. *J Am Chem Soc* **2016**, *138* (47), 15323–15335. <https://doi.org/10.1021/jacs.6b10272>.
- (146)Strodel, B. Energy Landscapes of Protein Aggregation and Conformation Switching in Intrinsically Disordered Proteins. *J Mol Biol* **2021**, *433* (20), 167182. <https://doi.org/10.1016/j.jmb.2021.167182>.
- (147)Kriwacki, R. W.; Hengst, L.; Tennant, L.; Reed, S. I.; Wright, P. E. Structural Studies of p21Waf1/Cip1/Sdi1 in the Free and Cdk2-Bound State: Conformational Disorder Mediates

- Binding Diversity. *Proc Natl Acad Sci U.S.A.* **1996**, *93* (21), 11504–11509. <https://doi.org/10.1073/pnas.93.21.11504>.
- (148)Uversky, V. N. Analyzing IDPs in Interactomes. In *Intrinsically Disordered Proteins*; Kragelund, B. B., Skriver, K., Eds.; Methods in Molecular Biology; Springer US: New York, NY, 2020; Vol. 2141, pp 895–945. https://doi.org/10.1007/978-1-0716-0524-0_46.
- (149)Spolar, R. S.; Record, M. T. Coupling of Local Folding to Site-Specific Binding of Proteins to DNA. *Science* **1994**, *263* (5148), 777–784. <https://doi.org/10.1126/science.8303294>.
- (150)Dyson, H. J.; Wright, P. E. Coupling of Folding and Binding for Unstructured Proteins. *Curr Opin Struct Biol* **2002**, *12* (1), 54–60. [https://doi.org/10.1016/S0959-440X\(02\)00289-0](https://doi.org/10.1016/S0959-440X(02)00289-0).
- (151)Fuxreiter, M.; Simon, I.; Friedrich, P.; Tompa, P. Preformed Structural Elements Feature in Partner Recognition by Intrinsically Unstructured Proteins. *J Mol Biol* **2004**, *338* (5), 1015–1026. <https://doi.org/10.1016/j.jmb.2004.03.017>.
- (152)Bertagna, A.; Toptygin, D.; Brand, L.; Barrick, D. The Effects of Conformational Heterogeneity on the Binding of the Notch Intracellular Domain to Effector Proteins: A Case of Biologically Tuned Disorder. *Biochem Soc Trans* **2008**, *36* (2), 157–166. <https://doi.org/10.1042/BST0360157>.
- (153)Wang, Y.; Fisher, J. C.; Mathew, R.; Ou, L.; Otieno, S.; Sublet, J.; Xiao, L.; Chen, J.; Roussel, M. F.; Kriwacki, R. W. Intrinsic Disorder Mediates the Diverse Regulatory Functions of the Cdk Inhibitor P21. *Nat Chem Biol* **2011**, *7* (4), 214–221. <https://doi.org/10.1038/nchembio.536>.
- (154)Mittag, T.; Marsh, J.; Grishaev, A.; Orlicky, S.; Lin, H.; Sicheri, F.; Tyers, M.; Forman-Kay, J. D. Structure/Function Implications in a Dynamic Complex of the Intrinsically Disordered Sic1 with the Cdc4 Subunit of an SCF Ubiquitin Ligase. *Structure* **2010**, *18* (4), 494–506. <https://doi.org/10.1016/j.str.2010.01.020>.
- (155)Tompa, P. The Interplay between Structure and Function in Intrinsically Unstructured Proteins. *FEBS Letters* **2005**, *579* (15), 3346–3354. <https://doi.org/10.1016/j.febslet.2005.03.072>.
- (156)Trivedi, R.; Nagarajaram, H. A. Intrinsically Disordered Proteins: An Overview. *Int J Mol Sci* **2022**, *23* (22), 14050. <https://doi.org/10.3390/ijms232214050>.
- (157)Piovesan, D.; Tosatto, S. C. E. Mobi 2.0: An Improved Method to Define Intrinsic Disorder, Mobility and Linear Binding Regions in Protein Structures. *Bioinformatics* **2018**, *34* (1), 122–123. <https://doi.org/10.1093/bioinformatics/btx592>.

- (158)Vymětal, J.; Vondrášek, J.; Hlouchová, K. Sequence Versus Composition: What Prescribes IDP Biophysical Properties? *Entropy* **2019**, *21* (7), 654. <https://doi.org/10.3390/e21070654>.
- (159)Cornish, J.; Chamberlain, S. G.; Owen, D.; Mott, H. R. Intrinsically Disordered Proteins and Membranes: A Marriage of Convenience for Cell Signalling? *Biochem Soc Trans* **2020**, *48* (6), 2669–2689. <https://doi.org/10.1042/BST20200467>.
- (160)Schroeder, R.; Barta, A.; Semrad, K. Strategies for RNA Folding and Assembly. *Nat Rev Mol Cell Biol* **2004**, *5* (11), 908–919. <https://doi.org/10.1038/nrm1497>.
- (161)Young, J. C.; Agashe, V. R.; Siegers, K.; Hartl, F. U. Pathways of Chaperone-Mediated Protein Folding in the Cytosol. *Nat Rev Mol Cell Biol* **2004**, *5* (10), 781–791. <https://doi.org/10.1038/nrm1492>.
- (162)Kovacs, D.; Tompa, P. Diverse Functional Manifestations of Intrinsic Structural Disorder in Molecular Chaperones. *Biochem Soc Trans* **2012**, *40* (5), 963–968. <https://doi.org/10.1042/BST20120108>.
- (163)Sugase, K.; Dyson, H. J.; Wright, P. E. Mechanism of Coupled Folding and Binding of an Intrinsically Disordered Protein. *Nature* **2007**, *447* (7147), 1021–1025. <https://doi.org/10.1038/nature05858>.
- (164)Mughal, F.; Caetano-Anollés, G. Evolution of Intrinsic Disorder in the Structural Domains of Viral and Cellular Proteomes. *Sci Rep* **2025**, *15* (1), 2878. <https://doi.org/10.1038/s41598-025-86045-4>.

Chapter 2

METHODS

Biological Samples and Techniques

Peptides

Multiple synthetic Nsp1-CT peptides were used throughout this work, and all of them were obtained through Genscript Biotech Corporation. All the peptides were prepared with N-terminal acetylation (Ac-). The following peptides were used in these studies:

Nsp1-CT₃₃: Ac- ELGTDPYEDFQENWNTKHSSGV TRELMRELNGG

Nsp1-CT₃₃ Y154(YNO₂): ELGTD(P(YNO₂))EDFQENWNTKHSSGV TRELMRELNGG

Nsp1-CT₃₃ H165A: Ac- ELGTDPYEDFQENWNTKASSGV TRELMRELNGG

Nsp1-CT₁₀: Ac-ENWNTKHSSG.

Protein Expression and Purification

Multiple medias were used in the process of protein expression. Below are the medias and their formulations. Bacto-tryptone and bacto-yeast were purchased from ThermoFisher Scientific Gibco.

SOC Media: To a flask add 20g bacto-tryptone, 5g bacto-yeast, 0.5g NaCl, 10 mL KCl (250mM), 0.2mL NaOH (5M) then adjust the volume to 1L and autoclave. After autoclaving add 5mL sterile MgCl₂ (2M). Once it has cooled add 20 mL of a sterile 1 M solution of glucose.

LB Media: To prepare the LB media, add 10g of bacto-tryptone, 5g of bacto-yeast, and 10g of NaCl to a flask along with 1L of MilliQ- water. Adjust the pH to 7 using 5M NaOH. Sterilize by autoclaving before use.

2XYT Media: To prepare the media, add 8 g of Bacto- tryptone, 5 g of Bacto- yeast extract, and 2.5 g of NaCl to 500 mL of Milli-Q water in a flask. Adjust the pH to 7.0 using 5 M NaOH. Sterilize the solution by autoclaving.

LB Agar Plates with antibiotic resistance: To a flask add 4g tryptone, 2g yeast, 4g NaCl, 6g Agar, 80 μ L NaOH (5M), and adjust with water to 400mL. Autoclave. Once the solution is approximately 55°C antibiotics can be added. Pipet 25mL into each plate, allow to cool and store at 4°C. Antibiotic used was Kanamycin (preparation for antibiotic samples is below).

M9 Minimal Media Preparation for ^{15}N Labeling: To prepare the minimal media, a 5X M9 salt solution was first prepared by dissolving 64 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 15 g of KH_2PO_4 , 2.5 g of NaCl, and 5.0 g of NH_4Cl in 1 L of Milli-Q water. For ^{15}N labeling experiments, NH_4Cl was substituted with ^{15}N -labeled ammonium chloride (Cambridge Isotope Laboratories Inc., Item No. NLM-467-5). The salt solution was sterilized by autoclaving. Separately, the following components were prepared and sterilized by autoclaving: 1 M MgSO_4 solution, 1 M CaCl_2 solution, and a 40% (w/v) glucose solution. To prepare 1 L of final minimal media, the following were combined: 200 mL of the 5X M9 salt solution, 800 mL of Milli-Q water, 2 mL of 1 M MgSO_4 , 0.1 mL of 1 M CaCl_2 , and 10 mL of the 40% glucose solution.

Antibiotic preparation: Kanamycin antibiotic stocks were prepared at a concentration of 100 mg/mL to achieve a final working concentration of 100 $\mu\text{g}/\text{mL}$ when added to media.

Plasmid details: The plasmid for the wild type protein was obtained from Genscript as powder. The plasmid (0.3 μg) was dissolved in 50 μL of TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0). From the solution 0.5 μL was used for transformations.

His6-Nsp1 pET-24a(+) plasmid sequence:

TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAAGCGCGGCGGGTGTGGTG
GTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTC
GCTTTCCTTCCCTTCCTTCTCGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAA
ATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCA
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TAGGCGACGAGCTTGGCACTGATCCTTATGAAGATTTTCAAGAAAACCTGGAAC

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 CCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAAC
 CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGT*TTT*TGCTGAAAGGAGGAACT
 ATATCCGGAT

Transformation of *E. coli* DH5 α with His₆-Nsp1_pET-24a(+) Plasmid: *E. coli* DH5 α competent cells were thawed on ice from -80°C . Simultaneously, 300 μL of SOC medium and an LB agar plate containing kanamycin were warmed to 37°C . In a sterile 15 mL tube, 100 μL of DH5 α cells were combined with 0.5 μL of the His₆-Nsp1_pET-24a(+) plasmid. The tube was gently tapped several times and incubated on ice for 5 minutes. Following this, the cells were heat-shocked at 42°C for 1 minute and immediately returned to ice for an additional 2 minutes.

After cooling, 200 μL of pre-warmed SOC medium were added to the transformation mixture. The tube was then incubated at 37°C for 45 minutes with shaking at 250 rpm. Subsequently, 50 μL of the transformed culture were plated onto the pre-warmed LB agar plate containing kanamycin and incubated at 37°C for 16 hours.

Several resulting colonies were selected for plasmid propagation. Each colony was inoculated into 5 mL of LB medium supplemented with kanamycin and grown for 16 hours at 37°C . Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Cat. No. 27104) following the manufacturer's protocol. Isolated plasmids were submitted to Laragen Inc. for sequencing verification.

To express the His₆-Nsp1_pET-24a(+) plasmid, a second transformation was performed using *E. coli* BL21(DE3) cells. The procedure followed was identical to the one described previously for DH5 α cells.

Creation of Glycerol Stocks for Protein Expression: To streamline future protein expression experiments and avoid repeated transformations, glycerol stocks of the transformed BL21(DE3) cells were prepared. A single colony from the transformed LB-kanamycin plate was inoculated into 5 mL of LB medium supplemented with 5 μL of kanamycin. The culture was incubated at 37°C with shaking at 250 rpm for 16 hours. For long-term storage, 875 μL of the overnight

culture were mixed with 125 μ L of sterile 80% glycerol in sterile Eppendorf tubes. The mixture was gently agitated to ensure even distribution before being stored at -80°C . For subsequent protein expression, a frozen glycerol stock was briefly placed on ice, and a sterile inoculating loop was used to streak a small amount of the culture onto a pre-warmed LB agar plate containing kanamycin. The plate was incubated overnight at 37°C (16 hours). Stocks were returned to the -80°C freezer immediately after use, provided they had not thawed.

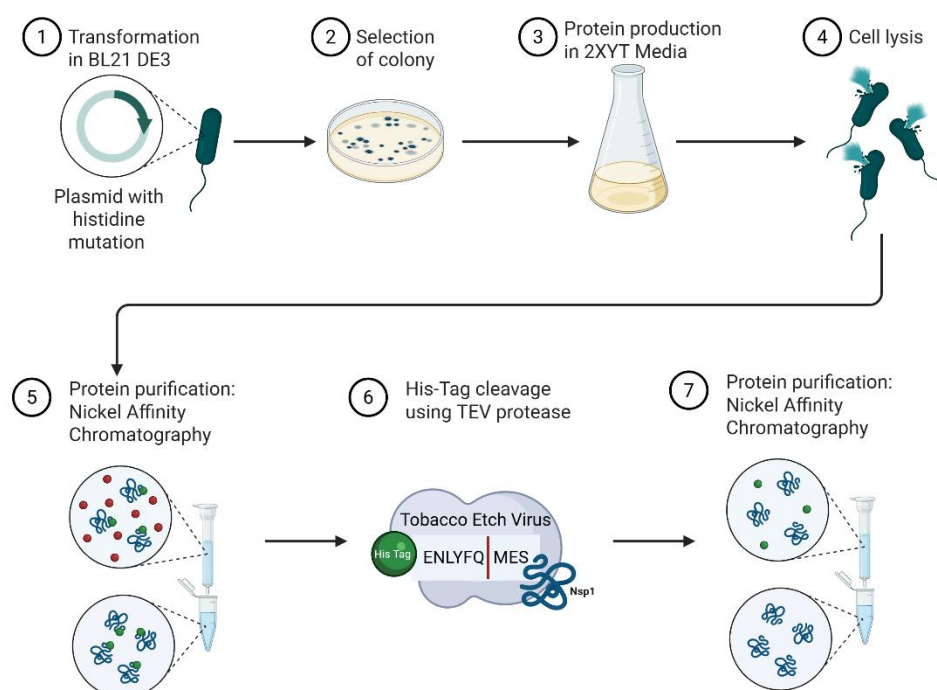


Figure 2.1 Expression and purification methods of full length Nsp1. Figure created with BioRender.com.

Expression of Wild-Type and Mutant Nsp1 Proteins: In order to express the wild type and mutant proteins the following procedure was adapted from Schubert et al.¹ Colonies from the previously transformed *E. coli* BL21(DE3) plates were selected to inoculate small-scale starter cultures.

For each expression, a 5 mL small culture of 2×YT medium supplemented with kanamycin was inoculated with a single colony and incubated at 37°C with shaking at 180 rpm for approximately 8 hours. After this initial growth phase, the entire small culture was transferred

into 500 mL of pre-warmed 2×YT medium containing kanamycin to initiate the large-scale expression culture.

The large cultures were incubated at 37 °C with shaking at 180 rpm until an optical density at 600 nm (OD₆₀₀) of 0.6–0.8 was reached (approximately 4 hours). At this point, protein expression was induced by the addition of 1 M IPTG to a final concentration of 0.5 mM. The temperature was then reduced to 18 °C, and the cultures were allowed to express protein overnight (16 hours) with shaking at 180 rpm.

To harvest the expressed cultures, cells were pelleted by centrifugation at 4000 rpm for 10 minutes at 4 °C. The supernatant was discarded. Pellets were either stored at –80 °C for later purification or processed immediately.

Purification of Wild-Type and Mutant Nsp1 Proteins: Cell pellets from expression cultures were resuspended in Buffer A (50 mM HEPES-KOH, pH 7.6, 500 mM KCl, 5 mM MgCl₂, 10% [w/v] glycerol, 0.5 mM TCEP). The suspension was sonicated using a probe sonicator for 5 minutes (3 s on, 17 s off, 80% amplitude). Lysozyme, DNase I, RNase A, and protease inhibitors were added, and the lysate was gently stirred at 4 °C for at least 1 hour.

Following lysis, the mixture was centrifuged at 12,000 rpm for 30 minutes at 4 °C. The supernatant was collected, and the pellet was discarded. The clarified lysate was then incubated with Ni-NTA resin overnight at 4 °C for affinity purification of the His₆-tagged Nsp1.

The next day, the resin was transferred to a gravity column. After collecting the flow-through, the resin was washed sequentially with the following: 5 column volumes (CV) of Buffer A, 5 CV of Buffer A containing 20 mM imidazole, 5 CV of Buffer A containing 40 mM imidazole.

His₆-Nsp1 was eluted using 1 CV of Buffer A containing 300 mM imidazole. The eluate was immediately buffer-exchanged back into Buffer A to remove excess imidazole prior to tag removal.

Removal of His₆-Tag: To cleave the His₆-tag, TEV protease from New England Biolabs (Catalog #P8112S) was added according to the manufacturer's instructions. The cleavage mixture was incubated overnight at 4 °C.

Following cleavage, the mixture was incubated once again with Ni-NTA resin overnight at 4 °C. The next day, the flow-through containing the untagged Nsp1 was collected after loading the mixture onto the column.

Protein purity was assessed via SDS-PAGE. If necessary, further purification was performed using size exclusion chromatography. Final samples were concentrated using Amicon Ultra-15 centrifugal filter units (3 kDa molecular weight cutoff), flash-frozen in liquid nitrogen, and stored at -80 °C until use.

¹⁵N labelled Nsp1 Expression

The same expression and purification procedures described previously were followed, with the only modification being the use of M9 minimal media instead of 2XYT. Colonies from the BL21 (DE3) transformation plates were used to inoculate small-scale cultures. Specifically, 5 mL of M9 minimal media containing kanamycin were inoculated with a single colony and incubated at 37 °C with shaking at 180 rpm until an OD₆₀₀ of ~0.6 was reached (approximately 2 days).

The entire 5 mL culture was then transferred into a large-scale culture consisting of 500 mL of M9 minimal media with kanamycin. These cultures were incubated under the same conditions (37 °C, 180 rpm) until they reached an OD₆₀₀ of 0.6–0.8, which typically required approximately 4 days.

Once the desired optical density was reached, protein expression was induced by adding 1 M IPTG to a final concentration of 0.5 mM. The temperature was then reduced to 18 °C, and the culture was incubated with shaking (180 rpm) for 22 hours.

Cell harvesting and protein purification were carried out following the same protocol as used for the wild-type and mutant Nsp1 proteins expressed in 2XYT media.

Site Directed Mutagenesis

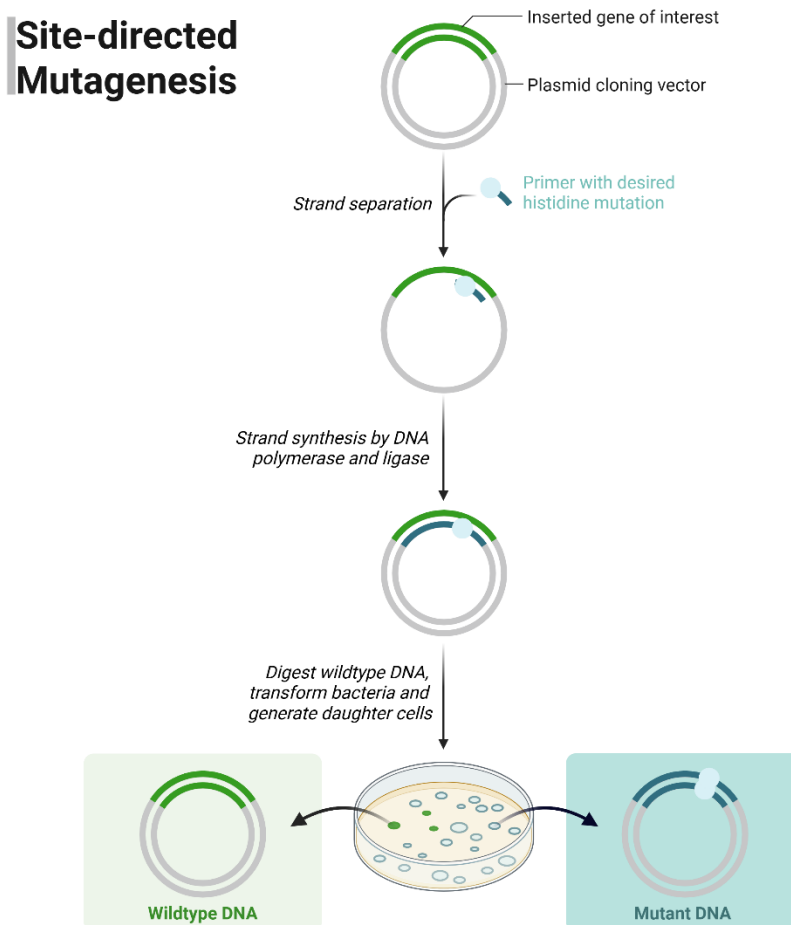


Figure 2.2 Visual representation of site-directed mutagenesis process. Figure created with BioRender.com.

Site-directed mutagenesis was performed to individually mutate each of the seven histidine residues in the Nsp1 protein to generate single-point mutants. Initially, each histidine was mutated to alanine. If the alanine substitution failed to yield soluble or properly expressed protein, alternative substitutions were attempted in the following order: glutamine, then asparagine.

Primers for each mutation were designed accordingly and ordered from Thermo Fisher Scientific. The sequences of the primers used for generating these mutants are listed below:

H165A Primers

Forward: TGGAACACTAAAGCGAGCAGTGGTGT

Reverse: AACACCACTGCTCGCTTTAGTGTTCCA

H134A Primers

Forward: GGAGCTGGTGGCGCGAGTTACGGCGCC

Reverse: GGCGCCGTAACTCGCGCCACCAGCTCC

H110A Primers

Forward: GTCCITGTCCCTGCGGTGGGCGAAATA

Reverse: TATTTGCCCCACCGCAGGGACAAGGAC

H83Q Primers

Forward: GCACCTCATGGTCAGGTTATGGTTGAG

Reverse: CTCAACCATAACCTGACCATGAGGTGC

H81N Primers

Forward: CGAACTGCACCTAACGGTCATGTTATG

Reverse: CATAACATGACCGTTAGGTGCAGTTTCG

H45A Primers

Forward: GAGGCACGTCAAGCGCTTAAAGATGGC

Reverse: GCCATCTTTAAGCGCTTGACGTGCCTC

H13Q Primer

Forward: AACGAGAAAACACAGGTCCAACTCAGT

Reverse: ACTGAGTTGGACCTGTGTTTCTCGTT

Mutagenesis was carried out using Thermo Scientific's Phusion Site-Directed Mutagenesis Kit (Catalog No. F541), following the manufacturer's recommended protocol. After transformation, colonies were selected, and plasmids were isolated using the Qiagen QIAprep Spin Miniprep Kit as previously described. The resulting plasmids were then sent to Laragen Inc. for sequencing to confirm the presence of the intended mutations.

***In vitro* translation assay**

Nsp1 is known for shutting down host translation by blocking the 40S ribosomal subunit.¹ To evaluate potential inhibitors, we monitored host translation using a cell-free *in vitro* translation system derived from HeLa cell lysates.

For these studies, the Thermo Scientific 1-Step Human Coupled IVT Kit – DNA (Catalog No. 88881) was modified. Samples were prepared according to the manufacturer's protocol for GFP expression, followed by the addition of test compounds.

Reagents were added to sterile PCR tubes in the following order using sterile pipette tips: HeLa Lysate (12.5 μ L), Accessory Proteins (2.5 μ L), Reaction Mix (5 μ L), pCFE-GFP DNA (0.5 μ g/ μ L, 2 μ L), the test compound or control, and nuclease-free water to a final volume of 25 μ L. Samples were gently spun down and then incubated at 30 °C with shaking at 180 rpm for 6 hours.

For Nsp1 inhibition studies, 1 μ M of purified Nsp1 was added to each reaction. Inhibitor concentrations varied depending on the compound being tested. Following incubation, each sample was diluted with 75 μ L of MOPS buffer (20 mM, pH 7.5), transferred to an NMR tube, and GFP fluorescence was measured.

Fluorescence measurements were performed using a fluorimeter with excitation at 488 nm and emission monitored at 512 nm. A long-pass filter was used to reduce background signal from the excitation source.

Synthetic Chemistry

Synthesis of Copper(II) Iminodiacetate

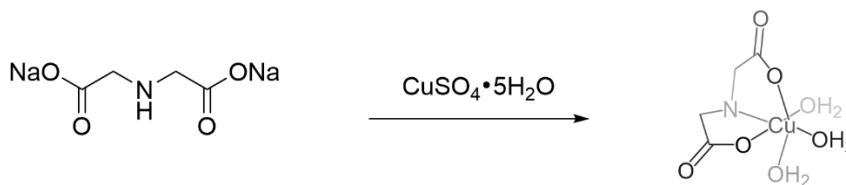


Figure 2.3 Synthetic scheme for Cu(II)IDA.

To make copper(II) iminodiacetate, equimolar amounts of cupric sulfate pentahydrate and sodium iminodiacetate dibasic monohydrate were dissolved in MilliQ water.

Synthesis of Copper(II)Histidinate

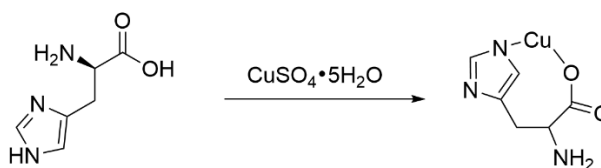


Figure 2.4 Synthetic Scheme for Copper(II) Histidinate.

To make copper(II) iminodiacetate, equimolar amounts of cupric sulfate pentahydrate and L-histidine were dissolved in MilliQ water.

Synthesis of Co(III)EDTA

The preparation was performed following the method described by Bürgisser and Stone². Co(II)EDTA was synthesized by mixing equimolar amounts of cobalt(II) chloride hexahydrate and sodium ethylenediaminetetraacetic acid (EDTA). Hydrogen peroxide was then added to the Co(II)EDTA solution, and the mixture was heated to 90 °C to facilitate oxidation. Upon cooling, a precipitate formed, which was collected and washed with methanol. The resulting deep purple crystals were purified through repeated water–methanol recrystallization and subsequently dried.

Synthesis of $[\text{NaCo(II)(NTA)(H}_2\text{O)}]_n$

Preparation was repeated according to Zhang et al.³ A mixture of nitrilotriacetic acid (0.9mmol), Cobalt(II) acetate tetrahydrate (0.5mmol) and water (20mL) was stirred for 30 min. Sodium hydroxide (0.5M) was then added to bring the pH up to 5.5. Three weeks later pink crystals were obtained.

Synthesis of $\alpha\text{-[KCo(III)(NTA)(OH)(H}_2\text{O)]}\cdot(\text{H}_2\text{O})$

Preparation was repeated according to Mori et al.⁴ 2g of nitrilotriacetic acid were dissolved in 12.5mL of 2.5M potassium bicarbonate. 2.5g of cobalt chloride hexahydrate and 500uL of 30% hydrogen peroxide were added and the mixture was stirred in an ice bath for 5 hours. The mixture was filtered and washed with cold water to reveal blue/purple crystals. Crystals were recrystallized in hot water containing potassium acetate followed by washing with ether.

Synthesis of $\text{Co(III)(acacen)(NH}_3)_2$

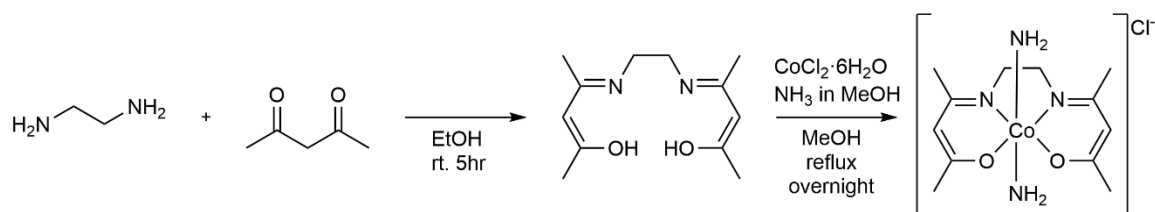


Figure 2.5 Synthetic scheme for acacen and $\text{Co(III)(acacen)(NH}_3)_2$.

Synthesis was based on the literature.⁵

Ligand Synthesis: Ethylenediamine (2.71 g, 45.1 mmol) was dissolved in ethanol (50 mL). A solution of freshly distilled acetylacetone (9.21 mL, 90.2 mmol) in ethanol was added drop-wise over 2 h with continuous stirring. The mixture was stirred for an additional 3 h, then filtered. The solid was washed successively with water (3×20 mL) and diethyl ether (3×20 mL) and dried under vacuum to give the acacen ligand.

^1H NMR (400 MHz, CDCl_3) δ 10.90 (s, 2H), 4.99 (s, 2H), 3.49 – 3.36 (m, 4H), 2.00 (s, 6H), 1.91 (s, 6H).

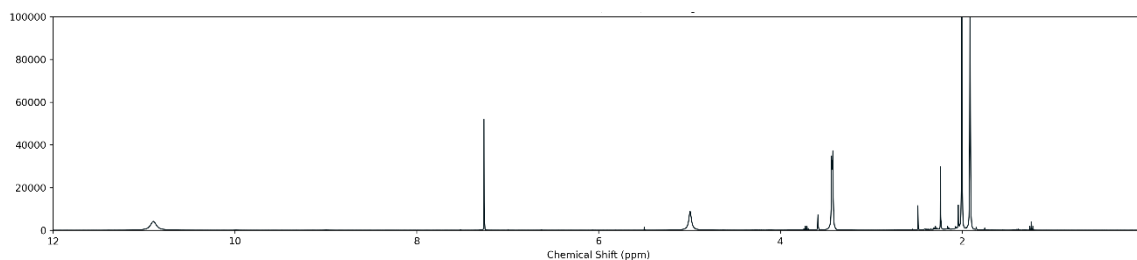


Figure 2.6 NMR spectrum of acacen ligand. Measured in CDCl_3 .

Metal Complexation: The isolated acacen ligand (500 mg, 2.22 mmol) was dissolved in methanol (50 mL). Cobalt(II) chloride hexahydrate (541 mg, 2.27 mmol) was added under a nitrogen atmosphere, and the solution was heated to 60 °C with stirring for 30 min. Saturated ammonia in methanol (0.25 mL) was then introduced, and the mixture was refluxed for 1 h. An additional 2.5 mL of saturated ammonia in methanol was added, after which oxygen was bubbled through the reaction mixture overnight. The resulting solid was collected by filtration, washed with cold methanol (3×10 mL) followed by diethyl ether (3×10 mL), and dried under vacuum to yield $\text{Co}(\text{acacen})(\text{NH}_3)_2 \cdot \text{Cl}$.

^1H NMR (400 MHz, D_2O) δ 5.15 (s, 2H), 3.61 (s, 4H), 2.25 (s, 6H), 2.09 (s, 6H).

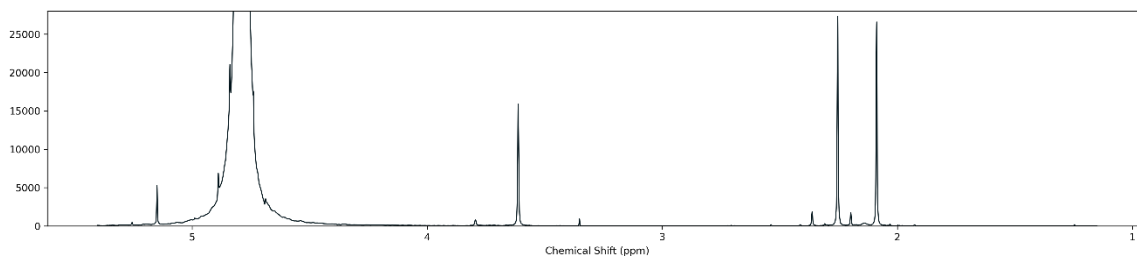


Figure 2.7 NMR spectrum of $\text{Co}(\text{acacen})(\text{NH}_3)_2$. Measured in D_2O .

Synthesis of Co(III)(acacen)(Imidazole)₂

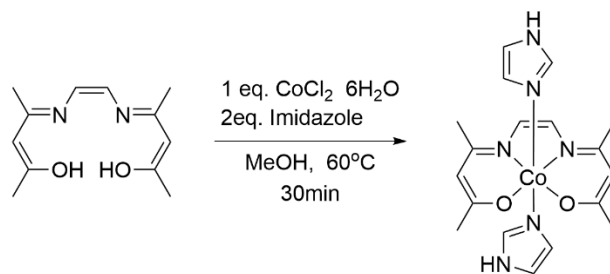


Figure 2.8 Synthetic scheme for Co(III)(acacen)(Im)₂.

The isolated acacen ligand (500 mg, 2.22 mmol) was dissolved in methanol (50 mL). Cobalt(II) chloride hexahydrate (541 mg, 2.27 mmol) was added under a nitrogen atmosphere, and the solution was heated to 60 °C with stirring for 30 min. 302 mg (4.44mmol) of imidazole dissolved in methanol were added and the reaction was stirred for three hours, after which oxygen was bubbled through the reaction mixture overnight. The resulting solid was collected by filtration, washed with cold methanol (3 × 10 mL) followed by diethyl ether (3 × 10 mL), and dried under vacuum to yield Co(acacen)(Im)₂·Cl.

¹H NMR (400 MHz, D₂O) δ 7.45 (d, *J* = 1.5 Hz, 3H), 7.02 (t, *J* = 1.6 Hz, 2H), 6.68 (t, *J* = 1.5 Hz, 2H), 3.43 (s, 4H), 2.25 (s, 6H), 2.06 (s, 6H).

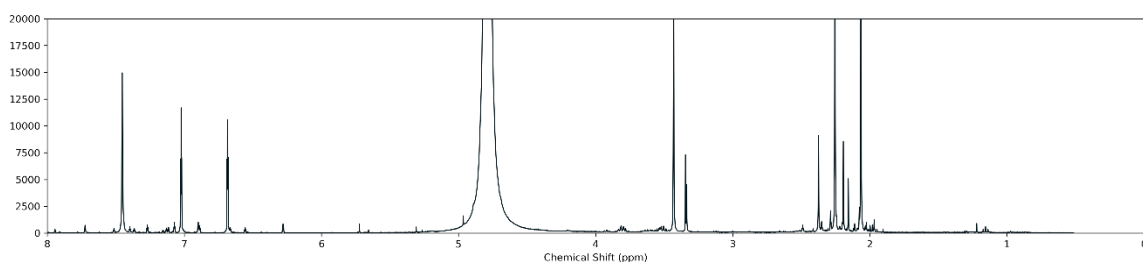


Figure 2.9 NMR spectrum of Co(III)(acacen)(Im)₂. Measured in D₂O.

Spectroscopic Techniques

UV-Vis

Absorption spectra were collected on an Agilent 8453 UV-Vis spectrophotometer.

Luminescence Spectroscopy

Fluorescence spectra were recorded on a modified Jobin Yvon Fluorolof-3 using two Ocean Optics QEPro CCD spectrometers as detectors spanning the 300-900nm range. The excitation wavelength used for tryptophan emission spectra was 280nm. The excitation wavelength used for GFP emission when conducting the in vitro assays was 488nm.

Circular Dichroism

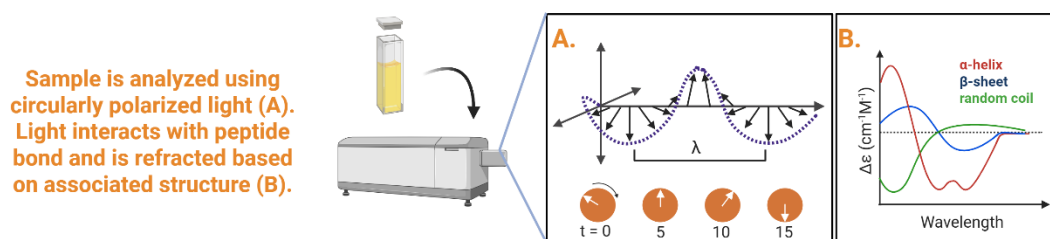


Figure 2.11 Circular dichroism description and schematic. Figure created with Biorender.com.

Circular dichroism spectra were recorded on an Aviv Model 430 spectropolarimeter. Spectra were collected from 190-250nm.

Time Resolved Fluorescence Energy Transfer

For the tryptophan fluorescence decay kinetics the excitation source was the fourth harmonic (266nm, 10ps, 10Hz) of a regeneratively amplified passively mode-locked Nd:YAG laser. Tryptophan fluorescence was collected using reflective optics, filtered through a monochromator, and detected with a picosecond streak camera (Hamamatsu C5680)

Nuclear Magnetic Resonance

NMR measurements were performed at room temperature, obtained using a Varian 400 MHz and the Bruker 400MHz spectrometer. ^1H chemical shifts were reported in ppm relative to tetramethylsilane, using residual proton and ^{13}C resonances from solvent as an internal standard.

For ^{59}Co NMR studies, a Bruker Neo 400 with a broadband iProbe was used. It operates at 95.79 MHz for ^{59}Co . The standard parameters called for 16,000 scans with a 10 microsecond observe pulse, a 20 millisecond acquisition time, a 100 millisecond relaxation delay, and a 100,000 Hz (1,044 ppm) sweep width.

Electron Paramagnetic Resonance

X-band CW-EPR spectra were collected using a Bruker EMX spectrometer at 77 K with a quartz liquid nitrogen immersion dewar. Samples were prepared as frozen glasses in 30% glycerol. Pulse ENDOR and HYSCORE spectroscopy were conducted on a Bruker ELEXSYS E580 pulse EPR spectrometer, equipped with either a Bruker MD4 (X-band) or D2 (Q-band) resonator. Temperature control was maintained via a ColdEdge ER 4118HV-CF5-L Flexline Cryogen-Free VT cryostat, coupled with an Oxford Instruments Mercury ITC temperature controller. CW-EPR spectra were simulated using EasySpin's least-squares fitting algorithm (esfit) with the solid-state simulation function (pepper). HYSCORE spectra were simulated using the solid-state pulse EPR module (saffron).

Computational Techniques

Computational Modelling in Copper Studies

For the computational methods used for the copper studies Moon Young Yang and William A. Goddard have provided the following description.

All QM computations were performed using Orca 5.⁶ Geometry optimizations were carried out with the B3LYP functional.⁷⁻⁹ The conductor-like polarizable continuum (CPCM) solvation model was used to account for solvent effects of water.¹⁰ Scalar relativistic effects were treated

using the zeroth-order regular approximation (ZORA).^{11,12} The ZORA-recontracted¹³ version of the def2-TZVPP basis set¹⁴ was used for Cu and the ZORA-def2-TZVP for all other atoms, along with fully decontracted def2/J auxiliary basis sets.¹⁵ For the calculation of hyperfine coupling and g-tensors, the B2GP-PLYP double-hybrid functional was used¹⁶, which is known to accurately predict the EPR properties for Cu(II) complexes.¹⁷ NoFrozenCore option and tight convergence criteria were used for the calculations with double-hybrid functionals. VMD¹⁸ and Avogadro¹⁹ programs were used for visualization, and EPR simulations were performed using the EasySpin software package.

Computational Modelling in Cobalt Studies

For the computational methods used for the cobalt studies Moon Young Yang has provided the following description.

Computational Co-59 NMR chemical shifts ($\delta^{59}\text{Co}$) of Co(III) complexes were computed using the Gaussian16 program.²⁰ The chemical shift was defined as:

$$\delta^{59}\text{Co} = \sigma_{\text{ref}} - \sigma_{\text{calc}}$$

where σ_{ref} is the calculated shielding constant of the reference complex, $[\text{Co}(\text{CN})_6]^{3-}$, and σ_{calc} is the shielding constant of the Co(III) complexes investigated in this study.

Geometry optimizations were performed at the BLYP/def2-SVP level of theory under the IEF-PCM implicit solvent model. The Co-59 shielding constants were then calculated using the Gauge-Independent Atomic Orbital (GIAO) approach at the GIAO-LC- ω PBE/NMR-DKH level of theory under the IEF-PCM implicit solvent model. This computational protocol has been previously validated for a benchmark set of 34 Co(III) complexes.²¹

For the *in silico* virtual screening of Nsp1 inhibitors, Joo Youn Lee provided the following descriptions.

Structure-based Virtual Screening

Structure-based virtual screening (SBVS) was performed to identify small-molecule inhibitors against Nsp1. Molecular docking was conducted for three binding sites around Nsp1 using Schrödinger Suite 2023-2 (Schrödinger LLC, New York, USA, 2023). The cryo-electron microscopy structure of Nsp1 bound to the human 40S ribosomal subunit (PDB ID 6ZLW) was obtained from the Protein Data Bank (<https://www.rcsb.org>) and prepared using Protein Preparation Wizard in Maestro v13.6. A receptor grid box of 25 Å x 25 Å x 25 Å was generated centered on Lys164 and His165, two residues located in C-terminal domain of Nsp1 that are involved in the interaction with the 40S proteasome. A chemical library comprising approximately 6.2 million compounds from ChemBridge, ChemDiv, and Enamine suppliers was employed for virtual screening. All compounds were prepared using LigPrep with OPLS_2005 force field to generate low-energy 3D conformations and appropriate ionization states. Molecular docking was performed using Glide v9.9 with standard precision (SP) mode. Based on the docking score and visual inspection of the binding modes, 340 compounds were selected for further *in silico* analysis.

Molecular Dynamics (MD) simulations

To further refine the docking results and predict the binding free energy for 340 compounds in complex with Nsp1 structure, molecular dynamics (MD) simulations were performed using Desmond v7.4 with OPLS_2005 force field in Schrödinger Suite 2023-2 (Desmond Molecular Dynamics System; D. E. Shaw Research: New York, NY, 2024). The System Builder was used for solvation, employing predefined TIP3P water molecules in an orthorhombic box with dimensions of 10 Å x 10 Å x 10 Å. The overall system was neutralized by adding Cl⁻ counterions placed at least 25 Å from the ligand. The NaCl concentration was adjusted to 0.15 mol/L. Short MD simulations of 20 ns were conducted under periodic boundary conditions in the NPT ensemble at normal temperature (300 K) and pressure (1.01325 bar). Energy calculations and trajectory data were recorded at intervals of 1.2 and 500 ps, respectively.

Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) calculation

The final equilibrium states for 340 protein-ligand docking poses were rescored using the MM-GBSA approach, as implemented in the Prime MM-GBSA module in the Schrödinger Suite 2023-2 (Schrödinger LLC, New York, USA, 2023). The OPLS_2005 force field, VSGB solvation model, and default Prime parameters were used for the MM-GBSA calculations. Ligand binding free energies were calculated from 20 frames extracted from the final 20 ns of each MD trajectory. The top 31 compounds were selected based on the predicted binding free energies and visual inspection of binding poses.

AlphaFold3

AlphaFold predictions were performed using the AlphaFold3 server by inputting the amino acid sequence of the peptide or protein, followed by the inclusion of relevant metal ions.²²

Data Analysis

Data analysis was performed using MATLAB (MathWorks Inc.) or Python within JupyterLab, unless otherwise specified. NMR data were analyzed using MestReNova. Chemical structures were drawn using ChemDraw. Protein visualizations presented in this thesis were created using a combination of PyMOL, Discovery Studio Visualizer, and BioRender.com. All citations were managed and generated using Zotero.

References

- (1) Schubert, K.; Karousis, E. D.; Jomaa, A.; Scaiola, A.; Echeverria, B.; Gurzeler, L.-A.; Leibundgut, M.; Thiel, V.; Mühlemann, O.; Ban, N. SARS-CoV-2 Nsp1 Binds the Ribosomal mRNA Channel to Inhibit Translation. *Nat Struct Mol Biol* **2020**, 27 (10), 959–966. <https://doi.org/10.1038/s41594-020-0511-8>.
- (2) Bürgisser, C. S.; Stone, A. T. Determination of EDTA, NTA, and Other Amino Carboxylic Acids and Their Co(II) and Co(III) Complexes by Capillary Electrophoresis. *Environ Sci Technol* **1997**, 31 (9), 2656–2664. <https://doi.org/10.1021/es970080f>.

- (3) Zhang, Q.-Z.; Lu, C.-Z.; Yang, W.-B. Synthesis and Structures of Two Cobalt Complexes $[\text{NaCo}^{\text{II}}(\text{NTA})(\text{H}_2\text{O})]_n$ and $\text{NH}_4[\text{Co}^{\text{III}}(\text{IDA})_2] \cdot 2\text{H}_2\text{O}$. *J Coord Chem* **2006**, *59* (8), 837–844. <https://doi.org/10.1080/00958970500412081>.
- (4) Mori, M.; Shibata, M.; Kyuno, E.; Okubo, Y. Studies on the Synthesis of Metal Complexes. IV. The Cobalt (III) Complexes of Ammoniatriacetic Acid. *Bull Chem Soc Jpn* **1958**, *31* (8), 940–944. <https://doi.org/10.1246/bcsj.31.940>.
- (5) Takeuchi, T.; Böttcher, A.; Quezada, C. M.; Simon, M. I.; Meade, T. J.; Gray, H. B. Selective Inhibition of Human α -Thrombin by Cobalt(III) Schiff Base Complexes. *J Am Chem Soc* **1998**, *120* (33), 8555–8556. <https://doi.org/10.1021/ja981191x>.
- (6) Neese, F.; Wennmohs, F.; Becker, U.; Riplinger, C. The ORCA Quantum Chemistry Program Package. *J Chem Phys* **2020**, *152* (22), 224108. <https://doi.org/10.1063/5.0004608>.
- (7) Becke, A. D. Density-Functional Exchange-Energy Approximation with Correct Asymptotic Behavior. *Phys Rev A* **1988**, *38* (6), 3098–3100. <https://doi.org/10.1103/PhysRevA.38.3098>.
- (8) Lee, C.; Yang, W.; Parr, R. G. Development of the Colle-Salvetti Correlation-Energy Formula into a Functional of the Electron Density. *Phys Rev B* **1988**, *37* (2), 785–789. <https://doi.org/10.1103/PhysRevB.37.785>.
- (9) Becke, A. D. A New Mixing of Hartree–Fock and Local Density-Functional Theories. *J Chem Phys* **1993**, *98* (2), 1372–1377. <https://doi.org/10.1063/1.464304>.
- (10) Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. Universal Solvation Model Based on Solute Electron Density and on a Continuum Model of the Solvent Defined by the Bulk Dielectric Constant and Atomic Surface Tensions. *J Phys Chem B* **2009**, *113* (18), 6378–6396. <https://doi.org/10.1021/jp810292n>.
- (11) Van Lenthe, E.; Baerends, E. J.; Snijders, J. G. Relativistic Total Energy Using Regular Approximations. *The Journal of Chemical Physics* **1994**, *101* (11), 9783–9792. <https://doi.org/10.1063/1.467943>.
- (12) Lenthe, E. V.; Baerends, E. J.; Snijders, J. G. Relativistic Regular Two-Component Hamiltonians. *J Chem Phys* **1993**, *99* (6), 4597–4610. <https://doi.org/10.1063/1.466059>.

- (13) Pantazis, D. A.; Chen, X.-Y.; Landis, C. R.; Neese, F. All-Electron Scalar Relativistic Basis Sets for Third-Row Transition Metal Atoms. *J Chem Theory Comput* **2008**, *4* (6), 908–919. <https://doi.org/10.1021/ct800047t>.
- (14) Weigend, F.; Ahlrichs, R. Balanced Basis Sets of Split Valence, Triple Zeta Valence and Quadruple Zeta Valence Quality for H to Rn: Design and Assessment of Accuracy. *Phys Chem Chem Phys* **2005**, *7* (18), 3297–3305. <https://doi.org/10.1039/B508541A>.
- (15) Weigend, F. Accurate Coulomb-Fitting Basis Sets for H to Rn. *Phys Chem Chem Phys* **2006**, *8* (9), 1057–1065. <https://doi.org/10.1039/B515623H>.
- (16) Karton, A.; Tarnopolsky, A.; Lamère, J.-F.; Schatz, G. C.; Martin, J. M. L. Highly Accurate First-Principles Benchmark Data Sets for the Parametrization and Validation of Density Functional and Other Approximate Methods. Derivation of a Robust, Generally Applicable, Double-Hybrid Functional for Thermochemistry and Thermochemical Kinetics. *J Phys Chem A* **2008**, *112* (50), 12868–12886. <https://doi.org/10.1021/jp801805p>.
- (17) Drosou, M.; Mitsopoulou, C. A.; Orio, M.; Pantazis, D. A. EPR Spectroscopy of Cu(II) Complexes: Prediction of g-Tensors Using Double-Hybrid Density Functional Theory. *Magnetochemistry* **2022**, *8* (4), 36. <https://doi.org/10.3390/magnetochemistry8040036>.
- (18) Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual Molecular Dynamics. *J Mol Graph* **1996**, *14* (1), 33–38. [https://doi.org/10.1016/0263-7855\(96\)00018-5](https://doi.org/10.1016/0263-7855(96)00018-5).
- (19) Hanwell, M. D.; Curtis, D. E.; Lonie, D. C.; Vandermeersch, T.; Zurek, E.; Hutchison, G. R. Avogadro: An Advanced Semantic Chemical Editor, Visualization, and Analysis Platform. *J Cheminform* **2012**, *4* (1), 17. <https://doi.org/10.1186/1758-2946-4-17>.
- (20) Frisch, M. J.; Trucks, G. W.; H. B. Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; B. G. Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams-Young, D.; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery, Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.;

Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; and Fox, D. J.. Gaussian16, 2016.

(21) Gomes, M. G. R.; De Souza, A. L. F.; Dos Santos, H. F.; De Almeida, W. B.; Paschoal, D. F. S. Assessment of a Computational Protocol for Predicting Co-59 NMR Chemical Shift. *Magnetochemistry* **2023**, *9* (7), 172. <https://doi.org/10.3390/magnetochemistry9070172>.

(22) Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A. J.; Bambrick, J.; Bodenstein, S. W.; Evans, D. A.; Hung, C.-C.; O'Neill, M.; Reiman, D.; Tunyasuvunakool, K.; Wu, Z.; Žemgulytė, A.; Arvaniti, E.; Beattie, C.; Bertolli, O.; Bridgland, A.; Cherepanov, A.; Congreve, M.; Cowen-Rivers, A. I.; Cowie, A.; Figurnov, M.; Fuchs, F. B.; Gladman, H.; Jain, R.; Khan, Y. A.; Low, C. M. R.; Perlin, K.; Potapenko, A.; Savy, P.; Singh, S.; Stecula, A.; Thillaisundaram, A.; Tong, C.; Yakneen, S.; Zhong, E. D.; Zielinski, M.; Židek, A.; Bapst, V.; Kohli, P.; Jaderberg, M.; Hassabis, D.; Jumper, J. M. Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3. *Nature* **2024**, *630* (8016), 493–500. <https://doi.org/10.1038/s41586-024-07487-w>.

*Chapter 3***COPPER BINDING TO NSP1 PEPTIDES**

Introduction

Nsp1 is a potent SARS-CoV-2 virulence factor and a promising antiviral drug target. However, its C-terminal polypeptide lacks a well-defined folded structure, making conventional structure-based drug discovery approaches ineffective. Many viral proteins contain intrinsically disordered protein regions (IDPRs), which contribute to their adaptability and survival in dynamic host environments.¹ These flexible regions often undergo disorder-to-order transitions upon binding to host factors, but their lack of stable pockets makes them difficult to inhibit with traditional small-molecule therapeutics.

Given these challenges, alternative approaches such as metal-based inhibition are promising routes for drug discovery. Transition metals can interact with disordered protein regions via coordination to specific amino acid side chains, potentially stabilizing transient conformations or disrupting native interactions. The C-terminal region of Nsp1 (residues 148–180, ELGTDPYEDFQENWNTKHSSGVTRELMRELNGG) contains several potential metal-binding residues, including histidine, aspartate, and glutamate. In this chapter, we explore how Cu(II) interacts with Nsp1-derived peptides using circular dichroism spectroscopy, fluorescence measurements, electron paramagnetic resonance (EPR) techniques, and computational modelling to assess structural changes upon metal binding. These studies provide insight into the coordination environment of Cu(II) and its potential impact on Nsp1 function.

Copper in Biology

Life has evolved through the interactions of both organic and inorganic compounds. Organic components such as amino acids, nucleotides, and carbohydrates form the backbone of biological macromolecules. In contrast, inorganic compounds, particularly those involving metal ions, play essential roles in enzymatic catalysis, structural stabilization, and electron transport. Examples include heme groups, vitamin B12, and metalloenzymes such as lytic polysaccharide monooxygenases.

Copper is a d-block, first-row transition metal with two biologically relevant oxidation states: its reduced state, Cu(I), or its oxidized state, Cu(II).² Cu(I), a soft metal center, exhibits high affinity for thiol and thioether groups, commonly coordinating with cysteine and methionine residues. Cu(II), on the other hand, is more inclined to bind oxygen donors, imidazole rings of histidines,

and amine nitrogen atoms.³ According to the Irving-Williams series, Cu(II) forms more stable complexes than most other biologically relevant divalent metal ions, with the general trend being $\text{Mn} < \text{Fe} < \text{Co} < \text{Ni} < \text{Cu} > \text{Zn}$.⁴

Organisms have evolved tightly regulated copper homeostasis systems to create a balance and avoid cytotoxicity. These mechanisms involve a network of transporters, chaperones, and storage proteins that maintain copper at optimal concentrations. In humans, copper uptake occurs primarily through the intestine via CTR1 (Copper Transporter 1), after which intracellular chaperones like ATOX1, CCS, and COX17 direct the metal to its target destinations, including cuproenzymes in the mitochondria, cytosol, and Golgi apparatus. Excess copper is sequestered or exported by ATP7A and ATP7B, P-type ATPases critical for preventing toxic accumulation.⁵ Disruptions in these pathways can lead to copper-related disorders: Wilson's disease results from mutations in ATP7B, leading to hepatic copper buildup and neurological symptoms, while Menkes disease arises from ATP7A mutations and manifests as copper deficiency in the brain and connective tissues.^{6,7} Copper's toxicity is a result of its redox activity and ability to catalyze the formation of reactive oxygen species (ROS) which damages DNA, lipids, and proteins.^{8,9} This delicate balance between copper's utility and toxicity highlights the importance of tightly controlled homeostasis in both health and disease.

Copper's high affinity for biological ligands makes it both an essential micronutrient and a potential cytotoxin. At physiological levels, copper is used by the immune system to enhance the phagocytic and bactericidal activity of neutrophils and macrophages.¹⁰ However, excess copper can disrupt cellular function, outcompete other metal ions for binding sites, and catalyze the formation of reactive oxygen species. Acute copper toxicity can result in nausea, vomiting, hematemesis, hypotension, jaundice, and gastrointestinal distress. Chronic exposure can cause liver and kidney damage.¹¹ Disorders such as Wilson's disease, a rare autosomal recessive condition, impair copper excretion and result in toxic accumulation, particularly in the liver.⁶ Conversely, copper deficiency can cause anemia, neutropenia, and neurological symptoms, as seen in Menkes disease, a disorder of impaired copper transport.⁷

Historically, copper has long been recognized for its antimicrobial properties. Ancient Egyptians used copper vessels to purify drinking water and treat wounds as early as 2600 BCE. Greek physicians employed copper compounds for sanitation and the treatment of infections.¹² In

19th-century Europe, workers in the copper industry were noted to have lower incidence of cholera, hinting at copper's protective effects.¹³ Although antibiotics became the standard of care in the 20th century, copper remains relevant for infection control, particularly in the face of rising antibiotic resistance.

Today, copper continues to be used in various biomedical applications. It has been shown to possess bactericidal, fungicidal, and antiviral properties. Copper surfaces drastically reduce the survival time of viruses such as Influenza A and SARS-CoV-2 compared to stainless steel or plastic.^{14,15} Copper complexes of non-steroidal anti-inflammatory drugs (NSAIDs) have demonstrated enhanced anti-inflammatory and anti-ulcer effects.¹⁶ Copper-based chemotherapeutics and contraceptive devices such as copper intrauterine devices (IUDs) are in clinical use and used by many around us, showcasing copper's therapeutic versatility.¹⁷

Given its versatile coordination chemistry, redox activity, and deep evolutionary significance, copper occupies a unique niche in biological systems, acting as both a vital micronutrient and a potent cytotoxin. The fine balance between its beneficial and harmful effects is tightly regulated by sophisticated homeostatic mechanisms across all domains of life. Disruptions in this balance are linked to numerous human diseases and can also be strategically exploited for therapeutic purposes. The following chapter explores the complex roles of copper in biology, with a particular focus on its interactions with SARS-CoV-2 Nsp1. Through spectroscopic and computational approaches, these studies investigate how copper's unique chemical properties may be leveraged to disrupt viral function and inform future antiviral strategies.

Results and Discussion

Insights on Intrinsically Disordered Region of Nsp1

The C-terminal of Nsp1 is intrinsically disordered and fluctuates in shape. Using Alphafold3, predictions indicate that it is expected to be mostly helical (Figure 3.1). When the C-terminal is bound to the 40S ribosome this is the case. It adopts a helix-turn-helix shape in the process of blocking the mRNA entry channel. However, prediction and experimentation can vary, therefore obtaining insights into this region of Nsp1 are important for future targetability and drug design when responding to SARS-CoV-2.



Figure 3.1 AlphaFold3 prediction of the C-terminal of Nsp1. The blue regions indicate a region of high confidence in the prediction and yellow signifying weaker confidence.

Circular dichroism (CD) spectroscopy provides valuable insights into protein secondary structure, as distinct structural elements such as α -helices and β -sheets exhibit characteristic spectral features in the far-UV region. The far-UV CD spectrum of the Nsp1 C-terminal domain (Nsp1-CT₃₃) displays a pronounced negative ellipticity minimum at 200 nm and a shoulder near 230 nm (Figure 3.2). The molar ellipticity minimum at 200 nm is indicative of a significant random coil content ($\sim 40\%$), with a likely positive contribution from β -sheet or turn structures offsetting the signal. Spectral deconvolution into three components— α -helix, β -sheet, and random coil—reveals that the peptide predominantly adopts β -sheet conformations, with minimal α -helical content ($<5\%$) (Figure 3.2).

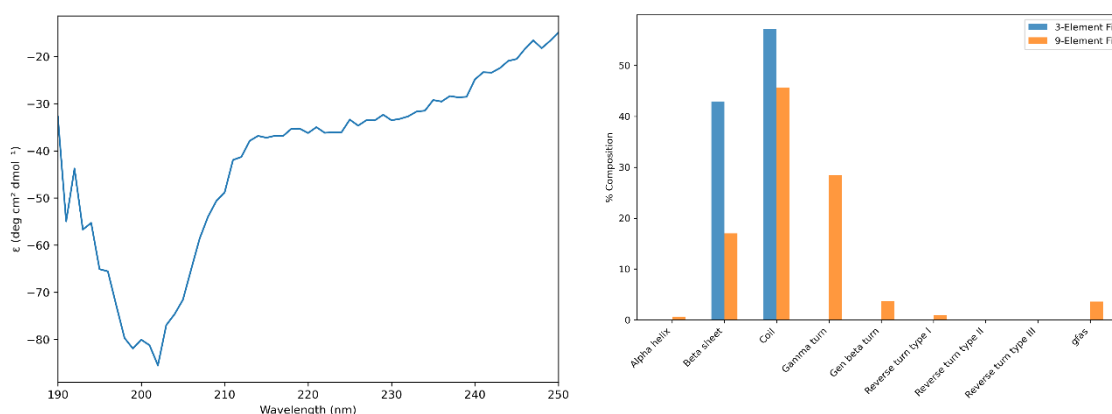


Figure 3.2 The circular dichroism spectrum of Nsp1-CT₃₃ can be seen on the left and on the right is the are the elemental fit analysis results that break down the spectrum into its different secondary structures.

The addition of metal ion salts at varying equivalents induces structural changes in the peptide, as evidenced by shifts in the circular dichroism (CD) spectrum. Notably, Cu(II) causes a

significant spectral shift (Figure 3.3), suggesting that its binding promotes the formation of a more defined secondary structure.

Fluorescence spectra were also collected for different metal ions across a range of concentrations, monitoring tryptophan fluorescence. Cu(II) binding results in significant fluorescence quenching, indicating direct interaction with the peptide's fluorophores. In contrast, other metal ions cause minimal quenching or even slight fluorescence enhancement, suggesting weaker or nonspecific interactions. The pronounced spectral changes observed in both CD and fluorescence measurements upon Cu(II) binding suggest a strong coordination with the peptide, warranting further investigation.

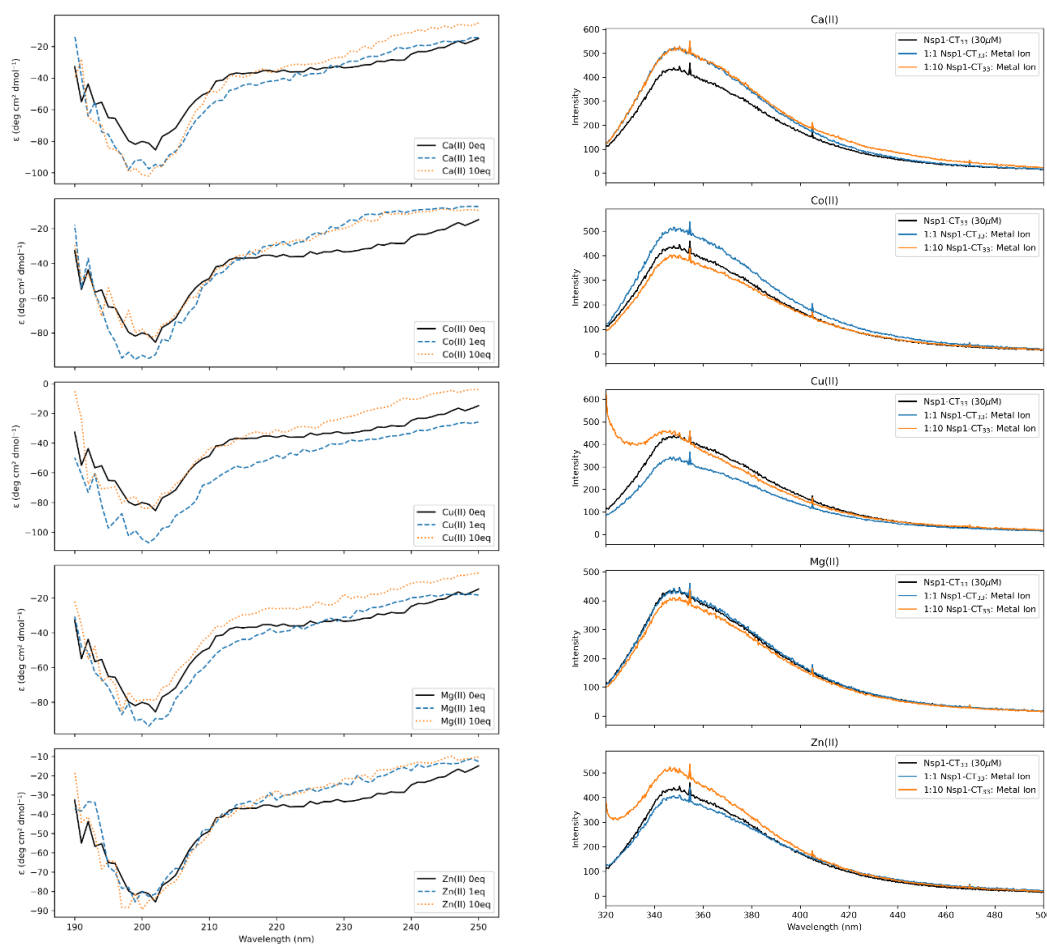


Figure 3.3 On the left the circular dichroism spectra for Nsp1-CT₃₃ with different metal ions can be found and on the left is the fluorescence spectra. For the circular dichroism spectra, 0eq refers to no metal ion presence only Nsp1-CT₃₃.

Due to the intrinsically disordered nature of Nsp1, studying its structure under different conditions can provide insights into its conformational preferences and potential functional roles. Trifluoroethanol (TFE) is known to stabilize α -helical conformations, mimicking conditions that may arise upon binding to other biomolecules. This can help determine whether the peptide has an inherent helical propensity that could be relevant in biological contexts. TFE is a slightly stronger proton donor than water but a much weaker proton acceptor. It primarily disrupts non-local hydrophobic interactions while slightly enhancing α -helical properties.¹⁸

Since intrinsically disordered regions (IDRs) often undergo disorder-to-order transitions upon interacting with ligands or binding partners, TFE-induced α -helicity could indicate whether metal binding stabilizes a more structured state. Figure 3.4 shows the effects of adding 30% TFE to the peptide, as well as the impact of Cu(II) binding.

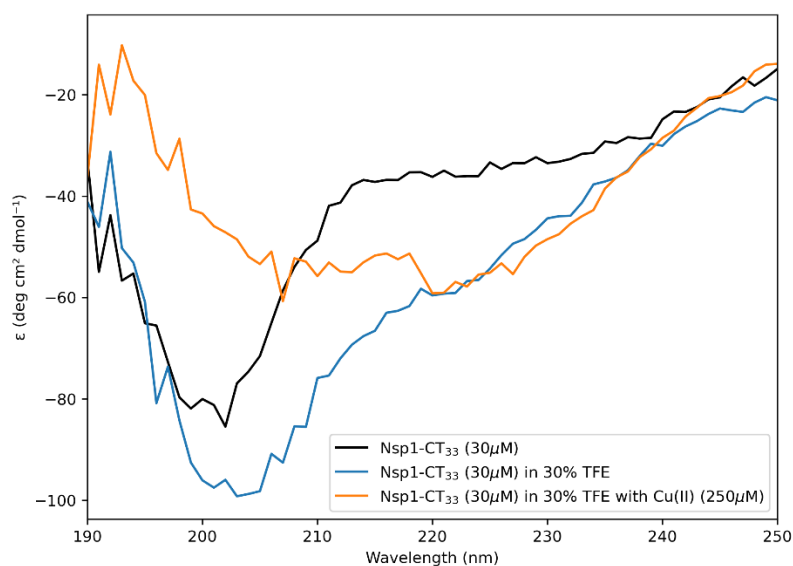


Figure 3.4 The circular dichroism spectra of Nsp1-CT₃₃ in the presence of trifluoroacetic acid (TFE) and the effects of adding copper.

The CD spectrum of Nsp1-CT₃₃ shows a strong negative peak near 200 nm, characteristic of a predominantly random coil conformation. The absence of a peak at 222 nm confirms the lack of α -helical structure. Upon addition of TFE, the negative peak at 200 nm decreases, while new negative peaks emerge at 208 nm and 222 nm, indicating a transition toward a more ordered state with partial α -helical formation. TFE, a helix-stabilizing solvent that mimics hydrophobic

environments, promotes this structural shift by stabilizing transient helical segments within the disordered protein. Finally, the addition of Cu(II) further enhances helicity, as seen by a deeper 222 nm peak. This suggests that Cu(II) stabilizes α -helical conformations, and the observed peak broadening or shift may indicate the formation of tertiary contacts induced by metal coordination.

All of this work led to further studies on the impact of Cu(II) binding on Nsp1-CT₃₃. While CD spectroscopy demonstrated that Cu(II) promotes helicity and may induce tertiary contacts, it does not provide direct information about specific binding interactions. To gain further insight into these interactions, fluorescence spectroscopy was employed to assess changes in the local environment of aromatic residues. Monitoring tryptophan fluorescence allows for the detection of binding-induced quenching, which can indicate direct coordination or conformational rearrangements upon Cu(II) addition.

Tryptophan fluorescence serves as a sensitive indicator of the polarity of the surrounding environment, offering insight into structural changes upon ligand binding.¹⁹ In Nsp1-CT₃₃, W161 exhibits fluorescence with a maximum at 350 nm, consistent with a solvent-exposed indole group. The addition of increasing concentrations of Cu(II) results in fluorescence quenching (Figure 3.3), suggesting direct interaction between Cu(II) and the tryptophan residue.

The fluorescence decay kinetics of the indole chromophore require an exponential function for accurate modeling, reflecting the complex dynamic environment of the residue (Equation 3.1).

$$\langle \tau \rangle = \int_0^{\infty} \frac{I(t) - I(\infty)}{I(0) - I(\infty)} dt \quad (\text{Equation 3.1})$$

The effective fluorescence decay time of W161 in Nsp1-CT₃₃ is approximately 2.4 ns, which is shorter than the 2.9 ns decay time observed for W4 in α -synuclein.²⁰ This suggests the presence of an additional quenching pathway for the singlet excited state of the indole chromophore in Nsp1-CT₃₃. To further investigate the structural dynamics of the peptide, fluorescence resonance energy transfer (FRET) was utilized using tryptophan and 3-nitrotyrosine as a donor-acceptor pair. This pair has a Förster distance of 26 Å, making it suitable for modeling the distance between W161 and Y154 (Figure 3.5).²¹

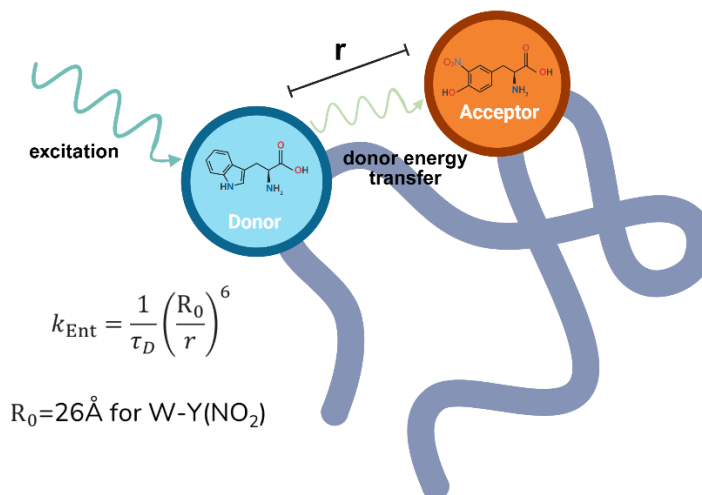


Figure 3.5 Visual representation of using time-resolved energy transfer for measuring distances between donor acceptor pairs.

In the cryo-EM structure of Nsp1 bound to the 40S ribosomal subunit, the centroid-to-centroid distance between W161 and Y154 is 15.4 Å.²² A β -sheet model of Nsp1-CT₃₃ predicts a distance of 29.7 Å. Applying the simplest Förster energy transfer model to Nsp1-CT₃₃-Y(NO₂)154 yields a W161-Y154 distance of approximately 24 Å. However, given that Nsp1-CT₃₃ is an intrinsically disordered polypeptide, a single static distance is unlikely to accurately capture the conformational ensemble. Instead, a distribution of distances provides a more appropriate model for W161–Y(NO₂)154 interactions and the fluorescence decay of *W161.

In order to analyze the data, a minimum entropy model was employed to extract distances between the Förster energy transfer pair. The tryptophan luminescence decay kinetics were modeled to the function in Equation 2.2. Where $I_o(t)$ is the tryptophan fluorescence decay in the absence of the Tyr(NO₂). $P(r)$ is the probability of observing a conformation with donor-acceptor distance r , and $k_{\text{et}}(r)$ is the rate of the energy transfer at distance r .

$$I(t) = I_o(t) \int P(r) \exp(-k_{\text{et}}(r)t) dr \quad (\text{Equation 3.2})$$

Distances were determined directly from the decay kinetics through numerical inversion of the Laplace transform describing $I(t)$, with the key constraint that $P(r)$ must remain nonnegative. To achieve this, a linear least-squares algorithm with a nonnegativity constraint was employed, yielding the narrowest $P(k)$ distributions from fluorescence kinetics. These $P(k)$ distributions

were then converted into distance distributions using the Förster equation $r = D_0(\kappa_R/\kappa_{et})^{1/6}$, where $D_0 = R_0\Phi_D^{1/6}$ and κ_R represents the measured radiative decay rate constant of excited N-Acetyl-L-Tryptophanamide (NATA). The method allows for distance measurements up to an experimental limit of approximately 40 Å.

By extracting minimum entropy, model-independent distributions of W161–Y(NO₂)154 distances from energy-transfer kinetics (Figure 3.6), three distinct populations were identified: ~13 Å (29%), ~17 Å (46%), and ~28 Å (25%). The presence of a significant fraction of extended structures between W161 and Y(NO₂)154 aligns with CD spectra, which indicate β -sheet formation within the peptide.

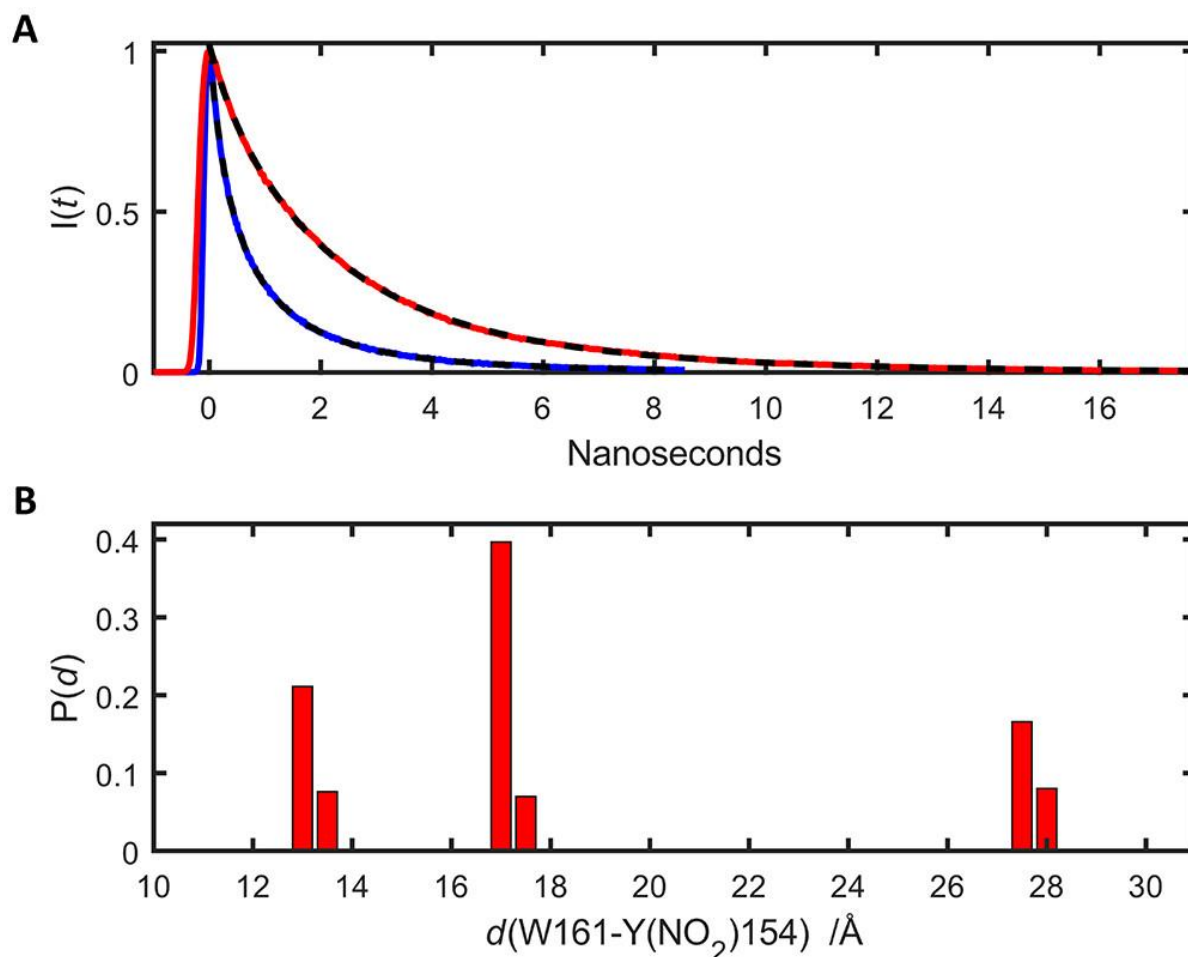


Figure 3.6 Time-Resolved Fluorescence and Distance Distributions Reveal W161–Y(NO₂)154 Energy Transfer Dynamics in Nsp1-CT₃₃ (A) Nsp1-CT₃₃ (red) and Nsp1-CT₃₃-Y(NO₂)154 (blue) W161 fluorescence decay kinetics ([Nsp1-CT₃₃] = 30 μ M, pH 6.5, MOPS 20 mM). Dashed black lines are fits to multiexponential decay functions with lifetime values of 2.4 and 0.9 ns. (B) Probability distribution of W161–Y(NO₂)154 distances extracted from W161 energy transfer kinetics.

Copper(II) Binding to Nsp1-CT

At pH 6.5, Nsp1-CT₃₃ contains multiple residues capable of coordinating metal ions, including eight carboxylate groups and one imidazole. W161 in Nsp1-CT₃₃ is particularly sensitive to low concentrations of Cu(II), as evidenced by the fluorescence quenching observed in Figure 3.7.

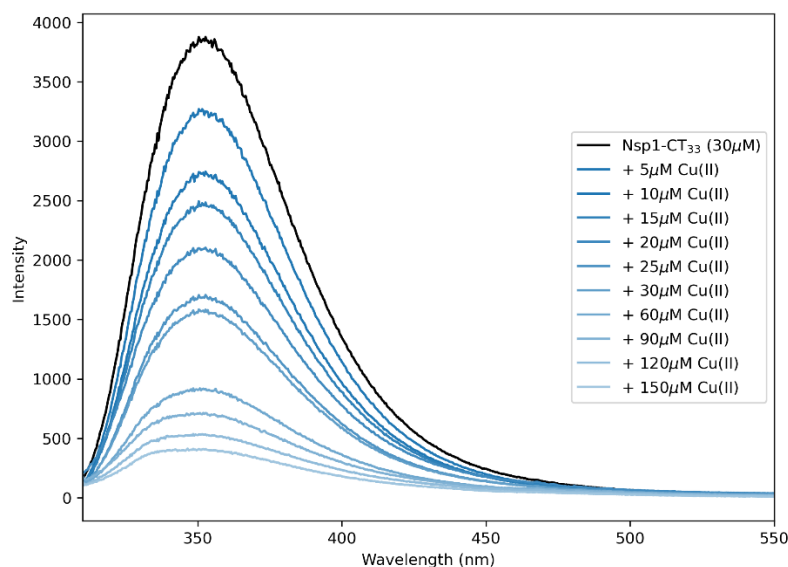


Figure 3.7 Tryptophan fluorescence quenching can be observed upon addition of Cu(II) to Nsp1-CT₃₃. Measurements were done in 20mM MOPS at pH 6.5.

At a peptide concentration of 30 μM , the addition of one molar equivalent of aquo Cu(II) results in a 25% decrease in W161 fluorescence intensity. Cu(II) also systematically reduces the effective W161 fluorescence lifetime. Modeling the decrease in the lifetime using a single Cu-binding-site model yields a dissociation constant of 9.7 μM at pH 6.5 (Figure 3.8). If the H165 is replaced with an alanine, the peptide is only weakly quenched, with a dissociation constant of 106 μM at pH 6.5, this makes it clear that the H165 is the coordination site for the Cu(II) (Figure 3.8).

Aquo Cu(II) appears to quench *W161 fluorescence via electron transfer (ET), as indicated by the observed decrease in effective lifetime and a quenching rate constant of $5 \times 10^8 \text{ s}^{-1}$. In structured proteins, an ET rate of this magnitude typically corresponds to a donor–acceptor distance of 10–14 Å.²³ This is consistent with the 14 Å separation between the W161 indole and the N ϵ of H165 in the helix-turn-helix conformation of Nsp1 bound to the 40S ribosome.^{22,24,25} However, the far-UV CD spectrum does not show a significant increase in α -helical content

upon Cu(II) binding (Figure 3.2), suggesting that the Cu(II):Nsp1-CT₃₃ complex adopts a distinct conformation in solution.

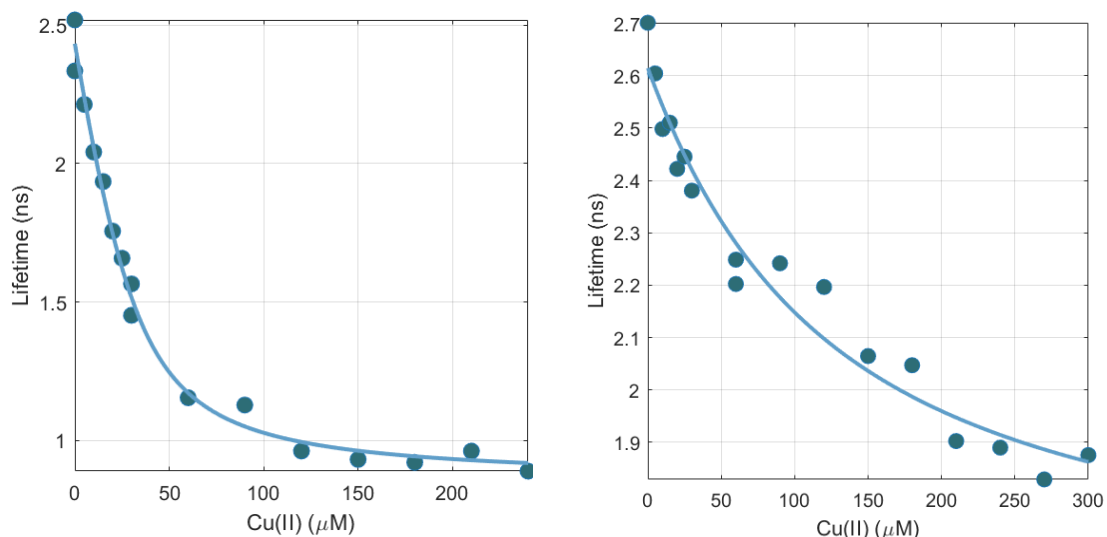


Figure 3.8 Variation in Nsp1-CT₃₃ W161 effective fluorescence decay time (Lifetime (ns)) as a function of Cu(II) concentration. On the left is the Nsp1-CT₃₃ and on the right is Nsp1-CT₃₃ H165A. Solid line is a fit to a single binding site model with $K_d = 9.7\mu\text{M}$ ($[\text{Nsp1-CT}_{33}] = 30\mu\text{M}$, pH 6.5, MOPS 20 mM, 30% glycerol) and $K_d = 106\mu\text{M}$ ($[\text{Nsp1-CT}_{33} \text{ H165A}] = 30\mu\text{M}$, pH 6.5, MOPS 20 mM, 30% glycerol).

Another possible explanation for the W161 quenching rate constant is that it represents the rate of intrachain diffusion, where the Cu(II)-bound residue transiently interacts with *W161. The quenching rate constant for the peptide-bound Cu(II) complex ($\sim 5 \times 10^8 \text{ s}^{-1}$) is at least an order of magnitude higher than the fastest reported rates of tertiary contact formation in disordered polypeptides.^{26–29}

To further investigate the interactions between Cu(II) and the peptide, electron paramagnetic resonance (EPR) spectroscopy serves as a valuable tool. The Cu(II) ion, with its $3d^9$ electron configuration and $S=1/2$, is paramagnetic and thus detectable by EPR. This property enables the characterization of Cu(II) metal complexes, providing insights into their electronic structure and coordination environment.³⁰

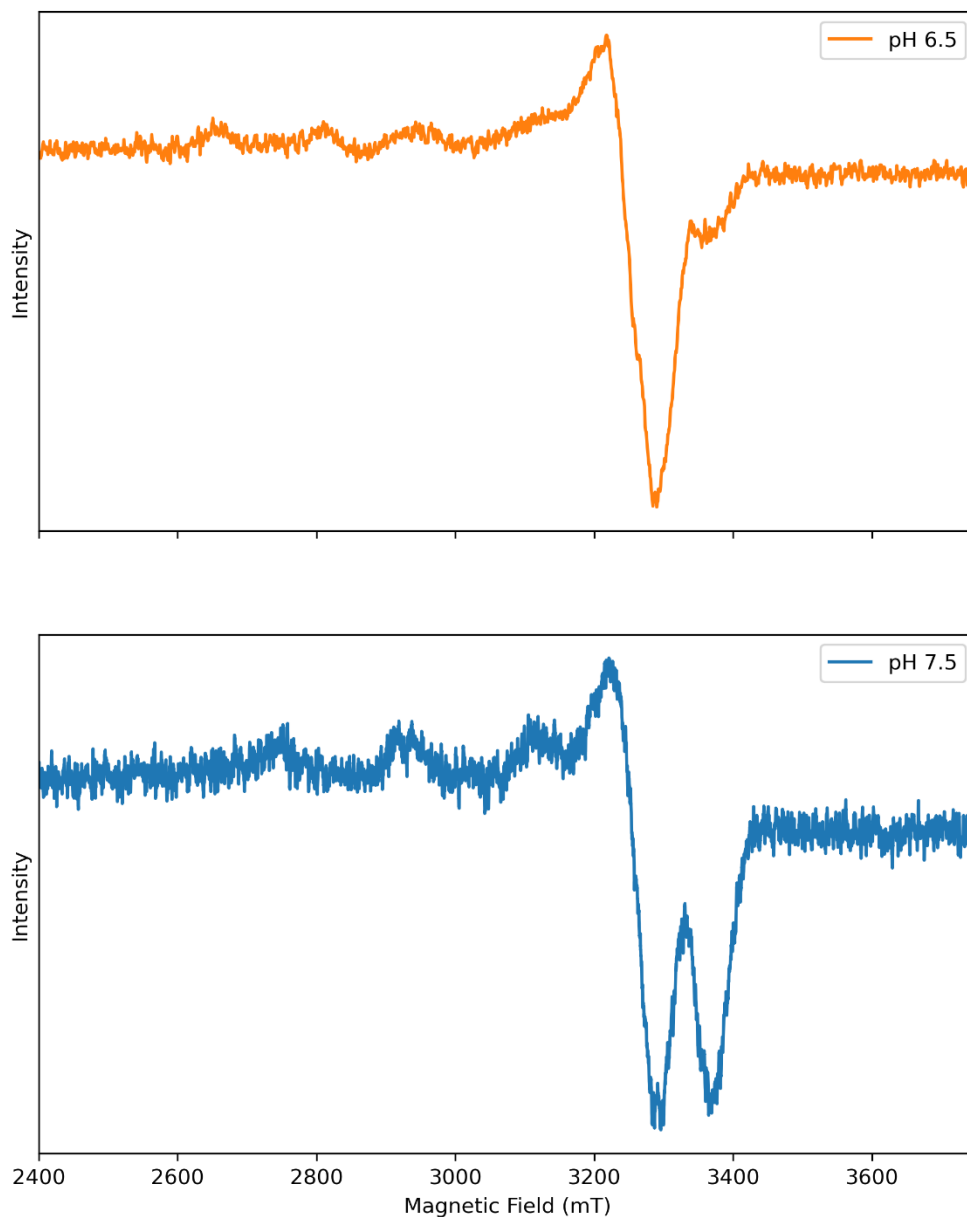


Figure 3.9 X-band EPR spectra of $[Cu] = [Nsp1-CT] = 100 \mu M$, MOPS 20 mM, 30% glycerol) at pH 6.5 (orange) and 7.5 (blue).

EPR spectroscopy offers a sensitive probe of the Cu(II) coordination environment. The continuous-wave X-band spectrum of Nsp1-CT (100 μM) in the presence of one equivalent of Cu(II) at pH 6.5 reveals a predominant species with near-axial Cu(II) coordination ($g_1 = 2.060$, $g_2 = 2.058$, $g_3 = 2.332$). The hyperfine coupling constant for the low-field resonance (g_3) is $A_3 = 457$ MHz. Notably, the spectrum shows no evidence of unbound aquo Cu(II), indicating that under these conditions, Cu(II) binds to a single Nsp1-CT₃₃ peptide (Figure 3.9).

At pH 7.5, the Cu(II) EPR spectrum exhibits increased rhombicity, with g -values shifting to higher magnetic fields ($g_1 = 2.040, g_2 = 2.067, g_3 = 2.219; A_3 = 583$ MHz), suggesting a less symmetric yet stronger ligand field around Cu(II) (Figure 3.9).

To interpret these spectral changes, Peisach-Blumberg correlations were applied. Cu(II) complexes typically adopt a distorted octahedral geometry, where four ligands form a strong equatorial plane, while two axial ligands are less tightly bound. These axial ligands contribute minimally to the magnetic and optical properties of the complex.³¹

In Cu(II) complexes with a 2N2O coordination, $g_{||}$ values tend to lie between 2.2 and 2.3 generally range from 2.2 to 2.3, depending on the overall charge, closer to 2.2 for a 2^- species and nearer to 2.3 for a 2^+ species. The corresponding $A_{||}$ values range between 450 MHz and 600 MHz, increasing toward 600 MHz for negatively charged species and decreasing toward 450 MHz for positively charged species.

When Cu(II) is coordinated by three oxygen and one nitrogen (3O1N), the complex tends to carry a positive charge, with $g_{||}$ values shifting higher (~ 2.2 – 2.4) and $A_{||}$ values decreasing (~ 374 – 450 MHz) relative to the 2N2O case. This pattern is observed for Nsp1-CT₃₃ at pH 6.5, where $A_{||} = 457$ MHz and $g_{||} = 2.332$, supporting a 3O1N coordination mode.

In contrast, when Cu(II) is bound to three nitrogens and one oxygen (3N1O), the charge varies, but the $A_{||}$ values remain similar to those in 2N2O systems. However, $g_{||}$ shifts between 2.15 and 2.3, increasing with more positive charge. This pattern aligns with Nsp1-CT₃₃ at pH 7.5, where $A_{||} = 583$ MHz and $g_{||} = 2.219$, indicating a 3N1O coordination environment.

To further analyze the coordination of the Cu(II) peptide complex, pulsed EPR techniques were employed. ENDOR (Electron Nuclear Double Resonance) enhances the resolution of EPR by directly probing hyperfine interactions between the unpaired electron and nearby nuclear spins. This technique is particularly effective for studying Cu(II) complexes as it provides detailed information about the local ligand environment. It gives insights into the identity, coupling strengths, and spatial arrangement of coordinating atoms such as nitrogen and oxygen. ENDOR resolves the hyperfine and quadrupolar interactions enabling precise characterization of the electronic and geometric structure of the complex.

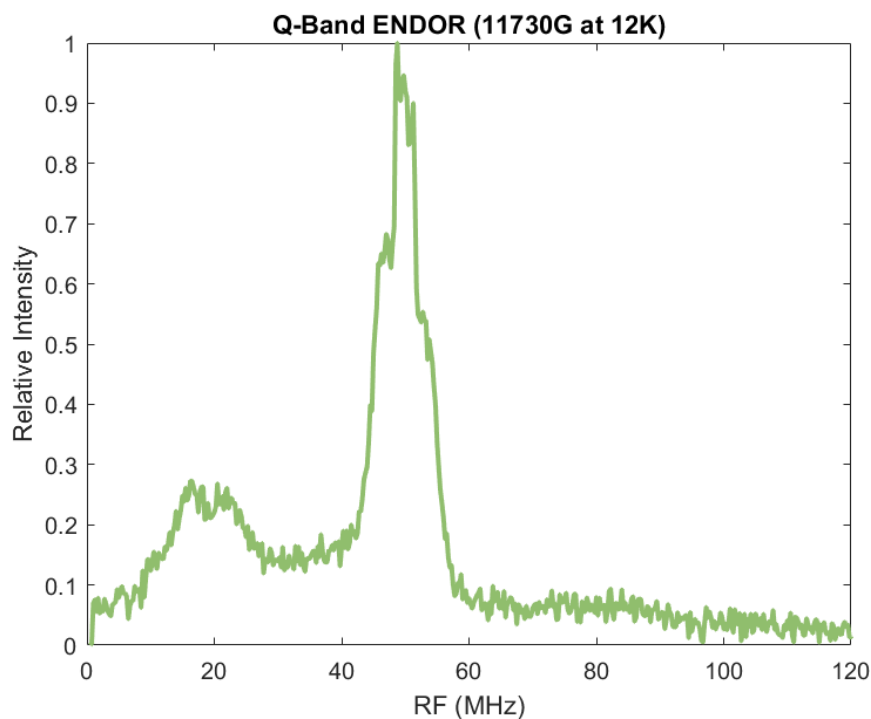


Figure 3.10 The Q-Band ENDOR spectrum was collected of Cu(II):Nsp1-CT₃₃ ([Cu] = [Nsp1-CT] = 100 μ M, MOPS 20 mM, 30% glycerol) at pH 7.5.

The ENDOR spectrum (Figure 3.10) exhibits two prominent features: a strong peak near 45 MHz and a lower-frequency feature in the 10–30 MHz range. At Q-band and a magnetic field of 11,730 G, the Larmor frequency of protons (^1H) is approximately 45 MHz. This suggests that proton nuclei are strongly coupled to the unpaired electron of Cu(II), likely originating from water, hydroxyl groups, or hydrogen-containing ligands. In this case, the observed proton coupling arises from the use of water as a solvent.^{32,33}

Additionally, the ENDOR spectrum displays two peaks split by the Larmor frequency of nitrogen, centered around 20 MHz. These lower-frequency features likely correspond to nitrogen nuclei interacting with the Cu(II) center. Since ^{14}N has a nuclear quadrupolar moment, its signals are broader and weaker compared to those of protons, explaining the reduced intensity observed in the spectrum. The 3–4 MHz spacing likely arises from weakly coupled nitrogen ligands,

HYSCORE (Hyperfine Sublevel Correlation Spectroscopy) is a two-dimensional EPR method that enhances the detection of weak hyperfine and quadrupolar interactions by correlating

nuclear frequencies. This technique is particularly valuable for Cu(II) complexes, as it provides detailed information about nearby nuclei. It helps resolve overlapping signals that may be difficult to distinguish in one-dimensional spectra. It offers improved spectral resolution and sensitivity, which enables a more comprehensive characterization of the electronic structure and ligand interactions.

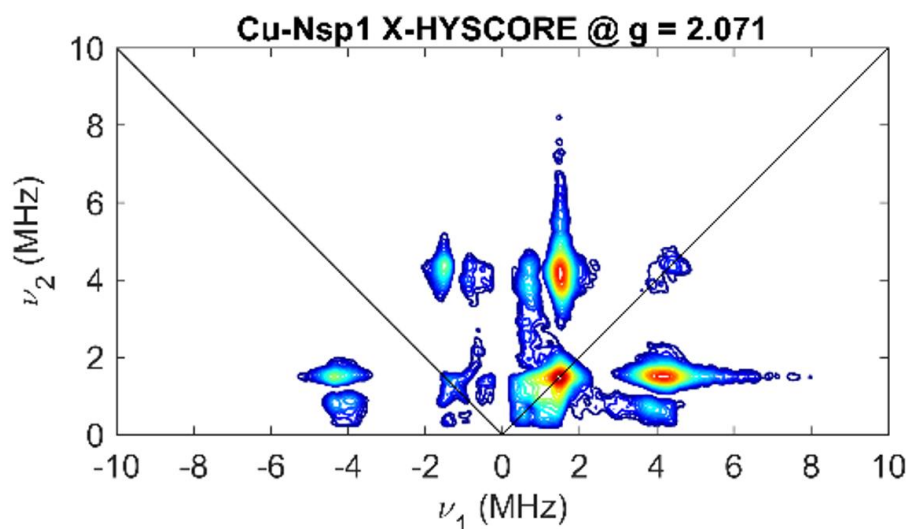


Figure 3.11 The HYSCORE spectrum was collected of Cu(II):Nsp1-CT₃₃ ([Cu] = [Nsp1-CT] = 100 μ M, MOPS 20 mM, 30% glycerol) at pH 7.5.

The collected HYSCORE spectra collected at $g=2.071$ (Figure 3.11), reveals a well-defined cross-peak in the low frequency region, this is indicative of hyperfine interactions with nearby nuclei. The presence of the peaks in the 2-4MHz range suggests coupling to nitrogen nuclei consistent with coordination from an imidazole group of a histidine residue. The cross-peaks are a result of the nuclear quadrupole interaction of nitrogen further confirming the involvement of an imidazole nitrogen Cu(II) coordination.

The characteristic splitting pattern observed in the (ν_1, ν_2) quadrants aligns with previously reported spectra of Cu(II)-imidazole complexes. The distinct correlation peaks at the ± 2 MHz and the a symmetry of the peak intensity suggest a nitrogen environment typical of histidine coordination.³⁴

To narrow down the coordination of the Cu(II) with H165, a smaller decapeptide was employed, Nsp1-CT₁₀. This was done so that computational modelling could be used and compared to experimental results.

W161 was important for reporting on Cu(II) binding to Nsp1-CT₃₃. To further identify potential inner-sphere ligands in the intrinsically disordered protein region, the W161 fluorescence was monitored in the presence of Cu(II) in Nsp1-CT₁₀. The average W161 fluorescence decay time was determined by integrating a normalized W161 fluorescence decay curve. In the absence of Cu(II), Nsp1-CT₁₀ has a lifetime of 2.1ns, after the peptide is fully bound to Cu(II) the lifetime decreases to 0.95ns. Dissociation constants of the Cu(II) from the Cu-bound peptide were calculated by measuring the observed lifetime as a function of Cu(II). The dissociation constants can be found in Table 3.1 and noticeably vary at different pH.

<i>pH</i>	6.5	7.5	8.5
K_d	10.1	5.05	<1

Table 3.3 Aquo-Cu(II) dissociation constants extracted from W161 fluorescence of Nsp1-CT₁₀.

Similar to the studies conducted with Nsp1-CT₃₃ and Cu(II), EPR can also be used to gain insights on the coordination. The X-band EPR of Nsp1-CT₁₀ (100 μ M, 77 K) in the presence of 1 equivalent of Cu(II) at pH 6.5 can be modeled with two nearly axial Cu(II) spin systems (Spin 1, 66%: $g_1 = 2.044$; $g_2 = 2.080$; $g_3 = 2.401$; $A_3 = 303$ MHz. Spin 2, 33%: $g_1 = 2.054$; $g_2 = 2.062$; $g_3 = 2.231$; $A_3 = 581$ MHz). The spectra (Figure 3.12) recorded at pH 7.5 and 8.5 are nearly identical consistent with a single $S = \frac{1}{2}$ spin center, with somewhat greater rhombic symmetry ($g_1 = 2.0426$, $g_2 = 2.068$, $g_3 = 2.219$, $A_3 = 564$ MHz). Using the Peisach-Blumberg correlation it suggest that at pH 6.5 the Cu(II) coordination is to one nitrogen and three oxygens for the dominant spin system. For the pH 7.5 and pH 8.5 spectra, the g_3 is consistent with a stronger ligand field indicating that most likely there are more nitrogens coordinating to the Cu(II).³¹

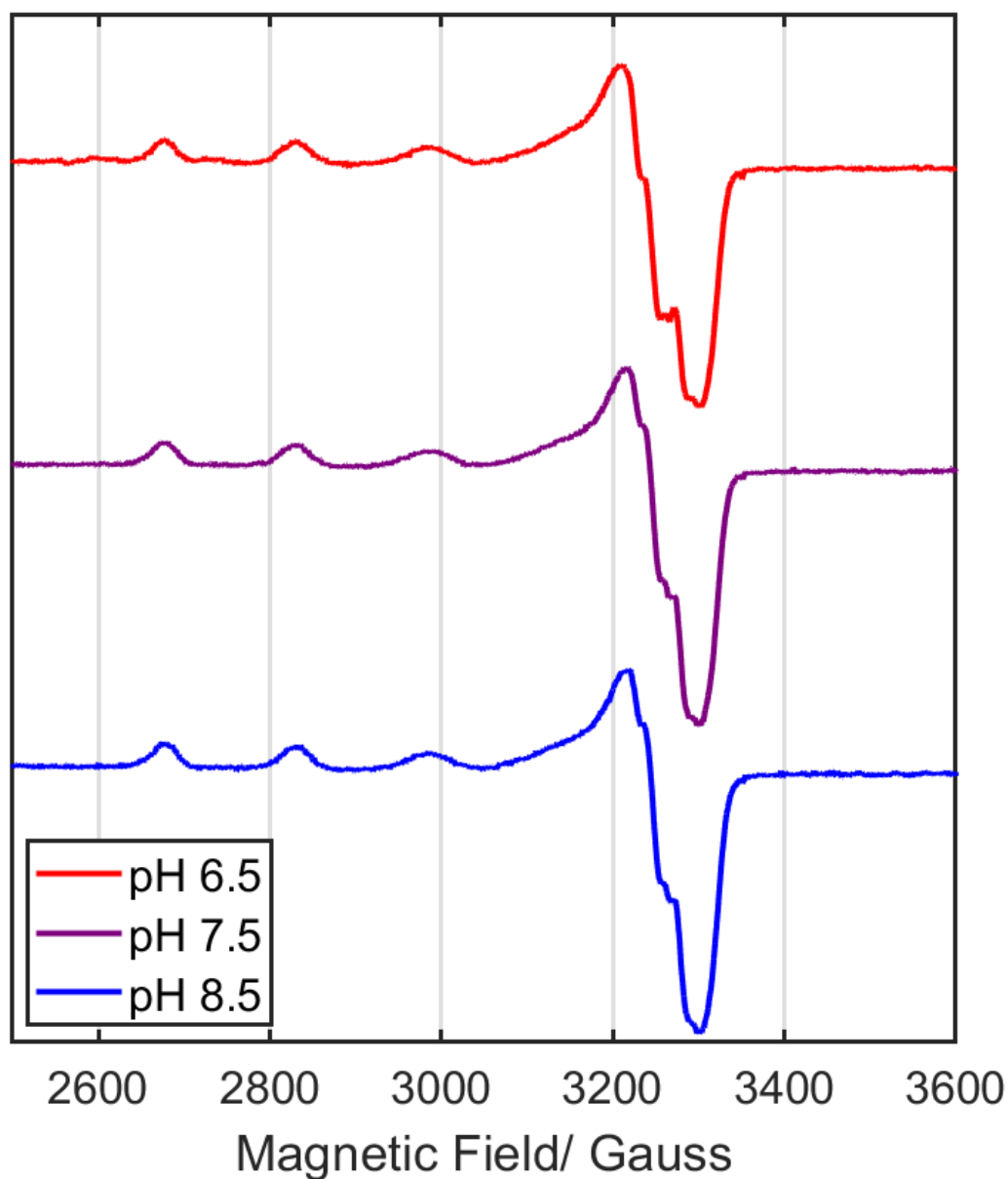


Figure 3.12 X-band EPR spectra of Cu(II): Nsp1-CT₁₀ ([Cu]=[Nsp1-CT₁₀] = 100 μ M, MOPS 20 mM, 30% glycerol, 77 K).

To further confirm the coordination of the inner sphere ligands quantum mechanical calculations were employed. This work was done in collaboration with Professor William A. Goddard and the calculations were carried out by Moon Young Yang. The Cu(II)-peptide structures were modeled with one or two explicit water molecules depending on the pH of the system. The optimized structures can be seen in Figure 3.13. Conducting calculations on the full

Nsp1-CT peptide sequence would be extremely costly therefore the modeled structures only include residues that form direct contact with Cu(II) and/or hydrogen networks with solvents.

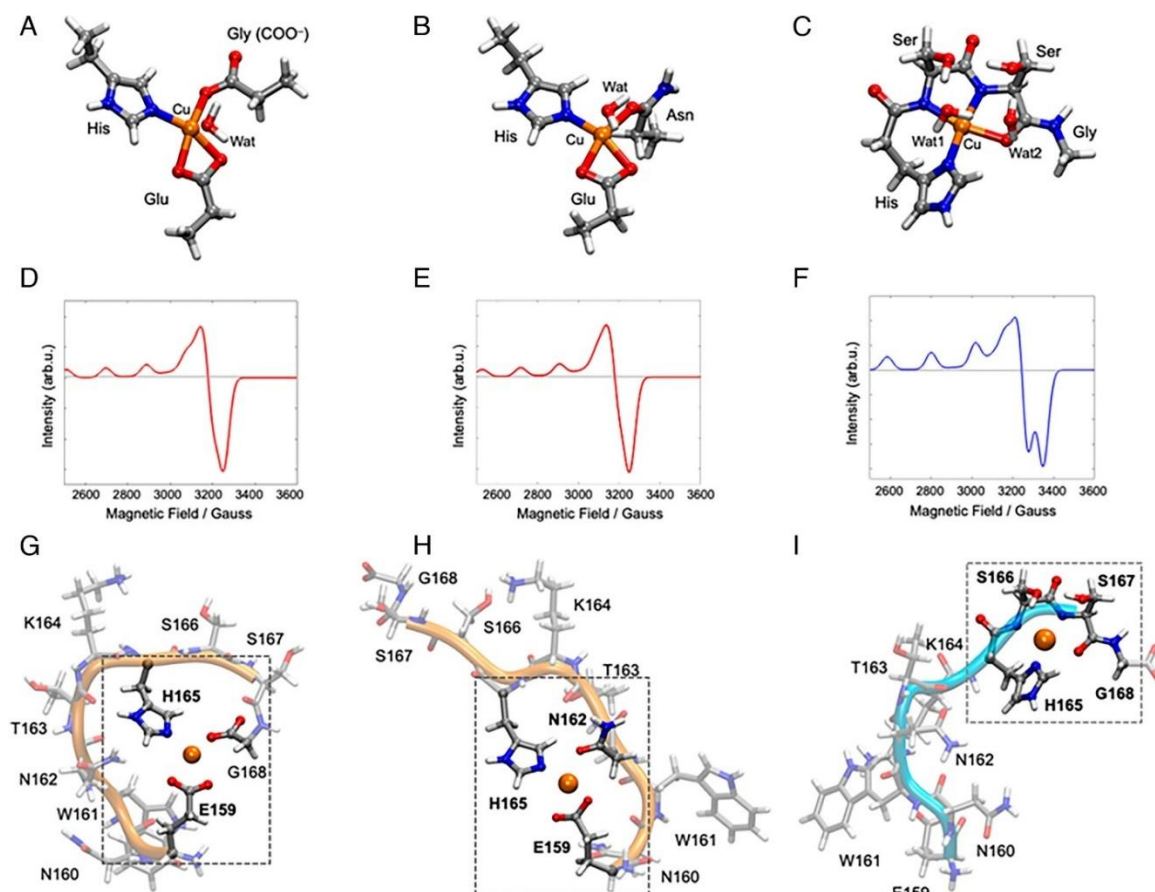


Figure 3.13 Computational models of the Cu(II) coordination environment at (A and B) pH 6.5 and (C) pH 8.5, and (D–F) their simulated EPR spectra, respectively. (G–I) Model structures of Cu(II):Nsp1-CT₁₀ constructed based on QM-optimized active sites (A–C), where. Water molecules were omitted and the nonactive sites were shown with transparency for clarity. Thick orange and cyan lines represent the backbone of the peptide.

From the calculations there are two structural models that are consistent with prominent components of the experimental spectrum measure at pH 6.5 (Figure 3.12). Both models include Cu(II) coordinated to one nitrogen and three oxygen atoms from the peptide. Normally, the N-terminal of the peptide might bind to Cu(II) however the peptide is N-terminally acetylated. In one model the Cu(II) is coordinated to the imidazole of H165 and the oxygen donors from E159 and the terminal carboxylate of G168, along with one water molecule. In the other model, one of the O-donors is from N162 instead of the terminal carboxylate of G168. The calculated

g-tensor components for the first model and second model of the pH 6.5 complex are also in reasonable agreement with the experimental results (model 1: $g_1 = 2.103$, $g_2 = 2.109$, $g_3 = 2.403$ / model 2: $g_1 = 2.100$, $g_2 = 2.114$, $g_3 = 2.387$).

If Cu(II)-peptide is compared to the prion octarepeat it is observed that it contains a similar HGGG sequence, compared to HSSG.³⁵ There is a similar coordination of inner-sphere ligands at pH 7.5 and 8.5 when compared to the prion octarepeat. Cu(II) is coordinated to the imidazole in the H165 and the amide nitrogens in S166 and S167. With the inclusion of an axial water molecule, the coordination environment is square pyramidal. The S166 and S167 form a hydrogen bond network with the two water molecules. In the calculated spectrum the g-tensor components are ($g_1 = 2.064$, $g_2 = 2.085$, $g_3 = 2.307$), which are in reasonable agreement with the experimental results.

Copper(II) Complexes for Binding to Nsp1

To identify a system with stronger binding affinity than aquo copper(II), attention was directed toward copper(II) complexes. Two complexes were selected for investigation: copper(II) iminodiacetate (Cu-IDA) and copper(II) histidinate. Iminodiacetate (IDA) is a tridentate ligand that forms a stable 1:1 complex with Cu(II) ($K_{Cu|IDA} = 10^{10.6}$), leaving three coordination sites available for additional interactions, such as binding to Nsp1.³⁶

Using the Nsp1-CT₃₃ peptide, it was observed that Cu-IDA effectively quenched tryptophan fluorescence, yielding a dissociation constant $K_d = 2.82 \mu\text{M}$ at pH 7.5 (Figure 3.14). This represents a significantly tighter binding interaction compared to aquo copper(II), which exhibited a $K_d = 7.67 \mu\text{M}$ under the same conditions. These results suggest that pre-coordination with IDA enhances copper's binding affinity toward Nsp1, likely by stabilizing the metal center while maintaining available sites for peptide coordination.

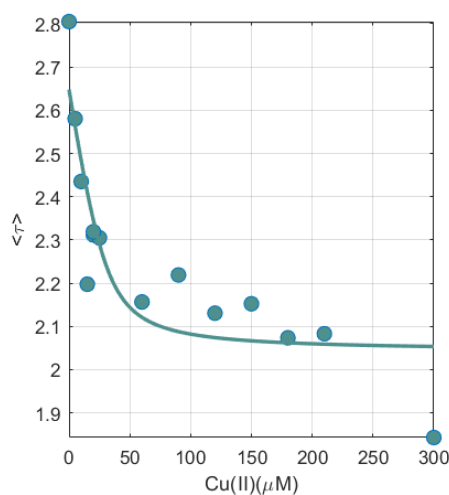


Figure 3.14 Average tryptophan fluorescence lifetimes as a function of increasing Cu-IDA concentration. [Nsp1-CT₃₃]=30 μM, samples were made in MOPS buffer (20mM, pH 7.5).

To further characterize the Cu-IDA complex and its interaction with Nsp1-CT₃₃, electron paramagnetic resonance (EPR) spectroscopy was employed to monitor changes in the copper coordination environment upon peptide binding. The resulting spectra, both for Cu-IDA alone and in the presence of peptide, are shown in Figure 3.15.

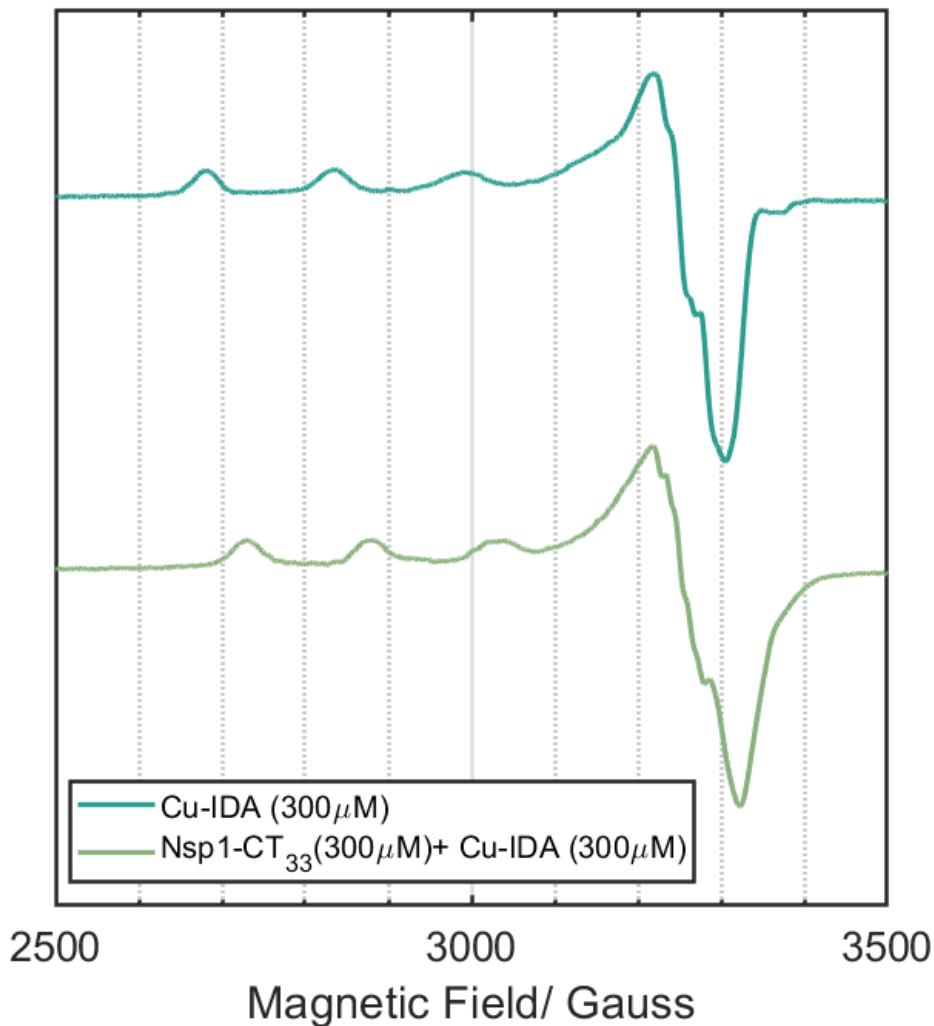


Figure 3.15 EPR spectrum of Cu-IDA and Nsp1-CT₃₃ with Cu-IDA. Samples were made at 300uM concentration in MOPS buffer (20mM pH7.5) with 30% glycerol. Measurements were taken at 77K.

Several notable changes were observed in the EPR spectrum upon the addition of Nsp1-CT₃₃ to the Cu-IDA complex. In the absence of peptide, the Cu-IDA complex exhibits parameters of $g_1 = g_2 = 2.06$, $g_3 = 2.31$, $A_1 = 12$ MHz, $A_2 = 55$ MHz, and $A_3 = 489$ MHz. Upon addition of one equivalent of Nsp1-CT₃₃, the spectrum shifts to $g_1 = g_2 = 2.06$, $g_3 = 2.26$, $A_1 = -14$ MHz, $A_2 = 74$ MHz, and $A_3 = 500$ MHz. These shifts include a decrease in g_3 and an increase in A_3 , along with broadening of the perpendicular (g_1/g_2) features, consistent with a strengthening of the equatorial ligand field around the copper(II) center.

These spectral changes suggest direct coordination of donor atoms from the peptide, likely involving imidazole nitrogens, backbone amide groups, or carboxylates, which displace solvent molecules in the coordination sphere. The resulting inner-sphere complex introduces enhanced equatorial bonding interactions and a slight perturbation of the axial symmetry. Together, the EPR data supports a model in which the peptide significantly alters the local geometry of the Cu(II) center through direct coordination. In chapter 5, we further explore this compound and test if it can inhibit Nsp1.

The second complex investigated was copper(II) histidinate, a compound currently used in the treatment of Menkes disease, a genetic disorder caused by mutations in the ATP7A copper transport gene, resulting in systemic copper deficiency.³⁷ Given its established clinical safety profile, copper(II) histidinate was selected to evaluate its potential for coordinating with Nsp1. For these studies, the Nsp1-CT₁₀ peptide was used, and tryptophan fluorescence quenching was monitored across increasing concentrations of copper(II) histidinate (Figure 3.16). The measured dissociation constant $K_d=30\text{ }\mu\text{M}$ at pH 7.5, indicating significantly weaker binding compared to aquo copper(II), which exhibited a $K_d=5\text{ }\mu\text{M}$ at pH 7.5. Despite its lower binding affinity, copper(II) histidinate was retained for further evaluation as a potential Nsp1 inhibitor due to its clinical relevance; these studies are discussed in Chapter 5.

These results highlight contrasting behaviors between the two copper(II) complexes: Cu-IDA demonstrated enhanced binding affinity relative to aquo copper(II), while copper(II) histidinate showed reduced affinity. This comparison demonstrates how ligand environment affects copper's interaction with Nsp1-derived peptides and provides a foundation for selecting metal-based candidates for functional inhibition studies.

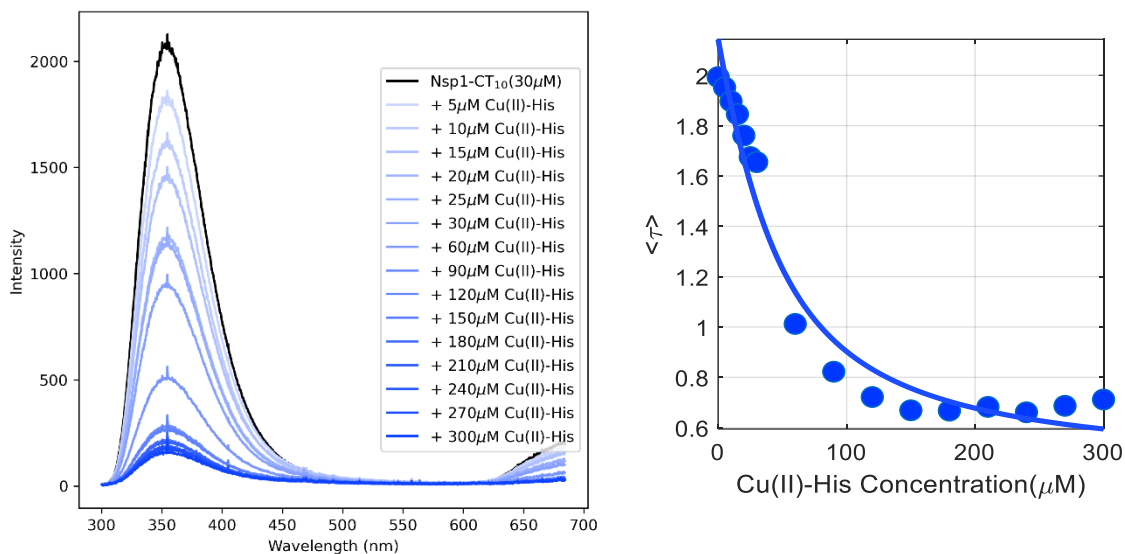


Figure 3.16 On the left is the steady state tryptophan fluorescence quenching of Nsp1-CT₁₀ upon addition of Copper(II)Histidinate (Cu(II)-His) and on the right is the change in the average lifetime as a function of concentration of Cu(II)-His. Measurements were done in MOPS buffer (20mM, pH 7.5).

Summary and Outlook

These studies demonstrate that Cu(II) coordinates to the C-terminal region of Nsp1, specifically targeting H165. The coordination environment is pH-dependent, shifting from a 3O1N coordination at lower pH to a 1O3N coordination at higher pH. At pH ≥ 7.5 , both experimental and computational analyses suggest that backbone amide chelation further stabilizes the complex.

In addition to aquo copper(II), investigations into copper(II) complexes revealed that ligand pre-coordination significantly influences binding affinity toward Nsp1 peptides. The Cu-IDA complex exhibited enhanced binding compared to aquo Cu(II), while copper(II) histidinate showed weaker interaction, despite its clinical relevance. These results highlight the importance of ligand design in modulating metal–protein interactions and suggest that copper(II) complexes could improve specificity and stability in targeting Nsp1.

Overall, these findings provide a foundation for the rational development of metal-based drug candidates with optimized binding properties for the C-terminal of Nsp1. By leveraging both the flexibility of intrinsically disordered regions and the tunable coordination chemistry of

copper complexes, this work opens avenues for novel therapeutic strategies aimed at disrupting Nsp1 function in SARS-CoV-2.

Supplementary Information

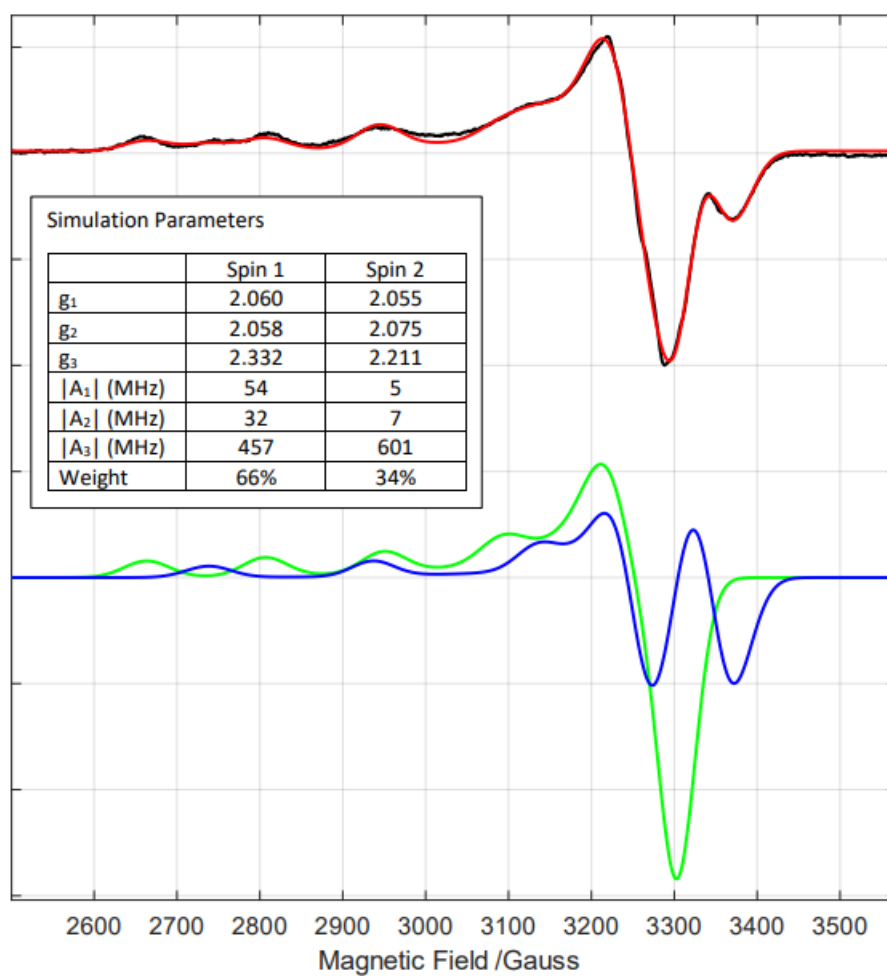


Figure S3.1 Cu(II):Nsp1-CT₃₃ ([Cu] = [Nsp1-CT₃₃] = 100 μ M, MOPS 20 mM, pH 6.5, 30% glycerol) X-band EPR spectrum (black) and simulation (red). Individual simulation components are shown in green (Spin 1) and blue (Spin 2). Acquisition parameters: MW frequency = 9.391-9.394 GHz, MW power = 2.2 mW, modulation amplitude = 8 Gauss, conversion time = 10.72 ms.

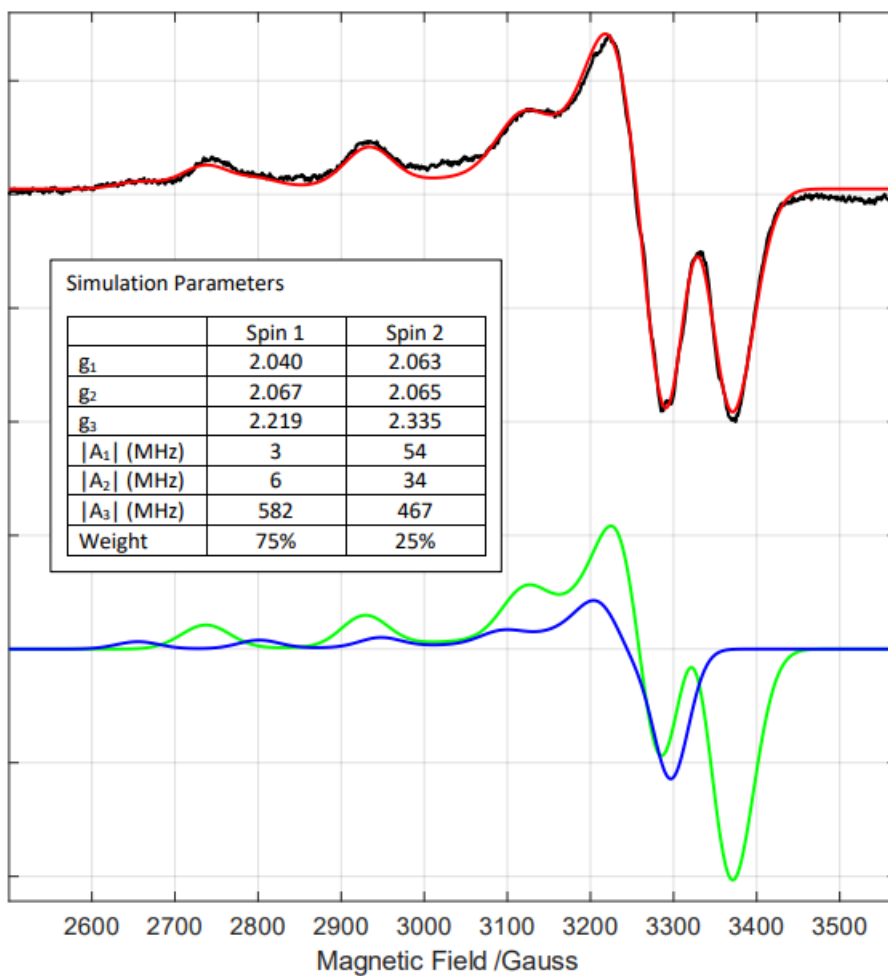


Figure S3.2 Cu(II):Nsp1-CT₃₃ ([Cu] = [Nsp1-CT₃₃] = 100 μ M, MOPS 20 mM, pH 7.5, 30% glycerol) X-band EPR spectrum (black) and simulation (red). Individual simulation components are shown in green (Spin 1) and blue (Spin 2). Acquisition parameters: MW frequency = 9.391-9.394 GHz, MW power = 2.2 mW, modulation amplitude = 8 Gauss, conversion time = 10.72 ms.

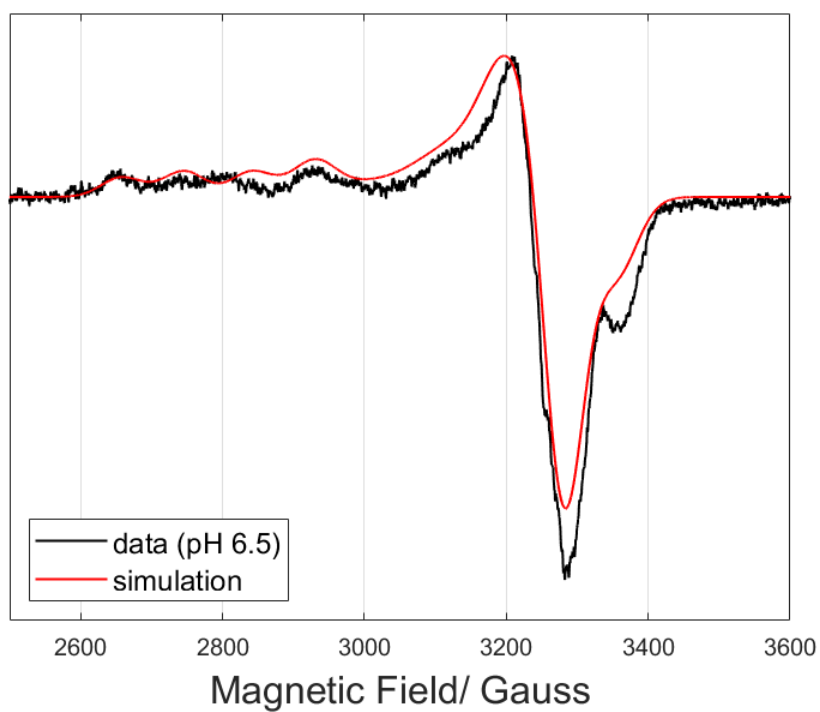


Figure S3.3 EasySpin simulation of the pH 6.5 Cu(II):Nsp1-CT₁₀ EPR spectrum.

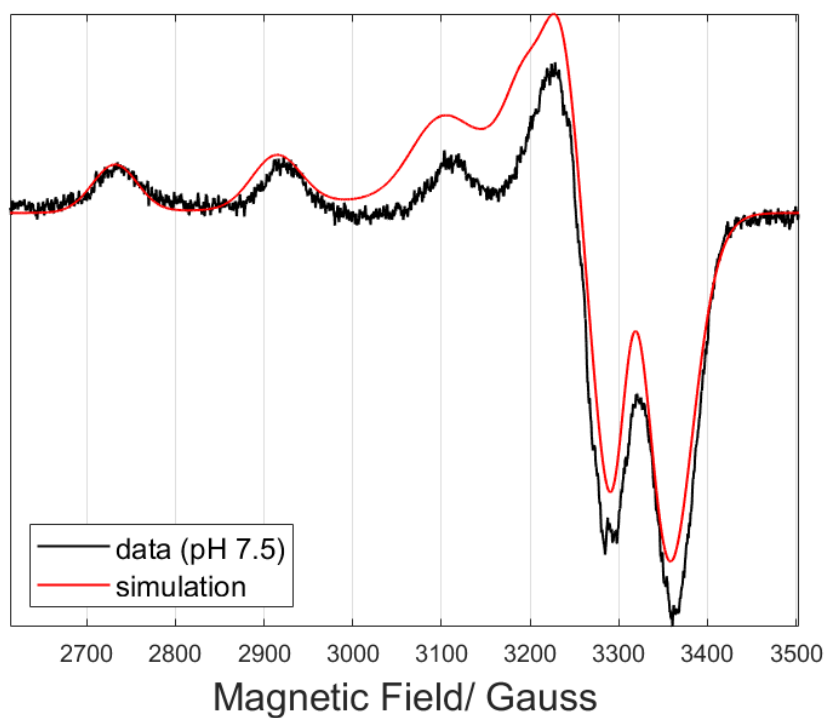


Figure S3.4 EasySpin simulation of the pH 7.5 Cu(II):Nsp1-CT₁₀ EPR spectrum.

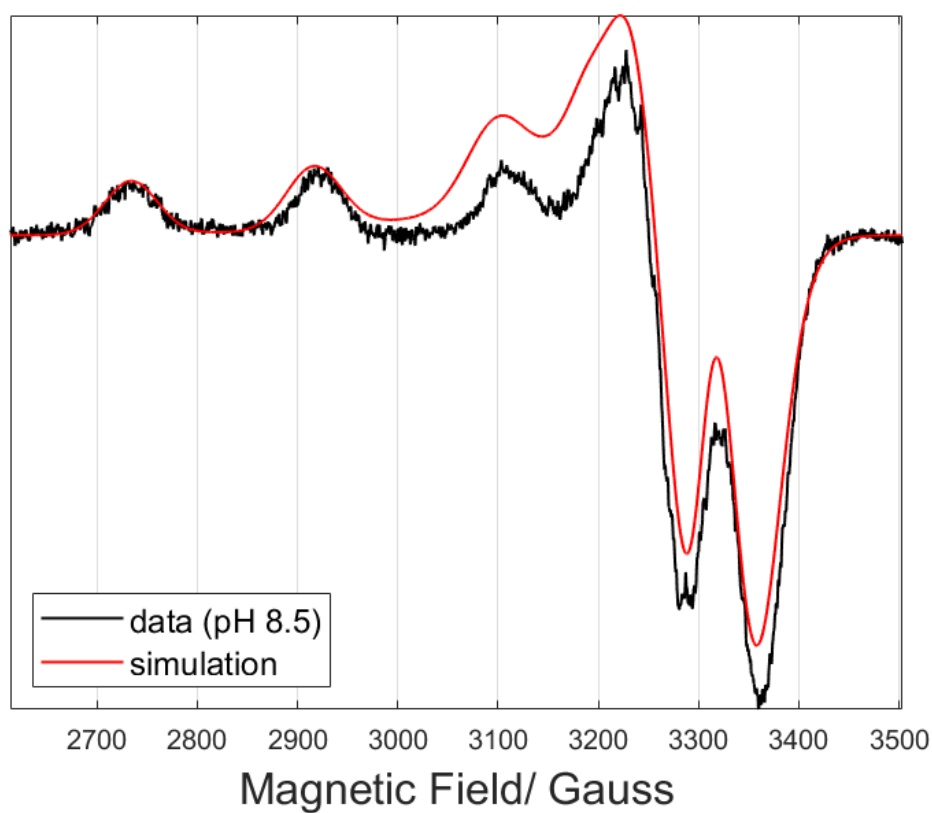


Figure S3.5 EasySpin simulation of the pH 8.5 Cu(II):Nsp1-CT₁₀ EPR spectrum.

Table S3.1 Cu(II):Nsp1-CT₁₀ EasySpin EPR fitting parameters.

<i>pH</i>	<i>6.5</i>		<i>7.5</i>	<i>8.5</i>
<i>Spin</i>	Spin 1	Spin 2	Spin 1	Spin 1
<i>Population (%)</i>	66	33	100	100
<i>g₁</i>	2.044	2.054	2.075	2.075
<i>g₂</i>	2.080	2.062	2.043	2.043
<i>g₃</i>	2.401	2.231	2.234	2.230
<i>Linewidth (mT)</i>	6	6	3	4
<i>A₁ (MHz)</i>	26	-7	86	85
<i>A₂ (MHz)</i>	46	-7	-5	4
<i>A₃ (MHz)</i>	303	581	554	553
<i>g₁-strain</i>	0.007	0.004	0	0
<i>g₂-strain</i>	0.005	0.012	0	0
<i>g₃-strain</i>	0.006	0.046	0.046	0.042
<i>A₁-strain</i>			191	217
<i>A₂-strain</i>			0	0
<i>A₃-strain</i>			37	23

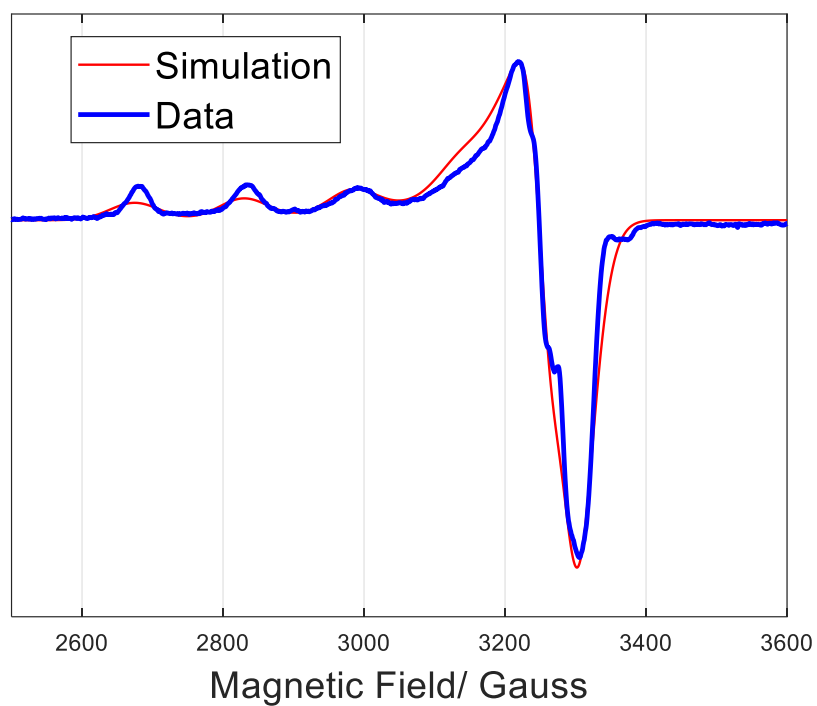


Figure S3.6 EasySpin simulation of the pH 7.5 Cu(II)IDA EPR spectrum.

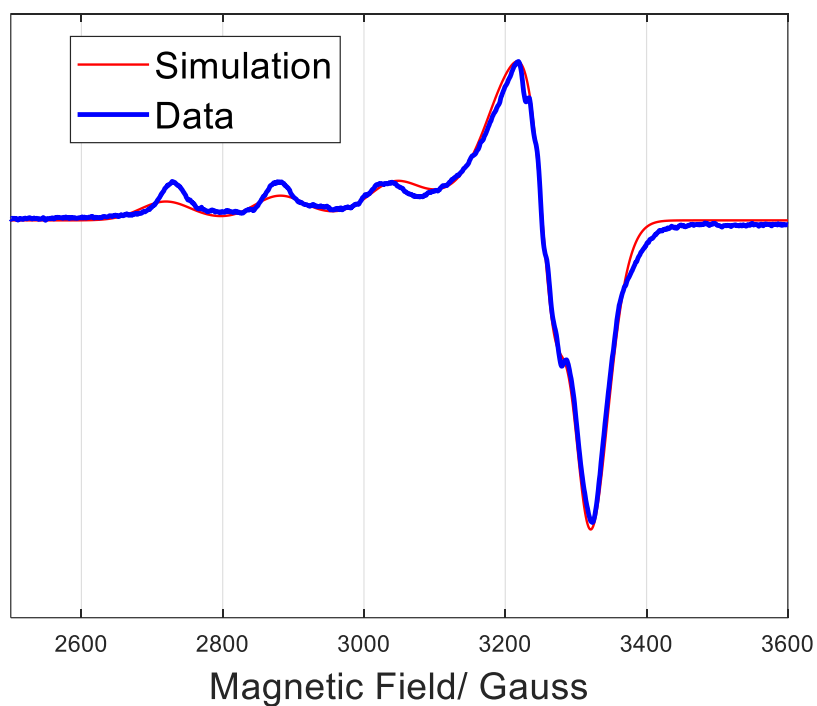


Figure S3.7 EasySpin simulation of the pH 7.5 Cu(II)IDA:Nsp1-CT₃₃ EPR spectrum.

Table S3.2 Cu(II)IDA:Nsp1-CT₃₃ EasySpin EPR fitting parameters for experiments done at pH 7.5.

	<i>Cu(II)IDA</i>	<i>Nsp1-CT₃₃-Cu(II)IDA</i>
g_1	2.063	2.061
g_2	2.062	2.057
g_3	2.309	2.267
A_1	12.005	-14.222
A_2	55.156	74.236
A_3	488.971	500.328

References

- (1) Tokuriki, N.; Oldfield, C. J.; Uversky, V. N.; Berezovsky, I. N.; Tawfik, D. S. Do Viral Proteins Possess Unique Biophysical Features? *Trends Biochem Sci* **2009**, *34* (2), 53–59. <https://doi.org/10.1016/j.tibs.2008.10.009>.
- (2) Solomon, E. I.; Heppner, D. E.; Johnston, E. M.; Ginsbach, J. W.; Cirera, J.; Qayyum, M.; Kieber-Emmons, M. T.; Kjaergaard, C. H.; Hadt, R. G.; Tian, L. Copper Active Sites in Biology. *Chem Rev* **2014**, *114* (7), 3659–3853. <https://doi.org/10.1021/cr400327t>.
- (3) Festa, R. A.; Thiele, D. J. Copper: An Essential Metal in Biology. *Curr Biol* **2011**, *21* (21), R877–883. <https://doi.org/10.1016/j.cub.2011.09.040>.
- (4) Irving, H.; Williams, R. J. P. 637. The Stability of Transition-Metal Complexes. *J Chem Soc* **1953**, 3192. <https://doi.org/10.1039/jr9530003192>.
- (5) Prohaska, J. R. Role of Copper Transporters in Copper Homeostasis. *Am J Clin Nutr* **2008**, *88* (3), 826S–9S. <https://doi.org/10.1093/ajcn/88.3.826S>.
- (6) Ala, A.; Walker, A. P.; Ashkan, K.; Dooley, J. S.; Schilsky, M. L. Wilson's Disease. *The Lancet* **2007**, *369* (9559), 397–408. [https://doi.org/10.1016/S0140-6736\(07\)60196-2](https://doi.org/10.1016/S0140-6736(07)60196-2).
- (7) Al-Chalabi, M.; Fathy, A.; Kelley, A.; Selim, L. A.-M.; Ramadan, A.; Noor, M.; Lakhani, S.; Mahfooz, N. Menkes Disease Treatment with Copper Chloride: Case Report. *Med Rep* **2023**, *1*, 100001. <https://doi.org/10.1016/j.hmedic.2023.100001>.
- (8) Husain, N.; Mahmood, R. Copper(II) Generates ROS and RNS, Impairs Antioxidant System and Damages Membrane and DNA in Human Blood Cells. *Environ Sci Pollut Res Int* **2019**, *26* (20), 20654–20668. <https://doi.org/10.1007/s11356-019-05345-1>.
- (9) Cervantes-Cervantes, M. P.; Calderón-Salinas, J. V.; Albores, A.; Muñoz-Sánchez, J. L. Copper Increases the Damage to DNA and Proteins Caused by Reactive Oxygen Species. *Biol Trace Elem Res* **2005**, *103* (3), 229–248. <https://doi.org/10.1385/BTER:103:3:229>.

- (10) Djoko, K. Y.; Ong, C. Y.; Walker, M. J.; McEwan, A. G. The Role of Copper and Zinc Toxicity in Innate Immune Defense against Bacterial Pathogens. *J Biol Chem* **2015**, *290* (31), 18954–18961. <https://doi.org/10.1074/jbc.R115.647099>.
- (11) De Romaña, D. L.; Olivares, M.; Uauy, R.; Araya, M. Risks and Benefits of Copper in Light of New Insights of Copper Homeostasis. *J Trace Elem Med Biol* **2011**, *25* (1), 3–13. <https://doi.org/10.1016/j.jtemb.2010.11.004>.
- (12) Dollwet, H. H. A.; Sorenson, J. R. J. Historic Uses of Copper Compounds in Medicine. *J Trace Elem Med Biol* **2**, 80–87.
- (13) Walusinski, O. The Scientific Illusion of Victor Burq (1822–1884). *Eur Neurol* **2018**, *79* (3–4), 135–149. <https://doi.org/10.1159/000487667>.
- (14) Noyce, J. O.; Michels, H.; Keevil, C. W. Inactivation of Influenza A Virus on Copper versus Stainless Steel Surfaces. *Appl Environ Microbiol* **2007**, *73* (8), 2748–2750. <https://doi.org/10.1128/AEM.01139-06>.
- (15) Poggio, C.; Colombo, M.; Arciola, C. R.; Greggi, T.; Scribante, A.; Dagna, A. Copper-Alloy Surfaces and Cleaning Regimens against the Spread of SARS-CoV-2 in Dentistry and Orthopedics. From Fomites to Anti-Infective Nanocoatings. *Materials* **2020**, *13* (15), 3244. <https://doi.org/10.3390/ma13153244>.
- (16) Weder, J. E.; Dillon, C. T.; Hambley, T. W.; Kennedy, B. J.; Lay, P. A.; Biffin, J. R.; Regtop, H. L.; Davies, N. M. Copper Complexes of Non-Steroidal Anti-Inflammatory Drugs: An Opportunity yet to Be Realized. *Coord Chem Rev* **2002**, *232* (1–2), 95–126. [https://doi.org/10.1016/S0010-8545\(02\)00086-3](https://doi.org/10.1016/S0010-8545(02)00086-3).
- (17) Tisato, F.; Marzano, C.; Porchia, M.; Pellei, M.; Santini, C. Copper in Diseases and Treatments, and Copper-based Anticancer Strategies. *Med Res Rev* **2010**, *30* (4), 708–749. <https://doi.org/10.1002/med.20174>.
- (18) Shiraki, K.; Nishikawa, K.; Goto, Y. Trifluoroethanol-Induced Stabilization of the α -Helical Structure of β -Lactoglobulin: Implication for Non-Hierarchical Protein Folding. *J Mol Biol* **1995**, *245* (2), 180–194. <https://doi.org/10.1006/jmbi.1994.0015>.
- (19) Pan, C.-P.; Muñio, P. L.; Barkley, M. D.; Callis, P. R. Correlation of Tryptophan Fluorescence Spectral Shifts and Lifetimes Arising Directly from Heterogeneous Environment. *J Phys Chem B* **2011**, *115* (12), 3245–3253. <https://doi.org/10.1021/jp111925w>.

- (20) Lee, J. C.; Gray, H. B.; Winkler, J. R. Copper(II) Binding to α -Synuclein, the Parkinson's Protein. *J. Am. Chem. Soc.* **2008**, *130* (22), 6898–6899. <https://doi.org/10.1021/ja711415b>.
- (21) Lee, J. C.; Langen, R.; Hummel, P. A.; Gray, H. B.; Winkler, J. R. α -Synuclein Structures from Fluorescence Energy-Transfer Kinetics: Implications for the Role of the Protein in Parkinson's Disease. *Proc Natl Acad Sci U.S.A.* **2004**, *101* (47), 16466–16471. <https://doi.org/10.1073/pnas.0407307101>.
- (22) Schubert, K.; Karousis, E. D.; Jomaa, A.; Scaiola, A.; Echeverria, B.; Gurzeler, L.-A.; Leibundgut, M.; Thiel, V.; Mühlemann, O.; Ban, N. SARS-CoV-2 Nsp1 Binds the Ribosomal mRNA Channel to Inhibit Translation. *Nat Struct Mol Biol* **2020**, *27* (10), 959–966. <https://doi.org/10.1038/s41594-020-0511-8>.
- (23) Winkler, J. R.; Gray, H. B. Long-Range Electron Tunneling. *J Am Chem Soc* **2014**, *136* (8), 2930–2939. <https://doi.org/10.1021/ja500215j>.
- (24) Thoms, M.; Buschauer, R.; Ameismeier, M.; Koepke, L.; Denk, T.; Hirschenberger, M.; Kratzat, H.; Hayn, M.; Mackens-Kiani, T.; Cheng, J.; Straub, J. H.; Stürzel, C. M.; Fröhlich, T.; Berninghausen, O.; Becker, T.; Kirchhoff, F.; Sparrer, K. M. J.; Beckmann, R. Structural Basis for Translational Shutdown and Immune Evasion by the Nsp1 Protein of SARS-CoV-2. *Science* **2020**, *369* (6508), 1249–1255. <https://doi.org/10.1126/science.abc8665>.
- (25) Yuan, S.; Peng, L.; Park, J. J.; Hu, Y.; Devarkar, S. C.; Dong, M. B.; Shen, Q.; Wu, S.; Chen, S.; Lomakin, I. B.; Xiong, Y. Nonstructural Protein 1 of SARS-CoV-2 Is a Potent Pathogenicity Factor Redirecting Host Protein Synthesis Machinery toward Viral RNA. *Mol Cell* **2020**, *80* (6), 1055–1066.e6. <https://doi.org/10.1016/j.molcel.2020.10.034>.
- (26) Chang, I.-J.; Lee, J. C.; Winkler, J. R.; Gray, H. B. The Protein-Folding Speed Limit: Intrachain Diffusion Times Set by Electron-Transfer Rates in Denatured Ru(NH₃)₅(His-33)-Zn-Cytochrome *c*. *Proc Natl Acad Sci U.S.A.* **2003**, *100* (7), 3838–3840. <https://doi.org/10.1073/pnas.0637283100>.
- (27) Hagen, S. J.; Hofrichter, J.; Eaton, W. A. Rate of Intrachain Diffusion of Unfolded Cytochrome *c*. *J Phys Chem B* **1997**, *101* (13), 2352–2365. <https://doi.org/10.1021/jp9622997>.
- (28) Szabo, A.; Schulten, K.; Schulten, Z. First Passage Time Approach to Diffusion Controlled Reactions. *J Chem Phys* **1980**, *72* (8), 4350–4357. <https://doi.org/10.1063/1.439715>.

- (29) Thirumalai, D. Time Scales for the Formation of the Most Probable Tertiary Contacts in Proteins with Applications to Cytochrome *c*. *J Phys Chem B* **1999**, *103* (4), 608–610. <https://doi.org/10.1021/jp982362n>.
- (30) Garribba, E.; Micera, G. The Determination of the Geometry of Cu(II) Complexes: An EPR Spectroscopy Experiment. *J Chem Educ* **2006**, *83* (8), 1229. <https://doi.org/10.1021/ed083p1229>.
- (31) Peisach, J.; Blumberg, W. E. Structural Implications Derived from the Analysis of Electron Paramagnetic Resonance Spectra of Natural and Artificial Copper Proteins. *Arch Biochem Biophys* **1974**, *165* (2), 691–708. [https://doi.org/10.1016/0003-9861\(74\)90298-7](https://doi.org/10.1016/0003-9861(74)90298-7).
- (32) Van Camp, H. L.; Wei, Y. H.; Scholes, C. P.; King, T. E. Electron Nuclear Double Resonance of Cytochrome Oxidase: Nitrogen and Proton Endor from the ‘Copper’ EPR Signal. *BBA Proteins and Proteomics* **1978**, *537* (2), 238–246. [https://doi.org/10.1016/0005-2795\(78\)90507-X](https://doi.org/10.1016/0005-2795(78)90507-X).
- (33) Scholl, H. J.; Huettermann, J. ESR and ENDOR of Copper(II) Complexes with Nitrogen Donors: Probing Parameters for Prosthetic Group Modeling of Superoxide Dismutase. *J Phys Chem* **1992**, *96* (24), 9684–9691. <https://doi.org/10.1021/j100203a023>.
- (34) Ritterskamp, N.; Sharples, K.; Richards, E.; Folli, A.; Chiesa, M.; Platts, J. A.; Murphy, D. M. Understanding the Coordination Modes of [Cu(Acac)₂(Imidazole)_{*n*=1,2}] Adducts by EPR, ENDOR, HYSCORE, and DFT Analysis. *Inorg Chem* **2017**, *56* (19), 11862–11875. <https://doi.org/10.1021/acs.inorgchem.7b01874>.
- (35) Van Doorslaer, S.; Vinck, E. The Strength of EPR and ENDOR Techniques in Revealing Structure–Function Relationships in Metalloproteins. *Phys Chem Chem Phys* **2007**, *9* (33), 4620. <https://doi.org/10.1039/b701568b>.
- (36) De Andrade, J. C. D.; Silva, L. A. T.; Lima-Junior, C. G.; Chojnacki, J.; Vasconcellos, M. L. A. D. A.; Da Silva, R. B.; Alves Júnior, S.; Da Silva, F. F. Copper and Copper-Manganese 1D Coordination Polymers: Synthesis Optimization, Crystal Structure and Preliminary Studies as Catalysts for Baylis–Hillman Reactions. *Inorganica Chimica Acta* **2021**, *514*, 119985. <https://doi.org/10.1016/j.ica.2020.119985>.
- (37) Kaler, S.; Munim, S.; Chen, M.; Niecestro, R.; Yam, L. eP195: Safety and Efficacy of Copper Histidinate (CUTX-101) Treatment for Menkes Disease Caused by Severe Loss-of-Function Variants in ATP7A. *Genet Med* **2022**, *24* (3), S121. <https://doi.org/10.1016/j.gim.2022.01.231>.

*Chapter 4***COBALT BINDING TO NSP1 STUDIES**

Introduction

Previous studies have demonstrated that Cu(II) can bind to Nsp1,^{1,2} however, the dynamic dissociation of these ions often limits the formation of stable metal–protein complexes. In contrast, Co(III), a low-spin d^6 ion, typically forms kinetically inert octahedral complexes, particularly with multidentate ligands such as nitrilotriacetic acid (NTA). This property makes Co(III) an appealing candidate for probing metal–protein interactions due to its ability to form strong, permanent coordination bonds. Such interactions have the potential to tightly bind and disrupt the activity of Nsp1, a SARS-CoV-2 nonstructural protein known to suppress host immune responses.

In this work, the binding of cobalt(III) nitrilotriacetic acid (Co(III)NTA) and Co(III) Bis(amine)(N,N'-ethylenebis(acetylacetonimine)) ([Co(acacen)(NH₃)₂]⁺) to the C-terminal region of Nsp1 was investigated. While earlier reports showed that metal ions like Cu(II) and Mn(II) associate with Nsp1, their complexes often lack stability. Co(III), with its inert coordination sphere, was hypothesized to form a more robust complex with the Nsp1 peptides and protein. Spectroscopic analysis as well as *in vitro* assays, including UV-Vis and ⁵⁹Co NMR, provided evidence for strong coordination between Co(III) complexes the peptides/proteins. UV-Vis spectra revealed ligand-to-metal charge transfer and d–d transitions characteristic of Co(III) coordination, while time-dependent spectral analysis supported stable complex formation. Furthermore, ⁵⁹Co NMR confirmed the presence of a low-spin d^6 Co(III) species, consistent with a stably bound complex. These findings offer valuable insight into the potential use of Co(III) complexes to modulate Nsp1 function and present a novel strategy for therapeutic interference in SARS-CoV-2 pathogenesis.

Cobalt in Biology

Cobalt is an essential trace element for life and plays a variety of biochemical and medical roles despite its relatively low abundance in biological systems compared to metals like iron and copper. As a first-row transition metal, cobalt exhibits versatile coordination chemistry and commonly exists in two biologically relevant oxidation states: Co(II) and Co(III), with occasional occurrences of Co(I) and Co(IV) under specific conditions. Co(II) typically forms labile, high-spin octahedral complexes, while Co(III) forms more inert, low-spin complexes that are both

thermodynamically and kinetically stable. These properties allow cobalt to participate in both dynamic and persistent interactions with biological ligands. Its redox flexibility and coordination preferences have also made cobalt a valuable element in therapeutic and diagnostic applications, particularly where stability and selective binding are desirable traits.

Cobalt's most well-known biological role is as the central metal ion in vitamin B12 (cobalamin), the only vitamin that incorporates a metal. Cobalamin is critical for DNA synthesis, red blood cell formation, fatty acid metabolism, amino acid synthesis, and maintaining proper neurological function. Its most metabolically active forms include methylcobalamin and adenosylcobalamin.³ Cyanocobalamin, while the most stable form of vitamin B12, is biologically inactive and is converted to active forms upon entry into the body.⁴ Vitamin B12 acts as a cofactor for two essential enzymes: methionine synthase, which catalyzes methyl group transfers in amino acid metabolism, and methylmalonyl-CoA mutase, which is involved in the breakdown of certain fatty acids and amino acids.⁵ Through these functions, cobalt contributes to a wide array of homeostatic processes and nervous system maintenance.⁶

Beyond its biological roles, cobalt has also found applications in modern medicine. Cobalt-chromium alloys are widely used in prosthetics due to their mechanical strength and durability.⁷ The isotope ⁶⁰Co is used as a γ -ray source employed in cancer radiotherapy and sterilization techniques.⁸ Cobalt-based nanoparticles have shown promise in biomedical research due to their high antimicrobial activity at low concentrations, suggesting cobalt's emerging potential in nanomedicine.⁹

Like many other things, maintaining proper cobalt homeostasis is critical, as both deficiency and excess can lead to physiological dysfunction. In humans, cobalt is primarily acquired through dietary intake of vitamin B12, and its distribution is tightly regulated to ensure adequate supply for cobalamin-dependent enzymes while preventing toxic accumulation.¹⁰ Transport and storage of cobalt are largely mediated through transcobalamin proteins and cellular uptake pathways specific to cobalamin.¹¹

Cobalt toxicity can arise from excessive exposure, such as metal refining or through poorly regulated dietary supplementation. High levels of cobalt can affect cellular respiration and redox homeostasis, leading to oxidative stress, mitochondrial dysfunction, and inflammation.¹²

Chronic exposure has been associated with cardiomyopathy, polycythemia, thyroid dysfunction, and neurological symptoms.¹³ Cobalt deficiency, on the other hand, though rare, can occur in cases of severe malnutrition or gastrointestinal disorders that impair vitamin B12 absorption. Such deficiencies may result in megaloblastic anemia, neuropathy, and cognitive impairments.¹⁴ Understanding the mechanisms that regulate cobalt uptake, usage, and excretion is essential for safely harnessing its biological and therapeutic potential.

Cobalt's unique chemical versatility, such as its redox activity and stable coordination geometry, makes it an attractive candidate for therapeutics. Its ability to form substitution-inert Co(III) complexes that slowly release ligands under physiological conditions has inspired the design of prodrugs that are selectively activated in hypoxic or diseased tissues.^{15,16} Cobalt-based compounds have shown promise in anticancer, antimicrobial, and enzyme-inhibition contexts, often through mechanisms that involve disruption of cellular redox balance, inhibition of key enzymes, or interaction with nucleic acids.^{17–20} Cobalt Schiff base complexes have been studied for their cytotoxicity toward tumor cells.²¹ Beyond drug development, cobalt is also being explored in biomaterials and diagnostic imaging agents, where its tunable coordination environment allows for targeted interaction with biomolecules.²² Together, these emerging applications reflect cobalt's growing significance in medicinal chemistry and underscore its potential as a tool for therapeutics.

Given cobalt's unique ability to form substitution-inert, low-spin Co(III) complexes, its application in biological systems presents a powerful strategy for studying protein–metal interactions. Unlike Cu(II), which often forms dynamic and reversible complexes that readily dissociate, Co(III) can “lock in” to target sites with high thermodynamic and kinetic stability. This property is especially advantageous in probing and potentially disrupting metal-binding proteins such as SARS-CoV-2 Nsp1, which contains accessible histidine residues in its C-terminal domain. The following chapter explores the design and application of Co(III) coordination complexes to investigate and modulate Nsp1 activity. These studies not only illuminate fundamental aspects of cobalt–protein binding but also suggest that Co(III)-based compounds may serve as promising drug candidates for targeting viral proteins with critical structural or regulatory roles.

To explore cobalt binding to Nsp1, two complementary strategies were pursued. The first approach involved treating Nsp1 as a polyamino–polycarboxylate-type ligand capable of coordinating to Co(II). This coordination is expected to lower the formal redox potential of the metal center, thereby facilitating *in situ* oxidation to Co(III) and generating a substitution-inert Co(III)–peptide complex. The second approach began with a pre-formed Co(III) complex, designed to undergo direct ligand exchange with Nsp1, displacing labile axial ligands to form a stable coordination complex. This chapter investigates both strategies in detail, using spectroscopic techniques to probe coordination, redox behavior, and the structural features of the resulting cobalt complexes.

⁵⁹Co NMR

Under most chemical conditions, cobalt is found in the oxidation state of Co(II) which is paramagnetic, however in situations where the cobalt atom is coordinated by six donor ligands cobalt(III) becomes relatively more stable. These low spin d⁶ complexes have been studied in ⁵⁹Co NMR. Cobalt-59 has a nuclear spin, I=7/2 and a quadrupole moment of 0.4x10⁻²⁸cm², the odd number nuclear spin makes it an excellent candidate for NMR detection of Co(III) species and the magnitude of the quadrupole moment makes ⁵⁹Co linewidths sensitive to the electric field gradients at the cobalt and the symmetry about the cobalt atom. Chemical shifts in cobalt span a range of 18000ppm.^{23–25} This technique provides a unique approach to monitor cobalt(II) oxidation to cobalt(III).

Results and Discussion

Preliminary Studies

Preliminary studies, including tryptophan fluorescence measurements with cobalt(II) at pH 7.5 and circular dichroism, suggested that Co(II) had minimal interaction with Nsp1 under neutral conditions (Chapter 3, Figure 3.3). However, fluorescence experiments at higher pH using the C-terminal decapeptide of Nsp1 (Nsp1-CT₁₀) revealed tryptophan fluorescence quenching upon titration with Co(II), indicating some level of interaction (Figure 4.1). This effect was enhanced at alkaline pH, where deprotonation of amino acid side chains, particularly lysine and histidine residues, could promote coordination to the Co(II) ion=

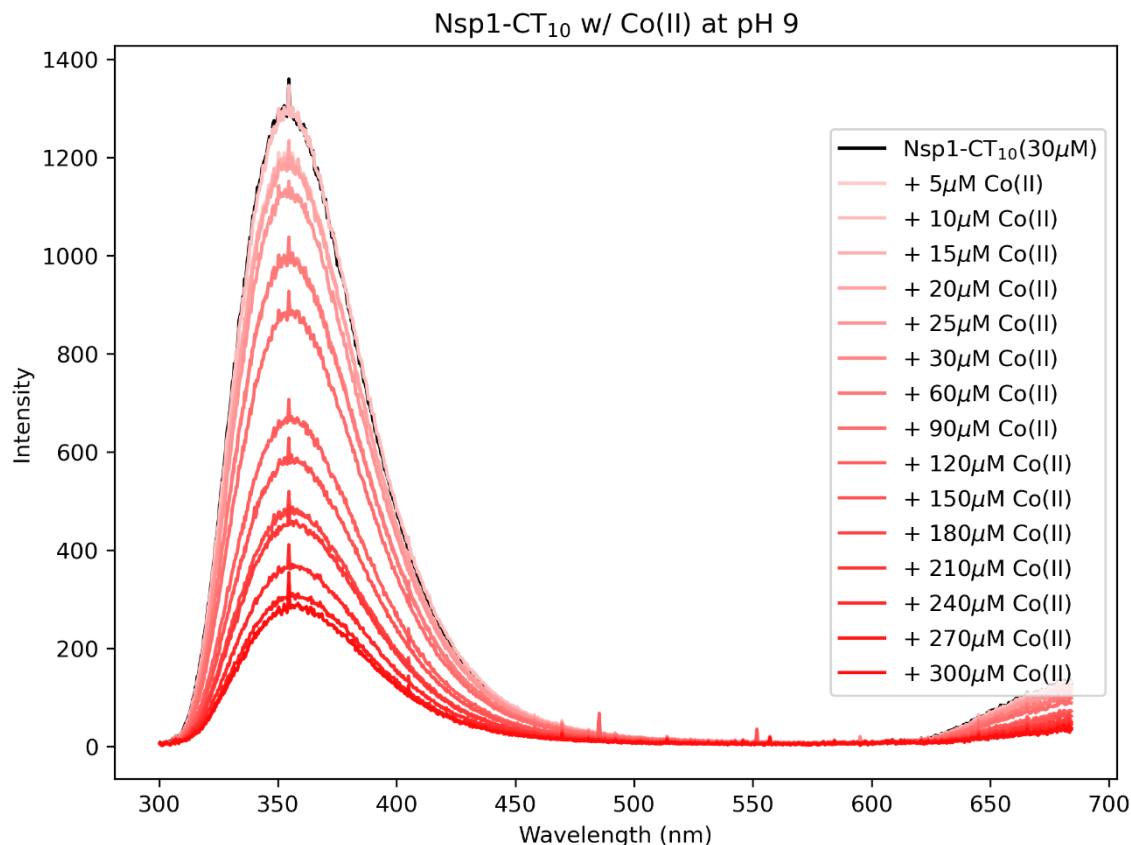


Figure 4.1 Tryptophan emission quenching upon addition of Co(II) to Nsp1-CT₁₀ at pH 9. Measurements were done in 20mM Tris-HCl.

These observations raised the question of whether the observed fluorescence quenching resulted from direct Co(II) binding or from in situ oxidation of Co(II) to Co(III) facilitated by the peptide. The oxidation of Co(II) to Co(III) is a critical step for generating kinetically inert cobalt complexes with biological ligands, including peptides. The standard electrode potential for the Co(III)/Co(II) redox couple in aqueous solution is approximately +1.81 V vs. SHE for the hexaaquo complex $[\text{Co}(\text{H}_2\text{O})_6]^{3+}/^{2+}$ and $[\text{Co}(\text{H}_2\text{O})_6]^{3+}$ is not stable at neutral pH. The $\text{Co}^{3+/2+}$ potential can be significantly lowered by the coordination of strong-field or multidentate ligands that stabilize the low-spin d^6 Co(III) state.

Polyamino-polycarboxylate ligands are known to bind strongly to Co(II) and more strongly to Co(III), leading to dramatic reductions in $\text{Co}^{3+/2+}$ potentials for the ligated Co(II) ions.^{26–28} Amino acid side chains like histidine and cysteine can facilitate this conversion by both stabilizing the oxidized state and promoting inner-sphere electron transfer. The peptide environment, particularly under mildly basic pH conditions, may provide suitable coordination sites that favor

oxidation. Previous studies involving copper revealed that Nsp1-CT₁₀ can act as a polyamino-polycarboxylate ligand. In these systems, the peptide coordinated to copper through both backbone amide nitrogen atoms and nearby carboxylate side chains, forming a stable inner-sphere coordination environment.

Consequently, selecting oxidants for Co(II) $\rightarrow$ Co(III) conversion requires balancing redox potential with biocompatibility, minimizing peptide oxidation or degradation. To clarify whether Co(II) or Co(III) was being observed in these studies, a series of chemical oxidants was tested to intentionally convert Co(II) to Co(III) under controlled conditions. This approach aimed to better understand the role of oxidation state in metal-peptide interactions and to develop experimental strategies for generating and stabilizing Co(III)-peptide complexes. These efforts laid the foundation for a broader investigation into the coordination behavior and redox manipulation of cobalt within biologically relevant systems.

Before attempting oxidation of Co(II), EDTA was used as a chelator to scavenge free Co(II). The rationale was that, upon chelation by EDTA, the coordination state could be inferred based on changes in fluorescence lifetime. Exposure of Co(II)-EDTA complexes to oxygen results in slow oxidation to Co(III).²⁹ To minimize this oxidation, measurements were conducted using degassed samples. Due to rapid ligand exchange kinetics, EDTA readily chelates Co(II), ensuring efficient sequestration before analysis.

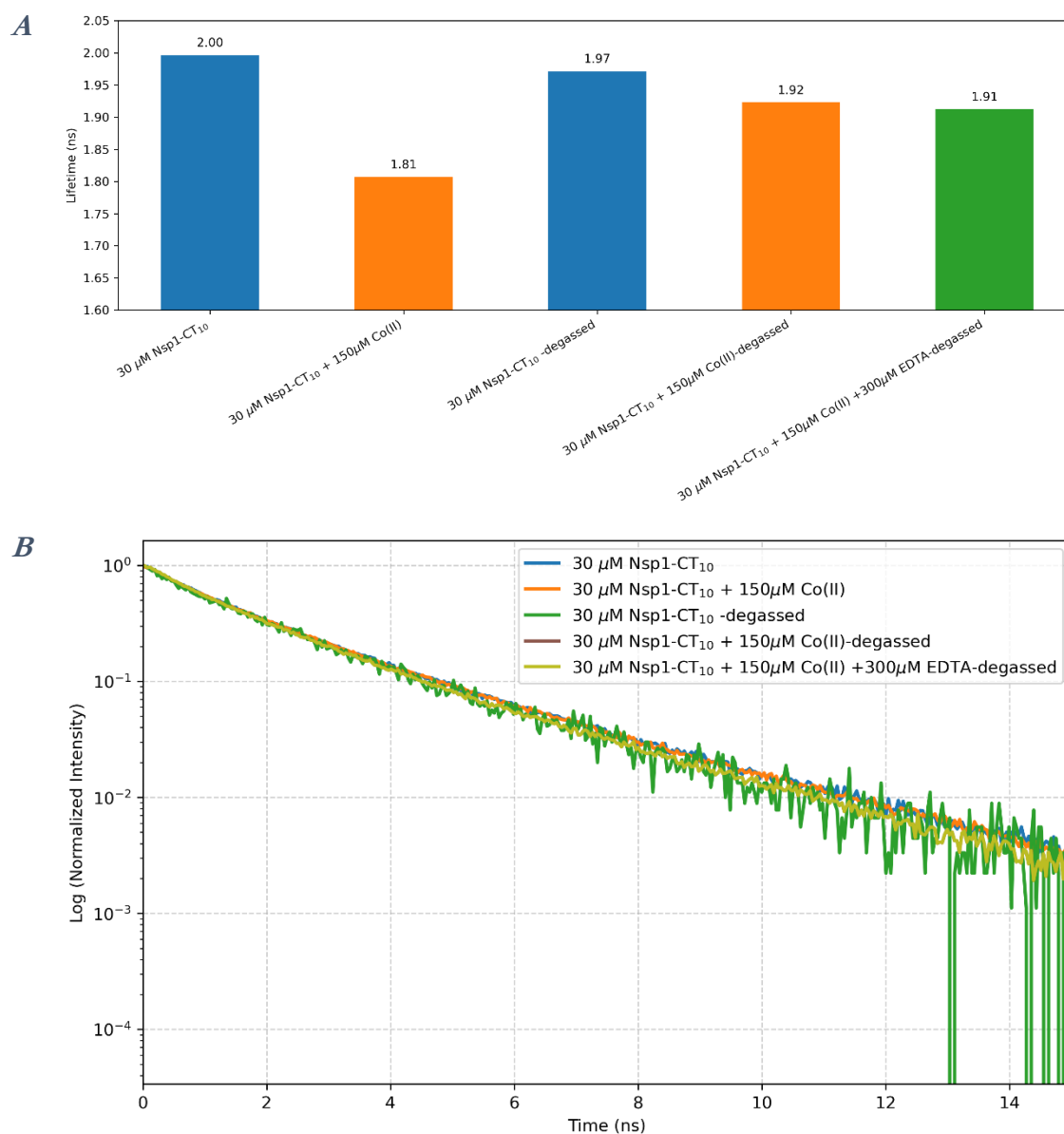


Figure 4.2 Tryptophan Fluorescence Lifetime Changes in Nsp1-CT₁₀ Upon Co(II) Binding A) Tryptophan lifetime changes upon addition of Co(II) to Nsp1-CT₁₀. B) Lifetime decay curves plotted on a log axis. Measurements were done at in 20mM Tris HCl at pH 8.

To probe Co(II) binding to the Nsp1-CT₁₀ peptide, the tryptophan fluorescence lifetimes were measured to assess Co(II) binding and reversibility. The lifetime of the peptide alone was 2 ns. Upon addition of Co(II), a marked decrease to 1.81 ns was observed, indicative of some quenching.

To investigate whether this effect was due to oxidative processes or oxygen-sensitive interactions, the experiment was repeated under degassed conditions. The degassed peptide showed a slightly reduced baseline lifetime of 1.97 ns. Upon addition of Co(II) under these conditions, the lifetime remained quenched at 1.92 ns, which is still within the experimental error. Notably, treatment with EDTA did not restore the lifetime; instead, a similar value of 1.91 ns was observed even after EDTA addition under anaerobic conditions.

Due to relatively minor changes in tryptophan fluorescence lifetimes, variations that were inconsistent across replicates and not reversed by EDTA addition or degassing, this approach was deemed inconclusive for reliably monitoring Co(II) coordination or oxidation state. As a result, the focus shifted to an alternative strategy that treated Nsp1 as a polyamino–polycarboxylato-type ligand, capable of directly coordinating to Co(II). This coordination was expected to lower the formal redox potential of the metal center, thereby enabling *in situ* oxidation to Co(III) and formation of a substitution-inert Co(III)–peptide complex. To investigate this redox transformation, various oxidants were used to study the Co(II) to Co(III) transition in this system.

Nsp1 as polyamino–polycarboxylato-type ligand

To better understand the role of cobalt oxidation state in peptide coordination, a series of oxidants were screened to facilitate the conversion of Co(II) to Co(III). This strategy aimed to generate kinetically inert Co(III)–peptide complexes and to check whether oxidation could promote more stable binding interactions compared to the labile Co(II) species. Although free Co(II) in aqueous solution has a relatively high standard oxidation potential (1.8 V vs SHE), this value can shift significantly when Co(II) is coordinated to biological ligands such as histidine or other peptide residues. Upon binding to the Nsp1-CT₁₀ peptide, the coordination environment stabilizes the metal center and lowers the oxidation potential, making Co(II) more susceptible to oxidation by milder oxidants. With this in mind, oxidants with intermediate redox potentials were selected with the goal of oxidizing Co(II) to Co(III) *in situ*, once bound to the peptide. By converting Co(II) to its low-spin Co(III) form, the metal would “lock in” and become a kinetically inert state, thereby probing stable coordination and enabling further characterization of the metal–peptide interaction.

The selected oxidants were chosen based on their standard redox potentials, solubility in aqueous buffers, and potential compatibility with peptide systems. Table 4.1 summarizes the oxidants tested along with their respective standard reduction potentials.^{30,31}

Table 4.4 Standard reduction potentials of potential Co(II) oxidants that were measured.

<i>Oxidant</i>	<i>Abbreviation</i>	<i>Standard Reduction Potential (E°) vs. SHE</i>
<i>Tris(1'10'-phenanthroline) Co(III) chloride</i>	[Co(phen) ₃]Cl ₃	0.61V
<i>Hydrogen Hexachloroiridate</i> ³²	H ₂ [IrCl ₆]	0.87V
<i>Potassium ferricyanide</i> ³³	K ₃ [Fe(CN) ₆]	0.436V

Testing of tris(1,10-phenanthroline)cobalt(III) chloride [Co(phen)₃]Cl₃ yielded UV-Vis spectra (Figure 4.3) that showed no significant spectral changes, suggesting a lack of oxidation under the experimental conditions. This conclusion is supported by the stability of the ligand-to-metal charge transfer (LMCT) bands observed between 320–350 nm, which remained largely unchanged. The absence of spectral shifts may result from insufficient redox potential or steric hindrance preventing efficient interaction between Co(phen)₃³⁺ and the Co(II)-peptide complex.

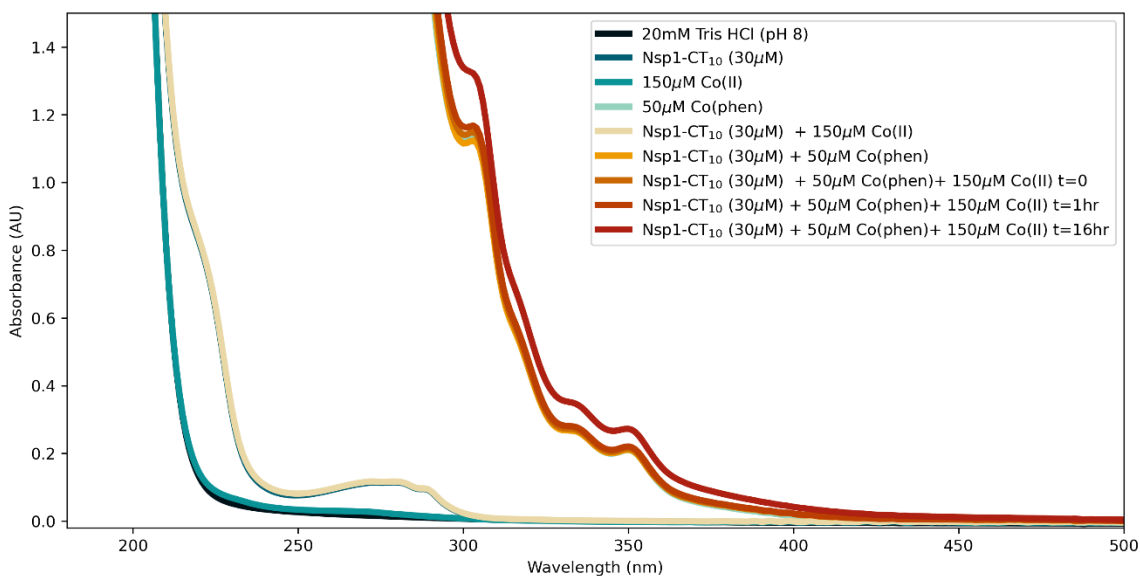


Figure 4.3 UV-Vis spectra of Nsp1-CT₁₀ upon addition of Co(II) with the use of Co(phen)₃ as an oxidant.

To identify an oxidant with a stronger reduction potential, hexachloroiridate(IV) $[\text{IrCl}_6]^{2-}$, which has a standard reduction potential of +0.87 V, was selected. UV-Vis spectral changes indicated that $[\text{IrCl}_6]^{2-}$ was capable of oxidizing both Co(II) and the peptide. In the spectra of samples containing hexachloroiridate (Figure 4.4), the LMCT bands between 400–500 nm decrease in intensity upon reduction of Ir(IV) to Ir(III), consistent with successful redox activity under the experimental conditions. These changes suggest that Co(II) was oxidized, but also raise the possibility that the peptide itself could have possibly been oxidized as well.

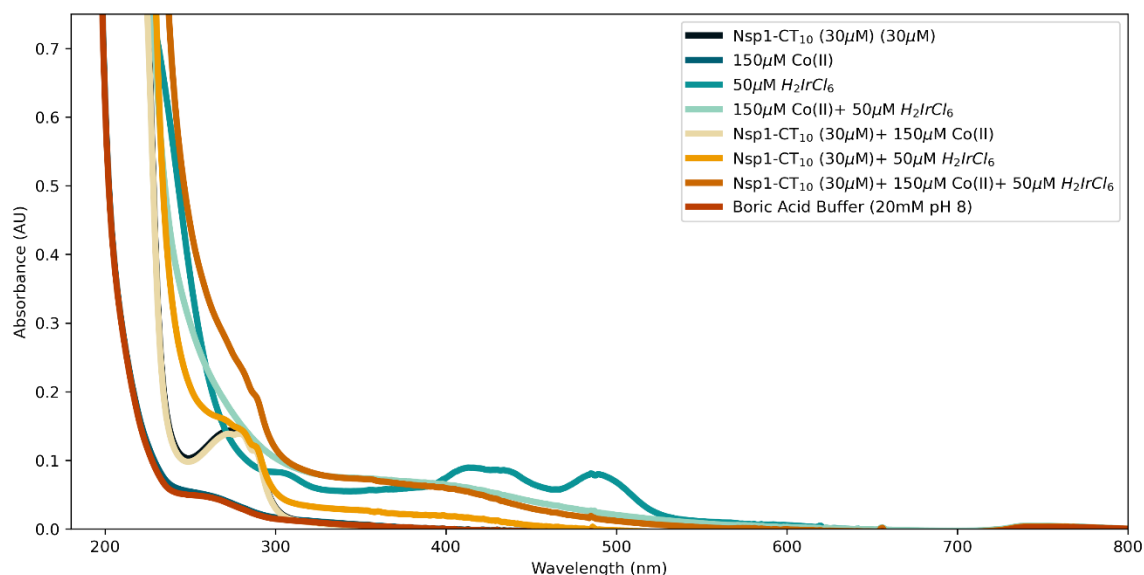


Figure 4.4 UV-Vis spectra of Nsp1-CT₁₀ upon addition of Co(II) with the use of hexachloroiridate as an oxidant.

Further inspection of the spectral data at varying concentrations of hexachloroiridate revealed concentration-dependent changes (Figure 4.5). The most pronounced effects were observed in the 260–290 nm region, where absorbance features associated with the peptide underwent significant alterations as oxidant concentration increased. These changes suggest oxidation-induced modification of the peptide, further supporting that the oxidant was not only targeting Co(II) but also affecting the peptide backbone or side chains. These shifts suggest possible oxidative changes to aromatic residues, such as tryptophan or tyrosine, which could alter peptide structure or metal-binding capacity.

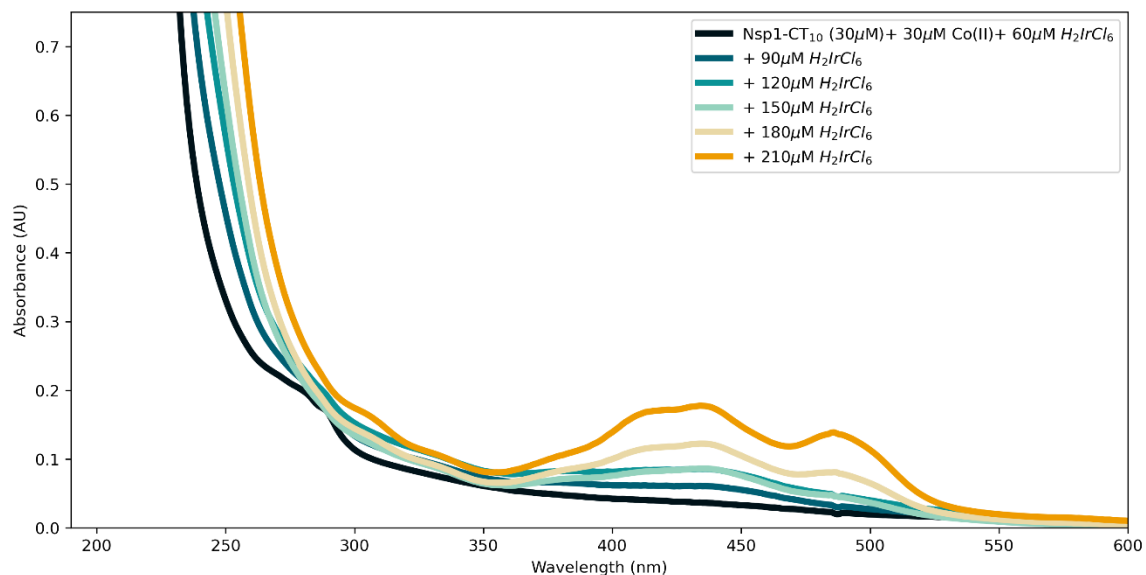


Figure 4.5 UV-Vis spectra of Nsp1-CT₁₀ upon addition of Co(II) with the use of hexachloroiridate as an oxidant in increasing concentrations.

The final oxidant tested was ferricyanide. Similar to hexachloroiridate, significant spectral changes were observed in the 260–290 nm region, corresponding to the peptide absorbance band. These changes indicate that the peptide was also being oxidized under these conditions. These findings indicate that both oxidants, while capable of promoting Co(II) oxidation, lack specificity and risk damaging the peptide scaffold.

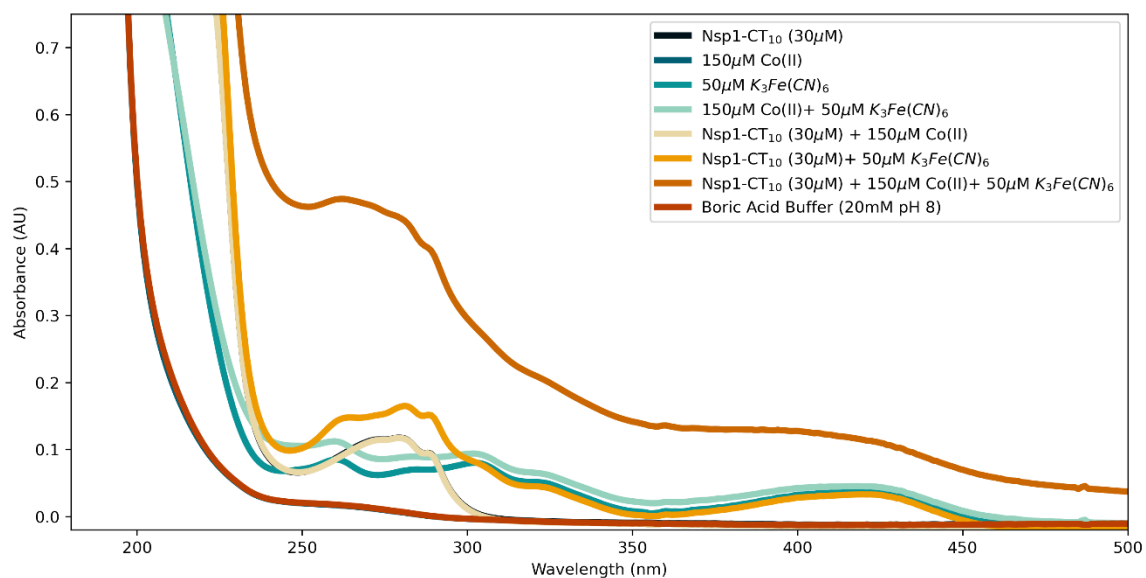


Figure 4.6 UV-Vis spectra of Nsp1-CT₁₀ upon addition of Co(II) with the use of potassium ferricyanide as an oxidant.

Although both hexachloroiridate and ferricyanide were effective in inducing spectral changes, the concurrent oxidation of the peptide indicated poor selectivity for Co(II), ultimately compromising peptide integrity. These results highlight the challenge of achieving selective *in situ* oxidation of Co(II) within this peptide system. The non-specific oxidation observed suggests that direct chemical oxidation may not be a viable strategy for generating Co(III)–peptide complexes under biologically relevant conditions.

Distinguishing between Co(II) and Co(III) complexes can be done using ^{59}Co NMR which has a nuclear spin of $7/2$. Co(III) is a low-spin d^6 diamagnetic system, making it readily observable by NMR, while paramagnetic Co(II), has no detectable signal under standard conditions. Therefore, the presence of a ^{59}Co NMR signal is indicative of successful oxidation to Co(III), while the absence of a signal suggests the complex remains in the Co(II) state. Initial measurements (see Supplemental Information, Figure S4.1-4.2) indicated that relatively high sample concentrations would be required for reliable detection. As a result, preliminary experiments were conducted using imidazole instead of the peptide to test coordination behavior under simplified conditions. This approach assumed that cobalt complexes would primarily coordinate to histidine residues within the peptide, with imidazole serving as a structural and electronic analogue of the histidine side chain.

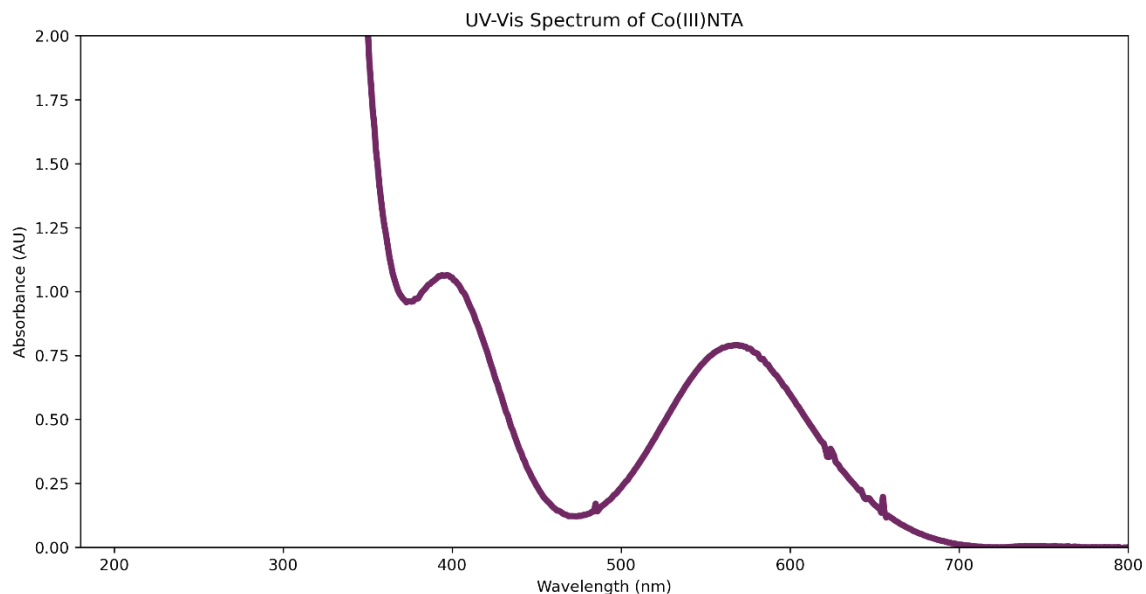


Figure 4.7 Co(III)NTA UV-Vis spectrum depicting bands at 400nm-and 575nm corresponding to d-d transitions.

The first complex we decided to evaluate was cobalt(II/III) nitrilotriacetic acid (NTA). First we synthesized Co(II)NTA (See Supplemental Information for synthesis). The UV-Vis spectrum of Co(III)NTA shows two prominent bands with absorption maxima at 400nm and with rising absorption to the ultraviolet (Figure 4.7). The rising absorption in the 350 nm region corresponds to ligand-to-metal charge transfer (LMCT). These transitions are usually stronger in intensity compared to d-d transitions. The bands at 400 and 575nm correspond to d-d transitions. In Co(III)NTA the complex has an octahedral or distorted octahedral geometry. The 575 nm band likely represents an electronic transition between split d-orbitals ($t_{2g} \rightarrow e_g$) caused by the ligand field around the Co(III) ion. In an octahedral field, the Co(III) ion (which has a low-spin d^6 electron configuration) exhibits these lower-energy d-d transitions, leading to absorption in the visible range.

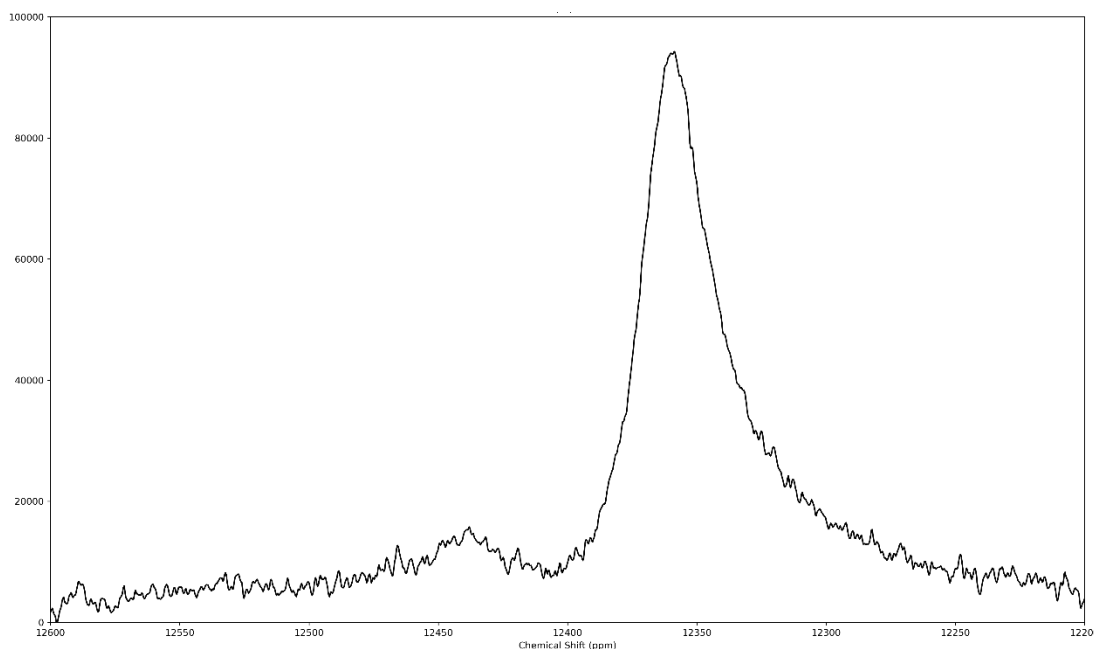


Figure 4.8 ^{59}Co NMR of Co(III)NTA, the sample concentration was 1.5mM. Sample was made in 20mM MOPS buffer at pH 8.5.

The ^{59}Co NMR spectrum of Co(III)NTA exhibits a strong, well-resolved peak at 12,358 ppm (Figure 4.8), consistent with literature-reported values for six-coordinate low-spin Co(III) complexes.²³ Oxygen-donor ligands produce the least shielding due to the large paramagnetic contribution which makes it into the chemical shift. Nitrogen-donor ligands tend to produce higher shielding. The nitrilotriacetic ligand consists of three oxygens and one nitrogen indicating that the cobalt is probably experiencing weak shielding, the single nitrogen in the nitrilotriacetic ligand is most likely responsible for the large chemical shift in the spectra.

Co(II)NTA features two open coordination sites, as the remaining four coordination positions are occupied by three oxygen atoms and one nitrogen atom from the NTA ligand. This arrangement presents the potential for binding to proteins or other ligands. To assess the feasibility of generating Co(III)NTA *in situ*, Co(II)NTA was introduced into a solution containing two equivalence imidazole and subsequently oxidized using hydrogen peroxide at 0.3% by volume. Prior to peptide-based measurements, imidazole was used as a model ligand to simulate potential coordination with histidine residues. Samples were prepared containing 1.5 mM Co(II)NTA, 3 mM imidazole, and 0.3% hydrogen peroxide. The resulting ^{59}Co NMR spectrum displayed a shifted peak at 11,880 ppm (Figure 4.9), suggesting the formation of a

distinct Co(III)-imidazole complex. The reduced signal intensity relative to Co(III)NTA alone indicates that oxidation was incomplete under these conditions, and that a portion of the cobalt remained in Co(II) state. Additionally, the observed chemical shift raised the question of whether one or two imidazole ligands were coordinating to the Co(III) center.

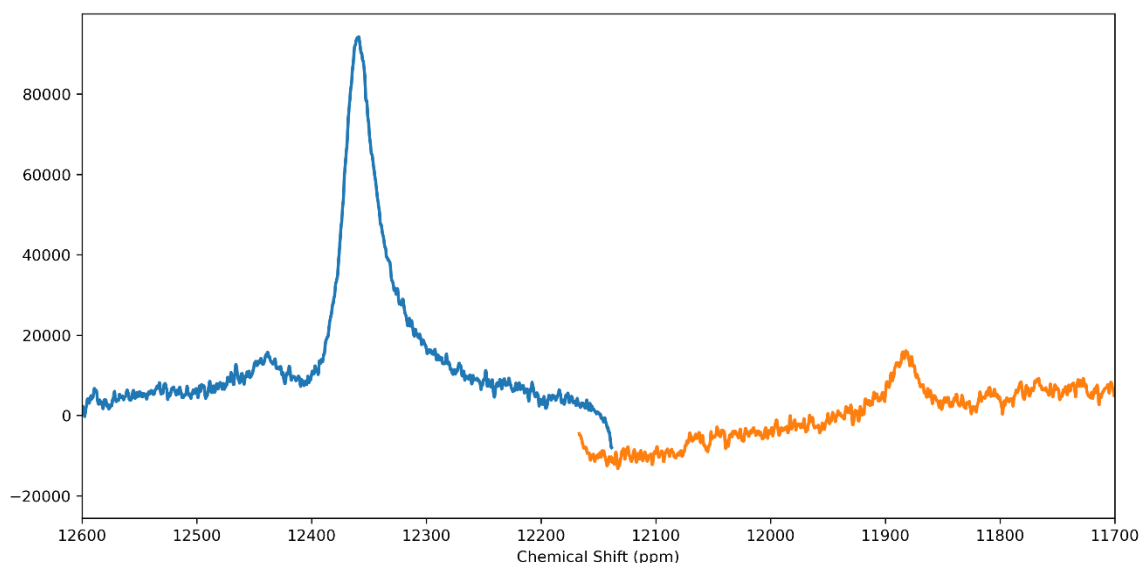


Figure 4.9 ^{59}Co NMR of Co(III)NTA and a sample containing Co(II)NTA, 2eq. Imidazole, and 0.3% hydrogen peroxide. Sample was made in 20mM MOPS buffer at pH 8.5.

Based on the promising results observed with the imidazole model system, similar conditions were applied to the peptide Nsp1-CT₁₀. The resulting ^{59}Co NMR spectrum displayed a peak at 11,880 ppm, matching the chemical shift observed for the Co(III)-imidazole complex (Figure 4.10). This result supports the formation of a Co(III)-peptide complex under the oxidation conditions used. However, the data did not resolve whether one or two histidine residues were coordinating to the cobalt center, leaving the question of coordination stoichiometry unanswered.

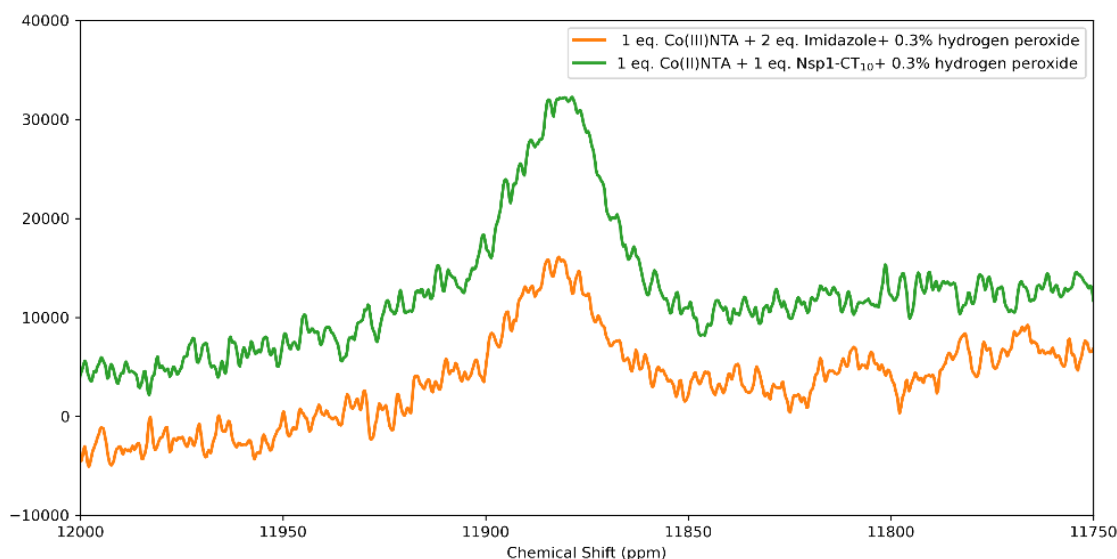


Figure 4.10 ^{59}Co NMR of 1 eq.Co(II)NTA, 2eq. Imidazole, and 0.3% hydrogen peroxide compared to a sample containing Nsp1-CT₁₀ with 1 eq. of imidazole, and hydrogen peroxide. Sample was made in 20mM MOPS buffer at pH 8.5.

There is a known correlation between the electronic spectra of cobalt complexes and their ^{59}Co NMR chemical shifts, specifically with the energy of the first absorption maximum, which reflects the d–d transition. This relationship is explained by the second-order paramagnetic contribution to the chemical shift, as described by crystal field theory.²⁵ The ^{59}Co NMR chemical shift consists of both diamagnetic and paramagnetic components, with the paramagnetic term dominated by the energy of the lowest excited electronic state corresponding to the d–d orbital splitting.

To investigate differences in ligand field strength between cobalt complexes, UV–Vis and ^{59}Co NMR data were compared. Co(III)NTA with a d–d transition maximum at 575 nm (Figure 4.7) exhibited a ^{59}Co chemical shift of 12,358 ppm (Figure 4.8) , while the peptide complex with a 500 nm absorbance for the d–d band transition (Figure S4.3) displayed a shift of 11,880 ppm (Figure 4.10). This trend reflects the established correlation between d–d transition energy and the paramagnetic contribution to the ^{59}Co chemical shift. The paramagnetic term is inversely related to the energy of the lowest excited state.³⁴ A transition at 575 nm corresponds to a lower-energy d–d excitation and a weaker ligand field, resulting in a more downfield NMR shift. Which is expected as the Co(III)NTA has water at the two open coordination sites. The water ligands are weaker field ligand compared to imidazole. Conversely, the 500 nm transition indicates a

stronger ligand field and produces a more upfield chemical shift, corresponding to the imidazole binding.

To determine whether one or two imidazole molecules were coordinating to the Co(III)NTA complex, computational methods were employed to predict ^{59}Co NMR chemical shifts. The calculated chemical shift for the unbound Co(III)NTA complex was approximately 11,839 ppm, differing from the experimental value by roughly 500 ppm. Additional computational calculations predicted that a Co(III)NTA complex coordinated by one imidazole and one water molecule at the open axial positions would yield a signal near 11,610 ppm, approximately 300 ppm lower than the experimentally observed shift. These results suggest that, under experimental conditions, the most likely species is a Co(III)NTA complex coordinated by one imidazole and one water molecule (Table 4.2).

Table 4.2 Predicted ^{59}Co NMR chemical shifts for Co(III)NTA. Ligand corresponds to the possible options aside from the NTA ligands already coordinated. Calculations were carried out by Moon-Young Yang (Prof. William A Goddard's Group).

<i>Ligands in Open Sites</i>	<i>Predicted NMR Chemical Shift (ppm)</i>
<i>Imidazole-Imidazole</i>	10715
<i>Imidazole-H₂O</i>	11610
<i>H₂O- H₂O</i>	11897
<i>H₂O-OH</i>	11839

Given the inconclusive nature of the initial approach, the focus shifted to the second strategy aimed at distinguishing between Co(II) and Co(III) species with greater specificity. This second approach employed a pre-formed Co(III) complex engineered to undergo ligand exchange with Nsp1, wherein labile axial ligands would be displaced to yield a stable and well-defined coordination complex.

Cobalt (III) complexes and direct ligand exchange

To explore cobalt complexes with greater clinical relevance and established ligand substitution behavior, attention shifted to a cobalt(III) Schiff base complex previously evaluated in clinical studies. These complexes were considered promising candidates for inhibiting Nsp1 due to their known ability to bind histidine residues in biological systems. $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ a cobalt(III) Schiff base complex, was selected based on prior investigations into its activity against ocular herpes. Cobalt(III) Schiff base complexes are known to bind histidine residues on enzyme surfaces and within active sites, often without strict sequence specificity.^{35,36} The reactivity and

binding behavior of these complexes are largely controlled by axial ligand substitution, which affects both ligand exchange rates and coordination selectivity.³⁷

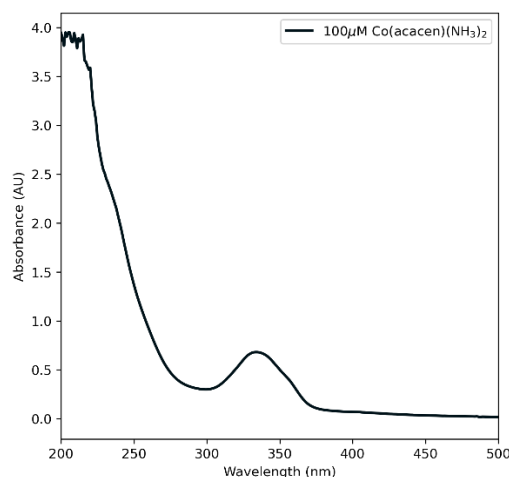


Figure 4.11 UV-Vis spectrum of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$.

The UV-Vis spectrum of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ (Figure 4.11) exhibits prominent features in the 300–360 nm range, which correspond to ligand-to-metal charge transfer (LMCT) transitions from the acacen donor orbitals to the Co(III) d-orbitals. While these spectra do not display any pronounced absorption beyond this region, additional weaker bands near 425 nm, only seen at higher sample concentrations (Figure 4.12) correspond to d–d transitions within the low-spin Co(III) center.

As in previous studies where imidazole interactions were modeled, imidazole was added to $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ to investigate ligand substitution (Figure 4.12). The resulting spectral changes include an increase in the intensity of the weak d–d bands. This enhancement is attributed to changes in ligand field strength, consistent with the progressive substitution of axial ammonia ligands by imidazole. Since imidazole is both a stronger σ -donor and a better π -

acceptor than ammonia, its coordination leads to a stronger ligand field, which results in increased intensity of the d–d transitions.

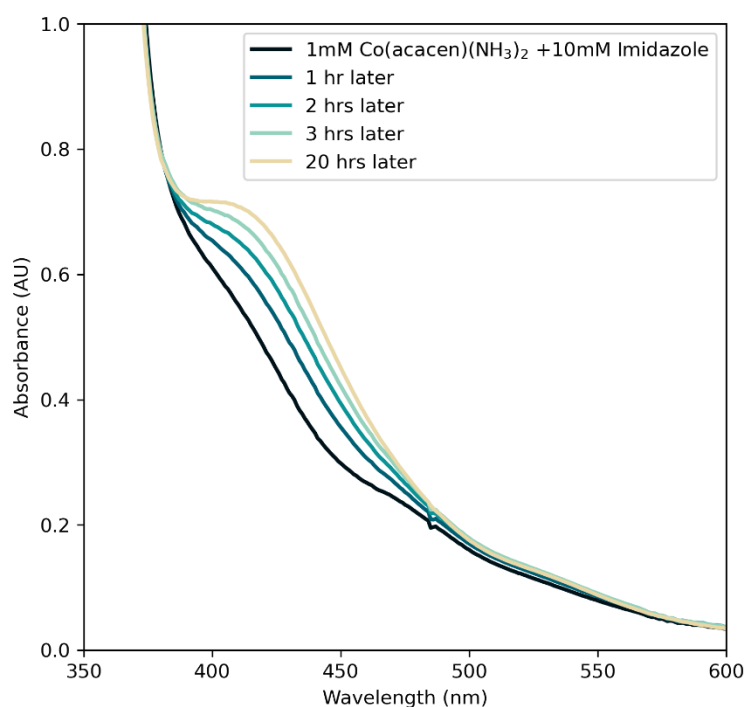


Figure 4.12 UV-Vis Spectrum of $\text{Co}(\text{acacen})(\text{NH}_3)_2$ upon addition of 10-fold excess imidazole throughout the course of 20 hours.

Previous studies³⁸ have reported that displacement of the axial ammonia ligands in $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ by imidazole occurs through a relatively rapid exchange process. However, the current findings suggest otherwise. This was further supported by the ^{59}Co NMR spectrum obtained following imidazole addition, which indicated incomplete or slower substitution under the conditions used.

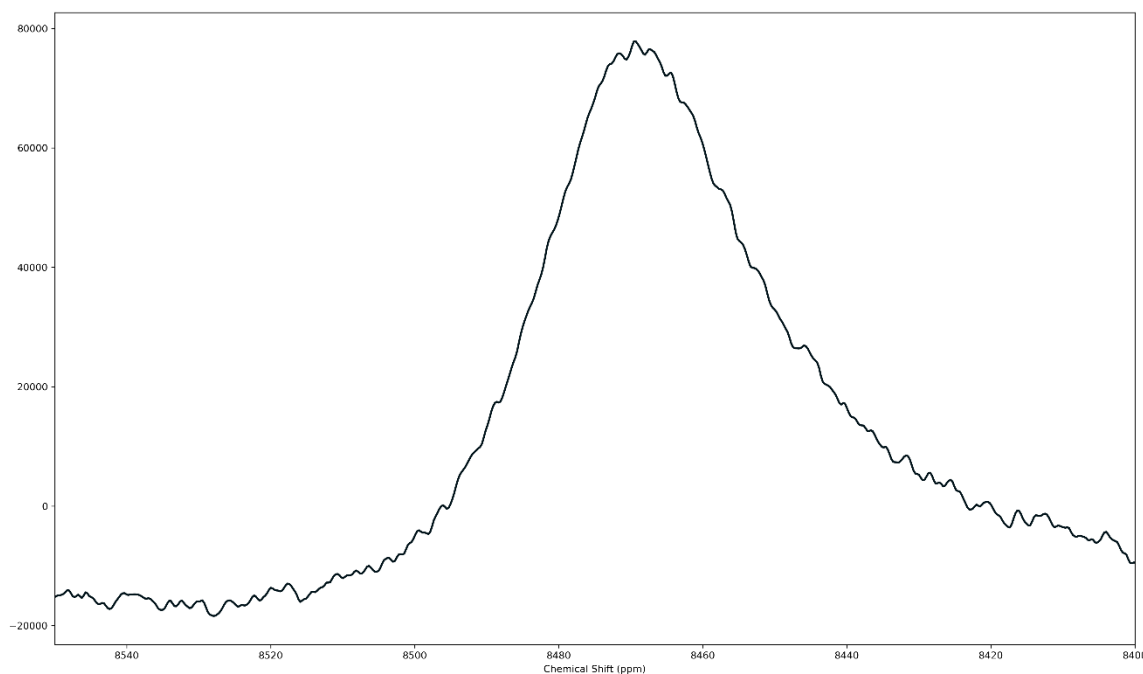


Figure 4.13 ^{59}Co spectrum of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ (10mM). Sample as made in D_2O .

The ^{59}Co NMR spectrum of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ exhibits a single peak at 8468 ppm (Figure 4.13), consistent with the expected low-spin Co(III) complex. Upon addition of a 10-fold excess of imidazole, multiple new signals appeared. The original peak at 8468 ppm remained, indicating the presence of unreacted $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$, while two additional peaks emerged at 8511 ppm and 9086 ppm (Figure 4.14). These new signals suggested the formation of substituted species, potentially involving imidazole and/or aquo ligands. To explore whether aquo substitution was responsible for the 9086 ppm peak, the complex was dissolved in D_2O containing NaOH to adjust the pH to 3.1 prior to data acquisition (Figure 4.15). Under these conditions, the resulting spectrum displayed two peaks: one at 8468 ppm and a second at 9081 ppm. Notably, the 9081 ppm signal did not exhibit the splitting observed previously at neutral pH, however, this may be attributed to a lower conversion rate to the corresponding species, resulting in a weaker signal. The presence of this peak under acidic conditions suggests the formation of a complex containing at least one aquo ligand. The species may involve one ammonia and one water molecule or two water molecules occupying the axial coordination sites. Coordination of water in either position is consistent with the observed downfield shift, as water

is a weaker field ligand compared to ammonia or imidazole and would deshield the cobalt nucleus to a greater extent.

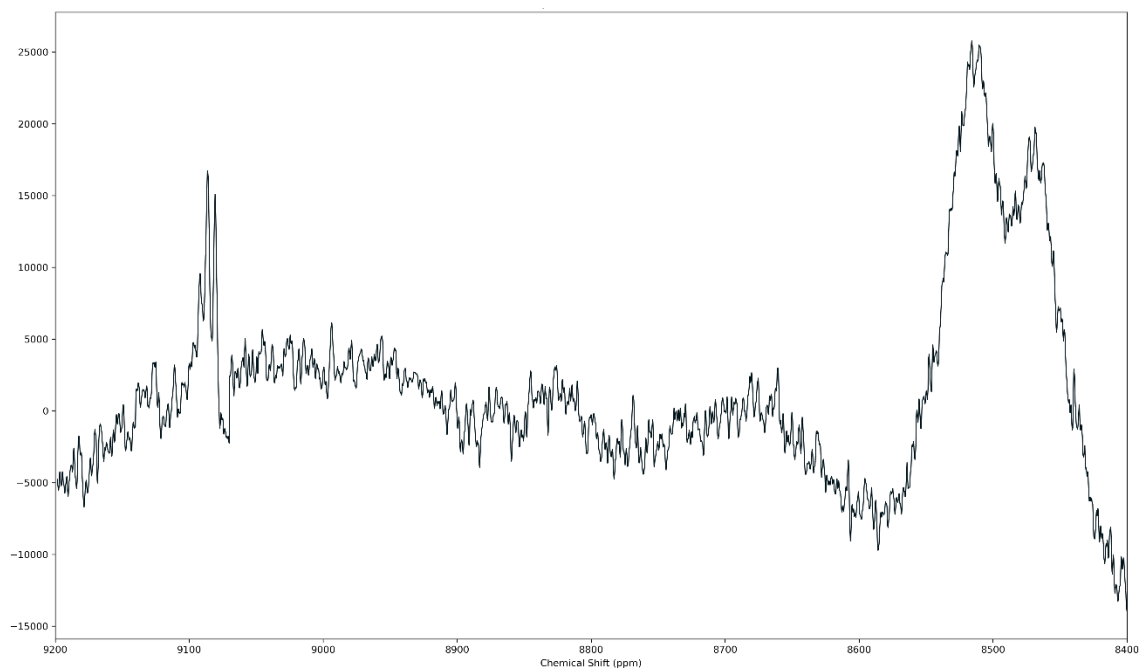


Figure 4.14 ^{59}Co NMR of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ containing a 10-fold excess of imidazole, the sample was measured in D_2O .

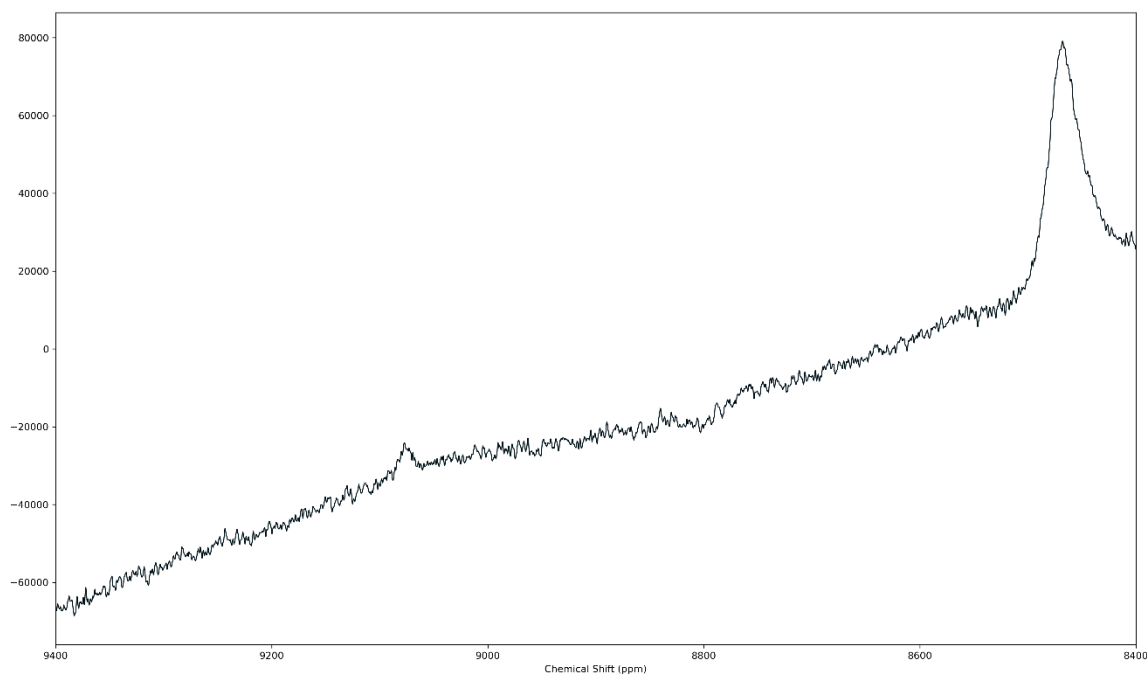


Figure 4.15 ^{59}Co NMR of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ measured in D_2O with the pH adjusted to 3.1 using sodium hydroxide.

The identity of the peak at 8511 ppm remains unresolved. To clarify the assignment of the bis-imidazole complex, $\text{Co}(\text{acacen})(\text{Im})_2$ was independently synthesized, eliminating the need for in situ ammonia displacement. The resulting ^{59}Co NMR spectrum displayed a single peak at 8648 ppm, confirming the identity of the bis-imidazole species (Figure 4.16). While this clarified the assignment of the 8648 ppm signal, it did not resolve the nature of the 8511 ppm peak. Ongoing experiments at the time of writing are aimed at further characterizing this intermediate species.

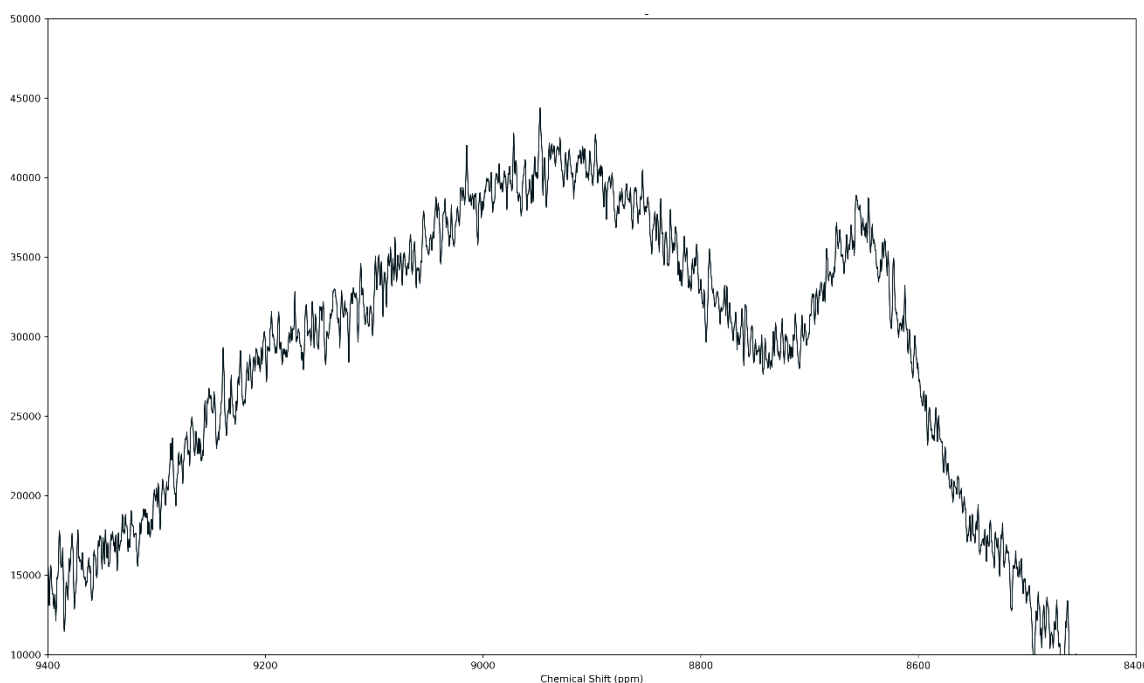


Figure 4.16 ^{59}Co NMR of $\text{Co}(\text{acacen})(\text{Im})_2$ in D_2O .

Computational studies were conducted to investigate the nature of the axial ligands in $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ with the results summarized in Table 4.3. However, many of the calculated chemical shift values deviated significantly from the experimental data, or were too similar across different coordination models, making it difficult to confidently assign each observed peak to a specific species.

Table 4.3 Predicted ^{59}Co NMR chemical shifts for Co(III)(acacen) . Ligand corresponds to the possible options for the axial ligands. Calculations were carried out by Moon-Young Yang (Prof. William A Goddard's Group).

<i>Axial Ligand</i>	<i>Predicted NMR Chemical Shift (ppm)</i>
<i>$\text{H}_2\text{O}-\text{H}_2\text{O}$</i>	12493
<i>$\text{H}_2\text{O}-\text{NH}_3$</i>	9772
<i>$\text{H}_2\text{O}-\text{Imidazole}$</i>	10251
<i>NH_3-NH_3</i>	8505
<i>$\text{NH}_3-\text{Imidazole}$</i>	8752
<i>$\text{Imidazole}-\text{Imidazole}$</i>	9240

Although the Co(acacen) system yielded valuable insights, the results remain partially inconclusive due to the presence of multiple variables and unresolved coordination states. Nonetheless, $[\text{Co(acacen)(NH}_3)_2]^+$ emerged as a promising complex for binding to Nsp1. The following chapter explores this hypothesis through direct experimentation.

Summary and Outlook

This work highlights the potential of cobalt(III) complexes as stable coordination complexes for targeting intrinsically disordered regions of Nsp1. Early attempts to oxidize Co(II) in situ revealed the inherent challenge of balancing redox strength with selectivity, as common oxidants not only facilitated cobalt oxidation but also induced undesirable peptide modifications. These findings highlighted the need for alternative strategies. This led to the successful application of ^{59}Co NMR as a powerful tool to directly distinguish cobalt oxidation states and confirm coordination events.

The exploration of pre-formed Co(III) complexes, particularly Co(III)NTA and $[\text{Co(acacen)(NH}_3)_2]^+$, provided valuable insights into ligand exchange dynamics and histidine-targeted binding. While certain coordination states remain unresolved, such as unidentified species observed in NMR studies, these complexities are still being researched.

Beyond confirming cobalt's ability to engage Nsp1-derived peptides, this study lays important groundwork for advancing metal-based antiviral strategies. The unique stability and inertness of Co(III) complexes offer a compelling platform for disrupting viral protein function, especially within flexible, poorly structured regions that evade traditional small-molecule approaches. In

chapter 5 these findings will be studied in relation to full-length Nsp1, elucidating the structural and functional consequences of cobalt binding, and addressing key challenges such as specificity, capability of inhibiting Nsp1.

Looking forward, these studies will be extended to full-length Nsp1 to evaluate the functional impact of cobalt binding on Nsp1's inhibitory activity could provide critical insights into the therapeutic potential of Co(III)-based compounds. Overall, this work establishes a foundation for the development of cobalt-centered strategies to modulate viral protein function through stable metal coordination.

Supplemental Information

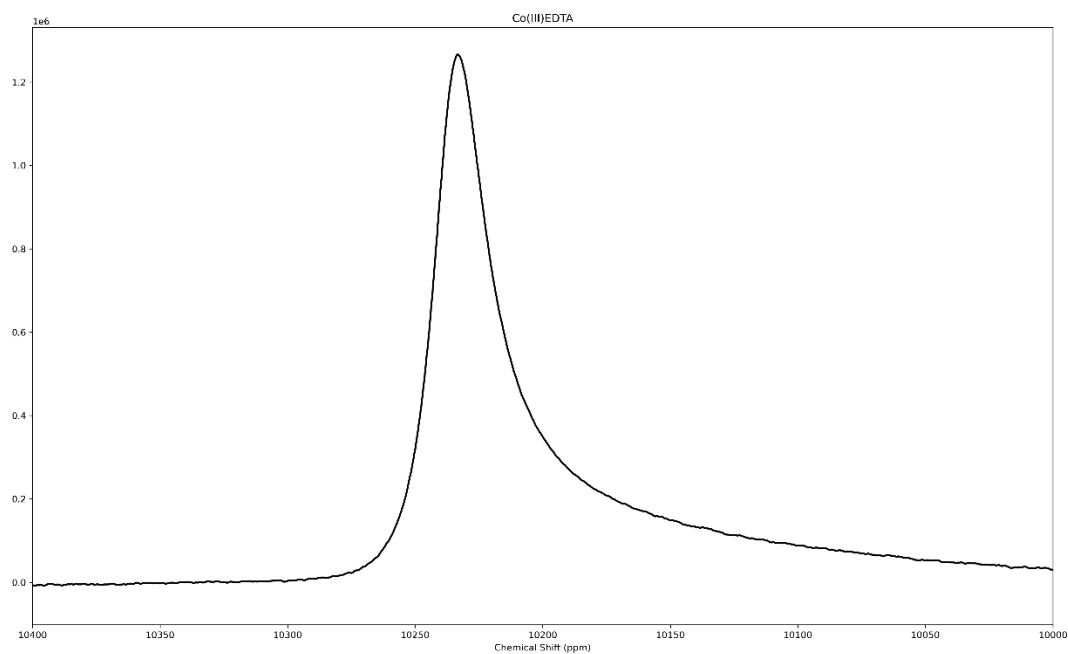


Figure S4.1 ^{59}Co NMR spectrum of Co(III)EDTA . Sample was 40mM Co(III)EDTA in MOPS buffer (20mM pH8.5). Peak is at 10233ppm.

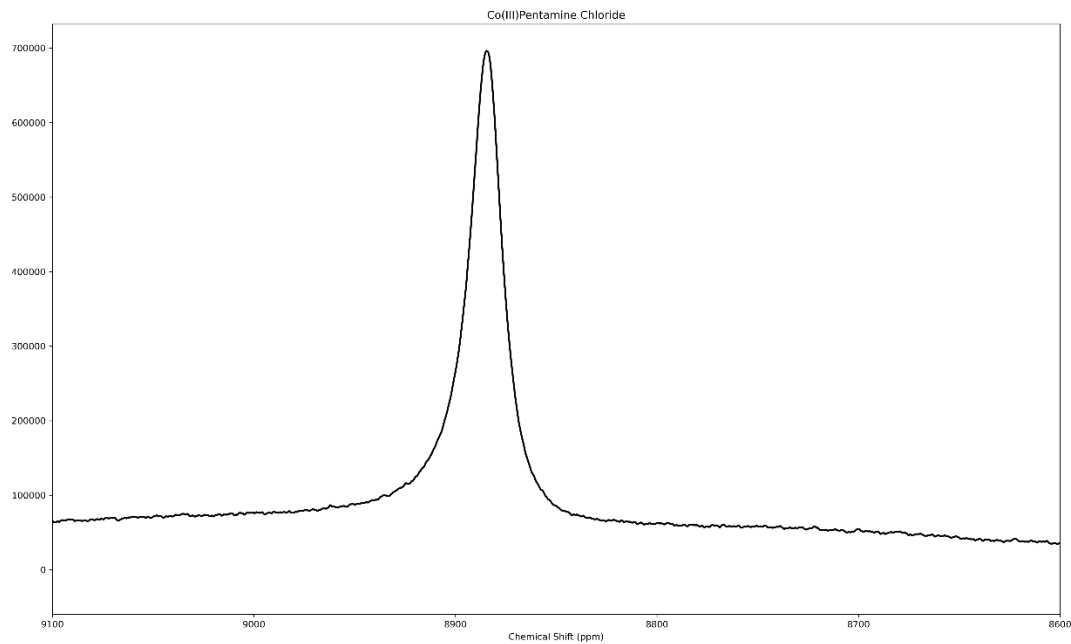


Figure S4.2 ^{59}Co NMR spectrum of $\text{Co(III)(NH}_3)_5\text{Cl}$. Sample was 15mM $\text{Co(III)(NH}_3)_5\text{Cl}$ in MOPS buffer (20mM pH8.5). Peak is at 8884ppm.

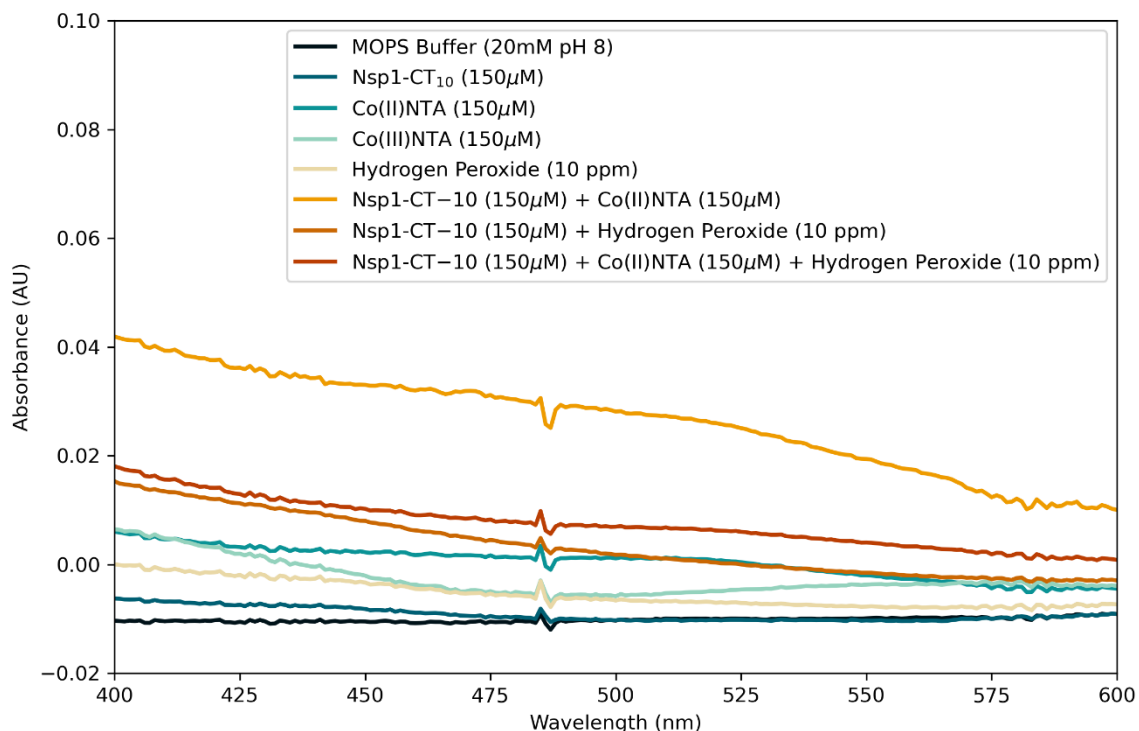


Figure S4.3 Highlighted is the Nsp1-CT₁₀ complex with Co(II)NTA with a 500 nm absorbance for the d-d band transition.

References

- (1) Morales, M.; Ravanfar, R.; Oyala, P. H.; Gray, H. B.; Winkler, J. R. Copper(II) Binding to the Intrinsically Disordered C-Terminal Peptide of SARS-CoV-2 Virulence Factor Nsp1. *Inorg Chem* **2022**, *61* (24), 8992–8996. <https://doi.org/10.1021/acs.inorgchem.2c01329>.
- (2) Morales, M.; Yang, M. Y.; Goddard, W. A.; Gray, H. B.; Winkler, J. R. Copper(II) Coordination to the Intrinsically Disordered Region of SARS-CoV-2 Nsp1. *Proc Natl Acad Sci U.S.A.* **2024**, *121* (20), e2402653121. <https://doi.org/10.1073/pnas.2402653121>.
- (3) Santos, A. J. M.; Khemiri, S.; Simões, S.; Prista, C.; Sousa, I.; Raymundo, A. The Importance, Prevalence and Determination of Vitamins B6 and B12 in Food Matrices: A Review. *Food Chem* **2023**, *426*, 136606. <https://doi.org/10.1016/j.foodchem.2023.136606>.
- (4) Guéant, J.-L.; Guéant-Rodriguez, R.-M.; Alpers, D. H. Vitamin B12 Absorption and Malabsorption. In *Vitamins and Hormones*; Elsevier, 2022; Vol. 119, pp 241–274. <https://doi.org/10.1016/bs.vh.2022.01.016>.

- (5) Mascarenhas, R.; Gouda, H.; Ruetz, M.; Banerjee, R. Human B12-Dependent Enzymes: Methionine Synthase and Methylmalonyl-CoA Mutase. In *Methods in Enzymology*; Elsevier, 2022; Vol. 668, pp 309–326. <https://doi.org/10.1016/bs.mie.2021.12.012>.
- (6) Calderón-Ospina, C. A.; Nava-Mesa, M. O. B Vitamins in the Nervous System: Current Knowledge of the Biochemical Modes of Action and Synergies of Thiamine, Pyridoxine, and Cobalamin. *CNS Neurosci Ther* **2020**, 26 (1), 5–13. <https://doi.org/10.1111/cns.13207>.
- (7) Rangrazi, A.; Mirmortazavi, A.; Imani, R.; Nodehi, D. Effect of Ozone on Corrosion Behavior of a Cobalt–Chromium Alloy Used in Removable Partial Denture Framework: An In Vitro Study. *J Adv Oral Res* **2021**, 12 (2), 304–309. <https://doi.org/10.1177/23202068211015748>.
- (8) Wooten, H. O.; Green, O.; Yang, M.; DeWees, T.; Kashani, R.; Olsen, J.; Michalski, J.; Yang, D.; Tanderup, K.; Hu, Y.; Li, H. H.; Mutic, S. Quality of Intensity Modulated Radiation Therapy Treatment Plans Using a 60 Co Magnetic Resonance Image Guidance Radiation Therapy System. In *J Radait Oncol Biol Phys* **2015**, 92 (4), 771–778. <https://doi.org/10.1016/j.ijrobp.2015.02.057>.
- (9) Eleraky, N. E.; Allam, A.; Hassan, S. B.; Omar, M. M. Nanomedicine Fight against Antibacterial Resistance: An Overview of the Recent Pharmaceutical Innovations. *Pharmaceutics* **2020**, 12 (2), 142. <https://doi.org/10.3390/pharmaceutics12020142>.
- (10) Simonsen, L. O.; Harbak, H.; Bennekou, P. Cobalt Metabolism and Toxicology—A Brief Update. *Sci Total Environ* **2012**, 432, 210–215. <https://doi.org/10.1016/j.scitotenv.2012.06.009>.
- (11) Blanco, A.; Blanco, G. Vitamins. In *Medical Biochemistry*; Elsevier, 2017; pp 645–687. <https://doi.org/10.1016/B978-0-12-803550-4.00027-6>.
- (12) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide. *IARC Monogr Eval Carcinog Risks Hum* **2006**, 86, 1–294.
- (13) Swaminathan, S. Trace Elements, Toxic Metals, and Metalloids in Kidney Disease. In *Nutritional Management of Renal Disease*; Elsevier, 2013; pp 339–349. <https://doi.org/10.1016/B978-0-12-391934-2.00023-0>.
- (14) Peters, N.; Dichgans, M.; Surendran, S.; Argilés, J. M.; López-Soriano, F. J.; Busquets, S.; Dittmann, K.; Rodemann, H. P.; Sindrilaru, A.; Sunderkötter, C.; Watanabe, H.; Roden, D. M.; Feuerstein, G.; Ruffolo, R.; Knöll, R.; Sen-Chowdhry, S.; Ward, D.; McKenna, W. J.; Mogensen, J.; Nadkarni, M. A.; Martin, F. E.; Jacques, N. A.; Hunter, N.; Böhm, M.; Luger, T. A.; Grune,

T.; Longo, N.; Filippo, C. A. D. S.; Schwarz, E. L.; Pasquali, M.; Erasmus, E.; Mienie, L. J.; Deschauer, M.; Zierz, S.; Loots, D. T.; Denson, L. A.; Su, H. C.; Lenardo, M. J.; McDermid, H. E.; Eisenhofer, G.; Calle-Martin, O. D. L.; Casamitjana, N.; Woellner, C.; Grimbacher, B.; Schuppan, D.; Lisch, W.; Seitz, B.; Janecke, A.; McCarthy, T. V.; Wallgren-Pettersson, C.; Haan, J.; Wunderlich, M. T.; Revencu, N.; Vikkula, M.; Honda, A.; Lalani, S. R.; Belmont, J. W.; Borissoff, J. I.; Cate, H. T.; Kasai, T.; Markovich, D.; Trauner, M.; Selmi, C.; Gershwin, M. E.; Trauner, M.; Trauner, M.; Lyons, M. A.; Maurer, K. J.; Carey, M. C.; Denson, L. A.; Lammert, F.; Sauerbruch, T.; Denson, L. A.; Jansen, P. L. M.; Jansen, P. L. M.; Jansen, P. L. M.; Sudhoff, H.; Dahl, S. V.; Ameis, D.; Faiyaz-Ul-Haque, M.; Ejaz Zaidi, S. H.; Silve, C.; Pavone, P.; Trifiletti, R. R.; Asmus, F.; Weckerle, P.; Schwanitz, G.; Busert, B.; Thiagarajah, T.; Muir, W.; Pickard, B.; Cleare, A. J.; Scharnagl, H.; März, W.; Kubitz, R.; Häussinger, D.; Schwenzer, N.; Leung, A. K. C.; Robson, W. L. M.; Wong, A. L.; Güzelcan, Y.; Trotta, F.; Monaco, A. L.; Leung, A. K. C.; Sauve, R. S.; Rozen, T. D.; David, G. L.; Zeldin, D. C.; Rao, P. S.; Molloy, A. M.; Scott, J. M.; Somay, G.; Meo, S. A.; Meo, S. A.; Fierer, J.; Berneburg, M.; Schwarz, T.; Schölmerich, J.; Lampe, A. K.; Bushby, K.; Speake, W. J.; Simpson, J.; Uronis, H. E.; Blobe, G. C.; Franco, D.; Aránega, A.; Stockfleth, E.; Nindl, I.; Hamel, C.; Riepe, F. G.; Strauss, E. C.; Oji, V.; Traupe, H.; Frieling, T.; Cavani, A.; Girolomoni, G.; Brehler, R.; Steinlein, O. K.; Uriu-Adams, J. Y.; Uriu-Adams, J. Y.; Deybach, J.-C.; Puy, H.; Ma, M. L. S.; Ma, P. T. S.; Leung, A. K. C.; Leung, A. A. C.; Ma, P. T. S.; Leung, A. K. C.; A.; Wierzba, J.; Selicorni, A.; Kodolitsch, Y. V.; Ito, W.; Maulik, N.; Voisard, R.; Teragawa, H.; Chayama, K.; Guerrini, R.; Marini, C.; Parrini, E.; Storch, A.; Schwarz, J.; Ständer, S.; Traupe, H.; Leung, A. K. C.; Hon, K.-L. E.; Chu, C.-W. W.; Bodamer, O. A.; Stöckler-Ipsiroglu, S.; Kondoh, T.; Shimokawa, O.; Harada, N.; Moriuchi, H.; Jansen, P. L. M.; Schölmerich, J.; Schulmann, K.; Pox, C.; Schmiegell, W.; Crowe, J. E.; Hatch, N.; Bothwell, M.; Leung, A. K. C.; Willenberg, H. S.; Bornstein, S. R.; Urban, Z.; Borgia, F.; Guarneri, F.; Vaccaro, M.; Metze, D.; Kunzelmann, K.; Mall, M.; Leung, A. K. C.; Leung, A. K. C.; Hon, K.-L. E.; Choi, P. C.-L.; Gahl, W. A.; Knoll, T.; Hesse, A.; Jaksch, M.; Leung, A. K. C.; Sauve, R. S. Cobalt Deficiency. In *Encyclopedia of Molecular Mechanisms of Disease*; Lang, F., Ed.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2009; pp 380–381. https://doi.org/10.1007/978-3-540-29676-8_365.

(15) Mathuber, M.; Schueffl, H.; Dömötör, O.; Karthaler, C.; Enyedy, É. A.; Heffeter, P.; Keppler, B. K.; Kowol, C. R. Improving the Stability of EGFR Inhibitor Cobalt(III) Prodrugs. *Inorg Che.* **2020**, *59* (23), 17794–17810. <https://doi.org/10.1021/acs.inorgchem.0c03083>.

- (16) Failes, T. W.; Cullinane, C.; Diakos, C. I.; Yamamoto, N.; Lyons, J. G.; Hambley, T. W. Studies of a Cobalt(III) Complex of the MMP Inhibitor Marimastat: A Potential Hypoxia-Activated Prodrug. *Chemistry A European J* **2007**, *13* (10), 2974–2982. <https://doi.org/10.1002/chem.200601137>.
- (17) Ma, Y.; Lin, W.; Ruan, Y.; Lu, H.; Fan, S.; Chen, D.; Huang, Y.; Zhang, T.; Pi, J.; Xu, J.-F. Advances of Cobalt Nanomaterials as Anti-Infection Agents, Drug Carriers, and Immunomodulators for Potential Infectious Disease Treatment. *Pharmaceutics* **2022**, *14* (11), 2351. <https://doi.org/10.3390/pharmaceutics14112351>.
- (18) Jungwirth, U.; Kowol, C. R.; Keppler, B. K.; Hartinger, C. G.; Berger, W.; Heffeter, P. Anticancer Activity of Metal Complexes: Involvement of Redox Processes. *Antioxid Redox Signal* **2011**, *15* (4), 1085–1127. <https://doi.org/10.1089/ars.2010.3663>.
- (19) Abass, A. A.; Abdulridha, W. A. M.; Alaarage, W. K.; Abdulrudha, N. H.; Haider, J. Evaluating the Antibacterial Effect of Cobalt Nanoparticles against Multi-Drug Resistant Pathogens. *J Med Life* **2021**, *14* (6), 823–833. <https://doi.org/10.25122/jml-2021-0270>.
- (20) Heffern, M. C.; Yamamoto, N.; Holbrook, R. J.; Eckermann, A. L.; Meade, T. J. Cobalt Derivatives as Promising Therapeutic Agents. *Curr Opin Chem Biol* **2013**, *17* (2), 189–196. <https://doi.org/10.1016/j.cbpa.2012.11.019>.
- (21) Kar, K.; Ghosh, D.; Kabi, B.; Chandra, A. A Concise Review on Cobalt Schiff Base Complexes as Anticancer Agents. *Polyhedron* **2022**, *222*, 115890. <https://doi.org/10.1016/j.poly.2022.115890>.
- (22) Doğan, Ş.; Ince, M.; Sogutlu, F.; Avcı, C. B.; Özel, D.; Yurt, F. Photodynamic Therapy Potential of Cobalt Phthalocyanine in Triple-Negative Breast Cancer. *Polyhedron* **2023**, *245*, 116617. <https://doi.org/10.1016/j.poly.2023.116617>.
- (23) *NMR and the Periodic Table*; Harris, R. K., Mann, B. E., Eds.; Academic Press: London ; New York, 1978.
- (24) Griffith, J. S.; Orgel, L. E. Ligand-Field Theory. *Q Rev Chem Soc* **1957**, *11* (4), 381. <https://doi.org/10.1039/qr9571100381>.
- (25) Yamasaki, A. Cobalt-59 Nuclear Magnetic Resonance Spectroscopy in Coordination Chemistry. *J Coord Chem* **1991**, *24* (3), 211–260. <https://doi.org/10.1080/00958979109407886>.
- (26) Cheng, K. L. Redox Behavior of Cobalt Chelates of Nitrilotriacetic Acid. *Anal Chem* **1958**, *30* (6), 1035–1039. <https://doi.org/10.1021/ac60138a007>.

- (27)Pyreu, D.; Bazanova, M. Thermodynamics of Mixed-Ligand Complex Formation of Metal (II) Complexonates with Imidazole in Aqueous Solution. *Inorganica Chimica Acta* **2016**, *453*, 687–691. <https://doi.org/10.1016/j.ica.2016.09.027>.
- (28)Bürgisser, C. S.; Stone, A. T. Determination of EDTA, NTA, and Other Amino Carboxylic Acids and Their Co(II) and Co(III) Complexes by Capillary Electrophoresis. *Environ Sci Technol* **1997**, *31* (9), 2656–2664. <https://doi.org/10.1021/es970080f>.
- (29)Szecsody, J. E.; Zachara, J. M.; Bruckhart, P. L. Adsorption-Dissolution Reactions Affecting the Distribution and Stability of CoII-EDTA in Iron Oxide-Coated Sand. *Environ Sci Technol* **1994**, *28* (9), 1706–1716. <https://doi.org/10.1021/es00058a024>.
- (30)CRC Handbook of Chemistry and Physics, 88th Ed Editor-in-Chief: David R. Lide (National Institute of Standards and Technology) CRC Press/Taylor & Francis Group: Boca Raton, FL. 2007. 2640 Pp. \$139.95. ISBN 0-8493-0488-1. *J Am Chem Soc* **2008**, *130* (1), 382–382. <https://doi.org/10.1021/ja077011d>.
- (31)Bard, A. J.; Parsons, R.; Jordan, J. *Standard Potentials in Aqueous Solution*, 1st ed.; Routledge, 2017. <https://doi.org/10.1201/9780203738764>.
- (32)Weusten, S. J. C.; De Groot, M. T.; Van Der Schaaf, J. A Comparative Study of the Stability of Hexachloroiridate and Hexacyanoferrate in Electrochemical Mass Transfer Measurements. *Journal of Electroanalytical Chemistry* **2020**, *878*, 114512. <https://doi.org/10.1016/j.jelechem.2020.114512>.
- (33)O'Reilly, J. E. Oxidation-Reduction Potential of the Ferro-Ferricyanide System in Buffer Solutions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1973**, *292* (3), 509–515. [https://doi.org/10.1016/0005-2728\(73\)90001-7](https://doi.org/10.1016/0005-2728(73)90001-7).
- (34)Bramley, R.; Brorson, M.; Sargeson, A. M.; Schaeffer, C. E. Cobalt-59 NMR Chemical Shifts of Cobalt(III) Complexes; Correlations with Parameters Calculated from Ligand-Field Spectra. *J Am Chem Soc* **1985**, *107* (9), 2780–2787. <https://doi.org/10.1021/ja00295a034>.
- (35)Louie, A. Y.; Meade, T. J. A Cobalt Complex That Selectively Disrupts the Structure and Function of Zinc Fingers. *Proc Natl Acad Sci U.S.A.* **1998**, *95* (12), 6663–6668. <https://doi.org/10.1073/pnas.95.12.6663>.
- (36)Blum, O.; Haiek, A.; Cwikel, D.; Dori, Z.; Meade, T. J.; Gray, H. B. Isolation of a Myoglobin Molten Globule by Selective Cobalt(III)-Induced Unfolding. *Proc Natl Acad Sci U.S.A.* **1998**, *95* (12), 6659–6662. <https://doi.org/10.1073/pnas.95.12.6659>.

- (37) Takeuchi, T.; Böttcher, A.; Quezada, C. M.; Simon, M. I.; Meade, T. J.; Gray, H. B. Selective Inhibition of Human α -Thrombin by Cobalt(III) Schiff Base Complexes. *J Am Chem Soc* **1998**, *120* (33), 8555–8556. <https://doi.org/10.1021/ja981191x>.
- (38) Manus, L. M.; Holbrook, R. J.; Atesin, T. A.; Heffern, M. C.; Harney, A. S.; Eckermann, A. L.; Meade, T. J. Axial Ligand Exchange of *N*-Heterocyclic Cobalt(III) Schiff Base Complexes: Molecular Structure and NMR Solution Dynamics. *Inorg Chem* **2013**, *52* (2), 1069–1076. <https://doi.org/10.1021/ic302379j>.

Chapter 5

FULL LENGTH NSP1 STUDIES

Introduction

All of the previous studies have focused on using smaller parts of the whole Nsp1 protein, either a decapeptide (Nsp1-CT₁₀), the 33-residue peptide (Nsp1-CT₃₃), or even using imidazole to model histidine interactions. However, a part is never the whole, meaning that while the studies might predict certain behaviors within the protein it might not be the case in the context of its entirety.

Nsp1 is a 180-amino-acid protein with a molecular weight of approximately 19.8 kDa. Structurally, it consists of three regions: an N-terminal domain, a central linker region, and a disordered C-terminal domain. The core of the protein is composed of a seven-stranded β -barrel, with an α -helix positioned laterally along the barrel and two 3_{10} -helices spanning one end of the structure (Figure 5.1).¹

The protein contains seven histidine residues: H13, H45, H81, H83, H110, H134, and H165 (Figure 5.1). Of these, H13, H45, H81, H83, and H110 are located within the structured N-terminal domain (residues 1–127), while H134 resides in the linker region and H165 is positioned in the disordered C-terminal domain. Notably, H45 lies within a region of high electronegative potential and increased residue clustering.¹ Structural analysis indicates that several histidines, specifically H45, H81, H83, H134, and H165, are surface exposed, potentially facilitating interactions with small molecules or metal complexes. In contrast, H13 and H110 are buried within the globular core of the N-terminal domain. The accessibility and spatial distribution of these histidine residues are particularly relevant to the metal-binding studies described in this chapter, as previous results have demonstrated the capacity of copper and cobalt complexes to coordinate with histidine side chains.

Sequence conservation analyses have revealed that, among 47,427 Nsp1 sequences deposited in the GISAID database as of June 2020, 97.6% share an identical amino acid sequence without mutations. In the minority of sequences containing mutations, these are typically limited to one or two residues. The most frequently observed variant is the D75E substitution, present in only 0.7% of all entries. These findings indicate that the Nsp1 region is highly conserved across SARS-CoV-2 strains.² Additionally, when compared to its homolog in SARS-

CoV, Nsp1 exhibits 84% amino acid sequence identity, suggesting that it maintains similar structural and functional characteristics.

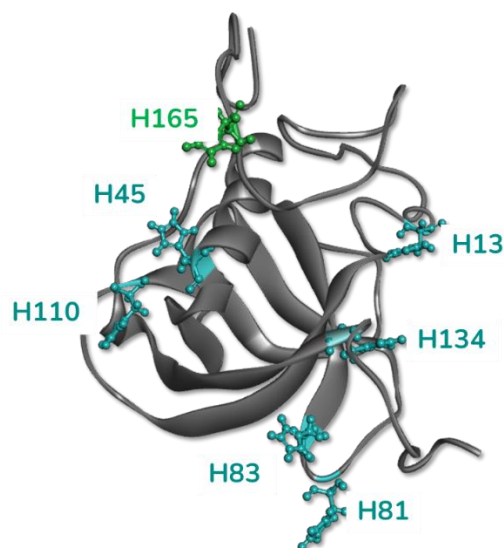


Figure 5.1 Structure of Nsp1, obtained from solution NMR. All histidine residues have been highlighted. In green is His165. PDB:8AOU.

Nsp1 is one of the first viral proteins produced following SARS-CoV-2 infection. After viral entry, the ORF1ab polyprotein is translated and subsequently autoproteolyzed into 16 nonstructural proteins (nsps). Nsp1 is the first of these nsps to be released and rapidly localizes to the host ribosomal machinery. It inhibits translation by binding to the 40S ribosomal subunit, where its intrinsically disordered C-terminal region adopts a helix–turn–helix conformation that occludes the mRNA entry channel.³ This interaction effectively suppresses host protein synthesis while permitting the selective translation of viral RNA.⁴ Within this C-terminal region, residues K164 and H165 are conserved between SARS-CoV and SARS-CoV-2 which are critical for ribosome binding and are essential for the inhibitory activity of Nsp1.⁵

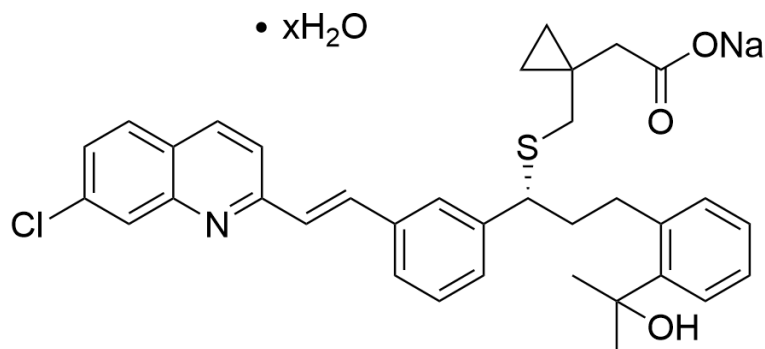


Figure 5.2 Structure of Montelukast sodium hydrate, an FDA approved drug for asthma patients that binds to Nsp1 *in silico* and *in vitro*.

Nsp1 has emerged as a promising drug target for the treatment of SARS-CoV-2, making it important to identify small molecules capable of inhibiting its function. Both *in silico* and *in vitro* studies have identified montelukast sodium hydrate (montelukast) as a potential inhibitor. Montelukast, an FDA-approved drug used to alleviate respiratory symptoms in asthma patients (Figure 5.2),⁶ has demonstrated favorable binding characteristics with Nsp1. Computational docking studies indicate that montelukast interacts with the C-terminal region of Nsp1, particularly at residues P153, P157, N160, K164, H165, and R171—sites that overlap with the ribosome-binding interface. Experimental data further support this interaction, with *in vitro* studies reporting a binding affinity of $K_D = 10.8 \mu\text{M}$.⁷ Although these findings suggest therapeutic potential, additional studies are required to evaluate the efficacy and specificity of montelukast in a physiological context. Moreover, the continued search for alternative drug candidates remains critical to ensure broad treatment accessibility and to support the development of diverse antiviral strategies.

This chapter focuses on investigating the full-length Nsp1 protein through multiple complementary approaches. The first section evaluates a series of drug candidates identified through computational screening, with the goal of preventing Nsp1-mediated inhibition of host translation. Subsequent sections explore the potential of copper(II) and copper(II)-based complexes to modulate Nsp1 function, followed by analogous studies examining the interactions and inhibitory potential of cobalt(II) and cobalt(III) complexes. These studies aim to provide insight into the inhibition of Nsp1 through both small molecule inhibitors and metal-based coordination chemistry.

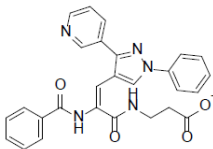
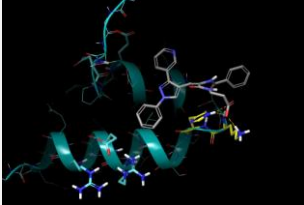
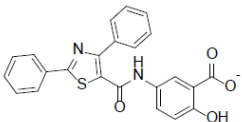
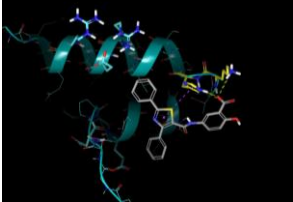
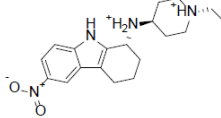
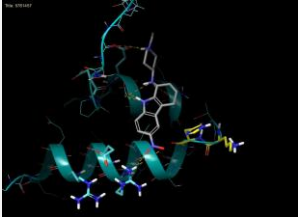
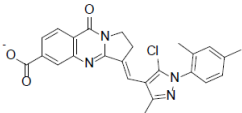
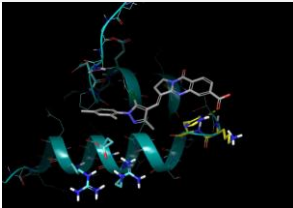
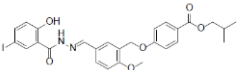
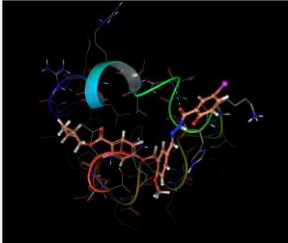
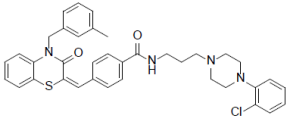
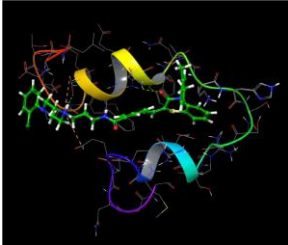
Results and Discussion

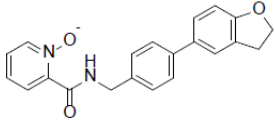
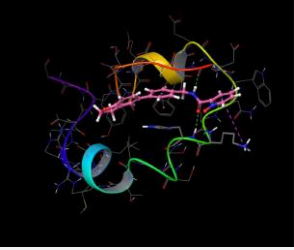
Computationally Screened Nsp1 Inhibitors

A logical initial strategy for identifying small molecules capable of inhibiting Nsp1 function is to perform computational screening of existing compound libraries to identify candidates that may block Nsp1–ribosome interactions. To pursue this, a virtual structure-based screening campaign was carried out in collaboration with Professor William A. Goddard’s group, with Joo-Youn Lee leading the computational work. The cryo-electron microscopy (cryo-EM) structure of Nsp1 bound to the 40S ribosomal subunit (PDB: 6ZLW) was used to generate a receptor grid centered around residues K164 and H165, which are critical for ribosomal binding. A chemical library of approximately 6.2 million compounds from ChemBridge, ChemDiv, and Enamine were screened using molecular docking techniques. Candidate compounds were prioritized based on docking scores and visual inspection of binding poses (see Methods). The top-ranking compounds were subsequently evaluated *in vitro* using a human coupled *in vitro* translation assay (ThermoFisher Scientific), designed to assess the inhibition of Nsp1-mediated translation suppression (see Methods).

The top candidate compounds identified through virtual screening are listed in Table 5.1. These compounds were evaluated using an *in vitro* translation assay. In this system, green fluorescent protein (GFP) is expressed and monitored through its intrinsic fluorescence. In the presence of Nsp1, translation is inhibited, resulting in a marked reduction or complete loss of GFP fluorescence. Therefore, compounds capable of disrupting Nsp1’s inhibitory interaction with the translation machinery would be expected to restore GFP production, as evidenced by a recovery of fluorescence intensity.

Table 5.1 The following data was provided by Joo-Youn Lee in Prof. Goddard's group. The results of the virtual screen computational analysis yielded the following compounds that could potentially inhibit Nsp1 binding to the 40S ribosomal subunit. These compounds were then tested using *in vitro* translation assay.

Structure	Supplier	Catalog No.	Average ΔG	Predicted Binding Position
	Enamine	Z56787287	-67.57	
	Enamine	Z5776335691	-56.07	
	ChemBridge	5781457	-55.45	
	Enamine	Z46646004	-50.23	
	ChemDiv	K087-0282	-79.25	
	ChemDiv	K788-7953	-85.48	

	Enamine Z952743434 -81.99	
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The selected compounds were tested in sets, as each in vitro translation assay kit allowed for eight samples per experiment. As a result, comparisons can only be made within each individual experimental set, relative to its corresponding controls. Two primary controls were included in each assay: the GFP control, consisting of an unaltered reaction that expresses GFP, and the Nsp1 control, which contains 1 μ M Nsp1 and is expected to significantly suppress GFP fluorescence due to inhibition of protein translation. The concentration of 1 μ M Nsp1 was selected based on prior studies demonstrating that even at low micromolar levels, Nsp1 is sufficient to inhibit translation.⁴

Each compound was also tested individually in the absence of Nsp1 to confirm that the compound alone did not affect GFP expression or fluorescence. To evaluate whether a compound could inhibit Nsp1 activity, fluorescence recovery, compared to the Nsp1 control, was used as an indicator of translation restoration.

The results of these in vitro assays are shown in Figure 5.3. Although some variability was observed across the three experimental sets, likely due to differences between assay kits, consistent inhibition of GFP expression was observed in all Nsp1-containing samples, confirming the assay's functionality.

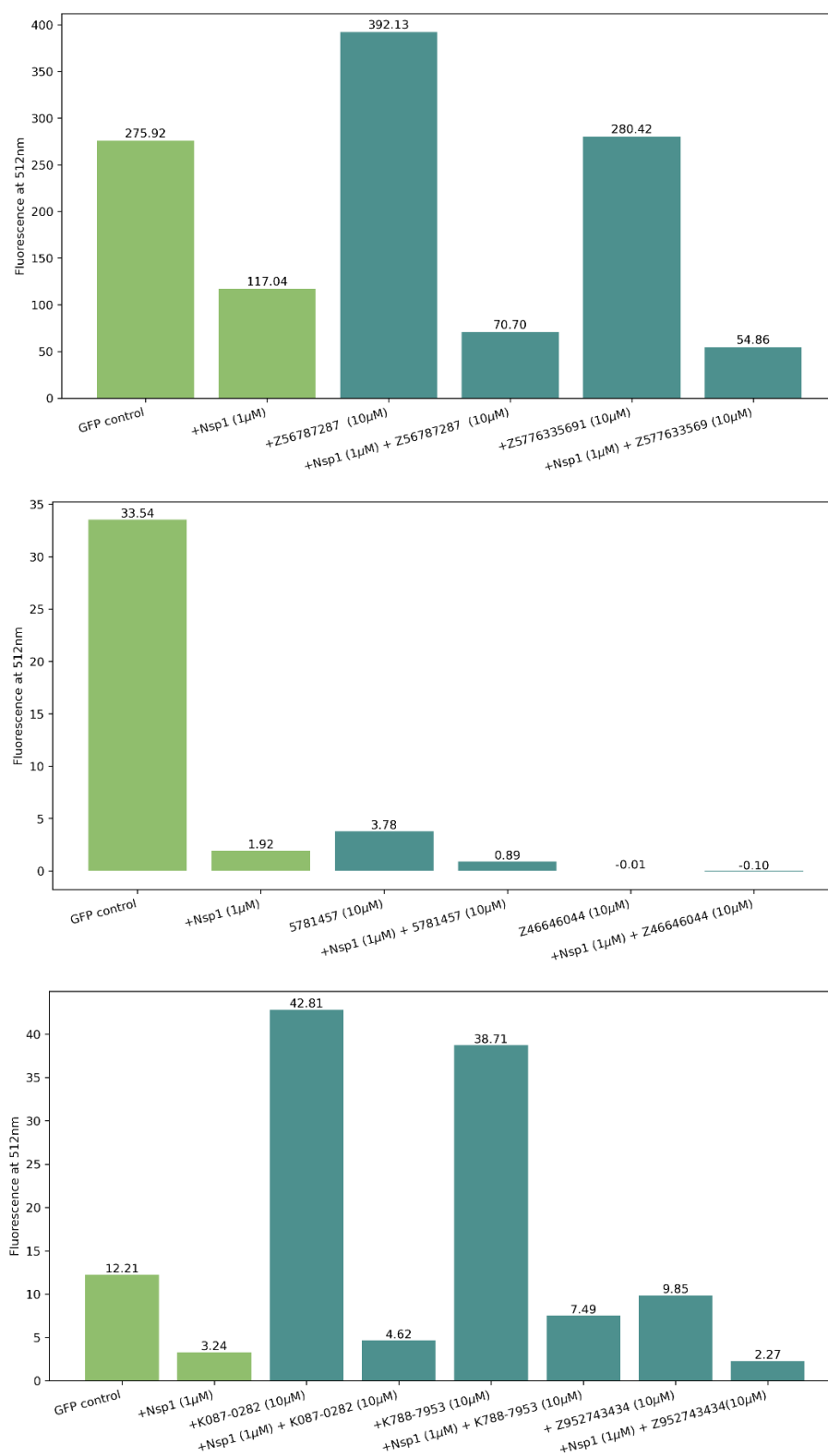


Figure 5.3 Above are the results of the 7 compounds that Joo-Youn Lee had screened as potential Nsp1 inhibitors. Experiments were done as three separate sets due to the limitations of the assay. All compounds were dissolved in MiliQ water unless insoluble in water then the samples were dissolved in dimethyl sulfoxide (DMSO).

The results from the in vitro translation assays showed varied outcomes and are summarized below.

In experiments involving compounds Z56787287, Z5776335691, K087-082, and K788-7953, a consistent trend was observed: each compound, when tested alone, led to an increase in GFP fluorescence relative to the GFP control. This suggests that these compounds may enhance translation efficiency or stabilize GFP expression under assay conditions. However, when tested in the presence of Nsp1, none of these compounds were able to restore fluorescence to the level of the GFP control. This indicates that they are ineffective at counteracting Nsp1-mediated translation inhibition.

Compounds 5781457 and Z952743434 displayed a different behavior. In the absence of Nsp1, both resulted in reduced GFP fluorescence compared to the control, suggesting that they may themselves interfere with the translation process. When combined with Nsp1, an even further reduction in fluorescence was observed, indicating that these compounds not only inhibit translation but also fail to prevent Nsp1-induced translational shutdown.

Compound Z4664604 exhibited the most severe inhibitory effect. On its own, it completely suppressed GFP expression, and the addition of Nsp1 had no significant further impact. The observed negative fluorescence value may be attributed to experimental variability or baseline correction error.

One important consideration that arose during these studies was the solubility of certain compounds. Several candidates were not soluble in aqueous buffer and were therefore dissolved in DMSO prior to their addition to the reaction mixture. To determine whether DMSO affected the assay, control experiments were performed using equivalent volumes of DMSO in the absence of compounds. These controls confirmed that DMSO had no effect on GFP fluorescence (Supplemental Information, Figure S5.1).

In conclusion, none of the computationally screened compounds tested in this study were capable of inhibiting Nsp1 function or restoring translation in the presence of Nsp1. This highlights the challenges of targeting Nsp1.

The lack of promising results from the computationally screened compound libraries prompted a shift toward metal-based inhibitors. The following sections focus on the exploration of copper(II) and cobalt(II/III) complexes as potential candidates for binding to Nsp1. These studies were designed to investigate whether metal coordination could disrupt Nsp1 function and offer a novel avenue for therapeutic intervention.

Aquo Copper(II) Binding to Full Length Nsp1

Previous studies demonstrated that aquo copper(II) complexes could bind to Nsp1 peptides, particularly through coordination with histidine 165 (H165). However, these initial studies were performed using truncated peptides in which H165 was the only histidine present. In contrast, the full-length Nsp1 protein consists of 180 amino acids and contains seven histidine residues: H13, H45, H81, H83, H110, H134, and H165.

Initial studies employed AlphaFold3⁸ to predict potential copper(II) binding sites within the full-length Nsp1 protein. The top-ranked model suggested that the copper(II) ion localizes near H110 and H45, however, no direct coordination to histidine residues was observed (Figure 5.4).

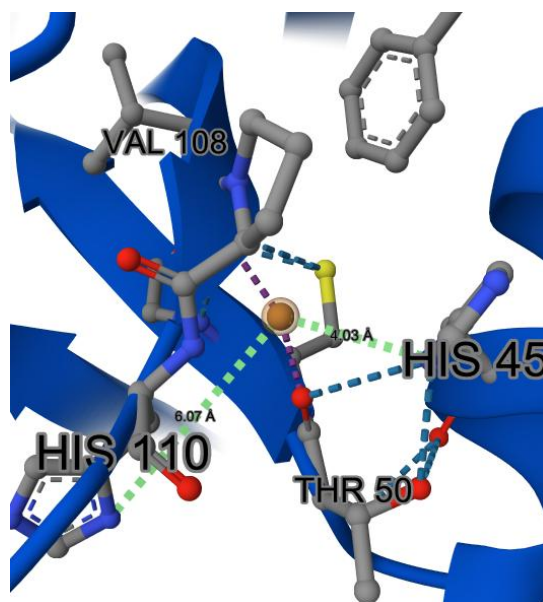


Figure 5.4 AlphaFold3 prediction of aquo copper(II) binding to Nsp1. The closest histidine residues to the modeled copper ion are H110 and H45. Although no direct coordination is predicted, the distance between the imidazole nitrogen of H110 and the copper ion is 6.07 Å, and 4.03 Å for H45. Dashed green lines indicate these distances. The only predicted metal coordination involves the carbonyl oxygens of Val108 and Thr50, shown as dashed purple lines.

While AlphaFold3 predictions placed copper(II) near H110 and H45, the observed distances, 6.07 Å from the imidazole nitrogen of H110 and 4.03 Å from that of H45, are significantly longer than typical metal–ligand coordination bonds, which for Cu(II) usually range from 2.0 to 2.5 Å.^{9,10} These distances suggest that, under the modeled conditions, direct coordination to these histidine residues is unlikely. The only predicted coordination interactions involve the carbonyl oxygens of Val108 and Thr50, highlighting potential backbone-mediated binding rather than side chain coordination.

Due to the proximity of several histidine residues to the modeled copper center, experimental studies were necessary to determine which, if any, of these residues contribute directly to copper coordination. To investigate whether copper(II) maintains its preference for H165 in the full-length context or whether alternative histidine residues could serve as binding sites, full-length Nsp1 was expressed in *E. coli*, and a site-directed mutagenesis was employed.

Seven single-point mutants were designed, each substituting one histidine residue to probe its role in copper coordination. For residues H45, H83, H110, H134, and H165, histidine was replaced with alanine, following standard mutagenesis practice. However, for H13 and H81, attempts to generate alanine mutants via site-directed mutagenesis were unsuccessful, as colonies failed to grow after transformation. As a result, alternative amino acids were used for substitutions at these positions.

To validate the critical role of H165 in Nsp1-mediated translational inhibition, its relevance was assessed using the same *in vitro* translation assay applied to the computationally screened drug candidates. This approach allowed for direct evaluation of the impact of the H165 mutation on Nsp1 function. The results of this assay are presented in Figure 5.5.

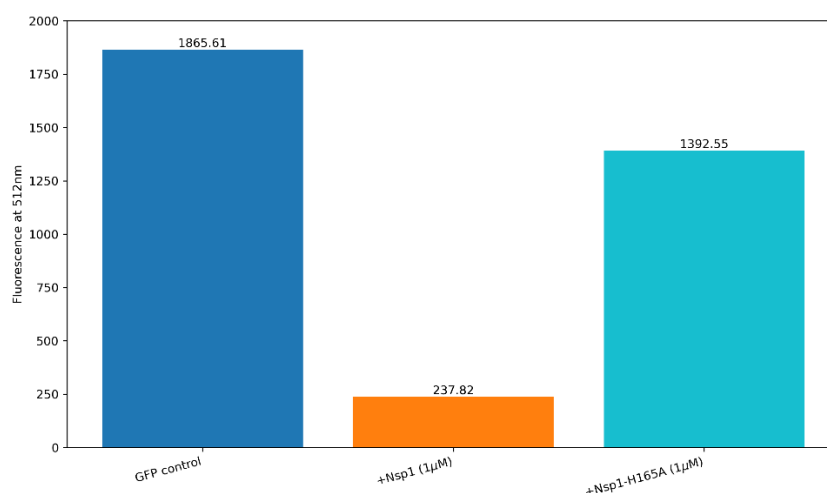


Figure 5.5 *In vitro* assay results when testing the His 165 mutant.

The results from the assay support previous findings that identify H165 as a critical residue for Nsp1-mediated translational inhibition.¹¹ Mutation of H165 led to partial recovery of GFP fluorescence, indicating a reduction in Nsp1's inhibitory activity. However, the fluorescence did not return to control levels, suggesting that additional factors contribute to translation suppression. Residue K164 has also been shown to play an important role in Nsp1 function, which likely accounts for the residual quenching observed in the H165 mutant.

Each histidine-to-alanine mutant was evaluated for copper(II) binding using tryptophan fluorescence quenching and electron paramagnetic resonance (EPR) spectroscopy. EPR measurements were conducted on five of the seven mutants; H81 and H45 were excluded due to insufficient protein yield following expression and purification. The EPR spectra for the remaining mutants revealed no significant differences compared to wild-type Nsp1 (Supplemental Information, Figure S5.2).

To gain further insight, tryptophan fluorescence quenching experiments were performed on all seven mutants. These studies enabled the detection of changes in copper(II) binding affinity that might not be captured by EPR alone. The results, summarized in Figure 5.6, were used to assess whether the copper-binding behavior observed in peptide models translates to the

full-length Nsp1 protein. Dissociation constants were calculated from the quenching curves to quantify the relative binding affinities of copper(II) to each mutant.

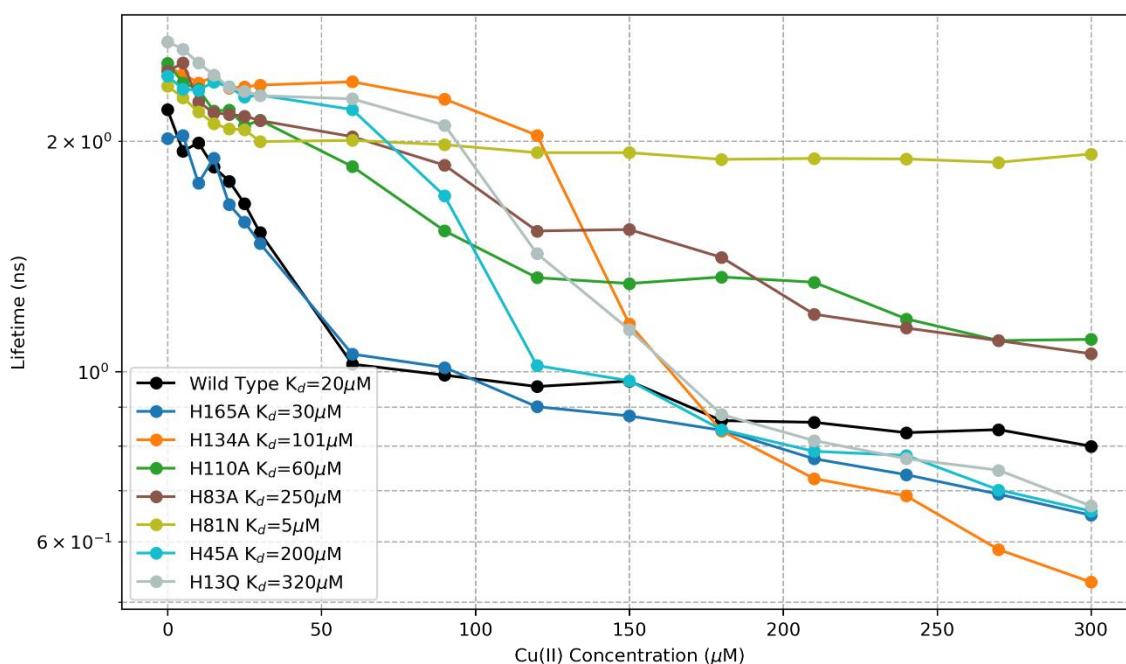


Figure 5.6 Tryptophan fluorescence quenching upon addition of aquo copper(II) to single mutants of Nsp1. The initial protein concentration is 30 μ M, all samples were made in MOPS buffer (20mM, pH 7.5).

It is difficult to draw definitive conclusions about the precise copper(II) binding sites from the tryptophan quenching data alone, however, several key observations can be made. First, H165 does not appear to be involved in copper(II) coordination. This is supported by the fact that the fluorescence quenching profile of the H165A mutant (dark blue trace in Figure 5.5) closely resembles that of the wild-type protein (black trace), with nearly identical kinetics and dissociation constants. In contrast, mutations at H13, H45, H83, and H134 significantly impair copper(II) binding, as seen by much higher dissociation constants relative to the wild type. These results suggest that these four residues play a critical role in coordinating copper(II), either directly or by maintaining a structural environment favorable for binding.

The H81N mutant presents a particularly interesting case. Unlike other mutations that weaken copper binding, this mutation appears to increase affinity, as seen by a lower dissociation constant compared to the wild type. Moreover, the quenching curve for H81N shows an initial steep decrease followed by a pronounced plateau, which may suggest that copper(II) binds

tightly to a high-affinity site before saturating or losing access to additional lower-affinity sites. It is possible that the H81N mutation induces a local conformational change that enhances accessibility or stabilizes a high-affinity binding geometry elsewhere in the protein. Alternatively, removing H81 may eliminate a weak or non-productive interaction, allowing copper(II) to preferentially engage with more effective ligands.

An important consideration in these copper studies was determining whether the observed quenching resulted primarily from intrachain diffusion or electron transfer. To address this, experiments were conducted under conditions of increased solvent viscosity by the addition of glycerol (Figure 5.7). The decrease in quenching efficiency observed with higher viscosity indicates that intrachain diffusion contributes significantly to the quenching mechanism. In this case, intrachain diffusion describes the ability of the tryptophan residue to sample conformations that bring it into close proximity with the copper (II) binding site. As solvent viscosity increases, this internal motion becomes slower, reducing the likelihood of such encounters.¹² This behavior is consistent with a conformationally gated process, where quenching occurs only when the protein adopts a configuration that allows close contact between the donor (tryptophan) and acceptor (copper). In this context, electron transfer (ET) refers to the transfer of an electron from the excited-state tryptophan to the copper (II) center, leading to quenching of fluorescence. If the rate-limiting step were the electron transfer itself, quenching would not be as strongly affected by solvent viscosity. Instead, the data suggests that the rate-limiting step is the conformational rearrangement required to bring the donor and acceptor into contact.

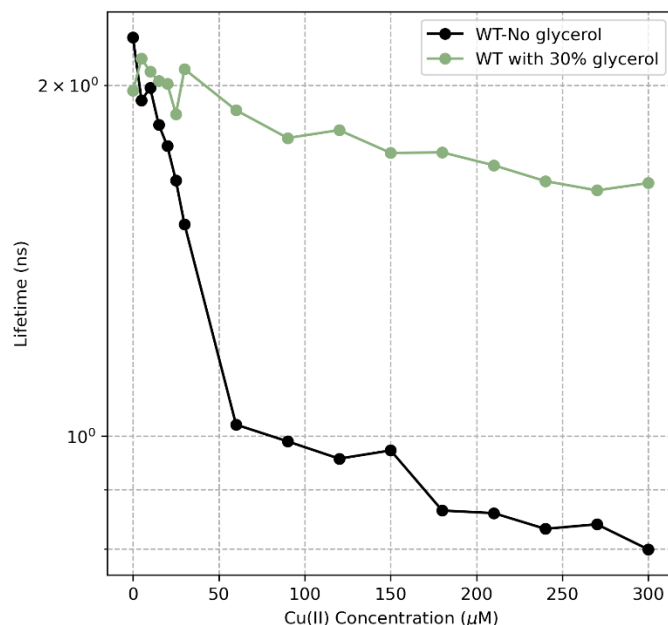


Figure 5.7 Viscosity-dependent quenching of tryptophan fluorescence in wild-type Nsp1 upon copper(II) addition. Increasing solvent viscosity resulted in decreased quenching efficiency, suggesting that the quenching process is limited by intrachain diffusion.

At the time of writing, there are still no definitive conclusions regarding which histidine residue(s) copper(II) coordinates to. However, a collaboration has been established with Dr. Paola Turano at the Magnetic Resonance Center and the Department of Chemistry at the University of Florence. Ongoing experiments are being conducted using uniformly ^{15}N -labeled Nsp1 to monitor copper(II) binding. Since Nsp1 has previously been assigned by NMR, similar experiments are being planned to detect residue-specific perturbations upon copper(II) coordination.¹³

Copper(II) Complexes and Nsp1 Inhibition

Following the approach used to evaluate computationally screened compounds, two copper(II) complexes previously studied with Nsp1-CT peptides were selected for testing in the full-length protein system. The goal was to determine whether these complexes could coordinate with histidine residues within Nsp1 and thereby prevent its interaction with the ribosome. The two selected complexes were copper(II) iminodiacetate (Cu-IDA) and copper(II) histidinate. Preliminary studies using peptide models demonstrated that Cu-IDA exhibited stronger binding affinity to Nsp1-CT33 compared to aquo copper(II), while

copper(II) histidinate showed slightly weaker binding than aquo copper(II) when tested with Nsp1-CT₁₀.

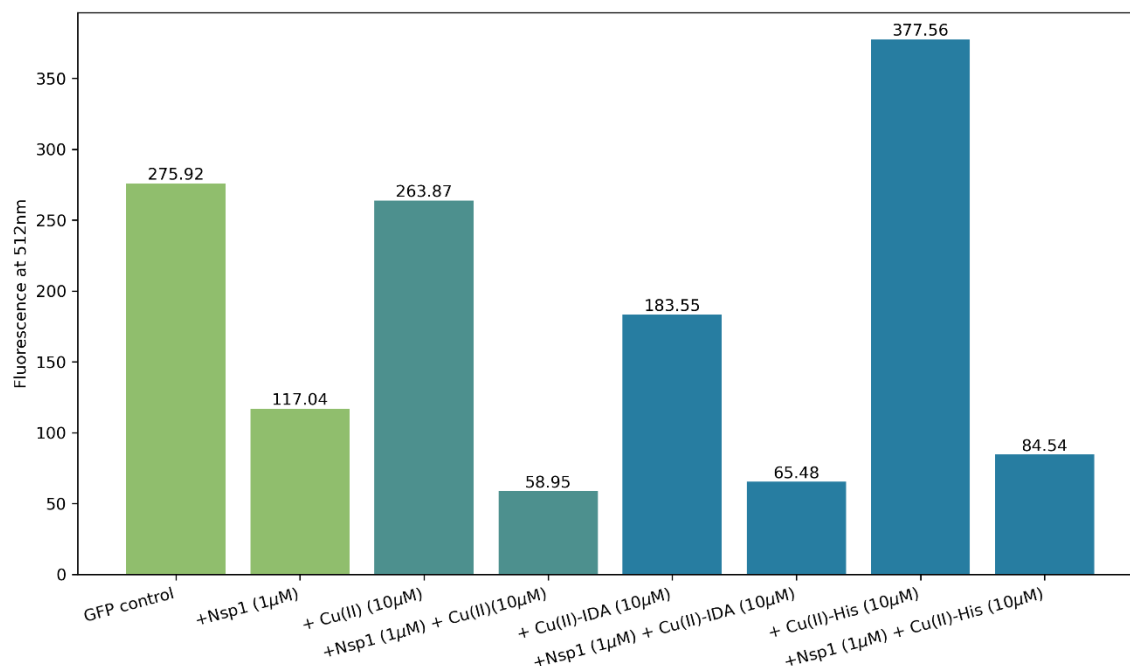


Figure 5.8 *In vitro* assay results for aquo copper(II), copper(II) iminodiacetate, and copper(II) histidinate are depicted as well as the controls.

As observed in previous assays, the system functioned as expected, with a clear reduction in GFP production upon addition of Nsp1 alone, confirming its inhibitory effect on translation. The results obtained with aquo copper(II) and the copper(II) complexes, however, revealed distinct behaviors.

Aquo copper(II) showed minimal impact on GFP production when tested independently, indicating that it does not significantly interfere with translation under these conditions. However, in the presence of Nsp1, GFP fluorescence was further reduced compared to the Nsp1 control, suggesting that aquo copper(II) does not inhibit Nsp1 function and may even exacerbate translational suppression.

Copper(II) iminodiacetate (Cu-IDA) exhibited a different profile. When tested alone, Cu-IDA impaired GFP production, as evidenced by decreased fluorescence relative to the GFP control, indicating a general inhibitory effect on translation. Similar to aquo copper(II), Cu-IDA failed

to restore GFP production in the presence of Nsp1, demonstrating that it is not effective as an Nsp1 inhibitor.

Interestingly, copper(II) histidinate enhanced GFP fluorescence when tested alone, suggesting a potential stimulatory effect on translation. Despite this, copper(II) histidinate did not prevent Nsp1-mediated inhibition, as GFP production was still markedly reduced in its presence.

These results indicate that while copper(II) complexes can influence general translation processes, none of the tested compounds effectively counteracted Nsp1's inhibitory activity, highlighting the challenge of achieving selective Nsp1 inhibition through metal-based strategies.

Cobalt(II/III) binding to full length Nsp1

Previous studies in Chapter 4 demonstrated that $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ is a promising candidate for binding to Nsp1, based on its coordination behavior with Nsp1-derived peptides. This section evaluates whether these findings extend to the full-length Nsp1 protein by examining the interaction of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ and its potential to modulate Nsp1 function in a biologically relevant context.

Tryptophan fluorescence quenching experiments were initially conducted to assess the binding behavior of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ with full-length Nsp1. The results, presented in Figure 5.9, reveal a complex quenching profile suggestive of multiple binding events. In the concentration range between 0 and 1 equivalent of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$, a rapid, near-linear decrease in the average tryptophan fluorescence lifetime was observed. This behavior is indicative of high-affinity binding, likely corresponding to a primary, specific interaction site on Nsp1.

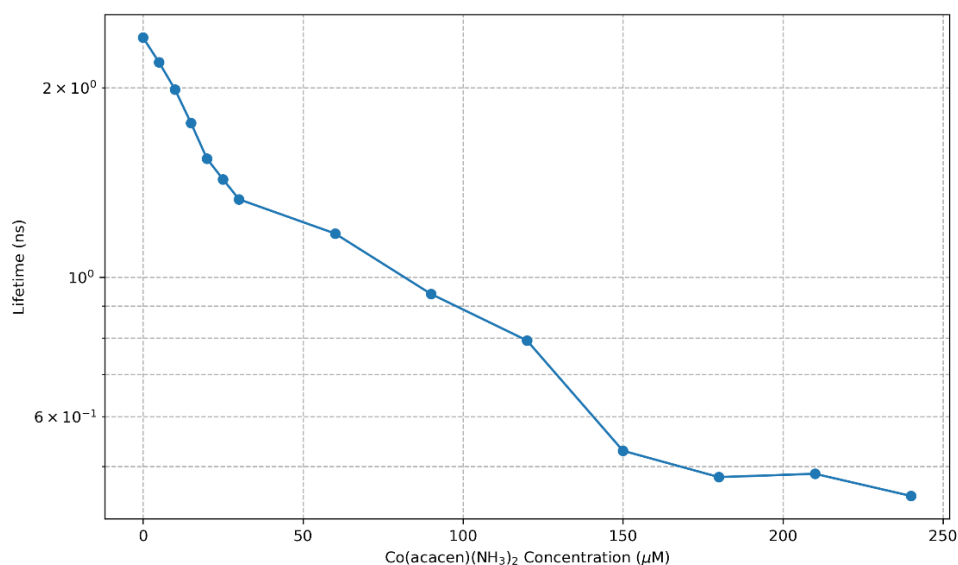


Figure 5.9 Tryptophan fluorescence quenching upon addition of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ to Nsp1. The initial protein concentration is $30\mu\text{M}$, all samples were made in MOPS buffer (20mM , $\text{pH } 7.5$).

At higher concentrations ($60\text{--}150\mu\text{M}$), a more gradual decline in fluorescence lifetime was detected, suggesting the presence of a secondary binding site with lower affinity for $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$. Beyond $150\mu\text{M}$, the quenching curve begins to plateau, consistent with saturation of available binding sites. The excess $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ beyond this point likely remains unbound in solution.

These observations point toward a two-phase binding mechanism, where initial high-affinity coordination is followed by weaker, nonspecific interactions at elevated ligand concentrations. We also wanted to check if this complex was capable of inhibiting Nsp1 and proceeded to test it with the *in vitro* assay (Figure 5.10).

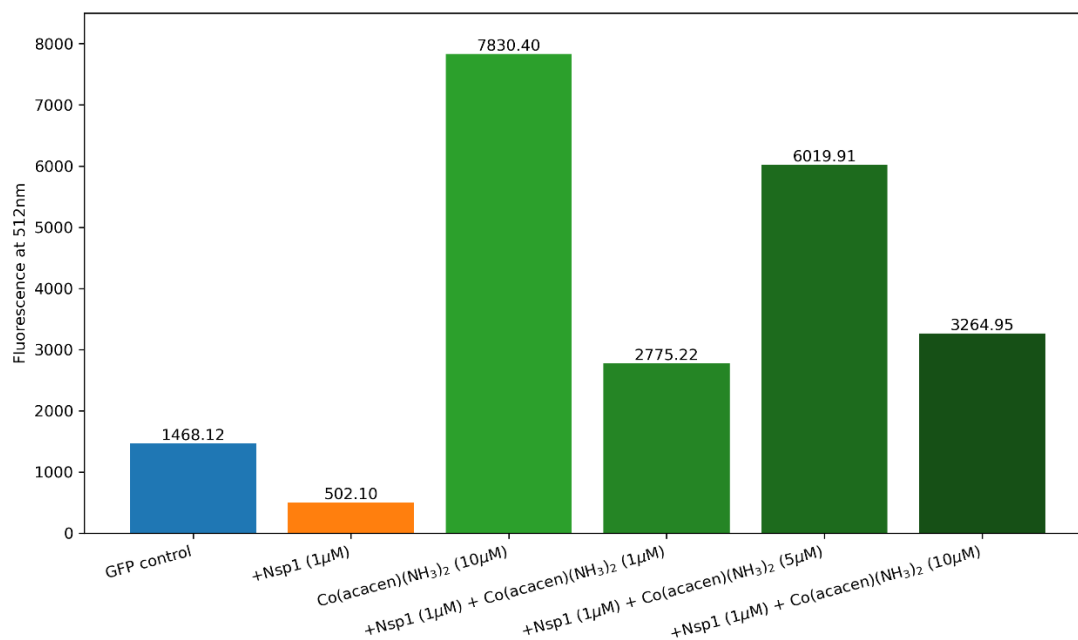


Figure 5.10 *In vitro* assay results when testing $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ at different concentrations.

In contrast to previous studies, $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ was observed to significantly enhance GFP production in the *in vitro* translation assay, resulting in a pronounced increase in fluorescence. Since $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ does not exhibit intrinsic fluorescence, this unexpected amplification of GFP signal suggests a potential interaction with the translation machinery or assay components that promotes protein synthesis. This characteristic complicates the interpretation of experiments involving Nsp1, as it makes it difficult to distinguish between normal GFP expression and fluorescence enhancement induced by $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$. The results of these assays are inconclusive and would need further investigation.

Given the evidence of multi-site binding observed in tryptophan fluorescence quenching experiments, along with the unusual behavior in the translation assay, ongoing studies are focused on elucidating the binding interactions of $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ with Nsp1. Current efforts involve the use of histidine mutants to identify specific coordination sites by analyzing changes in kinetic traces, with the goal of determining the residues responsible for cobalt binding.

Building on previous studies involving the oxidation of Co(II)NTA to Co(III)NTA using hydrogen peroxide (Chapter 4), Co(II)NTA was also evaluated for its potential to inhibit Nsp1 in the *in vitro* translation assay. In this case, no external hydrogen peroxide was introduced, with the expectation that oxidation to Co(III) might occur spontaneously due to the oxidative environment provided by components within the assay mixture.

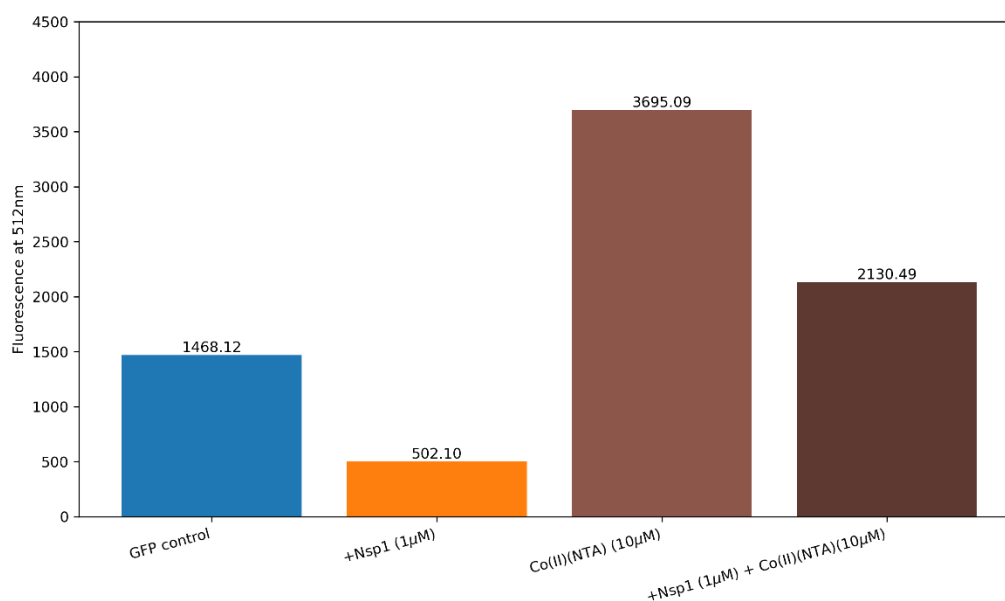


Figure 5.11 *In vitro* assay results when testing Co(II)NTA compared to controls.

The results mirrored those observed with $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ (Figure 5.11) where the addition of Co(II)NTA led to a substantial increase in GFP production beyond control levels. This unexpected enhancement of translation suggests that Co(II)NTA, similar to $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$, may interact with elements of the translation system, potentially stabilizing or stimulating protein synthesis pathways. However, this pronounced effect on GFP expression prevents clear assessment of Nsp1 inhibition, rendering the assay results inconclusive for determining the compound's ability to counteract Nsp1 function.

These findings highlight a recurring challenge when evaluating metal complexes in biological assays, the potential for off-target effects that influence cellular machinery independent of the intended inhibitory mechanism. Further studies will be necessary to dissect the underlying cause of this translation enhancement.

Summary and Outlooks

This chapter explored the challenges and opportunities associated with targeting full-length Nsp1 through both small molecule inhibitors and metal-based coordination strategies. Initial efforts focused on computationally screened compounds, but none of the candidates demonstrated the ability to inhibit Nsp1-mediated translation suppression in vitro, highlighting the difficulty of disrupting this highly conserved viral protein through standard drug discovery approaches.

Investigations shifted toward leveraging the metal-binding potential of Nsp1, particularly with the histidine residues. While aquo copper(II) and selected copper complexes exhibited binding to Nsp1, neither proved effective in reversing translational inhibition. Fluorescence quenching and mutagenesis studies revealed complex binding behaviors, highlighting the involvement of multiple histidine residues beyond the initially targeted H165. These findings emphasize the dynamic nature of metal coordination within full-length Nsp1 and the need for refined strategies to exploit these interactions for drug discovery.

Cobalt complexes, particularly $[\text{Co}(\text{acacen})(\text{NH}_3)_2]^+$ and Co(II)NTA , displayed unexpected behavior by enhancing GFP production in translation assays. While this characteristic complicates direct assessment of Nsp1 inhibition, it raises the question about how these metal complexes interact with cellular machinery. The observed multi-site binding requires more research to clarify potential off-target benefits. It is possible that off target binding might induce structural changes that could potentially still inhibit Nsp1.

Overall, these studies highlight the complexity of translating peptide-based findings to the full-length Nsp1 protein. While definitive Nsp1 inhibitors were not identified, valuable insights were gained regarding metal coordination behavior, histidine involvement, and the interplay between metal complexes and biological systems. Future directions will focus on residue-specific characterization of metal-binding sites, leveraging advanced NMR techniques and mutant libraries, as well as exploring modified metal complexes designed for greater selectivity and reduced interaction with host translation pathways. These efforts aim to refine metal-based strategies for Nsp1 inhibition and contribute to broader antiviral therapeutic development.

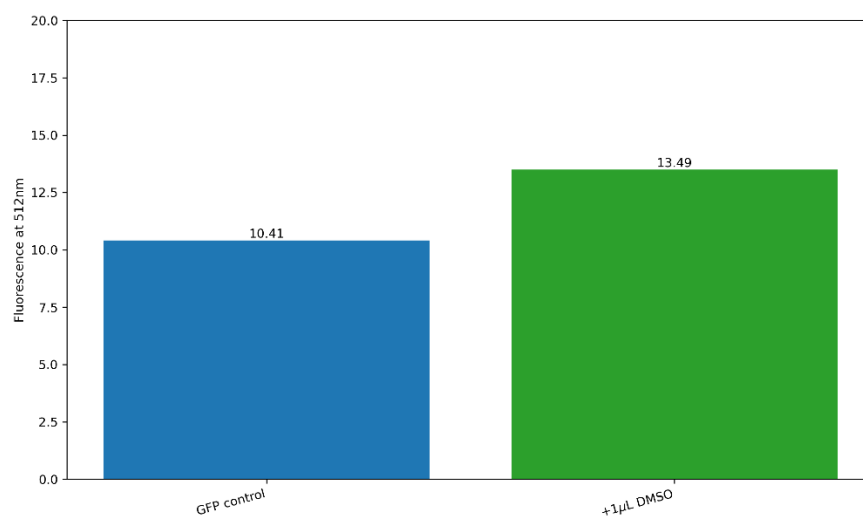
Supplemental Information

Figure S5.1 *In vitro* assay results for running a sample with DMSO. Results were within experimental error and show that DMSO does not negatively affect the production of GFP in the *in vitro* assay.

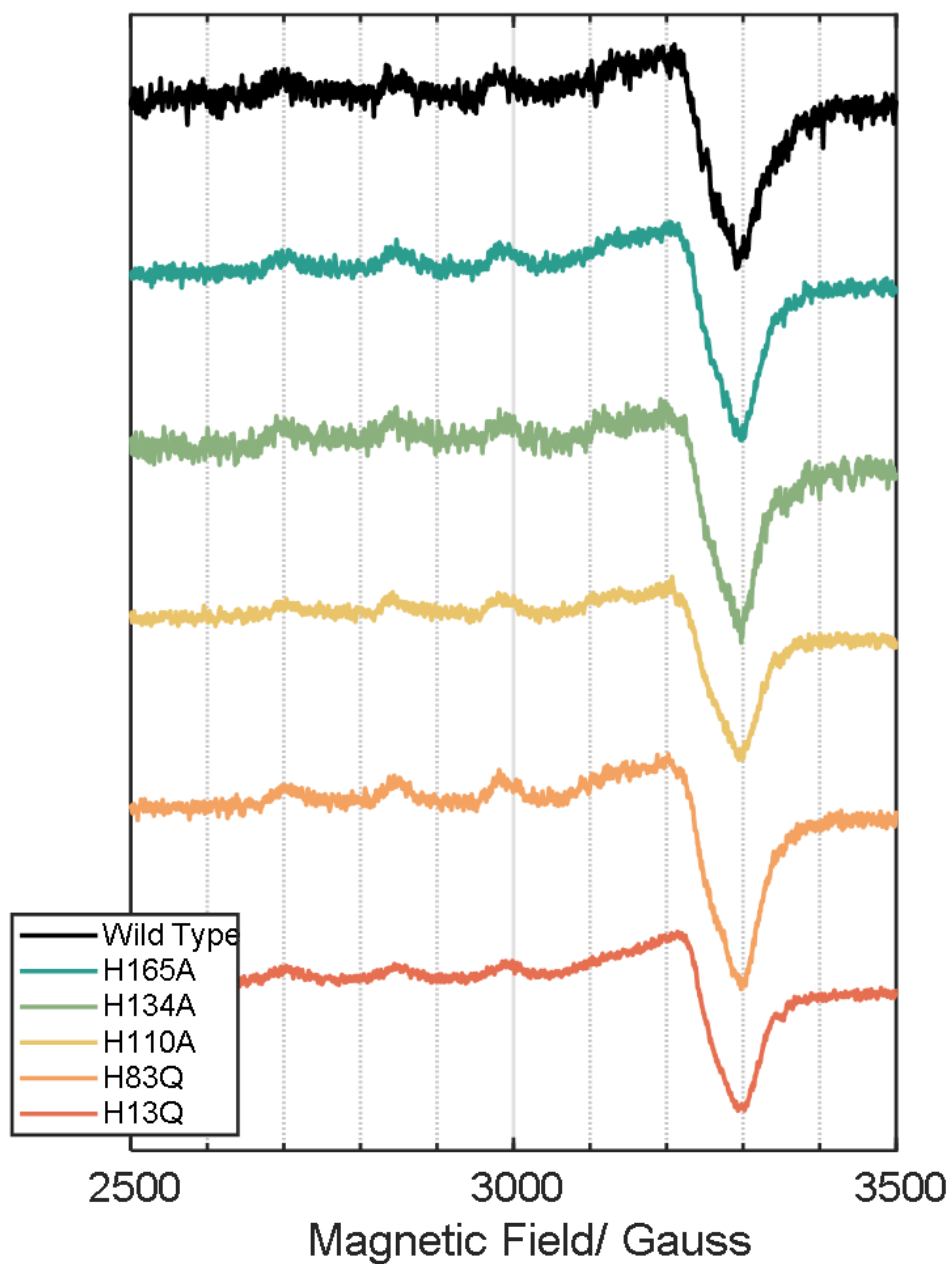


Figure S5.2 EPR spectra were collected of the histidine mutants that had high enough yields for quantification. Samples were made with 100 μ M protein and 100 μ M aquo copper(II). Samples were made in MOPS buffer (20mM pH 7.5) and with 30% glycerol as a glassing agent. Spectra were recorded at 77K. Amongst the five mutants that were recorded there were no significant changes in the spectra compared to the wild type.

References

- (1) Clark, L. K.; Green, T. J.; Petit, C. M. Structure of Nonstructural Protein 1 from SARS-CoV-2. *J Virol* **2021**, *95* (4), e02019-20. <https://doi.org/10.1128/JVI.02019-20>.
- (2) Min, Y.-Q.; Mo, Q.; Wang, J.; Deng, F.; Wang, H.; Ning, Y.-J. SARS-CoV-2 Nsp1: Bioinformatics, Potential Structural and Functional Features, and Implications for Drug/Vaccine Designs. *Front. Microbiol.* **2020**, *11*, 587317. <https://doi.org/10.3389/fmicb.2020.587317>.
- (3) Schubert, K.; Karousis, E. D.; Jomaa, A.; Scaiola, A.; Echeverria, B.; Gurzeler, L.-A.; Leibundgut, M.; Thiel, V.; Mühlemann, O.; Ban, N. SARS-CoV-2 Nsp1 Binds the Ribosomal mRNA Channel to Inhibit Translation. *Nat Struct Mol Biol* **2020**, *27* (10), 959–966. <https://doi.org/10.1038/s41594-020-0511-8>.
- (4) Finkel, Y.; Gluck, A.; Nachshon, A.; Winkler, R.; Fisher, T.; Rozman, B.; Mizrahi, O.; Lubelsky, Y.; Zuckerman, B.; Slobodin, B.; Yahalom-Ronen, Y.; Tamir, H.; Ulitsky, I.; Israely, T.; Paran, N.; Schwartz, M.; Stern-Ginossar, N. SARS-CoV-2 Uses a Multipronged Strategy to Impede Host Protein Synthesis. *Nature* **2021**, *594* (7862), 240–245. <https://doi.org/10.1038/s41586-021-03610-3>.
- (5) Thoms, M.; Buschauer, R.; Ameisemeier, M.; Koepke, L.; Denk, T.; Hirschenberger, M.; Kratzat, H.; Hayn, M.; Mackens-Kiani, T.; Cheng, J.; Straub, J. H.; Stürzel, C. M.; Fröhlich, T.; Berninghausen, O.; Becker, T.; Kirchhoff, F.; Sparrer, K. M. J.; Beckmann, R. Structural Basis for Translational Shutdown and Immune Evasion by the Nsp1 Protein of SARS-CoV-2. *Science* **2020**, *369* (6508), 1249–1255. <https://doi.org/10.1126/science.abc8665>.
- (6) Paggiaro, P.; Bacci, E. Montelukast in Asthma: A Review of Its Efficacy and Place in Therapy. *Ther Adv Chronic Dis* **2011**, *2* (1), 47–58. <https://doi.org/10.1177/2040622310383343>.
- (7) Afsar, M.; Narayan, R.; Akhtar, M. N.; Das, D.; Rahil, H.; Nagaraj, S. K.; Eswarappa, S. M.; Tripathi, S.; Hussain, T. Drug Targeting Nsp1-Ribosomal Complex Shows Antiviral Activity against SARS-CoV-2. *eLife* **2022**, *11*, e74877. <https://doi.org/10.7554/eLife.74877>.
- (8) Abramson, J.; Adler, J.; Dunger, J.; Evans, R.; Green, T.; Pritzel, A.; Ronneberger, O.; Willmore, L.; Ballard, A. J.; Bambrick, J.; Bodenstein, S. W.; Evans, D. A.; Hung, C.-C.; O'Neill, M.; Reiman, D.; Tunyasuvunakool, K.; Wu, Z.; Žemgulytė, A.; Arvaniti, E.; Beattie, C.; Bertolli, O.; Bridgland, A.; Cherepanov, A.; Congreve, M.; Cowen-Rivers, A. I.; Cowie, A.; Figurnov, M.; Fuchs, F. B.; Gladman, H.; Jain, R.; Khan, Y. A.; Low, C. M. R.; Perlin, K.; Potapenko, A.; Savy, P.; Singh, S.; Stecula, A.; Thillaisundaram, A.; Tong, C.; Yakneen, S.; Zhong, E. D.; Zielinski, M.

- Židek, A.; Bapst, V.; Kohli, P.; Jaderberg, M.; Hassabis, D.; Jumper, J. M. Accurate Structure Prediction of Biomolecular Interactions with AlphaFold 3. *Nature* **2024**, *630* (8016), 493–500. <https://doi.org/10.1038/s41586-024-07487-w>.
- (9)Carrera, F.; Marcos, E. S.; Merklings, P. J.; Chaboy, J.; Muñoz-Páez, A. Nature of Metal Binding Sites in Cu(II) Complexes with Histidine and Related N-Coordinating Ligands, as Studied by EXAFS. *Inorg Chem* **2004**, *43* (21), 6674–6683. <https://doi.org/10.1021/ic049699q>.
- (10)*Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklár, Z., Eds.; Springer Netherlands: Dordrecht, 1993. <https://doi.org/10.1007/978-94-011-6875-5>.
- (11)Yuan, S.; Balaji, S.; Lomakin, I. B.; Xiong, Y. Coronavirus Nsp1: Immune Response Suppression and Protein Expression Inhibition. *Front Microbiol* **2021**, *12*, 752214. <https://doi.org/10.3389/fmicb.2021.752214>.
- (12)Rhee, Y. M.; Pande, V. S. Solvent Viscosity Dependence of the Protein Folding Dynamics. *J Phys Chem B* **2008**, *112* (19), 6221–6227. <https://doi.org/10.1021/jp076301d>.
- (13)Wang, Y.; Kirkpatrick, J.; Zur Lage, S.; Korn, S. M.; Neißner, K.; Schwalbe, H.; Schlundt, A.; Carlomagno, T. 1H, 13C, and 15N Backbone Chemical-Shift Assignments of SARS-CoV-2 Non-Structural Protein 1 (Leader Protein). *Biomol NMR Assign* **2021**, *15* (2), 287–295. <https://doi.org/10.1007/s12104-021-10019-6>.

I can do all things through Christ who strengthens me.

Philippians 4:13