Investigations into the Enzymology and Biotechnology of the Hyperthermophilic Carboxypeptidase (PfuCP) from the Archaeon *Pyrococcus furiosus*

Thesis by

Timothy C. Cheng

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, CA

2003

(Defended September 27, 2002)

© 2003

Timothy C. Cheng

All Rights Reserved

Acknowledgements

There are many people to thank.

My parents, who have always been there... for all the times I can remember, all the times that I don't, and all the times I never knew about. The day is fast approaching when two round-the-world cruise tickets will magically appear on their doorstep...

My advisor, Sunney Chan...

Patience. Generosity. Unflagging support.

Vision. Wisdom.

For taking a chance on a project that was risky, but which ultimately taught more than could be expected, and for having that rare combination of encouragement and trust that young scientists need to grow and develop as individuals. It is both an honor and a privilege to be his final graduate student at Caltech as he ends an illustrious and prolific research career that spanned many projects and many interests.

Shao-Ching Hung, for showing me how to handle an EPR machine and for good company and good conversation on many an occasion. Brian Schultz for never tiring of being a sounding board that didn't just bounce back noise but which filtered and added music to the mix. Good friends, Andrew Yeh, Lance Christensen, Jeremy Kua, Bac Hoang... the time at Caltech was made all the better by their company. Hung-Kay Lee, Sean Elliot, David Zhang and other labmates past who were always ready to lend an ear or a hand. Drs. Gary Hathaway and Mona Shahgholi for their generous advice on matters mass spect, and Dr. Peter Snow for his heroic efforts in the original cloning of the PfuCP gene. Prof. Michael Chan and his trusty grad student Joseph Arndt, for their spectacular

work on the crystal structure. Profs. Judy Campbell, Douglas Rees, Jack Beauchamp, and Mitchio Okumura for sitting on yet another thesis committee and for their kind words of encouragement.

And last but not least, the scores of scientists both present and long gone, who put something of themselves into the ever changing fabric of knowledge and understanding, and who have provided both ample inspiration and rich working material to which future generations can add their own humble contributions.

Abstract

A novel metallocarboxypeptidase (PfuCP) from the hyperthermophilic archaeon *Pyrococcus furiosus* was purified and characterized to investigate its dependence on metal ion cofactors and to evaluate its suitability as a biotechnological tool for protein sequencing at elevated temperatures.

The crystal structure reveals a dimer of primarily α-helical subunits that bears no resemblance to the α/β-hydrolase morphology of typical carboxypeptidases and which defines a new family of HEXXH metalloproteases (M32) based on primary sequence alignments. A deep active site groove appears to function not only in size-selection of substrates but also in modulating the activity and substrate affinity through complicated allosteric effects involving ambient ligands which may play a role in regulatory metabolism.

Two forms of the enzyme were observed; one which retains stabilizing metal(s) that confer structural thermostability and a remarkable retention of activity to the dimer, and another demetallated form which has lost stability with regards to both dimeric integrity and activity. Difficulties in expressing a properly folded recombinant necessitated refolding of the expressed clone from inclusion bodies and further suggest that *in vivo* the stabilizing metal(s) may participate in folding a metastable enzyme.

The apparent paradox of activation by only Co^{2+} and not Zn^{2+} is resolved into two issues, uncompetitive inhibition by the latter as seen in steady-state kinetic experiments, and intrinsic, electronic aspects of a catalytic Co^{2+}. Several explanations are proposed for the intrinsic rate enhancement of Co^{2+} over Zn^{2+} including the ability of Co^{2+} to modulate

the potential energy surface for both reactants and transition states by virtue of its greater mobility within the protein framework.

The broad amino acid specificity and rapid digestion by PfuCP in peptide sequencing trials show promise, and high-temperature protein sequencing has now been demonstrated for the first time.

Table of Contents

Chapter 3

Chapter 4

Chapter 5

List of Illustrations and Tables

Tables

Figures

Abbreviations and Glossary

CP	Carboxypeptidase
ICPMS	Inductively coupled plasma mass spectrometry
IMAC	Immobilized metal affinity chromatography
PES	Potential Energy Surface
Pf	*Pyrococcus furiosus*
Standard Assay Conditions	0.1 M KMes pH 6.5, 2 mM substrate, 400 μM Co^{2+}, 4 μL enzyme
TSS	Transition State (Hyper)Surface
ZAX	N-Carbobenzoxy-Alanyl-Xaa (X amino acid, N-blocked dipeptide)