

ENZYME-SUBSTRATE INTERACTION BY
NUCLEAR MAGNETIC RESONANCE

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The chemical shifts of the nuclear magnetic resonances of N-acetyl-D-tryptophan are measured and compared to those observed when bound to α -chymotrypsin. Chemical shift changes of 1 Hz. are reported for a solution with 1% total substrate bound to the enzyme.

Introduction

The problem was to find out information about the structure of the active site of α -chymotrypsin through the analysis of the nuclear magnetic resonance spectrum of an enzyme-substrate complex containing α -chymotrypsin and N-acetyl-D-tryptophan. Analysis of such a spectrum and comparison of it with a spectrum of the pure substrate could give insight into binding sites in the active site itself as chemical shift differences between the peak in the spectrum of the complex and the corresponding peaks in the spectrum of the pure substrate could reveal where on the substrate magnetic shielding from binding electrons on the enzyme was occurring. This has been done with some success on lysozyme by Dr. Rafferty.

The first big obstacle confronted was obtaining a spectrum of pure enzyme-substrate complex. This is not possible for two basic reasons: 1) the equilibrium, $EI \xrightleftharpoons{K_1} E + I$, prevents having pure EI in an aqueous solution for n.m.r. observation, and 2) the large size of an enzyme molecule, and therefore the large size of a molecule of complex, prevents the proper spinning in the magnetic field of the spectrometer necessary to obtain sharp resonances. The first problem might be solved to a certain extent with a different solvent, but the second cannot be alleviated. Therefore, a different technique other than direct observation of a pure sample of EI was employed. This technique used the time averaging effect

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of the n.m.r. to extrapolate the spectrum of the complex. In order to do this, one must assume that the amount of time that the substrate spends bound to the enzyme is much smaller than

$$\sqrt{2}/2\pi(\nu_{ES} - \nu_S)$$

where $(\nu_{ES} - \nu_S)$ is the chemical shift difference between each peak of the pure complex and the corresponding peak of the pure substrate. Doing the following order-of-magnitude calculation,

$$\sqrt{2}/6.28(100\text{Hz}) = 1.4/628 \approx 5 \times 10^{-3} \text{ sec} \gg 10^{-5} \approx k_1^{-1}$$

one sees that the condition is satisfied, which means that the I and EI peaks will coalesce and form one peak at a chemical shift proportional to the amounts of each species present. Knowing the concentrations of the complex and substrate in the solution under observation, and also the chemical shift of the "average" peak, one can calculate the position of the pure EI peak if he has measured the position of the corresponding peak in the pure substrate. One should theoretically obtain a linear plot of shift changes against percent of total I bound to E, whose slope is the chemical shift of the pure EI if one takes spectra at many different concentrations of E and I.

In order to prevent new compounds arising from enzyme hydrolysis of the substrate, a competitive inhibitor was used. This allows binding at the active site and then dissociation with no chemical change, thus allowing the substrate to stay at constant total concentration without new peaks appearing from decomposition or hydrolysis products. The only serious problem was the denaturation of α -chymotrypsin after one hour at room temperature in a solution

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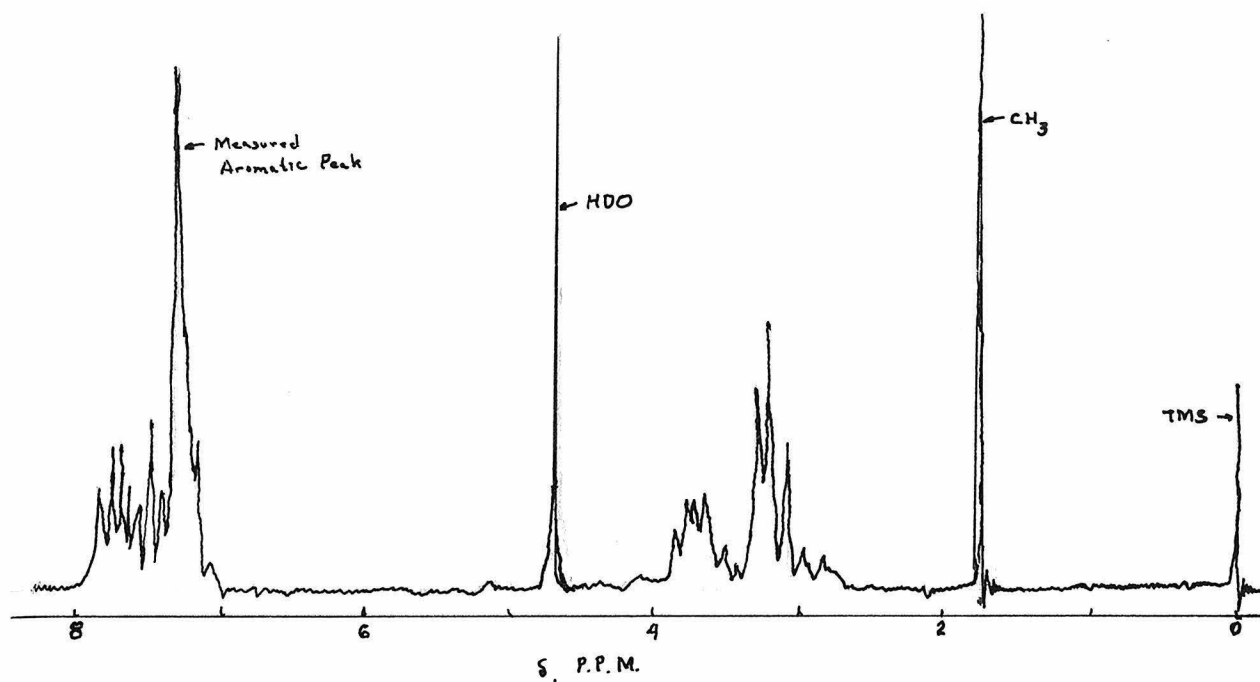


Figure 1: A typical spectrum of N-acetyl-D-tryptophan

of as high a concentration as was necessary for these observations, ie., 0.005 M. The inhibitor chosen was N-acetyl-D-tryptophan whose spectrum is shown above in figure 1. Some spectra of the L-enantiomer were taken for comparison. The former has a K_I of 4.8×10^{-3} M.² K_I is the dissociation constant in the system given on page one. Thus, as long as substrate concentrations stayed above 0.05 M, an assumption that all of the enzyme was bound to substrate could be used with $[E] = 0.005$ M, because doing the calculation of free enzyme with this assumption as follows,

$$[E] = (4.8 \times 10^{-3})(5 \times 10^{-3}) / 5 \times 10^{-2} = 4.8 \times 10^{-4} \text{ M}$$

shows that the free enzyme is only 10% of the total amount in solution. Any lower a substrate concentration would make this assumption risky. The K_I for the L-enantiomer was not found in the literature

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and will have to be determined for accurate comparisons of the binding of the two isomers on α -chymotrypsin. Since α -chymotrypsin seems to be more specific for L-forms of most amino acids, one would expect that the value of K_I would be lower than that of the D-forms of the N-acetyl tryptophan which would allow the use of lower substrate concentrations without affecting the binding assumption. An ultimate side-goal of the total investigation would be to explain the specificity of α -chymotrypsin for L-amino acids through a determination of the way in which it binds compared to the way in which the D-enantiomers bind.

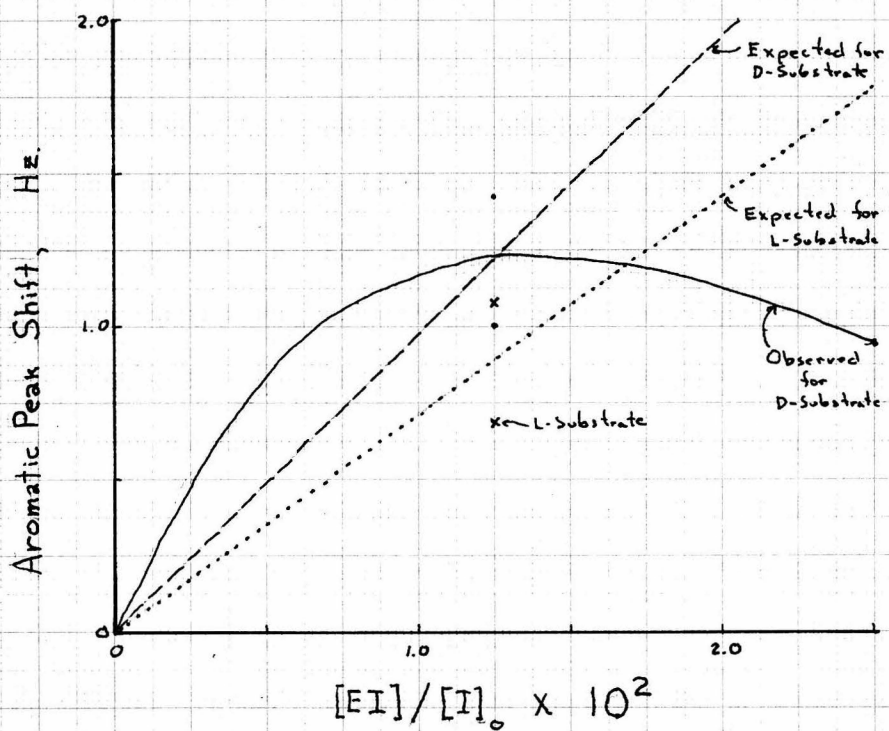
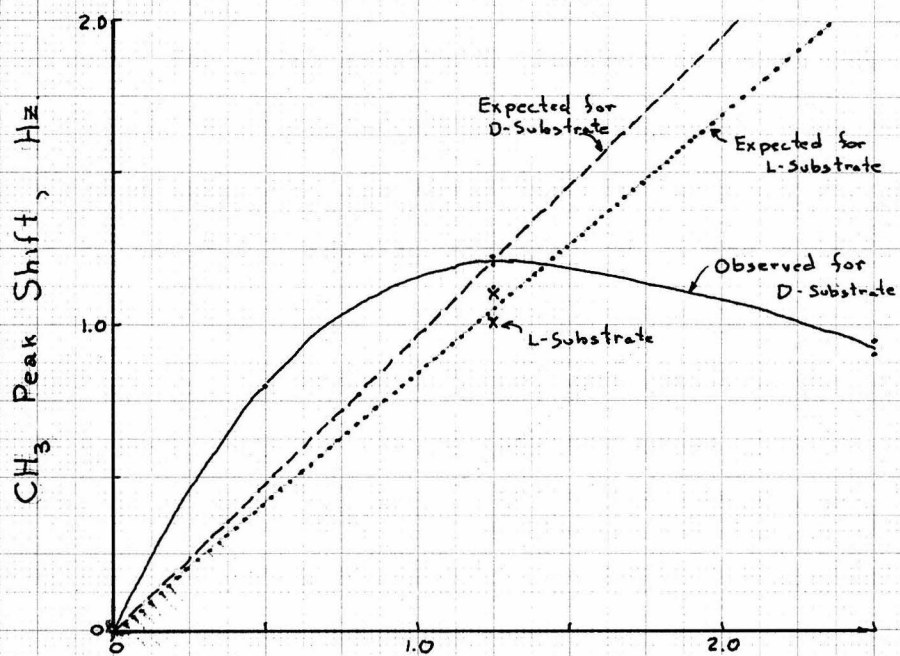
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Data

Data concerning the change in chemical shift was compiled for peaks in four regions shown in figure one. Only the two marked peaks showed any consistent interesting shifts. The plots on the following page show the variation of these chemical shifts with change in the relative concentration of the enzyme-substrate complex. Also shown are the expected linear shift variations.

The largest shift observed was on the order of 1.5 Hz. This is so small that it is difficult to claim that it is a definite peak shift for the magnetic field in the spectrometer varies enough to cause shifts of this order of magnitude. However, if this shift is assumed to be accurate and one uses the expected linear relationships, the total shift for pure EI complex is 95 Hz. and 98 Hz. for the methyl and aromatic peaks of the D-tryptophan derivatives, respectively.

Actually, this shift is rather impressive. If a higher concentration of EI were possible, some rather large shifts would be observed. However, this is difficult, if not impossible to do, since to do this one would have to increase [E] causing dimerization and denaturation of the enzyme, or decrease [I] making it impossible to observe the peaks on the spectrometer.



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Discussion of Results

Glancing at the plots on page 6, one notices that the results obtained were not the expected straight lines. There can be two explanations for this: 1) the theory predicting that the plots be straight lines is not totally correct, or 2) the data is inaccurate.

Considering the first possibility, one examines the ideas which predict that the plot should obey the equation,

$$\frac{1}{\delta} = \frac{k_I + I_0}{\Delta E_0}$$

δ = Measured chemical shift difference
 Δ = Extrapolated chemical shift difference

There is nothing obviously wrong with the derivation, which was obtained from Drs. Richards and Rafferty. In fact, a colleague (Gerald Swanson) performing the same experiment as this using N-acetyl tyrosine as the competitive inhibitor obtained a straight line. Also, later research by Dr. Richards using fluorine n.m.r. spectra also rendered straight-line plots.³

This leads one to consider the second explanation more seriously. One possibility in this area is that very few measurements were made at the 0.5 and 1.0% EI to I_0 ratio. Therefore, these readings cannot be regarded as entirely reliable since they were not reproduced. At 1.25%, the observations were numerous and the most recent and most carefully performed observations that were made show that his reading can be pinpointed very accurately. This leaves the points of higher EI percentage. The only percentage investigated extensively in this region was at 2.5%. The data here turns out to be very erratic and impossible to interpret.

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The only definite observation that can be made about these readings is that none of them were high enough to be on a straight line between the origin and the 1.25% points. However, it is possible that here, one has already gone beyond the limits of enzyme concentration or spectrometer sensitivity discussed in the experimental section under observation procedures. In short at dilutions needed to produce a ratio of 2.5% the high spectrum amplitudes and the corresponding settings on the RF field and filter bandwidth distort the peaks so much that accurate measurements are almost impossible. Until a computer of average transients (CAT) can be employed, little can be done to improve this situation as long as proton spectra are measured. The use of fluorine spectra could improve the measureability of the shifts at lower ratios since the shifts would be magnified using fluorine.

The other noteworthy point to be mentioned in regards to the inaccuracy of the data is the experiment done with DFP-treated chymotrypsin. These experiments showed a shift comparable to those using unblocked enzyme. This means that either there is binding at other sites than the active site, or the shifts are actually shifts in the spectrometer magnetic field. More accurate experiments in this area should be done to determine just how large the shifts are with the DFP-chymotrypsin, for they may be less than the shifts observed with untreated enzyme, which would mean that at least some of the shift is due to active-site binding.

Thus, future experiments in pursuit of the stated objective of determining something about the active site would include the

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following. First, further experiments should be performed to reproduce the above data or to obtain better data. Second, an investigation of the change in chemical shifts at higher EI percentages with the use of a CAT attachment. Third, further inquiries into the shift changes with DFP-chymotrypsin, along with other experiments trying to find the causes of the shifts observed with the DFP-chymotrypsin. Fourth, there is the possibility of making studies with chymotrypsin and its binding with other substrates such as phenylalanine derivatives which have simpler n.m.r. spectra and bind better with the enzyme than tryptophan. Fifth, the effects of changes in pH on the binding should be studied. Certainly, it might be better if the experiments could be run at a pH around 3.5, because dimerization would be less and actual binding might be greater than at pH 7.9. pH 7.9 was used because it is the pH of maximum enzymatic activity according to Niemann.⁴ Maximum activity does not necessarily bring about the best conditions for this experiment since activity means the breakdown of peptides and not binding.

Lastly, there is a very interesting study that could be made on the change in spin-spin coupling constants as well as the chemical shift on such functional groups as the CH₂ and CH groups adjacent to each other on tyrosine, tryptophan, and phenalanine (See Figure 2).

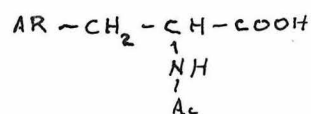


Figure 2: Areas of possible coupling constant study in tryptophan, tyrosine, and phenylalanine binding to α -chymotrypsin.

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Changes in the coupling constants for these groups could imply that something is happening to the dihedral angle between the bonds of these two groups. Such shifts have been observed although not reported in the data because of their uncertainty. If the shifts do indeed exist, an explanation for them would be that when the substrate is unbound, the bonds are free to rotate and an average of the dihedral angle is observed. When the substrate is bound to the enzyme, this dihedral angle becomes fixed as rotation is subject to steric hindrance. Thus, this could provide another method for studying the structure of the active site and enzyme-substrate binding by finding out which enzyme groups cause the steric effects.

In conclusion, it has been demonstrated by this investigation that there is an observable change in shift between the free substrate and the enzyme-substrate complex with α -chymotrypsin and that with further investigations and refinements of technique, nuclear magnetic resonance can be used to study the structure of the active sites of enzymes.

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Experimental

Substrates

The N-acetyl-D-tryptophan was obtained from Cyclo Chemical Corporation and used without further purification. In order to increase the solubility of the anion of the acid and to aid in pH control, a sodium salt of the acid was made by adding equimolar amounts of 0.1 M NaOH to the N-acetyl-D-tryptophan. The resulting neutral liquid was then lyophilized and the solid sodium salt obtained used as the substrate without further purification. 0.5 M, 0.2 M, and 0.1 M aqueous solutions of this salt were then prepared for use in the n.m.r. observations. The procedure for preparing these solutions was to add 0.0670 g., 0.0268 g., or 0.0134 g., respectively, of the salt to 0.5 ml. of Tris buffer #1.

The N-acetyl-L-tryptophan was also obtained from Cyclo Chemical Corporation and its solutions were prepared in the same manner as above for the D-enantiomer.

Enzyme

The α -chymotrypsin (bovine pancreas) was obtained from California Biochemical Company where it was two times crystallized and rendered salt free. 0.005 M solutions of this enzyme were prepared before adding to the substrate by adding 0.0313 g. of it to 0.25 ml. of buffer #2.

The diisopropylfluorophosphate (DFP) treated α -chymotrypsin was obtained from Worthington Biochemical Company. 0.005 M solutions were prepared before adding to substrate by adding 0.0314 g. of

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it to 0.25 ml. of Tris buffer #1. The end pH for both the untreated and the DFP-treated enzyme was 7.9.

Buffer Solutions

Buffer #1 was set at pH 7.9 using Tris-(hydroxymethyl)-aminomethane (THAM). A 0.1 M solution was prepared by adding 0.0920 g. of THAM and 0.1952 g. of THAM-hydrochloride (THAM-HCl) to 20 ml. of D₂O. (Note: pD for this buffer was 8.3).

Buffer #2 was prepared by adding 0.2534 g. of pure THAM to 20 ml. of D₂O. It was buffered at pH 7.9 (pD 8.3) when 0.0313 g. of salt free α -chymotrypsin (bovine pancreas) was added to 0.25 ml. of this solution.

Internal Standards

The internal standard used for the 50 Hz. sweeps had the following composition:

<u>Standard</u>	<u>Chemical Shift, Hz.</u>	<u>% in spectro. CCl₄</u>
Cyclohexane	95.4	7.4
1,2-dibromoethane	228.6	11.1
Bromoform	420.0	22.2

Some of the earlier spectra used dichloromethane as a standard with a chemical shift of 5.3 ppm. This standard was quite suitable for a 500 Hz. sweep width, but was inadequate for the shorter 50 Hz. sweeps.

The standards were sealed in a 10 cm. long 0.5 mm. capillary tube and then inserted in an n.m.r. tube before each observation.

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Ideally, the capillary tube should be concentric with the n.m.r. tube, but this is difficult and was not done. Hopefully, this did not have too much effect on the results of the experiment.

Observation Procedures

There were two basic procedures used in making observations on the n.m.r. spectrometer. The first used was one where successively larger amounts of enzyme were added to a constant initial substrate-concentration solution. This means that 0.5 ml. of a substrate solution with a concentration of, for example, 0.2 M was first observed without added enzyme. Then 0.25 ml. of 0.005 M α -chymotrypsin in buffer was added to the substrate, pH rechecked for 7.9, after which an n.m.r. spectrum was taken. Then 0.25 ml. more of enzyme was added, repeating the above procedure. This method proved to be poor since the extent of denaturation of the enzyme was different each time the procedure was repeated as different amounts of time were used. Furthermore, when 50 Hz. scans were used, the whole procedure could not be done within in an hour. This one hour time limit was critical since the enzyme seemed to become less active after this period of time. This procedure was revised by adding different and increasing amounts to different tubes of pure substrate solution. For example, one would start with three tubes of substrate, adding 0.25 ml. to the first tube, 0.50 ml. to the second, and 0.75 ml. to the third tube.

Concern over dilution problems using the enzyme as the dilutant,

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as well as excess enzyme cause a second method to be developed. This method varied the initial substrate concentration instead of the amount of enzyme added. Solutions of initial concentrations of 0.5 M, 0.2 M, and 0.1 M in substrate were used with a constant 0.25 ml. of 0.005 M α -chymotrypsin in buffer.

Accuracy problems produced the following method of using the n.m.r. spectrometer itself. Ten scans of each 50 Hz. region observed were taken--5 were for pure substrate and 5 were for enzyme in the solution. The chemical shifts at the up-field end for the 50 Hz. regions observed were 419 Hz., 227 Hz., 180 Hz., and 80 Hz. The settings on the Varian A60-A (60 Mc) spectrometer were 0.03 mG for the RF-field and 0.4 to 0.2 for the filter bandwidth. The spectrum amplitudes were highly sample dependent but in general had order of magnitude 100. Each 50 Hz. sweep took 100 sec. 500 Hz. standard-shift check sweeps were also run.

Data Compilation

The standard-shift check spectra were first measured for standard positions to make sure that all of the shift observed on the substrate peaks was due to the substrate itself and not a shift in the standard peak position. Then the shifts of the peaks from the standards for the non-diluted runs were measured and averaged over the five scans. The same was then done for the enzyme-addition observations. The two average values were then compared for each set of corresponding peaks.

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2. Ibid.
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