ENZYME-INHIBITOR INTERACTIONS STUDIED VIA FLUORINE NUCLEAR MAGNETIC RESONANCE. I. THE INTERACTION OF α-CHYMOTRYPSIN WITH DL-N-TRIFLUOROACETYLPHENYLALANINE

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With the advent of high resolution magnetic resonance spectrometers there has been a great expansion of the application of this powerful method to the study of protein and enzyme chemistry (Cohen and Jardetzky, (1968), and references cited therein). To date, however, the use of fluorine-19 magnetic resonance has not been widely applied to biological systems, and the work of Spotswood, Evans and Richards (1967) is among the first uses of this approach to the study of proteolytic enzymes. It is the purpose of this communication to present the preliminary results of a study of the interaction of DL-N-Trifluoroacetylphenylalanine (TFAP) with α-chymotrypsin (CT)[1].

Muller and Birkhahn (1967) have approached the problem of micelle structure by studying the F^{19} resonance of fluorinated surfactants in organic solvents and in aqueous solution above and below the critical micelle concentrations of the various surfactants. On the basis of

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[1] α-Chymotrypsin was purchased from Worthington Biochemical Corporation, Lot CDI 6137-8. TFAP was synthesized from DL-phenylalanine and trifluoroacetic anhydride according to procedure 10-40 of Greenstein and Winitz (1961). M.Pt 127.5-128 (corr.). Literature value 127-128. Anal. Calc: C-50.58 H-3.86 N-5.36; Found C 50.8; H 4.0; N 5.3.

their results they have developed an empirical relationship between the F^{19} chemical shifts of the fluorine nuclei in their various environments and the hydrophobic character of the micelle. Interestingly enough, their results imply that the micelle is intermediate between an aqueous environment and a non-polar environment, regardless of the surfactant[2].

It was thought that this approach might be applied to the study of enzyme active sites by studying the shift of a fluorinated inhibitor in organic solvents and in aqueous medium with and without an enzyme. CT was chosen as the first enzyme for study and TFAP was chosen as the inhibitor because of its sharp, simple, one peak F^{19} spectrum (see Figure 1). A similar approach to the environment of the active site

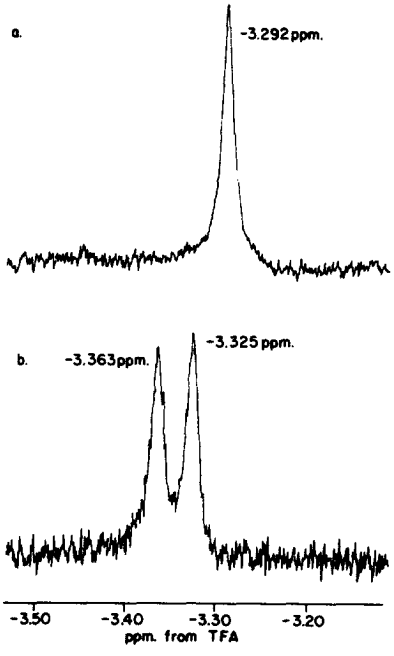

Figure 1. F^{19} nuclear magnetic resonance spectra of DL-N-Trifluoroacetylphenylalanine measured at 94.0776 MHz. a) without enzyme; b) with enzyme (CT). DL TFAP concentration is 4.0×10^{-2}M.

[2] In their studies, Muller and Birkhahn used ethanol as their model hydrophobic environment, since for those surfactants soluble in both ethanol and other organic solvents such as heptane, acetone, etc., there was little observed difference in the chemical shifts.

of CT has been made by Kallos and Avatis (1966) using the ultraviolet difference spectra of the nitrobenzenesulfonyl and toluenesulfonyl derivatives of CT. On the basis of their results they concluded that the environment around the nitrobenzene chromophore was about as hydrophobic as cyclohexane. However, even though the enzyme maintains a native conformation upon sulfonylation, the sulfonyl derivative itself is catalytically inactive and will not desulfonylate unless the sulfonyl compound is specially designed (Heidema and Kaiser, 1967). The present work complements the study of Kallos and Avatis by looking at the active site environment when the crucial serine hydroxyl group is not acylated.

The data obtained to date are presented in Table I. Figure 1 shows representative spectra obtained using a Varian Associates HA-100 NMR spectrometer operating in the frequency sweep mode at a fixed frequency of 94.0776 MHz. All spectra were recorded using the same tube and same TFA capillary as the lock signal to minimize susceptibility errors. It was found necessary to use a CAT (Varian C-1024) to record the spectra of some of the more dilute solutions. CT concentrations were determined by titration with cinnamoyl imidazole (Bender et al, 1966). All aqueous solutions were measured at pH 6.0 but for one sample at pH 2.0 which was measured in order to determine the effect of protonation of the carboxyl group in aqueous medium since this group is extensively ionized in aqueous solvents at pH 6.0, but associated in the organic solvents studied. The data in Table I show that the small effect of this pH change is probably attributable to a salt effect of the buffer rather than to the protonation of the carboxyl group.

The interesting and surprising results in Table I were completely unexpected. We had anticipated that the effect of the enzyme would be to move the F^{19} chemical shift of TFAP to a position intermediate between that in aqueous and organic solvents. However, as seen in Figure 1,

Table I

F^{19} Chemical Shifts of DL-N-Trifluoroacetylphenylalanine in Various Environments[a]

Solvent	Added Solute	[DL-TFAP] 10^2 x (M)	F^{19} Chemical Shift (ppm)[b]		
			Without CT	With CT	
			DL	D or L	L or D
Ethanol	-	4.00	-1.645		
p-Dioxane	-	~ 4.0[c]	-2.130		
85% Dioxane-15% Benzene	-	~ 4.0[c]	-2.174		
50% Dioxane-50% Benzene	-	~ 4.0[c]	-2.249		
15% Dioxane-85% Benzene	-	~ 4.0[c]	-2.206		
Benzene	-	~ 4.0[c]	-2.019		
Buffer, pH 6.0[d]	-	8.00	-3.302		
Buffer, pH 6.0	-	4.00	-3.292[e]		
Buffer, pH 6.0	-	2.00	-3.286[e]		
Buffer, pH 6.0	-	1.00	-3.290		
Buffer, pH 6.0	-	0.30	-3.285		
Buffer, pH 6.0	CT[f]	8.00		-3.351	-3.331
Buffer, pH 6.0	CT	4.00		-3.363	-3.325
Buffer, pH 6.0	CT	2.00		-3.370	-3.329
Buffer, pH 6.0	CT	1.00		-3.387	-3.331
Buffer, pH 6.0	CT	0.30		-3.387	-3.337
Buffer, pH 6.0	0.5M NaCl	0.30	-3.374[e]		
Buffer, pH 6.0	0.5M NaCl	2.00	-3.377[e]		
Buffer, pH 6.0	1.0M NaCl	2.00	-3.465[e]		
Buffer, pH 6.0	2.0M NaCl	2.00	-3.638[e]		
Buffer, pH 6.0	4.0M NaCl	2.00	-4.007[e]		
Buffer, pH 6.0	5.4M NaCl	2.00	-4.257[e]		
pH 2.0-HCl-KCl (0.1M)	-	2.0	-3.313		

[a] All shifts are in ppm relative to an external capillary of trifluoroacetic acid. Negative sign indicates a downfield shift from the external reference.

[b] Measurements are considered accurate to within 0.001 ppm.

[c] Due to very slight precipitation (removed before measurement) these concentration values are only approximate. Other concentrations are accurate to within 1%

[d] 0.10M citrate buffer.

[e] Used for Figure 3.

[f] Operational molarity of all solutions was 2.14 (+ 0.04)x10^{-3}M.

the TFAP peak positions of both the enantiomers in the presence of the enzyme move downfield from its position in pH 6.0 citrate buffer, while the position in organic solvents is well upfield from the buffer location. The shift is about twice as far upfield in ethanol as in benzene or dioxane. If we plot the data according to the equation of Spotswood, Evans and Richards (1967), i.e., $1/\delta_{obs} = \frac{K_I + I_o}{\Delta(E_o)}$, we obtain the curves shown in Figure 2. In this figure, K_I is the inhibition constant, I_o is the molar concentration of inhibitor, δ_{obs} is the chemical shift change caused by the addition of the enzyme at a given I_o, E_o is the active enzyme concentration and Δ is the maximum shift which would be observed if 100% of the inhibitor were complexed by the enzyme. As seen, the F^{19} resonances of both enantiomers are shifted downfield by the enzyme, and the plot allows an estimation of K_I and Δ. The values of K_I are 3.4×10^{-2}M for the more tightly bound enantiomer (larger shift) and 5.1×10^{-2}M for the less tightly bound enantiomer. These values are considered accurate only to within a factor of two since the presence of both enantiomers decreases the effective enzyme concentration experienced by either one. Work aimed

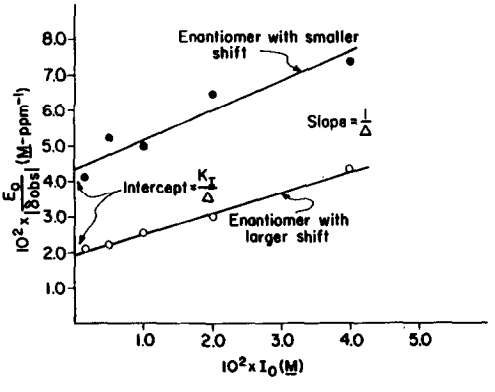

Figure 2. $E_o/|\delta_{obs}|$ vs. I_o. I_o scale is 0.5 x gravimetric concentration level since the shifts are for the enantiomers in the racemic solution.

at clearing up this point is in progress. The values of Δ are 1.75 ppm for the enantiomer with the larger shift and 1.18 ppm for the enantiomer with the smaller. Both of these are farther downfield (relative to the peak in buffer without enzyme) than are the upfield shifts observed in the non-polar solvents benzene and dioxane. Combined with the great difference between benzene and dioxane on the one hand and ethanol on the other, these results clearly rule out the applicability of an analysis similar to that of Muller and Birkhahn.

Two possible explanations of the downfield shifts produced by the enzyme are as follows.

The fluorine nuclei might be situated in an environment such that the anisotropy of adjacent groups (such as aromatic rings) causes a downfield shift.

Alternatively, if any one of the organic solvents used is considered to approach a hydrophobic environment (and they are all certainly more hydrophobic than 0.1 M citrate buffer), then one is led to consider that the environment of the trifluoroacetyl group when associated with the enzyme is more polar than when it is in the citrate buffer without enzyme[3]. To test this hypothesis, the F^{19} spectrum of TFAP was measured in varying concentrations of NaCl at pH 6.0. As seen in Table I, increasing concentrations of NaCl led to successively greater downfield shifts of the fluorine resonance signal. Figure 3 shows that this variation is linear with the salt concentration and allows an extrapolation to a salt induced shift of 1.75 ppm for the enantiomer of larger shift (1.18 ppm for the enantiomer of smaller shift). This value is 9.75 M NaCl (6.55 M NaCl for the enantiomer of

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[3] Preliminary work with DL-N-acetyl-m-fluorophenylalanine showed that the enzyme-induced shift was again downfield for both enantiomers, while the solvent shift was upfield. However, the complex splitting pattern (ABCDX system) and the weaker signal from the one F atom led us to temporarily abandon this compound in favor of TFAP.

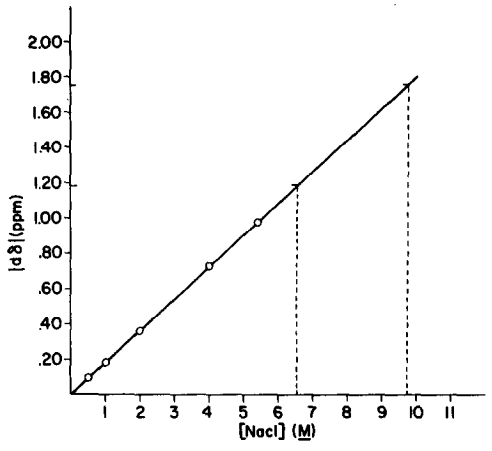

Figure 3. Change of chemical shift of 2.0×10^{-2} M TFAP caused by varying the concentration of NaCl. $|d\delta|$ values are calculated from the data of Table I by subtracting the chemical shift in absence of NaCl (-3.286 ppm) from the chemical shifts with NaCl.

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smaller shift); i.e., TFAP in a theoretical 9.75 M (or 6.55 M) NaCl solution would yield a downfield shift of the same magnitude as would be observed when TFAP is 100% complexed by CT. (The maximum solubility of NaCl in 0.1 M citrate buffer is 5.4 M.) A fluorescence study by Haugland and Stryer (1967) and an ultraviolet spectral study by Bender et al (1962) indicate that the environment of acyl-chymotrypsin is apparently highly polar[4].

Experiments designed to test which hypothesis is the correct explanation for this phenomenon are actively under way in our laboratories. The implications of the "polar environment before acylation" hypothesis with respect to the mechanism of chymotrypsin action are exceptional and this aspect of the problem is also being carefully examined.

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[4] One inconsistency in this argument is that ethanol, which presumably is less hydrophobic than benzene or p-dioxane, produces a greater upfield shift than these more hydrophobic solvents.

Summary: Fluorine (F^{19}) magnetic resonance has been used to examine the interaction of α-chymotrypsin and DL-N-Trifluoroacetylphenylalanine. The results show that the F^{19} chemical shift of the TFAP at pH 6.0 is moved downfield by the addition of the enzyme, while it is moved upfield when the compound is placed in the organic solvents ethanol, dioxane and benzene. In the absence of the enzyme the addition of NaCl causes a downfield shift of the F^{19} resonance peak, and the magnitude of the salt induced shift is directly proportional to the salt concentration. The results may be interpreted by either an anisotropic effect or by a "polar environment before acylation" hypothesis.

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