A NMR Study of the Interaction of N-Trifluoroacetyl-D-Phenylalanine with α-Chymotrypsin

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Zeffren and Reavill (1968) have recently reported the measurement by NMR methods of the dissociation constant (K_D) for N-trifluoroacetyl-D-phenylalanine[1] (TAPA) binding to α-chymotrypsin (α-CT) and the chemical shift (Δ) of the trifluoroacetyl ^{19}F resonance of TAPA upon binding to α-CT. Their results ($K_D = 3.4 \times 10^{-2}$ M and $\Delta = -1.75$ ppm at pH = 6.0) differ considerably from the results of Sykes (1968) ($K_D = 4.9 \times 10^{-2}$ M and $\Delta = +0.43$ ppm at pH = 7.8). The fact that the only difference between the two studies, besides the method of data analysis, was the pH suggests that the shift of the CF_3 fluorine resonance of TAPA upon binding to α-CT is very sensitive to changes in α-CT with changes in pH. Changes in the active site of α-CT at pH $\approx$ 7 have been shown to occur by several authors (Himoe, Brandt and Hess, 1967; Johnson and Knowles, 1966; and Moon, Stürtevant and Hess, 1965).

The chemical shift of the CF_3 fluorines of TAPA upon binding to α-CT could result from 1) proximity to an anisotropic group within the active site such as an aromatic residue

[1] The identification of the D enantiomer in the work of Zeffren and Reavill (1968) was provided by Dr. R. E. Reavill in a private communication.

(Johnson and Bovey, 1958), or 2) the relative polar or hydrophobic environment experienced within the active site. A polar environment could result from the electric field gradient of an ionizable residue in the active site (Buckingham, Schaefer, and Schneider, 1960); a hydrophobic environment could result from a cleft formed from non-polar residues (Spotswood, Evans, and Richards, 1967). The change in sign of Δ with change in pH from 6.0 to 7.8 thus implies that either the position of the inhibitor with respect to aromatic groups within the active site has changed or that the relative environment of the active site has changed, or some combination of the two effects. A possible explanation is the proposed protonation of His-57 below pH $\simeq$ 7.3 (Johnson and Knowles, 1966).

It is also worth pointing out that serious errors can enter into the analysis of the NMR data to obtain the parameters K_D and Δ. In the NMR fast exchange limit the observed chemical shift is the weighted average of the chemical shifts of the individual sites in the absence of exchange. Thus for the reaction

$$E + I = EI \tag{1}$$

the observed chemical shift is

$$\delta_{OBS} = \frac{[EI]}{I^o}\Delta + \delta_I \tag{2}$$

where δ_I is the chemical shift of the inhibitor in free solution and Δ is the shift of the inhibitor resonance upon binding to the enzyme. Typically, δ_{OBS} is measured as a function of initial inhibitor concentration at constant initial enzyme concentration. Thus if external referencing is used, δ_{OBS} must be corrected for any concentration dependence of the inhibitor resonance in the absence of binding (including a

change in bulk diamagnetic susceptibility with changing inhibitor concentration) and δ_I (measured in inhibitor solution without enzyme) must be corrected for the change in bulk diamagnetic susceptibility and solution properties upon the addition of enzyme. This second factor can be particularly difficult to determine, especially considering the sensitivity of nuclei such as ^{19}F to slight changes in solvent.

There are at least two approaches to obtain the parameters K_D and Δ from the observed chemical shifts. One is the method of Spotswood, Evans and Richards (1967) where the shifts are plotted according to equation 3

$$\frac{1}{\delta_{OBS} - \delta_I} = \frac{1}{\Delta}\left(\frac{I^o}{E^o}\right) + \frac{K_D}{\Delta E^o} \qquad (3)$$

Here the assumption $[I] \simeq I^o$ has been made.

A second approach (Sykes, 1968; Groves, Huck and Homer, 1967) is to fit the observed shifts to equations 2, 4 and 5

$$[EI] = \frac{(E^o + I^o + K_D) \pm \sqrt{(E^o + I^o + K_D)^2 - 4 E^o I^o}}{2} \qquad (4)$$

$$0 \leq [EI] / E^o \leq 1 . \qquad (5)$$

The value of K_D is chosen as the one with which a plot of the experimental values of δ_{OBS} versus $[EI] / I^o$ best fits a straight line. This solution gives K_D, Δ, and δ_I. This method has two advantages over the double reciprocal plot method of Spotswood, Evans, and Richards (1967). First, the value of δ_I, to which the double reciprocal plot is very sensitive, does not have to be measured. Second, the approximation $[I] \simeq I^o$ is not made and a wider range of concentrations can be used.

The chemical shift[2] of the trifluoroacetyl fluorines of TAPA[3] exchanging with α-CT, obtained in this study, is presented in Fig. 1. The inverse of the difference between the observed shift of the exchange averaged line and the corrected chemical shift of the inhibitor in free solution, δ_{OBS}^{FIT} (obtained from the fit of the observed shifts to equations 2, 4, and 5) is plotted versus the ratio of initial inhibitor to initial enzyme concentration. The error bars indicate an estimated accuracy of +0.10 Hz. In fact, the scatter in Fig. 1 is much less. There was no shift as a function of TAPA concentration for exchange of TAPA with chymotrypsinogen A, consistent with a large dissociation constant or a small chemical shift of the enzyme-inhibitor complex (Sykes, 1968). This also indicates that no correction to the observed shifts for exchange with α-CT is necessary because of a concentration dependence of the shift of TAPA from a source other than the binding process. The constants obtained from the fit of the observed shifts to equations 2, 4, and 5 are $K_D = 4.9 \times 10^{-2}$ M and $\Delta = +41$ Hz.

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[2]The chemical shifts were measured on a Varian HA-100 spectrometer operating at 94.1 MHz. A capillary of trifluoroacetic acid, held concentric with the axis of the NMR tube, was used as a reference signal. The same capillary was used for all samples. After temperature equilibrium was obtained, approximately 6 scans of the spectrum of the inhibitor were accumulated with a Varian C-1024 Time Averaging Computer while the spectrometer was locked on the trifluoroacetic acid reference. A sweep width of 10 Hz was accumulated at 0.1 Hz/sec. The chemical shift was determined by interpolation between markers which were calibrated in terms of the difference in frequency between sweep oscillator and the lock oscillator. The probe temperature of the spectrometer was $33 \pm 1^{\circ}$ C.

[3]Worthington three times crystallized α-CT (lot #CDI-6JF) was used without further purification. The concentration of active α-CT was determined by the 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate method of Erlanger and Edel (1964). TAPA was prepared by the method of Kerr and Nieman (1958). It had a melting point of 112-113.5° C (uncorr.) and a specific rotation of $[\alpha]_D^{25^{\circ}\,C} = -17.5$ (c=2, in ethanol). All solutions were made up in 0.1 M Tris-HCl buffer, pH = 7.8.

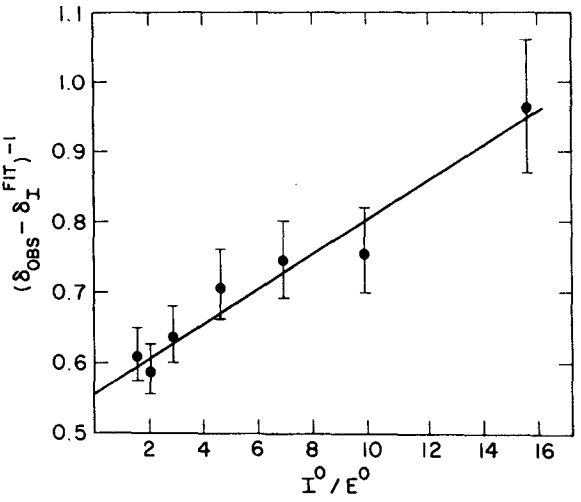

Fig. 1: The chemical shift of TAPA exchanging with α-CT as a function of I^o / E^o.

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SUMMARY:

The dissociation constant and bound chemical shift for N-trifluoroacetyl-D-phenylalanine exchanging with α-chymotrypsin has been determined by ^{19}F NMR. A computer method is presented for obtaining these constants from chemical shift data. The bound chemical shift appears to be a very sensitive probe for the active site of α-chymotrypsin and reflects changes in the active site with changes in pH.

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