Magnetic Resonance Studies of Protein–Small Molecule Interactions. Dynamics of Binding between *N*-Trifluoroacetyl-D-tryptophan and α-Chymotrypsin

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Abstract: Magnetic resonance techniques (specifically adiabatic half-passage) have been used to study the rates of association and dissociation of enzyme–inhibitor complexes (α-chymotrypsin–*N*-trifluoroacetyl-D-tryptophan). The principal contribution to relaxation for this system has been found to be the exchange process itself. At pH 5.0, $k_{on} = 14.9 \times 10^6$ l. mol^{-1} sec^{-1}, $k_{off} = 3.9 \times 10^3$ sec^{-1}. At pH 7.0, k_{on} 5.9×10^6 l. mol^{-1} sec^{-1}, $k_{off} = 3.5 \times 10^3$ sec^{-1}. Thus, at pH 5.0 the association rate is slower than that expected for a diffusion-controlled process only by a factor of about 50. The physical meaning of these rates and the relation of this work to other magnetic resonance studies of enzyme–inhibitor dynamics are discussed.

The dynamic events that occur when inhibitors bind to α-chymotrypsin have recently become amenable to experimental attack by magnetic resonance techniques which depend on adiabatic half-passage studies[1] or broadening of the observed resonance[2,3] when a molecule exchanges between solution and an enzyme. Two major sources can account for this broadening: (i) relaxation characteristics of the molecule under observation when bound to the enzyme and (ii) exchange broadening, a phenomenon which results when the rate of exchange is not very much faster than $\sqrt{2}\pi\Delta_{AB}$ (say a factor of ten), where Δ_{AB} is the difference in chemical shifts between the two environments.[4] If this exchange broadening contributes appreciably to the observed increase in line width when small molecules interact with proteins, the rate constants for formation and dissociation of the enzyme–small molecule complex can be determined.

In the previous work applied to chymotrypsin (studying the binding of *N*-trifluoroacetyl-D-phenylalanine), one group[1] has assumed that exchange broadening dominates; the other (using D- and L-tryptophan[2] and *trans*-cinnamate[3]) has analyzed line broadening exclusively in terms of the relaxation characteristics of the bound species.

The work of this paper uses the techniques of adiabatic half-passage to study the dynamics of binding of *N*-trifluoroacetyl-D-tryptophan to α-chymotrypsin. We conclude that the effect is caused by exchange and find the rate constant for dissociation of chymotrypsin–*N*-trifluoroacetyl-D-tryptophan at pH 5 to be: $k_{off} = 3.88 \times 10^3$ sec^{-1}. Taken together with a K_I for this complex of 2.6×10^{-4} *M*,[5] this gives an association rate constant $k_{on} = 1.5 \times 10^7$ mol^{-1} sec^{-1} which agrees well with values found from relaxation studies of furylacryloyl-L-tryptophanamide[6] ($k_{on} = 6.2 \times 10^6$ mol^{-1} sec^{-1}). Sykes on the other hand[1] has reported values for k_{on} which are slower by a factor of about 10^2–10^3.

A rough study of the pH dependence of these rates shows that k_{off} remains essentially constant whereas k_{on} decreases as the pH is raised from 5 to 7.

Principles of the Method. In a solution of enzyme and inhibitor, we assume the inhibitor can exist in two environments, free in solution or bound to the enzyme in an enzyme–inhibitor complex. Equations 1 and 2 describe this situation.

$$EI \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E + I \tag{1}$$

$$K_I = \frac{k_{off}}{k_{on}} = \frac{[E][I]}{[EI]} \tag{2}$$

If the rate of exchange between the two environments exceeds the nmr time scale,[4,7] the resonance of the inhibitor in the presence of enzyme will appear at a chemical shift, δ, that is the weighted average of the chemical shift for the inhibitor free in solution, δ_I, and the inhibitor in the enzyme–inhibitor complex, δ_{EI}. In eq 3 the quantities p_I and p_{EI} refer to the relative

$$\delta = p_I\delta_I + p_{EI}\delta_{EI} \tag{3}$$

populations or mole fractions of inhibitor in solution (p_I), or in the enzyme–inhibitor complex (p_{EI}).

On the other hand, the line width of the exchanging resonance will be the weighted average of that for the inhibitor in solution or in the enzyme–inhibitor complex *only if* the exchange process itself does not contribute to relaxation. Under nonsaturating conditions, the observed line width at half-peak height is related to the observed transverse spin–spin relaxation time, T_2, by eq 4, where $\delta\nu_{1/2}$ is the line width

$$1/T_2 = \pi\delta\nu_{1/2} \tag{4}$$

at half-maximum amplitude. For a resonance arising from exchange between two environments at a rate near the rapid exchange limit, T_2 is also given[4] by eq 5

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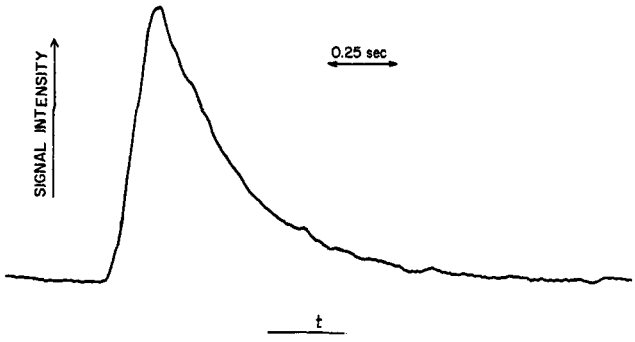

Figure 1. Typical exponential decay of the dispersion mode of a signal after adiabatic half-passage into the center of resonance. The time constant is $T_{1\rho}$ which becomes equal to T_2 as H_1 approaches zero. In this case H_1 is sufficiently small that $T_{1\rho} = T_2$. The solution was 1.92 m*M* α-chromotrypsin (corrected to active protein concentration), 30.6 m*M* *N*-trifluoroacetyl-D-tryptophan at pH 5.0 in 0.1 *M* citrate buffer. The decay defines $T_2 = 0.29$ sec.

$$\frac{1}{T_2} = \frac{p_I}{T_{2I}} + \frac{p_{EI}}{T_{2EI}} + p_I^2 p_{EI}^2(\delta_I - \delta_{EI})^2(\tau_I + \tau_{EI}) \quad (5)$$

where τ_I, τ_{EI} are the mean lifetimes and T_{2I} and T_{2EI} are the spin–spin relaxation times for inhibitor in solution (I) and in the enzyme–inhibitor complex (EI). Since

$$p_{EI} = \tau_{EI}/(\tau_I + \tau_{EI})$$

$$k_{off} = 1/\tau_{EI}$$

and

$$\Delta = \delta_I - \delta_{EI}$$

eq 5 reduces to eq 6.

$$\frac{1}{T_2} = \frac{p_I}{T_{2I}} + \frac{p_{EI}}{T_{2EI}} + \frac{p_I^2 p_{EI}\Delta^2}{k_{off}} \quad (6)$$

Only when the exchange broadening term $p_I^2 p_{EI}\Delta^2/k_{off}$ is unimportant ($k_{off} \gg \Delta^2$) does the observed transverse relaxation time represent the weighted average for the two sites and a plot of $1/T_2$ *vs.* EI/I_0 ($=p_{EI}$) will then be linear with an intercept of $1/T_{2I}$ and a slope of $1/T_{2EI} - 1/T_{2I}$. Unfortunately most nmr studies of enzyme–inhibitor interactions[1-3] have been done with $I_0 > E_0$ so that p_I usually varies only slightly from unity. Under these conditions, the contribution to $1/T_2$ from the chemical exchange term $p_I^2 p_{EI}\Delta^2/k_{off}$ will also be roughly proportional to EI/I_0 and dissecting the two sources of relaxation from a plot of $1/T_2$ *vs.* EI/I_0 will be difficult.

If, however, experiments are done under conditions such that p_I may differ considerably from unity and chemical exchange represents a significant contribution to nuclear relaxation, then a plot of $1/T_2$ *vs.* EI/I_0 should be clearly nonlinear.

Another method for distinguishing these two possible contributions to spin–spin relaxation involves measurement of the longitudinal or spin–lattice relaxation time, T_1, which, for the resonance of a nucleus exchanging between two sites, usually contains no significant contribution from the exchange process itself, but is, simply, the weighted average for the two sites; *i.e.*,

$$\frac{1}{T_1} = \frac{p_I}{T_{1I}} + \frac{p_{EI}}{T_{1EI}} \quad (7)$$

(Exchange can effect T_1 if the scalar coupling differs in the two environments for the nucleus being examined. In the present case, the CF_3 group is only weakly coupled to the α-C–H, $J \sim 0.4$ Hz, and this possible effect is therefore negligible.) If the correlation time, τ_c, for the group whose nuclei are being observed is less than the frequency of the nmr measurement (the Larmor frequency $\sim 10^{-9}$ sec in the present case) then the spin–spin (T_2) and spin–lattice (T_1) relaxation times will be equal.[8] For a protein the size of chymotrypsin, τ_c is about 10^{-8} sec[9] and if this is also the correlation time of the "reporting" group in the enzyme–inhibitor complex, then $T_{1EI} \neq T_{2EI}$.

As a result, differences between the observed values of T_1 and T_2 measured for a resonance of a nucleus exchanging between solution and a protein can arise from two independent phenomena: (i) $T_{1EI} \neq T_{2EI}$ because the correlation time of the reporting group in the inhibitor when bound to the enzyme exceeds the frequency of observation or (ii) the rate of exchange is such that the exchange broadening term $p_I^2 p_{EI}\Delta^2/k_{off}$ contributes significantly to the observed value of T_2.

How can one distinguish these two possibilities, in the absence of independent evidence either about the rate exchange or about the correlation time of the reporting group when bound to the protein? Basically two techniques can be employed. In one, the observed values of $1/T_2$ are plotted against EI/I_0. If the exchange term $p_I^2 p_{EI}\Delta^2/k_{off}$ makes no contribution, such a plot should be linear and intersect the $[EI]/[I_0]$ axis at a value of $1/T_{2I}$, which equals $1/T_{1I}$. On the other hand, if exchange contributes significantly to relaxation, such a plot will be nonlinear or if the best straight line is taken through the experimental points, the extrapolated intercept ($1/T_{2I_{app}}$) at $[EI]/[I_0] = 0$ will exceed $1/T_{1I}$ and the true value of $1/T_{2I}$. The nonlinear nature of the plot will be manifest only if $[EI]/[I_0] = p_{EI}$ is varied over a sufficiently broad range that the exchange term becomes nonlinear with p_{EI} because of the nonlinear dependence of $p_I^2 p_{EI} = (1 - p_{EI})^2 p_{EI}$ on p_{EI}. (In the present case, the extrapolated intercept ($1/T_{2I_{app}}$) exceeds $1/T_{1I}$ but the nonlinear nature of the curve as $[EI]/[I_0]$ decreases is just beginning to become apparent (Figure 1).)

The second method depends on the correlation times defined by the values of $1/T_1$ and $1/T_2$ extrapolated to the enzyme–inhibitor complex. As T_1 contains no contribution from the exchange process, T_{1EI} can be unambiguously obtained and this generally defines two possible correlation times (one to the right and one to the left of a point at $1/\omega_0$, where ω_0 is the frequency of observation). For the point to the left of $1/\omega_0$, $T_1 = T_2$; for the point to the right of $1/\omega_0$, $T_1 > T$ (see Figure 9-2 in ref 8). Accordingly, extrapolation of $1/T_2$ to the enzyme–inhibitor complex should, in the absence of relaxation caused by exchange, give either $T_{2EI} = T_{1EI}$ or, if $T_{1EI} \neq T_{2EI}$, a value of T_{2EI} which corresponds to the same correlation times as that defined by T_{1EI}. If this is not the case and the value of T_2 obtained by extrapolation is *greater* than that required for that correlation time to the right of $1/\omega_0$ defined by T_{1EI}, then this correlation time is eliminated and that

(8) Reference 4, Chapter 9.

(9) Based on the equation $\tau_c = 4\pi\eta a^3 3kT$, where η is the macroscopic viscosity of the solution and a the radius of a presumably spherical protein molecule, in this case 19.5 Å.[2]

value of τ_c to the left of $1/\omega_o$ unambiguously prescribed with the concomitant conclusions that $T_{1EI} = T_{2EI}$ and that the difference between the extrapolated values of T_1 ($= T_{1EI}$) and T_2 must arise from chemical exchange. (The results of the present work belong in this category.) On the other hand, if the extrapolated value of T_2 is *less* than that required for the correlation time to the right of $1/\omega_o$ defined by T_{1EI}, then some contribution to the extrapolated value of T_2 arises from chemical exchange, though whether the total difference between the extrapolated values of T_1 and T_2 are due to chemical exchange or whether some of this difference is due to chemical exchange and some due to a correlation time longer than $1/\omega_o$ is not unambiguously determined. However, the rate of chemical exchange cannot become too slow (the resulting difference between T_1 and T_2 becomes, then, large) or the observed spectrum will no longer be the broadened, weighted average of the nucleus in the two environments, but will begin to be resolved into two separate resonances.

The foregoing discussion has tacitly assumed that the actual correlation time has not become sufficiently long that T_2 has ceased to have a linear dependence on τ_c and has begun to level off. For ^1H, the point at which T_2 *vs.* τ_c levels off does not occur until around $\tau_c \sim 10^{-6}$ sec and the available data suggest similar behavior for ^{19}F. As this value τ_c is larger by about 10^2 than that for chymotrypsin, the assumption of the previous paragraph seems reasonable.

The distinction between exchange broadening and broadening due to molecular motions slower than ω can also be unambiguously made by observations of T_1 and T_2 at different field strengths. This will change the minimum in the T_1 *vs.* τ_c curve and, if the case in question is to the right of this minimum, a change in T_1 with field strength will be observed, whereas if the applicable τ_c is to the left of the minimum, T_1 will not depend on H_0. Moreover a change in H_0 will cause a change in Δ and this will be reflected in a change in the observed T_2 if the exchange broadening term $p_I^2 p_{EI}\Delta^2/k_{off}$ contributes significantly to T_2. In fact, in his study of the interaction of *N*-trifluoroacetyl-D-phenylalanine with α-chymotrypsin Sykes observed the dependence of T_2 and the independence of T_1 on H_0.[1]

Adiabatic Half-Passage. The technique of adiabatic half-passage involves sweeping rapidly into the center of the dispersion mode of the resonance of interest and observing the time dependence rate of decay of the signal; the time constant for this decay is T_2. If one sweeps repeatedly into the center of the resonance after varying time lags since the last sweep and observes the relationship between the length of the time lag and the maximum signal observed, the time constant relating the maximum observed signal strength to the time lag is T_1.[1,10,11]

After rapid passage into the center of the resonance, the initial magnetization in the x′ direction (x′ refers to the reference frame rotating at the frequency of the resonance in question), $M_{x'}$, is given by eq 8 where

$$M_{x'} = M_0(e^{-t\pi/2/T_r})(1 - e^{-t_1/T_1}) \qquad (8)$$

$t\pi/2$ is the time spent sweeping into the center of resonance, $1/T_r = (1/T_1 + 1/T_2)/2$, t_1 is the time off resonance and M_0 is $M_{x'}$ when $t\pi/2 = 0$ and $t_1 = \infty$. (In actual experimental practice, $t\pi/2$ can be made much shorter than either T_1 or T_2 so that the term $e^{-t\pi/2/T_r}$ is essentially unity. Indeed, in this work, $t\pi/2$ was less than 10^{-3} sec, while the smallest T_1 or T_2 was 0.1 sec.) Also, in practice t_1 can be made long relative to T_1 so that M_0 can be evaluated. From eq 8 this leads to

$$\ln (M_0 = M_{x'}) = \ln M_0 - t_1/T_1 \qquad (9)$$

A plot of $\ln (M_0 - M_{x'})$ against t_1 will, accordingly, be linear with a slope of $-1/T_1$ allowing ready determination of T_1.

Furthermore, each magnetization, $M_{x'}$, created by adiabatic half-passage will decay with a time constant, $T_{1\rho}$, as a function of time, t.

$$M = M_{x'}e^{-t/T_{1\rho}} \qquad (10)$$

The time constant, $T_{1\rho}$, though a complex function of the applied radiofrequency field H_1 becomes equal to T_2 as H_1 approaches zero. In practice, T_2 is taken as the limit to which $T_{1\rho}$ approaches as M_1 is diminished[1] and T_2 determined from the slope of the line obtained by plotting $\ln M$ *vs.* t as suggested by eq 11.

$$\ln M = \ln M_{x'} - 1/t_2 \qquad (11)$$

In this manner both T_1 and T_2 can be determined for various mixtures of enzyme and inhibitor. Analysis of these data by the extrapolative procedures discussed previously with appropriate account being taken of enzyme self-association[7] allows one to determine T_{1EI} and the extrapolated value of T_2 which, as discussed above, can in principle have contributions from T_{2EI} and the dynamics of solution–enzyme exchange.

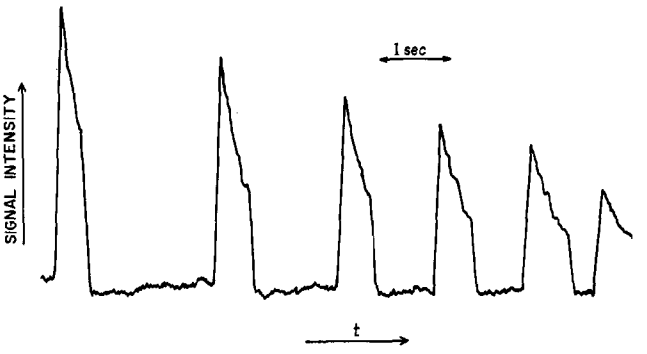

Figure 2. Typical series of excitations with varying intervening times off resonance. The solution was 1.92 m*M* α-chymotrypsin (corrected for active protein concentration), 30.6 m*M* *N*-trifluoroacetyl-D-tryptophan at pH 5.0 in 0.1 *M* citrate buffer. The decay defines T_1 = 1.34 sec.

Results

The techniques discussed above were used to measure T_1 and T_2 for the fluorine resonance of *N*-trifluoroacetyl-D-tryptophan at 5–30 m*M* concentrations in solutions containing 1.92 m*M* α-chymotrypsin. (This value is corrected for the 80% activity[6] of the commercial enzyme used.) Determinations were made at pH 5.0 and 7.0. As the values for the enzyme–inhibitor dissociation constant K_I and the enzyme dimerization constant were known from other work,[5,7] the values of EI/I_0 could be calculated independently. Figure 1 shows a typical exponential decay with the time constant T_2. Figure 2 shows a typical series of excitations

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(11) S. Meiboom, *J. Chem. Phys.*, **34**, 375 (1961).

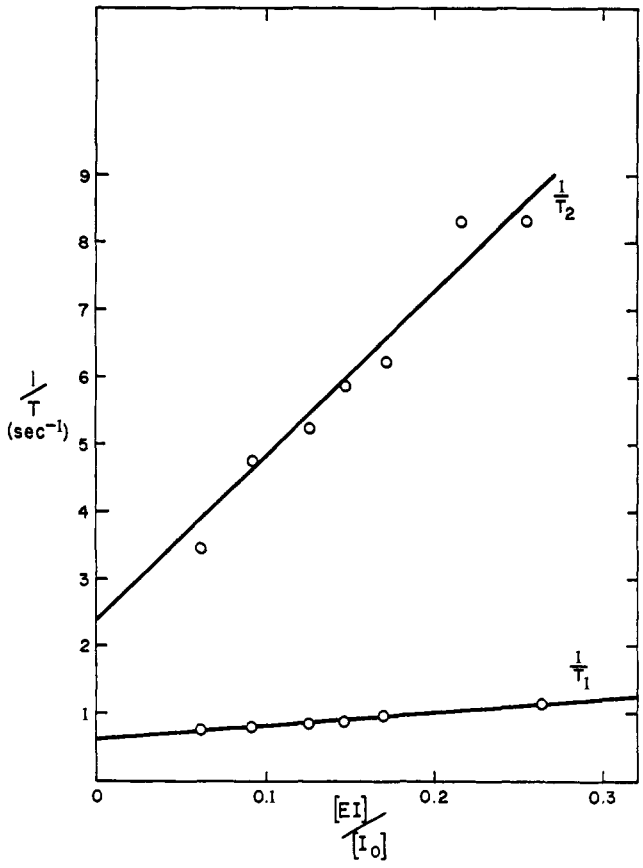

Figure 3. Plot of $1/T_1$ as a function of $[EI]/[I_0]$ at pH 5.0 in 0.1 M citrate buffer, 1.92 mM α-chymotrypsin; concentration of N-trifluoroacetyl-D-tryptophan varied from 5 to 30 mM.

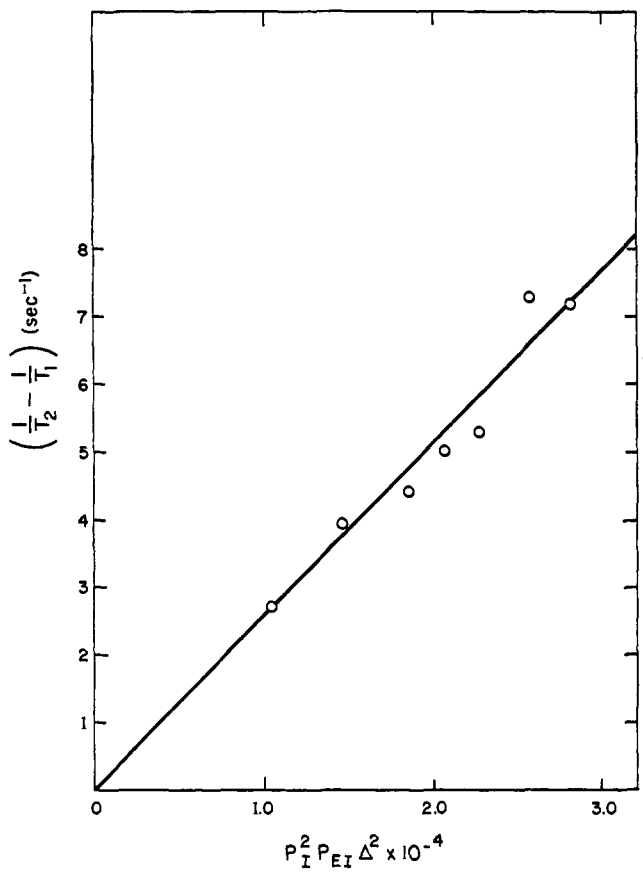

Figure 4. Plot of $(1/T_2 - 1/T_1)$ $vs.$ the exchange broadening term $p_I^2 p_{EI} \Delta^2/k_{off}$ at pH 5.0 in 0.1 M citrate buffer, 1.92 mM α-chymotrypsin; N-trifluoroacetyl-D-tryptophan concentration varied from 5 to 30 mM.

with varying intervening time periods. A logarithmic plot of the maximum intensity $vs.$ the time off resonance gives the time constant T_1. Figure 3 illustrates typical plots of $1/T_1$ and $1/T_2$ for the resonance in question at pH 5.0 and shows that T_1 is significantly larger than T_2.

We must now decide whether this inequality is due to the correlation time of the trifluoromethyl group of bound N-trifluoroacetyl-D-tryptophan being longer than $\sim 10^{-9}$ sec, or whether it stems from exchange dynamics because of the importance of the term $p_I^2 p_{EI} \Delta^2/k_{off}$.

Let us first assume the former, that is, exchange dynamics do not contribute, in which case eq 6 reduces to eq 12 and as $p_{EI} = EI/I_0$, a plot of $1/T_2$ $vs.$ EI/I_0 should

$$\frac{1}{T_2} = \frac{p_I}{T_{2I}} + \frac{p_{EI}}{T_{2EI}} \tag{12}$$

be linear and extrapolate to $1/T_{2I}$ at $EI/I_0 = 0$. This is the basis of the plot in Figure 1, the results of which are summarized in Table I. An important result is that $1/T_2$ and $1/T_1$ do *not* extrapolate to the same value for T_{1I} and T_{2I} which is a consequence of the importance of the exchange broadening term $p_I^2 p_{EI} \Delta^2/k_{off}$ which was assumed to be negligible in plotting $1/T_2$ of Figure 1. As experimental data were obtained at relatively high values of EI/I_0 (up to 0.28) this term is nonlinear with EI/I_0 (because of $p_I^2 = (I_0 - EI)/I_0$) and causes the true curve of $1/T_2$ $vs.$ EI/I_0 to begin to level off at higher values of EI/I_0. Thus a linear extrapolation back to $EI/I_0 = 0$ from these points will yield a value of $1/T_{2I}$ higher than the true value, which is just the present result.

Accordingly, the data were treated with exchange dynamics as the source of the inequality of T_2 and T_1. Substitution of eq 7 into eq 6 yields eq 13. Figure 4

$$\frac{1}{T_2} - \frac{1}{T_1} = \frac{p_I^2 p_{EI} \Delta^2}{k_{off}} \tag{13}$$

shows a plot of $1/T_2 - 1/T_1$ against $p_I^2 p_{EI} \Delta^2$ for the data at pH 5.0 (Δ was independently available[5]). The plot is convincingly linear with a zero intercept indicating that $T_{1I} = T_{2I}$ and supporting the present assumption that exchange dynamics are the origin of the inequality in T_2 and T_1. This leads to a determination of k_{off} and these results, together with those at pH 7.0, are collected in Table II (which includes values for K_1 from previous work[5] and k_{on}).

Table I. Relaxation Parameters for N-Trifluoroacetyltryptophan in Solution and Bound to α-Chymotrypsin Assuming Rapid Exchange (No Exchange Broadening)

pH	T_{1I}, sec	T_{1EI}, sec	T_{2I}[a]	T_{2EI}[a]
5.0	1.61	0.40	0.41	0.039
7.0	1.67	0.43	0.33	0.037

[a] These values assume that contribution to relaxation in the EI complex from $p_I^2 p_{DI} \Delta^2/k_{off}$ is negligible.

Discussion

We shall first examine the conclusion from above that the inequality of T_2 and T_1 is entirely due to ex-

Table II. Kinetic and Thermodynamic Parameters for the *N*-Trifluoroacetyl-D-tryptophan–α-Chymotrypsin Complex[a]

pH	k_{off}, sec^{-1}	k_{on}, M^{-1} sec^{-1}	K_I, M[b]
5.0	3.9×10^3	14.9×10^6	2.6×10^{-4}
7.0	3.51×10^3	5.9×10^6	5.9×10^{-4}

[a] Assumes $T_{2EI} = T_{1EI}$. [b] Reference 5.

change processes by examining in detail the correlation times implied by the results of this work.

The spin–lattice relaxation time, T_1, is not perturbed by the rates of exchange processes. Experimental values of T_1 may, therefore, be extrapolated to yield T_{1I} and T_{1EI} without need of assumptions or approximations as in the case of T_2. The value for T_1 so obtained for a given site can be related to the relaxation time τ_c for the nucleus at that site by the following relationships.[7] For values of $\tau_c < 1/\omega_o$, $\log T_1 = -\log \tau_c +$ constant. When $\tau_c = 1/\omega_o$, a minimum value for T_1 is attained. For $\tau_c > 1/\omega_o$, $\log T_1 = \log \tau_c +$ constant. In solution T_1 for the CF_3 group is $\leqslant 10^{-11}$ sec (where $T_1 \sim 2$ sec).[12] Accordingly, the observed value of $T_{1EI} \sim 0.4$ sec could represent $\tau_c \geqslant 10^{-8}$ or $\leqslant 10^{-10}$ sec (depending on whether the system lies to the right or left respectively of the minimum at $\tau_c = 1/\omega_o$).

The first possibility ($\tau_c \leqslant 10^{-10}$ sec) suggests a CF_3 group which is relatively free to rotate when the inhibitor is bound to the enzyme. As this correlation time is less than $1/\omega_o$, it represents the region where $T_{1EI} = T_{2EI}$.

In contrast, the second possibility ($\tau_c \geqslant 10^{-8}$ sec) implies a CF_3 group which has essentially the same correlation time as the protein to which the inhibitor is bound. If $T_{1EI} \geqslant 10^{-8}$ sec, one can estimate that by linear extrapolation $T_{2EI} \leqslant 0.006$ sec.[13] (Except for a slight nonlinear segment near $1/\omega_o$ and a leveling off at long correlation times, $\log T_2 = -\log \tau_c +$ constant over the entire range. Thus, T_2 *vs.* τ_c does not show the minimum at τ_c as does T_1.) For $\tau_c \geqslant 10^{-8}$ sec, this value of T_{2EI} (0.006 sec) represents the *maximum* permissible (a lower extrapolated value of T_2 could arise if there were additional relaxation by virtue of chemical exchange) and as it is less by almost an order of magnitude than the observed value (0.04 sec) leads to the conclusion that $\tau_c \leqslant 10^{-10}$ sec, that $T_{2EI} = T_{1EI}$ and that the inequality of T_2 and T_1 is thus due solely to exchange and not to a correlation time greater than $1/\omega_o$.

Therefore, in the case of *N*-trifluoroacetyl-D-tryptophan, virtually all the observed line broadening resulting from the reversible binding of this inhibitor to α-chymotrypsin is caused by the dynamics of the exchange process itself and very little (<10%) can be attributed to restricted molecular motion of the CF_3 group when the inhibitor is bound to enzyme.

One should realize that this correlation time ($\leqslant 10^{-10}$ sec) is for the CF_3 group and *not* the inhibitor molecule as a whole. Inhibitor may well be bound rigidly to the enzyme; the experimental observation means only that at least the CF_3 group can rotate relatively freely about its local C_3 axis. Less mobile regions of the inhibitor molecule may experience correlations greater than 10^{-10} sec, indeed may have $\tau_c \sim 10^{-8}$ sec and exhibit line broadening associated with T_{2EI} shortened by this fact. However, as k_{off} is clearly the same for every part of the inhibitor molecule, any nucleus for which $\Delta \geqslant 10$ Hz will show a significant contribution to the line width from exchange broadening and assumptions to the contrary[2] must be made with great care.[1]

The kinetic values obtained in this work differ by a factor of 10^2–10^3 from those obtained in an analogous study[1] of the binding of *N*-trifluoroacetyl-D-phenylalanine to chymotrypsin in which case exchange dynamics were likewise shown to be the dominant effect (as opposed to inhibitor immobilization). We feel the origin of these discrepancies arise because of the neglect in the earlier work of such perturbations as enzyme self-association and the assumption that enzyme was saturated with inhibitor. Moreover, the kinetic constants obtained from this work are in pleasing agreement with those found in a *T*-jump relaxation study[6] of the rapid binding–dissociation interaction between furylacryloyl-L-tryptophanamide (k_{on} 6.2×10^6 mol^{-1} sec^{-1} and k_{off} 2.7×10^3 sec^{-1}). Exact correspondence is, of course, not to be expected because in the two cases not only do the nature of the bound molecules differ, but also their chirality.

From previous studies on the dependence of binding on pH, we have concluded that carboxylate anion inhibitors bind only to a protonated form of chymotrypsin, the acidic group of importance (presumably His-57) showing a pK_a of 6.6. In the present study the value of k_{off} is observed to be unaffected by a change from pH 5.0 to 7.0. On the other hand, K_I increases by a factor of about 2.5 as the pH is raised from 5.0 to 7.0; k_{on} shows a corresponding decrease. Essentially this results because k_{on}, being a bimolecular constant, is sensitive to the fraction of the enzyme present in a reactive form. Between pH 5.0 and 7.0 this fraction decreases, because of deprotonation of the group with pK_a of 6.6, by a factor of 2.5; k_{on} decreases as a result by the same factor, that is, again only protonated chymotrypsin accepts an inhibitor molecule.

The value of k_{on} at pH 5.0 when all the enzyme is in the protonated reactive form of 1.5×10^7 mol^{-1} sec^{-1} should be compared with the diffusion-controlled rate constant for interaction between a small molecule and a protein of chymotrypsin's size of 10^9 mol^{-1} sec^{-1}.[14] The actual rate of binding, being slower by a factor of about 50, may reflect the molecular reorganizations necessary, for example for opening of the hydrophobic pocket and insertion of the aromatic ring. The small molecule enters the active site of the enzyme not as a solid, inflexible object fitting into a preformed hole, but as a result of conformational reorganization of both small molecule and protein adapting themselves for the most favorable binding interactions. The energetics of any such reorganizations deserve comment for a kinetic factor of 50 represents 2.3 kcal/mol, which emphasizes that these reorganizations do not impose a serious energy barrier to binding in the case of chymotrypsin.

For those regions of enzymes that bind hydrophobic groups, one would anticipate that, in the absence of substrate or inhibitor, the binding pocket would be closed, for nature abhors a vacuum and water is unlikely to find a favorable reception in an environ-

(12) T. E. Burke and S. I. Chan, *J. Mag. Resonance*, **3**, 55 (1970).
(13) E. W. Bittner and J. T. Gerig, *J. Amer. Chem. Soc.*, **92**, 5001 (1970).
(14) M. Eigen and G. B. Hammes, *Advan. Enzymol.*, **25**, 1 (1963).

ment as hydrophobic as, for example, the pocket in chymotrypsin into which aromatic or hydrophobic side chains bind. Accordingly, one will anticipate some molecular reorganization as the hydrophobic pocket opens with concomitant entry of a group from the small molecule which is binding. For hydrophilic regions of the active sites of enzyme, these comments may not apply, for, in these situations, water may occupy sites in the absence of substrates and binding may occur by displacement of water; thus hydrophilic regions of an active site may have geometries that differ little as a function of the presence or absence of substrate.

Even after being bound to the enzyme, certain portions of the small molecule may still retain considerable steric mobility, as, for example, does the trifluoromethyl group of *N*-trifluoroacetyl-D-tryptophan with a $\tau_c \leqslant 10^{-10}$ sec representing possibly only rotational freedom about the C_3 axis.

Experimental Section

Chemicals. *N*-Trifluoroacetyl-D-tryptophan was prepared as reported elsewhere.[5]

α-Chymotrypsin was obtained from Sigma Chemical Co. and contained 80% active protein as determined by titration.[7] Solutions were prepared in 0.1 *M* citrate buffer as previously described.[7]

Spectrometric Modification. All spectra were obtained on a modified Varian 100-15 spectrometer using a 12-mm probe. Modification consisted of adapting the control amplifier and phase detector in the Internal Reference Stabilization unit so that a field-frequency lock could be obtained while monitoring the dispersion mode of a resonance with the analytical channel. The sweep frequency oscillator was replaced by a stable voltage controlled oscillator of our own design that could either be swept linearly by a suitable ramp, the voltage of which is proportional to the memory address of a time averaging computer, or be swept rapidly by application of a sudden voltage jump.

The input voltage for the rapid sweep was obtained by monitoring the voltage drop across a capacitor while the capacitor was being charged or discharged. The circuitry was arranged in such a way that the time constant for charging or discharging the capacitor could be varied from 0.005 to 0.01 sec. The magnitude of the voltage jump was also variable so that the amount of frequency swept could be varied from 0 to 400 Hz. In practice, we commonly swept 200 Hz into the center of the resonance using a time constant for the capacitor of 0.01 sec. As the nuclei of interest become excited only in the last several cycles, $t\pi/2$ in eq 8 is much less than 0.01 sec (probably less than 0.001 sec) and accordingly negligible compared to T_1 or T_2.

The spectrometer was locked on a capillary of hexafluoroacetone and the lock frequency adjusted to center the resonance for a given state of capacitor charge. The frequency can then be moved off resonance by changing the state of charge on the capacitor and then, after an appropriate length of time, swept back to the position of resonance. More simply, the apparatus is so arranged that one is either on resonance with the capacitor charged and off resonance when the capacitor is discharged or *vice versa*. One, then, sweeps onto resonance by discharging a charged capacitor, or charging a discharged capacitor. Either procedure gives an equivalent result.

A Fabri-tek 1062 time averaging computer was used as the recorder because it provided a fast, accurate time base and an adequate frequency response. The spectra were transferred to paper for subsequent analysis.

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Catalysis of α-Hydrogen Exchange. XI. Monofunctional Catalysis of the Dedeuteration of Acetone-d_6[1,2]

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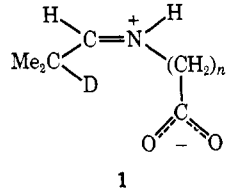
Abstract: The kinetics of the transformation of acetone-d_6 to acetone-d_5 and then acetone-d_4 in aqueous solution have been studied by a mass spectral method. As catalysts, hydrogen ion, hydroxide ion, trimethylamine, 1,4-diazabicyclo[2.2.2]octane, and methylamine buffers were studied. In the presence of methylamine buffers part of the reaction involves the rate-controlling removal of deuterium from the reversibly formed *N*-isopropylidenemethylammonium ion (the iminium ion). The rate constants reported for the attack of hydroxide ion on acetone-d_6 and of water and methylamine on the iminium ion differ significantly from earlier literature values.

Earlier papers in this series have described attempts to find bifunctional catalysis of the removal of the α-hydrogen atom of isobutyraldehyde in the presence of bifunctional species of the type B–R–NH_2, where B is a basic group and R is a divalent radical chosen to give an appropriate stereochemical relationship between B and the amino group. In the case of an ω-aminoalkanoic acid it was hoped that internal removal of the deuteron by the carboxylate anion group in the iminium ion **1** would yield bifunctional catalysis of the type desired. The fact that no such catalysis was observed with the amino acids for which n is 1–5 was attributed to the tendency of aldiminium ions to exist almost en-



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(2) For part X see J. Hine, M. S. Cholod, and J. H. Jensen, *J. Amer. Chem. Soc.*, **93**, 2321 (1971).