though it contains an arginine, an aspartic acid, and a glutamic acid residue. It is quite possible that salt bridges are present in secretin but that they are not directly revealed in the ORD and CD spectra, unless the gradual enhancement with increasing chain length in the values of the extrema are an expression of their contribution to a stable conformation. That the aspartic acid in position 3 has no major effect on the conformation is shown by the similarity of the spectra of S_{5-27} and secretin.

Conclusion

Our results suggest that a preferred conformation could exist in molecules as small as a tricosa- or heptacosapeptide even in the absence of strong conformation-determining factors such as disulfides or salt bridges. Rather the sum of subtle side-chain interactions seems to play an important role in determining the architecture. On the other hand, a certain number of amino acids with appropriate side chains must be present in the molecule before the weak interactions can result in a stable conformation. The 27-membered chain of secretin seems to have more than sufficient length to produce a secondary structure.[35]

(35) An inspection of a model of secretin assembled from space-filling (CPK) atomic models suggests that the side chain of leucine-26 is sandwiched between the side chains of phenylalanine-6 and leucine-10 and similarly the side chain of leucine-23 lies between those of leucine 10 and leucine-13. Of course, these suggestions can be considered only as possibilities and not as evidence. The secondary structure of secretin should be established by X-ray crystallography. In preliminary experiments secretin was crystallized both as a flavianate and as a salt of 4-hydroxyazobenzene-4′-carboxylic acid.

An Application of Transient Nuclear Magnetic Resonance Methods to the Measurement of Biological Exchange Rates. The Interaction of Trifluoroacetyl-D-phenylalanine with the Chymotrypsins

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Abstract: Transient nmr methods have been applied to the measurement of the rate constants of biological exchange reactions. The experimental method is based upon the measurement of the spin–lattice relaxation time in the rotating frame and is applied to the binding of trifluoroacetyl-D-phenylalanine to α-chymotrypsin, DFP-chymotrypsin, and chymotrypsinogen A. Trifluoroacetyl-D-phenylalanine is shown to bind to all three enzymes and the forward and reverse rate constants are determined for the first two. The rates are [k_1, M^{-1} sec^{-1} (k_{-1} sec^{-1})]: α-chymotrypsin, 1.0×10^4 (4.9×10^2); DFP-chymotrypsin, 1.6×10^4 (16.6×10^2). These rates are appropriate to conformational changes occurring in the enzymes upon inhibitor binding.

Nuclear magnetic resonance (nmr) methods have been extensively applied to the investigation of biological systems.[1] High-frequency nmr,[2] relaxation time and line width measurements,[3] and halide ion probe techniques[4,5] have been used to determine the structure, conformation, and motion of enzymes and proteins in solution. While interpretation of the nmr spectrum of the macromolecule is limited by its inherent complexity, much information can be obtained from the nmr spectrum of a small molecule that is able to probe the environment of the macromolecule by rapid exchange between free solution and attachment to the macromolecule. Hence, for an inhibitor exchanging between free solution and the active site of an enzyme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

an increase in the nmr line width of nuclei on the exchanging inhibitor molecule is often interpreted in terms of rotational restriction of the inhibitor within the active site, an increased rotational correlation time while bound to the enzyme, or an interaction with nuclei in the active site of the enzyme.

It has also been shown that a shift in the resonance frequency of nuclei on the inhibitor molecule can occur upon binding to the enzyme.[6,7] In principle, then,

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the nmr line widths also contain information about the rates of these exchange reactions. No attempt has been made to measure the rates of these reactions by nmr methods,[8] however, except in the case of enzymes with a metal ion in the active site.[9]

This paper presents an application of transient nmr methods to the measurement of the rate constants for biological exchange reactions. The experimental method is based upon the measurement of $T_{1\rho}$, the spin-lattice relaxation time in the rotating frame.[10-12] Contained in the method is a direct separation of the effects causing magnetic relaxation into those resulting from the exchange process and those occurring while the inhibitor is bound to the enzyme. The technique is used to measure the rate constants for the binding of N-trifluoroacetyl-D-phenylalanine (TAPA) to α-chymotrypsin, diisopropylphosphoryl-chymotrypsin (DFP-chymotrypsin), and chymotrypsinogen A.

The results indicate that a large number of rate constants, of considerable usefulness in the elucidation of enzyme specificity and the mechanisms of enzyme-catyzed reactions, can be directly and easily measured by nmr methods. The measurements require little special equipment and can be made on commercial nmr spectrometers.

Chemical Exchange

A necessary condition for the applicability of nmr to a specific system involving chemical exchange is that the exchange transfers the nuclei involved between sites of different local magnetic environment, characterized by different resonance frequencies. The exchange then is a relaxation mechanism for the nuclear-spin system. In the nmr fast-exchange region, where the rate of exchange is larger than the difference in resonance frequencies between sites, the individual resonances are averaged into a single resonance whose width is the weighted average of the widths of the individual resonances in the absence of exchange plus some residual broadening which decreases as the rate of exchange increases.

Consider an inhibitor binding to an enzyme. If a nucleus, or group of equivalent nuclei, on the inhibitor molecule experience a shift in resonance frequency while bound to the enzyme (because of the presence of an aromatic residue near the active site, for example), then the binding of this molecule to the enzyme exchanges these nuclei between positions of different resonance frequency. For inhibitors which are not irreversibly bound,[13] and for typical resonance frequency shifts,[14] these reactions will most often be in the fast-exchange limit and the rate information will be contained in the residual broadening of the averaged resonance.

This residual broadening is a function of the relative concentrations, the lifetimes, and the resonance frequency shifts of nuclei in the various sites. If exchange is the only slow process, then

$$\Delta\nu_{\text{residual}} = \frac{1}{T_2} - \frac{1}{T_1}$$

$$\frac{1}{T_1} = \sum_i \frac{P_i}{T_1(i)} \qquad (2)$$

where T_1 and T_2 are the longitudinal and transverse relaxation times, respectively, of the averaged resonance, P_i is the probability of a nucleus being in the site i, and $T_1(i)\,[= T_2(i)]$ is the longitudinal relaxation time of nuclei in the site i in the absence of exchange. A slow process implies that the rate of the effective motion causing relaxation is less than the resonance frequency of the nuclei involved. This limit is compatible with the fast-exchange limit

$$2\pi(\delta_i - \delta_j) < \frac{1}{\tau} < \omega_0 \qquad (3)$$

where $(\delta_i - \delta_j)$ is the difference in resonance frequency between sites i and j, $1/\tau$ is the rate of exchange, and ω_0 is the resonance frequency of the nuclei. For proton magnetic resonance, where typical shifts are 0–10^2 Hz and resonance frequencies are 10^2 MHz, this dual inequality is satisfied for rates in the range 10–10^6 sec^{-1}. The correlation time, τ, for most other interactions effective in liquids, except for the largest of macromolecules, will be less than 10^{-8} sec and these effects will contribute equally to T_1 and T_2.

Measurement of Relaxation Times

A direct and simple method, which has been previously used for the measurement of T_2,[10,11] is the spin-locking or rotating-frame experiment. This method is accurate for resonances with a low signal to noise ratio and is capable of measuring one resonance in a reasonably complex spectrum. Besides measuring T_2 in this manner, the rotating-frame experiment can also be used to measure T_1 under those experimental conditions. T_1's and T_2's in the range 10^{-1} to 10^1 sec can be conveniently measured.

The experiment is performed in the following manner. By adiabatic rapid passage into the center of the resonance, a magnetization of amplitude

$$M_x(t_1 + t_{\pi/2}) = M_0(1 - \exp(-t_1/T_1))\exp(-t_{\pi/2}/T_R) \qquad (4)$$

is aligned along the field H_1, where M_0 is the equilibrium magnetization, t_1 is the time spent off resonance after the previous passage into the center of the resonance, $t_{\pi/2}$ is the time required to pass into the center of the resonance, and $1/T_R = [(1/T_2) + (1/T_2)]/2$.[15]

This magnetization can be considered as resulting from a quantization of the nuclear spin states along the field H_1 in the interaction representation. It then relaxes, in a spin lattice fashion along H_1, toward a nearly zero equilibrium magnetization appropriate to a reso-

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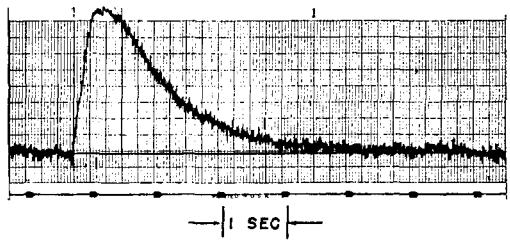

Figure 1. Typical ^{19}F $T_{1\rho}$ for trifluoroacetyl-D-phenylalanine binding to α-chymotrypsin; [trifluoroacetyl-D-phenylalanine] ≅ 0.048 *M*; 56.4 MHz.

nance experiment at a frequency $\omega_1 = \gamma H_1$ (see Figure 1). The decay is characterized by $T_{1\rho}$ which is not, in general, equal to T_2 (measured in the absence of H_1). For a system whose relaxation is caused, in part, by a slow motion, $T_{1\rho}$ has been shown to have the form

$$\frac{1}{T_{1\rho}(H_1)} = \langle A\rangle^2 \frac{\tau}{1 + (\omega_1\tau)^2} + \left(\frac{1}{T_1}\right)_{\text{other}} \tag{5a}$$

where $(1/T_1)_{\text{other}}$ is the contribution from relaxation processes which are not slow and $\langle A\rangle^2$ contains the physical constants of the slow relaxation mechanism. In comparison, T_2 has the form

$$\frac{1}{T_2} = \langle A\rangle^2\tau + \left(\frac{1}{T_1}\right)_{\text{other}} \tag{5b}$$

T_2 can thus be obtained by measuring $T_{1\rho}$ as a function of H_1 and extrapolating to $H_1 = 0$. Upon rearranging, eq 4 becomes

$$\ln\left[\frac{M_0\exp(-t_{\pi/2}/T_R) - M_x(t_1 + t_{\pi/2})}{M_0}\right] = \frac{t_1}{T_1} - \frac{t_{\pi/2}}{T_R} \tag{6}$$

and a plot of ln $(M_0 \exp(-t_{\pi/2}/T_R) - M_x(t_1 + t_{\pi/2}))$ *vs.* t_1 will have a slope $-1/T_1$. This is analogous to the 90–90° spin–echo pulse sequence.

The Form of $T_{1\rho}$ for Chemical Exchange

Consider the exchange of a nucleus, or group of equivalent nuclei, between two sites of different local magnetic environment

$$HA + B \underset{k_r}{\overset{k_f}{\rightleftharpoons}} A + HB \tag{7}$$

where H represents the nuclei exchanged and all spin–spin interactions involving H are negligible. In the fast-exchange limit

$$\tau_A/T_{1\rho},\ \tau_A/T_1(i),\ (\tau_A\delta_i) \ll 1 \qquad i = A, B \tag{8}$$

$T_{1\rho}$ is equal to

$$\frac{1}{T_{1\rho}(H_1)} = \sum_{i=A,B}\frac{P_i}{T_1(i)} + \frac{x\tau_A(\delta_A - \delta_B)^2}{(1 + x)^3 + (1 + x)\tau_A{}^2\omega_1{}^2} \tag{9}$$

where $x = [HA]/[HB]$, δ_i is the resonance frequency of H in site i, P_i is the probability of H being in site i, and

$$\frac{1}{\tau_A} = \frac{1}{[HA]}\frac{d[HA]}{dt} = k_f[B] \tag{10}$$

Similarly, in the limit

$$\tau_A/T_{1\rho},\ \tau_A/T_1(i),\ [HA]/[HB] \ll 1 \qquad i = A, B \tag{11}$$

$T_{1\rho}$ is equal to[16]

$$\frac{1}{T_{1\rho}(H_1)} = \sum_{i=A,B}\frac{P_i}{T_1(i)} + \frac{x\tau_A(\delta_A - \delta_B)^2}{(1 + x)^3 + (1 + x)\tau_A{}^2(\omega_1{}^2 + \delta_A{}^2)} \tag{12}$$

This expression is valid for all rates of exchange satisfying eq 11.

Making the following identifications between eq 1 and 7

$$EI \equiv HA,\ I \equiv HB$$

$$(\delta_A - \delta_B)^2 = (\delta_{EI} - \delta_I)^2 = \Delta^2 \tag{13a}$$

$$x = [EI]/[I] \ll 1$$

$$\frac{1}{\tau_A} = \frac{1}{[EI]}\frac{d[EI]}{dt} = k_{-1} \tag{13b}$$

the exchange contribution to relaxation in the fast-exchange limit becomes

$$\frac{1}{T_{1\rho}(O)} - \frac{1}{T_1} \simeq \frac{[EI]}{[I]}\left(\frac{1}{k_{-1}}\right)\Delta^2 \tag{14}$$

From eq 15

$$K_D = [E][I]/[EI] \tag{15}$$

the concentration of enzyme–inhibitor complex in the limit $[I] \cong I^\circ$ is equal to

$$[EI] \simeq I^0E^0/(K_D + I^0) \tag{16}$$

and

$$\frac{1}{T_{1\rho}(O)} - \frac{1}{T_1} = \frac{E^0}{K_D + I^0}\left(\frac{1}{k_{-1}}\right)\Delta^2 \tag{17}$$

It is important to remember that the units of Δ in eq 17 are radians sec^{-1}.

Determination of K_D and Δ

Obtaining the rate constants k_1 and k_{-1} thus requires the dissociation constant and the resonance-frequency shift of the enzyme–inhibitor complex. In the fast-exchange limit

$$\delta_{obsd} = \frac{[EI]}{[EI] + [I]}(\delta_I + \Delta) + \frac{[I]}{[EI] + [I]}\delta_I \tag{18}$$

This can be put in the form

$$\delta_{obsd} = ([EI]/I^0)\Delta + \delta_I \tag{19}$$

The concentration of enzyme–inhibitor complex is related to the known initial concentrations, E^0 and I^0, by

$$[EI] = \frac{(E^0 + I^0 + K_D) \pm \sqrt{(E^0 + I^0 + K_D)^2 - 4E^0I^0}}{2} \tag{20a}$$

$$0 \le [EI]/E^0 \le 1 \tag{20b}$$

K_D and Δ are obtained from the observed chemical shift of the exchange-averaged resonance as a function

(16) Equations 9 and 12 were first presented by Meiboom, whose results have been generalized to the case $T_1(i) \neq T_1(j)$.

of initial inhibitor concentration at constant initial enzyme concentration by a computer analysis.[17] The computer program works in the following manner. Taking the known E^0 and I^0's and an assumed K_D, the program calculates the values of [EI] according to eq 20 and plots δ_{obsd} *vs.* $[EI]/I^0$. It then calculates a least-mean-square straight line for the plot obtained and the root-mean-square deviation of the experimental points from the line. The best K_D, and hence Δ, is then chosen as the one giving the minimum deviation of the experimental points from a straight line.

Experimental Section

Worthington three times crystallized α-chymotrypsin (lot no. CDI-6JF) and chymotrypsinogen A (lot no. CG-F63) were used without further purification. Solutions of Worthington DFP-chymotrypsin (lot. no. CDDFP-208) were centrifuged before use. The concentration of active α-chymotrypsin was determined by the 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate method of Erlanger.[18] The concentrations of DFP-chymotrypsin and chymotripsinogen A were determined spectrophotometrically at 280 mμ, assuming a molar extinction coefficient of 5×10^4.[19] In experiments where the enzyme solutions were diluted, the concentration of enzyme was corrected for partial volume effects assuming a partial specific volume of 0.736.[20]

TAPA was prepared by the method of Kerr and Niemann.[21] The material had a melting point of 112–113.5° (uncor) and a specific rotation of $[\alpha]^{25}D$ − 17.5 (*c* 2, ethanol). All solutions were made up in 0.1 *M* Tris–HCl buffer, pH 7.8.

The relaxation time measurements were made at 56.4 and 94.1 MHz on slightly modified Varian nmr spectrometers (see ref 11). The modifications include a wide-band amplifier to provide a stronger H_1 field, a fast-response Sanborn recorder, and a field-pulsing circuit.[22] All filtering was kept to a minimum and the signal was recorded from the scope-out position of the V-3521 integrater-decoupler. A 15-mm insert was used at 56.4 MHz to increase signal strength.

The concentration of TAPA was kept constant for all relaxation time measurements so that no change in instrument settings was required between samples. The relaxation times T_1 and $T_{1\rho}$ (as a function of field strength H_1) were first measured for a stock inhibitor solution ($I^0 \cong 4.8 \times 10^{-2}$ *M* in 0.1 *M* Tris–HCl buffer, pH 7.8) and for the most concentrated enzyme–stock inhibitor solution used ($E0 \simeq 3 \times 10^{-3}$ *M*). A plot of relaxation times as a function of enzyme concentration was then obtained by successively diluting the enzyme solution with stock inhibitor solution.

All of the T_1's at 56.4 MHz were measured by the reversal of polarization method.[23] Each T_1 so measured represents the average of approximately ten measurements. T_1 was also measured at 94.1 MHz by the $T_{1\rho}$ method. Although more convenient than the reversal of polarization method since T_1 and T_2 can be measured simultaneously, and more sensitive since close to the full amplitude of the magnetization is measured, only a few measurements of T_1 by the $T_{1\rho}$ method are presented since this method was discovered after the main body of the experimental work was complete. The greatest advantage of this method of measuring T_1's over the reversal of polarization method is its ability to measure T_1's in the range 10^{-1} to 1 sec. T_2 was taken as the value of $T_{1\rho}(H_1)$ at a value of H_1 such that decreasing H_1 produced no shortening of $T_{1\rho}(H_1)$.

The chemical shifts were measured on a Varian HA-100 spectrometer operating at 94.1 MHz. Solutions were prepared by dissolving weighed amounts of inhibitor in a stock enzyme solution ($E0 \cong 3 \times 10^{-3}$ *M*, $I0 \cong 4 \times 10^{-3}$ to 4×10^{-2} *M* in pH 7.8 buffer).

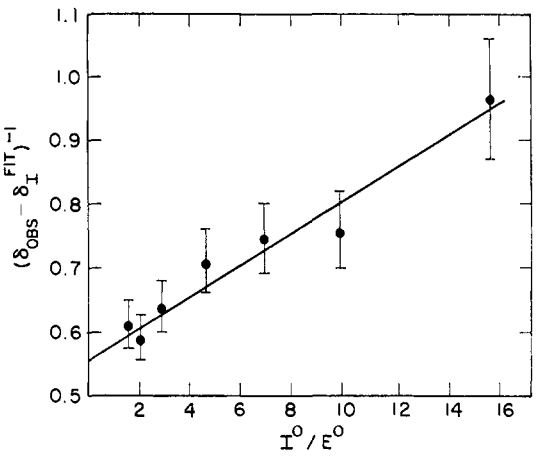

Figure 2. ^{19}F chemical shifts at 94.1 MHz for trifluoroacetyl-D-phenylalanine binding to α-chymotrypsin; [α-chymotrypsin] = 2.25×10^{-3} *M*; δ_I^{FIT} is the chemical shift of the inhibitor in free solution (see text); the error bars indicate an estimated accuracy of ±0.10 Hz.

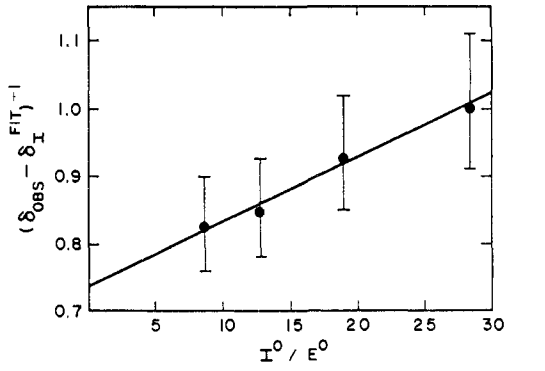

Figure 3. ^{19}F chemical shifts at 94.1 MHz for trifluoroacetyl-D-phenylalanine binding to DFP-chymotrypsin; [DFP-chymotrypsin] = 1.34×10^{-3} *M*; δ_I^{FIT} is the chemical shift of the inhibitor in free solution (see text); the error bars indicate an estimated accuracy of ±0.10 Hz.

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 six 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 the sweep oscillator and the lock oscillator. The probe temperature of both spectrometers was 33 ± 2°.

Results and Discussion

The chemical shifts of the CF_3 fluorines of TAPA exchanging with α-chymotrypsin and DFP-chymotrypsin are presented in Figures 2 and 3, respectively. The inverse of the difference between the observed chemical shift of the exchange-averaged line and the chemical shift of the inhibitor in free solution, δ_I^{FIT}, is plotted as a function of the ratio of initial inhibitor to initial enzyme concentration. δ_I^{FIT} was not measured directly but was obtained from the fit of the observed shifts as a function of initial inhibitor concentration at constant initial enzyme concentration to eq 19 and 20 since no suitable internal standard was available. The error bars indicate an estimated accuracy of ±0.10 Hz. In

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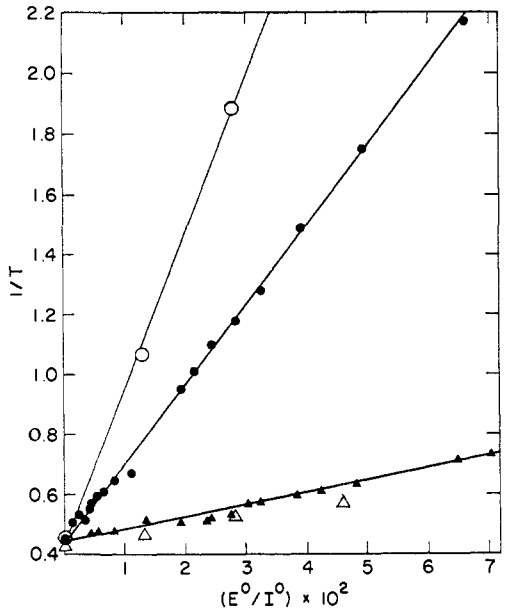

Figure 4. ^{19}F relaxation times for trifluoroacetyl-D-phenylalanine binding to α-chymotrypsin; [trifluoroacetyl-D-phenylalanine] $\cong$ 0.048 M; ●, $1/T_2$; ▲, $1/T_1$, at 56.4 MHz; ○, $1/T_2$; △, $1/T_1$, at 94.1 MHz.

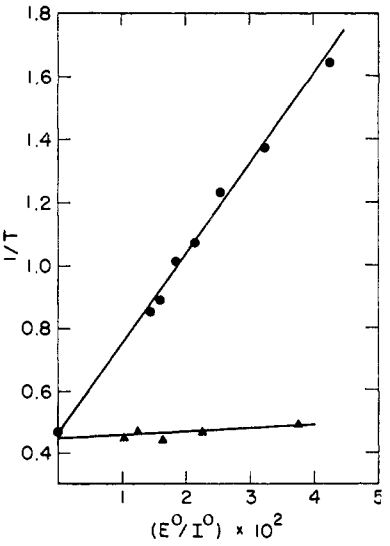

Figure 5. ^{19}F relaxation times for trifluoroacetyl-D-phenylalanine binding to DFP-chymotrypsin; [trifluoroacetyl-D-phenylalanine] $\cong$ 0.048 M; ●, $1/T_2$; ▲, $1/T_1$, at 56.4 MHz.

fact, the scatter in both plots is much less, The actual shifts for this system are quite small, however, as can be seen by taking the inverses of the data of Figures 2 and 3, and therefore large errors are possible at this point. There was no shift as a function of inhibitor concentration for exchange with chymotrypsinogen A, consistent with a large dissociation constant or a small chemical shift of the enzyme–inhibitor complex (see Figure 6). This also indicates that no correction to the observed chemical shifts for the other two enzymes is required because of a concentration dependence of the shift of TAPA from a source other than the binding process.

In the limit [I] $\simeq$ I^0, the plots of Figures 2 and 3 would be expected to be straight lines with slope $1/\Delta$ and intercept $K_D/E^0\Delta$.[7] The dissociation constants

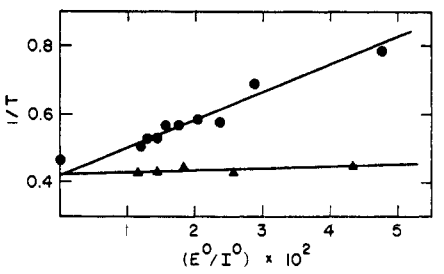

Figure 6. ^{19}F relaxation times for trifluoroacetyl-D-phenylalanine binding to chymotrypsinogen A; [trifluoroacetyl-D-phenylalanine] $\cong$ 0.048 M; ●, $1/T_2$; ▲, $1/T_1$, at 56.4 MHz.

and chemical shifts of the enzyme–inhibitor complexes, obtained from the fits that were used to select the values of δ_1^{F1T}, are presented in Table I for α-chromotrypsin

Table I. Dissociation Constants and Chemical Shifts at 56.4 MHz for Enzyme–Inhibitor Complexes

Enzyme	K_D, M	Δ_{EI}, Hz
α-Chymotrypsin	4.90×10^{-2}	24
DFP-chymotrypsin	1.06×10^{-1}	64

and DFP-chymotrypsin. The larger shift for the DFP-chymotrypsin–TAPA complex may reflect either an interaction of the CF_3 fluorines with the DFP group, a change in the position of inhibitor binding, or a change in the enzyme near the binding site for the inhibitor. The chemical shift of the CF_3 fluorines of TAPA bound to α-chymotrypsin is approximately two to three times larger than that of the CH_3 protons of N-acetyl-D-phenylalanine bound to α-chymotrypsin,[7] consistent with the enhanced shift of ^{19}F with respect to ^1H.[24] The dissociation constant for TAPA binding to α-chymotrypsin is larger than that for N-acetyl-D-phenylalaninamide,[25] consistent with the relatively poorer binding of the amino acid *vs.* the amide inhibitor above pH 7.3.[26] The trend in the dissociation constants is in agreement with the results of fluorescence depolarization studies.[27]

The relaxation times of the CF_3 fluorines of TAPA exchanging with α-chymotrypsin, DFP-chymotrypsin, and chymotrypsinogen A are presented in Figures 4, 5, and 6, respectively. The relaxation times T_1 and T_2 were measured at 94.1 and 56.4 MHz for exchange with α-chymotrypsin, and at 56.4 MHz for exchange with the other two enzymes. The T_1's were not a function of resonance frequency (see Figure 4), implying that the correlation times of the relaxation mechanisms other than exchange are shorter than 10^{-9} sec ($1/\omega_0$). The differences in the T_2's will be discussed later.

These relaxation time measurements indicate that

(24) W. Emsley, J. Feeney, and L. H. Sutcliffe, "High Resolution Magnetic Resonance Spectroscopy," Vol. 2, Pergamon Press, New York, N. Y., 1966.

(25) K's (average) = 7.8×10^{-3} M (A. Himoe, K. G. Brandt, and G. P. Hess, *J. Biol. Chem.*, 247, 3963 (1967)); $K_i = 12 \times 10^{-3}$ M (R. J. Foster, H. J. Shane, and C. Niemann, *J. Am. Chem. Soc.*, 77, 2378 (1955)); $K_i = 10.6 \times 10^{-3}$ M (D. W. Ingles and J. R. Knowles, *Biochem. J.*, 104, 369 (1967)).

(26) C. H. Johnson and J. R. Knowles, *ibid.*, 101, 56 (1966).

(27) D. A. Deranleau and H. Neurath, *Biochemistry*, 5, 1413 (1966).

there is binding of TAPA to all three enzymes, although the effect of the binding upon the relaxation times is quite different in each case. The difference in $1/T_2$ and $1/T_1$ for all three enzymes is directly proportional to initial enzyme concentration, as is predicted by eq 17. All processes such as active site to active site dimerization,[28,29] which would cause deviations from a linear dependence upon initial enzyme concentration, are thereby ruled out at this enzyme concentration, ionic strength, and pH.

The slope of $1/T_1 = P_I/T_1(I) + P_{EI}/T_1(EI)$ as a function of enzyme concentration is different for α-chymotrypsin, DFP-chymotrypsin, and chymotrypsinogen A. The ordering of the slopes is α-chymotrypsin > DFP-chymotrypsin $\gtrsim$ chymotrypsinogen A. While the shortening of T_1 could be due to a viscosity increase with increasing enzyme concentration, this is unlikely since solutions of the three enzymes would be expected to have approximately equal viscosities and the effect is very small for chymotrypsinogen A. The shortening of T_1, then, must arise from relaxation while the inhibitor is bound to the enzyme, $T_1(EI)$, because of a rotation restriction while bound,[5,30] or an interaction with nuclei in the active site. A rotational restriction is consistent with Koshland's idea of "induced fit."[31] The shortening of T_1 as a function of enzyme concentration for α-chymotrypsin is greater than that for DFP-chymotrypsin by approximately the amount expected by the increase in the P_{EI} term because of the smaller dissociation constant, suggesting that the inhibitor binds to the two enzymes in a similar fashion. The very small slope of $1/T_1$ for chymotrypsinogen A implies that the binding to chymotrypsinogen A is very weak or that the motion of the CF_3 portion of TAPA is not significantly restricted in the chymotrypsinogen–TAPA complex.

The effectiveness of the exchange process in causing relaxation, $(1/T_2 - 1/T_1)$, is also different for the three enzymes. The order is DFP-chymotrypsin > α-chymotrypsin $\gg$ chymotrypsinogen A. The enhanced effect for DFP-chymotrypsin with respect to α-chymotrypsin reflects the larger chemical shift of the DFP-chymotrypsin–TAPA complex.

Table II. Rate Constants for the Binding of N-Trifluoroacetyl-D-phenylalanine to α-Chymotrypsin and DFP-chymotrypsin

Enzyme	k_1, M^{-1} sec^{-1}	k_{-1}, sec^{-1}
α-Chymotrypsin	1.0×10^4	4.9×10^3
DFP-chymotrypsin	1.6×10^4	16.6×10^3

The rate constants obtained from the measured relaxation times and chemical shifts at 56.4 MHz, using eq 17, are presented in Table II. The interesting feature is that most of the change in the strength of the binding is reflected in k_{-1}. In other words, the effect of the addition of the DFP group to the active site of α-chymotrypsin is to reduce the interactions which stabilize the enzyme–inhibitor complex.

It is at first surprising that the rate constant k_1 is not near diffusional.[32] Much evidence exists, however, which suggests that the interaction of inhibitors and substrates with α-chymotrypsin is accompanied by conformational changes and that these rates are much slower.[33]

Consider the reaction mechanism proposed by Moon, Sturtevant, and Hess where an initial complex is formed at a rate near diffusional, followed by a conformational change to produce a second complex.[34,35]

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} (EI)_1 \overset{K_2}{\rightleftharpoons} (EI)_2 \tag{21}$$

$$K_1 = \frac{[E][I]}{[(EI)_1]} = \frac{k_{-1}}{k_1} \tag{22}$$

$$K_2 = \frac{[(EI)_1]}{[(EI)_2]} = \frac{k_{-2}}{k_2}$$

For such a scheme the observed chemical shift would be the weighted average of the shift for free solution, for inhibitor–enzyme complex 1, and for inhibitor–enzyme complex 2.

$$\delta_{obsd} = \frac{[(EI)_2]}{I^0}\Delta_{(EI)_2} + \frac{[(EI)_1]}{I^0}\Delta_{(EI)_1} + \delta_I \tag{23}$$

Under the assumption $[I] \cong I^0$, this becomes

$$(\delta_{obsd} - \delta_I) = \frac{E^0}{K_1K_2 + (1 + K_2)I^0}\Delta_{(EI)_2} + \frac{E^0K_2}{K_1K_2 + (1 + K_2)I^0}\Delta_{(EI)_1} \tag{24}$$

and

$$\frac{1}{\delta_{obsd} - \delta_I} = \frac{K_1K_2}{E^0(\Delta_{(EI)_2}K_2\Delta_{(EI)_1})} + \frac{(1 + K_2)}{(\Delta_{(EI)_2} + K_2\Delta_{(EI)_1})}\left(\frac{I^0}{E^0}\right) \tag{25}$$

For a plot of $(\delta_{obsd} - \delta_I)^{-1}$ *vs.* (I^0/E^0)

$$\frac{(\text{slope})}{(E^0)(\text{intercept})} = \frac{(1 + K_2)}{K_1K_2} \tag{26}$$

For TAPA binding to α-chymotrypsin, this ratio is

$$(1 + K_2)/K_1K_2 = 20 \tag{27}$$

For small K_2,[33,35] K_1 is large[34] and k_{-1} near diffusion, implying that there is little interaction of inhibitor with enzyme in the first complex. It is then reasonable to suppose that $\Delta_{(EI)_1} \approx 0$. The situation is then

$$\frac{1}{\delta_{obsd} - \delta_I} = \frac{1}{\Delta_{(EI)_2}}\left(\frac{I^0}{E^0}\right) + \frac{K_1K_2}{\Delta_{(EI)_2}E^0} \tag{28}$$

(28) F. J. Kézdy and M. L. Bender, *Biochemistry*, 4, 104 (1965).
(29) I. Tinoco, *Arch. Biochem. Biophys.*, 68, 367 (1957).
(30) L. J. Berliner and H. M. McConnell, *Proc. Natl. Acad. Sci. U. S.*, 55, 708 (1966).
(31) D. E. Koshland, Jr., *Science*, 142, 1533 (1963).
(32) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, 25, 1 (1963).
(33) H. L. Oppenheimer, B. Labonesse, and G. P. Hess, *J. Biol. Chem.*, 241, 2720 (1966), and references therein; B. H. Havsteen, *ibid.*, 242, 769 (1967).
(34) A. Y. Moon, J. M. Sturtevant, and G. P. Hess, *J. Biol. Chem.*, 240, 4204 (1965).
(35) R. Haynes and F. Reeney, *Biochemistry*, 7, 2879 (1968).

and[36]

$$\frac{1}{T_2} - \frac{1}{T_1} = \frac{[(EI)_2]}{I^0}\left(\frac{1}{k_{-2}}\right)\Delta_{(EI)_2}{}^2 = \frac{E^0}{K_1K_2 + I^0}\left(\frac{1}{k_{-2}}\right)\Delta_{(EI)_2}{}^2 \quad (29)$$

Table II can be reproduced by making the identification

$$k_{-1} \rightarrow k_{-2} \quad (30a)$$

$$k_1 \rightarrow k_2\left(\frac{k_1}{k_{-1}}\right) = \frac{k_2}{K_1} \quad (30b)$$

The point of this discussion is that the values of k_1 are apparent values dependent upon the total concentrations of species and the total binding process. This may include either a stepwise binding process as above or the fact that the enzyme exists in several ionization states, only one of which is capable of binding inhibitor.

A final check upon the measured rate constants and chemical shifts is provided by the differences in relaxation times as a function of resonance frequency (see Figure 4). The difference in relaxation times $(1/T_2) - (1/T_1)$ for trifluoroacetyl-D-phenylalanine binding to α-chymotrypsin at 94.1 MHz is greater than at 56.4 MHz because of the larger resonance frequency shifts at the higher frequency. The ratio

$$\frac{[(1/T_2) - (1/T_1)]_{94.1}}{[(1/T_2) - (1/T_1)]_{56.4}} \quad (31)$$

is not equal to $(94.1)^2/(56.4)^2$ as expected from eq 17, however, because of the following. At 56.4 MHz, the value of $(\tau\Delta)^2$ is $9.5 \times 10^{-2} < 1$. Assuming that all of the shortening of T_1 is due to the term $P_{EI}/T_1(EI)$ and none due to viscosity, for example

$$\frac{\tau_{EI}}{T_1(EI)} \cong \frac{2 \times 10^{-3}}{0.11} = 1.7 \times 10^{-2} \quad (32)$$

so that eq 8 is satisfied. Hence eq 9 and 17 are valid descriptions of the system. At 94.1 MHz, however, $(\tau\Delta)^2 = 0.26$. Since P_{EI} is never greater than $\sim 3 \times 10^{-2}$, eq 11 is satisfied at both resonance frequencies and the above ratio of relaxation time differences should be equal to

$$\frac{(94.1)^2(1.0 + 0.09)}{(56.4)^2(1.0 + 0.26)} = 2.4 \quad (33)$$

as compared to an experimentally obtained value of 2.1. The fact that these do not agree exactly presumably reflects experimental error and the approximations involved in the derivatives of eq 9 and 12.

Alternatively, since the chemical shift determination is subject to the largest errors, the rate constants for the exchange of trifluoroacetyl-D-phenylalanine with α-chymotrypsin can be obtained from the measurement of the relaxation times at two different resonance frequencies (see Figure 4) without the use of the chemical shift data, if the dissociation constant is assumed known. This also provides a check on the measured chemical shifts.

Using eq 12 and the data from Figure 4, the ratio of relaxation time differences (eq 31) is equal to

$$\frac{(94.1)^2(1.0 + (\tau\Delta)^2)}{(56.4)^2(1.0 + 2.78(\tau\Delta)^2)} = 2.09 \quad (34)$$

The solution to eq 34 is

$$(\tau\Delta)^2 = 0.23 \quad (35)$$

τ can then be obtained by substituting eq 35 into eq 36

$$\frac{1}{T_2} - \frac{1}{T_1} = \frac{E^0}{K_D + I^0}\left(\frac{1}{\tau}\right)\frac{(\tau\Delta)^2}{(1.0 + (\tau\Delta)^2)} \quad (36)$$

Assuming $K_D = 2.0 \times 10^{-2}$ M,[25,26] the solution to eq 36 is

$$\tau = 5.9 \times 10^{-3} \quad (37)$$

Hence,

$$k_{-1} = 1.7 \times 10^2 \text{ sec}^{-1}$$
$$k_1 = 8.5 \times 10^3 \ M^{-1} \text{ sec}^{-1} \quad (38)$$

and $\Delta = 14$ Hz. The agreement with the results presented in Tables I and II is satisfactory. The value of K_D has been assumed in eq 36 however, and is not presently available from other methods.

Conclusion

Nmr techniques have been demonstrated to be a direct, simple, and precise means of measuring the rate constants for biological exchange reactions. The method, presented in this paper for enzyme–inhibitor systems, has been applied to the binding of trifluoroacetyl-D-phenylalanine to α-chymotrypsin, DFP-chymotrypsin, and chymotrypsinogen A and yields rate constants appropriate to conformational changes occurring in the chymotrypsins upon inhibitor binding. It is important to remember, however, that the rate constants are apparent constants dependent upon the total concentrations of species and the total binding process. The method appears to be applicable to a large number of biological systems, and is limited only by the requirement of a resonance frequency shift upon binding.

The fact that the exchange contribution to the nmr line widths is not always negligible, even in the fast-exchange limit, also suggests that caution must be used in the interpretation of nmr line width data if chemical shifts are present.[38]

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(36) Equations 23 and 29 can be derived from the results of Swift and Connick[37] in the limik $\tau_i/T_1(i)$, $(\tau_i\Delta(i))^2 \ll 1$, K_2; $i = 1, 2$; $k_{-2} < k_{-1}$.

(37) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 37, 307 (1962).

(38) J. T. Gerig, *J. Am. Chem. Soc.*, 90, 2681 (1968)