At higher concentrations the tetrapeptide aggregates, and the fact that the three low field NH resonances have the same chemical shift and display the same temperature dependence (Figure 9, Table II) is indication that the chains unfold as the associations are formed. In these respects the data are quite different from those described above for the associated tripeptide, in which case neither the chemical shifts nor the temperature dependencies of the chemical shifts are the same for the downfield resonances. Whereas the tripeptide associations are best described as a network of extended "out-of-register" forms, the tetrapeptide associations are better described as primitive β sheets. The earlier CD and IR studies[3,4] substantiate our conclusion here that β-like structures begin to appear at the level of the tetrapeptide.

We are currently employing high resolution ^{1}H NMR to establish in detail the conformations of other homo-oligopeptide series in $CDCl_3$ solution in order to shed more light on the structural dependence of folding, already noted by Shields and co-workers[38] and Palumbo et al.[4] using IR absorption.

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Nuclear Magnetic Resonance Studies of *p*-Fluorocinnamate–α-Chymotrypsin Complexes

J. T. Gerig,* B. A. Halley, and C. E. Ortiz

Contribution from the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received December 27, 1976*

Abstract: Fluorine magnetic resonance spectroscopy has been used to characterize the complexes formed between *p*-fluorocinnamate anion and the proteolytic enzyme, α-chymotrypsin. Equilibrium binding constants determined by competitive inhibition kinetics or a dye-displacement technique agree with the value found by NMR. Consideration of protein-induced chemical shifts and spin-lattice and transverse relaxation rates strongly indicates that *p*-fluorocinnamate binds equally well to the monomeric, dimeric, and trimeric forms of the enzyme with the properties of the binding sites so revealed being indistinguishable. A substantial part (about 62%) of the fluorine-proton dipolar relaxation derives from interaction with protons of the enzyme.

Bender and his co-workers have nicely elucidated the mechanism of chymotrypsin catalysis especially as regards the hydrolysis of cinnamoyl esters and amides.[1] A minimal mechanism for this process is represented by

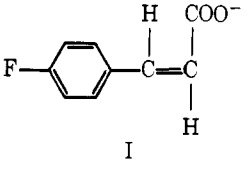
$$E + AL \rightleftharpoons [E{\cdot\cdot}AL] \underset{+L}{\overset{-L}{\rightleftharpoons}} \hat{E}A \underset{-H_2O}{\overset{H_2O}{\rightleftharpoons}} [E{\cdot\cdot}A] \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + A \quad (1)$$

This sequence portrays as the first stage of the reaction formation of an enzyme-substrate complex ([E··AL]) within which the acyl group of the substrate is transferred to the serine-195 residue of the protein, giving an acylated enzyme, $\hat{E}A$. Hydrolysis of this enzyme ester affords an enzyme-product complex ([E··A]) which then can reversibly dissociate to regenerate the free enzyme. When the acyl group A is cin-

namoyl the acylenzyme is stable enough to be isolated and studied at low pH.

A goal of a program in our laboratory is to compare the structures of the various intermediates found in eq 1 using magnetic resonance methods. The cinnamoyl-enzyme system is attractive in this regard because of the stability of the acylated enzyme form (ÊA). An early part of this work involved an examination of the interaction of *trans*-cinnamate with the enzyme in a process analogous to the last step of eq 1,[2] but this study is open to criticism on several fronts. In particular, no reliable evidence could be provided by the techniques of the earlier study about the rates of exchange in the equilibrium analogous to the last step of eq 1 and no indication of which oligomeric forms of the enzyme were involved in binding cinnamate was obtained. These data are necessary because the chemical shifts or relaxation times observed in an exchanging system can depend on the exchange rate as well as the chemical shift and relaxation times for the protein-bound form of the observed nucleus.[3] An incomplete analysis of the situation was hinted at by our calculation that the rotational correlation time, τ_c, for the protons of cinnamate in the cinnamate-chymotrypsin complex is about 60 ns,[2] while estimates of τ_c for the monomeric form of the enzyme by fluorescence polarization[4] or by ESR spectroscopy[5] give values in the range 11-16 ns, in reasonable agreement with expectations based on the Stokes-Einstein equation.[6]

In order to further illuminate the nature of the cinnamate-enzyme complex in solution it was decided to examine *p*-fluorocinnamate in this system by fluorine magnetic resonance (^{19}F NMR) spectroscopy.[7] Advantages of ^{19}F NMR particularly pertinent to the present system are that (1) the fluorine resonances of the small molecule can be observed under all concentration conditions without complications arising from signals due to nuclei of the protein, and (2) the rates of chemical exchange can be estimated by pulsed ^{19}F NMR techniques.[8]



I

Results

Kinetic Inhibition Studies. Although it is possible to obtain the dissociation constants of protein-small molecule complexes from magnetic resonance data,[9,10] we felt it advisable to have independent confirmation of parameters obtained in this way. Therefore, the ability of I to competitively inhibit the α-chymotryptic hydrolysis of glutarylphenylalanine *p*-nitroanilide (GPNA) was studied.[11] Conventional Lineweaver-Burk kinetics were observed and the fluorine-substituted cinnamic acid appeared kinetically to be a competitive inhibitor of the enzyme.[12] Inhibition (dissociation) constants (K_A) were reckoned from kinetic data obtained at three temperatures using ethanol or methanol as cosolvent and are recorded in Table I. The Michaelis constant for GPNA was approximately 1 mM under our experimental conditions but the temperature dependence of this parameter was not carefully studied as its value was not needed to determine K_A. It should be noted that the inhibition studies were carried out in the presence of an appreciable amount of organic cosolvent (2.5-3.2%); this was necessary because of the limited solubilities of the substrate and inhibitors.

Dye Displacement. In an attempt to define the dissociation constants under conditions where no organic solvents are present, the ability of *p*-fluorocinnamate to displace the dye Biebricht scarlet was examined. A characteristic difference spectrum is generated when this dye binds to the enzyme[13] that can be used to study the competitive displacement of the dye from the enzymic active site. No organic cosolvents were needed because of the high water solubility of Biebricht scarlet.

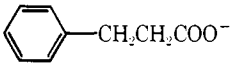
Table I. Dissociation Constants (K_I) for α-Chymotrypsin-*p*-Fluorocinnamate Complexes

Temp, °C	Cosolvent	K_A, mM	Comment
16	Ethanol	0.19	*a,b*
25	Ethanol	0.28	*a,b*
31	Ethanol	0.36	*a,b*
25	Methanol	0.34	*b,c*
30	None	0.18	*b,d*

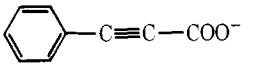
[a] Determined by inhibition of GPNA hydrolysis, 0.134 M phosphate buffer (μ = 0.2), pH 6.2 with 2.5% organic cosolvent present, [GPNA] = 0.07-0.2 mM. [b] Estimated uncertainty ±20%. [c] Same conditions as *a* but [GPNA] = 0.4-2.0 mM and 3.2% organic solvent present. [d] Determined by measuring the displacement of Biebricht scarlet, 0.1 M phosphate buffer at pH 7.0.

Experiments along these lines were partially thwarted when it was observed that the nature and apparent stability of the difference spectrum was time dependent over a 7-h period and that the order of mixing of the reagents seemed to affect the resulting absorbance. When attempts to determine the origin of these effects were not successful, the rigid protocol with a defined order of mixing and time for spectrophotometric readings was developed and used in estimate the binding constant given in line 5 of Table I. Because of the effects noted above, this datum must be regarded with caution.

Which Enzyme Forms Bind *p*-Fluorocinnamate? At the concentration used for the competitive inhibition and dye displacement studies (10^{-3}-10^{-2} mM) the enzyme exists essentially completely as the monomer.[14] At high concentrations in pH 6.2, 0.2 M phosphate buffer from 20 to 25 °C the enzyme associates into an equilibrium mixture of monomeric, dimeric, and trimeric forms.[14] Many small molecules, including specific substrates of the enzyme, bind or react only with the monomeric form of the enzyme.[8,15-20] However, β-phenylpropionate (II) and phenylpropiolate (III) appear to



II



III

bind equally well or nearly equally well to all forms of the enzyme with the dimeric form proffering two equivalent protein binding sites and the trimer, three.[19,21] Anion I is structurally intermediate between II and III and we must hold open the possibility that I can bind to the various forms of chymotrypsin in the same manner as II and III. We will consider several possible models for the interaction as regards binding to the oligomers of the protein, finally choosing the one with the smallest number of adjustable parameters which accounts for all of the data.

Binding to Monomer Only. We assume that the system is described by the equilibria

$$T \underset{+M}{\overset{-M}{\rightleftharpoons}} D \rightleftharpoons 2M \qquad (2a)$$

$$MA \underset{k_a}{\overset{k_d}{\rightleftharpoons}} M + A \qquad (2b)$$

$$K_A = \frac{[M][A]}{[MA]} \qquad (2c)$$

where M, D, and T represent the monomeric, dimeric, and trimeric structures of the protein, respectively, A is *p*-fluorocinnamate, and k_d and k_a are the rate constants for dissociation and association of the monomer complex.

When the fluorine signal from the fluorocinnamate in the presence of the enzyme is examined with proton decoupling only a single resonance is observed. The resonance position is shifted (downfield) in all cases and therefore the rate constants k_d and k_a must be sufficiently large that "intermediate" or "fast" exchange conditions apply to the binding of this small molecule to the protein. We assume that a simple two-site description of this process is appropriate and, under conditions where the concentration of one of the sites is in large excess over the other, Swift and Connick have shown that the observed change in chemical shift, $\Delta\delta$, induced by the enzyme is given by[3]

$$\Delta\delta = \frac{k_d^2 \Delta\omega \chi_B}{(R_{2,B} + k_d)^2 + \Delta\omega^2} = S\chi_B \qquad (3)$$

where $\Delta\omega$ is the fluorine chemical shift difference between the free and enzyme-bound forms of the small molecule, $R_{2,B} = 1/T_{2,B}$ is the transverse relaxation rate of the observed nucleus in the bound form, and χ_B is the mole fraction of bound material. Equation 3 should be valid for mole fractions as large as 0.2 when relaxation, shift, and exchange parameters of the orders of magnitude expected in this work obtain and we anticipate a linear variation of the observed signal position with mole fraction of inhibitor bound. High-resolution fluorine chemical shift data were collected using a wide range of enzyme (0.2–1.5 mM) and small molecule concentrations (0.2–20 mM) at 94.1 MHz by the Fourier transform technique with proton noise decoupling gated on during acquisition of the free induction decay.

At low concentrations of inhibitor, protein association should become competitive with inhibitor binding, and thus χ_B will be dependent upon the dissociation constant (K_A) for the enzyme-small molecule complex as well as the equilibrium constant(s) which characterize protein association. Following precedent, it was assumed that association could be taken into account adequately by defining an effective dimerization constant, K_D.[8,18] A computer program was used to adjust K_A and K_D over a reasonable range of values, and, for each set of equilibrium constants, a least-squares fit of the available chemical shift data to eq 3 was carried out;[8] the set of parameters which gave the best fit was assumed to be the one descriptive of the system. This approach was not very sensitive to the value of K_D but fairly cleanly defined the optimum value for the dissociation constant, K_A. Good agreement between observed and calculated chemical shift effects was obtained for all available experimental data ($0 < \chi_B < 0.4$). However, we noted that the optimum value of K_D suggested by the analysis (2 mM) could be varied by a factor of 2 with no pronounced effect on the values for K_A or S produced by the calculations (Table II). It should be noted that $K_D = 2$ mM is *not* in good agreement with estimates of this parameter obtained in other small molecule-chymotrypsin systems where K_D = 0.01–0.2 mM was typical.[8,18]

The observed fluorine spin-lattice relaxation rate, $R_{1,obsd} = 1/T_{1,obsd}$, will depend upon both the relaxation rate for the free fluorocinnamate, $R_{1,F}$, and the corresponding quantity for the α-chymotrypsin-bound form, $R_{1,B}$. Under the very loose condition

$$(k_d + k_a) \gg R_{1,F} + R_{1,B}$$

$$R_{1,obsd} = R_{1,F} + (R_{1,B} - R_{1,F})X_B \qquad (4)$$

if we assume a two-site exchange process.[22]

Fluorine spin-lattice relaxation rates were determined by a modified 180-τ-90 (inversion-recovery) pulse sequence at 22.8, 51.0, and 94.1 MHz.[23] Proton decoupling was not carried out in the experiments at 22.8 and 51.0 MHz and the decoupler was on only during data acquisition in the determinations at the highest frequency. Therefore, the relaxation curves are potentially nonexponential.[22,24] However, calculations show that the expected degree of nonexponentiality is slight until after 3 half-lives of the decay process and the obtainable signal-to-noise ratios were such that it was not possible to reliably follow the relaxation much beyond 2 half-lives. Therefore, the possible nonexponentiality in the relaxation plots was neglected and all curves were (well) fit assuming a single, exponential relaxation process. Using the K_1 and K_D values defined by the chemical shift data, the spin-lattice relaxation data were treated according to eq 4 and afforded R_1 parameters characteristic of the enzyme-bound fluorocinnamate (Table II).

Table II. Parameters for the Putative Monomeric α-Chymotrypsin-*p*-Fluorocinnamate Complex

a	$K_D = 2$ mM	$K_A = 0.21$ mM	$S = -242$ Hz (-2.57 ppm)[a]
b	Rf, MHz	R_{1B}, s^{-1} [b]	R_{2B}, s^{-1} [b]
	22.8	36.	86.
	51.0	20.	69.
	94.1	9.7	

[a] Obtained at 94.1 MHz, 25 °C, pH 6.2, 0.143 M phosphate buffer. [b] Obtained from plots of R vs. X_B, where X_B, the mole fraction of bound *p*-fluorocinnamate, was computed using the values of K_A and K_D shown, assuming binding to monomer only. Estimated uncertainty ±10%.

The observed NMR line width in exchanging systems such as the one under consideration here can reflect exchange rates as well as the transverse relaxation rates for the free ($R_{2,F} = 1/T_{2,F}$) and bound ($R_{2,B}$) nuclei. To sort out the exchange contribution we have applied the Carr-Purcell-Meiboom-Gill experiment for the determination of exchange rates that was originally propounded by Allerhand and Gutowsky.[25] In this experiment the observed transverse relaxation rate $R_{2,obsd}$ is given by[26]

$$R_{2,obsd} = \sum_i p_i R_{2,i} + f(p_i, k_i, R_{2,i}) + g(p_i, k_i, \Delta\omega_i^2, R_{2,i}, \tau_{cp}) \qquad (5)$$

The first term in eq 5 is the weighted average of the transverse relaxation rates, $R_{2,i}$, for all species in the system with the relative populations, p_i, serving as the weighting coefficients. The second term depends upon the populations, p_i, the rate constants, k_i, for exchange among the various forms, and the $R_{2,i}$. The final term is a function of these parameters as well as the chemical shift differences ($\Delta\omega_i$) and the time (τ_{cp}) between application of the refocusing 180° pulses in the Carr-Purcell-Meiboom-Gill sequence. The functional form of the last term is such that it approaches zero as the rate of pulsing becomes rapid ($\tau_{cp} \rightarrow 0$). One can show by calculation using reasonable values for the enzyme-*p*-fluorocinnamate system that the second term in eq 5 contributes less than 5% to $R_{2,obsd}$ and, therefore, in the rapid pulsing limit we expect $R_{2,obsd}$ to be, to a good approximation, simply the weighted average of all $R_{2,i}$ characteristic of the system. For a two-site exchange process such as that indicated in eq 2 we have

$$R_{2,obsd} = R_{2,F} + (R_{2,B} - R_{2,F})X_B \qquad (6)$$

Transverse relaxation rates of *p*-fluorocinnamate in the presence of α-chymotrypsin were determined as a function of t_{cp} for four values of χ_B at 51 MHz. All available data were simulataneously fit to the exact expression of Carver and Richards[27] describing a two-site system by a program which adjusted $R_{2,B}$, $\Delta\omega$, and k_α until a good match between theory and the experimental data was obtained.[8] Similar experiments

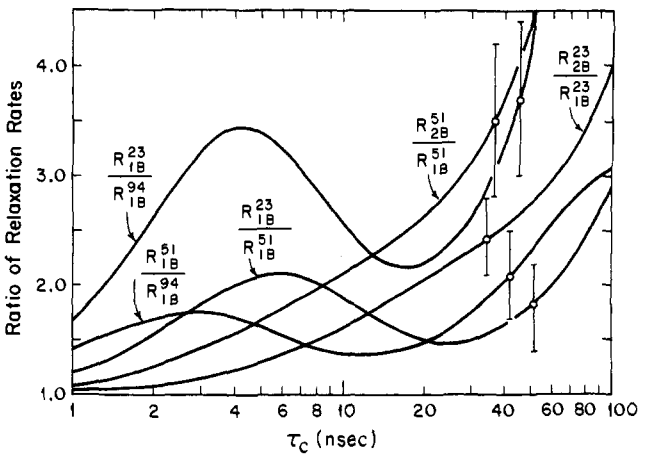


Figure 1. Relaxation rate ratios as a function of the correlation time τ_c. The experimental data from Table II are given as points while the error bars represent the estimated uncertainty of ±20%.

were carried out at 22.8 MHz but because the last term of eq 5 depends on the square of the chemical shift difference, the sigmoid character of $R_{2,obsd}$ vs. $1/\tau_{cp}$ plots found at 51 MHz was not observed. Table II records the R_{2B} at 22.8 MHz indicated by applying the (small) exchange correction consistent with the analysis at 51 MHz.

The relaxation of the protein-bound fluorine nucleus can potentially take place by three means: (1) dipole-dipole interactions with hydrogen nuclei in the vicinity of the fluorine, (2) a spin-rotation mechanism, or (3) chemical shift anisotropy (csa). Hull and Sykes have dismissed the spin-rotation mechanism for large molecules but present calculations and experimental data that show the importance of the chemical shift anisotropy mechanism.[28] Using their parameters and the expected correlation time for monomeric α-chymotrypsin, we calculate that the csa mechanism contributes negligibly to spin-lattice relaxation at all frequencies used in the present work. The csa contribution to transverse (R_2) relaxation is calculated to be less than 5% at 22.8 and 51.0 MHz but becomes nonnegligible (~11%) at 94.1 MHz. Thus, all spin-lattice relaxation data and transverse relaxation data at the lower frequencies can be interpreted using only the dipole-dipole mechanism.

To do so, we assume that *p*-fluorocinnamate (I) binds tightly enough to the monomeric enzyme that it cannot rotate within the complex.[4,5] It is further assumed that the immediate protein environment around the fluorine nucleus changes slowly on the time scale defined by the nuclear relaxation rates. Under these constraints R_{1B} and R_{2B} for the enzyme-bound fluorine are

$$R_{1B} = C_1 g_f \left[\frac{\tau_c}{1 + (\omega_H - \omega_F)^2\tau_c^2} + \frac{3\tau_c}{1 + \omega_F^2\tau_c^2} + \frac{6\tau_c}{1 + (\omega_H + \omega_F)^2\tau_c^2} \right] \quad (7a)$$

$$R_{2B} = C_{2gf} \left[4\tau_c + \frac{6\tau_c}{1 + \omega_H^2\tau_c^2} + \frac{\tau_c}{1 + (\omega_H - \omega_F)^2\tau_c^2} + \frac{3\tau_c}{1 + \omega_F^2\tau_c^2} + \frac{6\tau_c}{1 + (\omega_H + \omega_F)^2\tau_c^2} \right] \quad (7b)$$

where ω_H and ω_F are respectively the proton and fluorine resonance frequencies, expressed in radians/second, τ_c is the rotational correlation time for isotropic reorientation of the protein, C_1 and C_2 are collections of universal constants, and g_f is a geometrical factor given by[24]

$$g_f = \sum \frac{n_i}{r_i^6}$$

Here n_i is the number of protons at a distance r_i which interact with the fluorine nucleus; these protons include nuclei on the fluorocinnamate moiety as well as the protein. Because one cannot a priori define the geometrical factor, it is convenient to consider ratios of relaxation rates obtained at various radio frequencies. In such ratios the geometrical factors cancel leaving a quantity which depends in a defined way only upon the correlation time, the radio frequencies, and known constants.

In Figure 1 are depicted calculated relaxation rate ratios pertinent to this work as a function of τ_c. The experimental data in Table II are shown on this plot and it is seen that a correlation time of about 40 ns is consistent with the data. However, this correlation time is *not* consistent with the original assumption made in making this analysis, namely, that *p*-fluorocinnamate binds only to the monomeric form of the enzyme (τ_c = 11-16 ns). Allowing the cinnamate structure to move within the enzyme complex does not improve the situation, for such motion should lead to an apparent correlation time that is smaller than that expected for the monomeric protein. The binding to monomer only model thus leads to an unreasonable estimate of τ_c for the complex and we are forced to conclude that this model is not an adequate description of the binding of I to α-chymotrypsin.

Equivalent Binding to Monomer and Trimer. It is clear from the larger than expected correlation time obtained in the analysis described above that forms of the enzyme larger than monomer must also bind *p*-fluorocinnamate. In the crystalline state α-chymotrypsin is found as a dimer with the active sites of each partner in direct association[29] and the pH dependence of dimerization is consistent with the same type of interprotein interactions taking place in solution.[30,31] The solid state dimer resists binding most small molecules at the active site[20] and there is evidence for similar behavior in solution.[15-18] We therefore tried to fit the following binding mechanism to the data.[16]

$$M \rightleftharpoons D \rightleftharpoons T \quad (8a)$$

$$MI \rightleftharpoons M + I \quad (8b)$$

$$TI \rightleftharpoons MI + D \quad (8c)$$

$$TI \rightleftharpoons T + I \quad (8d)$$

It is assumed that trimer is formed by association of a monomer (free or complexed) with the dimer; the dimer is assumed to be incapable of binding the small molecule. We also assumed that the local binding sites for I on the monomer and trimer are structurally identical so that comparable internuclear distances in the vicinity of I at the active sites are the same. Exchange between M, D, and T is rapid,[14] and it was assumed that the dissociation rates of MI and TI are equal and rapid enough to average chemical shifts and relaxation times.

To account for the chemical shifts eq 3 can be expanded to[3]

$$\Delta\delta = \frac{k_d^2\Delta\omega X_{MI}}{(R_{2,MI} + k_d)^2 + \Delta\omega^2} + \frac{k_d^2\Delta\omega X_{TI}}{(R_{2,TI} + k_d)^2 + \Delta\omega^2} \quad (9a)$$

and if $k_d > R_{2,MI}$ or $R_{2,TI}$, as will surely be the case, eq 9a can be approximated

$$\Delta\delta = S' \chi_B \quad (9b)$$

where χ_B now represents the total mole fraction of bound I and S' has essentially the same significance as S in eq 3. Within these constraints, and using the dimerization and trimerization constants of Rao and Kegeles,[14] an analysis of the chemical shift data was carried out. An estimate of K_A was obtained in

Table III. Parameters for Oligomeric α-Chymotrypsin-*p*-Fluorocinnamate Complexes[a]

Parameter	Monomer + trimer	Monomer + dimer + trimer
K_A, mM	0.22	0.30
S', ppm	−2.67	−2.71
$g_f(R_1, 22.8) \times 10^2$ [b]	2.59	2.01
$g_f(R_1, 51.0)$	2.31	1.79
$g_f(R_1, 94.1)$	2.02	1.85
$g_f(R_2, 22.8)$	2.91	1.84
$g_f(R_2, 51.0)$	3.19	1.88

[a] Computed from same raw data as used in preparing Table II; K_D and K_T from ref 15 used in conjunction with K_A given and the models described in the text to evaluate the mole fraction bound to each form. [b] Computed assuming $\tau_{c,MI}$ = 15 ns. Units are Å^{-6}.

the same manner as described earlier and this along with the value of S' is given in Table III.

With this model the observed relaxation rates are averages of the rates characteristic of three states for I (I, MI, TI) so that for spin lattice relaxation

$$R_{1,obsd} = (1 - X_{MI} - X_{TI})R_{1,F} + X_{MI}R_{1,MI} + X_{TI}R_{1,TI} \quad (10)$$

with a similar equation applicable to the transverse relaxation rates determined in the fast pulsing limit. Using the assumptions mentioned above

$$R_{1,obsd} - (1 - X_{MI} - X_{TI})R_{1,F} = g_f[X_{MI}f(\omega_H, \omega_F, \tau_{c,MI}) + X_{TI}f(\omega_H, \omega_F, \tau_{c,TI})] \quad (11)$$

where $f(\omega_H, \omega_F, \tau_c)$ represents the function of spectral densities defined in eq 7. A similar equation describes the transverse relaxation rates. The unknowns in eq 11 are the geometrical factor g_f (assumed equivalent for both MI and TI) and the correlation times for MI ($\tau_{c,MI}$) and TI ($\tau_{c,TI}$). If the binding mechanism has been defined correctly it should be possible to find values for g_f and the correlation time which are consistent with all relaxation data at all frequencies examined. We assumed $\tau_{c,TI} = 3\tau_{c,MI}$ and, for a given $\tau_{c,MI}$, fit eq 11 to our relaxation data, evaluating g_f in each case. Some typical results are shown in Table III; there was no value of $\tau_{c,MI}$ in the range 14–19 ns which produced a single value of g_f for all relaxation data (R_1 at 22.8, 51.0, and 94.1 MHz, R_2 at 22.8 and 51.0 MHz). We thus concluded that the binding situation defined by eq 8 and the assumptions mentioned above are not appropriate to the interaction of α-chymotrypsin with *p*-fluorocinnamate.

Equivalent Binding to Monomer, Dimer, and Trimer. We again use the dimerization and trimerization constants of Rao and Kegeles[14,19] and postulate a single binding locus on the monomer, two on the dimer, and three on the trimer. The local environments of the inhibitor in each binding site are assumed to be identical.

Assuming that the dissociation rate constant, k_d, is identical for all complexes and that $k_d > R_{2,MI}$, $R_{2,DI}$ and $R_{2,TI}$, an equation identical in form with eq 9b will apply to the chemical shift data. K_A was again assumed equal for all binding sites and was varied until an optimum fit to the chemical shift data was obtained. The best K_A and S' so defined are given in Table III. This K_A was used to compute the mole fractions of the various bound forms in analysis of the relaxation data.

If the dimeric form of the enzyme also binds I, eq 11 must be expanded to

$$R_{1,obsd} - (1 - X_{MI} - X_{DI} - X_{TI})R_{1,F} = g_f[X_{MI}f(\omega_H, \omega_F, \tau_{c,MI}) + X_{DI}f(\omega_H, \omega_F, \tau_{c,DI}) + X_{TI}f(\omega_H, \omega_F, \tau_{c,TI}) \quad (12)$$

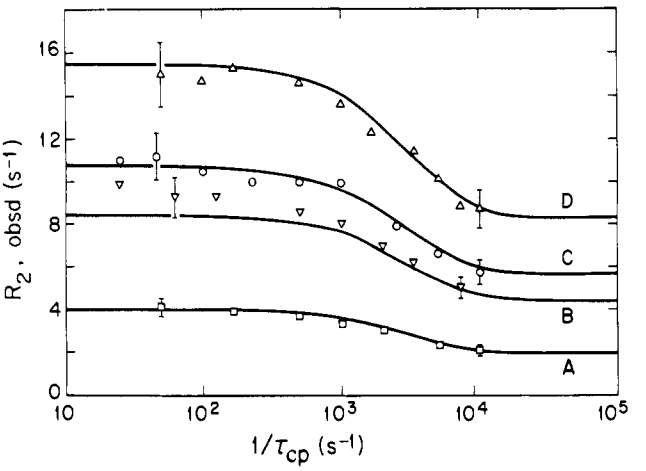


Figure 2. The observed transverse relaxation rate of the fluorine nucleus of *p*-fluorocinnamate in the presence of α-chymotrypsin at 51 MHz as a function of pulsing rate. Samples were prepared in 0.134 M phosphate buffer at pH 6.2, 25 °C. The solid curves are calculated assuming k_d = 9700 s^{-1}, $\Delta\omega$ = −2.65 ppm, and the model for binding discussed in the text. The error bars correspond to the experimental uncertainty of ±10%.

where the mole fractions X_{DI} and X_{TI} refer to all dimeric or trimeric forms, respectively, of the enzyme which contain bound I. A similar equation applies to the transverse relaxation data in the fast pulsing limit. Assuming that $\tau_{c,DI} = 2\tau_{c,MI}$ and $\tau_{c,TI} = 3\tau_{c,MI}$ the relaxation data were fit to eq 12 using values of $\tau_{c,MI}$ in the range 14–19 ns. In this case values of g_f for different sets of relaxation data agreed within 10% when $\tau_{c,MI}$ was 14–15 ns and at larger or smaller values of $\tau_{c,MI}$ the agreement was not as good. Thus, our relaxation data suggest that *p*-fluorocinnamate binds equivalently to one site of the protein monomer, two on the dimer, and three on the trimer with a value of $\tau_{c,MI}$ in good agreement with previous estimates. Taking $\tau_{c,MI}$ = 15 ns, the geometrical factor, g_f, characteristic of these complexes is 1.87 ± 0.06 × 10^{-2} Å^{-6}.

As noted earlier the dependence of $R_{2,obsd}$ on the pulsing rate ($1/\tau_{cp}$) in the Carr-Purcell-Meiboom-Gill experiment can lead to an estimate of the exchange rate. Our data were analyzed to obtain k_d by comparing calculated $R_{2,obsd}$ vs. $1/\tau_{cp}$ plots until a good match to the data was obtained. We assumed the four-site exchange process represented by eq 13; the mole

$$\begin{array}{ccccc} & & MI & & \\ & & \uparrow\downarrow k_d & & \\ TI & \overset{k_d}{\rightleftharpoons} & I & \rightleftharpoons & DI \\ & & & \underset{k_d}{} & \end{array} \quad (13)$$

fraction of each form was calculated using the dimerization and trimerization constants of Rao and Kegeles[14] and the K_A shown in Table III. Relaxation rates for I bound to the various enzyme forms were computed assuming only the dipolar mechanism; the average geometric factor mentioned above was used in these calculations. Both k_d and $\Delta\omega$ were varied to obtain the agreement between observed and computed transverse relaxation rates shown in Figure 2. This analysis gave k_d = 9.7 × 10^3 s and $\Delta\omega$ = 2.65 ppm. The chemical shift difference defined by this experiment at 51 MHz is in good agreement with the shift difference determined at 94.1 MHz (Table III).

The line width of the (averaged) *p*-fluorocinnamate signal observed in the high-resolution ^{19}F NMR spectra obtained at 94.1 MHz is dependent on the chemical shift difference at this frequency, the exchange rate, k_d, and the transverse relaxation rates for the fluorine nucleus in its various enzyme-bound forms. The proton-fluorine dipole-dipole contribution to each relaxation rate at 94.1 MHz was calculated using 1.87 × 10^{-2}

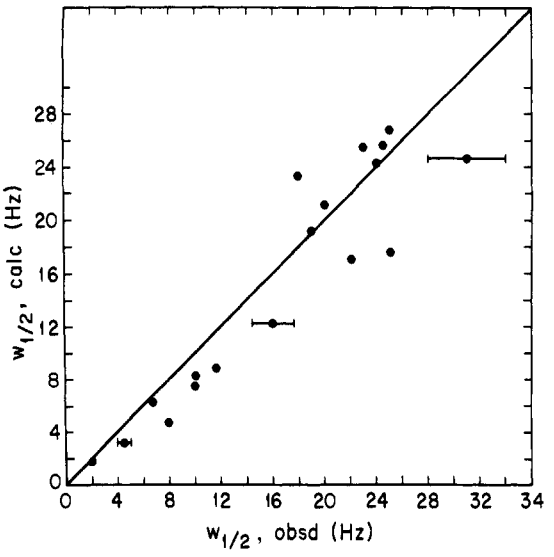


Figure 3. A comparison of observed and calculated ^{19}F NMR line widths at 94.1 MHz. The calculated points were obtained using the parameters and assumptions given in the text. The solid line is the curve expected if agreement was perfect. The bars represent 10% experimental errors.

as the geometrical factor and correlation times of 15, 30, and 45 ns for the protein monomer, dimer, and trimer, respectively. The csa contribution to relaxation was computed employing, unchanged, the parameters given by Hull and Sykes for a *p*-fluorophenyl residue.[28] Using the computed total transverse relaxation rates coupled with the values for $\Delta\omega$ and k_d noted above, the expected high-resolution line width for each sample used in the chemical shift experiments was calculated;[26] the exchange mechanism shown in eq 13 was assumed in these calculations. Figure 3 compares graphically the observed and computed ^{19}F NMR line widths. The agreement is reasonably good and indicates the essential correctness of the analyses we have presented, especially when the difficulties in accurately measuring broad line widths in noisy spectra are considered.

Discussion

Several considerations impinge on the credibility of our conclusion that *p*-fluorocinnamate binds equivalently to the various oligomeric forms of α-chymotrypsin known to exist under the conditions of our experiments. The arguments leading to this conclusion would be weakened if it were possible for the anion to bind at several locations on the enzyme surface in addition to the site identified as the active center. However, an extended series of experiments with *trans*-cinnamate show that strong binding occurs only at the active site and that, if binding at secondary sites does take place, the binding constants for interaction at these sites must be about two orders of magnitude larger than the interaction constant for the active site.[32] If a similar situation obtains with I, the binding at possible secondary sites must be minimal since the concentrations of I used in most experiments were well below the expected dissociation constants for these sites. Furthermore, I behaves as a competitive inhibitor and the K_A determined by ^{19}F NMR is in excellent agreement with the inhibition constants obtained in kinetic experiments; it is unlikely that this would be true if strong secondary interactions between I and the enzyme took place. We thus conclude that *p*-fluorocinnamate binds only at or near the active site of the protein.

It would, of course, be possible to fit our chemical shift and relaxation data to more complex models involving distinguishable sets of chemical shifts, relaxation rates, dissociation constants, and exchange rates for each binding site of each oligomeric form of the protein and we cannot exclude the possibility that a more complex mechanism is consistent with our data. It seems most likely that the differences between binding sites of the various oligomers are slight, however, and would most probably be reflected in the dissociation constant for binding at that site. There are indications that propiolate (III), for example, binds slightly less strongly to the dimer than the monomer,[19] but at the present level of accuracy, it is doubtful if experiments of the type described here could detect these subtle differences. The picture that emerges from this work is that all active sites in all oligomeric forms of α-chymotrypsin are essentially equivalent as regards binding *p*-fluorocinnamate. Oligomerization likely involves active site-active interactions to the extent that many small molecules cannot bind to the active sites of the oligomers.[15-20] However, the protein-protein interfaces must be such that hydrocinnamate, cinnamate, and propiolate anions can bind to the active centers *and yet exchange* with unbound molecules essentially unfettered.

Assuming that the proton-fluorine internuclear distances of the aryl group in I are the same as those found in fluorobenzene[34] one can compute the contribution of these proton-fluorine interactions in *p*-fluorocinnamate to the geometrical factor, g_f. The computed contribution is 0.706×10^{-2} Å^{-6}, and thus, 62% of the observed g_f must arise from protons of the protein interacting with the fluorine nucleus of bound I. We hope that nuclear Overhauser effect experiments in progress will identify the group or groups of the protein responsible for this relaxation effect.

The chemical shift change upon binding of I to the enzyme is in the same direction as that observed with *N*-trifluoroacetyl-*p*-fluorophenylalanine (−1.3 ppm) but over twice as large. The ortho and meta fluorocinnamate-chymotrypsin complexes exhibit large downfield fluorine chemical shifts as well.[35] The direction of the shift seems characteristic, then, of an aromatic fluorine nucleus within the hydrophobic cavity of the enzyme. Protein-induced fluorine chemical shifts are poorly understood at present and, since the para hydrogen of *trans*-cinnamate is shifted *upfield* when bound to the enzyme,[2] probably are not dominated by the same factors that produce the proton chemical shift changes in the enzyme-cinnamate complexes.

The rate of dissociation of *p*-fluorocinnamate-chymotrypsin complexes is very similar to that observed for the *N*-trifluoroacetyl-D-tryptophan-enzyme complex.[8, 18] The rate of dissociation is large enough that enzyme-induced chemical shifts are essentially in the fast-exchange limit. However, there remains an appreciable exchange contribution to the high resolution ^{19}F NMR line widths.

It is now clear that while our earlier conclusions regarding enzyme-induced proton chemical shift effects in the chymotrypsin-*trans*-cinnamate complex are correct, the relaxation data will have to be reconsidered in light of our finding that I and, likely, *trans*-cinnamate bind to the dimeric and trimeric forms of the enzyme.

Experimental Section

Materials. ***p*-Fluorocinnamic acid** was prepared as described previously.[36] **Glutaryl-L-phenylalanine *p*-nitroanilide** (GPNA) was used as received from Swartz/Mann. **Biebricht scarlet** was the multiply recrystallized material described previously.[37] Potassium phosphate (Mallinckrodt AR), phosphoric acid-d_3 (Ventron/Alfa Inorganics) and deuterium oxide (99.8% D, Stohler) were commercial reagents and were used without further purification. All water was deionized water which had been distilled in an all-glass apparatus before use.

α-Chymotrypsin (Worthington, three times recrystallized, salt free) was used without further purification. Active site titrations with *trans*-cinnamoylimidazole (five times recrystallized) were routinely carried out and showed 75–82% active sites from sample to sample. All enzyme concentrations were expressed as active site concentrations.

Instrumentation. All UV-visible spectra including the kinetic de-

terminations were carried out on a Beckman DU spectrometer with a Gilford 2000 accessory. The temperature of the cell compartment was controlled by circulating water through spacers on each side of the compartment. Sample temperatures were measured with a calibrated thermometer inserted through a hole in a modified compartment lid and are believed to be accurate and reproducible to better than 0.5 °C.

A Radiometer PHM 52 meter with a Radiometer micro combination electrode was used for all pH determinations.

Fluorine relaxation measurements at 22.8 and 51.0 MHz were performed on a Bruker 321S spectrometer which has been extensively modified locally.[26,38] Field-frequency stability was maintained by an external lock which used a sample of 2 M LiBr at the lower frequency, locking on the lithium resonance. At the higher frequency the deuterium resonance from a sample of doped D_2O was used as a lock signal. ^{19}F NMR at 94.1 MHz was carried out with a Varian Associates XL-100 spectrometer interfaced to a Nicolet Technology Corp. TT-100A accessory. Proton decoupling was achieved by use of a General Radio 1061 frequency synthesizer, Hewlett-Packard 3722 noise generator, and ENI 320L power amplifier coupled to the Varian V-4412 probe through a locally designed double-tuning network.[39] Temperature control on both instruments was via Varian controllers and was measured with a 5-mm o.d. thermometer supplied by Kontes Glass Co. Temperatures are believed to be accurate to ±1 °C. All samples were allowed to equilibrate at least 10 min in the spectrometer probe before starting an experiment.

Procedures. A. Samples for ^{19}F NMR experiments were prepared by weighing the protein into a 1-mL volumetric flask. Phosphate buffer (0.286 M phosphate, pH 6.2, 0.5 mL) was added. An appropriate amount of *p*-fluorocinnamic acid stock solution in water was added and the flask was filled to the mark with water. The final buffer concentration was thus 0.143 M (μ = 0.2 M).

The sample was mixed on the vortex mixer and then the pH was checked. After transfer of the sample to a sample tube, the solution was gently purged with a stream of nitrogen gas for 10–20 min; this purge did not affect enzyme activity as measured by the GPNA assay. Samples were tightly capped and placed in the spectrometer probe.

Samples for analysis at 94.1 MHz contained 2.5% D_2O to provide a lock signal; no corrections were applied to the pH meter reading, the samples being adjusted to apparent pH 6.2.

B. Determination of K_A for the *p*-fluorocinnamate–α-chymotrypsin complexes by displacement of the dye Biebricht scarlet takes advantage of a maximum in the difference spectrum which occurs at 550 nm.[13] This differential absorption, ΔOD_{550}, was used to determine the dissociation constant K_{BS}, of the dye and the difference extinction coefficient, $\Delta\epsilon_{550}$, of the complex. These were found to be 0.0426 mM and 14 196 M^{-1} cm^{-1}, respectively, in 0.1 M phosphate buffer at 30 °C.

Addition of competitive inhibitors leads to a decrease in ΔOD_{550} as the dye is displaced from the enzymic active site. In the presence of *p*-fluorocinnamic acid the decrease in ΔOD_{550} was observed to be time dependent, slowly increasing until reaching apparent equilibrium after 5–7 h. The overall change is small (~10%) and could be due to a slow conformational change of the enzyme. However, interpretation of experiments over this time span is uncertain because of the likely autolysis of the protein. Therefore, ΔOD_{550} was measured at the same time after mixing for all samples; the time chosen was such that most of the observed change in ΔOD_{550} had taken place but the extent of autolysis was minimal. *p*-Fluorocinnamic acid stock solutions in buffer were aliquoted into 25-mL volumetrics to which 3.0 mL of a 1 mM Biebricht scarlet solution in buffer had been added. The flask was made to the mark with buffer, and the contents were thoroughly mixed and then placed in a constant temperature bath. Solutions of enzyme in 1 mM HCl were maintained at the bath temperature. To prepare a sample for analysis, a 1.0-mL aliquot of the enzyme solution was placed in a test tube and 4.0 mL of the inhibitor–dye mixture was added. experiments with volumetric flasks showed that no volume change accompanied this mixing. After agitation on a vortex mixer, the assay tube was placed in the bath for 1.25 h whereupon the contents were transferred to a cuvette maintained at the bath temperature. The differential absorbance at 550 nm was read relative to a blank composed of 1.0 mL of distilled water plus 4.0 mL of the inhibitor–dye solution at 1.50 h after mixing. Three enzyme and three inhibitor concentrations were used to produce nine estimates of K_A; these were averaged to give the datum in Table I.

C. Inhibition of the enzyme-catalyzed hydrolysis of GPNA by *p*-fluorocinnamate was determined by dissolving an appropriate amount of GPNA in 0.4 mL of methanol or ethanol followed by dilution to 10 mL with buffer. Solutions of GPNA at lower concentrations were prepared by diluting this solution. An assay mixture was prepared in a 10-mm cuvette and typically consisted of 2.5 mL of buffer, 0.2 mL of GPNA solution, 0.2 mL of inhibitor solution in buffer, and 0.1 mL of enzyme contained in 1 mM HCl to give a final volume of 3.0 mL. The initial rate of absorbance increase at 410 nm was monitored and the reciprocal rate of the reaction in units of s/OD was computed. Reciprocal rates ($1/V$) were plotted against the reciprocal of the GPNA concentration ($1/S$). Data obtained without inhibitor present and all data with inhibitor in the reaction mixture gave nicely linear plots having a common intercept on the $1/V$ axis (competitive inhibition[40]). The dissociation constant (K_A) for the inhibitor was reckoned from the intercepts on the negative $1/S$ axis. All kinetic runs were made in triplicate and the individual values averaged to give the data used in the double reciprocal plots. The deviation from the mean did not exceed 5% in any case, leading to an estimated uncertainty in K_A of ±20%.

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Localized Orbitals for Polyatomic Molecules. 5. The Closo Boron Hydrides $B_nH_n^{2-}$ and Carboranes $C_2B_{n-2}H_n$

D. A. Dixon, D. A. Kleier, T. A. Halgren, J. H. Hall, and W. N. Lipscomb*

Contribution from the Gibbs Chemical Laboratory, Harvard University, Cambridge, Massachusetts 02138. *Received March 25, 1977*

Abstract: Wave functions calculated in the approximation of partial retention of diatomic differential overlap (PRDDO) are presented for the closo boron hydrides $B_nH_n^{2-}$ and carboranes $C_2B_{n-2}H_n$ for n = 5–12. The wave functions are examined in terms of both the canonical molecular orbitals and localized molecular orbitals (LMO's) obtained by the Boys criterion. Reactivity predictions are based on various properties related to the atomic charge. Valencies and bond orders are also presented. The LMO's for each molecule are discussed, and for the same n are compared in order to examine the effects of placing the electronegative carbons in the cluster. The LMO's are also compared to topologically allowed structures and are, in general, found to correspond either to sums of topologically allowed structures or to delocalized topologically allowed structures.

As a class of compounds, the carboranes and boron hydrides are, perhaps, the family of molecules that has been most completely studied by rigorous theoretical methods.[1] Previous theoretical studies employing wave functions of nearly ab initio quality have been made of all of the open boron hydrides and carboranes whose structures have been determined experimentally.[2,3] In order to complete this phase of our work on this interesting family of molecules, we have extended our theoretical studies by the PRDDO molecular orbital method[4] to the closo boron hydrides[5c,g] and dicarbacarboranes.[5] (The acronym PRDDO refers to partial retention of diatomic differential overlap.) A number of semiempirical calculations using a variety of methods[6,7] have been performed on these molecules, but only a few ab initio calculations are available, and these are for the smaller carboranes and boron hydrides.[8] We believe that our calculations described below are the best available wave functions for most of these molecules.

The most extensively studied carborane series[5] is the series $C_2B_{n-2}H_n$ for $5 \leq n \leq 12$. Molecular structure parameters have been obtained from microwave spectroscopy for 1,5-$C_2B_3H_5$,[9] 1,2-$C_2B_4H_6$,[10] 1,6-$C_2B_4H_6$,[11] and 2,4-$C_2B_5H_7$;[12] these molecules have also been studied by ab initio methods.[8] The structures of the *C,C'*-dimethyl derivatives of 1,7-$C_2B_6H_8$,[13] 1,6-$C_2B_7H_9$,[14] 1,6-$C_2B_8H_{10}$,[15] and 2,3-$C_2B_9H_{11}$[16] have been determined by x-ray diffraction techniques. The structures of 1,2-[17] and 1,7-$C_2B_{10}H_{12}$[18] have been determined from x-ray diffraction studies while a combination of ^{11}B NMR[19] and electron diffraction[20] were used in determining the structure of 1,12-$C_2B_{10}H_{12}$. The chemistry of the largest members of this series, 1,2-, 1,7-, and 1,12-$C_2B_{10}H_{12}$, has been the most fully explored.

The dicarba-*closo*-carboranes are isoelectronic and isostructural with the remarkably stable doubly charged negative ions, $B_nH_n^{2-}$ (n = 6–12). At the time of Lipscomb's comprehensive survey of boron hydride chemistry in 1963,[1] only $B_{12}H_{12}^{2-}$ had been structurally characterized.[21] Since then the structures of $B_6H_6^{2-}$,[22] $B_8H_8^{2-}$,[23] $B_9H_9^{2-}$,[24] and $B_{10}H_{10}^{2-}$,[25] have determined by x-ray crystallographic methods. The molecules $B_7H_7^{2-}$ [26] and $B_{11}H_{11}^{2-}$ [27] have been partially characterized by ^{11}B NMR studies, but $B_5H_5^{2-}$ has not yet been observed. The basic geometric structures of the $B_nH_n^{2-}$ series are usually closed polyhedra with high symmetry. In all known cases, the isoelectronic dicarbacarborane has the same basic polyhedral shape, but the symmetry is usually much lower.

One of the major techniques for gaining insight into the structure of polyhedral boron hydrides has been the notion of the three-center bond. The central three-center BBB bond has been found in localized molecular orbitals (LMO's) from good wave functions using either the Boys[28] or Edmiston–Ruedenberg (ER) criteria.[29] The study of localized molecular orbitals in these polyhedra is of particular interest because (1) the number of pairs of electrons is greater than the number of centers by one and (2) there are some difficulties when these electron pairs are placed in an environment with high symmetry. The relationship of these LMO bonding structures to the simple topological structures is especially of interest for development of a general theory of bonding in these polyhedra. As previously discussed the Boys and ER criteria disagree for one type of bonding arrangement: the open BCB bond is found in 1,2-$C_2B_4H_6$ by the ER procedure, whereas the Boys procedure yields only central three-center bonds. However, the Boys method is computationally so efficient that we employ it, rather than the ER procedure, in this study.

We present in this paper PRDDO calculations[4] on the $B_nH_n^{2-}$ and $C_2B_{n-2}H_n$ series of molecules, and we discuss the reactivity and structures of these molecules in light of the canonical molecular orbitals. Localized molecular orbitals are obtained using the Boys criterion in order to examine the valence bonds in these molecules and to compare the LMO's with simple topological structures. These highly symmetric molecules are also interesting because they may yield multiple maxima on the LMO hypersurface. Comparison of the LMO's of the $B_nH_n^{2-}$ with those in the isoelectronic carboranes allows for the examination of the effects on the bonding of placing electronegative atoms into these clusters. These polyhedral