Dynamics at the Active Site of Bis(4-fluorophenyl)carbamoyl-α-chymotrypsin

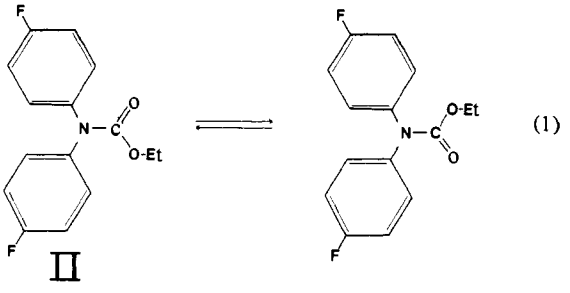
M. Cairi and J. T. Gerig*

Contribution from the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received November 29, 1982*

Abstract: Treatment of α-chymotrypsin with bis(4-fluorophenyl)carbamoyl chloride produces an inactive enzyme that has been stoichiometrically carbamoylated, presumably at the active site. Fluorine NMR spectra of this enzyme derivative show two broad signals separated by 3.4 ppm at 25 °C. A variety of NMR experiments suggest that these signals arise because of restricted rotation about the carbonyl carbon–nitrogen bond in the carbamate function; rate and activation parameters for this process have been determined, using these experiments. The fluorine chemical shift difference is much smaller (~0.2 ppm) when the protein is denatured in 8 M urea, but the activation parameters for the conformational interchange are scarcely altered by the loss of protein tertiary structure in this solvent. A rough analysis of fluorine T_1 and T_2 relaxation data indicate that the fluorophenyl rings are highly immobilized in the native acylated enzyme but become much freer in the denaturated structure. It is suggested that localized unfolding at the active site of the native protein is a necessary component of the mechanism whereby rotation of the bis(4-fluorophenyl)amino group takes place.

X-ray crystallographic investigations inform us as to the average positions of atoms in a protein crystal, and this information is invaluable in attempts to understand protein structure and function. However, X-ray studies may generate the misleading notion that protein tertiary structures are static and unique, although there is ample experimental evidence that proteins are flexible and can fluctuate rapidly between various conformational substates.[1-3] Diffraction data can be used to define regions of protein structures that are especially mobile in the solid state,[4-6] while a variety of techniques including NMR spectroscopy provide insight into structural changes in solution.

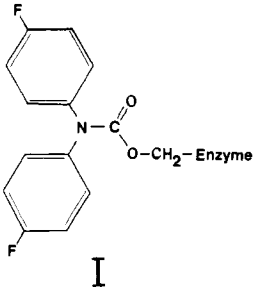
Erlanger and co-workers have shown that the proteolytic enzyme α-chymotrypsin may be stoichiometrically inactivated by attachment of the *N,N*-diphenylcarbamoyl group to the enzyme, presumably at the serine-195 residue of the active site.[7-11] Equation 1 indicates that the two phenyl rings of the diphenyl-



(1)

amino group are magnetically nonequivalent by virtue of restricted rotation about the carbonyl carbon–nitrogen bond of the urethane group. Consideration of a molecular model of this enzyme derivative in conjunction with X-ray structural data for several carbamoylated chymotrypsins,[12,13] suggests that one phenyl ring likely projects into the side-chain specificity pocket of the enzyme[14] while the other is oriented toward the surface of the protein. A region of polypeptide structure separates the rings in this view, and one anticipates that, if this arrangement is rigidly retained in solution, the energy barrier to rotation of the diphenylamino group would be very high. The purpose of the present work was to measure this barrier and thereby gain some impression of the flexibility of the tertiary structure near the active center of this enzyme derivative.

Proton or carbon NMR spectroscopy would be difficult to apply to this end because of the difficulty in resolving the resonances of the diphenylcarbamoyl group from many similar resonances originating from groups of the protein. We, therefore, prepared the fluorine-substituted system (I) indicated below to overcome



this problem. The results obtained are consistent with the expected orientation of the diphenylcarbanoyl group at the active site but suggest that rapid protein motions permit easy interchange of the phenyl (fluorophenyl) rings.

Experimental Section

Instrumentation. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Routine ^1H NMR spectra were recorded with a Varian T-60 spectrometer using acetone-D_6 (Merck) as a solvent and tetramethylsilane as internal standard. Visible and UV spectra were obtained on a Gilford 2000 or Cary 15 spectrometer. Mass spectra were obtained with a Micromass ZAB-2F instrument. A Perkin-Elmer 283 spectrometer was used to record infrared spectra. A Radiometer PHM 63 pH meter equipped with a Radiometer micro-

(1) Gurd, F. R. N.; Rothgeb, T. M. *Adv. Protein Chem.* **1979**, *33*, 74–156.
(2) Karplus, M.; McCammon, J. A. *CRC Crit. Rev. Biochem.* **1981**, *9*, 293–349.
(3) Careri, G.; Fasella, P.; Gratton, E. *CRC Crit. Rev. Biochem.* **1975**, *3*, 141–164.
(4) Frauenfelder, H.; Petsko, G. A.; Tsernoglou, D. *Nature (London)* **1979**, *280*, 558–563.
(5) Hartmann, H.; Parak, F.; Steigeman, W.; Petsko, G. A.; Ponzi, D. R.; Frauenfelder, H. *Proc. Nat. Acad. Sci. U.S.A.* **1982**, *79*, 4967–4971.
(6) Walter, J.; Steigeman, W.; Singh, T. P.; Bartunik, H.; Bode, W.; Huber, R. Acta Crystallogr., Sect. B **1982**, *B38*, 1462–1472.
(7) Erlanger, B. F.; Cohen, W. *J. Am. Chem. Soc.* **1963**, *85*, 348.
(8) Erlanger, B. F.; Castleman, H.; Cooper, A. G. *J. Am. Chem. Soc.* **1963**, *85*, 1872.
(9) Erlanger, B. F.; Cooper, A. G.; Cohen, W. *Biochemistry* **1966**, *5*, 190.
(10) Erlanger, B. F.; Buxbaum, S. N.; Sack, R. A.; Cooper, A. G. *Anal. Biochem.* **1967**, *19*, 542.
(11) Erlanger, B. F.; Edel, F. *Biochemistry* **1964**, *3*, 346.
(12) Robillard, G. T.; Powers, J. C.; Wilcox, P. E. *Biochemistry* **1972**, *11*, 1773.
(13) Brown, W. E. *Biochemistry* **1975**, *14*, 5079.
(14) Kraut, J. *Annu. Rev. Biochem.* **1977**, *46*, 331.

0002-7863/83/1505-4793$01.50/0

combination electrode was used to determine pH; kinetic experiments employed a Metrohm E526 titrator connected to a recorder.

Fluorine NMR spectroscopy at 94 MHz was performed with a Varian XL-100 spectrometer interfaced to a Nicolet TT-100A accessory. Heteronuclear proton decoupling on this instrument has been previously described.[15] Temperature control was achieved with the Varian controller and calibrated with a copper-constantan thermocouple.

Fluorine experiments at 282 MHz used a Nicolet NT-300 instrument. Sample temperature was controlled with the Nicolet system and calibrated using anhydrous methanol.[16]

Fluorine spectra at 470 MHz were obtained on the Bruker WH-500 instrument maintained by the Southern California Regional NMR Faculty at the California Institute of Technology. Sample temperatures were calibrated with an anhydrous methanol sample.

In all cases, deuterium oxide (~5%) in the samples served to provide a lock signal; 12-mm diameter samples were used at 94 MHz while 10-mm diameter samples were employed at the higher frequencies.

Materials. Common salts, buffers, and organic solvents were the highest grade obtainable commercially. All water was deionized and then distilled in glass. Deuterium oxide was purchased from Stohler Isotope Chemicals and used as received.

***N*,*N*-Bis(4-fluorophenyl)amine.** In a 125-mL round-bottom flask equipped with condenser and magnetic stirrer was placed 11 g (0.07 mol) of *p*-fluoroacetanilide (Fairfield Chemical), 8.3 g (0.06 mol) of K_2CO_3, 2.2 g (0.01 mol) of copper(I) iodide (Alfa), and 25 mL (0.22 mol) of *p*-bromofluorobenzene (Aldrich). The mixture was heated to vigorous reflux for 10 h whereupon the excess bromofluorobenzene was steam distilled from the system. The remaining reaction mixture was extracted four times with ether; the combined ether extracts were washed with water, dried with anhydrous $MgSO_4$, and evaporated in vacuo to leave a brown oil. This product was dissolved in 120 mL of 95% ethanol containing 15 g (0.27 mol) of KOH and the mixture heated to reflux for 1.5 h. The mixture was then poured into 500 mL of water. The aqueous phase was extracted three times with dichloromethane; the combined organic extracts were washed three times with water and dried over $MgSO_4$. Removal of the solvent in vacuo afforded 10 g (68%) of a brown oil which exhibited in the ^1H NMR spectrum two multiplets centered at 6.95 ppm. A small amount of this crude material was distilled at 1 mmHg to give a white solid, mp 36–37 °C (lit. mp 37.5 °C),[17] which gave a ^1H NMR spectrum identical with that of the crude liquid.

***N*,*N*-Bis(4-fluorophenyl)carbamoyl Chloride.** A three-necked round-bottom flask connected to two aqueous ammonia gas traps was charged with 7.5 g (0.036 mol) of crude *N*,*N*-bis(4-fluorophenyl)amine, 2.9 mL (0.036 mol) of pyridine, and 30 mL of chloroform. The system was purged with nitrogen and then the flask was cooled to −10 °C in an ice–salt bath. Phosgene (60 mL, Union Carbide) was condensed into the reaction vessel, and after removal of the cooling bath the mixture was allowed to stir for 3 h. Nitrogen was flushed through the system at the end of this period to purge the remaining phosgene, and the chloroform was distilled from the mixture. The residue was dissolved in benzene and the benzene-insoluble fraction removed by filtration. The benzene was removed in vacuo and the remaining solid recrystallized twice from ethanol to produce the desired product as white needles, mp 58–59 °C, in 53% yield (4.1 g). The ^1H NMR spectrum showed a complex multiplet centered at 7.2 ppm. A carbonyl absorption frequency at 1720 cm^{-1} was observed, the 35-cm^{-1} shift away from the corresponding frequency in *N*,*N*-diphenylcarbamoyl chloride being consistent with the expected influence of the ring substituents on the bond order of the carbonyl group.[18] The mass spectrum of the material showed parent ions at *m*/*e* 267 and 269 in the ratio 100:33 and was consistent with the desired structure.

Ethyl bis(4-fluorophenyl)carbamate was prepared by heating 1 g (60 mmol) of crude *N*,*N*-bis(4-fluorophenyl)amine and 2 mL (25 mmol) of ethyl chloroformate in a 25-mL round-bottom flask with reflux condenser at 92–95 °C for 2.5 h. The reaction mixture was taken up in benzene and the insoluble material removed by filtration. Removal of the benzene in vacuo gave 0.5 g (30%) of a brown oil. Distillation of the liquid at 1 mmHg afforded a product which solidified after standing 24 h at 4 °C. The melting point of this material was 60–62 °C; it showed a triplet at 1.1 ppm (3 H), a quartet at 4.1 ppm (2 H), and a multiplet at 7.1 ppm (8 H) in the ^1H NMR.

α-Chymotrypsin was a three-times recrystallized, salt-free product obtained from Miles Laboratories. Diisopropylphosphorylchymotrypsin was obtained from Worthington Biochemical. Tosylchymotrypsin was prepared following the procedure of Sigler et al.[19]

Enzyme Modification. Preliminary experiments demonstrated by UV spectroscopy that methanol stock solutions of bis(4-fluorophenyl)carbamoyl chloride were stable for at least 1 week at room temperature. At 22 °C in 3.2% methanol–water mixtures containing 0.005 M phosphate, the pseudo-first-order rate constant for hydrolysis of the acid chloride was 8.0×10^{-6} s^{-1}, while in 10% methanol–water the rate slowed, giving a pseudo-first-order constant of 3.5×10^{-6} s^{-1}. The hydrolysis rates were identical at pH 5.6 and 7.6, in agreement with the observation that diphenylcarbamoyl chloride hydrolysis is pH independent up to pH 10.[20]

To inactivate the enzyme, a solution of 1 g of α-chymotrypsin in 30 mL of water was prepared. A solution of *N*,*N*-bis(4-fluorophenyl)carbamoyl chloride (53 mg) in 1 mL of methanol was prepared, and 0.5 mL of this solution was added to the enzyme solution. The reaction mixture was stirred slowly and the solution pH maintained at 5.6 or 7.6 by addition of 0.01 M NaOH by a pH-stat/recorder system. Not all of the carbamoyl chloride is soluble under these conditions, and a fine precipitate appears as soon as the methanol solution is added to the reaction mixture. It was determined that the solubility of the chloride under these conditions was approximately 50 μM, and it was assumed that this concentration remained constant throughout the course of the reaction. The enzyme concentration in the final reaction mixture was typically 1.3 mM. The apparent first-order rate constant for the release of H^+ under these conditions was 2.1×10^{-3} s^{-1} at pH 5.6 and 30×10^{-3} at pH 7.6. After 2 h (~20 half-lives at pH 5.6), the reaction was halted and filtered to remove undissolved chloride. The enzyme concentration in the reaction mixture was determined by using the absorbance at 280 nm with ϵ_{280} = 50 000.[21] An assay of enzymic activity using the hydrolysis of *N*-glutarylphenylalanine *p*-nitroanilide[22] showed an activity of less than 0.5% toward this substrate. Fluorine NMR spectroscopy of these solutions generally showed the absence of resonances attributable to the acid chloride, and, by using integrations of the modified enzyme peaks and that of an internal standard (*p*-fluoroaniline) of known concentration, it was determined that there was present 0.93 ± 0.05 mol of difluorodiphenyl groups per mol of enzyme. The expected 1:1 stoichiometry of the carbamoylation was thus confirmed.

Both tosylchymotrypsin and diisopropylphosphorylchymotrypsin were treated with bis(4-fluorophenyl)carbamoyl chloride at pH 7.5 under the same conditions. There was no consumption of base by these reaction mixtures over the course of 0.5 h, indicating that these materials are unable to react with the chloride in the same manner as the native enzyme and providing an indication that it is the serine-195 residue of the native enzyme that is involved in the carbamylation.

Enzyme Stability. The stability of bis(4-fluorophenyl)carbamoyl-chymotrypsin to hydrolysis was determined by measuring enzymatic activity toward *N*-glutarylphenylalanine *p*-nitroanilide under standard assay conditions. In this way, it was demonstrated that the carbamoylated enzyme was stable for at least 11 days when stored at pH 5.6 or 7 and 4 °C; in all cases, recovered enzymatic activity under these conditions was less than 0.5%. At pH 7.6 and 22 °C, 20% activity was recovered after 8 days. The acyl-enzyme is thus stable enough toward hydrolysis for NMR experiments of several days duration. Samples for NMR experiments were stored at 4 °C and generally used within 2 weeks after preparation.

Fluorine NMR Spectroscopy. Samples of ethyl bis(4-fluorophenyl)-carbamate were approximately 0.1 M in acetone-d_6; *p*-fluoroacetanilide was present at a concentration of 0.1 M in these samples to provide an internal reference signal.

Three milliliters of native bis(4-fluorophenyl)carbamoyl-chymotrypsin solutions, prepared as described above, were mixed with 0.3 mL of D_2O to prepare enzyme samples for fluorine NMR experiments. Typically these samples contained ~1 mM protein, 10% D_2O and ~0.035 M NaCl at pH 5.6. A 0.1 M solution of *p*-fluoroaniline in methanol sealed in a capillary tube served as an external reference in this case. To prepare enzyme samples in ~100% D_2O, the enzyme modification reaction was carried out in this solvent.

Samples of denatured carbamoylated enzyme were prepared by adding sufficient solid urea or urea-d_4 to samples of the native enzyme to give an 8 M urea solution.

All spectral data were accumulated in the Fourier transform by application of 90° pulses. A delay of about $5T_1$ was utilized between accumulations. Spectra of the enzyme system were generally not proton decoupled as this made no detectable difference in the observed line

(15) Gerig, J. T.; Luk, K. F. S.; Roe, D. C. *J. Am. Chem. Soc.* **1979**, *101*, 7698–7704.
(16) Raiford, D. S.; Fisk, C. L.; Becker, E. D. *Anal. Chem.* **1979**, *51*, 2050.
(17) Leonard, N. J.; Sutton, L. E. *J. am. Chem. Soc.* **1948**, *70*, 1564.
(18) Lawrence, C.; Guiheneuf, G.; Wojtkowiak, B. *J. Am. Chem. Soc.* **1979**, *101*, 4793–4801.
(19) Sigler, P. B.; Jeffery, B. A.; Mathews, B. W.; Blow, D. M. *J. Mol. Biol.* **1966**, *15*, 175–192.
(20) Johnson, S. L.; Giron, H. M. *J. Org. Chem.* **1972**, *37*, 1383.
(21) Wilcox, P. E. *Methods Enzymol.* **1970**, *19*, 72.
(22) Bittner, E. W.; Gerig, J. T. *J. Am. Chem. Soc.* **1970**, *92*, 2114–2118.

Table I. Chemical Shifts and Activation Parameters

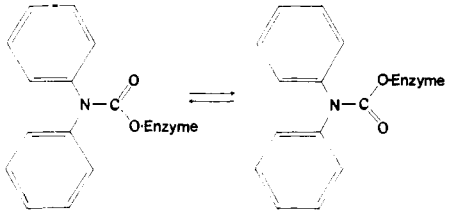
system	$\Delta\delta$	δ_L[a]	Δ_u[a]	k_{25},[b] s^{-1}	$\Delta G^{\ddagger}$, kJ mol^{-1}	$\Delta H^{\ddagger}$, kJ mol^{-1}	$\Delta S^{\ddagger}$, J deg^{-1} mol^{-1}
model II[c]	1.10	–	–	25 000	49.4 ± 0.4	49.8 ± 6.3	2 ± 20
model II[d]		13.73	13.73	–	–	–	–
native enzyme[e]	3.4	17.9	14.5	125	60.3 ± 0.8	82.0 ± 1.6	67 ± 40
denatured enzyme[f]	0.23	15.17	15.17	125	62.4 ± 0.6	90.8 ± 16	100 ± 40

[a] Approximate chemical shift at 25 °C relative to an external reference of 4 mM *p*-fluoroaniline in methanol, δ_L refers to the low-field signal. [b] Extrapolated or interpolated value for the rotation rate constant at 25 °C. [c] In acetone-d_6. The shift difference at −60 °C is given. [d] In 10% methanol/water at 25 °C. [e] In water containing 10% D_2O and 0.035 M NaCl at pH 5.6, 25 °C. [f] In 8 M urea in water containing 0.02 M NaCl and 6% D_2O at pH 5.6. An averaged signal was observed at 25 °C. The chemical shift difference at −10 °C is listed.

widths. Procedures for collection of the T_1 and $^{19}F[^1H]$ Overhauser effects were the same as described previously;[15] T_1 experiments at 282 MHz used a composite 180° pulse and phase cycling.[23,24] The two-dimensional exchange spectrum utilized the pulse sequence of Macura and Ernst[25] to suppress axial peaks and transverse interference. The saturation-transfer experiment was carried out as described previously.[26] Radio frequency power was adjusted to provide saturation of one signal of the enzyme without perturbation of the adjacent signal. Line shapes of the exchanging systems were analyzed for the rate of conformational interchange by visually comparing theoretical plots generated with a program based on a density matrix treatment of this situation to experimental spectra.[27] These analyses depend on four parameters: (1) the nonexchange chemical shift difference expressed in hertz, (2) the relative concentration of each exchangeable site, (3) the nonexchange line widths, and (4) the rate constant or lifetime for exchange. In some cases examined, the nonexchange chemical shift difference was clearly temperature dependent, and this complication was dealt with as described below. Errors in a given rate constant for rotation were estimated by noting the sensitivity of a simulation to these parameters when the parameters were allowed to vary over a reasonable range. The estimated errors in the rate constants were typically ±10–15% and were fit to the Eyring equation by the procedure described earlier.[55] Standard deviations estimated by the fitting program are given in Table I.

Results

Ethyl Bis(4-fluorophenyl)carbamate (II). In order to provide a baseline for expectations in the carbamoylated enzyme system, the kinetics of rotation of the urethane group in the model compound II were determined by line-shape methods. At room



(2)

temperature the fluorine spectrum of II in acetone-d_6 was a singlet, but as the sample temperature was lowered to −60 °C, the spectrum evolved into two resonances of equal intensity separated by 1.10 ppm. Spectra at intermediate temperatures were analyzed by line-shape simulation methods to give rate constant data which led to the activation parameters for urethane rotation presented in Table I.

Compound II is insoluble in water, and to provide information about the effects of a solvent environment more similar to that present in the enzyme systems, a sample of II dissolved in 10% methanol/90% water was examined. The freezing point of this solvent precluded measuring the rotational barrier in this medium. However, the chemical shift of the (averaged) observed signal relative to the same external reference as used with the enzyme system was determined. The temperature dependence of this chemical shift was small (0.008 ppm/deg) and linear over the temperature range 5–30 °C. At 25 °C the chemical shift observed was 13.73 ppm downfield relative to the signal from a capillary containing 5 mM *p*-fluoroaniline in methanol.

Native *N,N*-Bis(4-fluorophenyl)carbamoyl-α-chymotrypsin. Reaction of native α-chymotrypsin with bis(4-fluorophenyl)carbamoyl chloride was rapid relative to the rate of hydrolysis of the chloride under the reaction conditions. The solubility of the chloride in water was very low, and the system we finally developed for the modification reaction was heterogeneous. The reaction was rapid enough at pH 7.6 that it is doubtful if the titrimetric procedure used to follow the modification reaction produced reliable kinetic results.

Studies of the reactivation of the enzyme by hydrolysis of the carbamoyl group indicated that this decomposition reaction would be negligible at pH 5.6 for several weeks, certainly long enough for several NMR experiments with each preparation. 4,4′-Difluorodiphenylamine, the product of hydrolysis of the carbomoylated enzyme, has a distinctive fluorine chemical shift at 3.2 ppm to high field of the external reference, and any appreciable hydrolysis of the acyl-enzyme was immediately apparent in the fluorine NMR spectra.

The fluorine NMR spectrum of *N,N*-bis(4-fluorophenyl)carbamoyl-chymotrypsin at temperatures below 30 °C showed two broad signals of equal integrated intensity. As indicated in Table I, one of these signals was close to the shift observed for the model compound II while the second signal was about 3 ppm to lower field.

Fluorine NMR spectra of the modified enzyme were significantly temperature dependent (Figure 1) as regards both the shift *difference* between the two observed peaks and also the position of the resonances relative to the external reference. Over the temperature range 0–45 °C the shift difference changed nonlinearly from over 4 ppm at 1.5 °C to ca. 2 ppm at 40 °C (Figure 2). The position of the low-field resonance was constant at 17.9 ppm relative to the external reference up to 25 °C and then decreased rapidly after 25 °C to 17.2 ppm. The shift of the upfield peak increased monotonically from 14 ppm (1.5 °C) to about 15 ppm at 40 °C in the 470-MHz spectra.

It was clear from these initial observations that rotation of the carbamoyl group in the native enzyme must be very much slower than in the model compound II. While the change in peak positions with temperature is consistent with the onset of chemical exchange at the higher temperature, the unusual temperature dependence of the individual lines signaled the possibility that the NMR line-shape changes observed could simply be the result of overall protein structural changes with temperature variation and it was possible that no detectable exchange between the two 4-fluorophenyl environments was taking place.

The question whether or not exchange was present was examined in two ways. A saturation-transfer experiment was carried out in which one component of the NMR pattern observed was saturated and the intensity of the second resonance was monitored. After the spin system had come to equilibrium under these conditions[26]

$$M_0/M_\infty = kT_1 \tag{3}$$

where M_∞ is the observed signal intensity, M_0 is the intensity in the absence of saturation, k is the rate constant for interchange between the two sites represented by the signals, and T_1 is the spin–lattice relaxation time, assumed to be equal for the two sites. At 14 °C, the ratio M_0/M_∞ was observed to be 4.4. The value

(23) Freeman, R.; Kempsell, S. P.; Levitt, M. H. *J. Magn. Reson.* **1980**, *38*, 453–479.
(24) Cutnell, J. D.; Bleich, H. E.; Glasel, J. A. *J. Magn. Reson.* **1976**, *21*, 43.
(25) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95–117.
(26) Baine, P. B.; Gerig, J. T.; Stock, A. D. *Org. Magn. Reson.* **1981**, *17*, 41–45.
(27) Johnson, Jr., C. S. *Adv. Magn. Reson.* **1965**, *1*, 65.

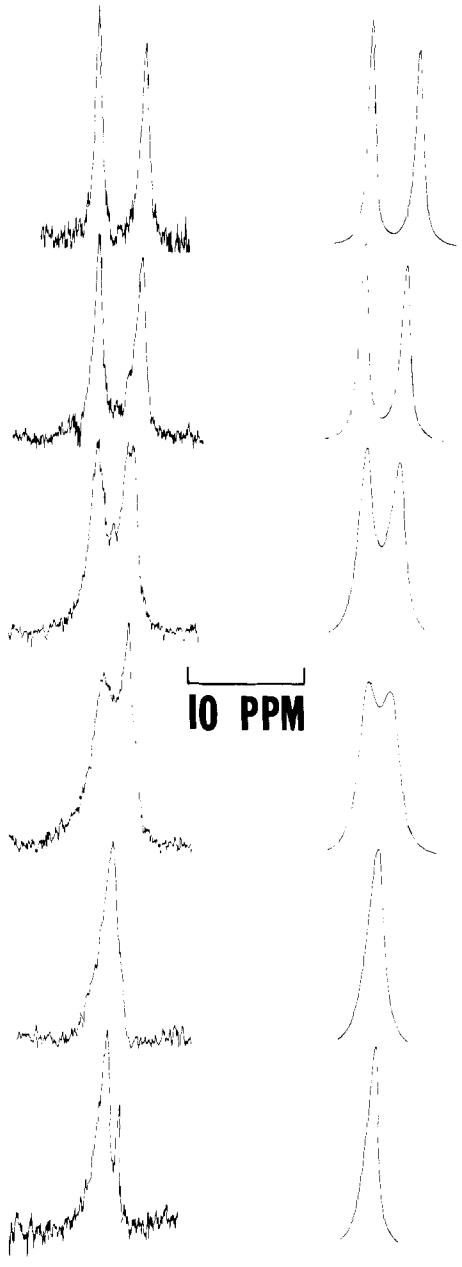


Figure 1. Fluorine NMR spectra obtained at 94 MHz of *N,N*-bis(4-fluorophenyl)carbamoyl-α-chymotrypsin at various temperatures. The smooth curves were those calculated using the assumptions described in the text. The sample temperatures, from top to bottom, were 1.5, 17, 30, 35, 43, and 46 °C.

of T_1 observed in an independent experiment (discussed below) was 0.16 s, leading to an estimated rate constant for exchange of 27 ± 7 s^{-1}. A more elaborate version of the saturation-transfer experiment was also carried out by two-dimensional NMR methods.[28] Although the very broad lines observed at 282 MHz might seem to preclude such experiments because of rapid transverse relaxation during the initial part of the pulse sequence, Figure 3 indicates that an acceptable exchange map can be obtained with this enzyme system. The appearance of cross peaks in the two-dimensional experiment is definitive evidence for rotation (interchange) of the two 4-fluorophenyl groups of the enzyme. Analysis of the heights of the cross peaks relative to the diagonal peaks according to eq 30 of Jeener et al.[28] suggested that the rate constant of exchange is 12 ± 4 s^{-1} at 5 °C.

The sample used for the two-dimensional experiment contained a detectable amount of nonnative carbamoylated protein which shows a single resonance at approximately 15.1 ppm from the external reference. Although the noise level in the 2D spectrum is high, there is no clear indication of cross peaks involving the nonnative enzyme, suggesting that this species is not on the itinerary that interconverts the fluorophenyl groups of the native enzyme.

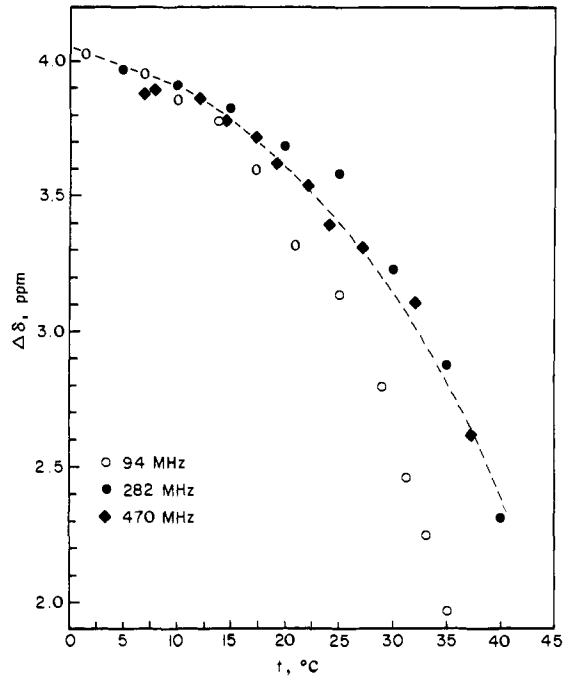


Figure 2. Temperature dependence of the observed chemical shift difference between the two fluorine signals of the native modified enzyme at 94, 282 and 470 MHz. The very broad lines observed at the higher frequencies made it difficult to determine the shift difference to better than ±0.05 ppm. The dashed line shows the assumed temperature dependence of the nonexchange chemical shift used in analyzing the data at 94 MHz for the rate constant for rotation of the diphenylcarbamoyl group.

It was thus clear that exchange was taking place, and we sought to estimate the rate of this process at other temperatures by line-shape analysis. In the slow exchange region, the contribution of exchange to the observed signal separation is given by[29]

$$\Delta\delta = \Delta\delta_0[1 - k^2/2\pi^2\Delta\delta_0^2]^{1/2} \quad (4)$$

where $\Delta\delta$, measured in hertz, is the observed separation in the presence of exchange and $\Delta\delta_0$ is the separation of signals in the absence of exchange. At a given temperature and exchange rate constant (k), the exchange-induced reduction of the signal separation will depend on the magnetic field used for the experiment since $\Delta\delta_0$ is field dependent; with increasing fields, the exchange contribution will be decreased and the observed separation will more closely approximate $\Delta\delta_0$ at that temperature.

Fluorine NMR spectra of the native enzyme were obtained at fluorine resonance frequencies of 94, 282, and 470 MHz. Data at the higher two frequencies were identical within experimental error, suggesting that the observed chemical shift differences measured in ppm at the higher frequencies represent the nonexchange, limiting chemical shift at each temperature. The fluorine signals at the higher fields were quite broad (~200 and ~280 Hz, respectively) so appreciable uncertainty attends a measurement of peak separation. A smooth curve was drawn through the available high frequency fluorine data (Figure 2), and the limiting shifts obtained in this way were used in a line-shape analysis of the data obtained at 94 MHz. There was no detectable dependence of the nonexchange line widths on temperature at any frequency, and this parameter was assumed to be constant in the line-shape calculations. Good agreement between observed and

(28) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546–4553.

(29) Becker, E. D. "High Resolution NMR", 2nd ed.; Academic Press: New York, 1980; p 243.

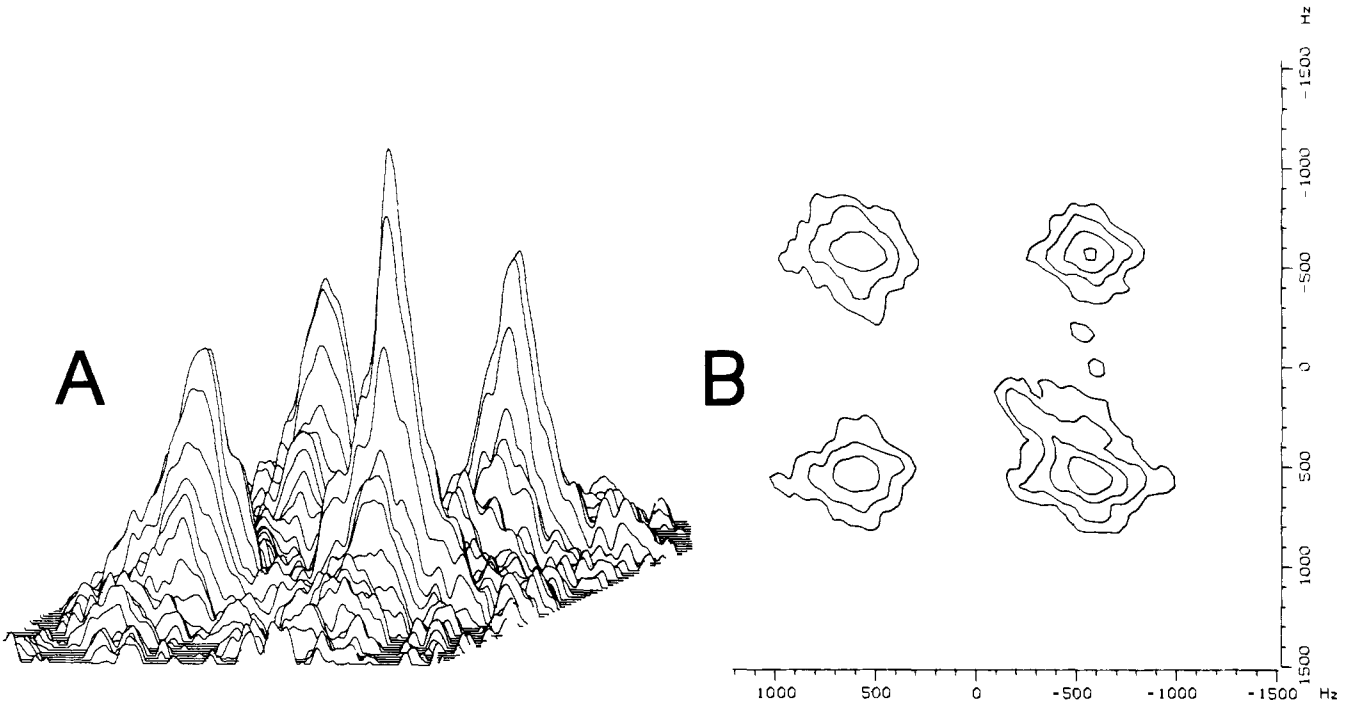


Figure 3. Two-dimensional exchange spectra of bis(4-fluorophenyl)carbamoyl-α-chymotrypsin obtained at 5 °C. The full-sweep width in both dimensions was 3000 Hz represented by 1K data points in the f_1 dimension, and 128 data points in the f_2 direction. (A) Stacked plot representation of the spectrum; (B) contour map generated from the data. The extra intensity in the foreground peak arises from a small amount of denatured enzyme.

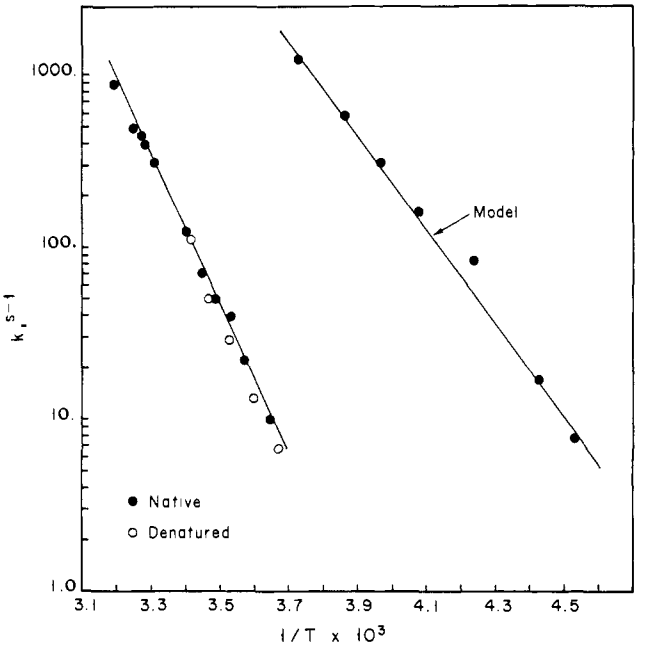


Figure 4. Plots of ln k vs. $1/T$ for carbamoyl group rotation in the native and denatured enzymes and in model compound II.

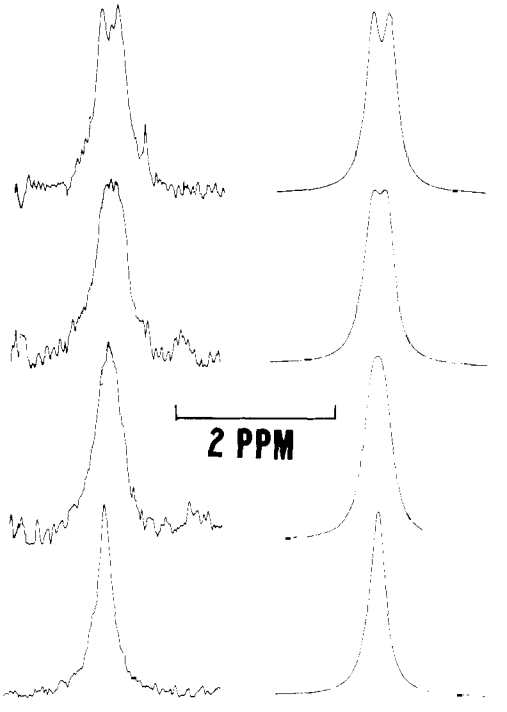


Figure 5. Fluorine NMR spectra at 94 MHz of *N,N*-bis(4-fluorophenyl)carbamoyl-α-chymotrypsin dissolved in 8 M urea. The smooth curves were calculated using the assumptions described in the text. The sample temperature, from top to bottom, were − 10, 5, 11, and 29 °C.

calculated fluorine NMR line shapes could be obtained using these assumptions (Figure 1), and it is believed that the rate constants for rotation so estimated are reliable to ±10%.

All temperature changes observed (Figure 1) were reversible except that when enzyme samples were kept at high temperatures (>30 °C) for extended periods of time a relatively sharp resonance appeared at approximately 15.1 ppm, overlapping the upfield fluorine signal from the carbamate group. This peak was assigned to nonnative ("denatured") acylated enzyme and was neglected in the line-shape analysis.

An Arrhenius plot of the exchange rate data is presented in Figure 4, and these data lead to the activation parameters given in Table I. A 200-fold reduction in the rate of carbamoyl group rotation at 25 °C was observed in the native enzyme relative to the rate of the corresponding process in the model urethane II; the rate change is due to increases in both the entropic and enthalpic factors.

Denatured Bis(4-fluorophenyl)carbamoyl-α-chymotrypsin. Presumably the increase in the barrier to rotation of the carbamoyl group in native chymotrypsin is related to constraints imposed by the tertiary structure of the protein, and to explore the effects of removing these constraints, we attempted to measure the rate of rotation in the denatured enzyme. It was found that aqueous solutions of the acyl-enzyme containing 8 M urea remained fluid

Table II. Relaxation Data[a]

	temp, °C	rf, MHz	$w_{1/2}$,[c] Hz	T_1,[d] s	NOE[e]
native enzyme	14	94	47, 57 (66)	0.16, 0.16 (0.10)	−0.8, −0.8 (−0.97)
	5	282[f]	200, 200 (163)	0.77, 0.69 (0.80)	−, − (−0.97)
	25	282	200, 200	0.67, 0.65	−0.8, −0.7
	30	470	275, 280 (348)	−, −	−, −
denatured enzyme[b]	30	94	3, 3	0.45, 0.45	−0.3, −0.3

[a] The first listing in each column is for the low-field fluorine signal. The values in parentheses were calculated as described in the text. [b] At 30 °C the spectrum is a single resonance, and the data given are those observed for this averaged resonance. [c] Observed line widths under conditions of a negligible rate of interchange of the fluorophenyl rings. There was no detectable difference in line widths when the spectra were obtained under conditions of proton decoupling. Estimated errors in the line widths are of the order of ±10%. The transverse relaxation time is given by $T_2 \simeq (1/\pi)w_{1/2}$. [d] Obtained by the inversion-recovery method in the absence of proton decoupling. Estimated error ±10%. [e] Obtained with noise-modulated proton decoupling; estimated error ±5%. [f] Interpolated data for 14 °C used in the relaxation time calculations.

enough at −15 °C to permit NMR spectroscopy. At 25 °C and 94 MHz only a single relatively sharp fluorine resonance was observed while at −15 °C two equally intense signals, separated by only 0.23 ppm, were evident (Figure 5). Given the small separation of these resonances and the substantial increase in the fluorine NMR line widths at higher magnetic fields, it was doubtful whether these signals would be resolvable at 282 or 470 MHz. We, therefore, assumed that the separation of resonances observed at 94 MHz represents the no-exchange chemical shift difference and analyzed the line shapes at other temperatures by assuming that this shift difference and the natural line widths of the signals are temperature invariant.

As indicated in Figure 4, the rate constants for rotation in this system are very similar to those observed with the native enzyme. A calculation of the activation parameters using the available data for the denatured system gave the results in Table I; these values are identical, within the uncertainties of the parameters, to the corresponding data for the native enzyme.

The denatured enzyme samples were prepared by adding sufficient solid urea to solutions of the native enzyme to provide 8 M urea. It was observed that the production of denatured enzyme as represented by the appearance of the signal at 15.1 ppm was not instantaneous; 12–24 h were required before the characteristic doublet signals of the native enzyme had completely disappeared. Thermal denaturation of this modified enzyme was also slow, with about 12 h required for denaturation at 45 °C. At 50 °C denaturation was complete in 2 h. Although these processes were not carefully studied and the times given are only approximate, it is clear that the carbamoylated enzyme is very resistant to both urea and thermally induced denaturation.

Solvent Effects on Chemical Shifts. Fluorine chemical shifts are sensitive to solvent, including the change from water to deuterium oxide.[30,31] Such changes have been used to suggest whether or not a particular fluorine nucleus in a protein structure is exposed to solvent. Generally, a 0.1–0.2 ppm upfield shift attends the change of solvent from H_2O to D_2O for fluorinated groups that are freely available to solvent.[33]

When measured relative to an external reference, there was no H_2O/D_2O solvent effect on either fluorine resonance of the nature carbamoylated enzyme when the solvent was changed from 6% D_2O in H_2O to 100% D_2O. However, a small change would have been difficult to detect because of the spectral line widths. Changing the solvent from 6% D_2O and 8 M urea in H_2O to 100% D_2O and 8 M urea-d_4 produced a 0.13 ppm upfield shift of the (averaged) resonance position in the denatured enzyme. These results are thus consistent with the notion that considerable unfolding of protein structure takes place under the denaturing conditions such that the fluorophenyl groups become more exposed to solvent. Both environments for fluorophenyl residues in the native enzyme appear to be well-shielded from solvent.

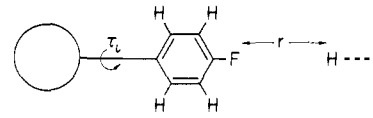


Figure 6. Representation of the model used in the relaxation analysis. The fluorophenyl ring is attached to a sphere which reorients isotropically with a correlation time τ_c.

Relaxation. Nuclear relaxation times can provide information about the time scale for molecular motions. Several determinations of fluorine spin–lattice relaxation times (T_1) and fluorine–proton nuclear Overhauser effects were made in the course of this work; the slow-exchange line widths used in the line-shape analyses provided an estimate of transverse relaxation rates (T_2). The data obtained are assembled in Table II.

To garner any appreciation of molecular dynamics from these relaxation data, it is necessary to assume a theoretical model for the system. As in previous work with the fluorophenyl ring,[34,35] we assumed that the fluorophenyl ring is attached to a sphere which tumbles isotropically with a correlation time τ_c (Figure 6). The ring is allowed to rotate about its symmetry axis freely in a motion describable by another correlation time, τ_i. The four protons of the fluorophenyl ring are explicitly included in the calculation while a single other proton at a distance r from the fluorine and on the C_1–C_4–F line was used to take into account relaxation due to other protons of the protein and solvent. Only proton–fluorine dipolar interactions and the contribution of fluorine chemical shift anistropy to relaxation were considered. Using the computational approach described previously,[34,35] the parameters τ_c, τ_i, and r were varied systematically until agreement between the observed relaxation parameters and those calculated at each frequency was optimized. Moderately good agreement between theoretical relaxation parameters and those observed experimentally for the native enzyme could be obtained (Table II) by using $\tau_c = 67 \pm 20$ ns, $\tau_i \cong 260$ ns, and $r = 0.2$ nm. For the denatured enzyme in 8 M urea, fewer data were available; the relaxation parameters at 94 MHz were consistent with $2 < \tau_c < 20$ ns and $2 < \tau_i < 10$ ns. There is ample evidence that the rotational correlation time of monomeric α-chymotrypsin is about 15 ns,[36,37] and the larger value suggested by the fluorine relaxation data obtained with bis(4-fluorophenyl)carbamoyl-α-chymotrypsin likely indicates that this protein is appreciably associated at the concentrations (1.1–1.4 mM) used in the present work. While a more detailed study of relaxation that takes into account the (likely) concentration dependence of the relaxation parameters and uses a more realistic model for local proton–fluorine interactions is desirable, it is clear qualitatively that the fluorophenyl rings of the carbamoyl group are highly immobilized in the enzyme derivative. Motion of these rings is significantly freer in the

(30) Muller, N. *J. Magn. Reson.* **1977**, *25*, 111–121.
(31) Lauterbur, P. C.; Kaufman, B. V.; Crawford, M. K. In "Biomolecular Structure and Function"; Agris, P. F. Sykes, B., Loeppky, R. Eds.; Academic Press: New York, 1978; pp 329–351.
(32) Hagen, D. S.; Weiner, J. H.; Sykes, B. D. *Biochemistry* **1979**, *18*, 2007–2012.
(33) Golovanov, I. B.; Gagloev, V. N.; Soboleva, I. A.; Smolyaninov, V. *J. Gen. Chem. USSR (Engl. Transl.)* **1973**, *43*, 642.
(34) Gerig, J. T.; Halley, B. A.; Ortiz, C. E. *J. Am. Chem. Soc.* **1977**, *99*, 6219–6226.
(35) Gerig, J. T.; Loehr, D. T. *Biopolymers* **1980**, *19*, 1827–1837.
(36) Kuznetsov, A. N.; Ebert, B.; Gyul Khandanyan, G. V. *Mol. Biol. (Moscow)* **1975**, *9*, 871.
(37) Vaz, W. L. C.; Schoellmann, G. *Biochim. Biophys. Acta* **1976**, *439*, 206.

denatured enzyme, possibly because protein association effects have been reduced in addition to disruption of protein tertiary structure.

Discussion

Solvolysis of Bis(4-fluorophenyl)carbamoyl Chloride. This acid chloride was observed to be highly stable to reaction with water and essentially unreactive toward methanol at room temperature. Under equivalent conditions of pH or solvent composition (methanol/water mixtures), the difluoro-substituted compound was one-sixth as reactive as the unsubstituted material, *N,N*-diphenylcarbamoyl chloride (M. Cairi, unpublished results). An S_N1-like mechanism has been advocated for diphenylcarbamoyl chloride solvolysis.[38] Our observations of the high sensitivity of the reaction to relatively small amounts of organic solvents (methanol) in the reaction mixture and the rate-retarding effect of fluorine substitution are consonant with this mechanism.

Temperature Dependence of Fluorine Shifts and Protein Structure. The changes in the chemical shifts of the two fluorine signals of native bis(4-fluorophenyl)carbamoyl-α-chymotrypsin are in the opposite directions as the temperature of the sample is increased. These changes are, therefore, unlikely due to some type of general medium effect, but rather reflect alterations in protein structure. Many experiments with chymotrypsin using spectrophotometric or chiroptical methods have been interpreted in terms of a possible transition between a low- and a high-temperature form of the enzyme with the transition taking place between 15 and 40 °C.[39-41] It has also been documented that the temperature dependence of many chymotryptic reactions is not simple,[42-45] and the nonlinear Arrhenius plots observed in these cases may signal a strongly temperature-sensitive protein structure.

Carbamoylation of α-chymotrypsin with *p*-nitrophenyl cyanate and octyl or butyl isocyanates occurs stoichiometrically at the serine-195 hydroxyl residue; the site of reaction was identified by crystallographic methods in the last two cases,[46,47] and by peptide mapping in the former.[12] In octylcarbamoyl-α-chymotrypsin, the octyl side chain was found exclusively within the hydrophobic substrate specificity pocket. If the diphenylcarbamate group in a model of *N,N*-diphenylcarbamoyl-chymotrypsin is oriented so as to match as closely as possible to the positions of the atoms in the octylcarbamoyl structure, one aromatic ring of the diphenylamino group extends into the same pocket while the other is located closer to the enzyme surface, proximal to the methionine-192 residue.

Derivatives of α-chymotrypsin that have *p*-fluorophenyl groups able to access the hydrophobic pocket usually exhibit large downfield fluorine shifts ranging from 4 to 7 ppm away from the positions of the same resonance observed when the protein is in a denatured state.[48] Recently, for example, Gorenstein and Shah have observed the fluorine shift of a fluorophenyl-containing transition state inhibitor of the enzyme at about 4 ppm to low field of the shift of the uncomplexed molecule.[49] It appears reasonable, therefore, to associate the low-field fluorine signal in the spectrum of the bis(4-fluorophenyl)carbamoylated enzyme with a fluorophenyl group resident in the substrate specificity pocket. The less-perturbed shift of the second resonance is consistent with a fluorophenyl ring being oriented away from close contacts with the protein surface. That there is no detectable H_2O/D_2O solvent effect on either chemical shift is consistent with the postulated position of the fluorophenyl ring in the solvent-poor hydrophobic pocket but suggests that in some way the second fluorophenyl ring is protected from strong interactions with the solvent.

Denaturation. If samples of *N,N*-bis(4-fluorophenyl)carbamoyl-chymotrypsin are maintained long enough at temperatures above 45 °C thermal denaturation takes place to a detectable extent, and a separate signal assigned to the (averaged) resonances of the unfolded enzyme is observed in the fluorine spectrum. Acylation of chymotrypsin with diphenylcarbamoyl chloride stabilizes the native-like conformation of the protein toward unfolding.[50,51] Since the unmodified enzyme is expected to be rapidly and nearly completely unfolded under these conditions,[52] our observations are consistent with operation of this stabilizing effect in the fluorine-substituted system.

Another indication of the stabilizing influence of the difluorophenylcarbamoyl group is the behavior of the protein in 8 M urea. In this medium, the native enzyme has a half-life appreciably less than 1 min[53] while we observed that the acylated enzyme used in this work required 12–24 h at room temperature for complete denaturation under these conditions. These results are in accord with previous reports that indoleacryloylchymotrypsin and various fluorocinnamoylchymotrypsins denature 2–3 orders of magnitude more slowly than the native enzyme in 8 M urea.[54,55]

An enhanced number of hydrophobic interactions between the aromatic rings of these acyl groups and the amino acids which define the specificity pocket of the enzyme presumably account for the increased kinetic stability toward denaturation of these acylenzymes. One can imagine a fairly tightly "locked" structure for the carbamoylated enzyme that is (1) resistant to denaturation, (2) strongly immobilizes the fluorophenyl rings, as indicated by the value of τ_c implied by the relaxation data, and (3) maintains those structural features that produce the characteristic fluorine chemical shift effect. Increasing temperature must "loosen" this structure for the chemical shift differences are reduced with increasing temperature.

Exchange Kinetics. In discussing the observed rotation kinetics in the carbamoylated enzyme, it is necessary to choose the appropriate reference system. Two such systems are available from the present work, the urethane II and denatured bis(4-fluorophenyl)carbamoyl-chymotrypsin. We find the rotation barrier ($\Delta G^{\ddagger}$) in the small molecule (II) to be about 14 kJ/mol smaller than the barrier found for the native enzyme while the rotation kinetics for the denatured carbamoylated enzyme are essentially indistinguishable within the limitations of our data from those of the native enzyme. A major difference between the two reference systems is the solvent. Free energy barriers to rotation in amides, urethanes, and similarly constituted molecules tend to be higher in aqueous compared to aprotic solvents, sometimes by as much as 19 kJ/mol.[56] Because of limited solubility and the solvent freezing points, the barrier in II could not be measured in highly aqueous media, but it appears reasonable to expect this barrier to be higher in this solvent than that observed in acetone (Table I). Thus, both reference systems probably have similar free energy barriers to carbamoyl rotation under comparable conditions.

The entropy of activation ($\Delta S^{\ddagger}$) appears to be larger for the protein systems than for II. While these positive $\Delta S^{\ddagger}$ values could be attributed to the influence of the protein matrix, interpreting the changes in this parameter must be approached cautiously for the value observed can be strongly influenced by systematic experimental errors. We have reported recently $\Delta S^{\ddagger}$ values for

(38) Johnson, S. L.; Giron, H. M. *J. Org. Chem.* **1972**, *37*, 1383.
(39) Havsteen, B.; Labouesse, B.; Hess, G. P. *J. Am. Chem. Soc.* **1963**, *85*, 796.
(40) Biltonen, R.; Lumry, R. *J. Am. Chem. Soc.* **1969**, *91*, 4251.
(41) Kim. Y. D.; Lumry, R. *J. Am. Chem. Soc.* **1971**, *93*, 1003.
(42) Wang, C. L. A.; Calvo, K. C.; Klapper, M. H. *Biochemistry* **1981**, *20*, 1401.
(43) Baggott, J. E.; Klapper, M. H. *Biochemistry* **1976**, *15*, 1473.
(44) Adams, P. A.; Swart, E. R. *Biochem. J.* **1977**, *161*, 83.
(45) Wedler, F. C.; Uretsky, L. S.; McLune, G.; Cenucla, J. *Arch. Biochem. Biophys.* **1975**, *170*, 476.
(46) Brown, W. E.; Wold, F. *Biochemistry* **1973**, *12*, 828.
(47) Brown, W. E.; Wold, F. *Biochemistry* **1973**, *12*, 835.
(48) Gerig, J. T. *Biol. Magn. Reson.* **1978**, *1*, 139.
(49) Gorenstein, D. G.; Shah, D. O. *Biochemistry* **1982**, *21*, 4679–4686.
(50) Lumry, R.; Biltonen, R. In "Structure and Stability of Biological Macromolecules"; Timasheff, S. N., Fasman, G. D., Eds.; Marrel Dekker: New York, 1969; p 140.
(51) Shiao, D.; Lumry, R.; Fahey, J. *J. Am. Chem. Soc.* **1971**, *93*, 2024–2035.
(52) Hollis, D. P.; McDonald, G.; Biltonon, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, 58, 758–765.
(53) Antonov, V. K.; Vorotyntseva, T. I. *FEBS Lett.* **1972**, *23*, 361–363.
(54) Gerig, J. T.; Halley, B. A. *Arch. Biochem. Biophys.* **1981**, *209*, 152–158.
(55) Rossi, G. L.; Bernhard, S. A. *J. Mol. Biol.* **1971**, *55*, 215–230.
(56) Jackman, L. M. In "Dynamic NMR Spectroscopy"; Jackman, L. M. Cotton, F. A., Eds.; Academic Press: New York, 1975; p 214.

rotation in a micellar urethane system in aqueous solution that are similar to those found for the protein systems here,[57] and we tend to believe that an appreciable positive $\Delta S^{\ddagger}$ parameter is simply characteristic of the rotation of this functional group within organized structures in water. With these considerations in mind, it is striking what little influence the native protein structure has on the rotation of the diphenylcarbamoyl group of the enzyme. Although the evidence discussed above suggests a rather highly ordered protein structure about the aromatic rings of this group, the rotation kinetics indicate that the group can easily become unencumbered of the enzyme and carry out rotational motion about the carbamyl carbon–nitrogen bond in essentially the same way as the denatured enzyme or the urethane model II. At least two easily accessible conformational states for the acylated enzyme are thereby suggested (eq 5). In one state (E_F), the diphenyl-

$$E_F \underset{}{\overset{\text{unfolding}}{\rightleftharpoons}} E_U \overset{\text{rotation}}{\rightleftharpoons} E_U \qquad (5)$$

carbamoyl group takes up the position in the structure suggested by the chemical shift data and is essentially immobilized. In a second state (E_U), the enzyme is locally unfolded so that rotation can take place easily; the chemical shifts and relaxation processes characteristic of this second state are likely different from those of the first. If the free energies of the two states, E_F and E_U, are different by ~4 kJ/mol and equilibrium between them is rapid, the (averaged) NMR parameters observed would largely be those of the first state. This free energy separation of the two states would add to the inherent barrier for rotation, but the increment is of the order of our experimental errors. In the denatured enzyme highly ordered states such as E_F would disappear, with the result that the large protein-induced chemical shift differences for the fluorophenyl rings would be greatly reduced. The diphenylcarbamoyl rotation process represented by the last part of eq 5 would take place at a rate largely defined by the local electronic structure of this group and should be similar for both native and denatured systems if structural constraints on this motion are removed.

The proposed conformation excursion E_F to E_U, or more elaborate schemes involving more conformational substates are compatible with notions of local mobility in protein structures that derive from a variety of experiments.[1,2] Our results allow us to put some rather broad limits on the time scale for these local motions in that the local unfolding must be more rapid than the time required for rotation but must be appreciably slower than the time required for molecular tumbling. Thus, the rate constant for overall unfolding must be between 2×10^2 s^{-1} and 2×10^7 s^{-1}.

Acknowledgment. This work was supported by Grant GM-25975 from the National Institutes of Health. The Southern California Regional NMR Facility was supported by the National Science Foundation (Grant CHE 79-16324A1), and the purchase of the 300-MHz instrument was aided by Grant CHE 80-018438 from the NSF. We tender our appreciation to these sponsors.

Registry No. II, 85710-94-7; *N,N*-bis(4-fluorophenyl)amine, 330-91-6; *p*-fluoroacetanilide, 351-83-7; *p*-bromofluorobenzene, 460-00-4; *N,N*-bis(4-fluorophenyl)carbamoyl chloride, 85710-93-6.

(57) Gerig, J. T.; Peyton, D. H.; Nicoli, D. *J. Am. Chem. Soc.* **1982**, *104*, 5034-5039.

Cyclohexanone Oxygenase: Stereochemistry, Enantioselectivity, and Regioselectivity of an Enzyme-Catalyzed Baeyer-Villiger Reaction[†1,2]

John M. Schwab,*[‡] Wu-bo Li,[‡] and Lawrence P. Thomas[§]

Contribution from the Department of Chemistry, The Catholic University of America, Washington, DC 20064, and New England Nuclear, Boston, Massachusetts 02118.
Received January 10, 1983

Abstract: Cyclohexanone oxygenase, from *Acinetobacter* NCIB 9871, has been incubated with (2*S*,6*S*)-[2,6-$^2H_2$]- and (2*R*)-[2-$^2H_1$]cyclohexanone. The resulting labeled ϵ-caprolactone (2-oxepanone) samples were degraded to 1-pentanol, which was esterified by using (−)-camphanyl chloride. Analysis of the camphanates by deuterium NMR spectroscopy, using Eu(dpm)$_3$, showed that the conversion of ketone to lactone had in each case proceeded with complete retention of configuration at the migrating carbon center. A similar degradation of (2*R*)-[2-$^2H_1$]cyclohexanone itself showed that reduction of [2-$^2H_1$]-cyclohex-2-enone by *Beauveria bassiana* ATCC 7159 is also completely stereoselective. A method has been developed for assessing the enantioselectivity of enzymes toward racemic substrate mixtures. (2*R*)-2-[*methyl*-$^2H_3$]- and (2*S*)-2-[*methyl*-^{13}C]methylcyclohexanone were synthesized and mixed in equal amounts, and the resulting mixture (a virtual racemate) was incubated with cyclohexanone oxygenase. The course of the reaction was followed by both ^{13}C and ^{2}H NMR spectroscopy, showing that the initial rate of oxidation of the 2*S* enantiomer was nearly twice that of the 2*R* enantiomer. (2*R*)- and (2*S*)-2-methylcyclohexanone were both converted by cyclohexanone oxygenase to 6-methyl-ϵ-caprolactone (7-methyl-2-oxepanone). Advantages of the virtual racemate/multinuclear NMR technique over existing methodology are described.

While the Baeyer-Villiger reaction[3] has been a standard tool of organic chemistry for the better part of a century, only in recent years has it become clear that nature makes widespread use of this reaction in biodegradative pathways.[4] Studies in several laboratories[4c-g,5] have revealed that Baeyer-Villiger enzymes are flavin-dependent monooxygenases, not requiring metal ions for

[†] Dedicated to Dr. Ulrich Weiss on the occasion of his 75th birthday.
[‡] The Catholic University of America.
[§] New England Nuclear.

(1) Presented in part at the 28th IUPAC Congress, Vancouver, Aug 1981, at the International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Kansas City, MO, June 1982, and at the 184th ACS National Meeting, Kansas City, MO, Sept 1982.

0002-7863/83/1505-4800$01.50/0