Deacylation of Fluorine-substituted *trans*-Cinnamoyl-α-chymotrypsins[1]

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Deacylation of unsubstituted and of *o*-F-, *m*-F, *p*-F, α-F, pentafluoro-, *p*-methyl-, and *p*-trifluoromethyl-substituted *trans*-cinnamoyl-α-chymotrypsins has been studied from p*H* 4 to 8. The deacylation rate constants were found to depend upon the ionization state of a group on the enzyme with an apparent p*K* in the range 6.3–7.3. The hydrolysis rates of the correspondingly substituted *p*-nitrophenylcinnamate esters were determined at p*H* 10.6. Correlation of the data for these model reactions with the corresponding rates of enzyme deacylation suggests that the *p*-methyl-substituted acylenzyme is about an order of magnitude more reactive than expected while *p*-trifluoromethyl substitution results in deacylation at a rate 10 times slower than expected. The remaining substituents exert about the anticipated rate effect on deacylation.

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On a étudié, entre les p*H* 4 et 8, la réaction de déacylation de *trans*-cinnamoyl-α-chymotrypsines non-substitués et substitués par des groupes *o*-F, *m*-F, α-F, pentafluoro, *p*-méthyle et *p*-trifluorométhyle. Les constantes de vitesse de déacylation dépendent de l'état d'ionisation d'un groupe de l'enzyme ayant un p*K* apparent entre 6.3 et 7.3. On a déterminé, à p*H* de 10.6, les vitesses d'hydrolyse des esters *p*-nitrophénylcinnamates substitués d'une façon correspondantes. La corrélation des données pour les réactions de ces modèles avec les vitesses des réactions de déacylation correspondantes catalysées par les enzyme suggère que l'acyle-enzyme substitué par un groupe *p*-méthyle est environ 10 fois plus réactif que l'on aurait pu l'attendre alors que la substitution par un groupe *p*-trifluorométhyle conduit à des résultats de déacylation qui sont environ 10 fois plus lent que prévu. Les autres substituants exercent sur la déacylation les effets anticipés. [Traduit par le journal]

Numerous studies have been carried out to elucidate the properties as well as the catalytic mechanism of the proteolytic enzyme, α-chymotrypsin (1–5). Equation 1 represents the minimal features of this mechanism in which enzyme (E) and substrate (S) first combine to form a Michaelis complex (ES). The substrate is pre-

$$[1] \quad E + S \rightleftarrows ES \rightarrow ES' \rightarrow EP_2 \rightleftarrows E + P_2 + P_1$$

sumed to consist of an alcohol, amine, or thiol moiety symbolized P_1 which is present as a derivative of a carboxylic acid (P_2). A large body of evidence points to the appearance of a covalent enzyme–substrate intermediate (ES′) along the reaction pathway, likely one in which the acyl group of the substrate is attached to serine-195 of the enzyme (6, 7). Generally, nonspecific substrates produce acylchymotrypsins that are relatively stable and, in several studies, these species have been directly observed or isolated (8–12). Acylenzymes have also been detected in the reactions of specific ester substrates but more rarely or under unusual conditions (13, 14). Hydrolysis of the intermediate enzyme–ester gives initially an enzyme–acid product complex (EP_2) which, upon dissociation, regenerates the native enzyme.

The goal of a program in our laboratory is to compare the structures of acylenzyme intermediates with the structures of the corresponding enzyme–P_2 complexes. Fluorine n.m.r. spectroscopy should be useful in such studies since fluorine chemical shift and relaxation time data can provide information about the local environment of the fluorine 'reporter' nuclei (15, 16).

The purpose of the present work was to determine the stabilities of various fluorine-substituted cinnamoyl-α-chymotrypsins in the hope that some of these acylenzymes would prove amenable to study by fluorine n.m.r.; experiments

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with the corresponding cinnamate–enzyme complexes are already underway (17). Previous kinetic investigations have shown that the electronic nature of the acyl group as well as its size are important in determining the rate of deacylation of the corresponding acylenzyme (11*a*, 18–20). In some cases unexpectedly large or small rates are observed, especially if fluorine substituents are present (20*b*). A second thrust of this work, then, was to compare the rates of deacylation of the fluorinated derivatives to their nonfluorinated analogs; such comparisons may give some indication of how representative of the nonfluorinated acylenzymes are the fluorine-containing ones.

Experimental

Materials

trans-Cinnamic acid (Matheson, Coleman and Bell), α-fluorocinnamic acid (Aldrich), and 4-methylcinnamic acid (Aldrich) were obtained commercially.

The other substituted cinnamic acids were prepared by heating at reflux the appropriate benzaldehyde (0.02 mol) and malonic acid (0.022 mol) in a solvent composed of pyridine (0.6 ml) and ethanol (0.4 ml). Upon heating, the malonic acid slowly dissolves; as the product forms, it precipitates from the solution until the mixture is pasty. At this point a few additional drops of ethanol can be added to maintain fluidity. Typical reaction times were 5–6 h. The cinnamic acids so produced were recrystallized from ethanol–water. Table 1 gives yields and melting points for these materials. Proton magnetic resonance spectra of these compounds uniformly showed a coupling constant between the vinyl protons of 15–17 Hz indicating a *trans* double bond.

The corresponding cinnamoyl chlorides were prepared by treating the appropriate cinnamic acid (0.03 mol) with thionyl chloride (0.11 mol, *ca.* 8 ml). After heating at reflux for 2 h and removal of excess thionyl chloride at atmospheric pressure, the products were distilled under vacuum. Table 1 records the boiling points and yields observed in these preparations.

p-Nitrophenylcinnamates were synthesized by dissolving the desired cinnamoyl chloride (0.006 mol) in dry benzene (25 ml) containing pyridine (1.2 ml). *p*-Nitrophenol (0.84 g, 0.006 mol) was added and the mixture heated at reflux for 1 h. Upon cooling the reaction mixture was washed five times with 5% potassium carbonate solution and then with water. (Addition of a small amount of ether was helpful when emulsion formation was a problem during these extractions.) After drying over anhydrous sodium sulfate, the solvent was stripped off *in vacuo* and the crystalline residue recrystallized from chloroform–cyclohexane. Data regarding yield, melting point, and microanalyses of these esters are given in Table 1.

The α-chymotrypsin used was a Worthington three-times crystallized product. Matheson spectroquality acetonitrile was used as co-solvent. Buffers were 0.05 *M* in buffer salts and contained 0.1 *M* KCl. Potassium phosphate was used at p*H* 6–8; at p*H* values lower than 6, acetate was used.

Kinetic Methods

Except for pentafluorocinnamoylchymotrypsin, the rates of deacylation of the various substituted cinnamoyl-enzymes were followed by monitoring the disappearance of the acylenzyme (12) at the wavelength indicated in Table 2 with a Beckman Model DUR spectrophotometer equipped with a Gilford 2000 recorder accessory. The temperature in the cell compartment was maintained at 30.2° by circulating thermostated water (±0.05°) through spacers on each side of the cell compartment. Stock solutions of the *p*-nitrophenyl esters at 0.54 m*M* were prepared

TABLE 1. Synthesis of substituted cinnamic acids and derivatives

					p-Nitrophenyl ester					
							Analysis[g]			
	Cinnamic acid		Cinnamoyl chloride				Calcd.		Found	
Substituent	Yield (%)	m.p. (lit.)	Yield (%)	b.p./mm Hg	Yield (%)[e]	m.p.	C	H	C	H
None	Commercial	132 —	94	87/0.1	68	145–146[f]	—		—	
o-F	88	178 (172[a])	88	84/0.2	70	154–155	62.72	3.51	62.39	3.12
m-F	95	161 (166[b])	39	84/0.05	52	149–150	62.72	3.51	62.42	3.04
p-F	78	208 (209[b])	90	90/0.15	53	148–149	62.72	3.51	62.51	3.16
α-F	Commercial	157 —	60	79/0.5	39	151–152	62.72	3.51	62.20	3.51
Pentafluoro	30	151 (153[c])	66	74/0.05	36	99–100	50.16	1.68	50.19	1.63
p-CH_3	Commercial	196 —	74	128/0.3	51	133–134	67.84	4.63	67.50	4.44
p-CF_3	88	229 (230[d])	82	90/0.1	55	110–111	56.98	2.99	57.53	2.84

[a]See ref. 30.
[b]See ref. 31.
[c]See ref. 32.
[d]See ref. 33.
[e]After three recrystallizations.
[f]Literature (34) m.p. 146°.
[g]Microanalyses by Chemalytics, Inc., Tempe, Arizona.

TABLE 2. Deacylation of substituted *trans*-cinnamoyl-α-chymotrypsins[a]

Substituent	Observed rate constant (k_ψ) × 10^5 s^{-1} (wavelength, nm)[b]				
	p*H* 7.99	p*H* 7.15	p*H* 6.13	p*H* 5.04	p*H* 4.13
None	1360 (310)	766 (305)	135 (304)	14.9 (304)	2.54 (305)
o-F	2170 (310)	1110 (306)	194 (306)	21.4 (306)	4.20 (307)
m-F	3660 (310)	1830 (303)	326 (303)	32.8 (303)	5.84 (303)
p-F	2450 (310)	1320 (307)	242 (306)	26.8 (306)	4.70 (307)
α-F	[c]	[c]	12100 (300)	1410 (300)	230 (300)
Pentafluoro	[c]	[c]	7220[d]	921[d]	[c]
p-CH_3	12300 (310)	5540 (310)	992 (310)	91.4 (310)	16.2 (315)
p-CF_3	436 (300)	265 (300)	64.4 (300)	7.75 (300)	2.25 (300)

[a]In water at 30.2°, 6.25% acetonitrile.
[b]Wavelength used to follow deacylation reaction by direct observation of acylenzyme.
[c]Could not be determined under these conditions.
[d]Dye-displacement technique.

in acetonitrile. An α-chymotrypsin stock solution at 1.37 m*M*, p*H* 3, was prepared assuming a molecular weight of 25 200 (21). An aliquot of the enzyme stock solution (0.4 ml) was diluted to 10 ml with the appropriate buffer solution; 3-ml aliquots of this solution were added to each cuvette, including the reference cell; 0.2 ml of acetonitrile was also added to the reference cell. The contents of the cells were allowed to reach thermal equilibrium at 30.2°. To initiate a reaction 0.2 ml of ester stock solution was added to a sample cuvette. The final reaction mixture thus had a total volume of 3.2 ml and was 0.034 m*M* in substrate, 0.051 m*M* in enzyme, and 6.25% in acetonitrile. Depending upon p*H* an initial change in absorbance due to acylation was noted followed by a slower exponential loss of absorbance as a result of deacylation.

Plots of ln ($A_\infty - A_t$), where A_∞ is the observed absorbance at infinite time and A_t is the observed absorbance at time *t*, *vs.* *t* were linear; least squares analysis of the data, using a program prepared for a Hewlett-Packard 9820A calculator, gave correlation coefficients in excess of 0.999. The slope of such a plot gives the apparent first-order rate constant, k_ψ, for deacylation.

Deacylation of the pentafluoro derivative was followed by a dye displacement technique (11*b*) using the dye Biebrich scarlet (22). Reaction conditions identical to those described above were used except that the dye was present in the reaction mixture and reference cell at a concentration of 5.96×10^{-5} *M*. With this technique no enzyme was added to the reference cuvette and the reappearance of the enzyme–dye complex as deacylation proceeded was followed at 550 nm.

The kinetics of base-catalyzed hydrolysis of the *p*-nitrophenyl esters were carried out as follows. Twenty milliliters of acetonitrile was diluted to 100 ml with 0.05 *M* potassium carbonate buffer. A 3-ml aliquot of this solution was placed in a cuvette and allowed to equilibrate at 30.2 °C. To start the reaction a 0.2-ml aliquot of the ester stock solution described above was added (because of decreased solubility, with the *p*-methyl compound 0.1 ml of stock solution and 0.1 ml of acetonitrile was used); an equivalent amount of acetonitrile was added to the reference cell. Total acetonitrile in the reaction system was 25%. The evolution of *p*-nitrophenol was followed at 400 nm and was strictly first order. The measured p*H* of the reaction medium was 10.59 and the pseudo first-order rate constants obtained were thus divided by $[OH^-] = 3.89 \times 10^{-4}$ *M* to obtain the second-order rate constant, k_{OH}, for hydroxide reaction.

Results

The kinetics of deacylation of *o*-fluoro-, *m*-fluoro-, *p*-fluoro-, α-fluoro-, *p*-methyl-, *p*-trifluoromethyl-substituted and unsubstituted cinnamoyl-α-chymotrypsins have been studied by direct spectrophotometric observation over the p*H* range 4–8. The α-fluoro derivative was examined only from p*H* 4 to 6 because the reaction is too rapid at higher p*H* values to be followed by the present techniques. An appropriate wavelength at which to observe the pentafluoro-substituted cinnamoylenzyme could not be found and a dye displacement method was used in this case. Deacylation in this system was also too rapid to be measured above p*H* 6 and at p*H* values below 5 the dye displacement technique was not usable because the dye precipitates from solution under these conditions. The results of these kinetic experiments are assembled in Table 2.

As expected, the deacylation reactions with all groups were found to be p*H* dependent. A mechanism of the form shown in eq. 2 was assumed where EH^+A and EA represent forms of the acylenzyme in which a critical group is proton-

$$\begin{array}{ccc} EA & \xrightarrow{k_3} & E + A \\ K \Big\| & & \\ EH^+A & \xrightarrow{k_3'} & EH^+ + A \end{array} \qquad [2]$$

ated or not protonated, respectively. For this mechanism, the observed first-order rate constant k_ψ is given by eq. 3*a*.

$$[3a] \qquad k_\psi = \frac{k_3 + \frac{k_3'[H^+]}{K}}{1 + \frac{[H^+]}{K}}$$

$$[3b] \qquad k_\psi = k_3 + \frac{k_3'}{K}[H^+] - \frac{k_\psi[H^+]}{K}$$

The available rate data were used to calculate the parameters k_3, k_3', and K which characterize the assumed mechanism by a least-squares technique using the rearranged form, eq. 3*b*. With the pentafluoro-substituted acylenzyme, data at only two pH values were available. In this case, k_3' was assumed to be zero in order to obtain an estimate of the equilibrium constant K and the first-order rate constant for deacylation, k_3. Table 3 presents the parameters found to describe the reaction of each cinnamoylchymotrypsin; these values for k_3, k_3', and K reproduce the experimentally observed rate constants well, usually with an accuracy of 5% or better. It should be noted that the accuracy of k_3' depends critically upon the accuracy of K (*cf.* eq. 3*b*).

The rates of hydroxide ion catalyzed hydrolysis of the *p*-nitrophenyl esters of the cinnamic acids used in this work were determined. At pH 10.59, these reactions are pseudo first-order with hydroxide ion in large excess concentration over the ester substrate. The second-order rate constants obtained from these experiments are recorded in Table 3.

Discussion

An unexpected aspect of our results in this work is the apparently substantial contribution reaction of the protonated form of the enzyme (EH^+A in eq. 2) makes to the overall deacylation process. While the rate constant, k_3', for this step is very small relative to k_3, at pH values below 6 this pathway for deacylation appears to generate an appreciable fraction of the total reaction observed. Although others have included steps analogous to the one defined by k_3' in eq. 2 in discussions of the chymotrypsin mechanism (23) we are unaware of estimates for the relative magnitudes of k_3 and k_3' from previous work. Rather, the usual assumption appears to be that $k_3' = 0$. Our results suggest that this assumption should be made with caution. However, it is possible that the complete mechanism for deacylation under our conditions is inadequately represented by eq. 1 and that additional terms perhaps involving the buffer components or other ionization constants may become necessary in a more refined description of this reaction.

The rate constants k_3 and k_3' for deacylation define steps in the mechanism that are essentially ester hydrolyses. A linear free energy relation between k_3, for example, and the rate constant for attack of hydroxide on a corresponding simple ester analog of the acylenzyme is expected and has been observed in a number of cases (18, 19). Substantial deviations from the regression line in these correlations can be taken to indicate interactions between an acyl group and the protein structure which significantly affect the enzyme reaction; previous work has shown that remote fluorine substitution can have an accelerating, a decelerating or no effect on the hydrolysis of acylchymotrypsins (20*b*).

Figure 1 shows a plot of the logarithm of k_3 for substituted cinnamoylchymotrypsin deacylation *vs.* the logarithm of k_{OH}, the rate constant for basic hydrolysis of the corresponding *p*-nitro-

TABLE 3. pH independent rate parameters for deacylation of substituted cinnamoylchymotrypsins[a]

Substitutent	pK	$k_3' \times 10^5$ s^{-1}	$k_3 \times 10^2$ s^{-1}	k_{OH}[b] l mol^{-1} s^{-1}
None	7.09	0.94	1.48	0.87
o-F	7.15	2.0	2.36	1.42
m-F	7.18	2.3	4.05	1.59
p-F	7.09	1.9	2.61	0.90
α-F	6.50	59	40.1	50.4
Pentafluoro	~6.3[c]	—	~18[c]	8.15
p-CH_3	7.23	5.4	13.5	0.57
p-CF_3	6.90	1.5	0.45	2.47

[a]At 30.2°, 6.25% acetonitrile.
[b]At 30.2°, 25% acetonitrile.
[c]Estimates based upon the reasonable assumption that k_3' is negligibly small.

phenylcinnamate esters. Excepting the data points for the methyl and trifluoromethyl derivatives, a reasonably linear relationship is found. This indicates that the steric and electronic effects of the other various fluorine substituents on the cinnamoyl group are being felt in both types of ester hydrolyses to about the same extent. The kinetics of deacylation of the various monofluorine substituted and the pentafluorinated cinnamoylchymotrypsins thus appear to be 'normal' by this criterion.

Placement at the *para* position of the cinnamoyl group of a methyl or trifluoromethyl group has unexpected kinetic consequences. Using the correlation shown in Fig. 1 as a guide, it appears that the *p*-methyl compound deacylates about a factor of 10 more rapidly than anticipated on the basis of the model reaction while the *p*-trifluoromethyl derivative reacts about a factor of 10 more slowly than expected. These results may be related in some way to the prior observation that a *p*-trimethylammonium substituent greatly retards the rate of deacylation (24), although there is no evidence that the acyl groups of these three *para*-substituted cinnamoylchymotrypsins are in similar environments at the enzyme active site.

A plot of the logarithm of k_3' against the logarithm of k_{OH} gives a plot similar to Fig. 1. The points for the *p*-methyl- and *p*-trifluoromethyl-substituted compounds again lie off the correlation line but the deviations are not so large as those in Fig. 1.

p*H*-reactivity studies of the action of α-chymotrypsin have repeatedly implicated a group on the enzyme with a p*K* of approximately 7 in the catalytic mechanism (4, 23). This group, which controls the rate of deacylation, has usually been assigned to histidine-57 (25, 26) although more recent evidence indicates that the buried carboxyl group of aspartate-102 may be this group (27). Interestingly, the apparent p*K* of the group involved in enzyme deacylation (determined kinetically) changes with the structure of the acyl group (11, 18, 23, 29). Bernhard *et al.* have indicated, for a variety of acyl groups, that this p*K* is linearly related to the p*K* of the corresponding free acid: the more acidic is the free acid, the lower is the p*K* observed in the deacylation reaction of the corresponding acylenzyme (11). Although we do not have available p*K* data for the acids employed in this study under our experimental conditions, the data in Table 3 are qualitatively consistent with the observations of

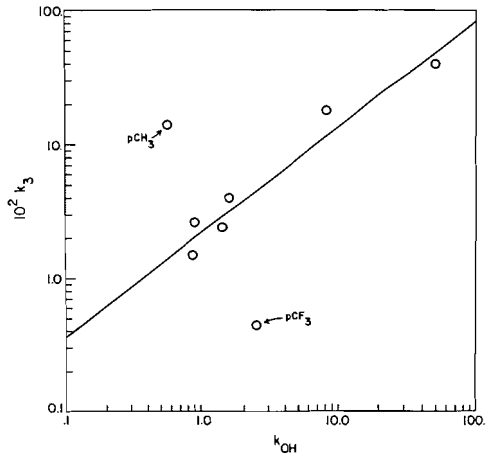

FIG. 1. Correlation of the rate of basic hydrolyses of various cinnamate *p*-nitrophenyl esters (k_{OH}) with the rate of deacylation of the corresponding acylchymotrypsin (k_3) (data from Table 3).

the Bernhard group. The pentafluoro- and α-fluorocinnamic acids are expected to be the most acidic of the group studied here because of the inductive effects of the fluorine atoms; in each of these cases the observed p*K* which determines the kinetics of deacylation of the corresponding acylenzyme is low. Similarly, the observed p*K*'s for the remaining cinnamoyl systems correlate reasonably well with Hammett's σ parameters.

There is little indication from this work that single substitution of fluorine atoms on the carbons of the double bond or the aromatic ring of the cinnamoyl group produce any special enzyme–cinnamoyl interaction that has kinetic consequences. In these cases, except for their electronic effect, fluorine atoms appear to be able to masquerade as hydrogen atoms. Substituents at the *para* position more bulky than a single fluorine do have an unexplained influence on the rate of deacylation; a methyl group in this location has an accelerating effect but if the hydrogen atoms of the methyl group are replaced by fluorine atoms, a substantial slowing of the deacylation reaction results. It seems likely, therefore, that contacts between the enzyme and the aromatic end of the cinnamoyl group are sufficient to significantly modify the positions of the atoms at the site of reaction for these cases. The abnormally low reactivity of the *p*-trifluoromethyl cinnamoyl enzyme warns that spectroscopic investigations of this protein by u.v. or magnetic resonance techniques may

not provide data that are particularly relevant to the nature of the other acylenzymes examined in this work. We hope that fluorine n.m.r. experiments in progress will illuminate this point.

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