The Stereospecificity of α -Chymotrypsin

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1. The rates of deacylation of acyl- α -chymotrypsins in which the hydrogen-bonding capacity of the acylamino group of the substrate has been systematically removed were measured. 2. The ratio of deacylation rates of L- and D-acyl-enzymes is found to depend largely on the existence in the substrate of an amido-NH- group. 3. The data presented agree with the postulate that the stereospecificity of α -chymotrypsin is exercised in catalytic rather than binding steps, and that the active site of the enzyme presents three loci to the substrate: the site containing the catalytic functionalities (including serine-195), the hydrophobic area for amino acid side-chain binding, and a hydrogen-bond acceptor site for acylamino group binding. 4. It is noted that, though the hydrogen-bonding site is crucial for the stereospecificity, the free energy of binding of substrates and inhibitors is dominated by the hydrophobic interaction. 5. It is tentatively proposed that α -chymotrypsin selects a high-energy conformation of the substrate when the latter binds at the enzyme's active site.

In an earlier paper (Ingles & Knowles, 1967) we proposed a model to account for the specificity and stereospecificity shown by α -chymotrypsin towards simple substrates with a single asymmetric centre. From this work it is apparent that (a) D-acyl-enzymes deacylate more slowly than L-acyl-enzymes, (b) the more hydrophobic the amino acid side chain the faster the L-acyl-enzymes deacylate and the slower the D-acyl-enzymes deacylate, and (c) there is a realizable limit to kinetic specificity (i.e. more effective side-chain binding does not ultimately result in a proportionately higher rate constant). These data were accommodated most simply in terms of a three-locus model of the enzyme's active site, in which the susceptible carbonyl group of the acyl-enzyme is oriented more or less favourably with respect to the catalytic functionalities of the enzyme. It was proposed that this orientation arises by virtue of (i) the anchor of the covalent ester link to serine-195 (Hartley, 1964), (ii) a hydrophobic interaction between the side chain of the amino acid comprising the acyl group and a corresponding non-polar region in the active site, and (iii) some interaction between the acylamino part of the acyl group and the enzyme. For effective discrimination between enantiomers containing a single asymmetric centre, at least three points of attachment are, of course, required.

There is now no doubt about the nature of the first two interactions mentioned above. Abundant evidence exists indicating that the acyl-enzyme intermediates are indeed esters of serine-195

(Oosterbaan, van Adrichem & Cohen, 1962; Shalitin & Brown, 1966). Secondly, the quantitative correlation between, for instance, the partitioning of competitive inhibitors (with various side chains) between water and chymotrypsin and the partitioning of analogous model compounds between water and organic solvent (see Knowles, 1965) leaves no doubt that a hydrophobic interaction of the amino acid side chain makes an important contribution to the free energy of substrate binding. However, no unequivocal evidence bears on the third interaction, that involving the acylamino group of the substrate. In our earlier paper an important role was attributed to the interaction of this group with the enzyme, the interaction being required to account for the stereospecificity of the deacylation step. The very nature of acylamino groups suggests that this interaction involves a hydrogen bond (or bonds). To confirm this point the hydrolysis catalysed by α -chymotrypsin of a number of analogues of *N*-acetylphenylalanine *p*-nitrophenyl ester, in which hydrogen-bonding potential has been systematically removed, was studied.

MATERIALS AND METHODS

All melting points quoted are corrected. Microanalyses were performed by Dr Weiler and Dr Strauss in this Department. Nuclear-magnetic-resonance (n.m.r.) spectra were measured on Perkin-Elmer R10 and R14 instruments. Measurements of optical rotation were carried out with a Perkin-Elmer P-E 141 polarimeter.

α -Chymotrypsin. This was a salt-free freeze-dried powder from three-times crystallized α -chymotrypsin purchased from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. The titration of α -chymotrypsin by the method of Schonbaum, Zerner & Bender (1961) with *N*-cinnamoyl-imidazole was used to obtain the operational normality of the enzyme solutions. The enzyme preparations used in this work were found to be 82–87% active on a weight basis, assuming a molecular weight of 25000.

Amino acids. The D-enantiomers of tryptophan, phenylalanine and leucine were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Acetonitrile. This was purified by the method of Cooper & Waters (1964).

Chloroform. This was freed from ethanol by shaking with two portions of conc. H_2SO_4 . The chloroform layer was washed several times with dilute NaHCO_3 solution and thoroughly with water and then dried over anhydrous MgSO_4 .

Buffers. All buffers were made up with A.R.-grade reagents and deionized water. pH measurements were made on a Radiometer instrument TTT1c with scale-expander PHA 630, standardized against standard buffer solutions (British Drug Houses Ltd., Poole, Dorset).

***p*-Nitrophenyl β -phenylpropionate.** β -Phenylpropionic acid (2.0g., 1mol.prop.) was dissolved in 50ml. of ethyl acetate and the solution cooled to 0°. *p*-Nitrophenol (2.22g., 1.25mol.prop.) and *NN'*-dicyclohexylcarbodi-imide (3.0g., 1.1mol.prop.) were added and the mixture was stirred at 0° for 2hr. and at room temperature for 20hr. The *NN'*-dicyclohexylurea precipitate was filtered off and washed with ethyl acetate. The combined ethyl acetate filtrates were washed with 1% NaHCO_3 solution and water and then dried over anhydrous MgSO_4 . The solvent was distilled off under reduced pressure to leave a bright-yellow powder. This was recrystallized twice from ethyl acetate–light petroleum (b.p. 40–60°) to give very pale-yellow crystals (2.88g., 80% yield) of the *p*-nitrophenyl ester, having m.p. 99–100° [McDonald & Balls (1957) give m.p. 97–98°]. (Found: C, 65.6; H, 5.0; N, 5.4. Calc. for $\text{C}_{15}\text{H}_{13}\text{NO}_4$: C, 66.3; H, 4.8; N, 5.2%).

***L*- β -Phenyl-lactic acid.** *L*-Phenylalanine was deaminated by the method of Dakin & Dudley (1914) to give *L*- β -phenyl-lactic acid (41% yield) as white needles from chloroform–light petroleum (b.p. 40–60°), having m.p. 124–125° and $[\alpha]_D^{20} - 27.5^\circ$ (c 1 in acetone) [Cohen & Weinstein (1964) give m.p. 126–127° and $[\alpha]_D^{25} - 27.8^\circ$ (c 3.78 in acetone)]. (Found: C, 64.8; H, 6.1. Calc. for $\text{C}_9\text{H}_9\text{O}_3$: C, 65.0; H, 6.0%).

***D*- β -Phenyl-lactic acid.** This was prepared analogously as described above for the *L*-enantiomer. Recrystallization from chloroform–light petroleum (b.p. 40–60°) gave white needles, m.p. 124–125.5° and $[\alpha]_D^{20} + 28.1^\circ$ (c 1 in acetone) [Cohen & Weinstein (1964) give m.p. 126–127° and $[\alpha]_D^{25} + 32.8^\circ$ (c 3.76 in acetone)] (Found: C, 65.2; H, 6.0. Calc. for $\text{C}_9\text{H}_9\text{O}_3$: C, 65.0; H, 6.0%).

***p*-Nitrophenyl *O*-acetyl-*L*- β -phenyl-lactate.** *L*- β -Phenyl-lactic acid (2.0g.) dissolved in 5ml. (excess) of acetic anhydride was heated on a boiling-water bath for 3hr. The mixture was cooled and diluted with water (100ml.). Solid Na_2CO_3 was added until the evolution of CO_2 ceased and the pH of the solution was above 8. The alkaline solution was extracted with ether and the aqueous layer acidified to Congo red with conc. HCl . The acid solution was then extracted with ether, and the ether extracts were dried over

anhydrous MgSO_4 and evaporated under reduced pressure to give 1.84g. (73%) of an almost colourless oil.

A solution of this oil (1.84g.) in ethyl acetate (50ml.) was cooled to 0°. *p*-Nitrophenol (1.23g.) and *NN'*-dicyclohexylcarbodi-imide (2.0g.) were added and the solution was stirred at 0° for 2hr. and then overnight at room temperature. The precipitate of *NN'*-dicyclohexylurea was filtered off and washed with ethyl acetate. The combined filtrates were dried over anhydrous MgSO_4 and evaporated under reduced pressure to leave a yellow oil. Excess of *p*-nitrophenol was removed by chromatography on a column of silica gel with ethanol-free chloroform as eluent. Two crystallizations from light petroleum (b.p. 60–80°) gave the *p*-nitrophenyl ester as white needles, m.p. 49–51° and $[\alpha]_D^{20} - 10.6^\circ$ (c 1 in chloroform) (Found: C, 62.6; H, 4.9; N, 4.4. $\text{C}_{17}\text{H}_{15}\text{NO}_6$ requires: C, 62.0; H, 4.6; N, 4.3%). The n.m.r. spectrum (in CDCl_3) confirmed the structure: acetyl CH_3 (singlet, τ 7.84), benzylic CH_2 (doublet, τ 6.72, *J* 6.5cyc./sec.) asymmetric CH (triplet, τ 4.63), aromatic protons *ortho* to NO_2 (doublet, τ 1.79, *J* 9cyc./sec.), aromatic protons *meta* to NO_2 (doublet, τ 2.94, *J* 9cyc./sec.) and aromatic protons of benzyl group (singlet, τ 2.69).

***p*-Nitrophenyl *O*-acetyl-*D*- β -phenyl-lactate.** This ester was prepared as outlined above for the *L*-enantiomer. Recrystallization from light petroleum (b.p. 60–80°) gave white needles, m.p. 50–52.5° and $[\alpha]_D^{20} + 9.4^\circ$ (c 1 in chloroform) (Found: C, 62.0; H, 4.3; N, 5.0. $\text{C}_{17}\text{H}_{15}\text{NO}_6$ requires: C, 62.0; H, 4.6; N, 4.3%).

β -Phenylpropionamide. Methyl β -phenylpropionate (prepared by the procedure of Curtius & Goebel, 1888) was converted into the amide by treatment with a saturated solution of dry NH_3 in methanol. The product (78%) had m.p. 97–100° after recrystallization four times from chloroform–light petroleum (b.p. 40–60°) [Huang & Niemann (1952) report m.p. 105–106°].

***O*-Acetyl-*D*- β -phenyl-lactamide.** *D*- β -Phenyl-lactic acid (2.0g.) was esterified by the procedure of Curtius & Goebel (1888) to give 1.92g. (82%) of the ethyl ester, m.p. 44–47° [Cohen & Weinstein (1964) give m.p. 46–47°]. A solution of ethyl *D*- β -phenyl-lactate (0.92g.) in dry ethanol (40ml.) was cooled to 0°, saturated with dry NH_3 and kept at 0° for 3 days. On removal of the solvent under reduced pressure, a pale-yellow solid was deposited. Recrystallization from ethyl acetate–light petroleum (b.p. 40–60°) yielded white crystals (0.445g., 57% yield), m.p. 110–113°. *D*- β -Phenyl-lactamide (0.445g.) was heated on a water bath with freshly distilled acetic anhydride (5ml.) for 5hr. After this solution had been cooled, 50ml. of water was added, followed by solid Na_2CO_3 until the evolution of CO_2 had ceased. The mixture was extracted with ethyl acetate, and the combined ethyl acetate extracts were washed twice with water and dried over anhydrous MgSO_4 . Removal of the solvent under reduced pressure yielded a pale-yellow oil. Purification by chromatography on silica gel gave an almost colourless oil, having $[\alpha]_D^{20} - 9.6^\circ$ (c 0.5 in chloroform), which could not be crystallized. This material gave only one spot on thin-layer chromatography (baked Kieselgel G plates eluted with ethyl acetate). The n.m.r. spectrum (in CDCl_3) confirmed the structure: acetyl CH_3 (singlet, τ 7.95), benzylic CH_2 (doublet, τ 6.85), asymmetric CH (triplet, τ 4.66), aromatic protons (singlet, τ 2.79) and amide NH_2 (broad singlet, τ 3.95) (Found: C, 63.8; H, 6.5; N, 6.7. $\text{C}_{11}\text{H}_{13}\text{NO}_3$ requires C, 63.8; H, 6.3; N, 6.8%).

Kinetic measurements. All kinetic measurements were

made as described previously (Ingles & Knowles, 1967). Reactions were carried out at 25° in aqueous 1.6% (v/v) acetonitrile solution.

RESULTS

Non-enzymic hydrolysis of the substrates. The second-order rate constants for the OH⁻ ion-promoted hydrolysis of the series of *p*-nitrophenyl esters were calculated from the slopes of plots of the pseudo-first-order rate constants ($k_{\text{obs.}}$) versus [OH⁻] and are listed in Table 1.

No specific buffer catalysis was observed for the runs performed in triethylamine buffers. Linear plots of $k_{\text{obs.}}$ versus [OH⁻] were obtained with intercepts close to zero on the $k_{\text{obs.}}$ axis. (Preliminary work on *p*-nitrophenyl β -phenylpropionate in a 0.1 M-carbonate-bicarbonate buffer system led to a markedly curved plot symptomatic of specific buffer catalysis. The 'limiting' slope, at high pH, gave a value for k_{OH^-} of 0.6 M⁻¹sec.⁻¹, in good agreement with the value obtained in triethylamine buffers.) Unfortunately, the reactivity of *p*-nitrophenyl *O*-acetyl β -phenyl-lactate does not permit the employment of the triethylamine buffer system for this substrate. The use of 0.2 M-collidine buffers (collidine being a tertiary amine of lower pK_a) gives a noticeably curved plot and the k_{OH^-} value quoted in Table 1 was obtained from a plot employing 0.05 M-tris-hydrochloric acid buffers. Above pH 8 this plot was reasonably linear (cf. Ingles & Knowles, 1967).

α -Chymotrypsin-catalysed hydrolysis of the substrates. The deacylation rate constants, k_{+3} and Michaelis constants, K_m (where determined), for the α -chymotrypsin-catalysed hydrolysis of the

series of *p*-nitrophenyl esters under investigation are shown in Table 2. The difficulties experienced with certain of the *N*-acetyl-amino acid *p*-nitrophenyl esters, where k_{+2} was not sufficiently greater than k_{+3} to give a clean 'burst' (Ingles & Knowles, 1967), were not encountered here. For the *p*-nitrophenyl esters of β -phenylpropionic acid and *O*-acetyl-D- β -phenyl-lactic acid, the observed bursts were in good agreement with the values calculated from enzyme concentrations. For all the substrates listed in Table 2, therefore, $k_{+2} \gg k_{+3}$. This means that k_{+3} can be determined either as k_0 from a Lineweaver-Burk plot where this is possible, or from the reaction velocity under saturation conditions (i.e. when $K_m \ll [S_0] \gg [E_0]$, $v = k_{+3}[E_0]$).

For *p*-nitrophenyl *O*-acetyl-L- β -phenyl-lactate the kinetic parameters quoted in Table 2 were obtained from the Lineweaver-Burk plot shown in Fig. 1. Non-enzymic hydrolysis of the ester was small (less than 3% of the enzymic rate), though the necessary corrections were applied. The deacylation rate constant for *p*-nitrophenyl *O*-acetyl-D- β -phenyl-lactate was obtained from measurements under saturation conditions. The non-enzymic hydrolysis of the substrate was negligible (less than 1% of the enzymic rate).

The deacylation rate constant for the *p*-nitrophenyl ester of β -phenylpropionic acid was also obtained under saturation conditions. The non-enzymic rate of hydrolysis was negligible. Over the concentration range studied the velocity of the reaction v_0 was almost independent of $[S_0]$. Thus at $[S_0] = 4.54 \times 10^{-5}$ M, $v_0 = 1.07 \times 10^{-7}$ M sec.⁻¹ (four experiments), at $[S_0] = 3.02 \times 10^{-5}$ M, $v_0 = 1.01 \times 10^{-7}$ M sec.⁻¹ (two experiments), and at $[S_0] = 1.08 \times$

Table 1. Rate constants for the OH⁻ ion-promoted hydrolysis of a series of *p*-nitrophenyl esters at 25° in 1.6% (v/v) acetonitrile

<i>p</i> -Nitrophenyl ester	pH range	Buffer type	10 ⁵ × Concn. of ester (M)	k_{OH^-} (M ⁻¹ sec. ⁻¹)
<i>N</i> -Acetylphenylalanine	6.94–8.41	0.1 M-Sodium phosphate and 0.05 M-tris-HCl	4.23	7400
<i>O</i> -Acetyl- β -phenyl-lactate	8.25–8.93	0.05 M-Tris-HCl	0.528	1920
β -Phenylpropionate	9.57–10.34	0.05 M-Triethylamine-HCl	1.08	22.3

Table 2. α -Chymotrypsin-catalysed hydrolysis of *p*-nitrophenyl esters of analogues of *N*-acetylphenylalanine

The reactions were carried out at 25° in 0.1 *I* phosphate buffer, pH 7.00 ± 0.02, in 1.6% (v/v) acetonitrile.

Acyl group	$[S_0]$ range (M)	$[E_0]$ (M)	k_{+3} (sec. ⁻¹)	K_m (M)
<i>N</i> -Acetyl-L-phenylalanyl	5.0×10^{-7} – 1.06×10^{-5}	6.67×10^{-9}	94.8	4.8×10^{-6}
<i>N</i> -Acetyl-D-phenylalanyl	6.88×10^{-5} – 2.63×10^{-4}	9.27×10^{-6}	0.0148	—
<i>O</i> -Acetyl-L- β -phenyl-lactyl	3.54×10^{-6} – 4.47×10^{-5}	4.90×10^{-8}	9.2	6.1×10^{-6}
<i>O</i> -Acetyl-D- β -phenyl-lactyl	1.99×10^{-5} – 3.98×10^{-5}	1.88×10^{-6}	1.03	—
β -Phenylpropionyl	1.08×10^{-5} – 4.54×10^{-5}	1.45×10^{-6}	0.074	—

Fig. 1. Lineweaver-Burk plot for the α -chymotrypsin-catalysed hydrolysis of *O*-acetyl-L- β -phenyl-lactate *p*-nitrophenyl ester. See Table 2 for experimental details.

Table 3. Inhibition constants for a series of amide analogues of *N*-acetyl-D-phenylalanine amide

The experiments were carried out at 25° in 0.1 *I* phosphate buffer, pH 7.00 $\pm$ 0.02, in 1.6% (v/v) acetonitrile with *N*-acetyl-L-leucine *p*-nitrophenyl ester as the substrate.

Inhibitor	[I ₀] (mM)	K _i (mM)
<i>N</i> -Acetyl-D-phenylalanine amide	21.5	10.6*
<i>O</i> -Acetyl-D- β -phenyl-lactamide	11.9	8.9†‡
β -Phenylpropionamide	23.6	4.55†

*Ingles & Knowles (1967).

†Owing to shortage of material this value is less accurate than the others.

‡This work.

10^{-5} M, $v_0 = 1.00 \times 10^{-7}$ Msec.⁻¹ (two experiments). Values of the kinetic parameters $k_{+3} = 0.0178$ sec.⁻¹ and $K_m = 2.3 \times 10^{-6}$ M [67 mM-phosphate buffer, pH 8.11, in 15% (v/v) acetone at 25°] have been reported for *p*-nitrophenyl β -phenylpropionate (Kézdy, Feder & Bender, 1967).

Inhibition of the α-chymotrypsin-catalysed hydrolysis of N-acetyl-L-leucine p-nitrophenyl ester by a series of analogues of N-acetyl-D-phenylalanine amide. The inhibition constants, K_i , for the amides of β -phenylpropionic acid and *O*-acetyl-D- β -phenyl-lactic acid were determined against *N*-acetyl-L-leucine *p*-nitrophenyl ester as the substrate and are listed in Table 3. The Lineweaver-Burk plots are shown in Fig. 2. An inhibition constant of 7 mM has been reported for β -phenylpropionamide (Foster & Niemann, 1955).

Fig. 2. Lineweaver-Burk plots for the α -chymotrypsin-catalysed hydrolysis of *N*-acetyl-L-leucine *p*-nitrophenyl ester: Δ , uninhibited; $\bullet$, in the presence of *O*-acetyl-D- β -phenyl-lactamide; $\circ$, in the presence of β -phenylpropionamide. For conditions see Table 3.

DISCUSSION

As in the earlier work, the choice of substrates was dictated by the requirements that deacylation be the rate-determining step and that each enantiomer be hydrolysed at a measurable rate. The substrates employed were the *p*-nitrophenyl esters of (a) *N*-acetyl-D- and -L-phenylalanine, (b) *O*-acetyl-D- and -L- β -phenyl-lactic acid and (c) β -phenylpropionic acid.

The deacylation rate constants for the series of substrates in Table 2 cannot be normalized for inherent reactivity differences by a procedure similar to that employed earlier (Ingles & Knowles, 1967). The mechanism of non-enzymic hydrolysis must change down the series since the cyclic intermediate formed in the OH⁻ ion-promoted hydrolysis of *N*-acetylphenylalanine *p*-nitrophenyl ester (Goodman & Stueben, 1962; Jersey, Kortt & Zerner, 1966) is not formed by the *p*-nitrophenyl esters of β -phenylpropionic acid and *O*-acetyl- β -phenyl-lactic acid. The use of *N*-benzyloxycarbonyl derivatives for the normalization of those substrates whose non-enzymic hydrolysis involves a cyclic intermediate cannot be applied here, since further electronic effects are introduced the magnitude of which cannot be predicted. For this reason the

results of Table 2 are best considered in terms of the stereospecificity ratios (k_{+3}^L/k_{+3}^D), which do not require normalized rate constants.

This ratio for the *N*-acetylphenylalanyl-chymotrypsins is 6400 and that for the *O*-acetyl- β -phenyllactyl-chymotrypsins is 9. This striking fall in stereospecificity accompanying the replacement of the amido -NH- group by an oxygen atom suggests that an important hydrogen-bonding interaction between the amido -NH- group and an enzymic group has been eliminated. The removal of the second hydrogen-bonding group, the amido -CO- group (in the β -phenylpropionyl-enzyme), results in a much smaller fall in stereospecificity (from 9 to 1). These results therefore suggest that the third

interaction responsible for the correct orientation of the susceptible carbonyl group in the deacylation reaction is a hydrogen bond between the -NH- of the substrate acylamino group and an unknown hydrogen-bond acceptor group in the active site of the enzyme. This conclusion is supported by the following data.

(a) Kaufman & Neurath (1949) showed that *N*-phthaloyl-DL-phenylalanine methyl ester, which possesses no amido hydrogen atom but retains the amido carbonyl group, is not measurably hydrolysed in the presence of α -chymotrypsin.

(b) Cohen and his co-workers have studied the effect of modification of the acylamino side chains in three different series of substrates having the

Table 4. *Effect of some substrate structure changes on specificity and stereospecificity*

References: *a*, Hammond & Gutfreund (1955); *b*, Cohen & Weinstein (1964); *c*, Laidler & Barnard (1956); *d*, Cohen, Crossley & Khedouri (1963*a*); *e*, Cohen, Crossley, Khedouri, Zand & Klee (1963*b*); *f*, Hein & Niemann (1962); *g*, Cohen, Neuwirth & Weinstein (1966); *h*, Cohen & Crossley (1964). Conditions: (i) 25°, pH 7.2, 0.1 M-NaCl; (ii) 25°, pH 7.8, 0.055 M-NaCl, 20% ethanol; (iii) 25°, pH 7.8, 0.075 M-NaCl, 20% ethanol; (iv) 25°, pH 7.8, 0.1 M-NaCl, 20% methanol; (v) 25°, pH 7.8, 0.1 M-NaCl; (vi) 25°, pH 7.9, 0.2 M-NaCl; (vii) 25°, pH 7.2, 0.095 M-NaCl.

R group	Config-uration	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (M ⁻¹ sec. ⁻¹)	Stereospecificity ratio	Reference	Conditions
CH ₂ ·C ₆ H ₅							
(a) Substrates of general formula: R·CH·CO·O·C ₂ H ₅							
CH ₃ ·CO·NH-	L	120	1.9	63000	630000	<i>a</i>	(i)
	D	Unreactive		< 0.1			
CH ₃ ·CO·O-	L	0.60	23	26	260	<i>b</i>	(ii)
	D	Unreactive		< 0.1			
HO-	L	4.1	2.7	1520	137	<i>b</i>	(iii)
	D	0.069	6.2	11.1		<i>b</i>	(iii)
H-	—	0.028	3.9	7.2	1	<i>c</i>	(v)
CH ₃							
(b) Substrates of general formula: R·CH·CO·O·C ₂ H ₅							
CH ₃ ·CO·NH-	L	0.34	250	1.36	13.6	<i>d</i>	(v)
	D	Unreactive		< 0.1			
CH ₃ ·CO·O-	L	0.00037	1.7	0.21	1.91	<i>e</i>	(i)
	D	0.02	180	0.11		<i>e</i>	(i)
C ₆ H ₅ ·CO·NH-	L*	0.26	9.8	27	8.2	<i>f</i>	(vi)
	D*	0.011	3.3	3.3		<i>f</i>	(vi)
C ₆ H ₅ ·CO·O-	L	0.003	16	0.19	0.053	<i>b</i>	(ii)
	D	0.021	5.8	3.6		<i>b</i>	(ii)
CH ₂ ·CO·O·C ₂ H ₅							
(c) Substrates of general formula: R·CH·CO·O·C ₂ H ₅							
CH ₃ ·CO·NH-	L	22	23	956	9560	<i>d</i>	(i)
	D	Unreactive		< 0.1			
CH ₃ ·CO·O-	L	0.98	27	36	1.44	<i>g</i>	(vii)
	D	0.30	12	25		<i>g</i>	(vii)
HO-	L	2.7	42	64	0.98	<i>g</i>	(vii)
	D	1.7	26	65		<i>g</i>	(vii)
H-	—	0.065	8.7	7.5	1	<i>h</i>	(i)

* Methyl esters.

general formula $R \cdot CH(R') \cdot CO \cdot O \cdot C_2H_5$. Their results are summarized in Table 4. In this work the meaning of the experimental kinetic parameter k_0 in terms of the individual rate constants for acylation, k_{+2} , and deacylation, k_{+3} , is not clear, and the following discussion is therefore in terms of the k_0/K_m ratios. This is in accord with the views of Bender & Kézdy (1965), who point out that the possibility of non-productive binding modes invalidates the use of kinetic parameters other than k_{+3} or k_0/K_m in structure-specificity correlations. Stereospecificity ratios (in this case the ratio $k_0^L K_m^D / k_0^D K_m^L$) may be calculated for most of the enantiomeric pairs of Table 4, and if we assume that the substrates designated as 'unreactive' have $k_0/K_m < 0.1$ (this is probably a generous upper limit; see Ingles & Knowles, 1966) then lower limits on the stereospecificity ratios of these substrates can also be fixed.

These ratios show the large fall in stereospecificity

accompanying the substitution of an oxygen atom for amido $-NH-$ of the acylamino group. The hydrogen-bonding role of the $-NH-$ group is also seen to be inadequately filled by an $-OH$ group occupying the same position. Though the ethyl α -hydroxy- β -phenylpropionates possess a stereospecificity ratio 137, the $-OH$ group of the diethyl malates appears to have no orienting function and the D- and L-enantiomers have identical reactivities. A similar observation has been made for the ethyl lactates (Cohen & Khedouri, 1961; Tinoco, 1958), and the symmetric ester diethyl α -hydroxymalonate is hydrolysed non-stereospecifically (Cohen & Khedouri, 1961). The results in Table 4 also demonstrate the much smaller fall in stereospecificity brought about by the removal of the second potential hydrogen-bonding group, the amido $-CO-$ group.

(c) Replacement of the amido $-NH-$ group of the acylamino side chain by a methylene group

Table 5. *Effect of acylamino group modification on the rate of catalysed hydrolysis*

References: *a*, Jones & Niemann (1963); *b*, Hein & Niemann (1961); *c*, Ingles & Knowles (1966); *d*, Wolf & Niemann (1963).

	Substrate	Configuration	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (m ⁻¹ sec. ⁻¹)	Reference
(i)	$\begin{array}{c} CH_2 \cdot C_6H_5 \\ \\ CH_3 \cdot CO \cdot NH \cdot CH \cdot CO \cdot O \cdot CH_3 \end{array}$	L	52.5	1.25	42000	<i>a</i>
	$\begin{array}{c} CH_2 \cdot C_6H_5 \\ \\ CH_3 \cdot CO \cdot CH_2 \cdot CH \cdot CO \cdot O \cdot CH_3 \end{array}$	DL	0.062	0.49	126	<i>b</i>
(ii)	$CH_3 \cdot CO \cdot NH \cdot CH_2 \cdot CO \cdot O \cdot CH_3$	—	0.046	300	0.153	<i>c</i>
	$CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CO \cdot O \cdot CH_3$	—	0.015	31.3	0.48	<i>d</i>

Table 6. *Effect on reactivity (k_0/K_m) of N-methylation of the amido group*

References: *a*, Peterson (1964); *b*, Peterson, Hubele & Niemann (1963); *c*, Cohen *et al.* (1963a); *d*, Ingles & Knowles (1966); *e*, Wolf & Niemann (1963). Conditions: (i) 25°, pH 7.9, 0.1 M-NaCl; (ii) 25°, pH 7.2, 0.1 M-NaCl.

	Substrate	Configuration	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (m ⁻¹ sec. ⁻¹)	Reference	Conditions
	$\begin{array}{c} CH_2 \cdot C_6H_4 \cdot OH \\ \\ CH_3 \cdot CO \cdot NH \cdot CH \cdot CO \cdot O \cdot CH_3 \end{array}$	L	117	0.32	360000	<i>a</i>	(i)
	$\begin{array}{c} H_3C \quad CH_2 \cdot C_6H_4 \cdot OH \\ \quad \\ CH_3 \cdot CO \cdot N \cdot CH \cdot CO \cdot O \cdot CH_3 \end{array}$	L	0.026	8.4	3.1	<i>b</i>	(i)
	$\begin{array}{c} CH_2 \cdot CO \cdot O \cdot C_2H_5 \\ \\ CH_3 \cdot CO \cdot NH \cdot CH \cdot CO \cdot O \cdot C_2H_5 \end{array}$	L	22	23	956	<i>c</i>	(ii)
	$\begin{array}{c} H_3C \quad CH_2 \cdot CO \cdot O \cdot C_2H_5 \\ \quad \\ CH_3 \cdot CO \cdot N \cdot CH \cdot CO \cdot O \cdot C_2H_5 \end{array}$	L	No observed hydrolysis		<0.1	<i>c</i>	(ii)
	$CH_3 \cdot CO \cdot NH \cdot CH_2 \cdot CO \cdot O \cdot CH_3$	—	0.046	300	0.153	<i>d</i>	(i)
	$\begin{array}{c} CH_3 \\ \\ CH_3 \cdot CO \cdot N \cdot CH_2 \cdot CO \cdot O \cdot CH_3 \end{array}$	—	No observed hydrolysis		<0.1	<i>e</i>	(i)

leads to a dramatic lowering of reactivity, wholly caused by a large fall in k_0 [see Table 5(i)]. When a similar substitution is made with a pair of substrates possessing a small amino acid side chain [Table 5(ii)], however, the results must be interpreted with care. In this case the substitution of $-\text{CH}_2-$ for $-\text{NH}-$ converts a typical acylamino side chain into a group possessing considerable hydrophobic character, which presumably now binds at the hydrophobic amino acid side-chain locus. The increase in reactivity observed here reflects the more favourable binding constant, k_0 being little changed.

(d) Substrates that are methylated at the amido nitrogen atom are orders of magnitude less reactive than the unmethylated analogues. The relevant data are summarized in Table 6. The values for the tyrosine derivatives again show that the fall in reactivity (here 10^5) is primarily a reflection of poorer catalysis, since the K_m values differ by only one order of magnitude.

The data quoted above (a-d) mostly refer to changes in the value of k_0/K_m . This is a valid parameter to use in such discussions (Bender & Kézdy, 1965), since it reduces in all cases to k_{+2}/K_s . However, one does not know whether the effects observed reflect kinetic specificity (affecting k_{+2}), or binding specificity (affecting K_s), or both. The results reported in the present work refer unequivocally to kinetic specificity, i.e. to specificity manifested by the enzyme in steps subsequent to initial binding.

Improved stereospecificity, which implies increased reactivity in the L-series and decreased reactivity in the D-series (reactivity being measured by either k_{+3} or the ratio k_0/K_m), is thus seen to result when the $-\text{NH}-$ of the substrate's acylamino group can form a hydrogen bond with the appropriate acceptor site in the enzyme's active site. This parallels the improved stereospecificity resulting from increasing the hydrophobic binding interaction between the amino acid side chain and the appropriate apolar locus at the active site (Ingles & Knowles, 1967). However, whereas improved hydrophobic binding is reflected directly in a lower binding constant (K_i or K_s), the possibility of hydrogen-bond formation from the acylamino group does not result in improved binding to the enzyme as measured by either of these constants. These facts are most clearly illustrated by the data in Table 7, from which it is apparent that, whereas stereospecificity in deacylation is dependent (as it must be) on both the hydrophobic binding of the amino acid side chain and the hydrogen-bonding interaction of the acylamino group, the free energy of binding of the molecule to the enzyme is dominated by the former. This is an important point of general significance that has been discussed in

Table 7. *Effects on stereospecificity, and inhibitor binding, of changes in amino acid side chain and in acylamino group*

	k_{+3}^L/k_{+3}^D for <i>p</i> -nitro- phenyl esters (sec. ⁻¹)	K_i for D-amides (mM)
(a) Alteration of amino acid side chain		
<i>N</i> -Acetyltryptophan	18300	1.48
<i>N</i> -Acetylphenylalanine	6400	10.6
<i>N</i> -Acetyl-leucine	148	141
<i>N</i> -Acetyl-glycine	1.0	692
(b) Alteration of acylamino group		
<i>N</i> -Acetylphenylalanine	6400	10.6
<i>O</i> -Acetyl- β -phenyl-lactate	9	8.9
β -Phenylpropionate	1.0	4.55

detail by Jencks (1966). He has proposed that the fact that the specificity of enzymes for good substrates is often manifested in rapid catalytic rates rather than in tight binding argues for the possibility of strain or distortion of the substrate on binding to the enzyme. In the present case, we see that the hydrogen-bonding interaction is not manifest in binding constants, but greatly affects the rate of deacylation of the acyl-enzyme (Table 7b). If the energy of the proposed hydrogen bond were small, the contribution to the free energy of substrate or inhibitor binding (as measured by K_s or K_i) could be negligible. However, the interaction of the acylamino group with the enzyme must be significant enough to produce the very large stereospecificities (as measured by k_{+3}^L/k_{+3}^D) listed in Table 7. On the basis of a rigid enzyme model (see Ingles & Knowles, 1967), we cannot say how much of the k_{+3}^L/k_{+3}^D ratios in Table 7(a) can be ascribed to the loss of the hydrogen-bonding interaction, and how much to the necessarily unfavourable steric interactions when two of the substituents on the asymmetric carbon atom of the D-enantiomer are transposed, as must happen for correct orientation of the susceptible carbonyl group of the substrate (see Fig. 7 of Ingles & Knowles, 1967).

A direct estimate of the energetic contribution of the hydrogen-bonding interaction can, however, be obtained from Table 7(b). Since the *N*-acetylphenylalanine derivatives are closely isosteric with the *O*-acetyl- β -phenyl-lactate compounds, the fall of k_{+3}^L/k_{+3}^D from 6400 to 9 is due simply to the loss of the acylamino group hydrogen-bond interaction. This is equivalent to nearly 4 kcal./mole, which is a reasonable value for a single hydrogen bond in a

relatively apolar environment (Pimentel & McClellan, 1960). An interaction energy of this magnitude cannot be considered negligible, is of the same order of magnitude as the overall binding free energy and would obviously not be unobservable in measurements of K_i if it contributed to initial binding.

There are two explanations that can account for these data. The first is that initial binding (as measured by K_i or K_s) does not involve a hydrogen bond, and only at the acyl-enzyme stage is this interaction either possible or detectable. This would explain the independence of the K_i values in Table 3 of the existence of an acylamino group in the listed inhibitors. The interaction of the acylamino group with the enzyme would only occur after the formation of the acyl-enzyme, and would be detected in k_{+3} (Table 7b). This explanation, however, does not readily accommodate the fact that stereospecificity (which demands a third interaction, i.e. the hydrogen-bond interaction of the acylamino group) is apparent in acylation as well as deacylation. Since this interaction is manifest in k_{+2} as well as k_{+3} , it is most likely to be present in the initial enzyme-substrate complex as well. The second explanation, which allows the hydrogen-bond interaction to contribute to initial binding, is therefore favoured, as is seen below.

It is feasible that the hydrogen-bonding interaction does contribute significantly to the overall binding free energy, but that it is only possible for a high-energy conformation of the substrate. That is, the extra binding free energy from the hydrogen bond is offset by a distortion of the substrate that arises from the selection of a high-energy conformer of the substrate. This second suggestion is supported by the work of Erlanger (1967), who has proposed that the geometry of the active site of α -chymotrypsin is such as to force an energetically expensive *cisoid* conformation on the acylamino group of the substrate. This proposal accounts for the rather surprising fact that β -phenylpropionamide binds to chymotrypsin rather better than *N*-acetyl-D-phenylalanine amide (Table 3). The K_i for the D-amide will be rather smaller than the K_s for the L-amide (Knowles, 1965), so it appears that the presence of an acylamino group in a substrate is actually a net hindrance to substrate binding. It should be pointed out that the present suggestion is not directly related to that of Charney & Bernhard (1967), who have proposed that chromophoric acyl- α -chymotrypsins (of the β -acryloyl-enzyme type) are in the 's-*cis*' configuration in the acyl-enzyme.

The present proposal, then, is that the apparent dissociation constant of enzyme complexes involving specific substrates is higher than expected on the basis of the known interactions, owing to

the selection of a high-energy conformer. It is unsatisfying in that there is no reason yet apparent why this distortion of the substrate should favour the catalytic process [compare the neat mechanistic advantage of the substrate distortion proposed by Blake *et al.* (1967) for lysozyme]. It may be important, however, that the acylamino group is kept well away from the catalytic functionalities and the susceptible carbonyl group of the substrate. This was indicated some time ago by Almond, Manning & Niemann (1962), who found that α -C-methyl-*N*-acyl- α -amino acid esters bind normally to α -chymotrypsin, but have catalytic constants at least 10^5 -fold lower than those of the corresponding substrates where the α -methyl group is replaced by hydrogen. Further information on this question must wait for more detailed crystallographic studies of substrate and inhibitor binding to the enzyme than are presently available (Matthews, Sigler, Henderson & Blow, 1967).

The observation of a marked inversion of stereospecificity for the D- and L-enantiomers of 3-methoxycarbonyldihydroisocarbostyryl (Hein & Niemann, 1962; Hein, McGriff & Niemann, 1960) has been a stumbling block to rationalizations of the origins of stereospecificity in α -chymotrypsin. The reactivity of the D-enantiomer is more than 1000 times that of the L-enantiomer and is close to that of the highly specific substrate *N*-acetyl-L-phenylalanine methyl ester. The situation has been greatly clarified by the observation that the oxygen analogue of 3-methoxycarbonyldihydroisocarbostyryl, methyl DL-3,4-dihydroisocoumarin-3-carboxylate, is hydrolysed stereospecifically in favour of the D-antipode, which has a reactivity very close to that of the D-amido compound (Cohen & Shultz, 1967). This suggests that the hydrogen-bonding interaction of the -NH- of the acylamino group, which is so important for the orientation of open-chain acyl- α -chymotrypsins, is not required when this orientation can be provided by the rigidity of cyclized substrates. On the basis of the model for acyl-chymotrypsins presented earlier (Ingles & Knowles, 1966), it can readily be shown that when the aromatic nucleus of D-3-methoxycarbonyldihydroisocarbostyryl is bound in the side-chain locus of the active site, preserving the hydrophobic interaction, the equatorial methoxycarbonyl group can be appropriately positioned close to the catalytic functionalities of the enzyme. In this conformation, with the orientation provided by the hydrophobic interaction and the tied-back amido group, the reactivity approaches that of *N*-acetyl-L-phenylalanine methyl ester, where the orientation is provided by both the hydrophobic and hydrogen-bond interactions. In L-3-methoxycarbonyldihydroisocarbostyryl the preservation of the hydrophobic interaction places the equatorial

methoxycarbonyl group some distance from the catalytic locus. The reactivity of the L-enantiomer is consequently much lower.

In summary, the results reported here show that the orientating interaction of the acylamino group of a chymotrypsin substrate is provided mainly by a hydrogen bond between the -NH- group of the acylamino group and an unknown acceptor group in the active site of the enzyme. This interaction orientates the susceptible carbonyl group of the acyl-enzyme either favourably (L-series) or unfavourably (D-series) for the subsequent deacylation step, but contributes negligibly to the free energy of binding as observed by K_t (or, presumably, K_s).

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