Biochem. J. (1972) **126**, 659–665
Printed in Great Britain

Comparative Studies of the Specificities of α-Chymotrypsin and Subtilisin BPN'

STUDIES WITH FLEXIBLE AND 'LOCKED' SUBSTRATES

By T. N. PATTABIRAMAN* and W. B. LAWSON
Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201, U.S.A.

(*Received* 26 *August* 1971)

Subtilisin BPN' hydrolysed *N*-acetyl-L-3-(2-naphthyl)-alanine methyl ester, *N*-acetyl-L-leucine methyl ester and *N*-acetyl-L-valine methyl ester, faster than α-chymotrypsin. Of eight 'locked' substrates tested, only methyl 5,6-benzindan-2-carboxylate was hydrolysed faster by subtilisin, whereas the other esters were better substrates for chymotrypsin. Compared with the values for chymotrypsin, the stereospecific ratios during the hydrolysis of the optically active locked substrates by subtilisin were decreased by one and two orders of magnitude for bi- and tri-cyclic substrates respectively. The polar groups adjacent to the α-carbon atom of locked substrates did not contribute significantly to the reactivity of the more active optical isomers, but had a detrimental effect on the less active antipodes during hydrolysis by both the enzymes. These studies show that the binding site of subtilisin BPN' is longer and broader than that of α-chymotrypsin.

In the preceding paper (Pattabiraman & Lawson, 1972*b*), the action of α-chymotrypsin and subtilisin BPN' on a series of α-acetamidoarylalkanoate esters and arylalkanoate esters was compared. From these studies we concluded that the hydrophobic site of subtilisin BPN' is longer than that of α-chymotrypsin. The faster rates of hydrolysis of *N*-acetyl-L-tryptophan methyl ester and its 1-naphthalene analogue by α-chymotrypsin indicated that the region is less curved in the case of subtilisin BPN'.

Additional evidence for these concepts is provided in the present paper by comparing the reactivity of locked substrates and additional flexible α-acetamido ester substrates (Fig. 1) for these enzymes. The results also indicate that, as was observed with α-chymotrypsin (Pattabiraman & Lawson, 1972*a*), polar groups or atoms adjacent to the α-carbon atom in locked substrates do not contribute significantly in enhancing the activity of the more active optical isomers, but cause a detrimental effect in the less reactive optical antipodes. The stereospecificity of substrates is shown to be determined by their overall geometry, as well as by interactions caused by the presence or absence of a polar group or atom.

Experimental

The experimental procedures and materials, except those described below, are the same as those given by Pattabiraman & Lawson (1972*b*).

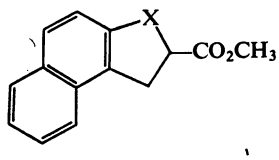
Preparation of compounds

Methyl dihydroisocarbostyril-3-carboxylate (compound VIII) was synthesized and resolved into its optical isomers as described by Hein & Niemann (1962). Methyl 3,4-dihydroisocoumarin-3-carboxylate (compound IX) was synthesized and resolved with α-chymotrypsin into the optical antipodes by the method of Cohen & Schultz (1968). Methyl indan-2-carboxylate (compound IV) and the optical isomers of methyl hydrocoumarilate (compound III) were made by established procedures as described by Lawson (1967) and Hayashi & Lawson (1969). The synthesis of *N*-acetyl-DL-3-(2-naphthyl)alanine methyl ester (compound VII) and the preparation of D- and L-methyl 1,2-dihydronaphtho[2,1-*b*]furan-2-carboxylate (compound I) were as described by Hayashi & Lawson (1969). The synthesis of methyl 2-acetamidoindan-2-carboxylate (compound V), methyl 5,6-benzindan-2-carboxylate (compound VI) and methyl 4,5-benzindan-2-carboxylate (compound II) and the resolution of the last compound with α-chymotrypsin were as described by Pattabiraman & Lawson (1972*a*).

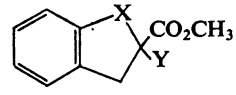
N-Acetyl-D-leucine methyl ester (*D-compound XI*). *N*-Acetyl-D-leucine (3.2g) [m.p. 182–183°C, and $[\alpha]_D^{25}$ +24.18° (*c* 1.47 in ethanol); Birnbaum *et al.* (1952) give m.p. 185°C and $[\alpha]_D$ + 21.3° in ethanol)] was esterified with methanol in the presence of thionyl chloride (Brenner & Huber, 1953) and the product was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give 2.7g of colourless cubes, yield 78%, m.p. 42.0–43.5°C and $[\alpha]_D^{26}$ +57.33° (*c* 2.44 in water) (Found: C, 57.41; H, 9.25; N, 7.64. $C_9H_{17}NO_3$ requires C, 57.73; H, 9.15; N, 7.48%).

N-Acetyl-L-leucine methyl ester (*L-compound XI*). This ester was prepared in an analogous way from *N*-acetyl-L-leucine [m.p. 183–184°C, and $[\alpha]_D^{25}$ −24.4° (*c* 1.1 in ethanol)] in 74% yield, m.p. 43–44°C and $[\alpha]_D^{26}$ −57.1° (*c* 2.09 in water) [Applewhite *et al.* (1958) report m.p. 43.0–44.5°C and Karrer *et al.* (1926) give $[\alpha]_D^{16}$ −17.22° in water].

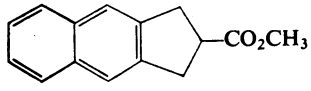
* Present address: Department of Biochemistry, College of Science, Osmania University, Hyderabad-7, India.



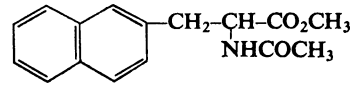
I: X = O
II: X = CH_2



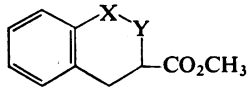
III: X = O, Y = H
IV: X = CH_2, Y = H
V: X = CH_2, Y = $NHCOCH_3$



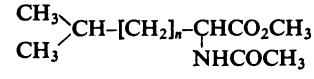
VI



VII



VIII: X = CO, Y = NH
IX: X = CO, Y = O



X: $n = 0$
XI: $n = 1$

Fig. 1. *Compounds I to XI*

N-Acetyl-D-valine methyl ester (*D-compound X*). *N*-Acetyl-D-valine [m.p. 163.5–165.0°C and $[\alpha]_D^{25}$ +19.95° (*c* 1.27 in water); Marshall *et al.* (1956) give m.p. 168°C] was esterified to yield *N*-acetyl-D-valine methyl ester as fine white needles in 80% yield, m.p. 60.5–62.0°C and $[\alpha]_D^{26}$ +48.02° (*c* 1.51 in water) (Found: C, 55.69; H, 8.55; N, 8.25. $C_8H_{15}NO_3$ requires C, 55.47; H, 8.73; N, 8.09%).

N-Acetyl-L-valine methyl ester (*L-compound X*). This ester was prepared in a similar way from *N*-acetyl-L-valine [m.p. 163–165°C and $[\alpha]_D^{25}$ −19.4° (*c* 1.2 in water) (Marshall *et al.*, 1956, give m.p. 168°C)] in 80% yield, m.p. 61.5–62.0°C and $[\alpha]_D^{25}$ −49.3° (*c* 0.58 in water) [Applewhite *et al.* (1958) give m.p. 61.3–62.5°C and $[\alpha]_D$ −47.6° in water.].

Optical resolution of N-acetyl-3-(2-naphthyl)alanine methyl ester (*compound VII*) *by subtilisin BPN'*. A solution of 1.1g (4.05mmol) of the ester in 50ml of dimethyl sulphoxide, 20ml of 1M-KCl and 130ml of water was treated with a solution of 5.64mg of subtilisin BPN' in 0.5ml of 0.1M-KCl at pH7.0 (substrate concentration, 20.2mM; enzyme concentration, 1.13 μM). In 30min 1.925ml of 1M-NaOH (1.925mmol, 47.5% hydrolysis) was consumed and the reaction then abated. $NaHCO_3$ (2g) was added and the solution was extracted five times with 40ml of ethyl acetate. The ethyl acetate extract was washed with water, then dried over Na_2SO_4 and evaporated. The crude precipitate was crystallized twice from ether–light petroleum (b.p. 30–60°C) to give 390mg (73% yield) of (−)-*N*-acetyl-3-(2-naphthyl)alanine methyl ester, m.p. 94–95°C and $[\alpha]_D^{25}$ −43.5° (*c* 0.59 in ethanol) (Found: C, 70.58; H, 6.25; N, 5.31. $C_{16}H_{17}NO_3$ requires C, 70.83; H, 6.32; N, 5.16%).

The aqueous layer was acidified to pH2.0 with HCl and extracted five times with 40ml of ethyl acetate. The extract was washed with water, then dried over Na_2SO_4 and evaporated. The crude material was crystallized from ethyl acetate to yield 420mg (81% yield) of (+)-*N*-acetyl-3-(2-naphthyl)alanine, m.p. 181–182°C and $[\alpha]_D^{25}$ +78.3 (*c* 0.69 in ethanol). This was esterified to give (+)-*N*-acetyl-3-(2-naphthyl)-alanine methyl ester in 83% yield, m.p. 92.5–94.0°C and $[\alpha]_D^{25}$ +44.6° (*c* 0.4 in ethanol) (Found: C, 70.69; H, 6.45; N, 4.98. $C_{16}H_{17}NO_3$ requires C, 70.83; H, 6.32; N, 5.16%).

Determination of the absolute configuration of (+)-N-acetyl-3-(2-naphthyl)alanine methyl ester. The optically active ester (150mg) was refluxed with 6ml of 3M-HCl for 16h. The solution was treated with charcoal while hot, filtered and neutralized with aq. NH_3. The amino acid (80mg) was obtained as white glistening plates, m.p. 240–244°C (softens at 210°C). (Found: C, 72.43; H, 6.10; N, 6.79. $C_{13}H_{13}NO_2$ requires C, 72.54; H, 6.09; N, 6.51%). $[\alpha]_D^{28}$ −73.02° (*c* 0.1 in 5mM-HCl), $[M]_D$ −156.99°; $[\alpha]_D^{28}$ −50.42° (*c* 0.43 in 0.04M-HCl), $[M]_D$ −108.40°; $[\alpha]_D^{28}$ −8.84° (*c* 0.43 in 0.08M-HCl), $[M]_D$ −19.0°; $[\alpha]_D^{28}$ −7.53° (*c* 0.43 in 1M-HCl), $[M]_D$ −16.19°. The positive shift in rotation with increasing acidity shows that the amino acid is of the L-configuration (Greenstein & Winitz, 1961).

Results

Substrates

The substrates used in these studies were prepared by conventional procedures. *N*-Acetyl-DL-3-(2-naphthyl)alanine methyl ester (VII) was resolved into its optical isomers with subtilisin BPN'. The reactive enantiomer was found to have the L-configuration according to the Clough–Lutz–Jirgensons rule (Greenstein & Winitz, 1961). The insolubility of 3-(2-naphthyl)alanine precluded measurement of optical rotation in water at neutral pH. However, the positive shift in rotation with increasing acidity is sufficient to show that it belongs to the L-series of amino acids.

Enzymic experiments

The intrinsic reactivities of the esters and the concentration of the enzymes and substrates used for evaluation of the kinetic parameters are listed in Table 1. The intrinsic reactivities are the ratios of the first-order hydrolyses of esters by OH^- relative to the rates of hydrolysis of *N*-acetyl-L-phenylalanine methyl ester, which is assigned a value of one. These values were obtained in aq. 50% dimethyl sulphoxide containing 0.1 M-NaCl. All kinetic experiments were performed in aqueous dimethyl sulphoxide, as many of the substrates used in these studies are insoluble in water. The effect of dimethyl sulphoxide on the activity of chymotrypsin and subtilisin is discussed by Pattabiraman & Lawson (1972*b*).

The kinetic parameters for the hydrolysis of flexible α-acetamido ester substrates are given in Table 2. *N*-Acetyl-L-3-(2-naphthyl)alanine methyl ester (L-compound VII) was hydrolysed 50 times faster by subtilisin than by chymotrypsin. Similarly *N*-acetyl-L-leucine methyl ester (L-compound IX) and *N*-acetyl-L-valine methyl ester (L-compound X) were hydrolysed 20 and 490 times, respectively, faster by

Table 1. *Intrinsic reactivities of esters and substrate and enzyme concentrations used in kinetic experiments*

Experimental details are given in the text.

		α-Chymotrypsin		Subtilisin BPN'	
Compound	Intrinsic reactivity	$10^7 \times [E]_0$ (M)	$10^3 \times [S]_0$ (M)	$10^7 \times [E]_0$ (M)	$10^3 \times [S]_0$ (M)
D-I				7.59	0.25– 2.5
	50.0*				
L-I				290	0.50– 5.0
(–)-II				342	0.25– 2.0
	0.423†				
(+)-II				342	0.25– 2.0
D-III				7.76	3.33–33.3
	34.4*				
L-III				7.76	3.33–33.3
IV	0.194†			68.4	2.0 –20.0
V	0.149†			342	4.0 –40.0
VI	0.321†			162	0.4 – 2.0
D-VII		544	5.0 – 8.0	648	4.0 –10.0
	1.0*				
L-VII		109	0.83– 8.3	6.48	0.83– 8.3
D-VIII		2.72	1.0 –10.0	16.2	2.0 –20.0
	9.15				
L-VIII		272	2.0 –20.0	324	2.0 –20.0
D-IX		0.680	1.6 –16.0	16.2	3.33–33.3
	34.0				
L-IX		54.4	2.0 –20.0	324	2.0 –20.0
D-X		544	20.0 –40.0	259	4.0 –40.0
	0.078				
L-X		272	2.0 –20.0	34.2	4.0 –40.0
D-XI		544	20.0 –40.0	0.684	8.0 –40.0
	0.357				
L-XI		13.6	4.0 –40.0	648	4.0 –40.0

* Hayashi & Lawson (1969). †Pattabiraman & Lawson (1972*a*).

Table 2. *Kinetic parameters for the hydrolysis of flexible substrates, at pH*8.0, 25°C, *in aq.* 41.7% *dimethyl sulphoxide*

Experimental details are given in the text.

Compound	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)	Normalized $k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)	Stereospecificity
			Subtilisin BPN'		
L-VII	26.8	22.2	1210	1210	34900
D-VII	—	—	0.035*	0.035	
L-X	4.23	145	29.1	378	146
D-X	0.134	667	0.201	2.59	
L-XI	619	364	1700	477	90700
D-XI	—	—	0.019*	0.053	
			α-Chymotrypsin		
L-VII	0.319	13.0	24.5	24.5	>16000
D-VII	—	—	<0.0015	<0.0015	
L-X	0.034	571	0.06	0.766	>40
D-X	—	—	<0.0015	<0.2	
L-XI	7.87	95.2	82.7	231	>40000
D-XI	—	—	<0.002	<0.006	

* $(v)/[E][S]$, average of four values.

subtilisin. Subtilisin displayed good stereospecificity in the hydrolysis of compounds VII and XI. The stereoselectivity, however, was limited with compound X. The stereospecific ratios for the hydrolysis of these esters by chymotrypsin could not be determined with accuracy because of the inertness of the D-isomers.

Table 3 shows the results on the hydrolysis of locked substrates. Methyl D-1,2-dihydronaphtho[2,1-*b*]-furan-2-carboxylate (D-compound I), (−)-methyl 4,5-benzindan-2-carboxylate [(−)-compound II], methyl D-hydrocoumarilate (D-compound III), methyl D-dihydroisocarbostyril-3-carboxylate (D-compound VIII) and methyl D-3,4-dihydroisocoumarin-3-carboxylate (D-compound IX) were hydrolysed faster by chymotrypsin than by subtilisin. In contrast methyl 5,6-benzindan-2-carboxylate (compound VI) was a better substrate for subtilisin. Chymotrypsin and subtilisin hydrolyse compounds D-I and D-III at rates comparable with those of their corresponding oxygenless analogues, compounds (−)-II and IV, respectively. The rates of hydrolysis of compounds L-I and L-III are slower than those of compounds (+)-II and IV, respectively. The stereospecific ratios for compounds VIII and IX with chymotrypsin in aq. 41.7% dimethyl sulphoxide reported here are comparable with the values observed by Hein & Niemann (1962) and Cohen & Schultz (1968) for these compounds in water. The stereospecific ratios observed with subtilisin for the optically active locked substrates are smaller than the values found for chymotrypsin. Methyl 2-acetamidoindan-2-carboxylate (compound V) is hydrolysed 4.7 and 11.7 times more slowly by subtilisin and chymotrypsin than methyl indan-2-carboxylate (compound IV).

Discussion

Morihara & Tsuzuki (1969) demonstrated that subtilisin BPN' hydrolyses *N*-acetyl-L-phenylalanine methyl ester, *N*-acetyl-L-tryptophan methyl ester and *N*-acetyl-L-leucine methyl ester (L-compound XI) at comparable rates, whereas chymotrypsin hydrolyses the last ester relatively slowly.

Evidence was given by Pattabiraman & Lawson (1972*b*) to show that the binding-site region of subtilisin BPN' is longer and less curved than that of α-chymotrypsin. The present experiments with 'locked' and flexible substrates provide supporting evidence for this conclusion. The linear substrates *N*-acetyl-3-(2-naphthyl)alanine methyl ester (compound VII) and methyl 5,6-benzindan-2-carboxylate (compound VI) are better substrates for subtilisin than for chymotrypsin. The corresponding curved analogues, *N*-acetyl-3-(1-naphthyl)alanine methyl ester (Pattabiraman & Lawson, 1972*b*) and methyl 4,5-benzindan-2-carboxylate, are hydrolysed faster by chymotrypsin

Table 3. *Kinetic parameters for the hydrolysis of locked substrates, at pH* 8.0, 25°C *in aq.* 41.7% *dimethyl sulphoxide*

Experimental details are given in the text.

Compound	$10^2 \times k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)	Normalized $k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)	Stereospecificity
			Subtilisin BPN'		
D-I	595	5.0	1190	23.8	
					290
L-I	20	48.8	4.1	0.082	
(−)-II	2.99	5.0	5.98	14.1	
					2.4
(+)-II	6.36	25.2	2.53	5.96	
D-III	455	100	45.5	1.33	
					9.8
L-III	58.8	126	4.67	0.136	
IV	14.3	156	0.915	4.73	
V	1.71	114	0.15	1.01	
VI	—	—	2.98*	8.92	
D-VIII	3350	200	167	18.4	
					781
L-VIII	4.29	200	0.214	0.024	
D-IX	1020	44.4	230	6.76	
					111
L-IX	17.3	83.3	2.08	0.061	
			α-Chymotrypsin		
D-I	588	1.46	4010†	338‡	
					37500
L-I	0.27	25.0	0.107†	0.009‡	
(−)-II	3.9	1.94	20.2†	202‡	
					209
(+)-II	0.28	28.6	0.097†	0.966‡	
D-III	222	14.3	155†	18.9‡	
					91
L-III	2.4	14.1	1.70†	0.208‡	
IV	0.44	10.9	0.407†	8.81‡	
V	0.025	9.52	0.026†	0.755‡	
VI	0.16	41.6	0.039†	0.797‡	
D-VIII	1555	7.0	2220	244	
					6980
L-VIII	5.1	160	0.319	0.035	
D-IX	5550	13.5	4100	121	
					838
L-IX	49.4	100	4.94	0.145	

* Average of six $(v)/[E]\,[S]$ values, determined at pH 8.0, in aq. 50% dimethyl sulphoxide. The extreme insolubility of compound VI precluded direct measurement in aq. 41.7% dimethyl sulphoxide. The value obtained in aq. 50% dimethyl sulphoxide (1.75) was corrected by multiplying by 1.637, the ratio of the $k_{cat.}/K_m$ values for the hydrolysis of *N*-acetyl-L-phenylalanine methyl ester in aq. 41.7% and 50% dimethyl sulphoxide.

† Pattabiraman & Lawson (1972a); in aq. 50% dimethyl sulphoxide at pH 7.0.

‡ These values were obtained from the reported $k_{cat.}/K_m$ values for these compounds (above reference), by multiplying by 4.217, the ratio of the measured $k_{cat.}/K_m$ values of D-compound I at pH 8.0, in aq. 41.7% dimethyl sulphoxide and at pH 7.0 in aq. 50% dimethyl sulphoxide.

than by subtilisin. *N*-acetyl-L-leucine methyl ester (L-compound XI) and the valine derivative (L-compound X) are hydrolysed faster by subtilisin than by chymotrypsin, suggesting that the binding site of subtilisin can accommodate the side chains of these two esters more productively, or alternatively that the steric hindrance caused during the accommodation of the side chains of these substrates is less with subtilisin.

Whereas a large array of locked substrates has been tested with chymotrypsin, only one such study has been reported for subtilisin. Dugas (1969) observed that methyl dihydroisocarbostyril-3-carboxylate (compound VIII) is hydrolysed by subtilisin with apparent absolute stereospecificity. The present study shows that compound VIII and other optically active 'locked' substrates are hydrolysed by subtilisin with decreased sterospecificity compared with chymotrypsin. Compounds D-I, (−)-II, D-VIII and D-IX, the geometry of which is more nearly complementary to that of the active site of chymotrypsin, are hydrolysed faster by this enzyme. In contrast, subtilisin hydrolyses the linear ester VI ten times faster than chymotrypsin does. The binding site of subtilisin does not differentiate compounds (−)-II, (+)-II and VI, as these three esters are hydrolysed at comparable rates. From these results, we suggest that the binding site of subtilisin is longer and less curved than that of chymotrypsin.

It was shown that the polar groups or atoms adjacent to the α-carbon atom in the more active optical isomers of 'locked' substrates do not contribute significantly in enhancing the reactivities during chymotryptic hydrolysis, but have a detrimental effect in the less active antipodes (Pattabiraman & Lawson, 1972*a*). Comparison of the reactivities of compounds D-I and (−)-II, D-III and IV, L-I and (+)-II and L-III and IV, shows that for subtilisin the same explanation also applies. The lower reactivity of compound V compared with that of compound IV, observed with both the enzymes, suggests that the α-acetamido group of compound V does not enhance the reactivity of this substrate and hence might be oriented away from the polar site of the active centre that accommodates the acetamido group of a typical substrate like *N*-acetyl-L-phenylalanine methyl ester (Pattabiraman & Lawson, 1972*a*).

An exact comparison of the stereospecific hydrolysis of flexible substrates VII, X and XI (Table 2) by chymotrypsin and subtilisin could not be made because of the inertness of the D-enantiomers with chymotrypsin. However, previous results (Pattabiraman & Lawson, 1972*b*) indicated that the ratios observed with subtilisin are equal to or greater than those observed with chymotrypsin for α-acetamido esters. A striking difference was observed in the decreased stereospecificities of locked substrates with subtilisin. The stereoselectivity is decreased by an order of magnitude for bicyclic substrates (III, VIII and IX), whereas for the tricyclic substrates (I and II) the decrease is roughly 100-fold.

The factors responsible for the stereospecificity observed during hydrolysis by subtilisin and chymotrypsin can be represented as follows. (*a*) A positive contribution of varying degree, due to the interaction of the α-acetamido group in the L-series of flexible substrates. In active 'locked' substrates (D-series) this contribution is minimal or lacking. The exact nature of the interaction of α-acetamido groups is not clear, but it appears that it could be caused by the bulk and electronic character of this group (Silver *et al.*, 1970; cf. also Pattabiraman & Lawson, 1972) rather than by a primary hydrogen-bonding interaction between the NH group of the substrate and a suitable residue on the surface of the enzyme (Blow, 1971). (*b*) A negative contribution, perhaps because of the steric hindrance of the α-acetamido or other polar groups in the D-series of flexible substrates. This deleterious effect is also high in the less-active 'locked' substrates (L-series). (*c*) The overall geometry of the substrate. This is probably of greater importance for the 'locked' substrates than for the flexible substrates because of the differences in the degree of freedom of orientation.

With rigid substrates, factors (*b*) and (*c*) appear to be responsible for the observed stereospecificity. In a compound of relative symmetry and small size like III, the major factor is (*b*) and the observed stereospecificity hence is limited. In compound II, the sole effective factor appears to be its geometry. Compound I has a geometry similar to that of compound II and in addition has a polar oxygen atom in the α-position. The stereospecific ratio observed with compound I is 100 times greater than that of compound II, and it is reasonable to conclude that the enhancement in stereospecificity is due to the additional factor (*b*). For compounds VIII and IX, again both factors (*b*) and (*c*) are responsible for the observed stereospecific ratios. The X-ray results (Blow, 1971; Kraut, 1971) and studies with model substrates (Glazer, 1967; Barel & Glazer, 1968; Morihara & Tsuzuki, 1969), have provided evidence that the geometry of the binding site of subtilisin is less well defined than that of chymotrypsin. The results in the present paper and in the preceding paper (Pattabiraman & Lawson, 1972*b*) suggest that the binding site in subtilisin is longer and perhaps also broader than that of chymotrypsin. Hence factor (*c*) appears to be of lesser importance in determining the reactivity and stereospecificity of 'locked' substrates like compounds I, II, VIII and IX with respect to subtilisin. Thus the decreased stereospecificity of these 'locked' substrates observed with subtilisin results from a less productive interaction of the more active optical isomers and from a relatively smaller steric hindrance in the case of their optical antipodes.

Because of these observations, it can be suggested that, although the active sites of α-chymotrypsin and subtilisin BPN′ have similar structures and functions of their catalytic region and their polar site (Pattabiraman & Lawson, 1972), the dissimilarities in the binding sites can account for the differences in the reactivity and stereospecificity of model substrates. In the X-ray work, a definite cleft, the 'tosyl hole', was found at the active site of chymotrypsin (Blow, 1971), whereas no pronounced depression at the active site of subtilisin BPN′ was detected (Wright *et al.*, 1969; Alden *et al.*, 1970).

We thank Mrs. Lindy Chao for synthesizing methyl dihydroisocarbostyril-3-carboxylate and methyl 3,4-dihydroisocoumarin-3-carboxylate. W. B. L. is grateful to Dr. D. M. Blow for the opportunity to inspect molecular models of chymotrypsin. This work was supported in part by Grant AM 05299 from the National Institutes of Health (U.S.A.).

References

Alden, R. A., Wright, C. S. & Kraut, J. (1970) *Phil. Trans. Roy. Soc. London Ser. B* **257**, 119

Applewhite, T. H., Waite, H. & Niemann, C. (1958) *J. Amer. Chem. Soc.* **80**, 1465

Barel, A. O. & Glazer, A. N. (1968) *J. Biol. Chem.* **243**, 1344

Birnbaum, S. M., Levintow, L., Kingsley, R. B. & Greenstein, J. P. (1952) *J. Biol. Chem.* **194**, 455

Blow, D. M. (1971) in *The Enzymes* (Boyer, P. D., ed.), 3rd. edn., vol. 3, p. 185, Academic Press, New York

Brenner, M. & Huber, W. (1953) *Helv. Chim. Acta* **36**, 1109

Cohen, S. G. & Schultz, R. M. (1968) *J. Biol. Chem.* **243**, 2607

Dugas, H. (1969) *Can. J. Biochem.* **47**, 985

Glazer, A. N. (1967) *J. Biol. Chem.* **242**, 433

Greenstein, J. P. & Winitz, M. (1961) *Chemistry of the Amino Acids*, p. 83, J. Wiley and Sons, New York

Hayashi, Y. & Lawson, W. B. (1969) *J. Biol. Chem.* **244**, 4158

Hein, G. E. & Niemann, C. (1962) *J. Amer. Chem. Soc.* **84**, 4487

Karrer, P., Esher, E. & Widmer, R. (1926) *Helv. Chim. Acta* **9**, 301

Kraut, J. (1971) in *The Enzymes* (Boyer, P. D., ed.) 3rd. edn., vol. 3, p. 547, Academic Press, New York

Lawson, W. B. (1967) *J. Biol. Chem.* **242**, 3397

Marshall, R., Birnbaum, S. M. & Greenstein, J. P. (1956) *J. Amer. Chem. Soc.* **78**, 4636

Morihara, K. & Tsuzuki, H. (1969) *Arch. Biochem. Biophys.* **129**, 620

Pattabiraman, T. N. & Lawson, W. B. (1972*a*) *J. Biol. Chem.* in the press

Pattabiraman, T. N. & Lawson, W. B. (1972*b*) *Biochem. J.* **126**, 645

Silver, M. S., Stoddard, M., Sone, T. & Matta, M. S. (1970) *J. Amer. Chem. Soc.* **92**, 3151

Wright, C. S., Alden, R. A. & Kraut, J. (1969) *Nature (London)* **221**, 235