The Interaction of α -Chymotrypsin with Isosteric Substrates of Different Charge Type

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1. The synthesis of three substrates of α -chymotrypsin of closely similar steric requirements but different charge type is reported. 2. The interaction of these compounds [SS-dimethyl-(L-3-carboxymethyl-3-acetamido)propyl sulphonium iodide, L-2-acetamido-5-methylhexanoic acid methyl ester and N-acetyl-L-glutamic acid α -methyl ester] with α -chymotrypsin has been studied. 3. For the charged substrates, values of k_0 are two orders of magnitude smaller than, and values of K_m two orders of magnitude larger than, the corresponding values for the uncharged isostere. 4. The results are interpreted in terms of the known specificity of the enzyme, and the relationship between binding and kinetic specificities is discussed.

Though the use of synthetic model compounds of closely similar steric requirements but different charge type is common in pharmacology (especially in the peptide hormone field) (see, e.g., Schwyzer, 1961), this approach has not been much followed (in the sense of using strict isosteric replacements) by enzymologists. Further to the thesis that the binding locus for the amino acid side chain of substrates of the enzyme α -chymotrypsin is simply hydrophobic (Knowles, 1965a; Hymes, Robinson & Canady, 1965), the present paper reports the synthesis, and details of the interaction with α -chymotrypsin, of three substrates of closely similar steric properties but of different charge type. The compounds concerned are SS-dimethyl-(L-3-carboxymethyl-3-acetamido)propyl sulphonium iodide (I), L-2-acetamido-5-methylhexanoic acid methyl ester (II) and N-acetyl-L-glutamic acid α -methyl ester (III).

MATERIALS

Three-times crystallized α -chymotrypsin, salt-free and freeze-dried, was purchased from Seravac Laboratories Ltd., Maidenhead, Berks. (batch 44). Titrations of the opera-

tional normality of enzyme solutions were performed with N-cinnamoylimidazole by the method of Schonbaum, Zerner & Bender (1961).

Isopentyl bromide was purchased from British Drug Houses Ltd., Poole, Dorset. The percentage of 1-bromo-2-methylbutane in the material was found by gas-liquid-chromatographic analysis to be less than 2%.

N-Acetyl-L-valine ethylester was prepared by the method of Waite & Niemann (1962) and had $[\alpha]_D^{20} -50.5^\circ$ (c 2 in water). Waite & Niemann (1962) give $[\alpha]_D^{25} -50.3^\circ$.

Acetic anhydride, triethylamine, iodomethane and dicyclohexylamine were redistilled before use.

SS-Dimethyl-(L-3-carboxymethyl-3-acetamido)propyl sulphonium iodide. N-Acetyl-L-methionine methyl ester was prepared from L-methionine methyl ester hydrochloride by the method of Waite & Niemann (1962). A solution of N-acetyl-L-methionine methyl ester (8.0g.) and iodomethane (17.4ml.) in acetic acid (80ml.) was refluxed for $\frac{1}{2}$ hr. and then kept at room temperature overnight. The mixture was added to 1 $\frac{1}{2}$ l. of ether and the resulting yellow precipitate recrystallized from methanol-ether. The white plates had m.p. 120–122°, $[\alpha]_D^{20} -7.4^\circ$ (c 2 in water). The yield was 70% (Found: C, 31.0; H, 5.3; I, 37.0; N, 3.8. C₉H₁₈INO₃S requires C, 31.1; H, 5.2; I, 36.6; N, 4.0%). The rotation of the material in water was unchanged after 48 hr. A solution of the material in 0.1M-tris-HCl buffer, pH 8.0, had $[\alpha]_D^{20} -6.5^\circ$ (c 2 in buffer), and after 24 hr. at room temperature had fallen to -1.0° .

D- and L-2-Acetamido-5-methylhexanoic acid methyl ester. The method used for the preparation of these compounds followed the approach of Jones, Kunitake, Niemann & Hein (1965). A mixture of ethyl acetamidomalonate (21.7g.), isopentyl bromide (17.5ml.) and sodium (2.3g.) dissolved in Grignard-dried ethanol (50ml.), was refluxed for 15hr. After removal of the solvent under reduced pressure the slurry was poured into ice-cold water (250ml.) to precipitate the alkylated acetamidomalonate. The precipitate was washed with ice-cold water until colourless, and recrystallized from water. It had m.p. 39–39.5° (Found: C, 57.8; H, 8.6; N, 4.6. $C_{14}H_{25}NO_5$ requires C, 58.6; H, 8.7; N, 4.9%). A mixture of the alkylated acetamidomalonate (23.4g.) and $NaHCO_3$ (20g.) in water (200ml.) was refluxed for 16hr. On cooling, and acidification to Congo Red, the decarboxylated material (DL-2-acetamido-5-methylhexanoic acid) was precipitated, and had m.p. 135–136° (Found: C, 58.1; H, 9.5; N, 7.3. $C_9H_{17}NO_3$ requires C, 57.9; H, 9.1; N, 7.5%). The racemic acid (8.5g.) was added to a solution of thionyl chloride (3.7ml.) in Grignard-dried methanol (14.3ml.) at -10° . After warming at 40° for 1½ hr. the mixture was refluxed for 2hr. and then left overnight. Excess of thionyl chloride and methanol were removed under reduced pressure, last traces of thionyl chloride being removed by addition of more methanol and re-evaporation. The resulting oil was dissolved in ethyl acetate and the solution washed successively with aq. $M-Na_2CO_3$ and water. After drying ($MgSO_4$) and evaporation of the solvent, the oil crystallized. Recrystallization from hexane gave white plates, m.p. 63–65°. Resolution of this acylated DL-amino acid ester was effected by α -chymotrypsin. Enzyme (40mg.) was added to ester (5.08g.) in 1l. of water, 0.1N-NaOH solution being added to keep the pH at 8. After 2hr. an amount of NaOH corresponding to 49–50% hydrolysis had been added. The reaction mixture was extracted with chloroform and the extracts, after drying, yielded a colourless oil on evaporation. After chromatography on a column of silica gel, a clear liquid was obtained that resisted all attempts at crystallization. This material (the D-ester) had $[\alpha]_D^{20} +19.7^\circ$ (c 2 in methanol) and $n_D^{25} 1.4588$ (Found: C, 59.6; H, 9.6; N, 8.4. $C_{10}H_{19}NO_3$ requires C, 59.7; H, 9.5; N, 7.0%). The aqueous layer from the enzyme digest was acidified with conc. HCl and extracted into ethyl acetate. Re-extraction of the L-acid into aq. 0.5M- $NaHCO_3$, followed by acidification and extraction back into ethyl acetate, gave the L-acid. This acid was re-esterified as described above and purified by column chromatography. The colourless oil of the L-methyl ester had an i.r. spectrum identical with that of the D-ester (above), $[\alpha]_D^{20} -18.9^\circ$ (c 2 in methanol) and $n_D^{25} 1.4583$.

N-Acetyl-L-glutamic acid α -methyl ester. L-Glutamic acid was benzyloxycarbonylated by the method of Bergmann & Zervas (1932), and converted into N-benzyloxycarbonyl-L-glutamic acid α -methyl ester dicyclohexylamine salt by the method of Nefkens & Nivard (1964). After removal of the dicyclohexylamine by the method of Nefkens & Nivard (1964), L-glutamic acid α -methyl ester was liberated by catalytic hydrogenation by the method of Klieger & Gibian (1962). The product had m.p. 143–146° and $[\alpha]_D^{20} +30.2^\circ$ (c 1 in methanol). Klieger & Gibian (1962) give m.p. 149–150° and $[\alpha]_D^{20} +29.7^\circ$ (c 1 in methanol). L-Glutamic acid α -methyl ester (10.2g.) and dicyclohexylamine (25.2ml.) were dissolved in chloroform (1l.) and the

solution was cooled in ice. With continuous stirring, acetic anhydride (approx. 5.75ml.) was added dropwise until the solution was negative to ninhydrin. Removal of the solvent under reduced pressure gave a yellow oil, which crystallized from methanol to give white crystals of N-acetyl-L-glutamic acid α -methyl ester dicyclohexylamine salt, m.p. 152–158°, $[\alpha]_D^{20} -11.8^\circ$ (c 1 in methanol). Further crystallization did not improve the rotation (Found: C, 62.4; H, 9.3; N, 7.5. $C_{20}H_{36}N_2O_5$ requires C, 62.5; H, 9.4; N, 7.3%). The free acid (N-acetyl-L-glutamic acid α -methyl ester) was liberated from its dicyclohexylamine salt by shaking an aqueous solution of the salt with 1.5-fold excess (in m-equiv.) of Dowex 50 (H^+ form). After removal of the resin by filtration, and of the solvent under reduced pressure, the free acid was obtained as a very pale-yellow oil. This oil was purified by chromatography on a column of silica gel to an almost colourless oil that would not crystallize. It had $[\alpha]_D^{20} -25.2^\circ$ (c 1 in methanol) (Found: C, 46.4; H, 6.8; N, 7.2. $C_8H_{13}NO_5$ requires C, 47.3; H, 6.4; N, 6.9%). The i.r. and nuclear-magnetic-resonance spectra were consistent with the structure. Examination in the mass spectrometer demonstrated the absence of any γ -methyl ester but showed a trace (approx. 0.5%) of the $\alpha\gamma$ -dimethyl ester [m/e (intensities relative to 102 peak): 43 (51%), 60 (4%), 84 (56%), 102 (100%), 144 (60%), 160 (4%), 203 (4%)].

METHODS

Microanalyses were performed by Dr Weiler and Dr Strauss in this Department.

Melting points were taken on a Kofler block and are uncorrected.

Optical rotations were measured with a Bendix-N.P.L. automatic polarimeter (type 143).

Mass spectra were recorded on an A.E.I. MS9 mass spectrograph.

All kinetic runs were followed at pH 7.90 and 25° by using a Radiometer pH-stat as described by Knowles (1965b).

Further details relevant to the particular compounds are set out below.

(a) *SS-Dimethyl-(L-3-carboxymethyl-3-acetamido)propyl sulphonium iodide.* Since this material is such a poor substrate, large concentrations of both substrate and enzyme were necessary. We have found (Ingles & Knowles, 1966) that, when the pH-stat is used, it is necessary to correct for enzyme autolysis for substrates for which the k_0/K_m ratio is smaller than approx. 0.5. Enzyme-blank corrections were therefore made for this substrate by the procedure outlined by Ingles & Knowles (1966). Substrate-blank corrections were applied in the usual way. The solutions for kinetic runs were made 0.1M in iodide ion by the addition of appropriate amounts of aq. NaI soln. Since the other substrates were run in solutions 0.1M in NaCl, kinetic runs for the sulphonium iodide were duplicated (for those runs at low $[S_0]$) in solutions 0.1M in NaCl. No effect due to changing the halide anion was apparent. Substrate concentrations were varied between 6 and 60mM; enzyme concentration was 0.142mM.

(b) *L-2-Acetamido-5-methylhexanoic acid methyl ester.* Because this material is a colourless liquid, it was necessary to test the aqueous stock solution for homogeneity. Two stock solutions, the concentrations of which differed tenfold, were prepared (5.25mM and 0.533mM). Rates of hydrolysis at a given $[S_0]$ from the two stock solutions

were identical and it was therefore concluded that the substrate had fully dissolved in both solutions. Since this compound is a good substrate, it was found that no substrate-blank correction was necessary. Substrate concentration was varied between 0.2 and 4 mM; enzyme concentration was 0.53 μ M.

(c) *N-Acetyl-L-glutamic acid α -methyl ester*. This substrate, like the sulphonium iodide, is a very poor one, and large corrections for enzyme autolysis and spontaneous substrate hydrolysis were required. These corrections essentially rule out the use of very low or very high initial substrate concentrations, respectively. This means in practice that it is impossible to work under conditions where the $[S_0]$ values used run either side of K_m . Though the gradient of the Lineweaver-Burk plot (k_0/K_m) is accurate to (say) 10%, the intercepts of this plot are so close to the origin that the values of k_0 and K_m in Table 1 for this compound are only estimates. [For a further discussion on the accuracy of kinetic parameters relating to very poor substrates, see Ingles & Knowles (1966).] Substrate concentration was varied between 8 and 200 mM; enzyme concentration was 33 μ M.

(d) *D-2-Acetamido-5-methylhexanoic acid methyl ester*. The K_i value for this compound was determined against *N*-acetyl-L-valine ethyl ester as substrate, by measurement of the effect of the inhibitor on the gradient of a Lineweaver-Burk plot (k_0/K_m), on the basis that the inhibition is competitive. A value of 16.3 mM was found. Checks on the homogeneity of stock solutions were made analogously to (b) above. Substrate concentration was varied between 27 and 200 mM; inhibitor concentration was 9.22 mM; enzyme concentration was 44.8 μ M.

Values of kinetic parameters were obtained from the raw data (except for esters I and III) by the computational procedures described by Knowles (1965b).

RESULTS AND DISCUSSION

Table 1 gives the kinetic parameters for the hydrolysis of substrates (I), (II) and (III) catalysed by α -chymotrypsin. The values for the neutral compound compare with those for the corresponding leucine compound (k_0 4.6 sec.⁻¹; K_m 2.9 mM; Hein, Jones & Niemann, 1962) and the straight-chain derivative of heptanoic acid (k_0 13.4 sec.⁻¹, K_m 1.64 mM; Jones *et al.* 1965). It can be seen from Table 1 that the introduction of a charge into the amino acid side chain of a substrate both decreases k_0 and increases K_m , in each case by about two orders of magnitude. Although substrate (III) is not sterically as close to substrate (II) as is substrate (I), the similarity is good enough to make any effect

due simply to differences in van der Waals boundaries unlikely (Fig. 1). A less-good comparison of the reactivity of substrate (III) (k_0/K_m 0.033 M⁻¹ sec.⁻¹) can be made with that of *N*-acetyl-L-glutamic acid diethyl ester where the side chain is $-\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{O}\cdot\text{C}_2\text{H}_5$, for which k_0/K_m is 640 M⁻¹ sec.⁻¹ (Cohen & Crossley, 1964).

Though it is clear from the values of k_0/K_m that charged substrates are very poorly catalysed by α -chymotrypsin, the multistep nature of the pathway of chymotrypsin-catalysed reactions (Bender & Kézdy, 1964) clouds the meaning of the individual parameters k_0 and K_m . Thus a direct comparison of the K_m values in Table 1 may not be valid, if there is a change in the rate-determining step of the catalysed reaction on going from one substrate to another. A change in K_m does not necessarily reflect a change in the true dissociation constant of ES, K_s (Knowles, 1965a), so we cannot unequivocally state that, because K_m for substrate (II) (2.83 mM) is less than K_m for substrate (I) or (III), substrate (II) binds more strongly to chymotrypsin than substrate (I) or (III). However, a reasonable estimate of K_s for substrate (II) can be obtained from the value of K_i for the corresponding D-enantiomer. This value is 16.3 mM. Since K_i values for D-inhibitors are commonly about 1½–3-fold smaller than the K_s values for the analogous L-substrates (Knowles, 1965a), 25–50 mM is the approximate value of K_s for substrate (II). We may therefore say with confidence that the introduction of a charge into the side chain of a chymotrypsin substrate increases the dissociation constant of the ES complex by at least an order of magnitude. This is expected from the known specificity of the enzyme, and further indicates the unimportance of exact steric complementarity between enzyme and substrate in the binding locus for the amino acid side chain. Such relatively indiscriminate behaviour can, in general, be anticipated in situations that primarily involve hydrophobic interactions, providing that the substrate side chain is not actually sterically larger than the binding locus.

From the k_0 values in Table 1, it is clear that the compound with a charge into the side chain has a rate constant for the rate-determining catalytic step lower by about two orders of magnitude than that for the neutral compound. The rate constants

Table 1. Kinetic parameters for the α -chymotrypsin-catalysed hydrolysis of isosteric substrates

Values relate to pH 7.90, 25° and 0.1 M-NaCl.

Substrate	Charge type	k_0 (sec. ⁻¹)	K_m (mM)	k_0/K_m (M ⁻¹ sec. ⁻¹)
(I)	Positive	0.074	350	0.212
(II)	Neutral	6.05 $\pm$ 0.29	2.83 $\pm$ 0.16	2140
(III)	Negative	$\sim$ 0.1	> 600	0.033

Fig. 1. Bond lengths (in Å) and angles of compounds related to substrates (I), (II) and (III), taken from *Tables of Interatomic Distances and Configuration in Molecules and Ions* (Chemical Society, 1959, 1965).

for OH^- -promoted hydrolysis of the three esters are close to each other: the large differences in k_0 values cannot arise from intrinsic reactivity differences of the esters, but must be ascribed to the kinetic specificity of the enzyme (Bender, Kézdy & Gunter, 1964).

Considering the relationship between K_m and k_0 for a series of substrates, two general situations can be envisaged: one where there is no detectable interdependence of these parameters, which vary independently of one another; the other where some definite relationship is apparent. The second situation is the one of interest, since the very existence of a relationship indicates that, for the series under consideration, the adsorptive forces of binding specificity are of the same type as those governing the specificity of the subsequent reaction. On the basis that a single kinetic constant is not dominating both of the operational parameters k_0 and K_m (for relevant arguments, see Knowles, 1965b), there are three ways in which K_m values can be related to k_0 values: (i) if the change in the free energy (going from one substrate to another) of binding of the reactant ($\delta\Delta G_{\text{ads.}}^{\text{r.s.}}$) is smaller than the change in the free energy of binding of the transition state of the rate-determining step ($\delta\Delta G_{\text{ads.}}^{\text{t.s.}}$); (ii) if $\delta\Delta G_{\text{ads.}}^{\text{r.s.}} > \delta\Delta G_{\text{ads.}}^{\text{t.s.}}$; and (iii) if $\delta\Delta G_{\text{ads.}}^{\text{r.s.}} = \delta\Delta G_{\text{ads.}}^{\text{t.s.}}$. In case (iii), the free energy of the Michaelis complex is decreased by the same amount as the free energy of the transition state (going from one substrate to the next), which results in a progressive decrease in K_m , while k_0 remains unchanged. In case (i), where $\delta\Delta G_{\text{ads.}}^{\text{r.s.}} < \delta\Delta G_{\text{ads.}}^{\text{t.s.}}$, decreases in K_m are paralleled by increases in k_0 . Finally, in case (ii), decreases in K_m are accompanied by decreases in k_0 .

It has been pointed out (Knowles, 1965a) that for the series of *N*-acetyl-L-amino acid ester substrates of chymotrypsin, case (i) appears to obtain, and 'better binding: better (subsequent) catalysis' is observed. The results reported in the present paper substantiate this view for three substrates, the interactions of which with the enzyme are relatively free from steric effects. The existence of such a relationship between binding specificity and kinetic specificity essentially demands that the adsorptive forces that lower K_s (resulting in better

binding) are of the same kind as those that affect the subsequent catalysis (raising k_0). This has been confirmed by the inverse correlation between the deacylation rate constants for a series of L-acyl-chymotrypsins and the inhibition constants for the corresponding D-amides (Ingles & Knowles, 1967). Moreover, as a result of the approximate inverse correlation between k_0 and K_m for a series of substrates differing only in their amino acid side chains, the adsorptive forces must affect the transition state of the rate-determining step more than the starting state. The identity of the rate-determining catalytic step is not in question here, though it should be pointed out that the mechanistic pathways for a number of hydrolytic enzymes now appear (Barman & Gutfreund, 1966; Moon, Mercouroff & Hess, 1965; Moon, Sturtevant & Hess, 1965) to involve a conformational isomerization of the Michaelis complex before formation of the acyl-enzyme. Clearly, any such isomerization of the ES complex may be subject to kinetic specificity, and the differences in k_0 values observed in the present work could arise from the rate-determining catalytic step being (or becoming) any of the following: isomerization of the ES complex, formation of the acyl-enzyme, or degradation of the acyl-enzyme.

There is no *a priori* reason why (even though the forces are similar in binding and catalysis) case (i) should obtain. Indeed, there are at least two areas where we should expect case (ii) ('better binding: worse catalysis') to be observed. If the interaction of substrate with enzyme involves significant contribution from 'wrong-way binding' modes (Huang & Niemann, 1952), then K_m will be lowered by favourable binding interactions in modes that are stereochemically unfavourable catalytically. Rapp, Niemann & Hein (1966) have designed a series of substrates that, for α -chymotrypsin, would be expected to have 'wrong-way binding' characteristics, and have demonstrated that the trend of 'better-binding: better catalysis' is not followed. The substrates used by these workers contain aromatic acylamino groups, however, and are not therefore good analogues of natural peptide substrates. For these materials to be bound in a productive ('right-way') mode, the forces of adsorption will differ from those of the non-productive mode. This will lead either to a tendency towards 'better binding: worse catalysis', or to a complete breakdown in the relationship between K_m and k_0 .

The second area in which we should expect case (ii) to be observed is really only an extension of the above, and concerns the values of the kinetic parameters for D-substrates. The D-substrate is, after all, the archetypal 'wrong-way-bound' substrate. Ingles, Knowles & Tomlinson (1966) have shown that as the binding interaction between the amino

acid side chain and the corresponding locus on the enzyme increases (in the work quoted, the series glycine, leucine, phenylalanine, tryptophan) the deacylation rate constant for the D-acyl-enzyme decreases. The details of the relationship between D- and L-acyl-enzymes have been discussed elsewhere (Ingles & Knowles, 1967).

The above arguments notwithstanding, for model L-substrates analogous to natural peptide substrates for α -chymotrypsin (where binding appears to be predominantly productive), it is evident that the postulate of 'better binding: better catalysis' holds.

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