SPECIFICITY IN DEACYLATION OF ACYL-α-CHYMOTRYPSINS

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Received April 13, 1966

Formally, there are two aspects of the specificity of α-chymotrypsin. First, the enzyme shows a marked predilection for derivatives of the more hydrophobic (notably aromatic) amino-acids, and second, amino-acid derivatives of the *L* configuration are substrates, while their *D* analogues are commonly competitive inhibitors. The former aspect can be treated in terms either of binding constants of substrates and inhibitors, or in terms of catalytic constants of substrates, and much of the binding and kinetic specificity displayed by α-chymotrypsin can readily be accounted for by the postulate that the active site of this enzyme is relatively apolar (Knowles, 1965a). The *stereo*specificity of the enzyme has, however, eluded detailed study, and all that could be said was that the K_s-values for *L* substrates appear to be slightly higher than the K_i-values of their *D* analogues.

Following a report by Zerner, Bond & Bender (1964), the present communication presents the rates of deacylation of some *D* and *L* acyl-chymotrypsins prepared from the optically pure N-acetyl-*D*- and *L*-amino-acid *p*-nitrophenyl esters. The results provide some further clues as to the origin of kinetic specificity (between amino-acids), and information on the problem of *stereo*specificity.

Materials and Methods: Optically pure N-acetyl *p*-nitrophenyl esters of the enantiomers of tryptophan, phenylalanine and leucine were prepared by a route exemplified by the following: N-carbobenzoxy-*L*-tryptophan (Cbz-*L*-Trp) $\xrightarrow[\text{DCCD}]{p\text{-nitrophenol}}$ Cbz-*L*-Trp *p*-nitrophenyl ester (Cbz-*L*-Trp-NPE) $\xrightarrow[\text{AcOH}]{\text{HBr}}$

L-Trp-NPE.HBr $\xrightarrow[\text{Ac}_2\text{O, } 0^\circ]{\text{1 equiv. of NEt}_3}$ N-acetyl-L-Trp-NPE (Ac-L-Trp-NPE).

All materials had satisfactory elemental analyses, and were crystallised to constant rotation. Ac-L-Trp-NPE had $[\alpha]_D$ = -46.3; D compound: +46.8 (c 1 in $CHCl_3$); Ac-L-Phe-NPE had $[\alpha]_D$ = -18.6; D compound: +17.4 (c 2 in $CHCl_3$); Ac-L-Leu-NPE had $[\alpha]_D$ = -55; D compound: $+54.1^\circ$ (c 1 in $CHCl_3$). For all kinetic runs, a Unicam S.P. 500 spectrophotometer fitted with a photomultiplier and coupled to a Smiths 'Servoscribe' pen recorder, was used, the appearance of p-nitrophenate at 400 mμ being followed.

Results and Discussion: The deacylation rates of the acyl-chymotrypsins are listed in Table 1. Before any comparisons can be made, however, these rates require normalisation, to take account of the differences (both steric and electronic) in inherent lability of the ester bond of the acyl-enzymes. One approach (see Bender, Kezdy & Gunter, 1964) is to use the saponification rates of the corresponding ethyl esters, but the relative inaccessibility of the ethyl ester rates, and the fact that the ethyl ester is not (as the acyl-enzyme must be) an 'active' ester, make p-nitrophenyl esters a more appropriate choice (the Hammett ρ-values for enzyme deacylation and saponification of p-nitrophenyl esters are 2.1 and 2.04 respectively). However, N-acetyl-amino-acid p-nitrophenyl esters almost certainly hydrolyse via oxazolone formation (Goodman & Stueben, 1962), and there is no evidence that such a mechanism obtains for enzymic deacylation (indeed, there are powerful arguments to the contrary). Accordingly, Table 1 contains the second-order hydroxide-ion-promoted hydrolysis rate constants for the Cbz-amino-acid p-nitrophenyl esters, and the observed deacylation rate constants are normalised taking the rate for N-acetylglycyl-chymotrypsin as unity. The value for acetyl-chymotrypsin is included in this table, and it is significant that it is higher than any of the D-acyl-enzymes. This implies that for the D compounds, the enzyme is effectively showing 'negative specificity', the origin of which will be discussed later.

A plot of log $k_3^{norm.}$ for L versus D acyl-enzymes is shown in Fig. 1. It is apparent from this figure that when one of the hydrogens of the α-carbon atom of glycine is replaced by an amino-acid side-chain, deacylation is favoured (the L series) and that when the other hydrogen is replaced, deacylation is disfavoured (the D series). Appropriately, the gradient of the line shows that the rates of deacylation are rather more sensitive to changes in the side-chain in the 'natural' L series.

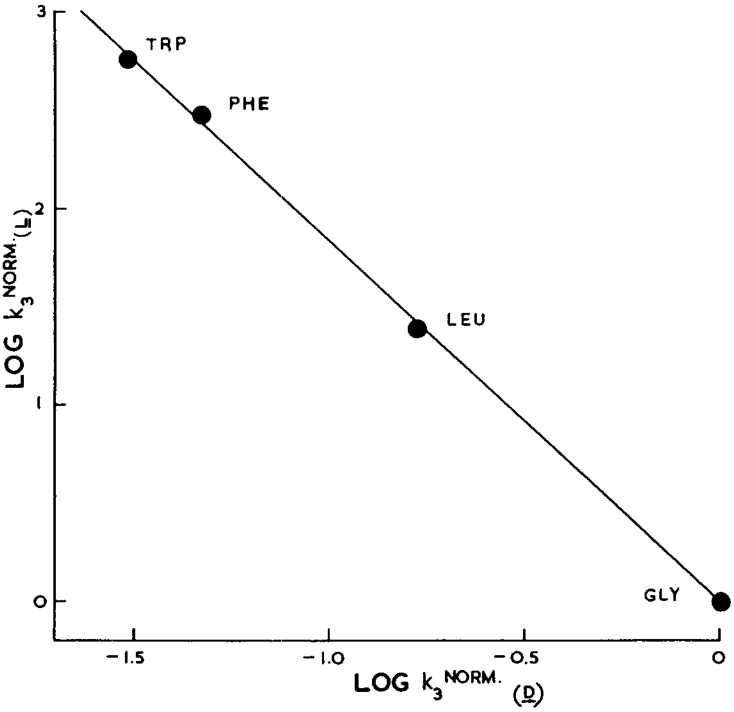

Fig. 1. Plot of log $k_3^{norm.}$ for L versus D acyl-enzymes.

These data can be interpreted in terms of a model of the acyl-enzyme (Fig. 2) where for good substrates, the acyl group is fixed at three loci: by the

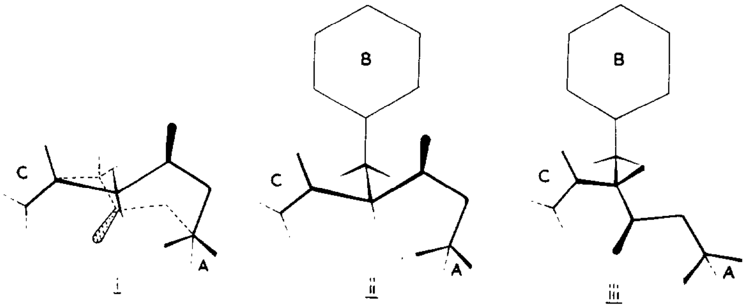

Fig. 2. Models for acyl-chymotrypsins. i: N-acetylglycyl-; ii: N-acetyl-L-phenylalanyl-; iii: N-acetyl-D-phenylalanyl-.

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covalent bond to the serine hydroxyl [A] of the enzyme (ser_{195}: Hartley, 1964), by the hydrophobic interaction between the side-chain (in Fig. 2, the benzyl group) and its corresponding locus at the active site [B] (Knowles, 1965a), and by the hydrogen bond(s) of the peptide link of the acylamino group [C] (Bender & Kezdy, 1965). For N-acetylglycyl-chymotrypsin (Fig. 2i) there are only two fixed points, and the susceptible carbonyl group can take up a number of orientations (one alternative is shown dotted in Fig. 2i). Replacement of one α-hydrogen by a benzyl group (giving the N-acetyl-L-phenyl-alanyl-chymotrypsin) fixes the orientation of the susceptible carbonyl group (Fig. 2ii), and replacement of the other α-hydrogen by benzyl (giving the N-acetyl-D-phenylalanyl-enzyme) also fixes the orientation of the carbonyl group, but in a different and (presumably) disfavoured way with respect to the catalytic functionalities of the enzyme.

This model accounts for the following facts: a) N-acetylglycyl-chymotrypsin (two points fixed) deacylates faster than acetyl-chymotrypsin (one point fixed), b) all the D acyl-enzymes deacylate more slowly than the glycyl-enzyme, c) the stronger the binding interaction of the amino-acid side-chain, the faster the L series deacylate and the more slowly the D series deacylate, [this provides some further experimental support for the ideas of 'better binding : better reaction' put

Table 1. Deacylation Rates of Acyl-α-Chymotrypsins[a]

acyl group	k_3 (sec.$^{-1}$)	k_{OH^-}[b] (M^{-1} sec.$^{-1}$)	$k_3^{norm.}$[h]
acetyl-L-Trp	52.0[c]	1.58	560
acetyl-L-Phe	94.8[c]	5.44	296
acetyl-L-Leu	5.02[c]	3.49	24.1
acetylglycyl	0.298[d]	5.08	1.0
acetyl-D-Leu	0.34[e]	3.49	0.166
acetyl-D-Phe	0.015[e]	5.44	0.0470
acetyl-D-Trp	0.0028[e]	1.58	0.0302
acetyl	0.00226[f]	0.208[g]	0.185

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[a] 25°, 0.1 μ-phosphate buffer, pH 7.00 ±0.02, 1.6% (v/v) acetonitrile, enzyme concentrations were obtained by the method of Schonbaum, et al. (1961).

[b] $\times 10^{-2}$. Values for the Cbz-amino-acid p-nitrophenyl esters.

[c] In these cases $k_2 \gg k_3$, so $k_o = k_3$. k_o -Values from Lineweaver-Burk plots following the computational procedures described in Knowles (1965b).

[d] In this case the k_2/k_3 ratio is low enough to affect the 'burst'. Accordingly, k_o-values ($= k_2k_3/k_2 + k_3$) from Lineweaver-Burk plots were combined with values of $(k_2 + k_3)/k_2$ obtained from plots of $1/\sqrt{\pi}$ versus $1/[S_o]$ (see Bender, Kezdy & Feder, 1965).

[e] The k_2/k_3 ratio in these cases is at least 20, and the small (< 5%) correction required was not applied here.

[f] Kezdy & Bender (1962).

[g] Daniels & Knowles, unpublished experiment.

[h] $k_3^{norm.i} = (k^{gly}_{OH^-} \cdot k_3^{i})/(k^{i}_{OH^-} \cdot k_3^{gly})$ where i indicates Trp, Phe or Leu.

forward earlier by one of us (Knowles, 1965a)], and d) the origin of the rate differences for deacylation of different acyl-chymotrypsins is largely entropic (Bender, Kezdy & Gunter, 1964).

One implication of the model proposed is that there should be a limiting deacylation rate. As the binding interaction of the side-chain with its locus improves, a point is reached where better binding cannot orientate the susceptible carbonyl group any 'more correctly'. This amounts to a proposal of 'specificity saturation' and there is some evidence that this is seen in the present series. In Fig. 3 are plotted values of log $k_3^{norm.}$ *versus* a measure of the efficacy of side-chain binding in α-chymotrypsin, namely the free energy of transfer of the amino-acids from water to 8M aqueous urea (Nozaki & Tanford, 1963). From this graph it appears that on going from Phe to Trp, while the binding interaction increases

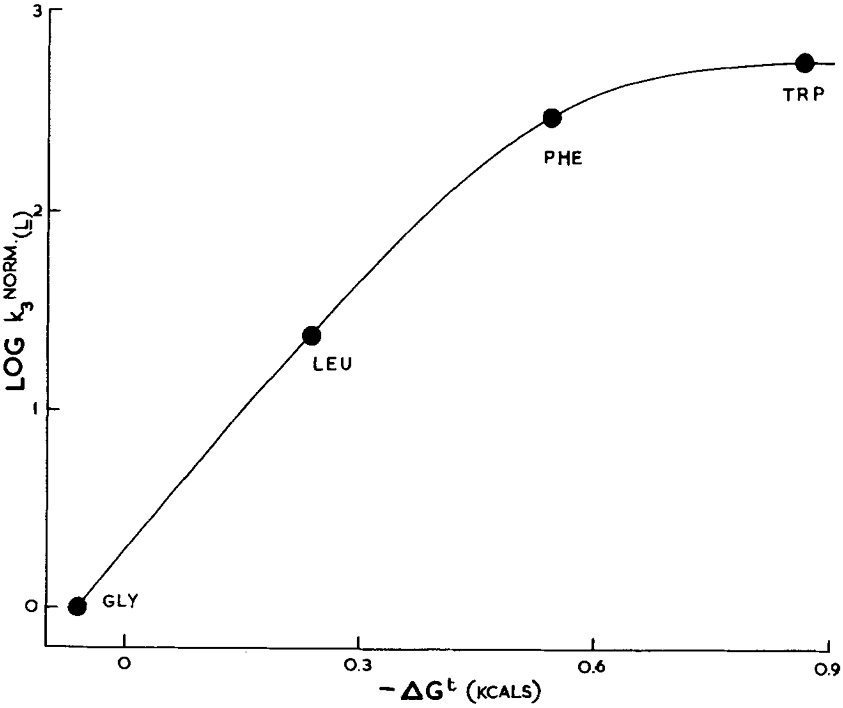

Fig. 3. Plot of log $k_3^{norm.}$ (L-series) *versus* ΔG^t for the transfer of the corresponding amino-acids from water to 8M urea.

markedly, the deacylation rate constant is only slightly increased. That is, the Phe and Trp derivatives are close to the maximum possible deacylation rate for N-acetyl-L-α-aminoacylchymotrypsins.

It must be pointed out that the above approach is based on an over-simplified situation involving a rigid enzyme and (in terms of bond lengths and bond angles) a rigid substrate. That is, induced fit or rack processes are not explicitly accounted for. At the present time, however, it seems more reasonable to take this approach, since the basic postulates should require little modification to be accommodated by a mechanism requiring enzyme and/or substrate flexibility.

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