SUBSTRATE ACTIVATION BY NON-COVALENT BINDING IN THE ACTIVE SITE OF CHYMOTRYPSIN*

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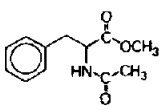
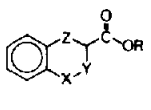
Received 10 June 1970

1. Introduction

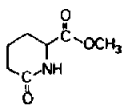
One of the possible reasons for the high efficacy of enzymic catalysis is activation of the substrate on its multidentate binding in the active site of the enzyme (so-called "rack mechanism" [1]). Such activation was demonstrated for lysozyme [2] and pyridoxal enzymes [3]. This paper presents evidence indicating that, in the case of α-chymotrypsin, multidentate binding of the substrate can also increase its intrinsic activity towards nucleophilic attack by the catalytically active enzyme groups. This suggestion is based on a comparative kinetics study of the alkaline and enzymatic hydrolysis of methyl *N*-acetyl-L-phenylalaninate, (I), and a number of its cyclic analogous, (II)–(VIII), with restricted conformation.



(I)



(II)–(VII)



(VIII)(DL)

(II) :	X–Y = CONH,	Z = CH_2,	R = CH_3(D)
(III) :	NHCO	CH_2	C_2H_5(DL)
(IV) :	COO	CH_2	CH_3(D)
(V) :	CON(Me)	CH_2	CH_3(D)
(VI) :	NHCO	NH	C_2H_5(DL)
(VII):	CH=CH	CH_2	CH_3(S)

* Part IV of a series on "Conformationally Restricted Substrates of Chymotrypsin"; Part III, L.D.Rumsh, L.I.Volkova and V.K.Antonov, FEBS Letters 9 (1970) 64.

2. Materials and methods

Compounds (I)–(IV), (VII) and (VIII) were prepared according to the procedures described in [4–9]. In order to isolate (+) - (III), the racemate was hydrolyzed with chymotrypsin at pH 7.2 (35°) in 20% aqueous ethanol. M.P. 138–139°, $[\alpha]_D^{20}$ + 43.2° (*c* 2 in $CHCl_3$). Compound (V) was prepared by methylation of (II) with CH_3I in the presence of Ag_2O. An oil was obtained of R_f 0.4 in ethyl acetate-hexane (1:2); ν:1660 (Amide I) and 1750 cm^{-1} (CO-ester). Compound (VI) was obtained by refluxing a mixture of *o*-phenylene diamine with bromo-malonic ester for 5 hr in $CHCl_3$–C_2H_5OH solution. M.P. 146–148°. α-Chymotrypsin was a crystalline preparation of the Leningrad meat-packing factory with an active enzyme content of 67–70%. The pK_a values were determined at 25° in 50% (v/v) aqueous ethanol. Alkaline hydrolysis was carried out in an aqueous KCl solution, containing 4% (v/v) CH_3CN, at pH 8.5–10.5. The k^{OH} values were calculated from the slope of the pseudo-first order linear plot of rate constants vs. pH. The course of the enzymic hydrolysis was followed with a Radiometer TTT Ic pH-stat at pH 7.2 in aqueous 0.1 M KCl solution containing 4% (v/v) of CH_3CN. The enzyme and substrate concentrations are presented in table 3. In the case of (III) the constants, obtained for the (±)-form, were corrected for inhibition by the (+)-form with K_i = 4.35 mM.

3. Results and discussion

We have recently [10] observed an anomalously

Table 1
Alkaline hydrolysis of esters (I)–(VIII) and pK_a of corresponding acids.

Compound	k^{OH} ($M^{-1}\,sec^{-1}$)	pK_a	Compound	k^{OH} ($M^{-1}\,sec^{-1}$)	pK_a
(I)	3.27	4.27	(V)	0.83	4.40
(II)	30.0	3.80	(VI)	13.7	3.90
(III)	0.26	4.55	(VII)	0.49	4.60
(IV)	117.0	3.50	(VIII)	48.4	3.77

Table 2
Activation parameters of alkaline hydrolysis.

Compound	T °K	$k' \times 10^5$ sec^{-1} (pH 8.5)	$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta F^{\ddagger}$ (kcal/mole)	$T\Delta S^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (e.u.)
(I)	298	0.5	15.4	27.1	11.7	−39.4
	303	0.68				
	307.5	1.03				
	312	1.62				
	321	3.95				
	330	8.95				
(II)	298	4.0	15.8	23.4	7.6	−25.5
	303	7.5				
	307.5	9.5				
	312	14.8				
	321	30.3				
	330	77.2				

Table 3
Enzymic hydrolysis at pH 7.2 in 0.1 M KCl with 4% (v/v) of acetonitrile.

Compound	$[S]_o$ $M \times 10^3$	$[E]_o$ $M \times 10^5$	k_{cat} sec^{-1}	$K_{m(app)}$ $M \times 10^3$	$k_{cat}/K_{m(app)}$ $M^{-1}\,sec^{-1}$
(I)	0.33–2.0	0.01	76.5	1.50	5.1×10^4
(II)	0.33–2.0	0.02	12.2	0.55	2.44×10^4
(III)*	0.4 –1.6	1.0	0.12	1.80	6.65×10^2
(IV)	0.33–2.0	0.01	35.0	0.70	5.0×10^2
(V)	2.0	1.0	does not react		
(VI)	2.0	1.0	does not react		
(VII)**	0.03	0.5			1.1×10^3

* (−)-Form. Data for the (±)-form have been corrected for competitive inhibition by (+)-(III) (K_i = 4.35 mM).
** The value $k_{cat}/K_{m(app)}$ was obtained under the conditions $[S]_o \ll K_{m(app)}$.

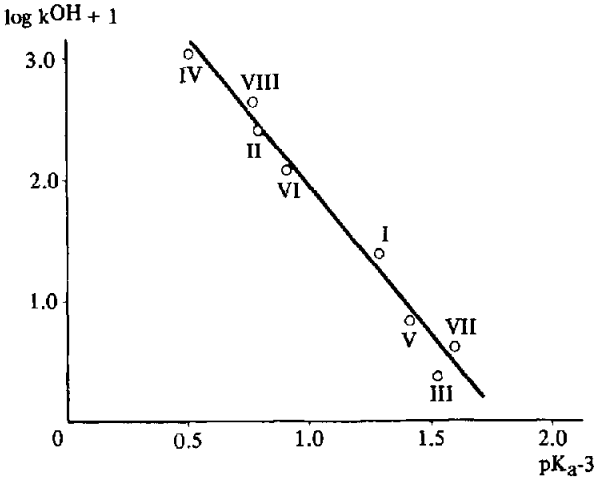


Fig. 1. Plot of the alkaline hydrolysis rates of compounds (I)–(VIII) vs. pK_a of corresponding acids.

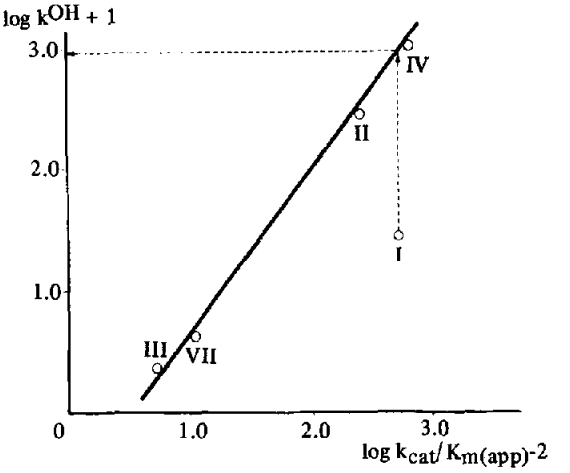


Fig. 2. Relation between chymotrypsin specificity to cyclic and linear substrates and their alkaline hydrolysis rates. Dashed line shows estimation of intrinsic reactivity of (I) on its binding in the enzyme's active site.

high rate of alkaline hydrolysis of carbomethoxydihydroisocarbostyryl, (II), in comparison with its acyclic analogue (I). We and, independently, Lawson et al. [11] have shown that this anomaly also occurs in a number of chymotrypsin cyclic substrates of the type mentioned. This is evident from the data presented in table 1.

The alkaline hydrolysis rate constants of the compounds under investigation show a satisfactory correlation with the pK_a values of the corresponding acids (fig. 1). It can be deduced, therefore, that the one and the same steric and electronic factors govern the hydrolysis rate of these compounds. In such cases it is rather difficult to distinguish between polar and steric effects; however, the following data show that the difference in the rates of alkaline hydrolysis of compounds (I) and (II) is due, mainly, to steric effects. We have studied the effect of temperature ranging from 25 to 57° on the rate of alkaline hydrolysis of these two compounds. Table 2 presents the activation parameters of the process calculated from these data, from which it can be seen that the difference in the alkaline hydrolysis rates of (I) and (II) is due to difference in the activation entropy. The transition from (I) to its conformationally restricted analogue (II) results in a change of the hydrolysis activation entropy by about 14 e.u. Now, it is known [12] that the freezing of rotation is associated with activation entropy changes of 4–6 e.u. per bond. Therefore, the activation entropy observed changes must be due to the restriction of the rotation about three bonds, viz. C_1–C_β, C_β–C_α and C_α–N.

The sorption of substrate (I) onto the active site of chymotrypsin also results in the restriction of free rotation about the same bonds due to hydrophobic bonding of the aromatic group and hydrogen bonding of the substrate's acyl NH with the corresponding enzyme loci. According to crystallographic data [13], when substrate (I) becomes attached to chymotrypsin the conformation of the former becomes similar to that of (II). One might expect that the binding of the substrate would increase its intrinsic reactivity up to a value at least commensurable with that of (II).

Table 3 presents the kinetic parameters for the enzymic hydrolysis of the compounds studied, determined under identical conditions. Only compounds (I)–(IV) and (VII) are endowed with substrate properties. For various reasons compounds (V), (VI) and (VIII) are not hydrolysed by chymotrypsin. Apparently compound (V) cannot form a productive complex since its carbomethoxy group is axial whatever the polarity of the medium [14]. Compound (III) not being aromatic does not bind to the enzyme.

Fig. 2 reveals a distinct correlation between the

rate of the enzymic hydrolysis ($\log k_{cat}/K_{m(app)}$) and alkaline hydrolysis ($\log k^{OH}$) for cyclic substrates from which the linear substrate (I) deviates. Such deviation is due apparently to the fact that the intrinsic reactivity of (I) in the active site of chymotrypsin differs from that in solution, whereas the intrinsic reactivity of the cyclic substrates (II)–(IV) and (VII) does not change since their conformation is the same in both solution and the enzyme complex.

We believe that this confirms the claim that the increase in reactivity of substrates of type (I) on binding in the enzyme's active site is only due to freezing of the internal rotation. This does not by any means exclude other ways of substrate activation by the enzyme. Such activation can be approximately evaluated since the reactivity of (I) in the enzyme active site should obey the correlation shown in fig. 2. From such an estimation it follows that sorption of the substrate leads to about a 30-fold increase in its intrinsic reactivity.

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