Phenyl Ester Hydrolysis catalysed by Alcalase. Case for Electrophilic Participation at Ester Carbonyl Oxygen

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Kinetic parameters for the hydrolysis of substituted phenyl acetates and hippurates catalysed by alcalase have been measured at 25° and 0·1M ionic strength. The pH-dependencies of the k_0/K_m parameter for 4-methoxy- and 4-chloro-phenyl hippurates and 4-nitrophenyl acetate are identical and sigmoid in shape for the three substrates. Acylation is probably the rate-limiting step in methyl hippurate hydrolyses because the value of k_0 is less than the essentially constant value for the aryl and benzyl esters. Acylation (k_0/K_m is used as a measure) is insensitive to the σ value for aryl hippurates ($\rho = 0{\cdot}35$, $r = 0{\cdot}788$) but more sensitive for the acetates ($\rho = 1{\cdot}14$, $r = 0{\cdot}868$). Electrophilic assistance by the enzyme at the ester carbonyl oxygen atom is advanced to explain part of the catalytic activity. Binding of the leaving group (aryl or benzyl) to the enzyme by lipophilic forces is also suggested to account for part of the efficiency.

ALCALASE † is a member of the subtilisin family of proteases which, unlike the digestive mammalian proteolytic enzymes, is unselective towards its amino-acid-type substrates. Subtilisins exhibit both peptidase [1] and esterase activities; [2] methyl esters of fatty acids [3] are hydrolysed as is 4-nitrophenyl acetate.[4] Dyes [5] and lipophiles such as indole and 3-phenylpropionic acid [2a] bind at the active site and inhibit the enzymes. Subtilisins are also active in hydrolysing esters of *N*-acylamino-acids [2b] which are similar in structure to the natural peptide substrates.

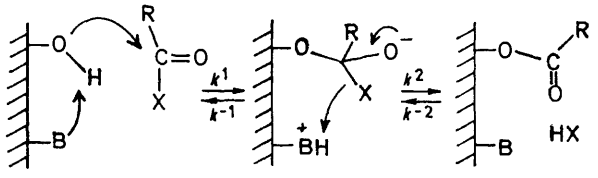
Alcalase is isolated from *Bacillus Subtilis* and is a single-strand polypeptide with 274 residues.[6] Subtilisin BPN′ (Nagarse) has a similar peptide sequence (differing at 83 residues) and 275 amino-acid residues; the *X*-ray crystallographic structure has been reported [7] and the three subtilisins, and a further enzyme, ' Novo Subtilisin ', have very similar tertiary structures.

There is much support for a three-step mechanism (1) involving an acyl–enzyme intermediate in alcalase-catalysed hydrolysis of esters and amides.[4,8] A serine hydroxy-group is implicated as the nucleophile [4,8,9] and

$$\begin{aligned} EOH + RCOX &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EOH \cdots RCOX \xrightarrow{k_2} EO{\cdot}COR + HX \\ EO{\cdot}COR &\xrightarrow{k_3} EOH + RCO_2H \end{aligned} \qquad (1)$$

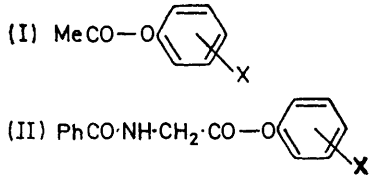
has been identified as residue 220 in alcalase.[6] An imidazolyl function is implicated in the mechanism on account of the pH-dependence of catalysis [2a,4] and is probably the side chain of histidine-63 in alcalase since the corresponding residue in subtilisin BPN′ (histidine-64) is in the active site region in the *X*-ray structure.[7]

It is a reasonable assumption that acylation of the enzyme by substrates involves, apart from the physically bound intermediate, a tetrahedral intermediate. Amide or alkyl ester substrates would be expected to require general acid catalysis to aid departure of the poor leaving group from the tetrahedral intermediate by analogy with other proteolytic enzymes.[10] Phenyl



(2)

ester substrates would involve a rate-limiting formation of the tetrahedral intermediate and thus acylation refers to the k^1 step. Any electrophilic assistance can therefore be assigned unequivocally to the k^1 step and the esters (I)—(VI) were studied with this aim.



X	X	X	X
a; H	d; 3-NO_2	g; 4-F	j; 4-NO_2
b; 4-Me	e; 4-Cl	h; 4-HCO	k; 4-NH_2
c; 4-MeO	f; 3-Cl	i; 4-Ac	

(III) $PhCO{\cdot}NH{\cdot}CH_2{\cdot}CO{-}OMe$

(IV) $PhCO{\cdot}NH{\cdot}CH_2{\cdot}CO{-}OCH_2Ph$

(V) $MeCO{\cdot}NH{\cdot}CH_2{\cdot}CO{-}OMe$

(VI) $MeCO{\cdot}NH{\cdot}CH_2{\cdot}CO{-}OCH_2Ph$

† Alternatively known as Carlsberg subtilisin.

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[9] F. Sanger and D. C. Shaw, *Nature*, 1960, **187**, 872.
[10] (*a*) A. Williams, *Biochemistry*, 1970, **9**, 3383; (*b*) A. Williams and G. Salvadori, *J. Chem. Soc.* (*B*), 1971, 1105; (*c*) G. Lowe and Y. Yuthavong, *Biochem. J.*, 1971, **124**, 117 (communicated prior to publication by G. Lowe); (*d*) References quoted in refs. 10*a*—*c*.

EXPERIMENTAL

Materials.—Alcalase was donated by Novo Industri (Copenhagen) as a freeze-dried solid. The aryl acetates (I) were prepared by Dr. G. Salvadori for a different study and their physical properties are recorded elsewhere.[11] The aryl hippurates (II) were prepared for a different investigation.[10a] The following esters were as previously described: methyl hippurate (III),[12] benzyl hippurate (IV),[13] methyl acetylglycinate (V),[12] and benzyl acetylglycinate (VI).[12] *N*-Cinnamoylimidazole was prepared according to Schonbaum *et al.*[14] and had m.p. 133—134° (lit.,[14] m.p. 133—133·5°). Acetonitrile was purified by the method of Lewis and Smyth [15] and only fractions with low aromatic absorption were used. Other materials were AnalaR grade and water was glass-distilled.

Kinetics.—Reactions with the aryl esters (I) and (II) were followed at a suitable u.v. wavelength (Table 1) in the thermostatted cell compartment of a Beckman-DBG spectrophotometer equipped with a linear-logarithmic converter and a Smith's Industries Servoscribe recorder. A typical experiment involved equilibration of buffer and substrate at 25° in a silica cell and the determination of a base line for buffer catalysis (usually minimal). Enzyme was added as a solution on the tip of a glass rod and the initial rate of hydrolysis was measured; total hydrolysis under identical conditions gave the change in extinction coefficient for the reaction. Hydrolysis of alkyl hippurates and acetylglycinates was followed using the pH-stat method (Radiometer, Copenhagen).[12] Michaelis–Menten parameters [see equation (3)] were determined from initial rates using an algorithm [16a] based on the Wilkinson [16b] method. Dartmouth Basic was also employed in these calculations using the Kent 'On-line' system (the assistance of Dr. N. J. Bridge is acknowledged) and a central Elliott 4130 computer. Enzyme concentration was determined by the method of Bender [17] at pH 4·6 using *N*-cinnamoylimidazole. pH-Profiles for 4-chloro- and 4-methoxy-phenyl hippurates were determined using $[S] < K_m$ [see equation (3)] so that measurements could be made using the same enzyme solution over a short time period, thus eliminating errors due to enzyme assay.

TABLE 1

Michaelis–Menten parameters for hippurate and acetate substrates of alcalase [a]

Substrate	λ/nm	Δε	k_0/K_N/l mol^{-1} s^{-1}	k_0/s^{-1}	10^3K_m/mol l^{-1}
			Hippurates [b]		
(IIk)	310	1140	0·499	1·6 ± 0·05	0·321 ± 0·01
(IIc)	290	2125	0·925	1·5 ± 0·05	0·162 ± 0·01
(IIb)	285	650	1·67	1·55 ± 0·06	0·0928 ± 0·005
(IIa)	270	1350	1·27	1·61 ± 0·05	0·127 ± 0·01
(IIg)	280	1630	1·32	1·70 ± 0·08	0·129 ± 0·01
(IIe)	275	897	1·58	1·62 ± 0·04	0·103 ± 0·01
(IIi)	325	4450	2·46	1·54 ± 0·04	0·626 ± 0·01
(IIj)	350	4950	2·30	1·58 ± 0·07	0·0687 ± 0·005
(IId)	355	1340	1·30	1·60 ± 0·04	0·123 ± 0·01
(III) [c,d]			0·140	0·77 ± 0·05	0·55 ± 0·04
(IV) [c]			3·68 × 10^{-4}	0·21 ± 0·01	57 ± 3
			Acetates		
(Ik)	310	1240	23 ± 1		
(Ib)	285	950	61 ± 3		
(Ij)	350	5000	1250 ± 19	0·49 ± 0·02	0·39 ± 0·01
(Ig)	280	1735	54 ± 2		
(Ic)	290	2940	28 ± 1	0·56 ± 0·03	2·0 ± 0·1
(Ia)	270	2185	62 ± 1	0·78 ± 0·03	12·5 ± 0·5
(Ih)	330	6590	912 ± 21	0·37 ± 0·01	0·40 ± 0·02
(Ie)	275	950	125 ± 5		
(Ii)	325	6280	408 ± 3	0·29 ± 0·01	0·72 ± 0·03
(Id)	355	3700	190 ± 4		
(If)	280	1450	145 ± 4		
			Acetylglycinates		
(V) [c,e,f]			2·76 ± 0·05		
(VI) [c,f]			88	3·68 ± 0·08	42 ± 3

[a] pH 7·00; phosphate buffer; 0·1M ionic strength, T = 25°. [b] k_0/K_m Has been multiplied by 10^{-4}. [c] Kinetic parameters determined with a pH-stat. [d] 10% CH_3·CN. [e] Concentration in substrate taken to 0·08M without saturation being observed. [f] We thank Mrs. H. C. Hawkins for assistance in measuring the kinetic parameters of these substrates.

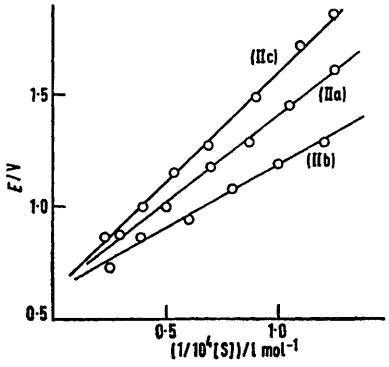

FIGURE 1 Typical kinetic data for hippurate ester substrates. Lines are theoretical from parameters in Table 1

RESULTS

Kinetics of alcalase-catalysed hydrolyses obeyed the rate law (3) although some of the aryl acetates did not

$$\text{Rate} = k_0[E][S]/([S] + K_m) \qquad (3)$$

exhibit 'saturation' kinetics at the concentrations employed. Results for pH 7·00 are recorded in Table 1 (Figure 1 illustrates typical Michaelis–Menten plots) and those for the pH-dependence of k_0/K_m for 4-chloro- and 4-methoxy-phenyl hippurate are in Table 2 (Figure 2). The pK_a values derived from the pH-profiles are almost identical and the profiles do not show a decrease at pH >9. Where comparisons are possible our kinetic results agree with those of other workers.

Apparent ionisation constants for 4-chloro- and 4-methoxy-phenyl hippurates agree with those previously determined,[2a,4] and as for 4-nitrophenyl acetate,[4] the pH-dependence is sigmoid up to pH 10.

[11] A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1967.
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[15] G. L. Lewis and C. P. Smyth, *J. Chem. Phys.*, 1939, **7**, 1085.
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[17] M. L. Bender, M. L. Begué-Cantón, R. L. Blakeley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, 1966, **88**, 5890.

The k_0 parameters for the hippurates are essentially constant, but the parameter k_0/K_m (a measure of acylation [18]) obeys a Hammett relationship with sensitivity

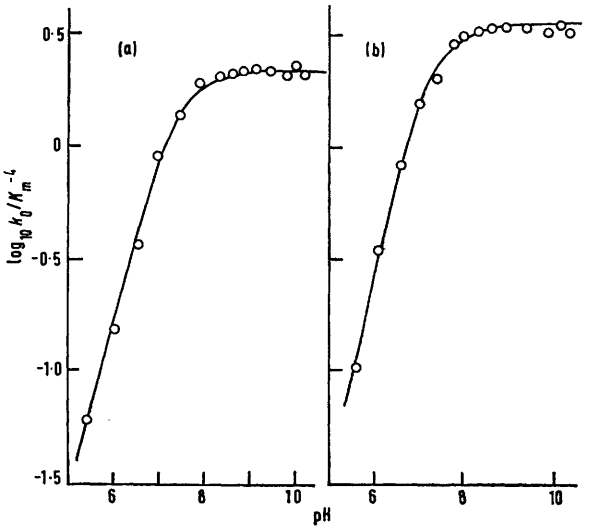

FIGURE 2 pH-Dependence of k_0/K_m for (a), 4-methoxyphenyl (IIc) and (b), 4-chlorophenyl hippurate (IIe). Data from Table 2; lines are theoretical from parameters in Tables 1 and 2

TABLE 2

pH-Dependence of k_0/K_m for hippurate ester substrates of alcalase [a]

pH	$10^{-4}k_0/K_m$/ l mol^{-1} s^{-1}	pH	$10^{-4}k_0/K_m$/ l mol^{-1} s^{-1}
4-Methoxyphenyl [f]		4-Chlorophenyl [f]	
5·43 [b]	0·0616	5·6 [b]	0·102
6·00 [c]	0·154	6·1 [c]	0·344
6·50 [c]	0·370	6·6 [c]	0·843
7·00 [c]	0·925	7·0 [c]	1·58
7·45 [c]	1·39	7·3 [c]	2·07
7·95 [d]	1·91	7·8 [d]	2·91
8·37 [d]	2·06	8·0 [d]	3·12
8·64 [d]	2·13	8·3 [d]	3·16
8·84 [d]	2·19	8·6 [d]	3·34
9·12 [d]	2·22	8·9 [d]	3·34
9·42 [e]	2·22	9·3 [e]	3·48
9·78 [e]	2·13	9·8 [e]	3·34
10·00 [e]	2·25	10·1 [e]	3·51
10·17 [e]	2·13	10·2 [e]	3·44

[a] T = 25°, 0·1M ionic strength. [b] Acetate buffer. [c] Phosphate buffer. [d] Tris(hydroxymethyl)aminomethane buffer. [e] Carbonate buffer. [f] pK_a 7·15 (4-methoxyphenyl), 7·10 (4-chlorophenyl).

0·35 (r = 0·788). The k_0 parameters for the phenyl acetates are not constant but the k_0/K_m parameter obeys a Hammett relationship with sensitivity 1·14 (r = 0·868). The linear free energy relationships are illustrated in Figures 3 and 4.

DISCUSSION

The substantially constant value of k_0 for aryl hippurates is in accord with an acyl–enzyme mechanism [equation (1)] where $k_3 < k_2$; k_0 is then identified as k_3 [equation (4)]. Provided the identical mechanism holds for the hydrolysis of aryl acetates then $k_2 \gtrsim k_3$ for

$$k_0 = k_2 \cdot k_3/(k_2 + k_3) \qquad (4)$$

these substrates because the value of k_0 is not constant.

The parameter k_0/K_m is essentially the bimolecular rate constant for acylation of free enzyme by free substrate; [18] for phenyl esters this is identified as k^1 [equation (2)] and includes both binding and catalytic terms. The sensitivity of this parameter to substituents on the phenyl leaving group (0·35) is very small for the hippurate series. Data for the alkaline hydrolysis of *N*-benzyloxycarbonylglycine aromatic esters [19] yield a Hammett ρ value of *ca.* 1·2 and these esters are sufficiently close electronically to the hippurates to be used as models. It is to be expected that general base-catalysed attack of water on such esters would have a higher Hammett sensitivity because the nucleophile is weaker than hydroxide; [20] general base-catalysed

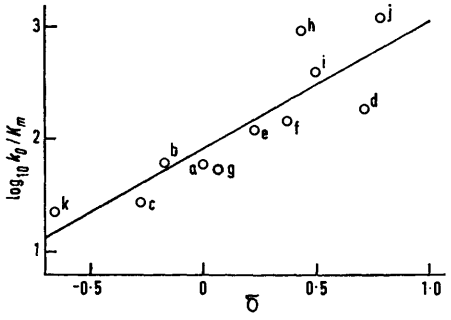

FIGURE 3 Dependence of k_0/K_m for phenyl acetates on the σ value of the substituents. Correlation line has slope ρ = 1·14 (r = 0·868); data from Table 1

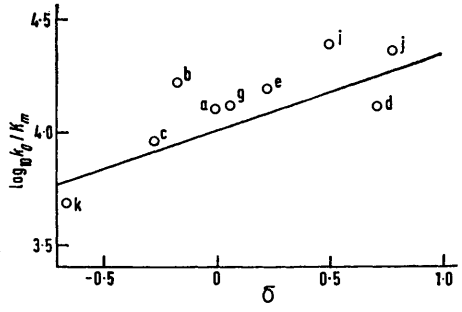

FIGURE 4 Dependence of k_0/K_m for phenyl hippurates on the σ value of the substituents. Correlation line has slope ρ = 0·35 (r = 0·788); data from Table 1

hydrolysis of substituted phenyl diphenylphosphinates [11] has a Hammett ρ value of 2·88 as opposed to one of 1·55 for the hydroxide reaction; also the Taft ρ* value for reaction of water with ethyl esters is 3·5 [21a] as opposed to the sensitivity of 2·5 [21b] for reaction of hydroxide ion. It might be expected that a variation of ρ between intramolecular (enzyme) and intermolecular

[18] See A. Williams and R. A. Naylor, *J. Chem. Soc.* (*B*), 1971, 1973, for a justification of this.
[19] J. F. Kirsch and M. Igelstrom, *Biochemistry*, 1966, **5**, 783.
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[21] (*a*) W. P. Jencks and J. Carriuolo, *J. Amer. Chem. Soc.*, 1961, **83**, 1743; (*b*) R. W. Taft, 'Steric Effects in Organic Chemistry,' ed. M. S. Newman, Wiley, New York, 1956, p. 556.

(model) reactions would occur, but work of Bruice and Benkovic [22] is against this. The sensitivity of the enzyme reaction (a general base-catalysed attack of alcohol) should therefore be $>1{\cdot}2$. We interpret the low sensitivity to the σ value as due to assistance in the k^1 step arising from stabilisation of the tetrahedral transition state (of the incipient alkoxide ion by an electrophile), binding of the ester carbonyl oxygen atom with an electrophile in the ground state, or both forms of interaction. Electrophilic interaction at the ether oxygen atom of the ester is possible but needs energy supplied from other sources as it tends to destroy the resonance energy of the ester bond. It is difficult to eliminate strain as the cause of the low sensitivity but again this mechanism requires energy to break the resonance hybrid.

Acylation by phenyl acetates has a larger sensitivity to the leaving group than that by the hippurates, and both sensitivities are close to the corresponding values for acylation of chymotrypsin.[10a,23] Although esters of the hippurate type are considerably more reactive to hydroxide ion than are acetates, sensitivities to substituents in the phenyl leaving group are identical. Thus it is probable that relatively less electrophilic participation at the ester carbonyl oxygen atom occurs in phenyl acetate hydrolysis, and we ascribe this to the different reactive binding mode which places the carbonyl in a non-electrophilic situation.*

Unlike chymotrypsin,[10a] alcalase exhibits a large difference between the k_0/K_m value for methyl and for 4-nitrophenyl hippurate. Benzyl esters also have a higher k_0/K_m value than the methyl ester (Table 1). The enhancement is partly electronic in the case of the former pair but the benzyl ester should be intermediate in reactivity between ethyl and methyl esters.[25] Possibly the active site contains a lipophilic region which binds lipophilic leaving groups and orientates them so that the ester carbonyl group is set up close to the nucleophilic serine. This is in contrast to chymotrypsin, where it is thought that the leaving group is not bound to the enzyme.[26] This study confirms the existence of a lipophilic site at the active site of the enzyme, suggested by the work of Glazer [2a,5] with dyes and inhibitors. Studies with papain [12,27] have shown that specific substrates $R^1CO{\cdot}NH{\cdot}CHR^2{\cdot}$-COX are more effective when R^1 is aromatic than when it is a methyl group, suggesting that R^1 binds to a corresponding lipophilic site in the active centre. The similarity of the k_0/K_m value for methyl acetylglycinate and hippurate (Table 1) suggests the absence of such a site in alcalase.

The pH-dependence of the k_0/K_m value confirms a further difference from chymotrypsin, namely the absence of a salt bridge maintaining the active conformation of the enzyme.[7,28] Since alcalase does not require an acidic group of pK_a 9 for activity with specific substrates, this provides further evidence that this pK_a value is not essential for the chemical mechanism of chymotrypsin or trypsin.

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* A Referee has suggested that the Hammett sensitivity to the leaving group supports the mechanism where the serine alcohol is unusually reactive owing to its deprotonation in an essentially non-aqueous environment (C. A. Vernon, quoted in ref. 24). This mechanism was originally proposed for chymotrypsin,[24] another member of the serine group of proteases. The observation of a *different* Hammett sensitivity for hippurates and acetates does not easily follow from a consideration of this mechanism although we do not in this paper discount the possible coexistence of the mechanism as a contributor to reactivity.

[22] T. C. Bruice and S. J. Benkovic, *J. Amer. Chem. Soc.*, 1963, **85**, 1.
[23] (*a*) R. E. Williams and M. L. Bender, *Canad. J. Chem.*, 1971, **49**, 210; (*b*) M. L. Bender and K. Nakamura, *J. Amer. Chem. Soc.*, 1962, **84**, 2577.
[24] D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, 1969, **221**, 337.
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[26] T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, 1969, **46**, 337.
[27] G. Lowe and E. C. Lucas, unpublished results quoted in G. Lowe, *Phil. Trans.*, 1970, *B***257**, 237.
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