

Proteolytic Enzymes: Lipophilic Binding Sites for Specific and Non-specific Substrates of α -Chymotrypsin¹

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Evidence is presented consistent with a lipophilic binding site for the leaving group and for the alkyl or aromatic function of the acylamino-group in specific substrates of α -chymotrypsin. Binding of small, non-specific substrates and inhibitors to α -chymotrypsin is shown, by comparison with elastase, to involve at the most only a small fraction of the 'tosyl-hole.'

THE selectivity of α -chymotrypsin towards aminoacyl residues possessing an aromatic side chain has been explained by a lipophilic interaction between a side chain and a 'pocket' close to the serine-195 residue called the 'tosyl-hole'.^{2a-d} This interaction contributes to binding and at the same time helps to align the acyl function correctly for acylation by the serine-195 residue of α -chymotrypsin.² A number of small compounds such as 4-nitrophenyl acetate³ not related to amino-acids also acylate α -chymotrypsin and dioxan inhibits the enzyme by binding in the 'tosyl-hole';^{2c,4} it is a reasonable proposal that small acylating agents act by virtue of a prior binding in the lipophilic pocket followed by an intra-complex acylation.

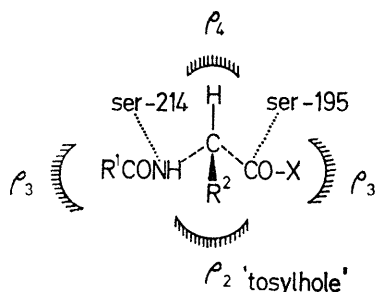


FIGURE 1 Binding of a specific substrate to α -chymotrypsin⁶

A binding site has been proposed for the attacking nucleophile and studies of alcoholysis⁵ have provided some evidence for this; the alcohol binds at a site on the enzyme excluding water. This site presumably corresponds to the ρ_3 -site using the nomenclature of Hein and Niemann (see Figure 1).^{6a} † Application of structure-reactivity relationships with varying leaving group^{7a} also indicates some binding of the leaving group (presumably related to the nucleophile binding by the

† In this study we define an L-amino-acid derivative as a specific substrate; presumably its binding action is related to the more physiological polypeptide substrate where binding sites removed from the 'active-centre' region have been postulated.^{7b}

‡ For details of Supplementary Publications see Notice to Authors No. 7 in *J.C.S. Perkin II*, 1975, Index issue. Items less than 10 pp. are supplied as full-size copies.

¹ A preliminary report of some of this work was given in a plenary lecture before the German Biochemical Society, Bochum, 1972; A. Williams and J. Udris, *Hoppe-Seyler's Z. Physiologische Chem.*, 1972, **353**, 688.

² (a) B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, 1967, **214**, 652; (b) P. B. Sigler, D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, 1968, **35**, 143; (c) T. A. Steitz, R. Henderson, and D. M. Blow, *ibid.*, 1969, **46**, 337; (d) R. Henderson, *ibid.*, 1970, **54**, 341; (e) J. Kallos and K. Avatis, *Biochemistry*, 1966, **5**, 1979.

³ F. J. Kezdy and M. L. Bender, *Biochemistry*, 1962, **1**, 1097.

principle of microscopic reversibility) and although there is not a large variation in lipophilic character in the leaving alcohols studied the interaction could be lipophilic in nature.^{7a}

Binding of the acylamino-group with the ρ_1 -site of the enzyme has been proposed to involve hydrogen bonding between the amido-NH of the substrate and the peptide carbonyl oxygen of serine-214;^{2d} Hansch and Coats^{7a} have indicated that the aromatic or alkyl function also has an effect on the interaction with ρ_1 but the available data were not sufficient to give a definite identity to the type of binding although there seems to be a trend favouring a polar type.

It is the purpose of this study to investigate the binding of small molecules and the nature of the ρ_1 and ρ_3 interactions.

EXPERIMENTAL

Materials.—The ester substrates were from work already reported^{8a-d} except the substituted methyl *N*-benzoylglycinates (m.p.s. in Table I) which were prepared *via* a

TABLE I

M.p.s for new substituted *N*-benzoylglycine methyl esters

Substituent	3-nitro	2-nitro	4-chloro	4-methyl
M.p./°C ^a	98–100	100–102	119–120	92–94

^a Measured with a Kofler Thermospan instrument.

Schotten-Baumann acylation of methyl glycinate with the corresponding benzoyl chloride. The benzenesulphonyl halides were prepared according to literature methods and the m.p. data together with references are given in Supplementary Publication No. SUP 21677 (2 pp.) ‡ which also contains analyses for new compounds. The identity of the substrates was checked using i.r. and n.m.r. (Perkin-Elmer R10 instrument) spectroscopy.

Chymotrypsin was obtained from Boehringer and Biebrich Scarlet (water soluble) from Hopkins and Williams was

⁴ R. P. Bell, J. E. Critchlow, and M. I. Page, *J.C.S. Perkin II*, 1974, 66.

⁵ M. M. Werber and P. Greenzaid, *Biochim. Biophys. Acta*, 1973, **293**, 208.

⁶ (a) G. E. Hein and C. Niemann, *J. Amer. Chem. Soc.*, 1962, **84**, 4487, 1195; (b) C. L. Hamilton, C. Niemann, and G. Hammond, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **55**, 664.

⁷ (a) C. Hansch and E. Coats, *J. Pharm. Sci.*, 1970, **59**, 731; (b) D. M. Segal, G. H. Cohen, D. R. Davies, J. C. Powers, and P. E. Wilcox, Brookhaven Symposia on Quantitative Biology, 1971, vol. 36, p. 85.

⁸ (a) A. Williams, E. C. Lucas, A. R. Rimmer, and H. C. Hawkins, *J.C.S. Perkin II*, 1972, 627; (b) A. Williams, E. C. Lucas, and A. R. Rimmer, *ibid.*, p. 621; (c) E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125; (d) G. Lowe and A. Williams, *Biochem. J.*, 1965, **96**, 199; (e) R. Jayaram and I. D. Rattee, *Trans. Faraday Soc.*, 1971, **67**, 884.

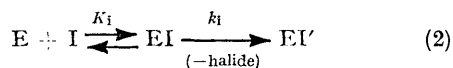
recrystallised according to the method of Jayaram and Rattee;^{8c} other reagents were from B.D.H. Acetonitrile was purified from the AnalaR material by distillation from P₂O₅. Twice distilled water (from glass) was used throughout this work.

Methods.—Substrates. The hydrolysis of esters catalysed by α -chymotrypsin was followed using a Radiometer pH-stat assembly (Titratigraph type SBR2c, titrator 11, pH meter 25, and syringe burette SBU1a). A stock solution of the enzyme (1 ml in 0.1M-NaCl) was added to 9 ml of a solution of substrate dissolved in acetonitrile (where appropriate) in a final NaCl concentration of 0.1M. The pH was kept at a constant value (see Table 2) by addition of 0.01M-NaOH titrant. Hydrolysis of substrate in the absence of enzyme was checked and the final initial rate corrected accordingly. The initial rates from these experiments were fitted to the Henri law [equation (1)] using the method of Wilkinson as modified by us for use with Basic Language.⁹ The Kent 'on-line system' was employed to compute k_0 and K_m values. In some cases the initial rate was proportional to substrate concentration over the total range used and the slope of the plot of initial rate *versus* concentration ($[E]k_0/K_m$) was used to give k_0/K_m . Enzyme concentration

$$\text{initial rate} = [E][S] k_0 / ([S] + K_m) \quad (1)$$

was estimated by titration with 4-nitrophenyl acetate¹⁰ using the absorption maximum of 400 nm at pH 7.00 and a Beckman-DGB spectrophotometer.

Sulphonyl halide inhibitors. The method employed to measure the reaction of sulphonyl halide with chymotrypsin involves the expulsion of a dye (Biebrich Scarlet) from the active site of the enzyme as sulphonylation proceeds; concomitant with dye expulsion is a spectral change at 550 nm which is exploited to follow the progress of the inhibition.¹¹ Two observations are important: (a) the effect of the inhibitor at zero time on the absorption at 550 nm and (b) the rate of decrease in absorption as dye is expelled from the active site. The former gives direct information (knowing the inhibitor, dye, and enzyme concentration and the enzyme-dye dissociation constant) about the enzyme inhibitor dissociation constant K_i [see equation (2)]. The



latter gives essentially the rate of inactivation of enzyme by inhibitor (k_i).

Dye solution (50 λ) was added to a solution of the enzyme in buffer and the trace at 550 nm was recorded on a Servoscribe potentiometric recorder. The inhibitor in acetonitrile (50 λ) was then added and the trace, involving expulsion of dye, measured. The magnitude of the dye-enzyme complex absorbance compared with dye alone was used as an internal measure of the enzyme concentration.¹¹ The values for K_i were calculated from the initial absorbances (estimated by extrapolation to zero time) and the k_i values from the decay portion of the trace as described by Gerig and Roe;^{11b} the values for the extinction coefficients of the dye, dye-enzyme complex, and the dissociation

constant for the latter are taken from Gerig and Roe's work.^{11b}

RESULTS

Most of the substrates hydrolyse according to the rate law of equation (1) but some have K_m values too high to enable dissection of k_0/K_m into its components. The parameters obtained are collected in Table 2 and a Hofstee plot¹² indicating the fit of the data for a typical substrate is illustrated (Figure 2). Agreement of results with other workers is good and is noted in Table 2.

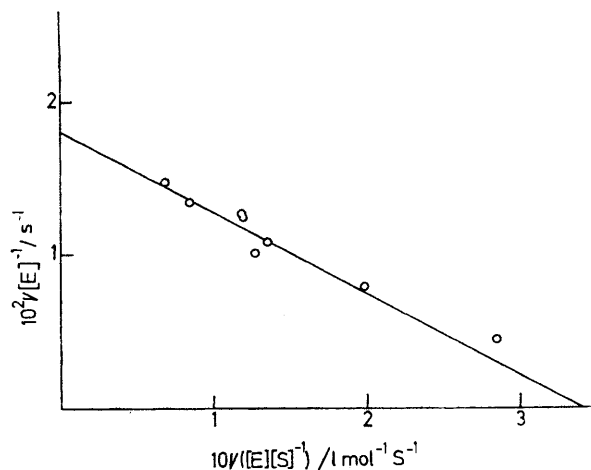


FIGURE 2 Methyl 3-nitrobenzoylglycinate as substrate for α -chymotrypsin; pH 7.00, ionic strength made up to 0.1M with NaCl, 25°

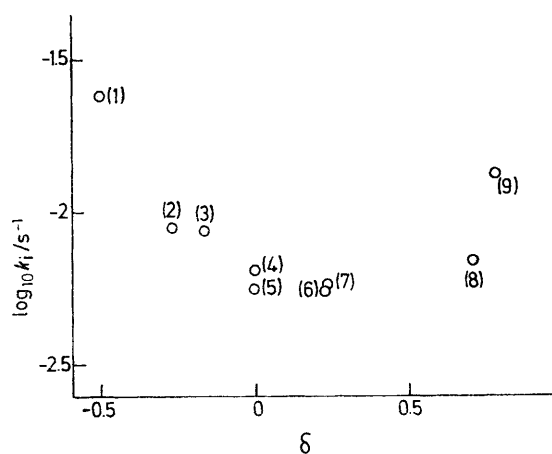


FIGURE 3 Dependence of k_i on σ for substituted benzenesulphonyl chlorides and α -chymotrypsin. Values of σ are from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75. Points are (1) 2,4,6-trimethyl; (2) 4-methoxy; (3) 4-methyl; (4) 4-acetamido; (5) unsubstituted; (6) 4-chloro; (7) 4-bromo; (8) 3-nitro; (9) 4-nitro

Inhibition reactions with sulphonyl halides followed first-order kinetics closely similar to those reported by Gerig and Roe^{11a} and values of k_i/K_i were obtained from the variation of the pseudo-first-order rate constant with inhibitor concentration; multiplication of this rate constant by K_i

¹¹ (a) A. N. Glazer, *J. Biol. Chem.*, 1967, **242**, 4528; (b) J. T. Gerig and D. C. Roe, *J. Amer. Chem. Soc.*, 1974, **96**, 233.

¹² Ref. 9, p. 17.

⁹ A. Williams, 'Introduction to the Chemistry of Enzyme Action,' McGraw-Hill, London, 1969, p. 119.

¹⁰ M. L. Bender, M. L. Begue-Canton, R. L. Blakeley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kezdy, J. V. Killheffer, T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, 1966, **88**, 5890.

TABLE 2
 Kinetic parameters for substrates of α -chymotrypsin^b

Substrate	k_o/s^{-1}	$10^{-3}K_m/mol\ l^{-1}$	$10^{-3}S/mol\ l^{-1}$	$k_o/K_m^{-1}\ l\ mol^{-1}\ s^{-1}$
Benzoylglycine methyl esters				
4-Chloro *			1—11	2.4
4-Nitro *			2—8	2.9
1 3-Nitro *	0.018	5.4	1—20	3.4
2-Nitro *	0.028	28.9	1—30	1.0
4-Methyl *			10—30	5.0
Parent	0.14 (0.205) ^a	4.2 (6.6) ^a	1—30	33 (31) ^a
Parent *	0.048	11	1—30	4.3
Benzyl formate *			0.5—20	4.1 (3 000) ^d
2 Methyl formate			0.2—4	< 0.01 (3 000) ^d
Benzyl acetate			0.2—20	< 0.01 (1) ^d
Benzyl acetylglucinate	0.038	5.3	1—10	7.1
3 Methyl acetylglucinate	0.0092	31	1—40	0.3 (0.437) ^e
Cyclohexylmethyl acetylglucinate			0.01—0.1	2.7
4 Benzyl benzoylglycinate *	0.092	0.21	0.01—0.5	446
(Methyl benzoylglycinate *) ^j				(4.3) ^j
Methyl benzoylglycinate) ^j				(33) ^j
5 (Methyl acetylglucinate) ^j				(0.3) ^j
Methyl cyclohexylacetylglucinate	0.75	102	10—200	7.6
6 (Ethyl benzoylglycinate) ^j				(4.9) ^j
(Ethyl Z-glycinate) ^{j,k}				(3.1) ^j
7 Methyl benzoyl-L-phenylalaninate *	200	0.18	0.01—0.5	1.1×10^6
Methyl acetyl-L-phenylalaninate	63	1.8	0.1—5	3.5×10^4
8 Methyl benzoyl-L-alaninate				24 ^g
Methyl acetyl-L-alaninate				1.72 ^e
9 Ethyl benzoylglycinate *	0.061	12.4	1—40	4.9
S-Ethyl N-benzoylthioglycinate *	0.23 ^h	0.85	0.2—4	272
10 Ethyl acetylglucinate ^e	0.039	0.389		0.101
S-Ethyl N-acetylglucinate ^e	0.23	0.041		5.61
Ethyl Z-glycinate ^f				3.08
11 S-Ethyl Z-thioglycinate ^f	0.276			420
4-Nitrophenyl Z-glycinate ^f	0.261			
12 Ethyl acetyl-L-phenylalaninate ^f				54 000
S-Ethyl N-acetyl-L-thiophenylalaninate ^f				1 000 000

* Values are for 10% acetonitrile solution; these are lower than in water (see methyl benzoylglycinate parameters in group 1).

^a T. H. Appleywhite and C. Niemann, *J. Amer. Chem. Soc.*, 1959, **81**, 2208. ^b Ionic strength made up to 0.1M with NaCl, pH 6.90, 25°, except where stated there is no added organic solvent. ^c J. P. Wolf and C. Niemann, *Biochemistry*, 1963, **2**, 82 (for pH 7.9). ^d Relative rate of reaction with hydroxide ion. ^e Ref. 24, pH 7.0. ^f L. Polgar, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 1972, **7**, 319 (pH 8.0, 1.7% acetonitrile). ^g J. R. Rapp, C. Niemann, and G. E. Hein, *Biochemistry*, 1966, **5**, 4100 (pH 7.9). ^h This value is probably close to k_3 [see equation (3)] because B. Zerner and M. L. Bender, *J. Amer. Chem. Soc.*, 1964, **86**, 3669 find k_o 0.5 s⁻¹ for 4-nitrophenyl benzoylglycinate in aqueous solution at pH 7.0, 25°. ⁱ Full details elsewhere in this Table. ^k Z = Benzyloxycarbonyl.

obtained from the dye displacement at zero time yields k_i and these data are collected in Table 3 and illustrated in Figure 3. The tosyl fluoride parameters from our work agree with those of previous workers^{11b} within the error limits to be expected for this type of work.

 TABLE 3
 Inhibition of α -chymotrypsin with aromatic sulphonyl halides^a

Inhibitor	$10^{-3}K_i/l\ mol\ l^{-1}\ e$	$10^{-3}k_i/s^{-1}\ e$	σ
Benzenesulphonyl chloride			
Parent	3	6.5	0
4-Bromo	3.3	5.7	0.232
4-Chloro	3.2	5.5	0.227
4-Nitro	2.9	13.5	0.78
3-Nitro	2.3	7.0	0.71
4-Methoxy	5.2	8.9	-0.27
4-Methyl	5.6	8.6	-0.17
4-Acetamido	6.5	5.5	0
2,4,6-Trimethyl ^d	5.0	23	(-3 × 0.17) ^b
Tosyl fluoride	5.7	23	

^a pH 7.00, ionic strength made up to 0.1M with NaCl, phosphate buffer, 5% CH₃CN. ^b See text for details. ^c Errors are $\pm 10\%$. ^d Owing to solubility difficulties this result has more error than the others.

DISCUSSION

It is difficult to measure individual rate constants in enzymatic reactions and the safest parameter to consider for the effect of structural change in substrates is the ratio k_o/K_m which involves no assumptions or complications due to non-productive binding.^{13,14}

The finding of this investigation that acylamino-substituents affect k_o/K_m in the order PhCH₂O ~ Ph ~ C₆H₁₁ > CH₃ (groups 5—8 in Table 2) seems to confirm the existence of a lipophilic interaction, not of the charge transfer or polar type in the ρ_1 -binding. It is unlikely that the observed effect is due to action on the hydrogen bonding of the amide NH functions of the substrate with the enzyme because the pK_a values of the acids benzoic, cyclohexanecarboxylic, and acetic are in the wrong order (4.17, 4.9, and 4.76 respectively). Also the difference in polarisability between cyclohexane

¹³ A. Williams and G. Woolford, *J.C.S. Perkin II*, 1972, 272. ¹⁴ (a) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, 1965, **34**, 49; (b) F. E. Brot and M. L. Bender, *J. Amer. Chem. Soc.*, 1969, **91**, 7187.

and aromatic rings would not seem to favour the polar interaction suggested by Hansch and Coats.^{7a}

The increase in k_o/K_m with decrease in Hammett σ for methyl hippurates substituted in the benzene nucleus (group 1 in Table 2) is to be expected since the lipophilic binding attraction of the ρ_1 -site would be greater for the methyl substituted and unsubstituted nuclei than for those with electron-withdrawing polar substituents. The hydrogen bonding interaction is not expected to be very sensitive to substituents especially ones so far removed from the bond in question; for a system $A-H \cdots B$ Brønsted α values close to 0.2 have been measured (where B is varied).¹⁵

These results for α -chymotrypsin provide further evidence for similarity in binding at the ρ_1 -site for a series of proteases. We have already discussed the lipophilic interaction between the acylamino-group of a substrate with papain^{8a} and Lowe and Yuthavong have provided evidence for the hydrogen bonding contribution.¹⁶ Alcalase possesses a lipophilic acylamino-binding site¹³ and subtilisin-BPN' a hydrogen bond interaction between the NH of the acylamino-group and the backbone peptide carbonyl of residue serine-125 (serine-221 is the nucleophilic species);¹⁷ alcalase and subtilisin-BPN' are closely similar enzymes. Elastase, an enzyme related to chymotrypsin, probably also possesses a lipophilic contribution to acylamino-binding since the ratio of k_o/K_m values for benzoyl- to acetyl-L-alanine methyl ester substrates is *ca.* 15.¹⁸ Model building, using Kendrew-type molecular models and the atomic co-ordinates for tosyl- α -chymotrypsin,^{19a} suggests that the acylamino-function comes somewhere between the imidazolyl group of histidine-57 and the tryptophan-215 indole group in the productive binding mode (the tryptophan is a phenylalanine in the elastase structure). The lipophilic interaction between the acylamino-group and the ρ_1 -site is probably closely related to the secondary binding site for the amino-acid residue adjacent to the peptide bond undergoing cleavage for extended peptide substrates^{19b} and has been denoted the P_2 -site by Kurachi *et al.*²⁰ A lipophilic interaction was proposed²⁰ between the side chain of the peptide residue adjacent to the cleaved residue (S_2) and the side chain of isoleucine-99 of α -chymotrypsin. A similar interaction exists for elastase and it is noted that residue-99 is a valine group which is still capable of participating in lipophilic binding. Presumably bovine trypsin and chymotrypsin-B will also possess this interaction with the acylamino-group on specific substrates since residue-99 is leucine and valine respectively^{21,22} both possessing lipophilic side chains.

The data reveal that substrates of chymotrypsin with

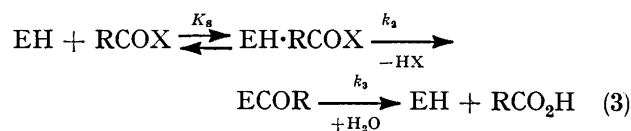
¹⁵ R. W. Taft, D. Gurka, L. Joris, P. von R. Schleyer, and J. W. Rakshys, *J. Amer. Chem. Soc.*, 1969, **91**, 4801.

¹⁶ G. Lowe and Y. Yuthavong, *Biochem. J.*, 1971, **124**, 107.

¹⁷ J. D. Robertus, J. Kraut, R. A. Alden, and J. J. Birktoft, *Biochemistry*, 1972, **11**, 4293.

¹⁸ (a) B. S. Hartley and D. M. Shotten, 'The Enzymes,' ed. P. D. Boyer, Academic Press, New York, 1971, vol. 3, p. 323; (b) A. Gertler and T. Hofmann, *Canad. J. Biochem.*, 1970, **48**, 384.

common acyl groups possess values of k_o/K_m which vary with the leaving group in the order: methyl < benzyl \sim cyclohexylmethyl (groups 2—4, 10—12 in Table 2). This ratio is not due to the different leaving abilities of the alcohols since the pK_a values are not dissimilar and there is little difference between methyl and benzyl esters towards hydroxide attack; there should be a smaller rate for the cyclohexylmethyl since this is more sterically hindered to nucleophilic attack than the methyl group. This reactivity series suggests the existence of a lipophilic interaction with the ρ_3 -site confirming the conclusions of Hansch and Coats.^{7a} The effect of changing thiol for oxygen ester (see Table 2, groups 9—12) increases k_o/K_m markedly. Hirohara *et al.*²³ were able to dissect the rate constants for a similar pair of esters and found the difference to reside in K_s , the pre-equilibrium binding constant. For all these esters the identity of k_o for a common acyl group indicated $k_2 > k_3$ for the three step mechanism (3) but the results of Ingles and Knowles²⁴ indicate $k_2 < k_3$ for ethyl *N*-acetylglycinate



since k_o is 0.039 and 0.23 s^{-1} for this ester and the corresponding thiol respectively. A similar observation is made here (Table 2, group 9) for the hippurate ($k_o^{\text{O}^2\text{-}p\text{-NO}_2\text{C}_6\text{H}_4} \sim k_o^{\text{SEt}} > k_o^{\text{Et}}$); thus the effect of changing from 'O' to 'S' is not all in the equilibrium term but does involve acylation and $k_2^{\text{O}} > k_2^{\text{S}}$. Since it is not likely that rate limiting decomposition of a tetrahedral intermediate is involved in acylation²³ we believe that lipophilic interactions contribute to both the pre-equilibrium binding of the substrate and the intracomplex acylation step; the latter giving rise to an enhanced k_2 for the sulphur ester. The model of α -chymotrypsin and a substrate bound with ρ_1 and ρ_2 interactions indicates that a leaving group such as benzyl alcohol could come close to the methionine-192 residue.

Both alcalase¹³ and papain^{8a} are thought to involve lipophilic leaving group sites and Table 4 summarises the lipophilic binding data for a series of closely related proteases.

Non-specific Substrates and Inhibitors.—Table 2 indicates that benzyl formate, a non-specific ester, is a substrate of chymotrypsin. The absence of reaction of methyl formate, an equally reactive ester towards hydroxide ion, shows that the activity is due to the

¹⁹ (a) J. J. Birktoft, B. W. Matthews, and D. M. Blow, *Biochem. Biophys. Res. Comm.*, 1969, **36**, 131; (b) G. L. Neil, C. Niemann, and G. E. Hein, *Nature*, 1966, **210**, 903.

²⁰ K. Kurachi, J. C. Powers, and P. E. Wilcox, *Biochemistry*, 1973, **12**, 771.

²¹ O. Mikes, V. Tomasek, V. Holeysovsky, and F. Sorm, *Biochim. Biophys. Acta*, 1966, **117**, 281.

²² L. B. Smillie, A. Furka, N. Nagabhushan, K. J. Stevenson, and L. O. Parkes, *Nature*, 1968, **218**, 343.

²³ H. Hirohara, M. L. Bender, and R. S. Stark, *Proc. Nat. Acad. Sci. U.S.A.*, 1974, **71**, 1643.

²⁴ D. W. Ingles and J. R. Knowles, *Biochem. J.*, 1966, **99**, 275.

benzyl portion rather than the reactive formyl group. Presumably binding in the benzyl acetate case (a non-substrate within the limits of our experimental error) is not sufficient to overcome the weak reactivity of the acetate group as measured by the hydroxide rate constant

TABLE 4

Lipophilic binding contribution to binding in some proteases

Enzyme	ρ_1	ρ_2	ρ_3
Chymotrypsin	+	+	+
Papain	+	-	+
Alcalase-subtilisin-BPN'	+	+	+
Elastase	+	+	?

(see Table 2). Other esters with poor leaving groups, methyl cinnamate and hydrocinnamate, and poorly reactive acyl functions relative to formate, are probably reasonably good substrates because of a binding *and* orientation factor. The catalytic action of chymotrypsin with small, non-specific, substrates may be explained by a pre-equilibrium binding of the aromatic nucleus in the 'tosyl-hole' in roughly the same position as dioxan or the tosyl group of tosyl- α -chymotrypsin; this is followed by an intracomplex acylation of the serine (residue-195) or imidazolyl group (histidine-57). This simple explanation is probably not valid because replacing residues glycine-216 and -226 by valine and threonine respectively blocks the 'tosyl-hole' in elastase while the rest of the active-site constituents retain the same positions as in chymotrypsin.¹⁸ Elastase is almost as active as chymotrypsin towards 4-nitrophenyl pivalate²⁵ and only ten-fold slower towards acetylation by 4-nitrophenyl acetate;^{3,26} both alcalase¹³ and trypsin²⁷ have a reactivity to the latter substrate similar to that of chymotrypsin (see Table 5). The lipophilic nature of the ρ_1 - and ρ_3 -binding sites discussed earlier suggests that these might contribute to small-substrate binding although the slightly greater activity of chymotrypsin than elastase to 4-nitrophenyl acetate suggests a contribution from 'tosyl-hole' binding. If glycine-216 and -226 are replaced by valine and threonine a model of chymotrypsin shows that there is still some space, at the 'mouth' of the 'tosyl-hole', ringed by lipophilic groups to provide binding which could contribute in the elastase case.

The absence of reactivity data for sulphonylation of

²⁵ (a) M. L. Bender and T. H. Marshall, *J. Amer. Chem. Soc.*, 1968, **90**, 201; (b) M. L. Bender and K. Nakamura, *ibid.*, 1962, **84**, 2577.

²⁶ T. H. Marshall and A. Akgun, *J. Biol. Chem.*, 1971, **246**, 6019.

elastase makes it difficult to compare with chymotrypsin; the selectivity for elastase (as opposed to water or other serine groups on the enzyme) infers that some sort of pre-equilibrium binding is operating but this cannot involve the full 'tosyl-hole' in the elastase case and this site is clearly not the only binding site of a lipophilic nature at the active-centre of chymotrypsin. Although the variation in K_i is very small there is a possibly significant trend to more efficient binding as the electron-withdrawing power of the substituent of the benzene ring is increased (Table 3). The variation of $\log_{10}k_i$ with σ (Figure 3) exhibits a minimum; if 2,4,6-trimethyl-benzenesulphonyl chloride may be assumed to have a σ value ($-3 \times 0.17 = -0.51$) there is a pronounced non-linearity which is not easy to interpret because k_i is affected by non-productive orientation modes to an

TABLE 5

Reactivity of some proteases towards 4-nitrophenyl pivalate and acetate

Protease	$k_o/K_m^{-1}/l \text{ mol}^{-1} \text{ s}^{-1}$	
	Pivalate	Acetate
Trypsin		71.4 ^a (corrected value 639)
Chymotrypsin	231 ^f	4 060, ^b 3 520 ^d
Alcalase		1 250 ^g
Elastase	137 ^e	470 ^{d,e}

^a Ref. 27. The solvent is 20% propan-2-ol-water (v/v); chymotrypsin in this solvent had $k_o/K_m = 454 l \text{ mol}^{-1} \text{ s}^{-1}$ and assuming a similar effect for trypsin the value in parenthesis is given by $7.1 \times 4 060/454$. ^b Ref. 3. ^c pH 7.26. ^d Ref. 26. ^e Ref. 25a. ^f M. L. Bender and G. A. Hamilton, *J. Amer. Chem. Soc.*, 1962, **84**, 2570. ^g Ref. 13.

extent not able to be estimated. If it may be assumed that these modes are negligible then the results are consistent with a mechanism changing from S_N2 to S_N1 timing (with respect to leaving and entering group) as the electron-donating power of the substrate increases. Steric release may also aid an S_N1 type of timing in the 2,4,6-trimethyl-substituted case. There is, however, no firm evidence,²⁸ despite a large literature, for S_N1 reactions of arenesulphonyl halide hydrolysis in chemical systems.

The S.R.C. is thanked for an advanced course studentship (J. U.) and for the purchase of spectroscopic equipment.

[5/1701 Received, 4th September, 1975]

²⁷ J. A. Stewart and L. Ouellet, *Canad. J. Chem.*, 1959, **37**, 751.
²⁸ See L. Senatore, L. Sagamore, and E. Ciuffarin, *J.C.S. Perkin II*, 1974, 722 for a lucid account of the present situation with regard to the S_N1 mechanism of sulphonyl halide hydrolysis.