# Proteolytic Enzymes: Lipophilic Binding Sites for Specific and Non-specific Substrates of α-Chymotrypsin [1]

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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 acetate [3] 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.

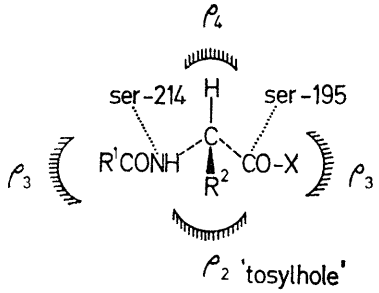


FIGURE 1 Binding of a specific substrate to α-chymotrypsin [6]

A binding site has been proposed for the attacking nucleophile and studies of alcoholysis [5] have provided some evidence for this; the alcohol binds at a site on the enzyme excluding water. This site presumably corresponds to the $\rho_3$-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 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 $\rho_1$-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 $\rho_1$ 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 $\rho_1$ and $\rho_3$ interactions.

## EXPERIMENTAL

*Materials.*—The ester substrates were from work already reported [8a-d] except the substituted methyl *N*-benzoylglycinates (m.p.s. in Table 1) which were prepared *via* a 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.

TABLE 1

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.

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

† 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.

[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;[8e] other reagents were from B.D.H. Acetonitrile was purified from the AnalaR material by distillation from $P_2O_5$. 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 $k_o$ 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 ($[E]k_o/K_m$) was used to give $k_o/K_m$. Enzyme concentration was estimated by titration with 4-nitrophenyl acetate[10] using the absorption maximum of 400 nm at pH 7.00 and a Beckman-DGB spectrophotometer.

$$\text{initial rate} = [E][S]\, k_o/([S] + K_m) \qquad (1)$$

*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

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow[(-\text{halide})]{k_i} EI' \qquad (2)$$

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

Dye solution (50λ) 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 (50λ) 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 $k_o/K_m$ into its components. The parameters obtained are collected in Table 2 and a Hofstee plot[12] 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.

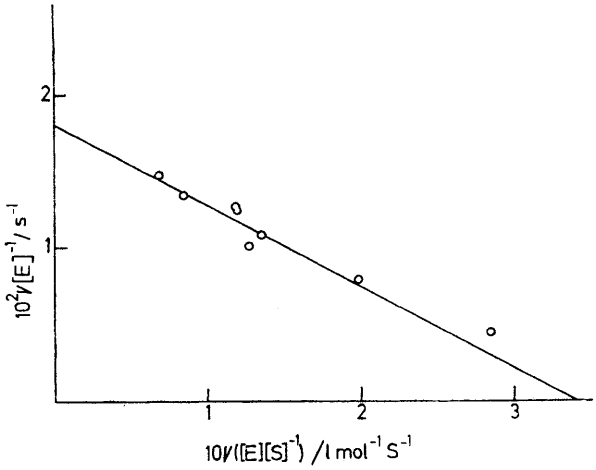


FIGURE 2 Methyl 3-nitrobenzoylglycinate as substrate for α-chymotrypsin; pH 7.00, ionic strength made up to 0.1M with NaCl, 25°

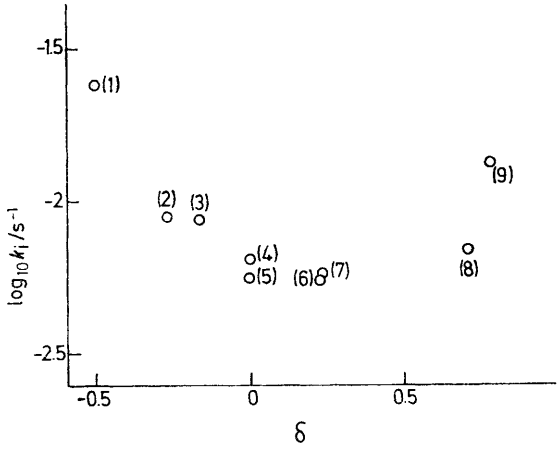


FIGURE 3 Dependence of $k_i$ on σ for substituted benzenesulphonyl chlorides and α-chymotrypsin. Values of σ are from G. B. Barlin and D. D. Perrin, *Quart. Rev.*, 1966, **20**, 75. Points are (1) 2,4,6-trimethyl; (2) 4-methoxy; (3) 4-methyl; (4) 4-acetamido; (5) unsubstituted; (6) 4-chloro; (7) 4-bromo;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

[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.

[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.

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 acetylglycinate | 0.038 | 5.3 | 1—10 | 7.1 |
| 3 | Methyl acetylglycinate | 0.0092 | 31 | 1—40 | 0.3 (0.437) [e] |
| | Cyclohexylmethyl acetylglycinate | | | 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 acetylglycinate) [j] | | | | (0.3) [j] |
| | Methyl cyclohexylacetylglycinate | 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 | $1.1 \times 10^6$ |
| | Methyl acetyl-L-phenylalaninate | 63 | 1.8 | 0.1—5 | $3.5 \times 10^4$ |
| 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 acetylglycinate [e] | 0.039 | 0.389 | | 0.101 |
| | *S*-Ethyl *N*-acetylglycinate [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. Applewhite 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°. [j] 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$^{-1}$ [c] | $10^{-3}k_i$/ s$^{-1}$ [c] | 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% $CH_3CN$. [b] See text for details. [c] Errors are ±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 $PhCH_2O \sim Ph \sim C_6H_{11} > CH_3$ (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 $\rho_1$-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 p$K_a$ 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 $\sigma$ for methyl hippurates substituted in the benzene nucleus (group 1 in Table 2) is to be expected since the lipophilic binding attraction of the $\rho_1$-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 $\alpha$ values close to 0.2 have been measured (where B is varied).[15]

These results for $\alpha$-chymotrypsin provide further evidence for similarity in binding at the $\rho_1$-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 site [13] 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-$\alpha$-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 $\rho_1$-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 proposed [20] between the side chain of the peptide residue adjacent to the cleaved residue ($S_2$) and the side chain of isoleucine-99 of $\alpha$-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 common acyl groups possess values of $k_o/K_m$ which vary with the leaving group in the order: methyl < benzyl ~ cyclohexylmethyl (groups 2—4, 10—12 in Table 2). This ratio is not due to the different leaving abilities of the alcohols since the p$K_a$ 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 $\rho_3$-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 Knowles [24] indicate $k_2 < k_3$ for ethyl *N*-acetylglycinate

$$\mathrm{EH} + \mathrm{RCOX} \underset{}{\overset{K_s}{\rightleftharpoons}} \mathrm{EH{\cdot}RCOX} \xrightarrow[-\mathrm{HX}]{k_2} \mathrm{ECOR} \xrightarrow[+\mathrm{H_2O}]{k_3} \mathrm{EH} + \mathrm{RCO_2H} \quad (3)$$

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 ($k_o^{O\text{-}p\text{-}NO_2C_6H_4} \sim k_o^{SEt} > k_o^{OEt}$); thus the effect of changing from 'O' to 'S' is not all in the equilibrium term but does involve acylation and $k_2^O > k_2^S$. Since it is not likely that rate limiting decomposition of a tetrahedral intermediate is involved in acylation [23] 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 $\alpha$-chymotrypsin and a substrate bound with $\rho_1$ and $\rho_2$ interactions indicates that a leaving group such as benzyl alcohol could come close to the methionine-192 residue.

Both alcalase [13] 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

[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.
[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 | $\rho_1$ | $\rho_2$ | $\rho_3$ |
|---|---|---|---|
| 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 pivalate [25] and only ten-fold slower towards acetylation by 4-nitrophenyl acetate;[3,26] both alcalase [13] and trypsin [27] have a reactivity to the latter substrate similar to that of chymotrypsin (see Table 5). The lipophilic nature of the $\rho_1$- and $\rho_3$-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 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-trimethylbenzenesulphonyl 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 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.

TABLE 5

Reactivity of some proteases towards 4-nitrophenyl pivalate and acetate

| Protease | $k_oK_m^{-1}$/l mol$^{-1}$ 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 mol$^{-1}$ 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. 25*a*. [f] M. L. Bender and G. A. Hamilton, *J. Amer. Chem. Soc.*, 1962, **84**, 2570. [g] Ref. 13.

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

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

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[28] See L. Senatore, L. Sagramore, 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.