ON THE RELATIONSHIP BETWEEN STRUCTURE AND REACTIVITY OF α-CHYMOTRYPSIN SUBSTRATES

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Hydrolysis of ester substrates by α-chymotrypsin proceeds through formation and decomposition of an intermediate compound, i.e. an acylated enzyme [1]:

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\searrow P_1$$

The overall rate of such a process is a function of the rate constants of the separate stages (i.e. k_2 and k_3) and of the equilibrium constant K_S. Therefore, to discuss the connection between substrate reactivity and its structure we must know these constants. In our work two methods of determining the individual constants have been used. The first method is based on adding a nucleophilic agent, 1,4-butanediol, to the reaction [2]; the second uses selective influence of salt (KCl) on the acylation stage (k_2) [3, 4].

We investigated the α-chymotryptic hydrolysis of a number of esters of *N*-acylated-L-amino acids [2] with the general formula:

$$R_1\text{—}C(O)NH\text{—}\underset{\displaystyle R_2}{\underset{|}{CH}}\text{—}C(O)OR_3$$

where R_1 is a radical of the acylamino-group, R_2 is the amino acid side chain and R_3 is a radical of the alkoxyl group. The results of the paper show that the paramount factor determining the rate constants of individual stages is the hydrophobic capacity of the substituents R_1 and R_2, which can be described by the value of standard free energy of transfer of these groups from water to a non-aqueous solvent, cf. [5]. For our work, the hydrophobicity of these substrate fragments is operationally defined by the water–octanol reference system [6].

Fig. 1 shows the dependence of the free energy of enzyme–substrate complex formation ($\Delta F_S = -2.3\ RT \log K_S^{-1}$) on the value of free energy increment of transfer of side chain R_2 of a number of substrates, methyl esters of *N*-acetyl-L-amino acids, from water into octanol. For most substrates a linear correlation is observed with a slope close to unity (see table 1). This once again proves quite convincingly that the processes of enzyme–substrate complex formation and transfer of the substrate side chain R_2 from water into a non-aqueous solvent are of the same nature [7–9]. Free energies of activation of acylation (k_2) and deacylation (k_3) reactions also show a linear dependence on the hydrophobic capacity of the side chain R_2, with slopes nearing unity (see table 1). This means that the transfer of a non-polar substrate side chain from water into an environment of the active centre of α-chymotrypsin makes an identical free energy contribution both in the acylation and the deacylation stages. It also indicates that processes of sorption and catalysis are interconnected [7, 8]. Thus, if we vary, within certain limits, the size of substrate side chain R_2 with a fixed α-acylamino group, Knowles's rule 'The better the binding – the better the reaction' [7] holds equally for both acylation and for deacylation stages of α-chymotrypsin.

The correlation becomes the exact opposite if we change the structure of *N*-acyl substituent R_1 with a fixed substrate side chain R_2. Fig. 2a–b shows that as the hydrophobicity of R_1 increases (i.e. as the negative value of the $\Delta F_{trans}^{(R_1)}$ increases) the true Michaelis constant improves sharply, but the acylation

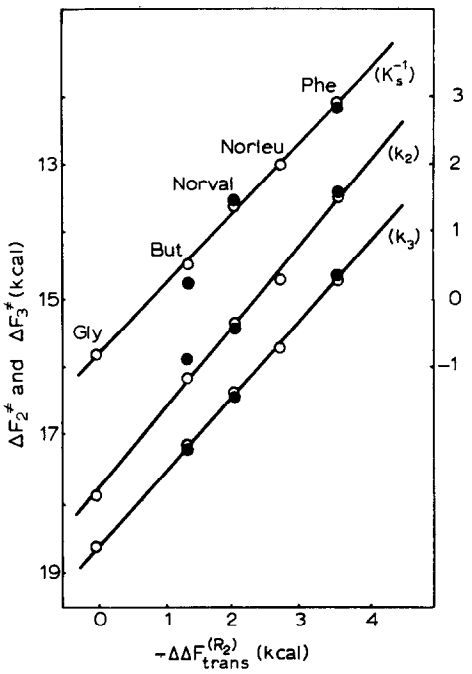

Fig. 1. Free energy of enzyme–substrate complex formation and free energies of activation of the stages of acylation and deacylation for α-chymotrypsin-catalyzed hydrolysis of methyl esters of *N*-acetyl-L-amino acids against free energy increment of the substrate side chain transfer from water into octanol. The open symbols are used for the data described in the accompanying paper [2]; the filled symbols represent the data obtained by means of the salt-method [3, 4].

rate constant (k_2) decreases, i.e. inverse dependence, 'the better the binding – the poorer the reaction', is observed. This cannot be explained by a difference in electrophilic nature of substrates under study. Proof of this is supplied, for instance, by the data of alkaline hydrolysis of methyl esters of some *N*-acetyl- and *N*-benzoyl-L-amino acids (see table 2). The decrease of the rate constants as the hydrophobicity of substituents R_1 increases can be explained by 'non-productive' (or 'wrong-way') binding [1, 10–12]. By present-day conception, the functional groups of the α-chymotrypsin model substrate (R_1, R_2 and R_3, see above) have complementary loci on the enzyme active centre (ρ_1, ρ_2 and ρ_3) [10]. When substrate binding to the active centre of α-chymotrypsin is productive, a correspondence of the interaction $R_1-\rho_1$, $R_2-\rho_2$ and $R_3-\rho_3$ is realized, and hydrolysis occurs. Analysis of the binding constants of various substrates

Table 1
Gradients of straight lines when plotting free energies of activation or free energy of binding vs. free energies of transfer of R_1 and R_2 substrate groups from water into non-aqueous solvent.

Reaction	Gradient with the variation of R_2	Gradient with the variation of R_1
Acylation, k_2	1.2 ± 0.3	−0.7 ± 0.1
Deacylation, k_3	1.1 ± 0.1	0 ± 0.2
Enzyme–substrate complex formation, K_S^{-1}	1.0 ± 0.1	1.3 ± 0.2
Over-all reaction, k_2/K_S	2.2 ± 0.2	0.6 ± 0.1

with α-chymotrypsin [10] leads to the conclusion that the hydrophobicity of ρ_2-locus exceeds that of ρ_1 (see also table 1, bottom line). Thus, if productive binding occurs the *N*-acyl group R_1 is bound to the ρ_1 locus of of a low hydrophobic nature (or, in terms of extraction, transfer of non-polar group R_1 to the hydrophobic locus ρ_1 is incomplete). As the hydrophobic nature of R_1 increases, the proportion of unproductive enzyme–substrate complexes such as $R_1-\rho_2$ increases, leading to a fall in the acylation rate constant k_2.

One might expect that with a fixed R_2 group, the hydrophobic nature of group R_1 would not influence the value of the deacylation rate constant (k_3), if exclusive $R_2-\rho_2$ productive binding to the acylenzyme is realized. Experimental data (fig. 2c) fully agree with the conclusion.

It is expected [1] that with fixed substituents, R_1 and R_2, the influence of the alkoxyl radical R_3 on substrate reactivity would be merely steric and electronic. Fig. 3 shows that a plot of the acylation rate constants of α-chymotrypsin with various esters (esters of *N*-acetyl-L-valine [2]) vs. the rate constants

Table 2
Rate constants of alkaline hydrolysis of methyl esters of some *N*-acyl-L-amino acids, $R_1CONHCH(R_2)COOCH_3$, pH 10 (pH-stat), 25°, 0.1 M KCl.

R_1	R_2	k_{OH^-} (M^{-1} sec^{-1})
CH_3	H	2.48 ± 0.12
C_6H_5	H	2.35 ± 0.11
CH_3	iso-C_3H_7	0.164 ± 0.007
C_6H_5	iso-C_3H_7	0.178 ± 0.009

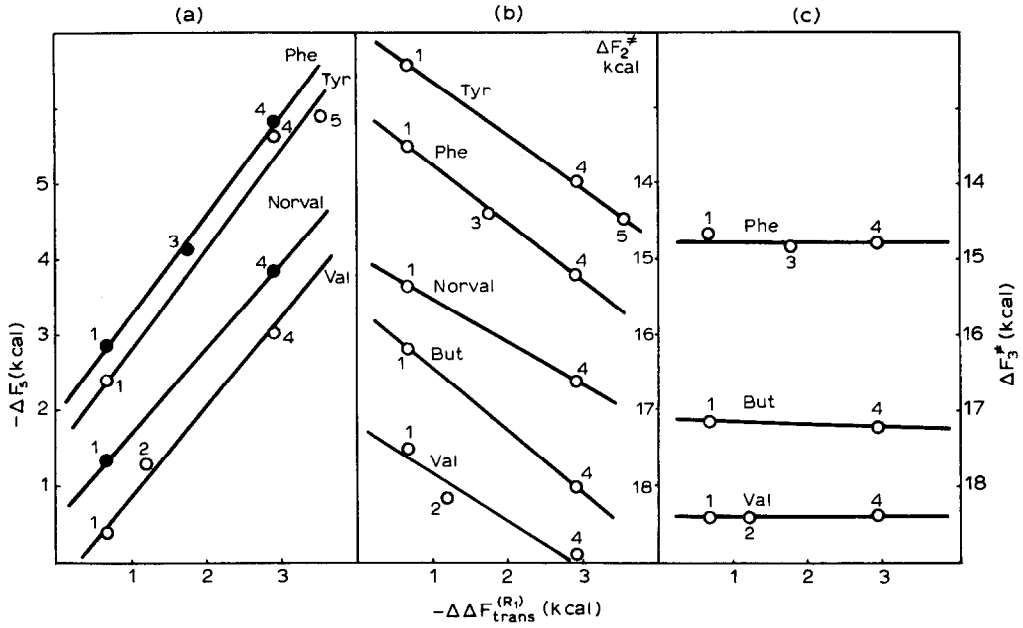

Fig. 2. Free energy of enzyme–substrate complex formation (a) and free energies of activation of acylation (b) and deacylation (c) stages in α-chymotrypsin-catalyzed hydrolysis of methyl esters of *N*-acyl-L-amino acids against free energy increment of transfer of hydrophobic radical R_1 of *N*-acyl substituent of the substrate from water into octanol. (1) acetyl, (2) chloroacetyl, (3) (3) acetyl-L-alanyl, (4) benzoyl, (5) acetyl-L-leucyl.

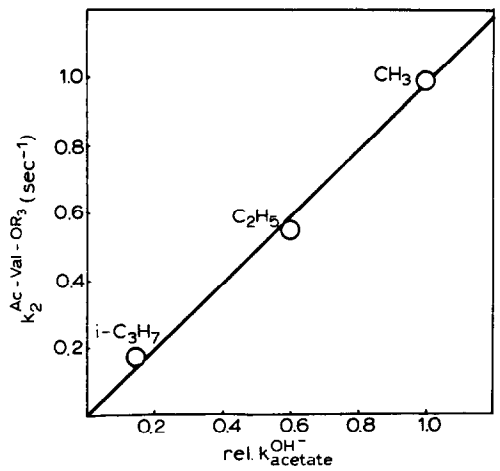

Fig. 3. The values of rate constant of α-chymotrypsin acylation by *N*-acetyl-L-valine esters against relative rate constants of alkaline hydrolysis of the corresponding esters of the acetic acid.

of alkaline hydrolysis [13] of the corresponding esters of acetic acid (fig. 3) is linear. This shows that the decrease in rate primarily reflects steric hindrance towards the approach of an attacking group, with the rate constants not subject to large steric influences due to the protein environment.

The above considerations indicate that there is a simple quantitative connection between structural characteristics of substrates and their reactivity at various stages of α-chymotryptic hydrolysis. Our results enable us to conclude that the earlier hypotheses 'The better the binding – the better the reaction' (fig. 1), 'The better the binding – the poorer the reaction' (fig. 2a–b) and 'The strength of binding has no influence on reaction' (fig. 2c) are all valid for the hydrolysis of esters by α-chymotrypsin but each in relation to the influence of corresponding structural parts of the substrate molecule on the respective stages of the enzymatic process. The physical meaning of this interconnection seems to be in stabilization of respective transition states of reactions, due to ancillary (mainly hydrophobic) interactions and in orientation effects [14, 15]. Above

all, the correlations described here (table 1) obviously allow us to carry out a sufficiently precise estimation of the individual constants of α-chymotrypsin-catalyzed reactions solely on the knowledge of the structure of substrates.

References

[1] M.L. Bender and F.J. Kézdy, Ann. Rev. Biochem. 34 (1965) 49.
[2] I.V. Berezin, N.F. Kazanskaya and A.A. Klyosov, FEBS Letters 15 (1971) 121.
[3] K. Martinek, A.K. Yatsimirski and I.V. Berezin, Molek. Biol. SSSR 5 (1971) no. 1.
[4] A.K. Yatsimirski, K. Martinek and I.V. Berezin, 7th International Symposium on the Chemistry of Natural Products (IUPAC), Abstract no. A69, Riga (USSR), June June 1970.
[5] I.V. Berezin, A.V. Levashov and K. Martinek, FEBS Letters 7 (1970) 20.
[6] C. Hansch, J.E. Quinlan and G.L. Lawrence, J. Org. Chem. 33 (1968) 347.
[7] J.R. Knowles, J. Theoret. Biol. 9 (1965) 213.
[8] I.V. Berezin and K. Martinek, FEBS Letters 8 (1970) 216.
[9] K. Martinek, A.V. Levashov and I.V. Berezin, Molek. Biol. SSSR 4 (1970) 517.
[10] G.E. Hein and C. Niemann, J. Am. Chem. Soc. 84 (1962) 4495.
[11] I.V. Berezin, N.F. Kazanskaya and A.A.Klyosov, Biokhimiya 36 (1971) No. 2.
[12] J.R. Rapp, C. Niemann and G.E. Hein, Biochemistry 5 (1966) 4100.
[13] L.P. Hammett, Physical Organic Chemistry (McGraw-Hill, New York, 1940) p. 211.
[14] I.V. Berezin and K. Martinek, Biokhimiya 34 (1970) 1254.
[15] I.V. Berezin and K. Martinek, Zh. Vses. Khim. Obshchestva im. D.I. Mendeleeva 16 (1971) no. 4.