Activation Parameters of Elastase and Chymotrypsin Catalyzed Hydrolysis. Differential Role of Favorable Enthalpy in Determining Reactivity[1]

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Abstract: The effects of temperature on the rates of porcine elastase and α-chymotrypsin catalyzed hydrolysis of a series of normal fatty acid *p*-nitrophenyl esters were studied. As reported previously, the rates at a given temperature increase monotonically to C-4 and C-7 with elastase and α-chymotrypsin, respectively, and then decrease monotonically. With the possible exception of the C-6 homolog with elastase, we find this specificity pattern results from the more reactive side chains having smaller enthalpies of activation. The apparent ionization constant of the essential group on the free elastase active site was measured at three temperatures, and the enthalpy and entropy of ionization were found to be consistent with that of a histidine side chain. Two specific *N*-acyl amino acid ester substrates of elastase were studied. In contrast to the importance of differences in enthalpy of activation found within the fatty acid series, their relative reactivity with respect to one another and greater reactivity than the less specific substrates result from less negative entropy of activation, their enthalpy of activation being within the range characterizing the less reactive fatty acid esters. Some of the lack of agreement of our results with those of previously published experiments with chymotrypsin may be related to a specific effect of Tris buffer. Our experiments showed that at pH 7.8 under turnover conditions the activation enthalpy for deacylation of acetyl chymotrypsin in Tris buffer is 5.5 kcal lower than in phosphate or barbital. Our finding that relative reactivity of normal fatty acid esters is controlled by differences in activation enthalpy may support the idea of conformational change in the enzyme–substrate complex or that differences in solvation are important. The specificity of *N*-acyl amino acid esters toward elastase may be related to the fact that they have two side chains and hence the ability for three-point attachment to the enzyme.

Since the early studies of Hofstee[2] the homologous series of normal fatty acid esters have been used to study the specificity of chymotrypsin. A recent study reveals that both elastase and chymotrypsin have monotonic reactivity–chain length relations with normal fatty acid nitrophenyl esters.[3] The basis of this reactivity pattern could lie in entropy control, where differences in entropy of activation determined relative reactivity within the series, or enthalpy control, where differences in enthalpy of activation determine specificity. Two types of entropy control have been reported for chymotrypsin reactions: enthalpy is essentially invariant over a range of substrates and specificity, *i.e.*, reactivity is determined solely by variation in entropy of activation;[4] and increase in enthalpy is more than compensated for by less negative entropy of activation in the more reactive substrates.[5] A clear understanding of activation parameters should be included in any general theory of enzyme specificity and mechanism. Entropy control has been interpreted as reflecting diflerences in ground state configurational entropy[4] and large enthalpy effects as suggesting conformational changes in the enzyme–substrate complex.[5a,6] Our results with chymotrypsin differ from some previously reported. We believe this may be due in part to an effect of Tris buffer on the activation parameters. Our results with elastase show that different patterns of activation parameters may appear depending on the structural class of substrates studied. The normal fatty acid ester substrates which minimize steric and polar effects showed enthalpy control of specificity toward both elastase and chymotrypsin. *N*-Acyl amino acid substrates showed important contributions of favorable entropy in their specificity toward elastase.

Our experiments were done under turnover conditions using a large excess of substrate. This complicates the experiment when a labile nitrophenyl ester is used. Spontaneous hydrolysis occurs in competition with enzyme-catalyzed hydrolysis if the pH is much above 7. We used a phosphate buffer slightly above neutrality. In this pH region the effect of temperature on the histidine ionization in the active site should be considered.[5b,7] We find that this correction changes the values of activation energies by as much as 3 kcal/mol but not the qualitative trends in activation parameters.

Experimental Section

Materials. Elastase was purchased from Worthington Biochemical Corporation, and from Whatman Biochemicals Limited. Stock solutions were prepared in pH 7.2 phosphate and kept on ice when not stored in the refrigerator. Stock concentration was determined by standard rate assay using *p*-nitrophenyl *N*-benzyl-

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(2) (a) B. H. J. Hofstee, *Biochim. Biophys. Acta*, **24**, 211 (1957); (b) *ibid.*, **32**, 182 (1959).

(3) T. H. Marshall, and Ay Akgün, *J. Biol. Chem.*, **246**, 6019 (1971).

(4) (a) M. L. Bender, F. J. Kezdy, and C. R. Gunter, *J. Amer. Chem. Soc.*, **86**, 3714 (1964); (b) M. J. Gilleland and M. L. Bender, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **31**, 1234 (1972).

(5) (a) W. P. Cane and D. Wetlaufer, Abstracts, 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966; (b) T. H. Fife and J. B. Milstien, *Biochemistry*, **6**, 2901 (1967).

(6) (a) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 298; (b) D. E. Koshland and K. E. Neet, *Annu. Rev. Biochem.*, **37**, 375 (1968).

(7) W. P. Cane, Ph.D. Dissertation, University of Minnesota, 1967.

Table I. Reactions of Elastase with *p*-Nitrophenyl Butyrate at pH 7.2 and 28° Analyzed by Different Procedures

		Hofstee plot[a]			Integrated eq 1		
E_0 ($\times 10^6$)	S_0 ($\times 10^4$)	k_{cat}, sec^{-1}	K_m, M ($\times 10^5$)	k_{cat}/K_m, M^{-1} sec^{-1} ($\times 10^{-3}$)	k_{cat}, sec^{-1}	K_m, M ($\times 10^5$)	k_{cat}/K_m, M^{-1} sec^{-1} ($\times 10^{-3}$)
2.28	0.37	0.077	1.37	5.6	0.087	1.60	5.4
1.83	0.176	0.073	1.62	4.5	0.091	2.18	4.2
4.50	0.848	0.088	2.06	4.3	0.087	2.02	4.3

[a] Plot of V/S *vs.* V.

oxycarbonylglycinate as substrate.[8] Levels of tyrosinate enzyme impurity were found to be negligible.[9] Chymotrypsin was purchased from Worthington Biochemical Corporation and solutions were prepared in distilled water and stored in the refrigerator. Their concentration was determined by titration with *trans*-cinnamoylimidazole.[10] The substrates have been described elsewhere[3] excpet for *p*-nitrophenyl *N-tert*-butyloxycarbonyl-L-alaninate which was Mann Lot T3777.

Kinetics. Rates of ester hydrolysis were followed by observing the rate of liberation of *p*-nitrophenol in a Cary 15 recording spectrophotometer at 400 nm. The cell holder was thermostated by means of a Lauda thermostat. Actual cell temperature was related to water bath temperature by a calibration curve.

Reactions which followed first-order kinetics, which included all of the elastase reactions except *p*-nitrophenyl butyrate, were plotted on semilog graph paper, and first-order rate constants were obtained from the slope. The second-order catalytic rate constant was obtained by dividing the observed first-order constant by the enzyme concentration. Michaelis-Menten kinetics, which were observed for the reaction of *p*-nitrophenyl butyrate with elastase and all the chymotrypsin reactions, were analyzed by using the integrated form of the Henri equation.

$$\frac{1}{t}\ln\frac{S_0}{S_t} = \frac{-1(S_0 - S_t)}{K_m}\frac{}{t} + \frac{V_m}{K_m} \qquad (1)$$

The raw data, which consisted of absorbance-time points, were analyzed by a computer program which converted them to concentration units and plotted them according to eq 1. The output of interest consists of the constants V_m and K_m. The plot is not linear if points earlier than 75% of reaction are used. This is expected since this equation requires the time to be very large with respect to the half-time for any intermediates.[11] In general, a five- to tenfold variation of substrate concentration was plotted. As a check on the validity of the overall procedure, three experiments using different substrate and enzyme concentrations were analyzed by making a Hofstee plot based on the differential form of the equation. The Michaelis-Menten parameters derived from the two methods of analyzing a single reaction are compared in Table I.

The effect of temperature on the phenol-phenolate equilibrium and hence on the molar absorbance was taken into account when necessary. Representative correction factors from the calibration curve are shown in Table II.

Table II.[a] Calibration Points Relating Temperature to Apparent Molar Absorption Coefficient

Temp, °C	Corr factor[b]	Temp, °C	Corr factor[b]
42.0	1.121	23.5	0.989
34.0	1.066	13.5	0.902
25.0	1.000	3.9	0.817

[a] pH 7.2 phosphate $\mu = 0.1$. [b] Factor by which the molar absorption coefficient at 25° should be multiplied to account for temperature effect on phenol-phenolate ionization.

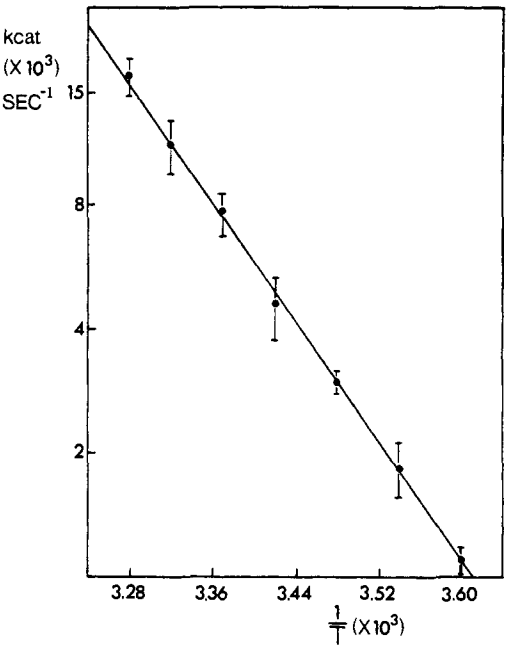

Figure 1. Arrhenius plot of chymotrypsin (1.16×10^{-6}) reacting with *p*-nitrophenyl butyrate ($2.4\text{-}2.9 \times 10^{-5}$); $\mu = 0.1$, pH 8.9 barbital, 0.3% (v/v) acetonitrile. Error limits indicate 1 standard deviation from least-squares analysis of integrated rate equation.

The active-site ionization constant of the free elastase was evaluated kinetically by plotting the reciprocal of the second-order catalytic rate constants *vs.* hydrogen ion concentration, the slope of which gives the kinetic pK_a at a given temperature. The enthalpy and entropy of ionization were evaluated from both a conventional van't Hoff plot and a plot of TpK_a *vs.* T. The two methods agree within 0.1 kcal/mol in enthalpy of ionization.

The net catalytic rate constants were corrected for the effect of temperature on the ionization state of the enzyme by dividing the observed rate constant by the mole fraction of the enzyme in the catalytically active (free base) form.[7] The effective pK_a at a given temperature was read from a calibration line of pK_a *vs.* temperature and used in the Henderson-Hasselbalch equation with slight additional corrections for the effect of temperature on the pH of the buffer if needed.[12] With phosphate buffers these latter corrections were so small as to be insignificant in their effect on the activation parameters.

Activation parameters were computed by plotting log k_c/T *vs.* $1/T$ according to eq 2, where h is Planck's constant, k is the Boltzmann constant, k_c the catalytic rate constant, R the gas constant, and T the absolute temperature. Enthalpy and entropy were derived from the slope and intercept, respectively, by means of a programmable Hewlett-Packard 9100B calculator giving least-squares analysis including standard errors for each quantity. As an additional check on the validity of this method of treating the data, a rearranged form of eq 2 was used to plot $T \ln k_c/T$ *vs.* T. This

$$\ln\frac{k_c}{T} = \ln\frac{k}{h} + \frac{\Delta S^{\pm}}{R} - \frac{\Delta H^{\pm}}{R}\frac{1}{T} \qquad (2)$$

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

(9) T. H. Marshall, J. R. Whitaker, and M. L. Bender, *Biochemistry*, **8**, 4665 (1969).

(10) G. P. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(11) H. Gutfreund, "An Introduction to the Study of Enzymes," Blackwell Scientific Publications, Oxford, 1965, p 45.

(12) "Handbook of Chemistry and Physics," 48th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1967, p D-80.

Table III.[a] Elastase Catalyzed Hydrolysis

p-Nitrophenyl ester	E_0 (10^6)	S_0 (10^5)	Temp, °C	k_2, M^{-1} sec^{-1} ($\times 10^{-3}$)	k_c[b], M^{-1} sec^{-1} ($\times 10^{-3}$)
Acetate	4.54	3.0	19.0	0.0192	0.0371
			27.7	0.0403	0.0708
			32.7	0.0533	0.0877
			37.6	0.0716	0.111
Propionate	4.20	6.39	9.8	0.110	0.239
			19.0	0.255	0.493
			27.7	0.445	0.782
			37.6	0.833	1.290
Butyrate	4.60	8.0	9.8	1.35	2.94
			19.0	2.62	5.08
			27.7	4.27	7.51
			37.6	6.64	10.3
Valerate	8.86	4.68	9.8	0.409	0.886
			19.0	0.798	1.54
			27.7	1.39	2.44
			37.6	2.37	3.67
Hexanoate	9.20	2.45	19.2	0.0185	0.0388
			23.5	0.0228	0.0456
			27.6	0.0309	0.0585
			33.0	0.0440	0.0765
			37.4	0.0550	0.0905
N-*tert*-Butyloxycarbonyl-L-alaninate	1.19	5.90	15.0	17.4	41.7
			19.0	25.6	57.9
			23.6	34.5	74.2
			28.0	44.4	89.7
			33.0	56.6	106.0
N-Benzyloxycarbonyl-glycinate	0.886	5.26	19.2	5.95	13.5
			23.6	8.59	18.5
			27.9	11.0	22.2
			32.6	14.7	27.5

p-Nitrophenyl ester	E_0 ($\times 10^6$)	S_0 ($\times 10^5$)	Temp, °C	k_{obsd}, sec^{-1} ($\times 10^3$)	k_c, sec^{-1}[c] ($\times 10^3$)
Deacylation of Chymotrypsin under Turnover Conditions					
Acetate	2.57	3.90	14.9	1.17 ± 0.15	2.48
			28.0	5.59 ± 0.48	9.85
			23.7	3.28 ± 0.61	6.13
			32.4	8.50 ± 1.4	14.0
Propionate	2.57	2.14	14.4	0.752 ± 0.013	1.63
			19.0	0.868 ± 0.045	1.73
			27.9	2.76 ± 0.24	4.86
			37.4	5.88 ± 0.54	8.95
Butyrate	2.54	2.60	23.7	2.84 ± 1.6	4.85
			28.3	4.96 ± 2.0	8.00
			32.8	8.08 ± 0.62	12.3
			37.9	12.0 ± 1.7	17.1
Valerate	2.64	4.35	23.5	5.82 ± 1.4	9.95
			27.9	10.4 ± 1.6	16.8
			32.7	15.4 ± 2.1	23.5
			38.7	28.2 ± 0.95	40.2
Hexanoate	1.15	1.07	10.3	5.06 ± 0.41	13.1
			19.2	13.1 ± 2.2	28.8
			27.7	30.8 ± 3.6	59.0
			37.6	84.1 ± 5.7	137
Heptanoate	0.269	0.43	10.1	7.21 ± 1.5	18.6
			18.9	18.0 ± 9	39.6
			29.5	50.2 ± 3.6	94.0
			37.4	87.0 ± 7.7	144
Octanoate	0.58	0.40	10.1	2.95 ± 0.11	7.6
			19.5	7.28 ± 1.5	16.0
			28.1	21.6 ± 0.9	40.5
			37.1	52.0 ± 10	36.5
Nonanoate	0.29	0.30	10.1	1.21 ± 0.21	3.12
			19.2	2.95 ± 0.82	6.49
			27.9	8.85 ± 0.079	16.9
			37.1	22.3 ± 2.2	36.4

[a] pH 7.3 phosphate, μ = 0.1, 3% or less acetonitrile. [b] Catalytic rate constant corrected for His ionization; $k_c = k_2$/His mole fraction. [c] Deacylation rate constant corrected for His ionization; $k_c = k_{obsd}$/His mole fraction.

latter form of eq 2 has been recommended to provide a reliable method of evaluating activation parameters without introducing compensating errors in enthalpy and entropy and thus producing spurious isokinetic relationships.[13]

Results

Catalytic rate constants are reported in Table III. First-order plots of the reaction between elastase and *p*-nitrophenyl butyrate showed nonlinearity after 2 half-times. Apparently this substrate is at the borderline between first-order and Michaelis–Menten behavior. All other reactions of elastase were found to obey first-order kinetics including that of *p*-nitrophenyl *N*-*tert*-butyloxycarbonyl-L-alaninate which has been reported to follow Michaelis–Menten kinetics.[14] We find that doubling the concentration of this substrate results in precipitation under our conditions.

All chymotrypsin deacylations were analyzed according to Michaelis–Menten kinetics.[2,3,15,16] The average standard deviation in the V_{max} was 10% (Figure 1).

The thermodynamics of ionization of the active site of elastase were determined by measuring the apparent kinetic pK_a at three temperatures using *p*-nitrophenyl valerate. The results are reported in Tables IV and V.

Table IV. pH Dependence of Elastase Catalyzed Hydrolysis of *p*-Nitrophenyl Valerate[a]

pH	Temp, °C	k_{obsd} ($\times 10^3$), sec^{-1}
7.26	10.1	2.7
7.39		3.2
7.94		4.7
8.25		5.2
6.48	23.6	2.3
6.86		4.2
7.20		7.5
7.33		8.0
6.48	37.6	5.4
6.86		11.0
7.20		15.0
7.33		15.0

[a] Phosphate buffer, μ = 0.1.

Table V. Thermodynamics of Ionization of Kinetic Group on Elastase

Temp, °C	pK_a	ΔH_i, kcal	ΔS_i, eu
10.1	7.46	5.87 ± 0.04	−13.3 ± 0.14
23.6	7.24		
37.6	7.05		

The heat of ionization is 5.9 kcal, similar to 7.0 determined by kinetic methods for the related enzyme trypsin.[17] The slight difference may be due to the fact that this measurement refers to ionization of the free elastase

(13) R. Lumry, and S. Rajender, *J. Phys. Chem.*, **75**, 1387 (1971).
(14) L. Visser and E. R. Blout, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **28**, 837 (1969).
(15) F. J. Kedzy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).
(16) It has been reported that the reaction between chymotrypsin and *p*-nitrophenyl acetate is bimolecular [L. Faller and J. M. Sturtevant, *J. Biol. Chem.*, **241**, 4825 (1966)]. However, this has been subsequently shown to be due to the low range of substrate concentrations used: M. J. Hardman, P. Valenzuela, and M. L. Bender, *ibid.*, **246**, 5907 (1971).
(17) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441, 1955.

Table VI. Effect of Active Site Ionization on Activation Parameters for Elastase Catalyzed Hydrolysis[a,b]

p-Nitrophenyl	$\Delta H^{\pm}$, kcal/mol	$-\Delta S^{\pm}$, eu[c]	$\Delta H^{\pm}$, kcal/mol[d]	$-\Delta S^{\pm}$, eu[d]
Acetate	12.4 ± 0.6	9.98 ± 2.09	10.2 ± 0.8	16.1 ± 2.54
Propionate	11.8 ± 0.4	6.98 ± 1.35	9.8 ± 0.5	12.7 ± 1.54
Butyrate	9.30 ± 0.4	11.0 ± 1.27	7.2 ± 0.5	16.8 ± 1.55
Valerate	10.3 ± 0.1	9.89 ± 0.25	8.3 ± 0.2	15.5 ± 0.55
Hexanoate	10.6 ± 0.4	12.0 ± 1.34	8.10 ± 0.4	18.9 ± 1.1
N-Benzyloxycarbonylglycinate	11.2 ± 0.7	2.82 ± 2.26	8.60 ± 0.82	9.98 ± 2.7
N-*tert*-Butyloxycarbonyl-L-alaninate	10.9 ± 0.65	1.02 ± 2.2	8.53 ± 0.76	7.49 ± 2.6
Effect of Ionization of Active Site on the Activation Parameters for Chymotrypsin-Catalyzed Hydrolysis[a]				
Acetate	19.3 ± 0.6	4.78 ± 2.0	16.8 ± 0.6	11.9 ± 2.0
Propionate	12.0[b]		6.60[b]	
Butyrate	18.4 ± 1.0	7.80 ± 3.4	16.0 ± 1.1	14.7 ± 3.5
Valerate	18.6 ± 0.9	5.92 ± 2.9	16.3 ± 0.9	12.5 ± 2.9
Hexanoate	17.2 ± 0.5	7.88 ± 1.7	14.4 ± 0.4	16.2 ± 1.4
Heptanoate	15.4 ± 0.4	13.6 ± 1.5	12.7 ± 0.6	21.3 ± 2.1
Octanoate	18.3 ± 0.8	5.5 ± 2.8	15.4 ± 0.8	13.9 ± 2.7
Nonanoate	18.5 ± 0.8	1.4 ± 2.7	15.6 ± 0.8	3.3 ± 2.7

[a] pH 7.3 phosphate μ = 0.1, 3% or less acetonitrile. Error limits are one standard error. [b] Based on bimolecular rate constant, k_{cat}/K_m. [c] Without correcting for ionization. [d] With correction for ionization.

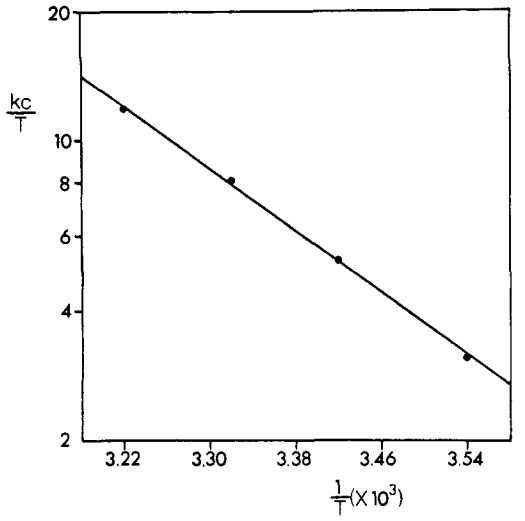

Figure 2. Effect of temperature on elastase catalyzed hydrolysis of *p*-nitrophenyl valerate plotted according to eq 2. Each rate constant is accurate to within ±2%.

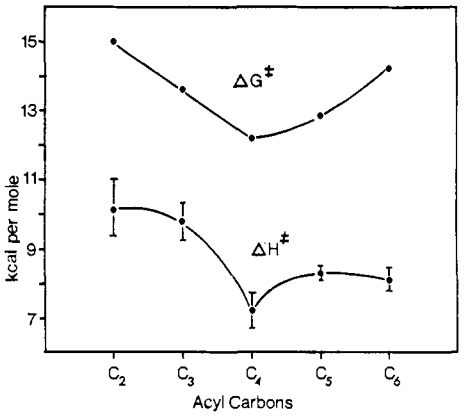

Figure 3. Correlation of free energy of activation at 25° and enthalpy of activation with fatty acid side chain length for elastase catalysis. The error limits indicated in the enthalpy represent the standard error in regression analysis of log k_c/T *vs.* $1/T$. The error in the free energy is ±25 cal/mol.

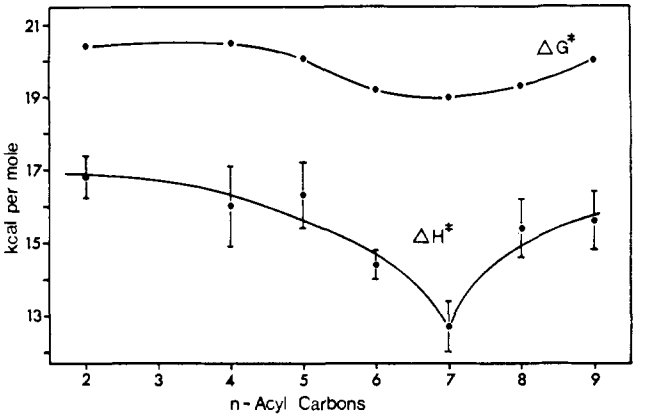

Figure 4. Relation of free energy of activation at 25° and enthalpy of activation to fatty acid chain length for chymotryspin deacylation. Error limits indicate 1 standard deviation from regression analysis of log k_c/T *vs.* $1/T$. The error in the free energy is ±60 cal/mol.

while the former refers to that of the acyl trypsin intermediate. These values are in the expected range for histidine side chain ionization and provide additional evidence that the kinetically important ionization in elastase may be a histidine.

A conventional Arrhenius plot for the reaction of chymotrypsin with *p*-nitrophenyl butyrate is shown in Figure 1. It is linear over a range of 27° and contains enough points that any nonlinearity should be apparent. Nonlinear Arrhenius plots, which are rather common in enzyme reactions,[18] have been reported for chymotrypsin.[19,20] A typical plot used to obtain activation parameters is shown in Figure 2. The effects of correcting for the changing ionization state of the active site with temperature are presented in Table VI. It lowers the activation enthalpy without affecting the qualitative trends. Within the homologous series of normal fatty acid esters enthalpy and free energies of activation are closely related for elastase (Figure 3) and chymotrypsin (Figure 4). The minima in both quantities occur with C_4 and C_7 for elastase and chymotrypsin, respectively. Our data suggest that for the elastase for the hexanoyl-C_6 side chain an unfavorable (negative)

(18) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press, New York, N. Y., 1964, p 158.
(19) H. Kaplan and K. J. Laidler, *Can. J. Chem.*, **45**, 547 (1967).
(20) D. M. Glick, *Biochim. Biophys. Acta*, **250**, 390 (1971).

entropy increment arises. On the other hand, for chymotrypsin, the preeminence of enthalpy in controlling reactivity seems to hold for the longer side chains beyond as well as before the optimal length of C-7. With both enzymes we have given special attention to the measurements involving the longer side chains, making repeated careful measurements so as to obtain as precise values as possible. Chymotrypsin shows some tendency for compensation in enthalpy and entropy since the minimum in the enthalpy profile is nearly three times as deep as that of the free energy profile. Our data may not, however, be precise enough to establish unambiguously linear compensation for this system.

Activation parameters were determined for acylation of chymotrypsin by *p*-nitrophenyl acetate under conditions of excess enzyme ($E_0 \gg S_0$). This system is expected to give first-order kinetics with a pseudo-first-order rate constant linearly proportional to enzyme concentration. The pseudo-first-order rate constant divided by E_0 is equal to k_2/K_s ($= k_{cat}/K_m$), the second-order acylation rate constant.[15] The kinetic results are reported in Table VII and the activation parameters in Table VIII. The slope gives the pH indepen-

Table VII. Kinetics of Chymotrypsin Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate under Conditions of Excess Enzyme

Temp, °C	pH 7.2[a] k_{obsd}, sec^{-1} ($\times 10^2$)	k,[b] M^{-1} sec^{-1} ($\times 10^{-3}$)
8.6	2.30	1.21
8.6	2.33	1.23
8.6	2.19	1.15
3.2	1.51	0.795
18.8	3.08	1.62
28.8	4.56	2.40
Temp, °C	**pH 5.05[c] k_{obsd}, sec^{-1} ($\times 10^4$)**	**k_c,[d] M^{-1} sec^{-1} ($\times 10^{-3}$)**
5.7	2.53	0.91
14.4	5.59	1.39
23.4	10.6	1.88
32.2	19.6	2.48

[a] Phosphate, $\mu = 0.1$, 0.2% (v/v) acetonitrile, $E_0 = 1.90 \times 10^{-5}$. [b] Pseudo-first-order rate constant divided by E_0. Not corrected for active site ionization. [c] Acetate, $\mu = 0.1$, 0.64% (v/v) acetonitrile. [d] Pseudo-first-order rate constant divided by E_0 and corrected for active site ionization.

dent value of k_2/K_m at 8.6° as 1.5×10^3 M^{-1} sec^{-1}, compared with 4.1×10^3 reported for 25°.[15] The enthalpy and entropy of activation for these reactions were evaluated by the same procedures as used for the deacylation reaction. The pH independent enthalpies of activation are more than twice as low as were found for deacylation under conditions of excess substrate. At pH 5.05 the correction for active site ionization is particularly important since the active site of the enzyme is essentially completely protonated. The enthalpy of ionization is as large as the (corrected) enthalpy of activation.

Discussion

The results reported here show that within this homogeneous series of apolar substrates, differences in enthalpy of activation play the dominant role in determining trends in reactivity. This pattern of enthalpy control of chymotrypsin specificity appears to vary from several published reports. Bender, *et al.*, found that within a range of specific and nonspecific substrates, relative rates of deacylation were determined by entropy of activation, the enthalpy being essentially constant.[4a] Gilleland and Bender extended this conclusion to the acylation reaction by specific substrates.[4b] Cane and Wetlaufer reported that increasing rates of deacylation within the C-2 to C-6 normal fatty acids resulted from large increases in enthalpy being overcompensated by favorable entropy.[5a] Fife and Milstien,[5b] whose experimental conditions were most similar to ours, reported slight increases in enthalpy to be overcome by less negative entropy of activation within the series C-2, C-3, C-4, C-6. Activation parameters for elastase have not been previously reported.

We believe that a major amount of the lack of agreement in relative and absolute values in activation parameters cited above is best attributed to a specific effect of Tris buffer. Table IX correlates our results

Table VIII. Activation Parameters for the Reaction of Excess Chymotrypsin with *p*-Nitrophenyl Acetate[a]

pH	$\Delta H^{\pm}$, kcal/mol	$-\Delta S^{\pm}$, eu
5.05	5.7 ± 0.3	24.0 ± 1.1
7.2	5.8 ± 0.7	24.0 ± 2.4

[a] Corrected for active site ionization. Errors are 1 standard deviation.

Table IX. Activation Enthalpies for Deacylation of *p*-Nitrophenyl Acetate Reacting with Chymotrypsin

$\Delta H^{\pm}$, kcal/mol	Conditions and buffer
15.7[a]	Turnover
16.8[b,c]	Turnover, phosphate
13.0[c,d]	Turnover, phosphate
8.5[e]	First order, Tris
9.7[f]	First order, Tris

[a] G. H. Dixon, W. J. Dreyer, and H. Neurath, *J. Amer. Chem. Soc.*, 78, 4810 (1956). [b] This research. [c] Corrected for effect of temperature on the ionization of the active site. [d] Reference 5b. [e] Reference 5a. [f] Reference 4a.

with those of several others in the literature. We suggest that the five independent measurements from different laboratories fall into two classes. Examination of the conditions employed suggests at least three possible sources for this: (1) first order *vs.* turnover kinetics; (2) presence or absence of excess substrate; (3) phosphate as opposed to Tris buffer. Our results tend to exclude explanation 2. We have measured activation parameters under conditions of excess substrate ($E_0 \ll S_0$) such that multiple substrate saturation is possible and excess enzyme ($E_0 \gg S_0$) such that this is not possible. The second-order rate constant in the first case corresponds to a turnover rate constant and in the latter instance to the acylation rate constant. Comparison of Tables III (for propionate reacting with chymotrypsin) and VIII (for acetate) shows that the two enthalpies are in the same range regardless of presence or absence of excess substrate. This implies

that the difference in activation enthalpy noted in Table IX may not be attributed to this factor. Our experiments favor explanation 3 since we find that under turnover conditions at pH 7.8, ionic strength 0.1, the enthalpy of activation for *p*-nitrophenyl acetate is some 5.5 kcal lower in Tris than in phosphate and barbital buffers. It is likely that this buffer effect, which has been noted before,[5b] influences values of and trends within activation parameters of other chymotrypsin reactions.

The first four normal fatty acid esters of the series react with elastase according to enthalpy control. However, the longest (C-6) has its reactivity reduced by an unfavorable entropy increment. This may be related to the special effect on the reactivity of substrates[21] and inhibitors[22] of longer chain lengths which has been interpreted as being related to limited conformational change in the region on direct ligand contact.[23]

Substrates of different structures may have entirely different patterns of activation parameters. For example, the two most specific substrates of elastase examined, *p*-nitrophenyl *N*-benzyloxycarbonylglycinate and *p*-nitrophenyl *N*-*tert*-butyloxycarbonyl-L-alaninate, have nearly identical enthalpies of activation. The entropies of activation differ slightly, distinguishing their reactivity, and are much less negative than those of the fatty acid esters. Thus we find entropy control, wherein differences in reactivity, *i.e.*, specificity, result from differences in entropy of activation. It may be significant that these *N*-acyl amino acids are capable of three-point attachment to the enzyme surface in the acyl enzyme whereas the normal fatty acids are capable of only two-point attachment.

The hypothesis that thermodynamic parameters are characteristic of a type of ligand structure is supported by the results of Belleau and DiTullio who studied the effect of varying chain length of *n*-alkyl tetramethylammonium activators on the methanesulfonylation of the acetylcholinesterase active site.[24] *n*-Alkyl substituents gave rise to bell-shaped curves (not unlike our system[3]) when acceleration was plotted against molar volume of the *n*-alkyl chain. Significantly, the enthalpy of binding, but not the free energy, showed some correlation with molar volume and with degree of acceleration. The hypothesis was submitted that binding enthalpy may reflect conformational changes such as would be initiated by ligand release of water from the binding cleft of acetylcholinesterase.[24] The observation that ligand binding to chymotrypsin displaces water may be of interest.[25] Regardless of the molecular mechanism, our results and those of Belleau and DiTullio suggest that enthalpy may be the fundamental thermodynamic parameter characterizing interaction between *n*-alkyl side chains and proteins regardless of whether the ligand is an activator or a substrate. We wish to suggest an analogy between enthalpy of binding and of activation.

It should be emphasized that it is difficult to interpret activation parameters in terms of a detailed mechanism. There has been a tendency to associate enthalpy effects with conformational or strain processes in the enzyme–substrate complex.[6,26] However, a recent discussion of solvation in enzyme-catalyzed reactions predicts interestingly that rate accelerations should result from favorable enthalpy of activation.[27] Recent studies of a model system whose rate enhancement was explained by "stereo-population control," or loss of configurational freedom in the substrate, revealed the rate acceleration was due to more favorable enthalpy of activation.[28] However, configurational restraint of the substrate was orginally offered as an explanation of entropy control of rates of deacylation of acyl chymotrypsins.[4a] Rate enhancement and specificity is a matter of differences in free energy of activation and it may be premature at this time to expect a unitary theory to be capable of accounting for all classes of substrates in terms of pure enthalpy or pure entropy effects.

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(21) (a) R. C. Thompson and E. R. Blout, *Proc. Nat. Acad. Sci. U. S.*, **67**, 1734 (1970); (b) A. Gertler and T. Hofmann, *Can. J. Biochem.*, **48**, 384 (1970).
(22) J. C. Powers and P. M. Tuhy, *J. Amer. Chem. Soc.*, **94**, 6544 (1972).
(23) D. M. Shotton, N. J. White, and H. C. Watson, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 91 (1972).
(24) B. Belleau and V. DiTullio, *J. Amer. Chem. Soc.*, **92**, 6320 (1970).
(25) T. A. Steitz, R. Henderson, and D. M. Blow, *J. Mol. Biol.*, **46**, 337 (1969).
(26) R. Lumry and R. Biltonen in "Structure and Stability of Biological Macromoles," S. N. Timasheff and G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 174.
(27) J. W. Larsen, *Biochem. Biophys. Res. Commun.*, **50**, 839 (1973).
(28) R. T. Borchardt and L. A. Cohen, *J. Amer. Chem. Soc.*, **94**, 9166 (1972).

Communications to the Editor

Stereochemistry of the Thermal Isomerization of 5-Methylenebicyclo[4.2.0]octa-2,7-dienes Leading to Tricyclo[4.3.0.0^{4,6}]nona-2,8-dienes[1]

Sir:

There remain some ambiguities on the reaction mechanism of the thermally induced isomerization of the fused cyclobutenes which concern ring opening.[2] For instance, a few contradictory arguments have been reported[3,4] against the proposal of antara–antara Cope

(1) Organic Thermal Reaction. XIX. For paper XVIII, see H. Tsuruta, T. Kumagai, and T. Mukai, *Chem. Lett.*, 981 (1972).
(2) (a) J. I. Brauman and D. M. Golden, *J. Amer. Chem. Soc.*, **90**, 1920 (1968); (b) J. E. Baldwin and G. D. Andrews, *J. Amer. Chem. Soc.*, **94**, 1775 (1972); (c) H. M. Frey and R. Walsh, *Chem. Rev.*, **69**, 103 (1969).
(3) J. E. Baldwin and M. S. Kaplan, *J. Amer. Chem. Soc.*, **93**, 3969 (1971), and references cited therein.
(4) H. J. Hansen and H. Schmid, *Chem. Brit.*, **5**, 111 (1969), and references cited therein.