Calorimetric Studies of the Activation of Chymotrypsinogen A*

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ABSTRACT: The enthalpy change in the trypsin-catalyzed "rapid activation" of chymotrypsinogen A to π-chymotrypsin (EC 3.4.4.5) at pH 7.4 and 25° is estimated by direct calorimetric measurements to be 0 ± 0.5 kcal mole^{-1}. This estimate is based on the observation that the activation of the zymogen in the presence of the chymotrypsin inhibitor hydrocinnamate, and the binding of hydrocinnamate to π-chymotrypsin, are accompanied by nearly the same enthalpy changes, approximately -6.5 kcal mole^{-1}. The heat of activation observed in various buffers in the presence of the inhibitor is independent of the heat of ionization of the buffer, indicating that there is no change in the extent of protonation of the protein, as is to be expected for the hydrolysis of a peptide bond to fully charged products. Activation in the absence of the inhibitor, on the other hand, is accompanied by enthalpy changes which depend linearly on the buffer ionization heat. The enthalpy of activation, corrected for buffer ionization changes, is $+16.8 \pm 0.4$ kcal mole^{-1} in the presence of 0.1 M NaCl and $+7.4 \pm 0.3$ kcal mole^{-1} in the presence of 0.05 M $CaCl_2$, reflecting the fact that the latter salt largely suppresses the autolysis of δ-chymotrypsin. The corresponding liberation of protons, as deduced from the calorimetric data, is 1.7 protons per molecule in NaCl solution and 1.1 in $CaCl_2$ solution. These results, together with estimates in NaCl and $CaCl_2$ solutions of the extent of autolysis of δ-chymotrypsin, give values of -2 ± 1 kcal mole^{-1} for the enthalpy of conversion of π-chymotrypsin to δ-chymotrypsin, and $+22 \pm 5$ kcal mole^{-1} for that of the conversion of the latter to its autolysis products. The chymotrypsinogen A–π-chymotrypsin equilibrium appears to favor π-chymotrypsin rather strongly, and thus to involve a significant decrease in free energy. This decrease must be primarily the result of an increase in entropy, perhaps stemming from a loosening of the structure of the protein. In view of the lack of valid representative values for the enthalpy changes to be expected for the various processes known to be involved in the activation of chymotrypsinogen, it is not possible to give a satisfactory rationalization of the results reported here.

The conversion of the bovine pancreatic zymogen, chymotrypsinogen A, to the various forms of active chymotrypsin (EC 3.4.4.5) has been shown to be a very complex process (Neurath, 1957; Desnuelle, 1960). Under conditions of relatively high trypsin:zymogen weight ratios, the Arg-15–Ile-16 bond is hydrolyzed to yield π-chymotrypsin. Rapid autolysis then leads to splitting of the Leu-13–Ser-14 bond, with liberation of the dipeptide Ser-Arg. The product of this reaction, δ-chymotrypsin, undergoes further autolysis to less active forms, one of which is an enzyme with the Tyr-146–Thr-147 bond hydrolyzed (Bettelheim and Neurath, 1955; Chervenka, 1962). A different activation process, usually initiated by a very low concentration of trypsin, yields the well-known α-chymotrypsin, and is relatively slow, making no significant contribution to the reaction as carried out in our experiments. The distribution of chymotrypsins in an activation mixture thus depends on the ratio of zymogen to trypsin and the duration of the reaction. The presence or absence of various substances such as $CaCl_2$ or the chymotrypsin inhibitor hydrocinnamate, as well as the temperature and the pH, also have effects on the composition of the product of the activation process.

In addition to the covalent changes occurring during activation, there is a variety of evidence that conformational changes parallel the appearance of enzymic activity (Neurath *et al.*, 1956; Chervenka, 1959; Raval and Schellman, 1965; Biltonen *et al.*, 1965; Fasman *et al.*, 1966; McClure and Edelman, 1967; Oppenheimer *et al.*, 1966). The details of these changes have been revealed by recent X-ray crystallographic studies (Sigler *et al.*, 1968; Freer *et al.*, 1970).

In view of the wide range of information concerning the activation of chymotrypsinogen which is now available, it is of interest to investigate the thermodynamics of the process. In this paper we report the results of direct calorimetric measurements of the changes in enthalpy accompanying some of the steps in the rapid activation of chymotrypsinogen A in the presence of relatively large concentrations of trypsin at pH 7.4 and 25°.

Materials and Methods

δ-Chymotrypsin, three-times-recrystallized chymotrypsinogen A, α-chymotrypsin, and two-times-recrystallized, salt-free trypsin were purchased from Worthington Biochemical Corporation, Freehold, N. J., and were used without further purification. δ-Chymotrypsin was also prepared using a standard procedure (Schwert and Kaufman, 1949). Solutions of π-chymotrypsin at low pH were obtained by the following procedure: 1 g of chymotrypsinogen A was dissolved in 40 ml of 0.005 M KH_2PO_4 buffer which was 0.15 M in KCl and 0.1 M in hydrocinnamate. The pH of the solution was adjusted to 7.4 and the solution was centrifuged and filtered. The zymogen solution was then activated for 60 min at 5° by the addition of 20 mg of crystalline trypsin, at which time the pH of the solution was adjusted to 3.0 with 2 N H_2SO_4. The solution was filtered, then dialyzed against 10^{-3} M HCl and 0.15 M KCl, and concentrated to about 0.5 the original volume using a Diaflo ultrafiltration apparatus.

Protein concentrations were determined by measuring the optical density of diluted samples at 280 nm; the molar

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absorptivity was assumed to be 5×10^4 M^{-1} cm^{-1} for chymotrypsinogen and chymotrypsin (Dixon and Neurath, 1957), and trypsin concentrations in milligram per milliliter were calculated by multiplying the optical density by 0.651 (data supplied by Worthington Biochemical Corp.). Chymotrypsin and trypsin activities were determined by the method of Schwert and Takenaka (1955), using *N*-acetyl-L-tyrosine ethyl ester and *N*-benzoyl-L-arginine ethyl ester, respectively, as substrates.

Hydrocinnamic acid was purchased from Matheson Coleman and Bell Co., East Rutherford, N. J., and was recrystallized from glass-distilled water. Piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Company, St. Louis, Mo., and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid from Calbiochem, Los Angeles, Calif.[1]

Calorimetric measurements of the activation of chymotrypsinogen were carried out using the Beckman Model 190 microcalorimeter. In a typical experiment 15.0 ml of the zymogen solution was mixed with 0.2 ml of a solution of trypsin in 10^{-3} M HCl. The reference vessel contained identical components with the exception of the zymogen. The apparent heat of the binding of hydrocinnamate to chymotrypsinogen and to π-, δ-, and α-chymotrypsin was measured with a flow modification of the Beckman Model 190 microcalorimeter (Sturtevant and Lyons, 1969; Velick *et al.*, 1971). In these experiments, a solution of the protein in 0.15 M KCl, adjusted to a pH between 3 and 5 to prevent autolysis, was mixed with 0.1 M phosphate buffer and 0.15 M KCl, with and without 0.1 M hydrocinnamate, to give a final pH of 7.4. The difference between the heat effects observed in the presence and absence of the inhibitor, corrected for the heat of dilution of the inhibitor, is the apparent heat of inhibitor binding. The heat of protein dilution, observed for α-chymotrypsin under similar conditions (Shiao and Sturtevant, 1969), is corrected by the blank experiment. The flow calorimeter was also used in the determination of the heats of ionization of Pipes and Hepes.

The kinetics of urea denaturation of chymotrypsinogen A, α-chymotrypsin, and various samples taken during activation was studied according to the method described by Chervenka (1962), using a Cary 14 spectrophotometer.

Results

Heats of Ionization of Pipes and Hepes. The use of buffers in calorimetric studies requires a knowledge of the heats of ionization (ΔH_i) of their ionizing groups in order to correct the observed heat effects for any proton release or uptake by the buffer during the reaction under study (Sturtevant, 1962). For the second ionization of phosphoric acid ΔH_i is +1.13 kcal mole^{-1} (G. D. Watt, 1968, personal communication), and for the ionization of Tris it is +11.3 kcal mole^{-1} (Öjelund and Wadsö, 1968). For Pipes and Hepes these quantities were determined by measuring the heats of titration of dilute solutions of the buffers in the presence of 0.1 M NaCl at 25° as a function of final pH, using the flow microcalorimeter. In these experiments, the final concentration of Pipes varied between 0.286 mM and 8.23 mM, and that of Hepes between 0.255 mM and 8.01 mM; 0.001 M and 0.01 M HCl solutions were used to titrate the buffer solutions from an initial pH of 9.0. The titration curves were analyzed according to eq 1 using a least-squares procedure. In eq 1,

$$\frac{1}{\Delta H_{tit}} = \left(\frac{K_d}{\Delta H_b} \times \frac{1}{[H^+]}\right) + \frac{1}{\Delta H_b} \qquad (1)$$

ΔH_{tit} is the experimentally observed heat, ΔH_b is the heat of protonation ($-\Delta H_i$) of the ionizing group, and K_d is its dissociation constant. The titration of the sulfonic acid groups of the buffers contributes to the ΔH_{tit} values at low pH, and therefore the results for final pH less than 3.9 were not used. In this way the heats of ionization were found to be +2.72 ± 0.06 and +4.93 ± 0.25 kcal mole^{-1} for Pipes and Hepes, along with 6.93 and 7.23 for the respective pK_d values. Since at pH 9.0 a small fraction of the buffers is still in the protonated form, the heats of ionization are corrected to be +2.74 and +5.01 kcal mole^{-1} for Pipes and Hepes, respectively. The experimentally determined titration results, along with the expected curves based on the above values for ΔH_i and pK_d are shown in Figure 1.

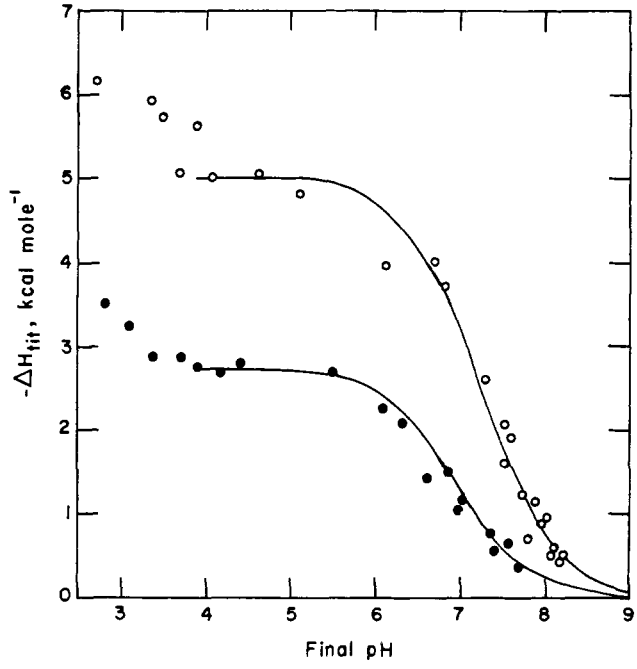

FIGURE 1: Thermal titration curves for Pipes (●) and Hepes (○). In each case, buffer at initial pH 9.00 was mixed with varying amounts and concentrations of HCl in the flow calorimeter. Final solution compositions were as follows: buffer concentration, 0.286 mM to 8.23 mM for Pipes and 0.255 mM to 8.01 mM for Hepes; NaCl, 10 mM. The solid curves correspond to the values for the heats of ionization and the pK's given in the text.

Enthalpy of Activation of Chymotrypsinogen in the Presence of Hydrocinnamate. The activation process can be stopped at the π-chymotrypsin stage by means of a chymotrypsin inhibitor. Calorimetric measurements of the activation were made in solutions containing 0.1 M hydrocinnamate as inhibitor, in various buffers containing either 0.1 M NaCl or 0.05 M $CaCl_2$, at pH 7.4 and 25°. Weight ratios of trypsin to zymogen of approximately 0.04 were employed. The data obtained in these experiments are summarized in Table I. The uncertainties given in the table, and elsewhere in this paper, are standard errors of the mean.

Although under the experimental conditions employed

[1] The following abbreviations will be used for the buffers: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl) aminomethane (Good *et al.*, 1966).

TABLE I: Apparent Heat of Activation of Chymotrypsinogen A in the Presence of 0.1 M Hydrocinnamate at pH 7.4 and 25°.

Initial Chymotrypsinogen Concn (mM)	Buffer Used (Concn, M)	Buffer Heat of Ionization, ΔH_i (kcal mole^{-1})	Observed Heat Evolution (mcal per 15.0 ml of Zymogen Solution)	$-\Delta Q_{app}$ (kcal per mole of Zymogen)
		Systems Containing 0.1 M NaCl		
0.384	Phosphate (0.05)	1.13	33.6	5.83
0.396	Phosphate (0.10)	1.13	35.6	5.99
0.395	Pipes (0.05)	2.74	42.0	7.09
0.367	Hepes (0.05)	5.01	35.3	6.41
0.390	Tris (0.05)	11.3	40.0	6.84
				Mean 6.43 ± 0.24
		Systems Containing 0.05 M $CaCl_2$		
0.398	Pipes (0.05)	2.74	46.8	7.84
0.364	Tris (0.05)	11.3	38.5	7.05
0.364	Tris (0.05)	11.3	41.9	7.67
				Mean 7.52 ± 0.30

the rate of activation was such that maximum enzymic activity was obtained within 10–15 min, nevertheless a total time of approximately 1 hr was usually required before the calorimeter output recorder had returned to baseline. The precision of data obtained in such prolonged experiments is somewhat less than expected on the basis of experience with instantaneous processes.

TABLE II: Enthalpy of Binding of Hydrocinnamate to α-, δ-, and π-Chymotrypsins and to Chymotrypsinogen A at pH 7.4 and 25°, in the Presence of 0.15 M KCl and 0.05 M–0.10 M Phosphate.

Protein	Protein Concn (mM)	Hydrocinnamate Concn (mM)	$-\Delta Q'_{app}$ (kcal mole^{-1})
α-Chymotrypsin	0.288	66.0	6.1
	0.363	50.0	7.5
	0.363	50.0	7.1
	0.370	66.0	7.1
	0.391	66.0	6.3
	0.433	50.0	7.0
		Mean	6.8 ± 0.2
δ-Chymotrypsin	0.306	66.0	5.9
	0.320	66.0	4.8
	0.460	50.0	7.2
	0.481	50.0	5.7
	0.481	50.0	6.3
		Mean	6.0 ± 0.4
π-Chymotrypsin	0.248	50.0	6.3
	0.421	50.0	6.0
Chymotrypsinogen A	0.356	66.0	−0.2
	0.371	55.0	−0.3
	0.373	50.0	−0.3
	0.535	50.0	−0.1
		Mean	−0.2 ± 0.1

Enthalpy of Binding Hydrocinnamate to Chymotrypsinogen A and to π-, δ-, and α-Chymotrypsins. Since, in the activation experiments outlined in the preceding paragraphs, the product, π-chymotrypsin, was saturated with hydrocinnamate, it was necessary to determine the enthalpy change associated with the interaction of the enzyme with the inhibitor, and also with the dissociation of any inhibitor bound to the zymogen at the start of the reaction. The results of experiments designed for this purpose are given in Table II. In most cases the procedure outlined in Materials and Methods was used, but in a few cases the direct method employed by Shiao and Sturtevant (1969) was applied, with closely agreeing results. The data for α-chymotrypsin are in agreement with those of Shiao (1970) obtained under slightly different conditions.

Enthalpy of Activation of Chymotrypsinogen in the Absence of an Inhibitor. The activation in the absence of an inhibitor was studied in a number of buffers with different heats of ionization (see Table I for the buffer heats of ionization), and in the presence of 0.05 M $CaCl_2$ or 0.1 M NaCl. These calorimetric experiments were complicated by the appearance of significant drifts, which continued for hours in the cases where the initial zymogen concentration was about 1%. An arbitrary procedure, in which the drift rate was calculated after 60 min of activation, and the total heat measured up to this time was corrected by assuming the drift to be constant throughout the reaction, was used to obtain some of the data in Table III. The results of these experiments agreed fairly well with activations carried out under similar conditions but using much less zymogen, in which cases the drift was usually negligible. This observation, along with numerous activity determinations at various times during the activations, suggests that under conditions of these experiments nonspecific autolysis takes place which results in heat effects which are not related to the primary processes of the activation reactions.

Dependence of the Distribution of Various Forms of Chymotrypsin on Salt Composition of Activation Mixtures. The significant differences between the observed enthalpies of

TABLE III: Enthalpy of Activation of Chymotrypsinogen A in Various Buffers with $CaCl_2$ or NaCl at pH 7.4 and 25°.

System: Buffer and Salt	Initial Chymotrypsinogen Concn (mM)	Observed Heat Evolution (mcal per 15.0 ml of Zymogen Solution)	ΔQ_{obsd} (kcal mole^{-1})
0.05 M Tris, 0.05 M $CaCl_2$	0.397	26.6	−4.46
	0.394	32.0	−5.41
	0.383	25.0	−4.37
	0.338	27.2	−5.37
	0.338	24.7	−4.87
	0.237	17.5	−4.90
	0.193	14.2	−4.90
	0.097	7.80	−5.38
0.05 M Hepes, 0.05 M $CaCl_2$	0.693	−13.5	1.30
	0.424	−19.4	3.05
	0.404	−6.44	1.06
	0.389	−7.94	1.36
	0.192	−6.34	2.20
	0.100	−2.35	1.56
	0.073	−2.46	2.23
	0.058	−2.82	3.20
0.05 M Pipes, 0.05 M $CaCl_2$	0.395	−23.0	3.87
	0.394	−24.0	4.09
	0.377	−27.0	4.78
	0.080	−5.60	4.64
0.05 M Tris, 0.1 M NaCl	0.350	12.4	−2.37
	0.274	7.99	−1.39
0.05 M Hepes, 0.1 M NaCl	0.350	−43.9	8.36
0.05 M Pipes, 0.1 M NaCl	0.410	−70.8	11.5
	0.409	−70.5	11.5
0.05 M Phosphate, 0.1 M NaCl	0.404	−89.6	14.8
	0.402	−88.3	14.6
	0.402	−97.3	16.1
	0.400	−94.4	15.7

activation in a particular system containing $CaCl_2$ or NaCl are paralleled by a difference in the level of enzymic activity after 60 min of activation, as can be seen from the data in Table IV. The activities given in the table are relative to the activity of δ-chymotrypsin prepared at 5° using a standard procedure. On this basis the activity of the sample which contained NaCl approximates the activity of commercial α-chymotrypsin. The kinetic assay developed by Chervenka (1962) was utilized to estimate the fraction of unautolyzed δ-chymotrypsin remaining after 60 min of activation in the presence of $CaCl_2$ and NaCl. As seen in Table IV, the distribution of active chymotrypsin among its various forms is strongly dependent on the presence or absence of $CaCl_2$. The importance of this finding in relation to the calorimetric results on these systems will be discussed below.

TABLE IV: Dependence of the Fraction of δ-Chymotrypsin Formed on the Salt Present in Inhibitor-Free Activation Mixtures at pH 7.4 and 25°.

	Relative Specific Activity[a]	Per Cent δ-Chymotrypsin[b]
Activations in presence of 0.05 M $CaCl_2$	0.92 ± 0.03	51.5 ± 1.2
Activations in presence of 0.1 M NaCl	0.65 ± 0.03	13.8 ± 4.2

[a] The activities are relative to the specific activity of δ-chymotrypsin prepared at 5° and are the averages of five separate determinations in each case. The samples were taken after 60 min of activation in the various buffer systems described in the text. [b] Determined according to the method described by Chervenka (1962); each value is the average of two separate experiments in which samples were taken after 60 min of activation.

Discussion

As seen in Table I, the heat of activation of chymotrypsinogen in the presence of hydrocinnamate is independent of the heat of ionization of the buffer used. This shows that there is essentially no change in the extent of protonation of the protein during the activation, and that any difference between the zymogen and the enzyme in the binding of phosphate is without significant enthalpy effect. Since the chemical process involved is the hydrolysis reaction

$$-NHCO- + H_2O \rightleftharpoons -NH_3^+ + {}^-OOC-$$

for which no change in protonation is expected, we may conclude that the subsequent conformational changes outlined below have no appreciable affect on the ionization constants of any groups in the protein with pK values near 7.4, such as His-57 at the active site.

The enthalpy of the reaction is approximately 1 kcal mole^{-1} more negative in the presence of 0.05 M $CaCl_2$ than in 0.1 M NaCl. A few calorimetric experiments on the addition of $CaCl_2$ to α- and δ-chymotrypsins to give a final concentration of 0.05 M led to an enthalpy value of about −2 kcal mole^{-1}, while similar experiments with chymotrypsinogen gave −1 kcal mole^{-1}. Although experiments of this sort have not been performed with inhibited π-chymotrypsin, it seems likely that these binding enthalpies account for the difference in activation enthalpies shown in Table I.

The mean enthalpy effect in NaCl solution is −6.43 ± 0.24 kcal mole^{-1}. Under similar conditions, the average difference in the apparent binding heats of the inhibitor to π-chymotrypsin and to chymotrypsinogen is −6.4 ± 0.4 kcal mole^{-1}. It thus appears that the conversion of chymotrypsinogen A to π-chymotrypsin is characterized by an enthalpy change of 0 ± 0.5 kcal mole^{-1}. This result is surprising in view of the variety of processes which are known to result from the initial peptide-bond hydrolysis.

In the presence of hydrocinnamate, π-chymotrypsin is the major product of the reaction, although the subsequent π- to δ-chymotrypsin conversion is not completely inhibited (Dreyer and Neurath, 1955; Bettelheim and Neurath, 1955). The hydrolysis of the Arg-15–Ile-16 bond at neutral pH

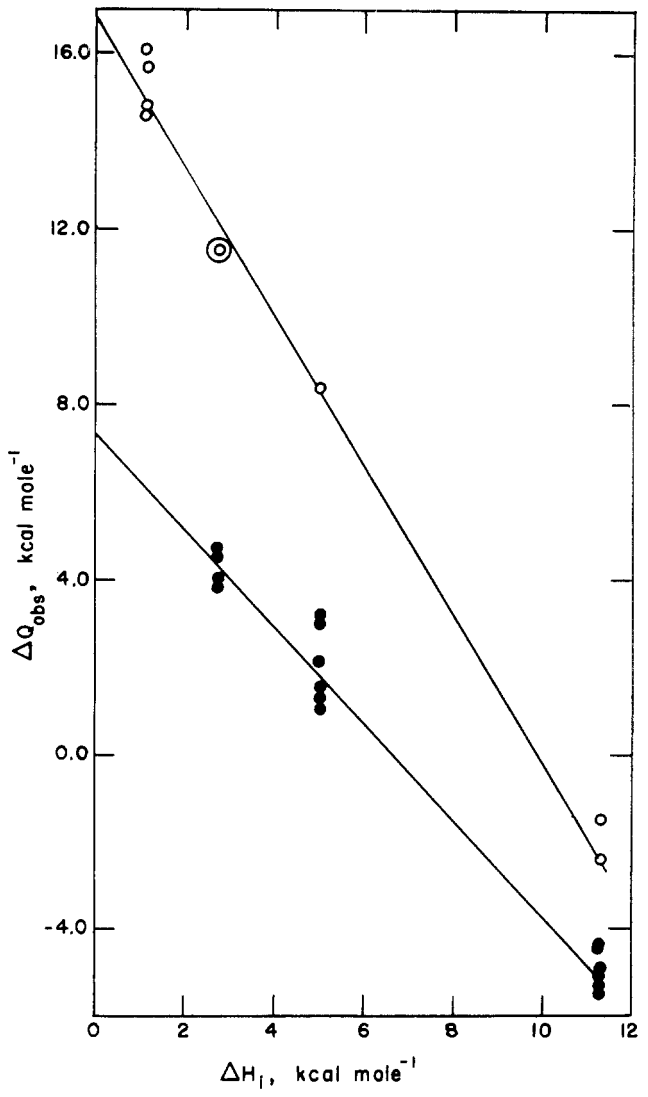

FIGURE 2: The variation at pH 7.4 and 25° of the enthalpy of activation of chymotrypsinogen A in inhibitor-free solutions with buffer ionization heat. In order of increasing ionization heat, the buffers employed were phosphate, Pipes, Hepes, and Tris, all at a concentration of 0.05 M. Added salts were 0.05 M $CaCl_2$ (●) and 0.1 M NaCl (○). Zymogen concentrations ranged from 0.06 mM to 0.69 mM.

results in the formation of an ion pair between the newly created positively charged N-terminus at Ile-16 and the negatively charged β-carboxyl group of Asp-194. The integrity of this ion pair has been shown to be crucial to the activity of the various chymotrypsins (Oppenheimer *et al.*, 1966), and its existence has been verified by X-ray studies (Sigler *et al.*, 1968). In addition to a number of optical studies of the activation, which resulted in some controversy about the extent and nature of conformational changes accompanying the process (Neurath *et al.*, 1956; Imahori *et al.*, 1960; Raval and Schellman, 1965; Biltonen *et al.*, 1965; Fasman *et al.*, 1966), recent reports on the results of X-ray studies of chymotrypsinogen A and α-chymotrypsin support the conclusion that the activation is accompanied by a number of local conformational changes (Sigler *et al.*, 1968; Freer *et al.*, 1970). According to the X-ray data these changes parallel the formation of π-chymotrypsin since the π, δ, and α forms of chymotrypsin are very similar in structure (Kraut *et al.*, 1967; Wright *et al.*, 1968; Matthews *et al.*, 1968; Cohen *et al.*, 1969; Davies *et al.*, 1969) and involve significant movements of the side chains of the following amino acid residues: Ile-16, Val-17, Asp-194, His-57, Ser-214, Ile-99, Arg-145 and Met-192. The changes in the positions of the first three are necessary for the formation of the important ion pair, while the movement of the remaining side chains is associated with the reorganization of the catalytic and binding sites of the enzyme. Some of these movements involve the transfer of hydrophobic side chains from the surface to the interior of the molecule (Ile-16 and Val-17), while the change in the position of others (*e.g.*, Met-192) is in the opposite direction. Changes in the positions of several other residues have also been noted, along with small changes in hydrogen bonding within the catalytic site of the enzyme. Since the measured enthalpy change for the activation includes contributions from all of the above molecular events that accompany the formation of π-chymotrypsin, we can only conclude that there is a nearly complete balance of exothermic and endothermic contributions. Any attempt at a quantitative analysis of this balance is premature because inadequate information concerning the enthalpy changes to be expected for the various contributions is available. The heats of peptide-bond hydrolysis which have been reported (Sturtevant, 1962), although small, are not directly applicable. Only rough estimates of the enthalpy changes in the formation of hydrophobic interactions are available (Kauzmann, 1959; Tanford, 1970). The enthalpy change due to the electrostatic interaction between Ile-16 and Asp-194 is determined in the usual Bjerrum approximation (see, for example, Rawitscher *et al.*, 1961) by the temperature coefficient of the effective dielectric constant in the vicinity of the charged groups, and this is, of course, a totally unknown quantity.

The formation of π-chymotrypsin appears to go practically to completion under the conditions employed in these experiments. Since the enthalpy change is very small, it is evident that the necessary decrease in free energy is largely the result of a substantial increase in entropy. As in the case of the enthalpy change, it is not possible to give a convincing rationalization for a positive value of ΔS.

The observed enthalpies of activation in the absence of inhibitor are strongly dependent upon the buffer and salt composition of the reaction systems (Table III). The variation of the observed heats with buffer heats of ionization indicates that there is proton release in the presence of either $CaCl_2$ or NaCl. In each case the observed heat, ΔQ_{obsd}, can be extrapolated to zero heat of buffer ionization using the relationship

$$\Delta Q_{obsd} = \Delta H_{app} + n\Delta H_i \qquad (2)$$

where ΔH_{app} is the extrapolated heat, ΔH_i the buffer ionization heat, and n is the number of protons involved in the reaction. Least-squares analysis of the data in Table III according to eq 2 yields for ΔH_{app} the values $+7.4 \pm 0.3$ and $+16.8 \pm 0.4$ kcal mole^{-1}, and for n the values -1.1 ± 0.1 and -1.7 ± 0.1 (protons are released) for the systems containing $CaCl_2$ and NaCl, respectively. The variation of the experimentally observed enthalpies with buffer heat of ionization, along with the calculated relationship based on the above parameters is shown in Figure 2. The adherence of the values in phosphate buffer to the upper line in the figure indicates that phosphate binding has no detectable enthalpy effect. When these results are compared with the results in Table IV, it can be seen that the difference in the values of ΔH_{app} parallels a difference

in the distribution of unautolyzed δ-chymotrypsin in activation mixtures containing $CaCl_2$ or NaCl.

If we assume that in each system the apparent extrapolated heat, ΔH_{app}, is the sum of the enthalpy changes for the following processes

$$\text{chymotrypsinogen A} \xrightarrow{\Delta H_1} \delta\text{-CT} + n_1H^+ \quad (3)$$

$$\delta\text{-CT} \xrightarrow{\Delta H_2} \delta\text{-CT (modified)} + n_2H^+ \quad (4)$$

where δ-CT (modified) represents all the autolyzed forms of δ-chymotrypsin, then for each case

$$\Delta H_{app} = \Delta H_1 + f_2\Delta H_2$$

In this equation f_2 is the fraction of δ-chymotrypsin which has autolyzed to the form(s) with lower specific activity. Using the data in Table IV and the above values for ΔH_{app},[1] we obtain -2 and $+22$ kcal mole^{-1} for ΔH_1 and ΔH_2, respectively. In a similar manner the number of protons released in each step, n_1 and n_2, are calculated to be 0.3 and 1.6, respectively. Due to the difficulties encountered in the calorimetric experiments on these systems, there is a large uncertainty (about 20%) associated with these results. ΔH_1 obtained in this manner is an estimate of the enthalpy change for all the processes included in the conversion of the zymogen to π-chymotrypsin, and in addition, the chrymotrypsin-catalyzed hydrolysis of the peptide bond between Leu-13 and Ser-14, resulting in the release of the dipeptide seryl-arginine and the formation of δ-chymotrypsin, and any other changes caused by the hydrolysis. Since the enthalpy of formation of π-chymotrypsin has been shown to be nearly zero, it follows that ΔH_1 gives an approximate value for the enthalpy change for the π- to δ-chymotrypsin reaction. ΔH_2, on the other hand, is associated with the formation of autolyzed products of δ-chymotrypsin, one of which has been proposed to be an α-chymotrypsin-like enzyme in which the Tyr-146-Thr-147 bond has been cleaved (Chervenka, 1962).

It is well known that the various forms of chymotrypsin undergo reversible polymerization reactions under a wide variety of conditions whereas chymotrypsinogen does not. Particularly at the high concentrations employed in the activation experiments, it might thus be expected that significant enthalpy contributions would arise from this source. The most pertinent data in this connection have been recently published by Shiao and Sturtevant (1969). These authors found that at pH 7.8 and 25°, in 0.05 M phosphate buffer containing 0.2 M KCl, α-chymotrypsin shows heats of dilution which can be accounted for in terms of a reversible dimerization with a dissociation constant of 3.5×10^{-4} M and a dissociation enthalpy of +17.7 kcal (mole of monomer)$^{-1}$. Experiments on the binding of inhibitors to partially "dimerized" α-chymotrypsin indicated that binding takes place only with the monomeric protein. If these observations are also valid for π-chymotrypsin at pH 7.4, it would appear that the data given in Table II contain very considerable contributions from the enthalpy of dissociation of dimers. On the other hand, Krigbaum and Godwin (1968) studied solutions of chymotrypsinogen A and various chymotrypsins by low-angle X-ray scattering and concluded that chymotrypsinogen and α- and δ-chymotrypsins are almost entirely monomeric at pH 7 in 0.1 M NaCl at protein concentrations up to 20×10^{-4} M. It seems clear that the nature of the polymerization process in the case of α-chymotrypsin is very different at pH 7.8 and at pH values of 6.2 and below (Morimoto and Kegeles, 1967), and it is conceivable that the extent of the reactions could depend strongly on pH. It is thus impossible on the basis of presently available data to take reliable estimates of the influence of aggregation on the activation ehthalpies given above. In the most interesting case, that of rapid activation to π-chymotrypsin, the enthalpy of activation at infinite dilution at worst would be approximately 9 kcal mole^{-1} more positive than the value 0 ± 0.5 kcal mole^{-1} quoted above, for the activation at 4×10^{-4} M, although the discrepancy might well be much smaller than this. Similar aggregation enthalpies are of course included in the activations run in the absence of inhibitor.

[1] The value $\Delta H_{app} = +7.4$ is corrected to +8.4 to allow for the difference, noted earlier, in the enthalpy of Ca^{2+} binding to the zymogen and the active enzymes.

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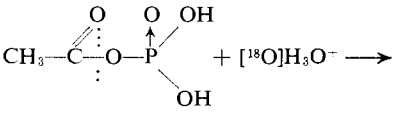
Oxygen-18 Studies to Determine the Position of Bond Cleavage of Acetyl Phosphate in the Presence of Divalent Metal Ions*

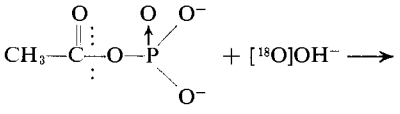
Judith Pollock Klinman† and David Samuel

ABSTRACT: The hydrolysis of acetyl phosphate has been carried out in [^{18}O]H_2O at 39° in the presence of Mg^{2+} and Ca^{2+} in the pH range 7.4–8.2. The objective of this study was to determine the position of bond cleavage of metal-complexed acetyl phosphate for each of the rate terms contributing to the metal ion catalyzed hydrolysis. The breakdown of divalent metal ion complexed acetyl phosphate (AcPM) by water occurs by both P–O and C–O cleavage; P–O cleavage is the predominant mode for the Mg^{2+} system, whereas for the Ca^{2+} system C–O cleavage occurs to a greater extent than P–O cleavage. The breakdown of AcPM by MOH^+ occurs by C–O cleavage. The data obtained from this study are consistent with a C–O mode of cleavage of AcPM by OH^- in the case that values for K, the association constant for the formation of AcPM, are close to those originally reported; the effect of significantly larger values for K, which have been recently reported, on the interpretation of this mode is discussed.

This study was undertaken to determine the effect of divalent cations on the position of bond cleavage of acetyl phosphate in order to understand better the catalytic role of metal ions in the hydrolysis of acyl phosphates. The uncatalyzed hydrolysis of acetyl phosphate has been studied in considerable detail (Lipmann and Tuttle, 1944; Koshland, 1952; Kurz and Gutsche, 1960; DiSabato and Jencks, 1961). By isotope labeling experiments, acetyl phosphate has been shown to undergo C–O or P–O cleavage, depending on the pH

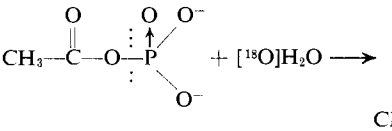


$$+ [^{18}O]H_3O^- \longrightarrow [^{18}O]CH_3COOH + H_3PO_4 + H_3O^+$$



$$+ [^{18}O]OH^- \longrightarrow [^{18}O]CH_3COO^- + HPO_4^{-2}$$

(Bentley, 1949; Park and Koshland, 1958). Under acidic or basic conditions, C–O bond cleavage occurs. At more neutral pH values (where acetyl phosphate exists as either a mono- or dianion), P–O bond cleavage is the predominant pathway.



$$+ [^{18}O]H_2O \longrightarrow CH_3CO_2^- + [^{18}O]H_2PO_3O^{-1}$$

Oestreich and Jones (1966) describe the catalysis of the breakdown of the divalent anion of acetyl phosphate by divalent metal ions by eq 1.[1] The present paper describes the results of

$$v_{cat} = k_2[\text{AcPM}] + k_3[\text{AcPM}][OH^-] \quad (1)$$

a determination of the amount of P–O and C–O cleavage contributing to each of the two rate terms in eq 1 for catalysis by Mg and Ca ions, utilizing the rate and equilibrium constants determined by Oestreich and Jones.

In a recent report by Briggs *et al.* (1970), these authors con-

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[1] The following abbreviations are used: acetyl phosphate dianion, AcP^{2-}; acetyl phosphate monoanion, AcPH; divalent metal ion, M^{2+}; divalent metal ion complexed acetyl phosphate, AcPM; sum of all species of acetyl phosphate, AcP.