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THE DENATURATION OF PEPSIN

IV. THE EFFECTS OF TEMPERATURE

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SUMMARY

The temperature coefficient of pepsin denaturation has been measured as a function of pH in solutions of neutral salt (KNO_3), guanidinium chloride and $Pb(NO_3)_2$. In KNO_3 the heat of activation has been found to decline uniformly by almost 50 kcal between pH 6.0 and 6.7. This is precisely the pH region where a number (5 or 6) of carboxyl groups, which appear to be hydrogen bonded in the native protein, are cleaved by a first order process when the enzyme is inactivated. In $6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ solutions the $\Delta H^{\ddagger}$ *vs.* pH curve is displaced by about 0.4 pH units in an acid direction. Guanidine also diminishes the heat of activation. In 1.18 *M* guanidine, the heat of activation shows a similar decline above pH $\sim$ 6.0 as observed in KNO_3.

INTRODUCTION

In earlier reports potentiometric titration[1] and inactivation rate data[2] have been presented from which it has been concluded that about 5 or 6 carboxyl groups are hydrogen bonded in pepsin. Moreover, these groups are thought of as forming an integral part of the network of secondary valence structures which are responsible for maintaining the enzyme in its active state. However, it was also shown that the environmental conditions which determine the pH stability of pepsin could be modified by either hydrogen bond-breaking reagents, such as urea or guanidine or by small amounts of ions possessing a high affinity for either the proton or the carboxylate anion of the hydrogen bonded carboxyl groups.

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It is the purpose of this paper to explore the temperature coefficients of pepsin denaturation when reagents are present which are known to accelerate the rate of inactivation by modifying the structure of the carboxyl hydrogen bonds.

METHODS AND MATERIALS

The methods used and the sources of materials have been reported in previous communications in this series[1-3].

Inactivation experiments, conducted at a series of temperatures, were generally performed in unbuffered solutions of pepsin in water containing at least 0.15 *M* KNO_3 to fix the ionic strength. The release of protons that occurred during denaturation was monitored and compensated for by a manually operated pH-stat arrangement with a Beckman Model GS pH meter. The pH meter was calibrated with phosphate buffers at the same temperature as the inactivation experiment. All pH values listed were measured at the temperature of the experiment, except where otherwise stated.

Pepsin activities were determined by enzyme assay using hemoglobin as substrate. A few experiments were performed in phosphate buffer, where rates were measured by both enzyme assay and a solubility method. All rates were first order. Heats of activation were evaluated from the slope of a plot of the logarithm of the first order velocity constant *vs.* the reciprocal absolute temperature.

A solubility method was developed, which was quite similar to that used by NORTHROP[4]. The procedure consisted of precipitating denatured pepsin with a mixture of 10 % trichloracetic acid, 0.2 *M* HCl and 1.0 *M* NaCl. The precipitate was separated immediately by centrifugation in a low speed clinical centrifuge. The O.D. of the supernatant was measured at 280 mμ. This method gave results that were identical to the hemoglobin assay at pH 6.65 and 7.00. At lower pH values and higher temperatures, denatured pepsin is more subject to hydrolysis by active pepsin and would complicate this assay. If we, therefore, define denaturation, in its most common usage, as the loss of solubility (at the isoelectric point) then we can speak of the denaturation or inactivation of pepsin interchangeably, since both processes proceed at identical rates.

Constant experimental temperature was maintained by circulating water from a constant temperature bath through a water-jacketed beaker which served as the titration vessel. The temperatures quoted are those of the solutions in the beaker, which differed slightly from that in the bath when working at temperatures other than that of the room.

RESULTS

The temperature coefficient of inactivation rates

KNO_3: One of the most characteristic features of protein denaturation reactions is their unusually high temperature coefficients. In this respect, pepsin denaturation is quite typical. Activation energies (E) may be calculated from the dependence of reactions on temperature by the Arrhenius equation. The heat of activation ($\Delta H^{\ddagger}$) is readily obtained[5] from the energy of activation by $\Delta H^{\ddagger} = E - RT$. Since RT is generally small compared with E, we have equated E with $\Delta H^{\ddagger}$.

Free energies of activation ($\Delta F^{\ddagger}$) were obtained from the relation $k = k'T/h$

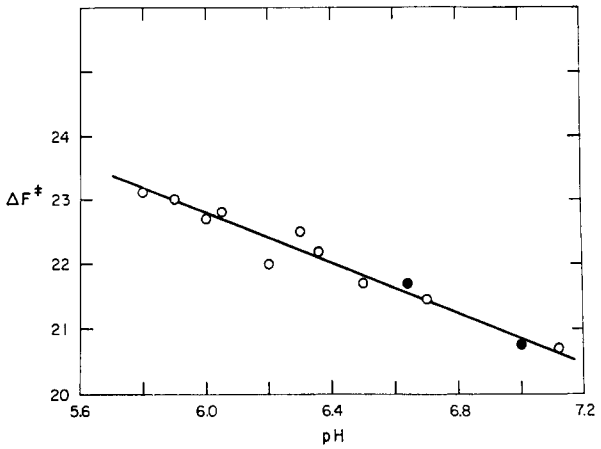

Fig. 1. Variation of the free energy of activation ($\Delta F^{\ddagger}$) with pH. ○, 0.15 *M* KNO_3; ●, 0.15 *M* NaCl–0.05 *M* phosphate.

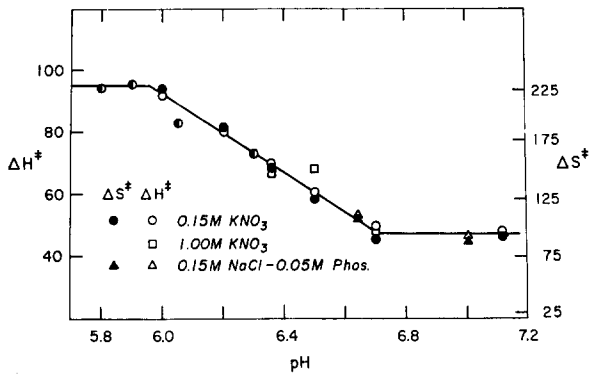

Fig. 2. Variation of the heat ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation of pepsin denaturation with pH.

$e^{-\Delta F^{\ddagger}/RT}$ and entropies of activation ($\Delta S^{\ddagger}$) from $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, where k is the first order rate constant in seconds, k' is the Boltzman constant, T, the absolute temperature, h, the Planck constant and R the gas constant. Thermodynamic activation parameters have been computed and appear in Figs. 1 and 2*.

Since the inactivation of pepsin goes to completion under all conditions presently investigated, only activation parameters can be obtained. The free energy of activation was found to decrease linearly with pH throughout the range investigated, *i.e.*, pH 5.80 to 7.15. This decline in $\Delta F^{\ddagger}$ reflects the rapid increase in rate that occurs in this pH zone. In contrast to the monotonic change in $\Delta F^{\ddagger}$, the variation of the heat (and entropy) of activation with pH (Fig. 2) shows two pH independent zones, one below 6.0 and one above 6.7, with a transition region between these values wherein

* The reported values of $\Delta F^{\ddagger}$ in both the Figures and the Tables are average values collected over a temperature range indicated in the Tables. Actually the values of $\Delta F^{\ddagger}$ increased as the temperature decreased-usually about 1 kcal over the temperature range investigated. Since the predominant effects are observed in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, which presumably do not vary with temperature, only average values of $\Delta F^{\ddagger}$ have been reproduced.

$\Delta H^{\ddagger}$ falls linearly by almost 50 kcal. Since $\Delta F^{\ddagger}$ changes by less than 3 kcal the variation in $\Delta S^{\ddagger}$ necessarily follows those in $\Delta H^{\ddagger}$ rather closely. This is shown graphically in Fig. 2 by plotting both functions on the same plot and adjusting the ordinate scale so that both sets of data fall on a single curve.

At several pH values (squares in Fig. 2) $\Delta H^{\ddagger}$ values were determined in 0.15 and 1.00 *M* KNO_3. Only at one value (pH 6.50) was there a significant difference ($\sim$ 10 kcal) between the two ionic strengths.

Phosphate–NaCl: The effect of temperature on the rate of pepsin inactivation at pH 6.65 and 7.00 was investigated in phosphate buffers. The activation parameters obtained in these buffered solutions agreed with those observed in unbuffered solutions of KNO_3 as shown in Fig. 2. Moreover, similar rates were obtained by either enzyme assay or by loss in solubility of the native pepsin (see METHODS Section).

Guanidine: Since guanidine has been shown to increase markedly the rate of pepsin inactivation[2], the effect of temperature was investigated also. The concentration of KNO_3 was increased to 0.50 *M* in this series of experiments. Some typical data are illustrated in Fig. 3. The data, in terms of activation constants, are summarized in Table I and Fig. 4. At all pH values studied, guanidine served to decrease the thermal energy barrier to denaturation. At pH 5.70 (and 5.90) the reduction in $\Delta H^{\ddagger}$ was approximately proportional to the concentration of guanidine. In 3.18 *M* guanidine, a $\Delta H^{\ddagger}$ value of 25 kcal was obtained. This value is in the range of activation energies observed in small molecule reactions and is 70 kcal less than occurs without guanidine at the same pH value.

In 1.18 *M* guanidine, between pH values of 5.7 and 6.5, the $\Delta H^{\ddagger}$ values were about 25 kcal less than were found in 0.15 *M* KNO_3. It is of significance that a similar break in the $\Delta H^{\ddagger}$-pH curve was obtained with 1.18 *M* guanidine as was observed in the corresponding KNO_3 curve. We may gather from this similarity in behavior that the intrinsic effect of pH is still present in 1.18 *M* guanidine solutions.

Pb$(NO_3)_2$: The thermodynamic activation parameters obtained in solutions of

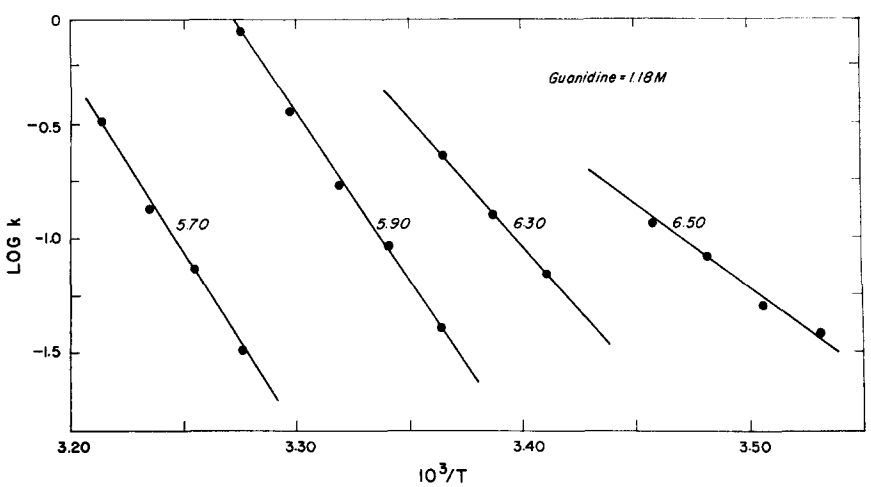

Fig. 3. The effect of pH on the temperature coefficient of pepsin inactivation in 1.18 *M* guanidine solutions. Pepsin concentration $\simeq$ 0.2 %; KNO_3 = 0.50 *M*. *k* is the first order velocity constant in reciprocal minutes.

$6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ are reported in Table II. In $1 \cdot 10^{-3}$ *M* $Pb(NO_3)_2$ at pH 5.9 the inactivation did not follow first order kinetics; therefore, the effect of Pb^{++} concentration on $\Delta H^{\ddagger}$ was not evaluated.

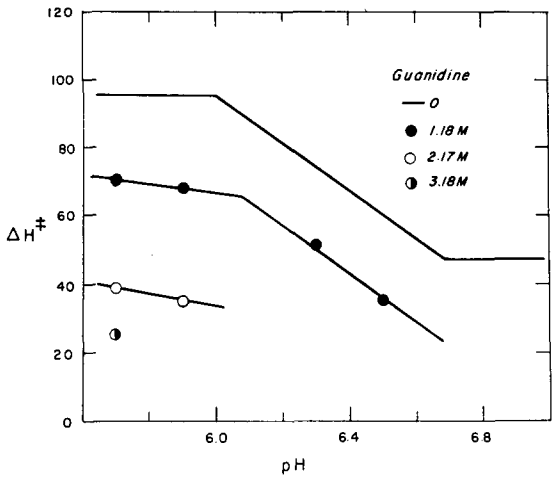

Fig. 4. Influence of pH on the heat of activation of pepsin in guanidine solutions. $KNO_3 = 0.50$ *M*.

TABLE I

THE EFFECT OF pH AND GUANIDINE ON THE ACTIVATION PARAMETERS OF PEPSIN DENATURATION*

pH	*KNO₃*	*Temperature range*	*Guanidine*	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
5.70	0.50	32–38°	1.18 *M*	71	160	21.9
		21–26	2.17	39	58	21.5
		10–17	3.18	25	16	20.7
5.90	7.15	41–49	0	95	226	23.0
	0.50	24–34	1.18	68	156	21.0
		13–19	2.17	35	50	20.6
6.30	7.15	31–37	0	73	164	22.5
	0.50	20–24	1.18	52	105	20.9
6.50	0.15	24–33	0	60	125	21.7
	0.50	10–16	1.18	35	49	21.1

* $\Delta F^{\ddagger}$ and $\Delta H^{\ddagger}$ in kcal/mole; $\Delta S^{\ddagger}$ in cal/mole/degree.

TABLE II

THE EFFECT OF pH ON THE ACTIVATION PARAMETERS OF PEPSIN DENATURATION*

pH	*Temperature range*	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
5.80	37–44°	74	164	22.8
5.90	32–36	76	176	22.0
6.00	30–36	67	148	21.8
6.20	22–30	57	120	21.5
6.40	18–30	40	64	21.1
6.60	14–22	42	75	20.7

* In $6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ ($KNO_3 = 0.15$ *M*).

The heats of activation are plotted as a function of pH in Fig. 5. where they may be compared with the corresponding values found in 0.15 *M* KNO_3. The two curves are similar in shape, with the Pb^{++} curve being displaced by about 0.4 pH units in an acid direction. However, the $\Delta H^{\ddagger}$ value observed at pH 5.7 did not fit on the displaced curve and was 20 kcal less than in 0.15 *M* KNO_3. The acid shift evident in the $\Delta H^{\ddagger}$-pH curve of Fig. 5 would appear to be related to a similar acid shift observed

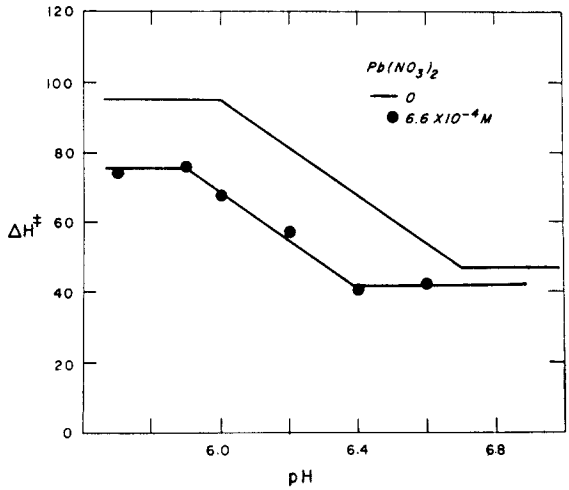

Fig. 5. Influence of pH on the heat of activation of pepsin in $6.6 \cdot 10^{-4}$ *M*. $Pb(NO_3)_2$. KNO_3 = 0.15 *M*.

in the irreversible segment of pepsin "titration curves" when conducted with Pb^{++} present[1]. When Zn^{++} was added at 7.5 times as large a concentration as Pb^{++}, a value of 65 kcal was found for $\Delta H^{\ddagger}$ at pH 6.4. This coincides with the value observed in 0.15 *M* KNO_3 solutions. This result is compatible with the much smaller effect of Zn^{++} that was observed on the rate of pepsin inactivation[2].

Ethyl alcohol: It has been established that the rate of pepsin inactivation increases with the 4.5th power of the molar concentration of ethyl alcohol[2]. Moreover, the pH dependence of inactivation remains constant between 0 and 38 % (v/v) ethyl

TABLE III

THE EFFECT OF ETHYL ALCOHOL ON THE ACTIVATION PARAMETERS OF PEPSIN* AND HEMOGLOBIN** DENATURATION

pH	*Temperature range*	C_2H_5OH	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
5.90*	41–49	0	95	226	23.0
	31–36	20 % (v/v)	88	215	22.0
	21–29	40 %	52	104	21.2
6.00–7.00**		0	76	153	25.1
		20 %	107	264	23.5
		30 %	117	309	22.5

* KNO_3 = 0.15 *M*.

** Hemoglobin data taken from work of BOOTH, ref.[16]. The activation parameters were similar between pH 6.00 and 7.00.

alcohol. It is assumed, therefore, that the mechanism of inactivation is unchanged in this range of alcohol concentration.

The kinetic activation constants in 20 and 40 % alcohol are included in Table III. In 20 % (v/v) alcohol the heat of activation is only slightly reduced from its value in water. However, in 40% alcohol, a drop of 43 kcal occurred.

The pH dependence of denaturation below pH 5.0

It was of interest to know whether the very marked pH dependence of inactivation rates observed above pH $\sim$ 5.5 continued to hold at lower pH values where the ionization of carboxyl groups are repressed. By raising the temperature to above 60° pepsin was inactivated by first order kinetics at pH values between pH 3.4 and 5.0. These data are recorded in Fig. 6. The pH values were determined at 25° at the end of the experiment. The pH was controlled by a pH-stat during the experiment.

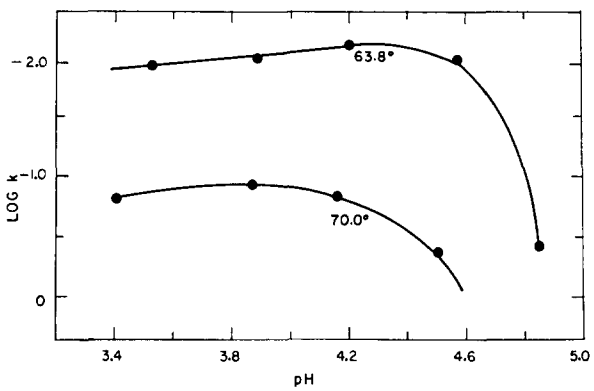

Fig. 6. Effect of pH on the first order velocity constant of pepsin inactivation at low pH values and high temperatures. k in reciprocal minutes.

It is apparent from Fig. 6 that between pH 3.4 and 4.6, the pH dependence is strikingly different from that found at higher pH values. In fact, the two curves illustrated in Fig. 6 show that the rate of inactivation first decreases slightly with increase in pH, then passes through a minimum in rate and finally increases very rapidly to give the exceptional pH dependence observed in the neutral range. It is interesting to note, that the heats of activation between pH 3.4 and 4.6 are high and agree approximately with those found between pH 5.7 and 6.0 (see Fig. 2). It may, therefore, be concluded that denaturation and not autolysis constitutes the principal pathway of inactivation between pH 3.4 and 4.6 at elevated temperatures.

DISCUSSION

Relationship to STEINHARDT's *data*

A discrepancy appears to exist between STEINHARDT's temperature data and those reported presently[6]. STEINHARDT's value of the activation energy of pepsin denaturation was obtained from the difference in rates at only two temperatures, *i.e.*, 25° and 15°. The pH dependence was linear (inverse fifth order) up to a rate of about $\log k = -2$ (min^{-1}); the $\Delta H^{\ddagger}$ was considered constant and equal to 63.5 kcal/mole, from pH 6.1 to 6.4, the range of overlap of his two curves. The results reported in

Fig. 2 show a decline from about 80 to 65 kcal in this pH interval. Over the larger pH range shown in Fig. 2, the $\Delta H^{\ddagger}$ was observed io fall gradually from about 95 to 48 kcal between pH 6.0 and 6.7 and then remain constant. A reduction in $\Delta H^{\ddagger}$ above pH 6.4 is evident also in STEINHARDT's data[6].

Various values for the pH dependence of pepsin inactivation rates have appeared in the literature[6]. Since the temperature coefficient is known to vary with pH, this effect would directly lead to different values for the variation of rate with pH. Thus the pH dependence would tend to decrease as the reaction temperature was increased.

The variation of $\Delta H^{\ddagger}$ with pH

There are several examples of protein denaturation in the literature where the activation energy is rather strongly dependent on pH. Thus there is a decrease of about 100 kcal in $\Delta H^{\ddagger}$ for ovalbumin when going from[7] pH 5.5–7.7 to[8] pH 1.0–1.7. HARRINGTON has shown recently that prototropic changes occur, apparently involving about 8-carboxylate-tyrosyl bonds, when ovalbumin is denatured in guanidine[9]. Hemoglobin also shows a similar decline in $\Delta H^{\ddagger}$ between neutrality and mildly acidic solutions, amounting to about 60 kcal[10]. The $\Delta H^{\ddagger}$ observed between pH 3.2 and 4.1 was constant at 15.7 kcal, which is rather low for a denaturation reaction[11]. In this pH zone ZAISER AND STEINHARDT[12*] have shown that about 36 acid-binding groups are liberated concurrently with the denaturation.

The pH range of special significance is from about 6.0 to 6.7, where the heat of activation falls rather uniformly by about 50 kcal. It is important to note that due to a large electrostatic factor the only groups that are undergoing dissociation in this pH region in native pepsin are carboxyl groups[1]. Since the heat of dissociation of free carboxyl groups is generally less than 2 kcal the ionic state (net charge) of pepsin should not change by much as the temperature is varied if the pH, ionic strength and other variables are kept constant. The observed heats of activation, therefore, would not include a significant contribution from their heat of ionization. Of the 63.5 kcal activation energy STEINHARDT attributed about 45 kcal to the heat of ionization of 5 amino groups which were assumed to be the critical charged groups involved in pepsin denaturation[6]. Since the critical groups have been shown to be carboxyl[1], the observed values of $\Delta H^{\ddagger}$ would, therefore, represent the thermal energy required to activate weak, noncovalent bonds in pepsin. Consequently, the decrease in $\Delta H^{\ddagger}$ found between pH 6.0 and 6.7 should be related to the activation of hydrogen-bonded carboxyl groups. On this basis, these groups require almost 50 kcal of activation energy. Above pH 6.7 where $\Delta H^{\ddagger}$ appears to be invariant with pH again only the remaining weak bonds (*i.e.*, α-helical, hydrophobic, etc.) need to be activated and they absorb about the same amount of thermal energy.

Below pH $\sim$ 5.5 only free carboxyl groups are dissociated and the rate of pepsin inactivation shows but a small dependence on pH. Above pH $\sim$ 5.5. the more stable hydrogen-bonded groups tend to ionize and the rate of inactivation becomes strikingly dependent on pH. With perturbations of these bonds resulting from the lower acidity of the medium, their dimensions would tend to increase and their strength to diminish. An increase in entropy would also result. The reduction in $\Delta H^{\ddagger}$ that occurs above

* In a recent report S. BEYCHOK AND J. STEINHARDT (abstracts of Papers, Am. Chem. Soc., Chicago, Ill. Sept. 7–12, 1958, p. 41C.) claim that 20% of these groups are due to electrostatic factors and 80% to unreactive acid binding groups.

pH 6.0 indicates that the ionizing tendency of hydrogen-bonded groups can facilitate their activation. The parallel decline that takes place in $\Delta S^{\neq}$ between pH 6.0 and 6.7 reflects a greater entropy of the initial state of the molecule provided that the structure of the activated state does not vary much with the small increase in pH.

In an earlier paper[1] it was suggested that the unusually large pH dependence of inactivation rates implied an acceptor group (to the carboxyl donor) which possessed a pK quite close to that of the donor. On this basis, we proposed that the acceptor group was also a carboxyl group which formed a double hydrogen bond with the donor group. The marked pH dependence of $\Delta H^{\neq}$ should provide additional support for this hypothesis. It is of interest consequently to try to correlate that part of the activation energy associated with this bond with the heat of the carboxyl–carboxyl hydrogen bond. In acetic acid, the energy of this homologous double bond is close to 14 kcal[13]. If three such bonds occur in pepsin, which is compatible with the titration data, a ΔH value of 42 kcal is obtained. The difference between this value and that of $\Delta H^{\neq}$ for these bonds is about 5 kcal which may readily be accounted for by a small positive heat of ionization, *i.e.*, $\sim$1 kcal per bond. The utilization of the full energy value of the carboxyl hydrogen bond assumes that these bonds are not significantly solvated. Since carboxyl hydrogen bonds are not very stable in an aqueous environment[14], they presumably would not be appreciably solvated in native pepsin.

Since guanidine (as well as urea) is considered to be a better hydrogen-bonding solvent than water, it is not surprising that pepsin is more labile in guanidine solutions. The increase in rate observed in guanidine is accompanied by a reduction in heat of activation (see Fig. 4). At pH 5.70 (and 5.90), the decrease in $\Delta H^{\neq}$ is approximately proportional to the concentration of guanidine. MIHALYI[15] has reported a similar decline in $\Delta H^{\neq}$ with urea concentration for fibrinogen denaturation. Since guanidine can reduce $\Delta H^{\neq}$ below the values observed in aqueous media, it seems likely that hydrogen-bonded groups in pepsin other than carboxyl are activated also by guanidine.

It has been demonstrated elsewhere that Pb^{++} (and other metals having a large affinity for carboxylate groups) can increase the acid strength of the hydrogen-bonded groups in pepsin[1]. By enhancing the tendency of these groups to ionize, and consequently to rupture hydrogen bonds, the thermal energy required for activation is decreased. The ability of Pb^{++} to reduce $\Delta H^{\neq}$ is essentially no greater that that accomplished by lowering the acidity of the solution, since both the Pb^{++} and KNO_3 curves reach a limiting value of 44 ± 3 kcal for $\Delta H^{\neq}$ and become independent of pH at higher values. The effect of Pb^{++} upon $\Delta H^{\neq}$ would therefore, be restricted to that part of $\Delta H^{\neq}$ required to activate carboxyl bonded groups.

It is instructive to compare the effect of ethyl alcohol on the kinetic behavior of pepsin with that of hemoglobin[8,15]. The rate of denaturation of both proteins is increased as alcohol is added to the aqueous protein solutions. However, the heat of activation increases with hemoglobin and decreases with pepsin as the concentration of ethyl alcohol increases. Since the reduction in the free energy of activation that occurs upon the addition of ethyl alcohol is fairly small, the entropy of activation must increase considerably for hemoglobin denaturation and accordingly decrease

* It is noteworthy that a larger entropy of activation ($\sim$ 135 e.u.) is associated with the activation of the carboxyl hydrogen-bonded groups than with the residual weak interactions where an increase of only 90 e.u. was calculated.

for pepsin inactivation (see Table III). The difference in $\Delta H^{\ddagger}$ between water and a 40 % ethyl alcohol solution of pepsin is about 43 kcal. A 30 % alcohol solution produces an increase in $\Delta H^{\ddagger}$ of about 40 kcal for hemoglobin denaturation[16]. Both changes in $\Delta H^{\ddagger}$ occur at pH values where $\Delta H^{\ddagger}$ in water is close to a maximum.

In solutions where the activity of water is reduced, as in water–alcohol mixtures, the solvent should be less effective than water in disrupting the hydrogen bonding in a protein solute. On the other hand, intramolecular bonding between hydrophobic side chain residues will tend to be less stable than in water due to the stronger solvating ability of the non-polar chain of the alcohol. It would appear, therefore, that the influence of alcohol on $\Delta H^{\ddagger}$ would depend on the relative contributions of the hydrogen and hydrophobic bonding situations to the intramolecular cohesive energy. If the 36 (or 18 per 34,000 molecular weight) acid binding groups liberated in hemoglobin denaturation are hydrogen-bonded in the native protein, these bonds would provide a major source of stability. This situation is to be contrasted with the relatively small number of carboxyl hydrogen bonds (5 or 6) in pepsin (M = 36,000). From the effect of alcohol on $\Delta H^{\ddagger}$, we may surmise that hydrophobic bonds provide a larger stabilizing force in pepsin than in hemoglobin. In this connection, it is interesting to note that the heat of activation for hemoglobin denaturation, in acid, is only 16 kcal, whereas in pepsin denaturation, above pH 6.7, it is still close to 50 kcal. The large increase in $\Delta S^{\ddagger}$ for hemoglobin denaturation in 30 % alcohol is suggestive of a much more ordered structure for native hemoglobin in this solvent. This could occur by more extensive hydrogen bonding, especially in the secondary structure (peptide hydrogen bonds), resulting from the rearrangement (or reduction) in the hydrophobic interactions.

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