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Thermodynamics of the Denaturation of Pepsinogen by Urea†

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ABSTRACT: The denaturation of swine pepsinogen has been studied as a function of urea concentration, pH, and temperature. The unfolding of the protein by urea has been found to be fully reversible under different conditions of pH, temperature, and denaturant concentration. Kinetic experiments have shown that the transition shows two-state behavior at 25 °C in the pH range 6-8 covered in this study. Analysis of the equilibrium data obtained at 25 °C according to Tanford (Tanford, C. (1970), *Adv. Protein Chem. 24,* 1) and Pace (Pace, N. C. (1975), *Crit. Rev. Biochem. 3,* 1) leads to the conclusion that the free energy of stabilization of native pepsinogen, relative to the denatured state, under physiological conditions, is only 6-12 kcal mol^{-1}. The temperature dependence of the equilibrium constant for the unfolding of pepsinogen by urea in the range 20-50 °C at pH 8.0 can be described by assigning the following values of thermodynamic parameters for the denaturation at 25 °C: ΔH = 31.5 kcal mol^{-1}; ΔS = 105 cal deg^{-1} mol^{-1}; and ΔC_p = 5215 cal deg^{-1} mol^{-1}.

We have been interested for some time in interactions in the swine pepsinogen molecule and the bearing of these on its mechanism of activation to pepsin (McPhie, 1975). Perlmann (1963) showed that high concentrations of urea transformed the zymogen into a form which could not be activated at low pH and that removal of urea restored the potential activity to 90% of the native value. Edelhoch and co-workers (1965) studied the kinetics of this transition and also showed its product to be an open, expanded form of the molecule (Frattali et al., 1965). More recently, Lapanje (1969) interpreted his measurements of the intrinsic viscosity of pepsinogen solutions in 8 M urea to indicate that the protein exists as a cross-linked random coil under these conditions. We report here a more thorough investigation of the thermodynamics of the unfolding of pepsinogen by urea, using well known methods (Tanford, 1968, 1970). We are interested in the structural determinants which give the zymogen greatly enhanced stability at neutral pH, as compared with pepsin.

Experimental Procedure

Pepsinogen (lots PG34D903 and PG36S810) obtained from Worthington Biochemical Corp. and UltraPure samples of urea purchased from Schwarz/Mann were used without further purification. Other chemicals were analytical grade.

Absorption spectra were measured in a Cary 15 spectrophotometer using tandem thermostated cells. Protein solutions for the equilibrium and kinetic studies of the denaturation of pepsinogen were prepared as follows. For denaturation experiments, known amounts of the stock protein, buffer, and urea solutions at the same pH were mixed in a 5-mL volumetric flask and incubated for a period that was found to be sufficient by kinetic experiments for the completion of the reaction. A similar procedure was employed in preparing the protein solutions for renaturation experiments with the only exception that pepsinogen was first denatured in concentrated urea solution and then diluted with buffer. Sodium phosphate buffer of ionic strength 0.15 M was used throughout this study unless stated otherwise. Pepsinogen concentration was determined using a value of 5.1×10^4 for the molar extinction of the native protein at 278 nm. Proteolytic activity was estimated with a 25 mg/mL solution of hemoglobin in 0.1 M hydrochloric acid, as described previously (McPhie, 1975).

The kinetics of urea denaturation at 25 °C were studied at pH values 6, 7, and 8 and at different urea concentrations. For denaturation experiments, a known volume of urea solution in phosphate buffer was taken in a cell kept in the spectrophotometer. To this, 100 to 200 μL of a concentrated solution of pepsinogen was added with the help of an "adder-mixer." The rate of denaturation was determined by following the change in absorbance at 292 nm immediately after mixing the two solutions in the cell. The rate of renaturation was measured similarly except that a concentrated solution of pepsinogen in urea of desired concentration was added to a known volume of buffer. Thus it was possible to record the progress of the denaturation and renaturation reactions from 3 s after mixing. The first-order rate constants were computed from standard

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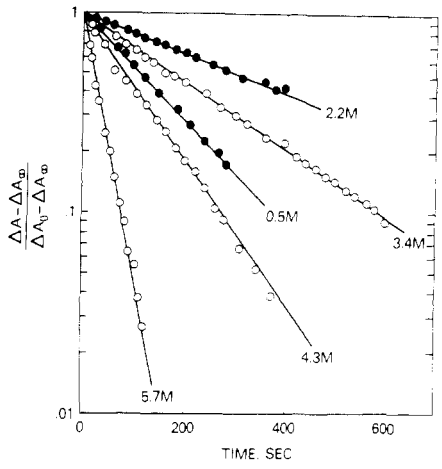

FIGURE 1: Representative kinetic plots for denaturation and renaturation of pepsinogen at several urea concentrations (pH 8.0, 25 °C), as measured by difference spectroscopy at 292 nm. Open circles represent denaturation and filled circles renaturation. Protein concentration was in the range 10–20 μM. ΔA represents the difference in optical density between protein in urea solution and protein in absence of urea.

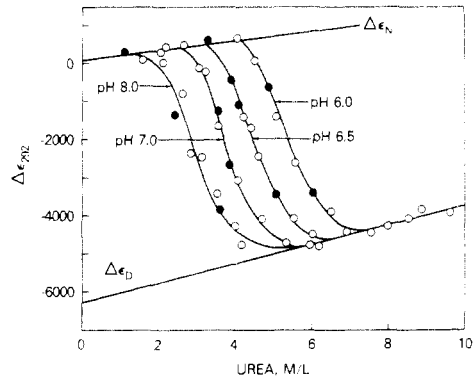

FIGURE 2: Effect of urea concentration on the difference spectral intensity of pepsinogen at 292 nm, 25 °C. The base line for $\Delta\epsilon_N$ is the average value for the entire range of pH, while the line for $\Delta\epsilon_D$ corresponds to the pH values 6.0 and 6.5. Open and filled circles have the same meaning as in Figure 1.

first-order plots of log $(\Delta A - \Delta A_\infty)/(\Delta A_0 - \Delta A_\infty)$ vs. time, where ΔA, ΔA_0, and ΔA_∞ are the values of the observed differences in absorption at a time t, zero time, and at equilibrium, respectively.

In the experiments on the pH dependence of urea denaturation, the pH of the nonbuffered protein solution was varied by adding appropriate amounts of concentrated HCl or NaOH solution. The pH of the solution was recorded with a Radiometer pH meter 26 using a GK-2321-C combined electrode.

Melting curves of pepsinogen in the presence of urea were obtained using the slow temperature jump apparatus described by Tsong et al. (1972). This method enabled us to complete each experiment within 30 min. This reduced time of exposure to high temperatures made the experiments performed at pH 8 completely reversible (cf. Perlmann, 1963). However, at lower pH values, the higher temperatures (>50 °C) required for complete unfolding produced increasing amounts of irreversible denaturation, as judged spectrophotometrically. Consequently, only melting curves obtained at pH 8 are reported here.

The results were fitted to the various equations, given below, using the MLAB system on a PDP 10 computer (Schrager, 1970).

Results

Absence of Stable Intermediate States. In order to extract meaningful thermodynamic parameters from equilibrium measurements of the denaturation of pepsinogen by urea, it is necessary to demonstrate the absence of states other than native (N) and denatured (D) protein. A decisive test for the presence of intermediates is to study the kinetics of refolding and unfolding of the protein. Measurements of this kind have already been reported for the urea denaturation of pepsinogen under various conditions of denaturant concentration, temperature and pH (Edelhoch et al., 1965). No evidence of stable intermediates was found. These kinetic experiments were limited to the pH range 6.0–7.2. We report here results obtained at pH 8.0; our findings at lower pH values are in excellent agreement with those reported earlier by Edelhoch et al. (1965) and, therefore, will not be presented here.

Figure 1 shows typical first-order kinetic plots, using difference spectral measurements, and they are seen to be linear. Furthermore, the intercepts at $t = 0$ all agree, within experimental error, with the expected values of $\Delta\epsilon$ for native state ($\Delta\epsilon_N$) or denatured state ($\Delta\epsilon_D$). Similar results were obtained under all conditions.

Isothermal Denaturation. The urea-induced transition of pepsinogen from the native to denatured state was followed at various pH values by measuring changes in $\Delta\epsilon$ (at 292 nm) as a function of increasing concentration of the denaturant. The results are depicted in Figure 2; $\Delta\epsilon$ represents the difference in molar absorbance between the protein in urea solution at a given pH and protein in the absence of urea at that pH. As can be seen in this figure, the experimental points obtained from denaturation and renaturation experiments lie on the same smooth curve, indicating that urea denaturation is reversible. The midpoints of the urea transitions at pH values 6.0, 6.5, 7.0, and 8.0 are, respectively, 5.4, 4.5, 3.8, and 3.0 M. $\Delta\epsilon_N$ and $\Delta\epsilon_D$ are found to depend on urea concentration but to be independent of pH. It is interesting to note that the value of $\Delta\epsilon_D$ obtained by extrapolation to 0 M urea is the same as that determined from the denaturation studies of pepsinogen by guanidine hydrochloride (Ahmad and McPhie, unpublished results).

It can be seen in Figure 2 that the denaturation transition shifts to lower urea concentrations as the pH is increased. It must therefore be possible to induce denaturation at constant urea concentration by increasing the pH. The results of following the transition in this way are shown in Figure 3. These results are also found to be reversible. The increases in $\Delta\epsilon$ observed above pH 8 proved to be due to ionization of exposed tyrosine residues (McPhie, 1975) not to reversal of the transitions. In order to maintain clarity, experimental points in 6 and 7.5 M urea above pH 8 are not shown.

In control experiments, it was shown that pepsinogen which was fully refolded, after exposure to concentrated urea, as judged by absorbance changes, could also be activated to give full activity against hemoglobin.

Dependence of K_D on pH. The absence of any contribution to the measured property from states other than N and D means that the data of Figures 2 and 3 can be represented by the equilibrium $N \rightleftharpoons D$ and the results can therefore be used to calculate the equilibrium constant K_D (=[D]/[N]) as described by Tanford (1968). The values of K_D at various urea concentrations are shown replotted in terms of log K_D as a function of pH (see Figure 4a).

Figure 4b shows the result of shifting the points of Figure 4a along the abscissa to make a master curve which is independent of the denaturant concentration. The shift factors are

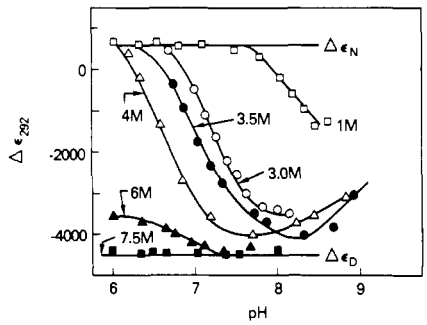

FIGURE 3: Effect of pH on difference spectral intensity, at 292 nm, of pepsinogen in different concentrations of urea at 25 °C. The lines for $\Delta\epsilon_N$ and $\Delta\epsilon_D$ are drawn using results at 1.0 and 7.5 M urea, respectively, and are used in the calculation of equilibrium constants.

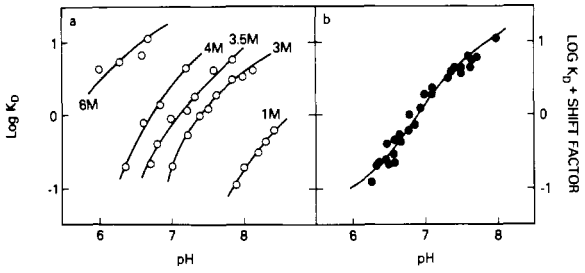

FIGURE 4: (a) The pH dependence of log K_D at constant urea concentration, 25 °C. The values of K_D, calculated from the results of Figure 3, vary in the range $0.1 \leq K_D \leq 1$. (b) The master curve for the pH dependence of log K_D drawn according to eq 1 with the values given in the text. Shift factors at 1.0, 3.0, 3.5, 4.0, and 6.0 M urea are 1.65, 0.50, 0.25, −0.25, and −1.30 pH units, respectively.

shown in the legend. Making use of the concept that the pH dependence of the equilibrium constant indicates a difference in the number of bound protons between the two states, due to changes in pKs of a few groups in the molecule, induced by the conformational change, the data of Figure 4b were fitted to eq 1:

$$F(a_H) = \frac{\prod_{i=1}^{n} (a_{H^+} + K_{i,D})}{\prod_{i=1}^{n} (a_{H^+} + K_{i,N})} \quad (1)$$

where $F(a_H)$ describes the dependence of K_D on pH. The results are completely described, in the pH range 6–8, using $n = 3$, $pK_{i,D} = 6.50$ and $pK_{i,N} = 7.32$.

Dependence of ΔG_D on Urea Concentration. Assuming that the free energy change for protein denaturation, ΔG_D, can be written as the sum of three mutually independent free energy changes, we may write

$$\Delta G_D = \Delta G_D^\circ + \Delta G(a_H) + \Delta G(a_u) \quad (2)$$

where ΔG_D° is the free energy change that represents the value of ΔG_D under conditions where both $\Delta G(a_H)$ and $\Delta G(a_u)$ are zero and depends only on temperature. $\Delta G(a_H)$ is $-RT \ln F(a_H)$ and $\Delta G(a_u)$ is the free energy change for denaturation which depends only on the activity of urea, a_u.

The difference between ΔG_D at each pH, calculated from the results of Figure 2, and $\Delta G(a_H)$ calculated from eq 1 at that pH, is plotted as a function of urea concentration in Figure 5. Using the linear extrapolation procedure recommended by Pace (1975), a slope of 1.18 kcal mol^{-1} M^{-1} and a value of 6.5 kcal mol^{-1} for ΔG_D° are obtained.

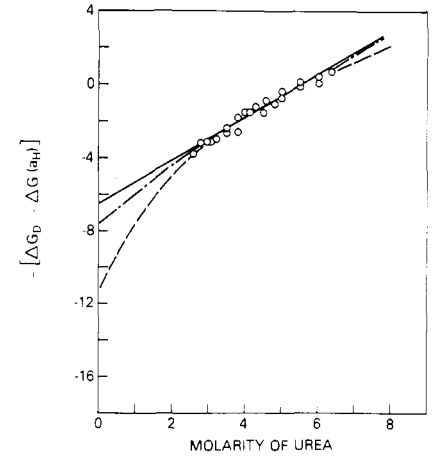

FIGURE 5: A plot of $[\Delta G_D - \Delta G(a_H)]$ as a function of urea concentration. (—) Linear extrapolation; (---) eq 3 with $k' = 1.0$; (-·-) eq 3 with $k' = 0.1$.

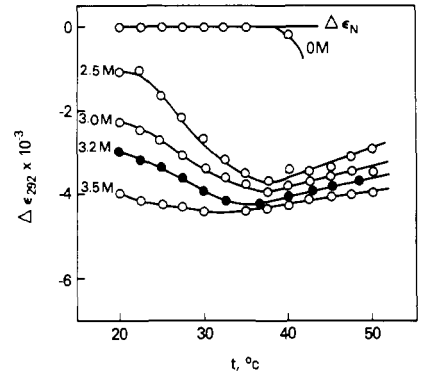

FIGURE 6: The effect of temperature on the urea denaturation of pepsinogen at pH 8.0. The concentration of the denaturant for each melting curve is shown in the figure.

The variation of ΔG_D with denaturant concentration can also be described in terms of a model which assumes increased binding of denaturant molecules to the protein after unfolding (Tanford, 1970). To obtain a suitable analytical function of $\Delta G(a_u)$, we first need to know the activity of urea as a function of its molar concentration. Values of a_u in aqueous solutions can be calculated from data in the literature (Scatchard et al., 1938; Bower and Robinson, 1963). In this way the data of Figure 5 were fitted to the equation

$$\Delta G(a_u) = RT \ln(1 + k' a_u)^{\Delta\nu} \quad (3)$$

where k' is the association constant with which each urea molecule binds to the protein and $\Delta\nu$ is the increase in number of binding sites on unfolding. The values obtained were $k' = 0.10$, $\Delta\nu = 30.9$, and $\Delta G_D^\circ = 7.60$ kcal mol^{-1}. The value of k' falls within the range found in model compound studies (Tanford, 1970). Pace (1975) found that, with a number of proteins, he could fit similar data equally well by setting $k' = 0.10$ or 1.0. Figure 5 shows this is also true in this case. The higher value for the association constant yields $\Delta\nu = 10.8 \pm 0.7$ and $\Delta G_D^\circ = 11.7 \pm 0.7$ kcal mol^{-1}. At present, there is no reason to prefer either value of k'.

Dependence of K_D on Temperature. The effect of temperature on the urea denaturation of pepsinogen was studied at pH 8.0 in the temperature range 20–50 °C (Figure 6). The curve at 0 M is taken to represent the temperature dependence of $\Delta\epsilon_N$; above 35 °C, $\Delta\epsilon$ increases on heating due to thermal denaturation. The difference spectral intensity in the post-

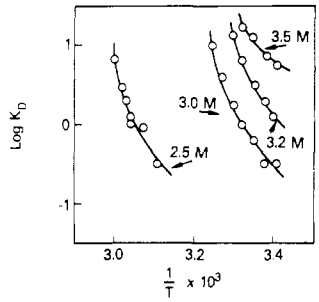

FIGURE 7: The temperature dependence of log K_D for denaturation of pepsinogen by urea. The line for each curve is drawn according to eq 4.

transition region, i.e., $\Delta\epsilon_D$, decreased on heating at all urea concentrations. The extrapolation of the results of each transition to 25 °C gave values in agreement with those shown in Figure 2. The values of $\Delta\epsilon_D$, computed from each curve obtained at constant denaturant concentration, were used to calculate the equilibrium constant at different temperatures. The transitions shown in Figure 6 were found to be fully reversible; heating protein solution in the presence of urea at 50 °C for 30 s, which is time enough for the completion of adsorbance changes, and cooling back to lower temperatures gave a value of $\Delta\epsilon$ identical with that obtained by heating directly to the same temperature. Solutions cooled back to room temperature showed full potential activity against acid denatured hemoglobin.

Exposure of protein to urea at higher temperatures for longer times brought about irreversible changes in conformation. Attempts were also made to follow the thermal urea denaturation at lower pH values, but it was found that heating of protein above 50 °C always resulted in some irreversible denaturation.

Figure 7 shows van't Hoff plots calculated from Figure 6. The temperature dependence of K_D can be described by an equation of the form

$$\log K_D = A + (\Delta C_p/R) \log T + B/T \qquad (4)$$

where A and B are temperature independent constants and ΔC_p is the heat capacity change for urea-unfolding of pepsinogen. Fitting the data obtained at a urea concentration of 2.5 M (Figure 7) to eq 4 gave $A = -7679$, $B = 3.308 \times 10^5$ deg, $\Delta C_p = 5.215$ kcal mol^{-1} deg^{-1}. Differentiation of eq 4 gave values of the other thermodynamic parameters, the enthalpy change, $\Delta H = 31.5$ kcal mol^{-1}, and entropy change, $\Delta S = 105$ cal mol^{-1} deg^{-1}, at 25 °C. Analysis of the other curves showed these parameters to be independent of urea concentration, over this range.

Discussion

There is no indication that pepsinogen undergoes any conformational changes at 25 °C, in the absence of denaturants, within the pH range 6.0–8.0 (Perlmann et al., 1967; McPhie, 1975). Consequently, the starting material in all the transitions reported here corresponds to the native protein (state N) with a compactly folded structure. Lapanje (1969) concluded that, at high concentrations of urea, pepsinogen behaves as a cross-linked random coil. However, he showed that reduction of the three disulfide bonds in the protein produced a further small increase in intrinsic viscosity. This might be taken to indicate the removal of the small constraints imposed by these cross-linkages. On the other hand, circular dichroic measurements have shown that S–S bonds can stabilize regions of local structure even under strongly denaturing conditions (Cortijo et al., 1973). The molecular dimensions derived from viscosity measurements have been shown to be remarkably insensitive to such structured regions (Miller and Goebel, 1968). Other workers have presented evidence for residual secondary structure in unfolded proteins (cf. White, 1976). With these reservations in mind, we will refer to the end product of urea unfolding as a cross-linked random coil (state D).

TABLE I: Thermodynamic Parameters Characterizing the Urea Denaturation of Pepsinogen at 25 °C.

Stability of the Native Pepsinogen Molecule	
Linear extrapolation	
$\Delta G_D°$, kcal mol^{-1}	6.5
Extrapolation by eq 3; $k' = 0.1$	
$\Delta G_D°$, kcal mol^{-1}	7.6
Extrapolation by eq 3; $k' = 1.0$	
$\Delta G_D°$, kcal mol^{-1}	11.7
ΔH, kcal mol^{-1}	31.5
$\Delta S'$, cal deg^{-1} mol^{-1}	105
ΔC_p, cal deg^{-1} mol^{-1}	5215
Principal groups responsible for the effect of pH in the region 6.0–8.0 (from eq 1)	
Number of groups	3
pK in native state	7.32
pK in unfolded state	6.50

On the basis of the kinetic studies of unfolding and refolding in urea solutions at 25 °C, we have assumed a two-state mechanism for this transition. The values of ΔG_D thus obtained represent the free energy change for the reaction native protein $\rightleftharpoons$ cross-linked random coil, and $\Delta G_D°$ measures the stability of pepsinogen in a native environment. If significant concentrations of stable intermediates are present, the calculated value of $\Delta G_D°$ derived by a two-state analysis will almost always be less than the true value of the free energy of stabilization (Tsong et al., 1972). The thermodynamic parameters derived from our analysis are summarized in Table I.

As is evident from Figure 5, it is difficult to obtain a reliable estimate of the intrinsic stability of the native protein. No matter what means are used to destabilize the native protein so that equilibria between native and denatured states can be observed, there is a sizable extrapolation to aqueous conditions. For urea denaturation of pepsinogen, measurement of equilibrium properties is possible only above 2.6 M denaturant concentration. One way to decrease the size of extrapolation would be to extend the urea denaturation studies above pH 8. However, above pH 8 pepsinogen no longer retains its native conformation (McPhie, 1975).

Assuming that the linear dependence of ΔG_D on urea concentrations continues to zero concentration of the denaturant, a value of 6.5 kcal mol^{-1} for the stability of the native conformation over the denatured one is obtained. Higher estimates are obtained if the extrapolation is based on models which assume that denaturation results because the unfolded state possesses a greater number of denaturant binding sites as compared with the native state (Tanford, 1970). Table I shows that regardless of which extrapolation procedure is used, the free energy by which the native state of pepsinogen is stabilized is small. Similar conclusions emerge from other unfolding studies in which the native globular conformation appears to be stabilized by only 4–15 kcal mol^{-1} (Tanford, 1970; Pace, 1975; Ahmad and Salahuddin, 1976).

We have found the effect of temperature on urea denaturation of pepsinogen to be reversible. Previous attempts to achieve a complete reversal of the changes used to follow the thermal denaturation of pepsinogen in urea were not successful (Perlmann, 1963). The main practical difficulty in demon-

strating the reversibility of this process may be due to decomposition of urea to cyanate at high temperatures and pH. This has been shown to react with sulfhydryl and amino groups of proteins (Stark et al., 1960), leading to irreversible chemical changes. In this study of the effect of temperature on the urea denaturation of pepsinogen, protein was never exposed to temperatures above 50 °C for more than 1 min. Preliminary studies indicate that reversal of denaturation was decreased if pepsinogen in urea solution was heated at higher temperature for longer times. This reversibility made possible the evaluation of the changes in enthalpy, entropy, and specific heat accompanying the unfolding of pepsinogen (Table I). Again, the values derived all fall within the range found in similar investigations on other proteins.

One might hope that a comparison of these parameters with those obtained by studying other proteins would give indications of the size and composition of the region of pepsinogen exposed to solvent after unfolding. This idea is reinforced by Chothia's (1975) comparative analysis of the crystal structures of 15 proteins. He found the proportion of polar groups forming intramolecular hydrogen bonds to be constant and that the amount of surface area lost by folding was a simple function of molecular weight. These will make major contributions to the free energy of folding, which should then vary in a similar way. However, our results lead to rather contradictory conclusions concerning the structure of pepsinogen.

Results such as those shown in Figure 5 can be analyzed in terms of interactions between the denaturant and the protein in its native and unfolded states. The linear treatment of such data was first proposed by Greene and Pace (1974). They believed that the slope of such a plot would indicate the gross change in exposure of peptide groups and side chains to solvent after unfolding. The slope should increase with the molecular weight of the protein as the fraction of residues buried in the native state increases. This was found to be the case with four typical proteins. Based on their results, our value of 1.18 kcal mol^{-1} M^{-1} is very low, suggesting that only a small portion of the polypeptide chain is unfolded in this transition. Tanford's (1970) method of analysis measures $\Delta\nu$, an apparent increase in the number of binding sites for denaturant on the protein after unfolding. Pace (1975) has analyzed the results referred to above in this way. Compared with his four examples, our values of $\Delta\nu$, 31 or 12, derived using two acceptable values for k', are again much smaller than expected for a protein of this size. A serious draw back to these methods of analysis is that they assume similar accessibilities to solvent of the native and denatured states of all proteins. This may not always be the case (Pace, 1975).

The other parameter which is often correlated with the size of the unfolding segment in protein denaturations is the large change in specific heat which accompanies unfolding. This is often ascribed to the hydrophobic effect. Unfolding of the protein exposes nonpolar side chains to solvent, with a resultant formation around these of cages of structured water, which have both low entropy and high heat capacity (Brandts, 1969; Tanford, 1968, 1970). Since larger proteins have a higher fraction of buried residues in the native state, this change in specific heat should increase with molecular weight. Such is indeed the case and compared with other values (Tanford, 1970), ΔC_p = 5215 cal mol^{-1} deg^{-1} suggests a large segment of the protein chain is unfolded in the case of pepsinogen. Sturtevant (1977) has recently considered the origins of heat capacity changes in protein reactions and concluded that it is an oversimplification to assign these only to the hydrophobic effect. He points out that increased degrees of freedom for intramolecular vibrations, resulting from breakdown of the closely packed protein interior on unfolding, can make a sizable contribution. This raises some doubt as to the usefulness of ΔC_p as a measure of the size of an unfolding domain.

It was hoped that analysis of the pH dependence of urea unfolding according to eq 1 would give a clearer indication of factors responsible for the increased stability of pepsinogen at neutral pH, compared with pepsin. Equations similar to eq 1 have previously been interpreted in terms of abnormally titrating groups involved in the structure of the native protein (Tanford, 1970). However, the values of the constants required to fit our results to this equation cannot readily be reconciled with the known properties of pepsinogen. Edelhoch et al. (1965) compared their results with earlier studies on pepsin and concluded that unfolding was triggered by the rupture of a number of stabilized carboxyl groups, hydrogen bonded in the native protein. Perlmann et al. (1967) found that the thermal unfolding of pepsinogen was accompanied by the release of three protons per molecule of protein. They pointed out that this is equal to the number of histidine residues and suggested that these were stabilized in the charged state by electrostatic interactions in the native protein. These would breakdown on unfolding, releasing the protons. In agreement with this we have found that none of these histidine residues react with diethyl pyrocarbonate in the pH range 6–8, unless the protein is first unfolded (unpublished observations). The values of $n = 3$ and $pK_{i,D} = 6.5$ are certainly compatible with this model. However, $pK_{i,N} = 7.32$ is much too low to explain these observations. It must be pointed out that it is only possible to study the properties of native pepsinogen in the range of pH values 6–8 (McPhie, 1975). Such a range is much too narrow for a rigorous determination of the pH dependence of the unfolding of the protein by urea. In the absence of more information, eq 1 must be regarded as strictly phenomenological, in this limited range.

Acknowledgments

We would like to thank Dr. F. H. White, Jr., for a critical review of this manuscript and a referee for correcting an error in our calculations of the activity of urea solutions.

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Hydrolytically Induced Allosteric Change in the Heavy Chain of Intact Myosin Involving Nonessential Thiol Groups†

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ABSTRACT: The two globular head portions, each bearing an active site, contain an uncleaved heavy chain when isolated by chymotrypsin from intact myosin. By specific labeling with radioactive *N*-ethylmaleimide the essential thiol 1 and thiol 2 groups were found to reside in this heavy chain. In intact myosin nonessential thiol 3 groups become the most reactive during ATP hydrolysis above 15 °C. These thiol 3 groups are located in a portion of the myosin heavy chain which appears as a fragment with an apparent molecular weight of 11 000 during proteolysis. The facts that this fragment is produced in an almost 1:1 molar ratio with the head heavy chain and that it bears unblocked N-terminal amino groups whereas the heavy chain does not and is not contained in the rod portion of the myosin molecule indicate that it may originate from the heavy chains in the neck region where the heads are joined to the rod. Since this fragment is removed by ion-exchange chromatography, it is not part of the functioning head and hence not involved in the active site. As its nonessential thiol 3 groups are rendered the most reactive of all thiol groups in the enzyme–product complex M**ADP·P_i, the hydrolytic step induces an allosteric conformational change in the neck region of intact myosin.

Monitoring the changes in reactivity of specific thiol groups has acquired widespread recognition as a method for probing protein conformational changes. These side groups may become even more reactive than they are in the free amino acid cysteine as a result of local environmental changes, which in turn indicate alterations in chain conformation (Gutfreund and McMurray, 1970). Enzymes are known to undergo conformational changes following the binding of substrate and the subsequent steps of their catalytic activity. These latter syncatalytic alterations in the protein structure can affect the environment of reactive side groups of specific amino acids as has been demonstrated for a certain cysteinyl residue of aspartate aminotransferase whose activity is markedly enhanced during catalysis (Birchmeier et al., 1973).

The hydrolysis of Mg–ATP by isolated myosin heads, the soluble proteolytic fragment of myosin bearing an active site, has been shown by fast kinetic techniques to proceed via seven elementary steps (Bagshaw and Trentham, 1974). The long lived species within this hydrolytic cycle is known to be an enzyme–product complex M**ADP·P_i[1] (Lymn and Taylor, 1970; Trentham et al., 1972). It can be distinguished from the complex occurring on direct binding of the product (M*ADP) which has also been shown to represent a reaction cycle intermediate (Bagshaw and Trentham, 1974), on the grounds of intrinsic tryptophan fluorescence (Werber et al., 1972; Mandelkow and Mandelkow, 1973), ultraviolet absorption (Morita, 1967; Malik and Martonosi, 1972), electron spin-label techinques (Seidel and Gergely, 1971), and circular dichroism (Murphy, 1974). A further approach is that of thiol group reactivity (Watterson and Schaub, 1973; Reisler et al., 1974) which does not involve spectral techniques and so may be done on insoluble myosin suspensions. It allows characterization of the different forms of the enzyme with or without ligand independent of a reference state, say the enzyme alone. This is achieved by following the sequence of blockage of different types of thiol groups, under a given condition at high or low ionic strength, in conjunction with the effect of this progressive blockage on the enzymic properties of myosin (Watterson et al., 1975).

Several types of thiol groups have been classified on the basis of the effect of their blockage on the myosin ATPase. Blockage of thiol 1 causes activation of the Ca^{2+}-dependent ATPase, when tested at high ionic strength, and concomitant inactivation of the K^+-dependent ATPase, while subsequent blockage of thiol 2 then inactivates the Ca^{2+}-dependent ATPase (Sekine et al., 1962; Sekine and Yamaguchi, 1963). Blockage of thiol 2 alone seems only to inactivate the K^+-dependent ATPase without appreciably affecting the Ca^{2+}-dependent ATPase (Reisler et al., 1974). These groups will be referred to as the essential thiol groups and they have recently been shown to

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[1] Abbreviations used: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); HMM, heavy meromyosin; HC, heavy chain; LC-1, light chain 1; LC-2, light chain 2; LC-3, light chain 3; P_i, inorganic phosphate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

0006-2960/78/0417-0246$01.00/0