Free Energy Changes in α-Lactalbumin Denaturation†

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ABSTRACT: Previous work has shown that native α-lactalbumin (N) is completely denatured by the addition of guanidine hydrochloride (conformation D) but that partially denatured conformations appear in other denaturants. In particular, conformation I appears when the pH is lowered from 5.5 to 2.2 ($I_{2.2}$) or when $LiClO_4$ is added at pH 5.5 ($I_{5.5}$). We have now determined the free energy changes for the processes N → $I_{5.5}$, N → $D_{5.5}$, and $I_{2.2}$ → $D_{2.2}$. We have also estimated the maximum value of the free energy change for the process N → $I_{2.2}$, and this allows us to estimate the changes for all conformational changes between any two of these five conformations.

An earlier paper from this laboratory reported on our investigation of the denatured states of bovine α-lactalbumin (Sharma & Bigelow, 1974) and compared the results to our previous work on egg-white lysozyme (Kugimiya & Bigelow, 1973). We showed that lactalbumin produces different denatured states in different denaturants and that the properties of these states are both qualitatively and quantitatively very similar to those found when lysozyme is treated with the same denaturants.

It was possible to prepare a tentative diagram showing how the various states were related to each other (see Figure 1). N represents the native molecule and state D the completely disordered conformation. States I, II, and III represent partially denatured conformations, that is to say, conformations which retain some of the native secondary and tertiary structure. The need for two pathways between N and IV is dictated by differences in the numerical changes observed for the various physical parameters studied (Sharma & Bigelow, 1974). The existence of two pathways between N and D is in contrast to previous results with bovine ribonuclease, where only one pathway had been needed to represent all the observed denatured states (Bigelow, 1964). It should not be inferred from the diagram that molecules of lactalbumin which are being converted from N to D (as in concentrated guanidine hydrochloride necessarily *go through* the intermediate states on the pathways. The diagram only shows that D has less residual structure than III, III than II, and II than N, by all the parameters studied. Also D has less residual structure than I, and I than N.

The diagram shown in Figure 1 was tentatively inferred from an incomplete set of experiments done with both α-lactalbumin and lysozyme, by UV difference spectrophotometry, optical rotatory dispersion (ORD), and viscometry. We feel that it will be of considerable interest and value to make more detailed studies of the individual denatured states, and in this paper we concentrate on the pathway N ⇄ I ⇄ D. State I was observed by Sharma & Bigelow (1974) when α-lactalbumin was denatured by $LiClO_4$, and they believed that acid denaturation, studied earlier by Kronman et al. (1965) led to the same conformation.

In this paper we study, by UV difference spectroscopy and circular dichroic spectroscopy (CD), the conversion of native lactalbumin to I or D and also the conversion of I to D, which was not previously examined. The data have been used to calculate standard free energy changes for each of the transitions, and an internally consistent set of values has been determined. In a future paper we will be reporting on studies of the pathway N ⇄ II ⇄ III ⇄ IV.

Experimental Procedures

Materials. Bovine α-lactalbumin was purified from a crude preparation of the Sigma Chemical Co. (Lot L-4379). This protein was dissolved in 0.15 M KCl which had been adjusted to pH 5.5 and passed through a 3 × 90 cm column of Sephadex G-100 at 6 °C. The lactalbumin peak was collected and shown to be homogeneous by polyacrylamide gel electrophoresis in 0.05 M Tris buffer at pH 8.3. Samples from the Sephadex column were lyophilized and stored in the deep freeze. Before use, a sample was dissolved in 0.15 M KCl, pH 5.5, and dialyzed over 18 h against three changes of the solvent. Each solution was tested for homogeneity by gel electrophoresis; deep freezing the protein caused no apparent problems.

Guanidine hydrochloride (Gdn·HCl) was an ultrapure reagent provided by the Schwarz/Mann Division of Becton, Dickinson, and Co. Anhydrous lithium perchlorate was from Matheson Coleman and Bell.

Methods. The UV measurements were made by using a Cary 118C automatic recording spectrophotometer. Matched tandem cuvettes with light paths of 0.431 cm in each half were used.

The CD measurements were made by using a Jasco J-20 spectropolarimeter. The instrument was calibrated with *d*-camphor-10-sulfonic acid by standard techniques. Cuvettes of 0.5- or 1.0-mm light path were used for the peptide region of the spectrum, and 1.0-cm cuvettes were used in the aromatic region. Mean residue ellipticity is calculated by using

$$[\theta]_\lambda = \theta_\lambda\left(\frac{115.3}{100lc}\right)$$

where θ_λ is the observed ellipticity at λ in degrees, 115.3 is the mean residue weight of α-lactalbumin, c is the protein concentration in grams per milliliter, and l is the light path of the cuvette in decimeters.

Both the spectrophotometer and the spectropolarimeter were equipped with thermostated cell holders which were maintained at 25 °C by circulating pumps.

Protein concentrations of stock solutions were determined spectrophotometrically by using a molar extinction of 28 440 at 281.5 mm (Aune, 1968). Experimental solutions contained 0.3–0.4 mg/mL of protein.

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Table I: Spectroscopic Parameters of the Native and Denatured States of α-Lactalbumin

state (or process)	denaturant	$-[\theta]_{208} \times 10^{-3}$	$-[\theta]_{222} \times 10^{-3}$	$-[\theta]_{274}$	$-\Delta\epsilon_{292}$
N		13.8	12.3	340	0
I ($N_{5.5} \rightarrow I_{2.2}$)	pH 2.2	16.8	12.2	20	2050
I ($N_{5.5} \rightarrow I_{5.5}$)	$LiClO_4$, pH 5.5	15.3	12.2	10	1800
D ($I_{2.2} \rightarrow D_{2.2}$)	Gdn·HCl, pH 2.2		2.5	20	900
D ($N_{5.5} \rightarrow D_{5.5}$)	Gdn·HCl, pH 5.5		2.5	20	3000

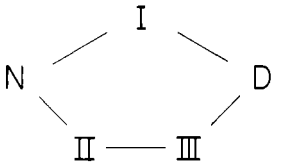

FIGURE 1: Scheme relating the native and denatured states of α-lactalbumin. See text for explanation.

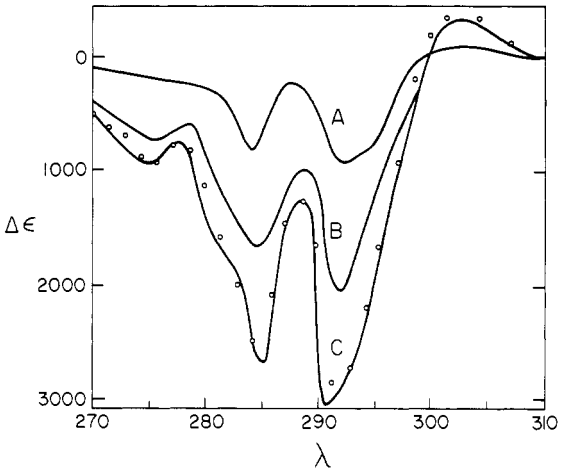

FIGURE 2: Difference spectra for various transitions of α-lactalbumin. (A) $N \rightarrow I_{2.2}$; (B) $I_{2.2} \rightarrow D_{2.2}$; (C) $N \rightarrow D_{5.5}$. The circles represent the sum of A and B, which is equal to C within the experimental error.

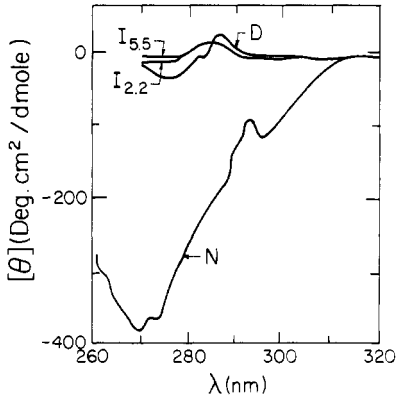

FIGURE 3: Aromatic circular dichroic spectra of α-lactalbumin.

Results

It is going to be necessary in what follows to distinguish state I molecules at pH 2.2 and pH 5.5, and likewise state D molecules. We will do this by the use of subscripts. For example, acid denaturation of α-lactalbumin, which we believe produces state I molecules at pH 2.2, will be indicated by $N \rightarrow I_{2.2}$. The $LiClO_4$ denaturation, which we believe produces state I molecules at pH 5.5, will be indicated by $N \rightarrow I_{5.5}$.

Figure 2 shows the difference spectra we have measured on the transitions $N \rightarrow I_{2.2}$, $N \rightarrow D_{5.5}$, and $I_{2.2} \rightarrow D_{2.2}$.

Figure 3 shows the aromatic CD spectra for N, $I_{2.2}$, $I_{5.5}$, and D, and Figure 4 shows the peptide CD spectra for the same species. $D_{2.2}$ and $D_{5.5}$ give the same CD spectra.

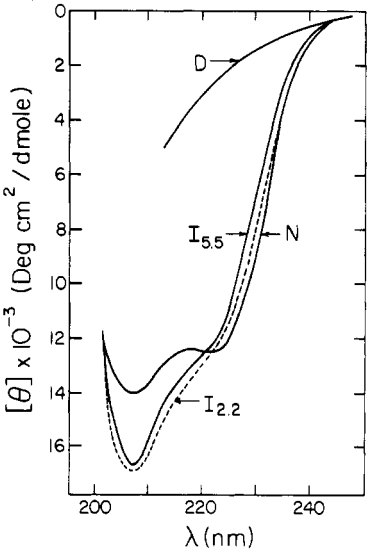

FIGURE 4: Peptide circular dichroic spectra of α-lactalbumin.

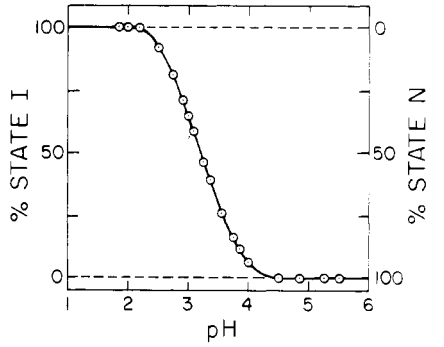

FIGURE 5: Normalized pH transition curve for $N \rightarrow I_{2.2}$.

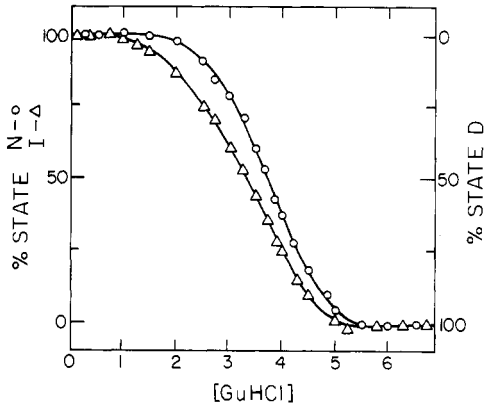

FIGURE 6: Normalized transition curves for $N \rightarrow D_{5.5}$ (O) and $I_{2.2} \rightarrow D_{2.2}$ (Δ).

Figures 5 and 6 show normalized transition curves for several of the denaturation processes. These have been prepared from UV spectral data and CD data measured at several pH values (Figure 5) or Gdn·HCl concentrations (Figure 6). In all cases the UV and CD data gave the same transition curve, supporting the idea that all the transitions are two-state processes, though this may not be correct (Kuwajima, 1977).

The essential spectroscopic data have been collected in Table I.

Discussion

Transition $N \rightleftarrows I$. As we said above, we believe this transition can be effected either by the addition of $LiClO_4$ at pH 5.5 ($N \rightarrow I_{5.5}$) or by lowering the pH ($N \rightarrow I_{2.2}$). Kronman et al. (1965) were the first to demonstrate an acid transition in α-lactalbumin by difference spectrophotometry. We have confirmed their results in the region 270–320 nm (Figure 2) and we have also carried out CD measurements for the transition in both the aromatic (Figure 3) and peptide (Figure 4) regions of the spectrum. Data from these and similar measurements carried out at several pH values were converted into the normalized transition curve shown in Figure 5.

The difference spectrum caused by acid is quantitatively very similar to that reported by Sharma & Bigelow (1974) for $LiClO_4$ denaturation. Figures 3 and 4 show the CD spectra of the state I molecules produced by $LiClO_4$ at pH 5.5 and by acid. It is clear that the CD spectra of $I_{2.2}$ and $I_{5.5}$ and by acid. It is clear that the CD spectra of $I_{2.2}$ and $I_{5.5}$ are very similar, qualitatively and quantitatively, in both regions of the spectrum, supporting the belief that the structures are similar. It is also evident from Figure 4 that the conformations are not very different from the N conformation, though it appears that they may contain slightly more α helix than N. It is interesting that the *aromatic* CD spectrum of the protein is essentially destroyed in both $I_{2.2}$ and $I_{5.5}$ and that two-thirds of the total possible difference spectrum is observed (Figure 2).

Figure 5 shows a normalized transition curve for $N \rightarrow I_{2.2}$.

Transition $I_{2.2} \rightleftarrows D_{2.2}$. It is clear from Figure 3 that states I and D are indistinguishable by *aromatic* CD and therefore that CD measurements in this region cannot be used to study the transition from I to D. On the other hand, difference spectroscopy and *peptide* CD measurements can be used, as is apparent from Figures 2 and 4.

Figure 2 shows the difference spectrum that is observed in the process $I_{2.2} \rightarrow D_{2.2}$. In this experiment lactalbumin has previously been partially denatured to $I_{2.2}$ by lowering the pH, and the further transition, which occurs on the addition of Gdn·HCl, has been studied.

Figure 6 shows normalized transition curves for the Gdn·HCl denaturation of both N and I, that is, for Gdn·HCl denaturation at pH 5.5 and 2.2.

Transition $N \rightleftarrows D$. Figure 2 also gives a difference spectrum for the Gdn·HCl denaturation of native lactalbumin at pH 5.5 ($N \rightarrow D_{5.5}$). Figures 3 and 4 show the CD spectra for $D_{2.2}$ and $D_{5.5}$.

Figure 2 shows that the difference spectra for the various processes are additive; that is, the difference spectrum observed for the process $N \rightarrow D$ is, within experimental error, equal to the sum of the spectra for the two partial denaturation steps ($N \rightarrow I$ and $I \rightarrow D$) which make it up.

Gdn·HCl destroys the aromatic CD spectrum of the protein and replaces the native CD spectrum with a typical random coil spectrum.

Figure 6 shows normalized transition curves for the Gdn·HCl denaturation of both N and $I_{2.2}$. It is clear from the data that state I unfolds at a slightly lower concentration of Gdn·HCl than does N; the concentration of Gdn·HCl is about 0.3 M smaller at the half point.

Spectral Properties of N, I, and D. The data presented in this paper confirm the conclusion of Sharma & Bigelow (1974) that α-lactalbumin can take up an intermediate state of denaturation, here called I. They also confirm that either $LiClO_4$ or dilute acid will bring about the conversion of N to I.

Table II: Calculated Secondary Structures of Native and Acid-Denatured α-Lactalbumin and Native Lysozyme[a]

protein	pH	α helix (%)	β structure (%)	random coil (%)
native lysozyme[a]	6.6	29	11	60
native α-lactalbumin	5.5	34	8	58
acid-denatured α-lactalbumin	2.2	43	1	56
perchlorate-denatured α-lactalbumin	5.5	41	0	59

[a] Greenfield & Fasman (1969).

These conclusions were reached tentatively by Sharma & Bigelow largely from the similarity of the difference spectrum they found in $LiClO_4$ and the values observed earlier by Kronman et al. (1965) in dilute acid. The b_0 values from optical rotatory dispersion measurements were also very similar. We have now been able to verify these quantitative similarities, and we have also shown that both denaturants cause very similar changes in the CD spectrum of the protein; in both cases we see the complete destruction of the aromatic CD and a rather small change in the peptide CD. The CD observations lead us to conclude that the peptide backbone of I is quite similar to that found in N but that some tryptophyl residues which are immobile in N are considerably loosened in I.

It is expected, of course, that state D is the completely unfolded molecule, and the CD and spectral data in Table I are consistent with this expectation. The aromatic CD spectrum no longer exists, and the peptide CD has been converted into the form typical of a random coil polypeptide.

As can be seen in Figure 2, the sum of the difference spectra observed in the steps $N \rightarrow I$ and $I \rightarrow D$ equals, within the experimental error, the difference spectrum for the process $N \rightarrow D$. The difference spectra in each step are due to the exposure of tryptophyl residues. The numerical data suggest that one or two tryptophyl residues are exposed in the process $N \rightarrow I$ ($\Delta\epsilon_{292} \simeq -2000$) and another in the step $I \rightarrow D$ ($\Delta\epsilon_{292} \simeq -900$).

Structure of I. It is reasonably clear that the state I molecule is not very different from the native molecule, even though the transition is accompanied by large changes in the mobility and environment of the aromatic chromophores which are demonstrated by the large difference spectra and by the destruction of the aromatic CD spectrum. There are only small changes in the peptide CD spectrum and, as Sharma & Bigelow (1974) found, in the relative viscosity of solutions of the protein. These two parameters are much more useful for estimating overall conformational changes in the molecular structure.

We have analyzed the low-wavelength CD spectra according to the methods of Greenfield & Fasman (1969) in order to estimate the contributions of α helix, β structure, and random coil to the N and I structures. The results of these calculations are given in Table II. It appears that when N is converted to I, there is a slight increase in α-helix content at the expense of β structure. This is a very small change in the spectrum, and we can only offer the interpretation as a tentative one, given the possible errors in the method of analysis (Greenfield & Fasman, 1969).

Denaturation of N and I by Gdn·HCl. It is clear from the data presented that denaturation by Gdn·HCl leads to the

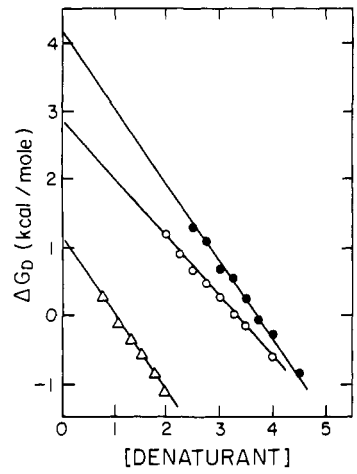

FIGURE 7: Free energy of denaturation as a function of denaturant concentration. (●) Denaturant is Gdn·HCl at pH 5.5; N → $D_{5.5}$. (○) Denaturant is Gdn·HCl at pH 2.2; $I_{2.2}$ → $D_{2.2}$. (△) Denaturant is $LiClO_4$ at pH 5.5; N → $I_{5.5}$.

same conformation whether the experiments are done on native molecules at pH 5 or $I_{2.2}$ molecules at pH 2. The processes differ because the *starting* conformations differ, and it is obviously of interest to compare the two conversions, if possible.

Figure 6 shows that the denaturation of the native molecule requires a higher concentration of Gdn·HCl and is a steeper function of the Gdn·HCl concentration than the denaturation of state $I_{2.2}$; in other words, N is more stable than $I_{2.2}$, and the denaturation of N is a more cooperative phenomenon than the denaturation of $I_{2.2}$, as one would expect.

Free Energy Calculations. The data in Figure 6 can be used to determine the free energy changes for the various transitions. First, the data are converted into apparent equilibrium constants for the processes N ⇄ D and I ⇄ D by the calculation

$$K = \frac{x}{100 - x} \tag{1}$$

at various concentrations of Gdn·HCl; x is the percentage of the molecules in state D at the concentration of Gdn·HCl in question.

The values of K are then converted into apparent molar free energy differences for the transitions (eq 2).

$$\Delta G_D = -RT \ln K \tag{2}$$

ΔG_D depends on the denaturant concentration of course, and it is a matter of some interest to try to understand the significance of the dependence. Several theoretical models have been proposed by Aune & Tanford (1969), and these have been compared for several proteins, using data from various laboratories, by Pace (1975). We will be comparing the various models in a subsequent paper (C. C. Bigelow and C. C. Contaxis, in preparation).

The simplest way to represent the data is to plot ΔG_D against the denaturant concentration by using eq 3. The intercept,

$$\Delta G_D = \Delta G_D^{H_2O} - m(\text{denaturant}) \tag{3}$$

$\Delta G_D^{H_2O}$, is the free energy change for the transition *in the absence of denaturant.*

Pace (1975) has discussed the use of eq 3 and the interpretation of the parameters $\Delta G_D^{H_2O}$ and m. In this paper we will analyze all of our results by using the linear extrapolation in eq 3 to determine $\Delta G_D^{H_2O}$ for the $LiClO_4$ transition N → $I_{5.5}$ and the Gdn·HCl transitions N → $D_{5.5}$ and $I_{2.2}$ → $D_{2.2}$.

$\Delta G_D^{H_2O}$ Values for the Various Transitions. Figure 7 shows plots of the values of ΔG_D against denaturant concentration for the different transitions caused by $LiClO_4$ and Gdn·HCl. As can be seen, they are all good straight lines in the transition regions (we have plotted ΔG_D values only in the regions between K = 0.1 and 10) which are readily extrapolated to zero denaturant concentration.

Table III: Thermodynamic Parameters for the Various Transitions between N, I, and D

process	denaturant	$\Delta G_D^{H_2O}$ (kcal mol^{-1})	m (kcal L mol^{-1})	$D_{0.5}$ (M)
$N_{5.5} \rightarrow D_{5.5}$	Gdn·HCl	4.2	1.27	3.75
$N_{5.5} \rightarrow I_{5.5}$	$LiClO_4$	1.2	1.13	1.05
$I_{2.2} \rightarrow D_{2.2}$	Gdn·HCl	2.9	0.89	3.26
$N_{5.5} \rightarrow I_{2.2}$	acid	6.4		

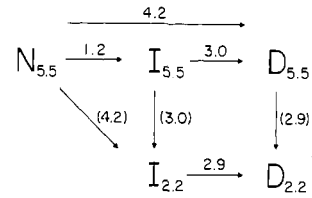

FIGURE 8: Scheme relating the denatured states studied in this paper. The free energies in kcal mol^{-1} are given; the values in parentheses are estimates (see text).

The resulting values of $\Delta G_D^{H_2O}$ and m, along with values of the denaturant concentrations at the transition midpoints ($D_{0.5}$), are listed in Table III. The $\Delta G_D^{H_2O}$ values are also entered in Figure 8, which relates the various conformations to each other.

It is seen from Figure 8 that $\Delta G_D^{H_2O}$ for N → $I_{5.5}$ is 1.2 kcal mol^{-1} and for N → $D_{5.5}$ is 4.2 kcal mol^{-1}. Therefore the value for the process $I_{5.5}$ → $D_{5.5}$ (which cannot actually be studied because precipitation occurs in the mixture of $LiClO_4$ and Gdn·HCl) is 3.0 kcal mol^{-1}. About 30% of the total conformational free energy stabilizing the native protein is lost in the conversion to I and about 70% in the conversion of I to D.

With just the three values of $\Delta G_D^{H_2O}$ measured for the chemical denaturants, we cannot determine ΔG_D values for the other possible processes in the diagram. However it is clear that this would be possible if we could determine $\Delta G_D^{H_2O}$ for any transition between a pH 5.5 state and a pH 2.2 state. We will now estimate the maximum possible value for the process $N_{5.5}$ → $I_{2.2}$, i.e., the acid transition for which we have presented a transition curve in Figure 5.

The pH dependence of the equilibrium constant in Figure 5 can be represented by eq 4, where a_H is the hydrogen ion

$$\frac{\delta \ln K_D}{\delta \ln a_H} = \Delta\nu \tag{4}$$

activity and $\Delta\nu = \Delta\nu_I - \Delta\nu_N$, the increase in the number of protons bound when N is converted to I (Hermans & Scheraga, 1961).

Equation 4 can be converted into eq 5, and Figure 9 shows

$$\frac{\delta \log K_D}{\delta \text{pH}} = -\Delta\nu \tag{5}$$

a plot of log K_D vs. pH for the conversion N → $I_{2.2}$. It is clear that a good linear relationship is found, which means that $\Delta\nu$ is constant over the pH range in which the acid transition is observed. We find that $\Delta\nu$ = 1.34, and we will assume that the constancy of $\Delta\nu$ will hold down to pH 2.2 and up to pH 4.5 (at which values essentially all of the molecules are in $I_{2.2}$ form or the N form, respectively (see Figure 5)) to allow us to integrate eq 5:

$$\log K_D = -\Delta\nu \text{pH} + C$$
$$= -1.34\ \text{pH} + C \tag{6}$$

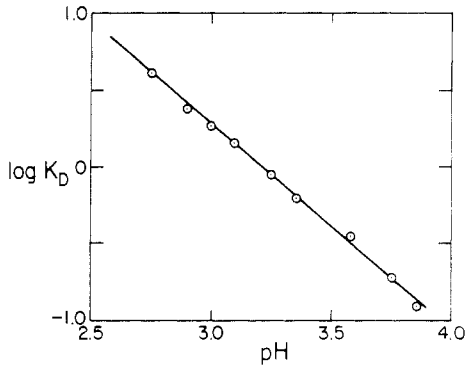

FIGURE 9: Plot of log K_D vs. pH for $N \rightarrow I_{2.2}$.

C is found to be 4.30 by substituting $K_D = 1$ at pH 3.21, and ΔG_D is found in the usual way:

$$\Delta G_D = -RT \ln K$$
$$= -1.36\ (-1.34\ \text{pH} + 4.30)$$
$$= 1.82\ \text{pH} - 5.85\ (\text{kcal mol}^{-1})$$

The transition is essentially complete between pH 4.5 and 2.2; at these values, $\Delta G_D = 2.34$ and -1.87 kcal mol^{-1}, respectively. The difference between them, 4.2 kcal mol^{-1}, is an estimate of $\Delta G_D^{H_2O}$ for the transition $N_{5.5} \rightarrow I_{2.2}$. It can only be regarded as an estimate because there will be a contribution to the total observed ΔG from the protonation reactions which take place during the titration.

Nevertheless, we have entered the figure 4.2 kcal mol^{-1} in Figure 8. This allows us to estimate the other values shown in parentheses. Thus it has been possible to describe five conformational states of the α-lactalbumin molecule and to measure or estimate the standard free energy changes (in water) that accompany the processes among them.

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Nature of the Fast and Slow Refolding Reactions of Iron(III) Cytochrome *c*†

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ABSTRACT: The fast and slow refolding reactions of iron(III) cytochrome *c* (Fe(III) cyt *c*), previously studied by Ikai et al. (Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73*, 165–184), have been reinvestigated. The fast reaction has the major amplitude (78%) and is 100-fold faster than the slow reaction in these conditions (pH 7.2, 25 °C, 1.75 M guanidine hydrochloride). We show here that native cyt *c* is the product formed in the fast reaction as well as in the slow reaction. Two probes have been used to test for formation of native cyt *c*: absorbance in the 695-nm band and rate of reduction by L-ascorbate. Different unfolded species (U_F, U_S) give rise to the fast and slow refolding reactions, as shown both by refolding assays at different times after unfolding ("double-jump" experiments) and by the formation of native cyt *c* in each of the fast and slow refolding reactions. Thus the fast refolding reaction is $U_F \rightarrow N$ and the slow refolding reaction is $U_S \rightarrow N$, where N is native cyt *c*, and there is a $U_S \leftrightarrow U_F$ equilibrium in unfolded cyt *c*. The results are consistent with the $U_F \leftrightarrow U_S$ reaction being proline isomerization, but this has not yet been tested in detail. Folding intermediates have been detected in both reactions. In the $U_F \rightarrow N$ reaction, the Soret absorbance change precedes the recovery of the native 695-nm band spectrum, showing that Soret absorbance monitors the formation of a folding intermediate. In the $U_S \rightarrow N$ reaction an ascorbate-reducible intermediate has been found at an early stage in folding and the Soret absorbance change occurs together with the change at 695 nm as N is formed in the final stage of folding.

Cytochrome *c* (cyt *c*)[1] was the subject of the first systematic study, in modern terms, of the kinetics of a protein folding transition. After finding conditions for reversible unfolding and refolding, Ikai et al. (1973) analyzed the kinetics by the theory of Ikai & Tanford (1973), which assumes that only one form of the unfolded protein is present. They concluded that the fast-folding reaction (0.1–0.2 s, pH 6.5, 25 °C, 0.5–2.7 M Gdn-HCl) probably represents the formation of one or more dead-end or abortive intermediates. The discovery of two forms of unfolded RNase A cast doubt on this conclusion. There is a slow equilibrium between a fast-folding form U_F and a slow-folding form U_S (Garel & Baldwin, 1973, 1975a,b; Brandts et al., 1975; Hagerman & Baldwin, 1976; Garel et al., 1976). If the same is true of cyt *c*, it could explain the

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[1] Abbreviations used: cyt *c*, ferricytochrome *c* (horse heart); RNase A, bovine pancreatic ribonuclease A; Gdn-HCl, guanidine hydrochloride; U_S and U_F, slow-folding and fast-folding forms of an unfolded protein; N, native form; τ, time constant of a reaction (reciprocal of the apparent rate constant).

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