Decrease in Stability of Human Albumin with Increase in Protein Concentration*

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The stability (reflected in denaturation temperature, $T_d$) of defatted human albumin monomer, monitored by differential scanning calorimetry, decreases with increasing protein concentration. This is shown to be compatible with a simple model in which reversible polymerization of denatured monomer promotes unfolding. This model also predicts an increase in transition cooperativity with decreasing protein concentration whereas experimentally cooperativity decreases because the rate of thermally induced polymerization of unfolded monomer is slow relative to the scan rate of the calorimeter. The denaturation of undefatted human albumin monomer, subsaturated with high affinity endogenous long-chain fatty acid (LCFA), was previously observed by differential scanning calorimetry to be a biphasic process. $T_d$ for the first endotherm, associated with the denaturation of LCFA-poor species, decreases with increasing protein concentration similar to that for defatted monomer whereas $T_d$ for the second endotherm, associated with denaturation of LCFA-rich species, is independent of concentration. The magnitude of the concentration dependence of $T_d$ relates directly to the extent of polymerization of denatured monomer, which decreases with increasing level of bound ligand. The bimodal thermogram observed for undefatted monomer persists upon simultaneous extrapolation of $T_d$ values to low concentration and low scan rate thereby demonstrating that this biphasic denaturation arising from ligand redistribution during denaturation is a true thermodynamic phenomenon and not an artifact of specific experimental conditions or the method used to induce denaturation.

In an earlier study, undefatted human albumin monomer, subsaturated with high affinity endogenous LCFA,1 was found to undergo biphasic denaturation, which was reflected as two endotherms in the DSC thermogram (1, 2). In order to elucidate the mechanism of the denaturation of albumin, the consequence of removing endogenous LCFA (defatting) on the observed biphasic unfolding of undefatted monomer has been investigated by DSC in the current study. In addition, the effects of protein concentration on the unfolding process of both undefatted and defatted monomer have been determined. The behavior of the denaturation temperature of defatted monomer as a function of concentration is shown to be compatible with an attendant polymerization of unfolded albumin monomer during thermal denaturation. In order to establish whether the undefatted monomer undergoes biphasic denaturation under conditions that better approximate those of equilibrium with minimal protein-protein interactions, a method is presented for simultaneously extrapolating the denaturation temperatures to low protein concentration and low scan rate.

MATERIALS AND METHODS

Protein Stock Solutions—Undefatted human albumin monomer was prepared by gel filtration chromatography from 7.3% fraction V albumin as before (1). This fraction V starting material was unheated, contained no stabilizers, and contained 0.50 mol of free sulfhydryl/mol of protein. The monomeric protein was concentrated by diafiltration and dialyzed to give undefatted monomer at 89.1 mg/ml in 150 mM NaCl, pH 7.7.

Defatted albumin monomer was prepared by the method of Chen (3, 4) from the above 7.3% fraction V material, concentrated by diafiltration, purified by the same gel filtration chromatography procedure used above, reconcentrated, and dialyzed to yield 78.9 mg/ml defatted monomer in 150 mM NaCl, pH 7.7.

Protein concentration was determined by using 5.50 and 5.45 for $A_{280}$ for undefatted monomer and defatted monomer, respectively. These values were obtained from spectrophotometric absorbance measurements at 280 nm of dilutions in 0.10 M NaPO₄ pH 7.0, of solutions the concentrations of which had been determined by differential refractometry at 546 nm with a value of 0.186 ml/g for dₕ/dc (6). The undefatted and defatted albumin preparations contained 1.43 and 0.84 mol of endogenous LCFA/mol of albumin monomer, respectively, which were determined by the method of Bergmann et al. (6).

High pressure liquid chromatography analysis with a Spherogel-TSK 3000 column (Beckman Instruments, Inc.) and polyacrylamide gel electrophoresis in sodium dodecyl sulfate under reducing and nonreducing conditions demonstrated that both the undefatted and defatted albumin preparations were ≥99% monomer. Cellulose acetate electrophoresis showed that >99% of both the undefatted and defatted monomer preparations migrated as albumin.

Solutions for Calorimetry—Solutions for DSC were prepared by dilution of the appropriate albumin monomer stock solution with 150 mM NaCl; concentrations of diluted solutions were determined from the weight and densities of the solvent and the protein stock solutions by using a partial specific volume of 0.735 ml/g for albumin (7). The amount of sample placed in the calorimeter was determined from the weight and appropriate density (8).

Calorimetry—Design, operation, and calibration of the differential scanning calorimeter, which is of the heat flow type, are described.

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1 The abbreviations used are: LCFA, long-chain fatty acid; DSC, differential scanning calorimetry; HHW, half-height width.
was loaded into the sample cell with the reference cell remaining unfilled and heated from 30 to 95 °C at 14.7 K/h. The solvent contribution to the heat capacity was determined separately and subtracted from the protein scan.

The differential voltage signal, V, was converted to excess heat capacity, Cex, in cal/(K·g protein) by the relation (11)

\[ C_{ex} = \epsilon(T) \cdot \frac{[V + r(T) \cdot dV/dt]}{(4.184 \cdot \Phi \cdot w)} \]  

where \( \epsilon(T) \) is the calorimeter calibration constant in watts/V, \( V \) is the differential voltage in V, \( r(T) \) is the time constant in s, \( dV/dt \) is the first derivative of the differential voltage with respect to time in V/s, \( \Phi \) is scan rate in K/s, and \( w \) is the mass of protein in g.

Enthalpy of denaturation values was determined by integrating the thermogram over the temperature interval of denaturation with respect to a linear base line drawn throughout this region from where the transition was judged to have started as evidenced by departure of the excess heat capacity curve from the sloping predenaturation base line to where it meets the nearly flat postdenaturation base line. This approach has been shown to be a good approximation for transitions characterized by a change in heat capacity (12) and is appropriate for thermograms comprised of multiple endotherms.

**Results**

**Experimental Conditions and Uncertainties**—DSC experiments were performed on defatted and undefatted (1.4 eq of LCFA/monomer) human albumin monomer in 150 mM NaCl, pH 7.0. The protein undergoes irreversible thermal denaturation at all concentrations.

Denaturation temperature, \( T_d \), is defined as the temperature at which a local maximum occurs in the excess heat capacity, \( C_m \). The reproducibility in the denaturation temperatures for the sharper endotherms (\( T_{d1} \) in Tables I and III and \( T_{d0} \) in Tables II and IV) is estimated as ±0.03 °C and that for the broader endotherms (\( T_{d0} \) in Tables II and IV) as ±0.1 °C. The uncertainty in each enthalpy of denaturation, \( \Delta H_a \), in Tables I and II is estimated as ±3%. The estimated uncertainties in \( C_{ex} \) (maximum excess heat capacity) and HHW in Tables I and III are ±2% and ±0.06 °C, respectively.

**Variation of \( T_d \) and \( \Delta H_a \) with Protein Concentration**—Thermograms for defatted human albumin monomer were measured as a function of protein concentration from 1.4 to 74 mg/ml at a scan rate of 14.7 K/h. Defatted monomer shows a single endotherm at all concentrations; a typical thermogram at 30 mg/ml is presented in Fig. 1, thermogram A. Values of \( T_d \), \( \Delta H_a \), \( C_{ex} \), and HHW at each concentration are given in Table I. With decreasing albumin concentration, \( T_d \) increases monotonically from 63.9 °C at 74.1 mg/ml to 66.4 °C at 1.43 mg/ml, whereas \( \Delta H_a \) appears to be constant with an average value of 4.18 cal/g. In addition, with decreasing protein concentration, the endotherm decreases in amplitude and broadens, i.e. \( C_{ex} \) decreases and HHW increases.

Thermograms for undefatted albumin monomer were measured as a function of concentration from 1.3 to 64 mg/ml at a scan rate of 14.7 K/h. At all protein concentrations studied, the thermogram for undefatted monomer is bimodal (Fig. 2); the lower denaturation temperature is designated \( T_{d0} \) and the higher \( T_{d1} \). Both \( T_{d0} \) and \( \Delta H_a \) appear independent of protein concentration with average values of 78.2 °C and 5.16 cal/g, respectively (Table II). With decreasing albumin concentration, \( T_{d0} \) increases monotonically from 68.3 °C at 64.1 mg/ml to 71.1 °C at 1.25 mg/ml (Table II). Thus, at a constant scan rate of 14.7 K/h, the two denaturation peaks tend to merge with decreasing protein concentration; however, even at 1.25 mg/ml the thermogram for undefatted monomer is bimodal (Fig. 2).

Sturtevant and co-workers (13–15) have demonstrated that a plot of the logarithm of protein concentration versus \( 1/T_d \) with \( T_d \) in K is linear with a negative slope when an oligomeric protein dissociates on denaturation. The positive slope observed for a similar plot of the data reported here for defatted monomer (Fig. 3, line A) suggests that albumin undergoes polymerization on denaturation. A simple model for such a chemical reaction may be described by

\[ N_d \cdot \epsilon = (1/m) \cdot D_m + n \cdot L \]  

where \( N_d \) is the native protein unit with \( n \) ligands bound that undergoes reversible two-state unfolding with an attendant dissociation of ligand and polymerization of \( m \) denatured
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Figure 2. Thermograms of undefatted human albumin monomer as a function of protein concentration. All measurements were made in 150 mM NaCl, pH 7.0, with a scan rate of 14.7 K/h. Undefatted monomer contained 1.4 mol of endogenous LCFA/mol of monomer. Excess heat capacity is expressed in mcal/K for 1.45 ml of protein solution; the protein concentrations are as indicated. Thermograms at the lower protein concentrations are plotted with an expanded ordinate scale.

All concentrations in Equation 3 are expressed in molarity. $(N)_o$ is the total concentration of protein expressed in terms of the molecular weight of the denaturing unit, $N_{Lm}$, and $(L)$ is the concentration of free ligand. The extent of reaction, $\alpha$, is defined as $\alpha = m - (D_o)/(N)_o$. By using similar assumptions and approximations as those of Sturtevant and co-workers (13), the overall temperature dependence of the equilibrium constant in Equation 3 gives

$$\Delta H_{eq}/(R \cdot T_o) + n \ln (L)_o - ((m - 1)/m) \cdot \ln (N)_o = \text{constant}$$

(4)

where $\Delta H_{eq}$, van't Hoff enthalpy, is assumed to be temperature independent and $(L)$ has been set equal to total ligand concentration, $(L)_o$ by assuming that $(L)_o \gg (N)_o$. Thus, $T_o$ shows a dependence on protein concentration if upon denaturation the protein undergoes polymerization or dissociation.

According to Equation 4 for denaturation/polymerization at constant ligand concentration, a plot of $\ln (N)_o$ versus $1/T_o$ will give a straight line with a positive slope of $n \cdot \Delta C_p/(m(m - 1))$. Such a plot for defatted monomer is linear and has a positive slope (Fig. 3, line A).

For undefatted monomer, $T_o$ data (Table II) are plotted as $\ln (N)_o$ versus $1/T_o$ in Fig. 3 also. A straight line with a positive slope results for $T_o^D$ (Fig. 3, line B), and a straight vertical line corresponding to 78.2 °C represents $T_o^P$ (Fig. 3, line C).

**Figure 3.** van't Hoff plot according to Equation 4 for DSC experiments at various concentrations of defatted and undefatted human albumin monomer preparations. All measurements were in 150 mM NaCl, pH 7.0, with a scan rate of 14.7 K/h. Undefatted monomer contained 1.4 mol of endogenous LCFA/mol of monomer. The data are plotted in a $\ln (N)_o$ versus $1/T_o$ format with $T_o$ in K; $(N)_o$ values are in mg/ml protein. Line A, $T_o$ data for defatted monomer (Table I). The experimental data (●) were fitted by linear least squares with $\ln (N)_o$ as the independent variable and $1/T_o$ as the dependent variable to give a slope of $+2.00 \times 10^5$ K and correlation coefficient of 0.957. Line B, $T_o^P$ data for undefatted monomer (Table II). The experimental data (●) were fitted by linear least squares as above to give a slope of $+9.76 \times 10^4$ K and correlation coefficient of 0.997. Line C, $T_o^P$ data for undefatted monomer (Table II). The average value of the experimental data (●), 78.2 °C, is represented by a vertical line.

**Table II.** Thermodynamic data measured by DSC for the thermal denaturation of undefatted human albumin monomer

| Protein concentration (mg/ml) | $T_o^P$ (°C) | $T_o^D$ (°C) | $\Delta H_{eq}$ (cal/g) |
|-----------------------------|--------------|--------------|------------------------|
| 0.1                        | 64.1         | 78.4         | 5.63                   |
| 0.2                        | 48.9         | 78.1         | 4.82                   |
| 0.3                        | 46.7         | 78.2         | 5.16                   |
| 0.4                        | 40.3         | 78.2         | 5.16                   |
| 0.5                        | 32.6         | 78.1         | 5.06                   |
| 0.6                        | 19.9         | 78.2         | 5.13                   |
| 0.7                        | 10.4         | 78.2         | 5.20                   |
| 0.8                        | 5.53         | 78.2         | 5.15                   |
| 0.9                        | 4.82         | 78.3         | 5.1                   |

$\Delta H_{eq}$ and $T_o$ show a dependence on protein concentration if upon denaturation the protein undergoes polymerization or dissociation.
where $\Delta H_{\text{st}}$ is assumed to be temperature independent. For given values of $(N)_0$, $m$, $\Delta H_{\text{st}}$, and $C_{\varepsilon}(T)$ can be computed from the appropriate value of $\alpha(T)$, which is determined from the overall equilibrium constant, $K_{\text{eq}}$, in Equation 3 with $n = 0$. The numerical value of $K_{eq}$ derives from the van't Hoff temperature dependence of the equilibrium constant at temperature $T$ in K

$$K_{\text{eq}}(T) = \exp[-\Delta H_{\text{st}}/(1 - T/\theta_0)/(R \cdot T)]$$ (7)

where $\theta_0$ in K is the transition temperature for the overall reaction, i.e. the temperature at which $K_{eq} = 1$.

A relationship between the van't Hoff and calorimetric enthalpies in terms of experimental parameters is obtained from Equation 6 by setting $T = \theta_0$

$$\Delta H_{\text{st}} = \left(1 + \alpha_0 (m - 1)\right) R \cdot T_{\theta_0} \cdot C_{\varepsilon}^0$$ (8)

where $C_{\varepsilon}^0$ is the maximum in the excess heat capacity curve, $C_{\varepsilon}(T_d)$, and $\alpha_0 = \alpha(T_d)$.

Values for $m$ and $\Delta H_{\text{st}}$ are obtained by simultaneously satisfying Equation 8 and the expression for the slope of a plot of $\ln(N)_0$ versus $1/T_\theta$ (see Equation 4) by fitting data for defatted monomer at 30 mg/ml (Table 1). The appropriate value of $\alpha_0$ is obtained from the endotherm computed with Equation 6, and $T_\theta$ is chosen to reproduce the experimental $T_\theta$. The denaturing unit is assumed to be monomer $(M_\theta = 66,500)$ throughout. The iterative computation gives $n = 1.68$ and $\Delta H_{\text{st}} = 161.5$ kcal/mol with $\alpha_0 = 0.43$ and $T_m = 60.6$ °C. Since $\Delta H_{\text{st}} = 278$ kcal/mol, $\Delta H_{\text{st}} < \Delta H_{\text{en}}$.

The experimental thermogram for defatted monomer at 30.4 mg/ml (Fig. 1, thermogram A) has a slightly elongated tail on the high temperature side with a $C_{\varepsilon}^0$ of 0.926 cal/(g-K) and a HHW of 2.46 °C. The calculated endotherm for the denaturation/polymerization reaction at 30.1 mg/ml protein (Fig. 1, thermogram B), computed with the input parameters determined above, is skewed to the high temperature side with a $C_{\varepsilon}^0$ of 0.948 cal/(g-K) and a HHW of 3.86 °C. By contrast the endotherm calculated for the two-state denaturation involving no polymerization (Fig. 1, thermogram C) is essentially symmetric with a $C_{\varepsilon}^0$ of 1.28 cal/(g-K) and a HHW of 2.88 °C.

Endotherms corresponding to protein concentrations from 1.4 to 74 mg/ml for the denaturation/polymerization reaction of defatted monomer were computed by assuming a temperature-dependent $\Delta H_{\text{st}}$. These calculated endotherms (data not shown) decrease in HHW, a reflection of increasing cooperativity, sharpen, and increase in amplitude with decreasing protein concentration. In contrast, experimental endotherms substantially broaden and decrease in maximum amplitude with decreasing protein concentration (Table 1).

Variation of $T_\theta$ with Scan Rate and Protein Concentration— The effect on $T_\theta$ of decreasing both scan rate and protein concentration has been investigated. At constant concentra-

$^a$ A. Shrake and P. D. Ross, manuscript in preparation.

![Image](https://via.placeholder.com/150)

**Table III**

| Protein Concentration (mg/ml) | Scan Rate (K/h) | $T_\theta$ (°C) | $C_{\varepsilon}^0$ (cal/(g-K)) | HHW (°C) |
|------------------------------|-----------------|-----------------|-----------------------------|----------|
| 30.4                         | 14.7            | 64.66           | 0.926                       | 3.46     |
| 30.4                         | 4.88            | 64.00           | 0.998                       | 3.11     |
| 4.99                         | 14.7            | 65.64           | 0.750                       | 4.40     |
| 4.99                         | 4.88            | 64.81           | 0.890                       | 3.68     |

with $T_\theta$, in K (17-19). $E_a$ is the temperature-independent Arrhenius activation energy in kcal/mol of denaturing unit; $R$ is the gas constant in kcal/(K-mol), and the first constant is a positive number. Equation 9 predicts that a plot of $\ln \Phi$ versus $1/T_\theta$ will give a straight line with a negative slope. Since for defatted monomer at different concentrations and scan rates (Table III) $\alpha$ is essentially constant at $T = T_\theta$ ($\alpha = 0.479 \pm 0.014$ (S.D.)), $T_\theta$ may be taken as $T_\theta$. The data...
for defatted albumin monomer show that at constant concentration $T_d$ decreases with decreasing scan rate (Table III) as predicted by Equation 9.

At constant scan rate $T_d$ increases with decreasing defatted monomer concentration; thus, the effects on $T_d$ of decreasing scan rate and protein concentration are in opposition. To extrapolate to low protein concentration and to low scan rate, predicted by Equation 9, $T_d$ values as a function of protein concentration and scan rate for undefatted human albumin monomer are presented in Table IV. $T_d^{(j)}$ is independent of scan rate and concentration, has an average value of 78.1 °C, and is represented as a horizontal line in Fig. 5, curve C. The $T_d^{(j)}$ values were extrapolated as those for defatted monomer and are plotted in Fig. 5, curve B. $T_d^{(j)}$ for undefatted monomer decreases from 68.3 °C at 30 mg/ml and 30 K/h to 64.8 °C at 0.30 mg/ml and 0.30 K/h.

$T_d$ values as a function of protein concentration and scan rate for undefatted human albumin monomer are presented in Table IV. $T_d^{(j)}$ is independent of scan rate and concentration, has an average value of 78.1 °C, and is represented as a horizontal line in Fig. 5, curve C. The $T_d^{(j)}$ values were extrapolated as those for defatted monomer and are plotted in Fig. 5, curve B. $T_d^{(j)}$ for undefatted monomer decreases from 68.3 °C at 30 mg/ml and 30 K/h to 64.8 °C at 0.30 mg/ml and 0.30 K/h in contrast to $T_d^{(j)}$. The important result is that under conditions that better approximate an ideal equilibrium process, $T_d^{(f)}$ and $T_d^{(j)}$ are further separated than under actual experimental conditions.

**DISCUSSION**

An earlier study of the stability of undefatted human albumin monomer revealed that the thermal denaturation of this protein is biphasic, i.e. the DSC thermogram consists of two peaks (1). The undefatted protein was subsaturated with high affinity LCFA (21, 22). An analysis of this albumin during the course of its irreversible thermal denaturation showed that the endotherm with the lower $T_d$ is associated with denaturation of LCFA-poor protein species whereas the endotherm with the higher $T_d$ is associated with denaturation of LCFA-rich protein species. This uneven distribution of bound LCFA was proposed to arise during denaturation due to increasing free ligand concentration (1).

In the current investigation, the thermal denaturation of defatted human albumin monomer, which has essentially all endogenous LCFA removed, is found to be monophasic at all concentrations studied. Since no ligand redistribution can occur during denaturation of defatted monomer, the occurrence of a single endotherm with this protein is compatible with the proposed origin of the biphasic denaturation of undefatted monomer (1). Tiktopulo et al. (23) have performed a DSC study of defatted human albumin monomer at pH 7.0 and observed a bimodal thermogram in contrast to the slightly asymmetric single endotherm observed in the present study. They have associated this bimodality with three independent domains within the molecule. However, the protein was modified by reacting the free sulfhydryl with L-cystine; in addition, experiments were performed at scan rates of 60 and 120 K/h, generally at low ionic strength and in the presence of low levels of phosphate, which binds to the protein. The bimodality observed in the present work in the thermogram for undefatted albumin monomer does not derive from sequential denaturation of domains within the same molecule (24) but rather from the denaturation of different kinds of molecules, LCFA-poor and -rich species (1).

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The thermal denaturation of human albumin is irreversible under conditions used in this study although we have utilized equilibrium thermodynamics to describe the denaturation/polymerization of albumin. However, Sturtevant and co-workers (14) have demonstrated that treatment of an overall irreversible process (F = U - I) yields results similar to those for the reversible process alone (F = U) when the irreversible step is slow compared to the rates of interconversion of species F and U. In the present study F = U corresponds to the reversible denaturation/polymerization reaction of Equation 2 whereas U - I corresponds to the slower irreversible aggregation of denatured albumin polymers. The appearance of the irreversibly denatured albumin in the presence and absence of LCFA was indicative of aggregation.
However, in the presence of high levels of LCFA, reversible polymerization does not occur since $T_d^{(1)}$ for undefatted monomer is independent of concentration. Furthermore, since $T_d^{(2)}$ is independent of both scan rate and concentration, the rate of irreversible aggregation (25–27) must be slow compared to the time scale of the calorimeter experiment.

Involvement of the denatured protein in polymerization shifts the equilibrium between the native and denatured species. In favor of denaturation in accordance with Le Chatelier's principle; therefore, with decreasing protein concentration, the effect of polymerization lessens and as a result $T_d$ should increase as is observed. For defatted monomer a plot of $\ln(N)_0$ versus $1/T_d$ gives a straight line with a positive slope as predicted by Equation 4, which was derived for the denaturation/polymerization reaction of Equation 2. This two-state reaction requires all denatured units to polymerize to the same degree, $m$. The actual degree of polymerization is not specific; thus, the parameter $m$ represents an average value. The significant result is that during thermal denaturation defatted monomer undergoes concomitant polymerization, which is manifested in a concentration dependence of $T_d$. The thermodynamics and kinetics of the denaturation of proteins may also be perturbed by excluded volume and electrostatic effects (28), but contributions from these effects are probably not large under our experimental conditions.

The thermogram for undefatted albumin monomer is bimodal at all protein concentrations and scan rates studied. $T_d^{(2)}$ is independent of concentration and scan rate whereas at constant scan rate $T_d^{(1)}$ increases monotonically with decreasing protein concentration; nevertheless, at the lowest concentrations bimodality is still apparent. $T_d^{(2)}$ data for undefatted monomer plotted as $\ln(N)_0$ versus $1/T_d$ show a straight line with a positive slope; this reflects polymerization of all these denatured LCFA-poor species. The corresponding $T_d^{(1)}$ plotted in the same manner are represented by a straight line with infinite slope; this indicates the lack of polymerization of these LCFA-rich species. The presence of increasing levels of fatty acid anion has been observed to correlate with a decrease in cooperativity. These computed results are in agreement with the oversimplified nature of the model. Nevertheless, the general features of the endotherms computed for denaturation/polymerization are qualitatively correct under experimental conditions devoid of kinetic effects. Thus, the observed slight skewing to the high temperature side of the experimental endotherm of defatted monomer relates to attendant polymerization on denaturation. The principle effect of the kinetic perturbation is a general broadening of the endotherm. $T_d$ values appear little affected by the relatively slow rate of polymerization of denatured monomer since a plot of $\ln(N)_0$ versus $1/T_d$ is linear as predicted by the equilibrium process described by Equation 4.

In order to gauge the significance of the bimodality observed in the thermograms for undefatted monomer, the thermograms must be examined under conditions as ideal and as close to equilibrium as possible. The effects on $T_d$ of decreasing protein concentration and scan rate act in an opposing manner and deaminate the observed data. In order to determine the net result of these two effects, $T_d^{(1)}$ and $T_d^{(2)}$ for undefatted monomer were extrapolated to various scan rates and protein concentrations. $T_d^{(2)}$ is independent of both concentration and scan rate, as anticipated for LCFA-rich species. However, $T_d^{(1)}$ data behave similarly to $T_d$ data for defatted monomer, as anticipated for LCFA-poor species, and are extrapolated analogously; on approach to ideal equilibrium conditions, $T_d^{(2)}$ also decreases. Thus, the bimodality observed for undefatted monomer at 15 K/h and 30 mg/ml not only persists at low scan rate and low protein concentration but is enhanced. Therefore, the biphasic denaturation of undefatted albumin monomer, reflected by the bimodal thermogram and arising from ligand redistribution during denaturation, is a true thermodynamic phenomenon and not an artifact of high protein concentration or high scan rate or of the means used to effect denaturation.

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