Biphasic Denaturation of Human Albumin Due to Ligand Redistribution during Unfolding*

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Denaturation of defatted human albumin monomer, monitored by differential scanning calorimetry, is monophasic as reflected by the single, resulting endotherm. With low levels of various ligands, biphasic or monophasic unfolding processes are manifested as bimodal or unimodal thermograms, respectively. The greater the affinity of native protein for ligand, the greater is the tendency for biphasic denaturation. We propose that such a biphasic unfolding process arises from a substantial increase in stability (transition temperature) of remaining native protein during denaturation. This increase in stability derives from the free energy of ligand binding becoming more negative due to the release of high affinity ligand by unfolding protein. The tendency for biphasic denaturation is greatest at low (subsaturating) levels of ligand where greatest increases in stability occur. Biphasic unfolding arising from such ligand redistribution results from denaturation of different kinds of protein molecules, ligand-poor and ligand-rich species, and not from sequential unfolding of domains within the same molecule. Differentiating between these two mechanisms is necessary for the correct interpretation of biphasic denaturation data. Furthermore, biphasic unfolding due to ligand redistribution occurs independently of the means used to effect denaturation.

The maximum increase in stability due to ligand binding relative to the stability of defatted albumin monomer alone occurs with the intermediate affinity ligand octanoate (22 °C) and not with the high affinity ligand hexadecanoate (15 °C). This indicates a much greater affinity of denatured albumin for hexadecanoate since increase in stability derives from the difference between free energy of ligand binding to folded and unfolded protein forms.

Elucidation of the mechanism of protein denaturation is important for an understanding of protein stability and also for an understanding of the unfolding pathway itself. In our initial study by DSC,† undeáfatted albumin monomer, containing subsaturating, high affinity, endogenous LCFA, was found to undergo biphasic denaturation, indicated by a bimodal thermogram (1, 2). For proteins consisting of a single polypeptide chain, multiphasic denaturation is often interpreted in terms of sequential unfolding of different domains within the same molecule (3). However, the two endotherms observed on heating undeáfatted albumin monomer were shown to be associated with the irreversible denaturation of LCFA-poor and LCFA-rich protein species, respectively. Thus, this biphasic unfolding does not relate to protein substructure but rather to denaturation of different kinds of protein molecules.

We proposed that the uneven distribution of protein species, varying in level of bound LCFA and responsible for the observed biphasic unfolding, does not preexist but rather is created during the denaturation process (1). Defatted human albumin monomer was shown by DSC in a more recent investigation (4) to have a single, almost symmetric endotherm in accord with the proposed mechanism of unfolding. For undeáfatted monomer, the observed bimodality becomes more pronounced upon extrapolation to very low protein concentration and very slow scan rate (4). Thus, this biphasic unfolding reaction is a thermodynamic phenomenon and is, therefore, independent of the means used to bring about denaturation.

Interpretation of data from the initial study by DSC is complicated because the effects of N-Ac-L-tryptophanate and octanoate (caprylate) are superimposed on those of the partially saturating, endogenous LCFA (1, 2), which is a mixture of fatty acids. To clarify the role of each ligand and investigate the hypothesis that the uneven distribution of protein species giving rise to biphasic denaturation arises during the unfolding process, we have carried out a study by DSC of the stabilization and denaturation of defatted human albumin monomer by the weaker binding ligands N-Ac-L-tryptophanate and caprylate and by the high affinity ligand hexadecanoate (palmitate), which is a major endogenous LCFA (5).

MATERIALS AND METHODS

Ligands—A stock solution of 163 mM sodium N-Ac-L-tryptophanate was prepared by dissolving N-acetyl-L-tryptophan (Sigma) in 150 mM NaCl by addition of 12.5 N NaOH until pH 7.2 was attained. After filtration, the concentration was determined by measuring A in 0.1 M NaPO₄, pH 7.0, with ε = 5250 M⁻¹ cm⁻¹ (6). A CD spectrum of the N-Ac-L-tryptophanate in the same buffer showed positive ellipticity from 225 to 240 nm with a maximum at 228.2 nm. The sodium caprylate (Sigma) was derivatized with bis(trimethylsilyl)trimethylsilyl)trifluoracetamide in pyridine and analyzed by gas chromatography with a 3% cyanopropylmethylphenylmethylpolysiloxane column. At 80 °C with a flow rate of 30 cm³/min, the chromatogram

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‡ The abbreviations used are: DSC, differential scanning calorimetry; LCFA, long-chain fatty acid; HHW, half-height width.
consisted of a single peak with a retention time of 7.0 min. A stock solution of 172 mM sodium caprylate was prepared by dissolving this material in 150 mM NaCl, adjusting the pH from 9.2 to 7.2 with 0.1 N HCl, and filtering the solution.

The melting point of the palmitic acid (Sigma) was sharp and occurred at 61.8°C (literature 63-64°C (7)). The palmitic acid was derivatized and analyzed by gas chromatography as above but with a column temperature of 150°C and exhibited a single peak with a retention time of 14.3 min.

Protein Stock Solutions—Defatted albumin monomer at 89.0 mg/ml in 150 mM NaCl, pH 7.7, was prepared as reported previously (4). Palmitate-refatted albumin monomer was prepared from defatted albumin monomer by the method of Spector and Horak (3) with palmitic acid-coated Celite. The refatted albumin solution was concentrated to 85 mg/ml and exhaustively dialyzed at 4°C against 150 mM NaCl to give 11 ml of palmitate-refatted albumin monomer at 66.1 mg/ml, pH 7.0. A value of 5.48 for $A_{280}$ was obtained from differential refractometry as before (4).

Both protein stock solutions were characterized as previously (4). The defatted and palmitate-refatted albumin monomer preparations contained 0.084 eq of endogenous LCFA/monomer and 6.63 eq of monomer with a molecular weight of 66,500, as determined in Table 1.

Palmitate—Defatted and palmitate-refatted albumin monomer preparations contained 0.084 eq of endogenous LCFA/monomer and 6.63 eq of monomer, respectively. Both preparations were ≥99% monomeric by high performance liquid chromatography and nonreducing polyacrylamide gel electrophoresis and ≥98% monomeric by reducing polyacrylamide gel electrophoresis. Cellulose acetate electrophoresis demonstrated that ~100% of the protein in both preparations migrated as albumin.

Solutions for Calorimetry—For DSC solutions containing submaximal concentrations of N-Ac-L-tryptophanate or caprylate, the ligand stock solution was diluted with 150 mM NaCl to an appropriate concentration; this solution was then used to dilute the defatted albumin monomer stock solution in order to decrease errors introduced by using small volumes of stock ligand solutions. Different levels of bound palmitate were obtained by mixing appropriate volumes of defatted and palmitate-refatted albumin monomer preparations and 150 mM NaCl. All solutions for calorimetry were adjusted to pH 7.0. Concentrations of diluted solutions were determined by weight with the appropriate densities as before (4); the partial specific volume of the ligand stock solution was diluted with 150 mM NaCl to an appropriate concentration; this solution was then used to dilute the defatted and palmitate-refatted albumin monomer at 89.0 mg/ml and exhaustively dialyzed at 4°C against 150 mM NaCl to give 11 ml of palmitate-refatted albumin monomer at 66.1 mg/ml, pH 7.0. A value of 5.48 for $A_{280}$ was obtained from differential refractometry as before (4).

In particular, enthalpy of denaturation was determined with respect to a sloping linear baseline.

RESULTS

Experimental Conditions and Uncertainties—All DSC experiments were carried out on defatted human albumin monomer at approximately 30 mg/ml in the absence and presence of the ligands N-Ac-L-tryptophanate, caprylate, or palmitate in 150 mM NaCl, pH 7.0, with a heating rate of 14.7 K/h. Under these conditions, the protein undergoes irreversible denaturation. Denaturation temperature, $T_d$, is defined as the temperature at which a local maximum occurs in the excess heat capacity. $T_d$ is determined by assuming that $C_{HHW}$ is the gas constant in kilocalories/(K.mol), $T_d$ is the denaturation temperature in K, $n$ is the number of binding sites on the protein for the ligand, and $L_0$ is the total ligand concentration, which has been set equal to the free ligand concentration, $L$, by assuming that $L_0 >>$ total protein concentration. Equation 1 predicts that a plot of $\ln(L_0)$ versus $1/T_d$ gives a straight line with a slope of $-\Delta H_{HHW}/(n-R)$.

Effect of N-Ac-L-tryptophanate—Thermograms for the denaturation of defatted monomer with added N-Ac-L-tryptophanate consist of single denaturation peaks (Fig. 1). With increasing N-Ac-L-tryptophanate concentration, $T_d$ increases with concomitant decrease in HHW and increase in $C_{HHW}$ and $\Delta H_d$ except at the highest level of ligand, 86 mM, where $T_d$ decreases slightly (Table 1 and Fig. 1). Furthermore, endotherms in the presence of this ligand are essentially symmetric even at the lowest ligand concentrations, which are subsaturating (13). At highest concentrations of added ligand, $\Delta H_d$ has a maximum value of ~5.1 cal/g and $T_d$ reaches a limiting value of ~90°C. The uncertainty in $\Delta H_d$ is ±2% and ±0.05°C, respectively.

The van't Hoff enthalpy of denaturation, $\Delta H_{vH}$, for individual thermograms was determined with respect to the sloping linear baseline from the slope of a plot of $\ln(\alpha(T)/[1-\alpha(T)])$ versus $1/T$ with $T$ in K where $\alpha(T)$ is the fractional extent of conversion of native to denatured protein and is determined from the thermogram by assuming that $\Delta H_d$ is temperature-independent. By assuming that the denaturing unit is albumin monomer with a molecular weight of 66,500, $\Delta H_{vH}$ is presented in Table 1 as the ratio $\Delta H_{vH}/\Delta H_d$ with an uncertainty of ±4%.

![Fig. 1. Thermograms for defatted human albumin monomer in presence of N-Ac-L-tryptophanate (N-Ac-L-Trp). Excess heat capacity in calories/(g·K) is plotted as a function of scanning temperature in °C. All measurements were made at 30 mg/ml protein in 150 mM NaCl, pH 7.0, with a scan rate of 14.7 K/h. The total concentrations of added ligand are as indicated.](image-url)
Table I

Thermodynamic data measured by DSC for the thermal denaturation of defatted human albumin monomer in the presence of N-Ac-L-tryptophanate

All measurements were made at 30 mg/ml protein in 150 mM NaCl, pH 7.0, at a scan rate of 14.7 K/h. The protein preparation used to determine the parameters in this table had been stored at +5°C for several months after defatting prior to use. For this protein in the absence of added ligand, $C_{\text{max}}^{\text{HHW}}$ was slightly smaller and HHW was slightly greater than corresponding parameters for freshly defatted monomer.

<table>
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<th>Total concentration of added ligand (mM)</th>
<th>$T_d$</th>
<th>$\Delta H_d$</th>
<th>$C_{\text{HHW}}^{\text{exo}}$</th>
<th>HHW</th>
<th>$\Delta H_{\text{HHW}}/\Delta H_d$</th>
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Fig. 2. Plots of $\ln(L)_0$ versus $1/T_d$ for DSC experiments. All measurements were made at a defatted human albumin monomer concentration of 30 mg/ml in 150 mM NaCl, pH 7.0, with a scan rate of 14.7 K/h. $T_d$ and $L_b$ values are plotted in K and mM, respectively. Equation 3 was fitted to the experimental data by nonlinear least squares to minimize the sum of the squares of the differences between experimental and calculated $L_b$ values. Line A, plot of Equation 3 with $n = 3.19$ and constant $= -1.19 \times 10^3$, which are values resulting from the best fit of experimental data in the presence of N-Ac-L-tryptophanate (O). Data for the highest ligand concentration (C) were excluded. Line B, plot of Equation 3 with $n = 8.36$ and constant $= -4.54 \times 10^3$, which are values resulting from the best fit of experimental data in the presence of caprylate (M). Data for the two highest ligand concentrations (D) were excluded.

value of ~74°C (Table I), corresponding to a maximum increase of ~9°C in $T_d$ relative to that for defatted monomer alone (Table I). A plot of $\ln(L)_0$ versus $1/T_d$ is reasonably linear as predicted by Equation 1 for the six lowest concentrations of N-Ac-L-tryptophanate (Fig. 2).

Effect of Caprylate—Thermograms for the denaturation of defatted monomer with added caprylate are presented in Fig. 3. All thermograms are composed of a single endotherm; however, at the lowest levels of added caprylate, which are subsaturating (14, 15), the peak is broadened and shortened, relative to monomer alone (Table II), and is asymmetric, skewed to the high temperature side (Fig. 3). With increasing caprylate concentration, $C_{\text{max}}^{\text{HHW}}$ and the $\Delta H_{\text{HHW}}/\Delta H_d$ ratio increase and HHW decreases. At a concentration of 5.1 or 6.5 mM caprylate, the asymmetry disappears (Fig. 3). $T_d$ reaches a maximum value of 87.0°C at a concentration of 60 mM caprylate (Table II). This is a 22.4°C increase in $T_d$ relative to that for defatted monomer alone (Table II). At concentrations >60 mM caprylate, the endotherm broadens and shortens and $\Delta H_d$ appears to decrease; at concentrations >60 mM caprylate, the $\Delta H_{\text{HHW}}/\Delta H_d$ ratio decreases (Table II); and at a level of 101 mM caprylate, $T_d$ is decreased 2.1°C from its maximum value (Table II). The decrease in $T_d$, $\Delta H_d$, and $C_{\text{max}}^{\text{HHW}}$ and increase in HHW at highest caprylate levels probably reflects an interaction of the ligand with the denatured protein. At 30 mM caprylate, HHW attains its smallest value, $C_{\text{max}}^{\text{HHW}}$ and $\Delta H_d$ are greatest, and the $\Delta H_{\text{HHW}}/\Delta H_d$ ratio is unity.
pH plotted
corresponding values for undefatted monomer (4). These differences may arise because palmitate comprises only
of the remainder of endogenous LCFA/monomer
protein denaturation may be described as a two-state process. At the highest levels of bound palmitate, the singe
 increases more rapidly than Td (Fig. 5). As a result, the endotherms coalesce at 6.0 eq of palmitate bound to
 give a single, asymmetric peak, skewed to the low temperature side (Fig. 4). The endotherm observed with 6.8 eq of
palmitate bound is similar to that observed with 6.0 eq of palmitate bound but is somewhat less asymmetric (Fig. 4).
The AHv/AHd ratio also increases from 0.72 for 6.0 eq of palmitate bound to 0.88 for 6.8 eq of palmitate bound. This
increase suggests that as the level of bound palmitate approaches full saturation the denaturation reaction approaches a two-state process. At the highest levels of bound palmitate, the single denaturation temperature approaches a limiting
value of ~79.4 °C, which is ~15 °C greater than that for defatted monomer alone (Table III), and AHd also approaches
a limiting value of 5.3 cal/g (Table III). In addition, with increasing levels of bound palmitate, the first endotherm decreases in area whereas the second endotherm, which is not present in the thermogram for the defatted monomer alone, increases (Fig. 4). At 2.6 eq of palmitate bound, the two endotherms have approximately the same amplitudes and areas.

The thermogram for defatted monomer refatted with palmitate to give 1.4 eq of palmitate/monomer (Fig. 4) is very similar to that observed for undefatted monomer with 1.4 eq of endogenous LCFA/monomer (4). At ~30 mg/ml albumin, \( \Delta H_d \) is 4.82 cal/g for the former and 5.06 cal/g for the latter; both \( T_d^{\prime} \) and \( T_d^{\prime\prime} \) for the former are 0.8 °C lower than corresponding values for undefatted monomer (4). These differences may arise because palmitate comprises only ~29% of the endogenous LCFA: oleate and linoleate constitute most of the remainder (5).

Change in Heat Capacity for Denaturation (\( \Delta C_p \))- \( \Delta H_d \) is plotted as a function of \( T_d \) in Fig. 6 for protein in the presence

### Table II

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<th>Total concentration of added ligand</th>
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<th>( \Delta H_d )</th>
<th>( C_p^{\text{ex}} )</th>
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### Table III

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<th>( T_d^{\prime\prime} )</th>
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of N-Ac-L-tryptophanate and caprylate (Tables I and II, respectively); data in the presence of palmitate (Table III) are presented only for those cases for which the single denaturation temperature is a reasonable estimate of transition temperature, i.e. where \(\alpha(T_d)\) = 0.5. Although scatter is present, principally a reflection of the uncertainty in the \(\Delta H_d\) values, all the data below 72.5°C were fitted with a straight line by linear least squares to give a slope (\(\Delta C_p\)) of 0.129 cal/(g·K), which is in the range of \(\Delta C_p\) values for denaturation reported for other proteins (16). Furthermore, this value agrees well with those estimated from individual thermograms at \(T_d\) by extrapolation of pre- and postdenaturation base lines. As a result of the fitting, \(\Delta H_d\) can be represented at lower ligand concentrations as a linear function of temperature by

\[
\Delta H_d(T) = \Delta H_d + \Delta C_p(T - T_d)
\]

where \(\Delta H_d = 5.16\) cal/g, \(T_d = 72.0\) °C, and \(\Delta C_p = 0.129\) cal/(g·K), and \(\Delta H_d\) is \(\Delta H_d\) at the arbitrary reference temperature, \(T_0\). The similar behavior of \(\Delta H_d\) as a function of \(T_d\) for these ligands in this temperature range suggests that the contribution to \(\Delta H_d\) from the dissociation of any of these ligands from native protein during denaturation is small compared to that from the denaturation of unliganded albumin. At higher concentrations of caprylate, represented by the dashed line in Fig. 6, there is substantial curvature in the plot and there is a suggestion of a similar curvature in the palmitate data. Possible explanations are a temperature dependent \(\Delta C_p\) and/or binding of ligand to denatured protein with a significant exothermic enthalpy of binding. However, the marked curvature at high concentrations of caprylate resulting in \(\Delta H_d\) being a double-valued function of \(T_d\) can not arise solely from a simple temperature dependence of \(\Delta C_p\).

**Number of Ligand Binding Sites**—If \(\Delta H_{\text{at}}\) is equated with the calorimetrically determined enthalpy of denaturation, \(\Delta H_d(T_d)\), which is expressed in Equation 2 as a linear function of temperature, Sturtevant and coworkers (10) have demonstrated that Equation 1 becomes

\[
\frac{(\Delta H_d - \Delta C_p T_d)}{R} = -\frac{\Delta C_p \ln(T_d/T_0)}{R} + n\ln(L) = \text{constant}
\]

where \(\Delta H_d\) and \(\Delta C_p\) are expressed per mol of denaturing unit, \(T_d\) and \(T_0\) are in K, and \((L)\), \(R\), and \(n\) are as in Equation 1. By assuming that monomer with a molecular weight of 66,500 is the denaturing unit, \(\Delta H_0 = 343\) kcal/mol and \(\Delta C_p = 8.56\) kcal/(mol·K). The parameters \(n\) and “constant” in Equation 3 were adjusted by a nonlinear least squares procedure to minimize the sum of the squares of the differences between corresponding experimental and computed \(\ln(L)\) values; the latter were determined by using the corresponding experimental \(T_d\) values in Equation 3.

The best fit value for the number of binding sites, \(n\), for N-Ac-L-tryptophanate is 3.19 and the resulting computed values of \(\ln(L)\) are plotted versus 1/\(T_d\) in Fig. 2, line A. Setting \((L) = (L) = (L) = (L)\) is a good approximation with N-Ac-L-tryptophanate. The best fit value of \(n\) for caprylate is 8.36; Fig. 2, line B, is a plot of the resultin \(\ln(L)\) values for caprylate calculated as a function of \(T_d\). The estimated number of binding sites for caprylate is an upper limit since at lower ligand concentrations \((L) < (L)\). The nonlinear least squares fit of Equation 3 to the data for both ligands does not significantly improve the agreement between computed and experimental \(\ln(L)\) values relative to a linear least squares fit of Equation 1 to the plot of \(\ln(L)\) versus 1/\(T_d\). However, in order to estimate the number of binding sites for each ligand, \(\Delta H_{\text{at}}\) must be equated with the calorimetric enthalpy of denaturation, \(\Delta H_d\), which shows a linear dependence on temperature; thus, Equation 3 must be used. In the presence of palmitate, neither Equation 1 nor 3 is applicable because \((L) \ll (L)\).

**DISCUSSION**

In a study of the stabilization of undefatted human albumin monomer by caprylate and N-Ac-L-tryptophanate with DSC, undefatted protein alone was observed to undergo biphasic denaturation, reflected in a thermogram comprised of two
endotherms (1). Human albumin has 7–12 binding sites for LCFA (17, 18); thus, undefatted protein, containing 1.4 eq of endogenous LCFA/monomer, is subsaturated with these ligands, for which albumin has very high affinity. The two denaturation peaks were demonstrated analytically to be associated with an uneven fatty acid distribution (1). At ~50 mg/ml undefatted monomer, the endotherm with the lower \( T_d \) corresponds to irreversible denaturation of ~70% of the total protein but this denatured albumin contains only ~20% of the total endogenous LCFA. The endotherm with the higher \( T_d \) arises from irreversible denaturation of the remaining ~30% of the protein, which has ~80% of the total LCFA bound. Thus, the observed biphasic unfolding process, which results from the denaturation of different protein molecules, LCFA-poor and LCFA-rich species, is related to the uneven fatty acid distribution. Such biphasic denaturation is in no way related to albumin substructure, i.e. to consecutive unfolding of domains within the same molecule (3). The uneven fatty acid distribution was proposed to arise during the course of the DSC experiment rather than to preexist (1) since LCFA binding data for albumin (17, 18) cannot account for such an uneven ligand distribution. An uneven fatty acid distribution can arise during denaturation if the affinity of the native protein for the LCFA is great enough and if initially the protein is subsaturated with ligand. As the more labile specialized ligand is released, the stability (transition temperature) as well as the saturation level of the remaining native protein increases due to the free energy of ligand binding becoming more negative (19, 20), which is the driving force for the biphasic denaturation observed for undefatted albumin. Thus, biphasic denaturation due to such a ligand redistribution, dictated by thermodynamics, can occur regardless of which variable (e.g. temperature, concentration of denaturant, or pH) is used to induce denaturation.

A substantial increase in stability must take place during denaturation in order for biphasic denaturation to occur. Therefore, the tendency for such a biphasic unfolding process is greatest when the ligand association constants of native protein are large, the number of binding sites is maximal, and the free ligand concentration is low, i.e. subsaturating, since under these conditions the increase in the free energy of denaturation during the course of denaturation is greatest.

Upon denaturation, defatted monomer alone shows a single, almost symmetric endotherm, indicative of monophasic denaturation, as does this protein on addition of palmitate with the presence of N-Ac-L-tryptophanate. These observations are compatible with the proposed mechanism of biphasic denaturation since for the two extreme cases, a high level of ligand and an absence of ligand, relatively little or no increase, respectively, in the free energy of denaturation of remaining native protein can take place.

The \( T_d \) data for defatted albumin monomer in the presence of N-Ac-L-tryptophanate and of caprylate are compatible with the van’t Hoff expression of Equation 3, which relates denaturation temperature to ligand concentration and permits an estimation of the number of binding sites on the protein by equating \( \Delta H_{AL} \) with the experimental enthalpy of denaturation, \( \Delta H_d(T_d) \), given by Equation 2. We find three sites for N-Ac-L-tryptophanate and eight sites as an upper limit for caprylate. McNemany (13) reports one higher affinity site and several lower affinity sites for N-Ac-L-tryptophanate at 18°C and pH 7.4. The stoichiometry of binding caprylate at 37°C and pH 7.4 is reported as 10 (14); however, after correcting the binding constants to a temperature in the denaturation region, 73°C, 3 of the 10 association constants are <200 M⁻¹. Sturtevant and coworkers (10–12) have demonstrated that both van’t Hoff expressions, Equations 1 and 3, predict the observed dependence of denaturation temperature on ligand concentration at saturating levels of ligand. In the presence of N-Ac-L-tryptophanate and caprylate these equations appear to apply in the subsaturating region as well, although more than one native albumin species undergoes denaturation under these conditions; this suggests that \( \Delta H_{AL} \) is approximately the same for the different native protein species, i.e. the contribution to \( \Delta H_{AL} \) from the dissociation of ligand upon denaturation is small compared to that from the denaturation of unliganded protein. The similar behavior of \( \Delta H_{AL} \) plotted as a function of \( T_d \) for \( T_d \) values up to ~72°C for all three ligands (Fig. 6) is in accord with this suggestion. Equations 1 and 3 are based on equilibrium thermodynamics although the overall denaturation process that they describe is irreversible. However, the thermal denaturation of albumin has been shown to be adequately described in terms of equilibrium thermodynamics (4).

After approximately correcting binding constant data to a temperature in the mid-denaturation region, 73°C, standard free energies of binding of ~12, ~44, and ~115 kcal/mol of albumin monomer are obtained for N-Ac-L-tryptophanate, caprylate, and palmitate, respectively; these correspond to average values of ~10.0, ~4.4, and ~9.6 kcal/mol of binding site, respectively. Although the average (on a per site basis) affinities of native albumin for N-Ac-L-tryptophanate and caprylate are approximately the same, the greater number of binding sites for the latter make its standard free energy of binding substantially more negative; thus, the tendency for biphasic denaturation is greater with caprylate than with N-Ac-L-tryptophanate.

Bimodality is clearly evident at the lowest levels of bound palmitate with the presence of a smaller second endotherm having a \( T_d \) greater than that of the major endotherm. With increasing level of bound ligand, both denaturation temperatures increase and the amplitude and area of the endotherm with the lower \( T_d \) decrease whereas those for the other endotherm increase. \( T_d^1 \) and \( T_d^2 \) increase such that the two denaturation peaks eventually merge. However, two discrete endotherms persist up to 5.2 eq of palmitate bound. At the highest levels of bound palmitate attainable, asymmetry in the single endotherm with a skewing to the low temperature side is obvious, the denaturation temperature and \( \Delta H_d \) approach limiting values, and the \( \Delta H_{AL}/\Delta H_d \) ratio increases toward unity.

Single endotherms are observed at all concentrations of added caprylate. However, at lowest concentrations of caprylate, which correspond to subsaturating ligand, the shortening and broadening (relative to monomer alone) and asymmetry of the endotherm are indicative of incipient bimodality. The skewing to the high temperature side suggests the presence of at least one additional smaller denaturation peak. The loss of asymmetry and narrowing of the endotherm, which reflects increasing cooperativity of the transition, at intermediate

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1 This increase in protein stability derives solely from the free energy of ligand binding and is not necessarily linked to the presence of a ligand-induced conformational change.

2 McNemany (13) presents data for the binding of N-Ac-L-tryptophanate to defatted monomer at 18°C and pH 7.4. We have fitted three binding constants to the data holding \( k_1 \) fixed at the given value and varying \( k_2 \) and \( k_3 \) subject to the constraint that \( k_3 = k_2 \). The van’t Hoff temperature dependence of \( k_3 \), \( \Delta H_{AL} = -8.5 \) kcal/mol (13), was used to correct each of the binding constants to 73°C. The 10 binding constants for caprylate at 37°C and pH 7.4 (14) were each corrected to 73°C with a \( \Delta H_{AL} \) value of ~4.6 kcal/mol, which had been determined for binding 1 eq/monomer to bovine albumin (13); the same value of \( \Delta H_{AL} \) was used to correct each of the 12 binding constants for palmitate at 37°C and pH 7.4 (18) to 73°C.
concentrations of caprylate show that the denaturation peaks have coalesced prior to an endotherm with a higher $T_d$ becoming the major peak in contrast to the situation with palmitate. With increasing ligand concentration, the $\Delta H_{Dd}/\Delta H_d$ ratio increases until at 30 mM caprylate it is unity suggesting a two-state process. At highest levels of caprylate and both $\Delta H_d$ and the $\Delta H_{Dd}/\Delta H_d$ ratio decrease from maximal values measured at 30 mM caprylate and $T_d$ decreases from its maximum at 60 mM ligand. An interaction of the ligand with denatured protein, which is weaker than that between the ligand and native protein, is probably responsible for destabilization of the protein at high concentrations of ligand.

At all levels of added N-Ac-L-tryptophanate, single, essentially symmetric endotherms are observed even though the lower levels of ligand are subsaturating. With increasing concentration of added N-Ac-L-tryptophanate, the endotherm increases in amplitude and narrows, i.e. cooperativity increases, and the $\Delta H_{Dd}/\Delta H_d$ ratio, $T_d$, and $\Delta H_d$ increase. At highest ligand concentrations $T_d$ and $\Delta H_d$ approach limiting values as the $\Delta H_{Dd}/\Delta H_d$ ratio approaches unity, although at the highest concentration of ligand $T_d$ and the ratio decrease; this suggests an interaction between N-Ac-L-tryptophanate and denatured protein similar to that with caprylate. Thus, bimodality plays no role in the thermal denaturation of albumin in the absence of exogenous ligand.

Earlier the concentration dependence of $T_d$ for defatted albumin monomer in the absence of exogenous ligand was demonstrated to be related to attendant polymerization of the protein on thermal denaturation (4). The kinetics of polymerization causes endotherm distortion, which precludes any attempt to calculate the thermodynamic parameters of the melting envelope. However, this kinetic phenomenon apparently has little effect on $T_d$ values (4). In the present study, $\Delta H_{Dd}$ values for defatted monomer in the presence of various levels of N-Ac-L-tryptophanate and caprylate have been determined from endotherm shape by assuming the simplest two-state unfolding process, i.e. no polymerization and no ligand redistribution. At lower levels of these ligands, $\Delta H_{Dd}$ is substantially lower than the corresponding experimental $\Delta H_d$ (Tables I and II). This lack of two-state character arises from the denaturation envelope at low (sub-saturating) levels of ligand being composed of contributions from more than one protein species and also may derive in part from polymerization effects on the endotherm. At higher levels of ligand, the highest levels of caprylate and 30 mM caprylate), the $\Delta H_{Dd}/\Delta H_d$ ratio is essentially unity (Tables I and II). This apparent two-state behavior probably arises both from the absence of polymerization and from the almost exclusive contribution of a single, saturated protein species to the denaturation envelope at high ligand concentrations.

The maximum increases in $T_d$ in the presence of N-Ac-L-tryptophanate, caprylate, and palmitate relative to $T_d$ for defatted monomer alone ($\Delta T_d^{max}$ values) are 9.1, 22.4, and 14.7 °C, respectively. The estimated standard free energy of binding palmitate to native protein is more negative than that for caprylate in the denaturation region yet caprylate has a substantially greater $\Delta T_d^{max}$ value. The decreased efficacy of palmitate in stabilizing albumin probably arises from an interaction of palmitate anion with denatured albumin since the ability of a ligand to thermally stabilize a protein is related to the difference in free energy of ligand binding to the native and denatured forms (19, 20); this is a consequence of Le Chatelier’s principle. An interaction of caprylate and of N-Ac-L-tryptophanate with denatured protein is suggested by the decrease in $T_d$ at highest ligand concentrations. Unfolded albumin apparently has a somewhat more negative free energy of binding palmitate than caprylate since $\Delta T_d^{max}$ with palmitate is smaller despite the fact that native albumin has a substantially more negative free energy of binding palmitate than caprylate. Deviation from linearity in a plot of $\Delta H_d$ versus $T_d$ (Fig. 6) of the palmitate data at relatively low $T_d$ values compared with the N-Ac-L-tryptophanate and caprylate data support the view that the free energy of binding palmitate to unfolded protein is more negative than that of either of the other ligands.

Any macromolecule may show biphosphatic denaturation due to ligand redistribution. Such biphasic behavior has been observed upon melting DNA in the presence of the dye acridine orange (21). The transition temperature increases with increasing dye concentration. At lower levels of added ligand, the breadth of the transition increases and one of these curves is clearly biphasic (22). At high dye concentrations, the breadth of the transition decreases. Stewart (21) ascribes the broadening to ligand release by unfolding DNA molecules accompanied by an increase in saturation of the remaining helix form with ligand.

Breslauer and coworkers (23) have studied by UV spectroscopy and DSC the thermally induced melting of poly[d(A-T)] in the presence of high affinity ligands. In the absence of ligand and at high (saturating) concentrations of ligand, the duplex melts monophonically and the increase in melting temperature correlates with the magnitude of the ligand binding constant (23). At low (sub-saturating) concentrations of ligand, the DNA melts biphasically and this biphasic character increases as the affinity of the DNA for the ligand increases (23).

McGhee (20) has carried out theoretical calculations of the helix-coil transitions of DNA in the presence of various levels of ligand. At high values of the single ligand binding constant, biphasic progress curves were computed at low (sub-saturating) levels of ligand both in the presence and absence of a cooperative interaction between bound ligands. McGhee (20) investigated the melting of poly[d(A-T)] in the presence of the antibiotic netropsin by UV spectroscopy, obtained biphasic melting curves at low concentrations of the ligand, and with a suitable choice of parameters was able to account for the experimentally observed biphasic behavior in terms of his model.

We have carried out theoretical calculations, based on thermodynamic principles, for albumin in the presence of varying amounts of each of the three ligands studied here. This computation, which will be presented elsewhere, results in calculated thermograms that emulate the behavior of the experimental thermograms. For a high affinity ligand, the contribution from each protein species to a calculated bimodal thermogram usually was decidedly non-two-state in nature. As a result, deconvolution of any similar experimental thermogram should be carried out with caution. The mechanism for biphosphatic denaturation that involves ligand redistribution is not limited to thermally induced denaturation but is operative independent of the means used to effect denaturation. Thus, this mechanism must be considered when studying macromolecular unfolding/folding reactions in the presence of any ligand whether it be a small molecule, ion, or another macromolecule. The foregoing underscore the necessity of understanding the chemistry of the denaturation process before attempting any theoretical interpretation of experimental data.

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REFERENCES