Ligand-induced Biphasic Protein Denaturation*

Andrew Shrae‡
From the Division of Blood and Blood Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

Philip D. Ross
From the Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

The results of a thermodynamic calculation of the excess heat capacity that is based on experimental observations and that incorporates the effects of ligand binding on the two-state, thermal denaturation of a protein are presented. For a protein with a single binding site on the native species and at subsaturating concentrations of ligand, bimodal or unimodal thermograms were computed merely by assuming a larger or smaller ligand association constant, respectively. The calculated thermograms for this simplified case show the salient features of those observed by differential scanning calorimetry for defatted human albumin monomer in the absence and presence of three ligands for which the protein has higher, intermediate, and lower affinity (Shrae, A., and Ross, P. D. (1988) J. Biol. Chem. 263, 15392–15399). The computation demonstrates that biphasic unfolding can result from a significant increase in the free energy of denaturation (and the transition temperature) during the course of unfolding due to a substantial increase in free ligand concentration caused by the release of bound ligand by denaturing protein.

Such ligand-induced biphasic denaturation does not relate to macromolecular substructure but derives from a perturbation, during unfolding, of the ligand binding equilibrium, which is coupled to the equilibrium between the folded and unfolded protein species. Thus, this bimodality is not limited to thermally induced unfolding but is operative independent of the means used to effect denaturation and therefore must be considered when studying any macromolecular folding/unfolding reaction in the presence of ligand.

We observed biphasic denaturation of defatted albumin monomer in an initial study of the thermally induced denaturation of human albumin by DSC (1). The defatted albumin contained subsaturating levels of endogenous LCFA (2) for which the protein has very high affinity (3, 4). Since albumin undergoes irreversible denaturation and precipitation due to aggregation, we were able to determine by chemical analysis the amount of ligand bound to the soluble and the precipitated protein at several temperatures in the denaturation region (1). At the temperature corresponding to the cusp between the two denaturation peaks, the fraction of the protein denatured was equal to the fraction of the total heat of denaturation absorbed (1). In addition, initially heating the protein to the temperature of the cusp, cooling, and then reheating this sample to a temperature above the denaturation range resulted in a thermogram exhibiting only the endotherm that is found above the cusp temperature (1). These observations rule out the possibility that a more labile domain of albumin irreversibly unfolds such that the partially unfolded species remains in solution at the cusp temperature but rather indicate that the two endotherms arise from the unfolding of different kinds of molecules. Furthermore, the endotherm with the lower denaturation temperature corresponded to denaturation of ~70% of the total protein present but this precipitated albumin contained only ~20% of the total endogenous LCFA, whereas the more stable ~30% of the protein, which gave rise to the second endotherm, contained ~80% of the total bound LCFA (1). Clearly the two denaturation peaks result from the denaturation of different types of albumin, namely protein with lower and higher levels of bound fatty acid.

The heterogeneity in the distribution of ligand among the protein molecules observed during the course of denaturation cannot be reconciled with the measured binding constants at 23 and 37 °C (3, 4). This observed uneven distribution of ligand in addition to the DSC behavior of defatted albumin in the presence of added ligands for which the protein has lower and intermediate affinity (1) led us to propose that this biphasic denaturation relates to a redistribution of ligand during unfolding as required by equilibrium binding (1).

In a more recent study, defatted human albumin monomer (5), which contained essentially no endogenous LCFA, gave a single endotherm on undergoing thermal denaturation, as anticipated. Furthermore, the addition of subsaturating concentrations of ligands for which the defatted monomer has higher, intermediate, and lower affinity resulted in bimodal, single (but asymmetric), and single (essentially symmetric) endotherms, respectively. The tendency toward bimodality was found to correlate with the magnitude of the ligand association constants and the number of ligand-binding sites on defatted albumin (5). We proposed that bimodality occurs if a substantial increase in the transition temperature (with a concomitant increase in the free energy of denaturation) of the protein takes place during denaturation due to the release of bound ligand by unfolding protein (5). Preliminary computations indicated the feasibility of using such a proposal for describing ligand-induced biphasic denaturation (6–8). In this paper, we present a rigorous thermodynamic calculation, which is conceptually simple and instructive and which simulates the most fundamental aspects of the thermal denatur-
In the absence of ligand, the simplest reaction for reversible protein denaturation involves an equilibrium between two forms, a folded or native species (N) and an unfolded or denatured species (D).

\[ N \rightleftharpoons D \]  

(1)

The equilibrium constant for this denaturation reaction may be written according to the law of mass action in terms of the concentrations of N and D or in terms of \( \alpha \), the fractional extent of denaturation, where \( \alpha = [D]/([N] + [D]) \).

\[ K_{\text{eq}} = \frac{[D]}{[N]} = \frac{1 - \alpha}{1 + \alpha} \]  

(2)

By rearranging Equation 2, \( \alpha \) may be expressed as a function of \( K_{\text{eq,0}} \)

\[ \alpha = \frac{K_{\text{eq,0}}}{K_{\text{eq,0}} + 1} \]  

(3)

\( K_{\text{eq,0}} \) may also be expressed thermodynamically as

\[ K_{\text{eq,0}} = \exp\left(\frac{-\Delta G_{\text{bound}}}{R \cdot T}\right) \]  

(4)

where \( \Delta G_{\text{bound}} \) is the free energy of denaturation in kilocalories/mole of protein. When \( \Delta G_{\text{bound}} = 0 \), \( K_{\text{eq,0}} = 1 \) (i.e. \([N] = [D])\). \( T \) is the temperature in Kelvin and \( R \) is the gas constant in kilocalories/mole/Kelvin. By assuming a temperature-independent enthalpy of denaturation (\( \Delta H_{\text{eq,0}} \)), a van’t Hoff treatment of the temperature dependence of \( K_{\text{eq,0}} \) as defined in Equation 4 gives the following expression for \( \Delta G_{\text{bound}} \)

\[ \Delta G_{\text{bound}} = \Delta H_{\text{eq,0}} \left(1 - \frac{T}{T_d}\right) \]  

(5)

where \( T_d \) is the transition temperature for denaturation, i.e. the temperature in Kelvin at which \( \Delta G_{\text{bound}} = 0 \). \( \Delta H_{\text{eq,0}} \) is expressed in kilocalories/mole of protein. The thermogram for the simple two-state denaturation process is generated from the excess heat capacity function, \( C_v(T) \), which is calculated (9) by

\[ C_v = \Delta H_{\text{eq,0}} \frac{d\alpha}{dT} \]  

(6)

In the absence of ligand, the following analytical expression for \( C_v \) is obtained from consideration of Equations 5, 4, and 6.

\[ C_v = \frac{\Delta H_{\text{eq,0}}}{R \cdot T^2} \left(\frac{K_{\text{eq,0}}}{K_{\text{eq,0}} + 1}\right)^2 \]  

(7)

In the presence of ligand, \( N' \), that is assumed to bind only to the native protein at a single site, the ligand binding equilibrium

\[ N' + L \rightleftharpoons N'\cdot L \]  

(8)

dictates the relative concentrations of ligand-free (N') and ligand-bound (N'\cdot L) native protein present. Thus, the denaturation equilibrium (Equation 1) is coupled to the ligand-binding equilibrium (Equation 8). In the presence of ligand, Equations 1–6 are correct as written.

\[ a = [D]/([N] + [D]) \]

\[ \alpha = \frac{K_{\text{bind}}}{K_{\text{bind}} + 1} \]

\[ K_{\text{eq,0}} = \exp\left(\frac{-\Delta G_{\text{bound}}}{R \cdot T}\right) \]

\[ \Delta G_{\text{bound}} = \Delta H_{\text{eq,0}} \left(1 - \frac{T}{T_d}\right) \]

\[ C_v = \Delta H_{\text{eq,0}} \frac{d\alpha}{dT} \]

\[ C_v = \frac{\Delta H_{\text{eq,0}}}{R \cdot T^2} \left(\frac{K_{\text{eq,0}}}{K_{\text{eq,0}} + 1}\right)^2 \]

\[ N' + L \rightleftharpoons N'\cdot L \]

\[ \alpha = \frac{K_{\text{bind}}}{K_{\text{bind}} + 1} \]

\[ K_{\text{eq,0}} = \exp\left(\frac{-\Delta G_{\text{bound}}}{R \cdot T}\right) \]

\[ \Delta G_{\text{bound}} = \Delta H_{\text{eq,0}} \left(1 - \frac{T}{T_d}\right) \]

\[ C_v = \Delta H_{\text{eq,0}} \frac{d\alpha}{dT} \]

\[ C_v = \frac{\Delta H_{\text{eq,0}}}{R \cdot T^2} \left(\frac{K_{\text{eq,0}}}{K_{\text{eq,0}} + 1}\right)^2 \]

In the presence of ligand, Equations 5, 4, and 3 and \( T_d \) in turn is a function of \( \alpha \), the analytical expression for \( d\alpha/dT \) becomes extremely complicated. Therefore, in the presence of ligand, we have chosen to obtain \( \alpha(T) \) by an iterative, numerical calculation, which gives a self consistent value of \( \alpha \) at each temperature. Then \( d\alpha/dT \) is determined by numerical differentiation in order to obtain \( C_v(T) \) with Equation 6. A description of the numerical computations is in the Appendix.

RESULTS AND DISCUSSION

Thermograms were calculated for a protein with a single binding site on the native species by assuming a molecular weight of 50,000 (taken as representative of a typical globular protein) and a concentration of 5 mg/ml (appropriate for a DSC experiment and corresponding to 100 \( \mu \)M total protein concentration). Thermograms were computed at various levels of added ligand, ranging in initial fractional saturation* or \( i_{\text{int}} \) (initial number of moles of ligand bound/mole of total protein) from 0.1 to 1. The temperature independent \( \Delta H_{\text{int}} \) was assigned a representative value of 6.0 cal/g of protein (12) (corresponding to 300 kcal/mol) and \( T_d \) was taken as 65.0 °C. The temperature independent binding constant for the single site on the protein was assumed to have values of \( 1.00 \times 10^4 \) M\(^{-1}\), \( 2.00 \times 10^4 \) M\(^{-1}\), and \( 1.00 \times 10^5 \) M\(^{-1}\). The resultant thermograms are presented in Fig. 1, A–C, respectively, with \( C_v \) in kilocalories/mole/Kelvin. Pre- and post-denaturation base lines are colinear since \( \Delta H_d \) was assumed to be independent of temperature. Denaturation temperature, \( T_d \), is defined as the temperature at which a local maximum in \( C_v \) occurs. \( C_v \) is the maximum amplitude of \( C_v \). \( \Delta H_d \) is the enthalpy of denaturation in kilocalories/mole determined from the area under the denaturation profile with respect to the base line, as would be obtained from a DSC experiment. For these calculated denaturation envelopes, \( \Delta H_d = \Delta H_{\text{eq,0}} \). Thus, \( \Delta H_d = 300 \) kcal/mol for each thermogram in Fig. 1. \( T_d \), \( C_v \), and \( \Delta H_d/\Delta H_{\text{eq,0}} \) values are tabulated for each binding constant as a function of \( i_{\text{int}} \) and initial free ligand concentration in Table 1. \( \Delta H_{\text{int}} \) in kilocalories/mole is computed from endotherm shape with the following expression (13) derived for simple two-state denaturation (i.e. \( T_d = \) independent of temperature) with \( T_d \) expressed in Kelvin.

\[ \Delta H_{\text{int}} = \frac{4R \cdot T^2 \cdot C_v \cdot \Delta H_{\text{eq,0}}}{\Delta H_d} \]  

(10)

The most striking result in Fig. 1 is the generation of biphasic thermograms at intermediate \( i_{\text{int}} \) values for the protein containing a higher affinity binding site (\( K_{\text{bind}} = 1.00 \times 10^6 \) M\(^{-1}\)) (Fig. 1C) in contrast to the nearly symmetric single endotherms computed at all levels of ligand with the protein containing a lower affinity site (\( K_{\text{bind}} = 1.00 \times 10^4 \) M\(^{-1}\)) (Fig. 1A). For the protein with an intermediate affinity site (\( K_{\text{bind}} = 2.00 \times 10^4 \) M\(^{-1}\)), single endotherms were calculated at all concentrations of added ligand (Fig. 1B) although the asymmetry present at lower \( i_{\text{int}} \) values is suggestive of nascent bimodality.

* Initial fractional saturation (or \( i_{\text{int}} \)) refers to values of fractional saturation (or \( \bar{i} \)) in a temperature range below that for denaturation.
behavior is reflected in the evolution of thermogram shape with increasing ligand concentration; the thermogram initially broadens and decreases in amplitude relative to ligand-free protein, becomes broadest and therefore has the smallest amplitude at $\tilde{v}_m \approx 0.5$, then sharpens (Fig. 1). The extent of broadening tends to correlate with the amount of observable bimodal character (Table I and Fig. 1); thus, essentially no broadening or bimodality is visible upon casual inspection of the thermograms for the protein having a lower affinity site (Fig. 1A).

In the presence of any ligand (i.e. high or low affinity), $T_m$ is increased relative to $T_m^0$ (Equation 9) prior to denaturation, and $\Delta G_{\text{ bound}}$ is thereby increased (Equation 5). This results in increased stabilization of the native species through the coupling of the denaturation and ligand binding equilibria (Equations 1 and 8), a manifestation of Le Chatelier’s principle. The extent of this stabilization is a function of $K_{\text{bind}}$ and free ligand concentration (Equation 9).

The occurrence of biphasic denaturation correlates with the extent of enthalpic broadening, which occurs because during the course of denaturation $T_m$ further increases relative to $T_m^0$. This additional increase in $T_m$ arises from the release of bound ligand by denaturing protein which increases $[L]$ (Equation 9) thereby further increasing $\Delta G_{\text{bound}}$ (Equation 5). The tendency toward bimodality increases as the increase in $T_m$ during unfolding becomes greater because the extent of broadening increases and because concomitantly the rate of change of $T_m$ relative to $T$ also increases.

For a single binding site and with a temperature-independent $\Delta H_m$, the maximum increase in $T_m$ during denaturation occurs at $\tilde{v}_m = 0.5$. For the higher affinity case $T_m$ increases by 2.49 °C (from 65.53 °C before denaturation to 68.02 °C after complete denaturation) and for the lower affinity case by only 0.17 °C (from 65.53 to 65.70 °C). These increases in $T_m$ during unfolding are associated with 1.0- and 1.5-fold increases in $[L]$, respectively, after complete denaturation relative to $[L]$ before denaturation (see Table I) and result in the presence (Fig. 1C) and absence (Fig. 1A) of bimodality, respectively. The greatest increase in $T_m$ during unfolding actually takes place when the ratio $[K_{\text{bind}}/[L]_m^0 + 1]/[K_{\text{bind}}/([L]_m^0 + 1)]$ is maximal where $[L]_m$ is the total ligand concentration, i.e. $[L]$ after complete denaturation, and $[L]_m$ is the value of $[L]$ prior to denaturation (see Equation 9). Thus, the increase in $T_m$ during unfolding, which correlates with the tendency for bimodality, is largest when the affinity of the native protein for ligand is maximal and when the protein is initially subsaturated. In such a situation, during denaturation the saturation level of the remaining native protein increases substantially. For the higher affinity case $\tilde{v}_m$ increases from 0.50 before denaturation to 0.90 just prior to complete denaturation but for the lower affinity case from 0.50 to only 0.60 (see Table I). Thus, the two denaturation peaks in Fig. 1C correspond to unfolding protein molecules with lower and higher levels of bound ligand.

Even for a protein with high affinity for ligand, at high levels of ligand ($\tilde{v}_m \approx 1$) $T_m$ is essentially constant throughout denaturation since the relative increase in $[L]$ during unfolding is small (Equation 9). Thus, for the two extreme situations, no ligand and the highest level of ligand considered (for each of the three values of $K_{\text{bind}}$), $T_m = T_m^0$ and $\Delta H_m = \Delta H_m^0$; hence, $\Delta H_m/\Delta H_m^0$ is equal to 1. (Table I) although the two values of $C_m^0$ are unequal (Table I). $T_m$ in the absence of ligand is less than that at the highest ligand concentration (Table I) and $\Delta H_m = \Delta H_m^0 = 300$ kcal/mol for all thermograms. Therefore, as a result of Equation 10, $C_m^0$ in the absence of ligand is greater than that at the highest ligand concentration. The simple thermodynamic calculation carried out here for the thermal denaturation of a protein with a single binding site and with different affinities for three ligands emulates the experimentally observed results for defatted human albumin monomer (5). The treatment assumes an equilibrium between the folded and unfolded forms although the thermal denaturation of albumin as measured in the reference cited is irreversible. However, the thermal denaturation of albumin has been shown to be adequately described in terms of equilibrium thermodynamics (14).

The enthalpy measured for defatted albumin monomers as a function of added N-acetyl-L-tryptophanamide (Fig. 1 and Table I of Ref. 5) behave very much like those computed for a protein with lower affinity for a ligand ($K_{\text{bind}} = 1 \times 10^4$ M$^{-1}$) (Fig. 1A and Table I). At all levels of added N-acetyl-L-tryptophanamide, single, essentially symmetric endotherms are observed even at subsaturating levels of ligand. However, with increasing N-acetyl-L-tryptophanamide concentration up to high ligand concentrations, $T_m$ and $C_m$ increase monotonically and $\Delta H_m/\Delta H_m$ first increases, then decreases, and finally increases to unity at high ligand concentration. In the absence of ligand, $\Delta H_m/\Delta H_m$ for defatted human albumin monomer is 0.7; this
Ligand-induced Biphasic Protein Denaturation

Table I
Thermodynamic data from calculated thermograms for thermally induced two-state denaturation of a protein in the presence of ligand for which the protein has a single binding site with various values for the ligand association constant

The computational procedure is described under "Materials and Methods." The input parameters are given in the legend of Fig. 1. The total protein concentration is 100 μM. For biphasic thermograms, the lower denaturation temperature is \( T_\text{d1} \) and the higher is \( T_\text{d2} \).

<table>
<thead>
<tr>
<th>( K_{\text{bind}} )</th>
<th>( T_\text{d1} )</th>
<th>( T_\text{d2} )</th>
<th>( C_{\text{m}} )</th>
<th>( \Delta H_{\text{m1}} )</th>
<th>( \Delta H_{\text{m2}} )</th>
<th>( \Delta C_{\text{p}} )</th>
<th>( \Delta H_{\text{p}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 1 \times 10^9 , \text{M}^{-1} )</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
</tr>
<tr>
<td>( 2 \times 10^9 , \text{M}^{-1} )</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
</tr>
<tr>
<td>( 1 \times 10^9 , \text{M}^{-1} )</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
<td>( 69.0 )</td>
<td>1.00</td>
<td>( 65.0 )</td>
</tr>
</tbody>
</table>

\( \Delta C_{\text{p}} \) has been shown to be compatible with a reversible polymerization of the unfolded monomer (14). Furthermore, the extent of polymerization decreases with increasing ligand concentration (14). Thus, the experimentally observed initial increase in \( \Delta H_{\text{m1}}/\Delta H_{\text{m2}} \) and lack of a decrease in \( C_{\text{m}} \) compared with the calculated behavior are due to the decrease in the extent of polymerization, which is not considered in the computation.

All thermograms for the denaturation of defatted monomer with caprylate (Fig. 3 and Table II of Ref. 5) are composed of single endotherms. However, at the lowest levels of added caprylate, which are subsaturating, the peak is broadened and shortened, relative to that for monomer alone. Moreover, it is asymmetric, skewed to the high temperature side, and \( C_{\text{m}} \) and \( \Delta H_{\text{m1}}/\Delta H_{\text{m2}} \) have minimum values here. At intermediate caprylate concentrations, the asymmetry disappears, and with increasing ligand concentration \( C_{\text{m}} \) and \( \Delta H_{\text{m1}}/\Delta H_{\text{m2}} \) increase and the endotherm sharpens. \( T_\text{d} \) increases monotonically but at highest caprylate concentrations, \( T_\text{d} \), \( C_{\text{m}} \), and \( \Delta H_{\text{m1}}/\Delta H_{\text{m2}} \) increase and the single endotherms. However, at the lowest levels of added caprylate (up to high concentrations) is very similar to that computed for a protein with intermediate affinity for a ligand (\( K_{\text{bind}} = 1 \times 10^9 \, \text{M}^{-1} \)) (Fig. 1B and Table I).

In the presence of bound palmitate, the thermograms for albumin are biphasic at the lowest levels of bound palmitate (Fig. 4 and Table III of Ref. 5). With increasing level of bound palmitate, \( T_\text{d1} \) and \( T_\text{d2} \) increase monotonically and the endotherm corresponding to \( T_\text{d} \) increases in amplitude whereas that corresponding to \( T_\text{p} \) increases in amplitude. At intermediate levels of bound palmitate, the two endotherms coalesce to form a single peak skewed to the low temperature side. As the level of bound palmitate increases further, the single endotherm becomes more symmetric and \( \Delta H_{\text{m1}}/\Delta H_{\text{p}} \) approaches unity. These observations are in accord with calculated results for a protein with higher affinity for a ligand (\( K_{\text{bind}} = 1 \times 10^9 \, \text{M}^{-1} \)) (Fig. 1C and Table I).

A consideration of the standard free energies of ligand binding for defatted monomer at a temperature in the mid-denaturation region indicates that albumin has lowest affinity for N-acetyl-l-tryptophanate, intermediate affinity for caprylate, and highest affinity for palmitate (5). Actually the binding constants for N-acetyl-l-tryptophanate and caprylate are approximately the same (5). The greater number of binding sites for caprylate makes its standard free energy of binding more negative than that for N-acetyl-l-tryptophanate. Thus, the tendency for biphasic denaturation to occur correlates both with the magnitude of the ligand binding constants and the number of binding sites on the protein. Although the calculation presented here assumes only a single ligand-binding site on the native protein, the experimental DSC behavior of albumin that has multiple binding sites for each of the three ligands studied (5) has been emulated in each set of computations by using a compensating value of \( K_{\text{bind}} \) which also compensates for the different concentrations of total protein used for the experiments (5) and computations, 30 and 5 mg/ml, respectively. However, the general accord between the experimental thermograms for albumin and those calculated for a protein with a single ligand-binding site should not be taken as an indication that the experimental binding isotherms for albumin can be adequately represented by assuming a single binding site. Furthermore, cooperative binding is not a necessary factor in ligand-induced biphasic unfolding since bimodal thermograms may be computed even with a single binding site.

With increasing levels of N-acetyl-l-tryptophanate, caprylate, and palmitate, \( T_\text{d} \) for albumin reaches a maximum value at high levels of each ligand and then decreases slightly (5). This is in contrast to the pattern shown by calculated \( T_\text{d} \) values, which increase without limit with increasing ligand concentration (Table I) because Equation 9 allows only for ligand binding to the native protein. This aspect of the experimental results reflects an interaction of the ligand with the unfolded protein, which at increasing high levels of ligand causes \( T_\text{m} \) to decrease and in turn \( T_\text{d} \) to decrease. This interaction is also responsible for the maximum \( T_\text{d} \) value for palmitate being less than that for caprylate even though the affinity of native albumin for palmitate is greater than that for caprylate. The ability of a ligand to affect \( T_\text{d} \) is related to the difference in free energy of ligand binding to the native and denatured forms. Unfolded albumin apparently has a
somewhat more negative standard free energy of binding palmitate than caprylate.

In order to explore the effect of molecular weight on the occurrence of biphasic denaturation, we have carried out computations for a smaller protein, having a molecular weight of 15,000 and a $\Delta H_m$ of 6.0 cal/g (now corresponding to 90 kcal/mol), at a concentration of 5 mg/ml assuming the same values for the single binding constant as used in the calculations above. The results (data not shown) are analogous to those obtained for the protein with a molecular weight of 50,000. At $\vartheta = 0.5$, the thermogram calculated with the largest value of $K_{bind}$ shows bimodality; however, the thermogram computed with the intermediate value of $K_{bind}$ now shows some bimodality also. and the thermogram calculated with the smallest value of $K_{bind}$ is comprised of a single essentially symmetric endotherm, which is diminished ~7% in amplitude relative to that for ligand-free protein.

The ligand-induced biphasic protein denaturation described here derives from a perturbation, during the course of thermal denaturation, of the ligand binding equilibrium, which is linked to the equilibrium between the native and denatured forms. The release of bound ligand by unfolding protein increases the free ligand concentration; thus, both $T_m$ and $\Delta C_{bind}$ of the protein increase. The greater these increases, the greater is the tendency for biphasic denaturation; therefore, any factor causing a substantial increase in free ligand concentration during denaturation increases the propensity for biphasic protein unfolding. Thus, at subsaturating concentrations of ligand, an increase in the $K_{bind}$ of native protein, in the number of binding sites on native protein, and/or in the total protein concentration will enhance the prospects for bimodal denaturation. Since biphasic denaturation is most prevalent at initially subsaturating levels of ligand, the increase in free ligand concentration during the course of thermal denaturation also causes an increase in the saturation level of remaining native protein. Therefore, the endotherm with the lower $T_d$ and that with the higher $T_a$ are associated with the unfolding of ligand-poorer and ligand-enriched protein forms, respectively. Moreover, ligand-induced biphasic denaturation is not restricted to thermally induced denaturation but operates independently of the means used to bring about denaturation. Thus, this phenomenon must be considered when studying any macromolecular unfolding reaction in the presence of ligand.

Acknowledgment—We would like to thank Dr. John S. Finlayson of the Division of Blood and Blood Products, Center for Biologies Evaluation and Research, Food and Drug Administration for his helpful comments during the preparation of the manuscript.

APPENDIX

The fractional extent of denaturation, $\vartheta$, is computed as a function of temperature in the following way. The temperature is incremented by a fixed amount (0.06°C). At each temperature, the previous final value of $\vartheta$ (from the previous temperature) is incremented by a fixed amount (0.00005); for each such increment in $\alpha$, the free ligand concentration, [L], is adjusted such that the saturation level as determined by $([L]_{tot} - [L])/(1 - \alpha \times \text{total molar protein concentration})$ equals that determined by $K_{bind} [L] / (K_{bind} [L] + 1)$ (within ±0.002%). With this value of free ligand concentration, $T_m$ is calculated from Equation 9; this in turn permits the value of $\alpha$ to be computed thermodynamically from Equations 5, 4, and 3 with $\Delta H_m$, $T_m$, and $T$; this value of $\alpha$ is then compared with that obtained from incrementing $\alpha$. The value of $\alpha$ from incrementing is increased stepwise by repeating the cycle until it just exceeds the thermodynamically calculated value; then it is decremented once to give the final value of $\alpha$ at the temperature under consideration. Such a procedure gives a self-consistent, final value of $\alpha$ within limits (±0.00005). After a self-consistent, final value of $\alpha$ is obtained, the temperature is incremented (by 0.05°C), and the entire process is repeated. Thus, one obtains $\alpha$ as a function of temperature throughout the range of denaturation. In order to obtain $C_m$, as a function of temperature, the numerical derivative of $\alpha$ is taken at each temperature by the method of Savitzky-Golay (15) for up to quartic accuracy by using five temperature points and then multiplied by $\Delta H_m$ (Equation 6).

In order to demonstrate that the increments used for temperature and for $\alpha$ were sufficiently small, an endotherm for protein denaturation in the absence of ligand was computed by the iterative, numerical procedure and also with the analytical expression (Equation 7). The envelope computed with the numerical algorithm using the increments for temperature and $\alpha$ given above had the same shape as that calculated analytically, with an acceptable scatter in the data for the former. The computer programs used were written in BASIC in double precision, compiled, and run on a desktop computer.

REFERENCES

Ligand-induced biphasic protein denaturation.
A Shrake and P D Ross


Access the most updated version of this article at http://www.jbc.org/content/265/9/5055

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/9/5055.full.html#ref-list-1