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Biophysical Chemistry 126 (2007) 43–49

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Ligand effects on protein thermodynamic stability

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Received 25 February 2006; received in revised form 15 May 2006; accepted 16 May 2006
Available online 14 June 2006

Abstract

A simple, partition-function formalism is used to describe the coupling between ligand binding and protein equilibrium unfolding. This general theoretical framework is shown to provide an adequate basis for the analysis of experimental ligand effects on the unfolding of complex protein systems. Nevertheless, the most important consequences of ligand binding for protein thermodynamic stability, as exposed by the partition-function approach, are found to be those demonstrated by Julian Sturtevant about 20 years ago.

Keywords: Protein stability; Ligand effects; Ligand binding; Partition-function formalism

1. Introduction

In principle, protein–ligand interactions could be easily detected on the basis of the effect of the low-molecular-weight substance on the thermal unfolding of the protein, since the denaturation temperature (here defined as the temperature at which 50% of the protein remains in the native state) is expected to change upon ligand binding. Naïve intuition would actually suggest that the denaturation temperature will increase or decrease with increasing ligand concentration, depending on the type of conformational change (stabilizing or destabilizing) induced in the protein by the binding of the ligand. Also, the denaturation temperature will not change further once the binding sites are essentially fully saturated with ligand. Consequently, a plot of denaturation temperature versus ligand concentration will show a plateau at saturating ligand concentrations and will have a shape that roughly reflects that of the ligand binding curve (degree of saturation versus ligand concentration).

The statements in the three last sentences above would certainly seem plausible. *They are, however, wrong*. Provided that the ligand binds to the native protein, but not to the unfolded protein (for a two-state equilibrium unfolding process), the denaturation temperature will always increase with ligand concentration, regardless of the kind of conformational change induced by the binding. Furthermore, no plateau will be seen in a plot of denaturation temperature versus ligand concentration; rather, the denaturation temperature will keep increasing with ligand concentration, even after all binding sites are saturated. Julian Sturtevant demonstrated these essential features about 20 years ago [1,2] and, in doing so, he established the basis for the rigorous analysis of ligand effects on protein thermodynamic stability.

Here, I will first expound Sturtevant's findings using a simple two-state model. Then, I will describe a general, partition-function formalism, which provides an adequate basis for the analysis of experimental ligand effects on the thermodynamic stability of complex protein systems. We will see, nevertheless, that the main results of this general analysis simply recapitulate Sturtevant's insight from 20 years ago.

2. A two-state model for ligand effects on protein stability

Assume that the thermal unfolding of a given protein can be adequately described by the two-state equilibrium model:

$$N \leftrightarrow U \tag{1}$$

where N is the native state and U is the unfolded state (actually an ensemble of more or less unfolded conformations) and K_0 is the unfolding equilibrium constant,

$$K_0 = \frac{[\mathrm{U}]}{[\mathrm{N}]} \tag{2}$$

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† Dedicated to the memory of Julian Sturtevant.

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doi:10.1016/j.bpc.2006.05.021

which changes with temperature according to the van't Hoff equation:

$$\frac{\partial \ln K_0}{\partial T} = \frac{\Delta H_N}{RT^2} \tag{3}$$

where ΔH_N is the unfolding enthalpy change.

Assume now that the native protein can bind stoichiometrically a certain ligand (L),

$$N + L \leftrightarrow NL \tag{4}$$

and that the binding is described in terms of a binding constant,

$$\beta = \frac{[NL]}{[N][L]} \tag{5}$$

which again changes with temperature according to the van't Hoff equation; i.e., according to Eq. (3) but with the ligand binding enthalpy (ΔH_L) in the right-hand-side instead of the unfolding enthalpy (ΔH_N), that is, $\partial \ln\beta/\partial T = \Delta H_L/RT^2$.

Finally, we can also pose an equilibrium constant for the unfolding of the ligand−bound native protein (unfolding coupled to ligand dissociation):

$$NL \leftrightarrow U + L \tag{6}$$

$$K = \frac{[U][L]}{[NL]} \tag{7}$$

with K changing with temperature according to the van't Hoff equation and the corresponding enthalpy change (ΔH_{NL}).

Of course, Eqs. (1), (4) and (6) do not describe independent equilibria. Rather, the corresponding equilibrium constants and enthalpy changes are related through:

$$K = \frac{K_0}{\beta} \tag{8}$$

$$\Delta H_{NL} = \Delta H_N - \Delta H_L \tag{9}$$

We now define the denaturation temperature ($T_{1/2}$) as that temperature at which half of the protein is in the unfolded state. Therefore, for $T_{1/2}$, the following concentration ratio must equal unity.

$$\rho = \frac{[U]}{[N] + [NL]} \tag{10}$$

Using Eqs. (2) and (5), (10) can be written as,

$$\rho = K_0 \cdot (1 - X_L) \tag{11}$$

where X_L is the fraction of native protein with bound ligand:

$$X_L = \frac{[NL]}{[N] + [NL]} = \frac{\beta[L]}{1 + \beta[L]} \tag{12}$$

The value of $T_{1/2}$ will of course depend on ligand concentration, but for all {[L], $T_{1/2}$} couples, the value of ρ is unity. In other words, in a plot of temperature versus ligand concentration (or versus logarithm of ligand concentration), the $T_{1/2}$ values define a line of constant ρ. The slope of this line can be computed on the basis of the well-known rules of partial differentiation:

$$\frac{dT_{1/2}}{d\ln[L]} = -\frac{(\partial \ln\rho/\partial \ln[L])_T}{(\partial \ln\rho/\partial T)_{[L]}} \tag{13}$$

The two derivatives on the right-hand-side of Eq. (13) can be evaluated from the equations above by straightforward (although, somewhat tedious) differentiation. The final result for the slope is:

$$\frac{dT_{1/2}}{d\ln[L]} = \frac{RT_{1/2}^2 X_L}{\Delta H_N - X_L \Delta H_L} \tag{14}$$

The denominator on the right-hand-side is bounded between ΔH_N (when $X_L = 0$) and ΔH_{NL} (when $X_L = 1$; see Eq. (9)). Both ΔH_N and ΔH_{NL} are unfolding enthalpies (for the N and NL species, respectively) and have positive values for heat denaturation (induced by temperature increase). Therefore, the denominator is a positive number and so is the numerator ($RT_{1/2}^2 X_L$). It follows that the slope $dT_{1/2}/d\ln[L]$ is necessarily positive. In other words, *the denaturation temperature for heat*

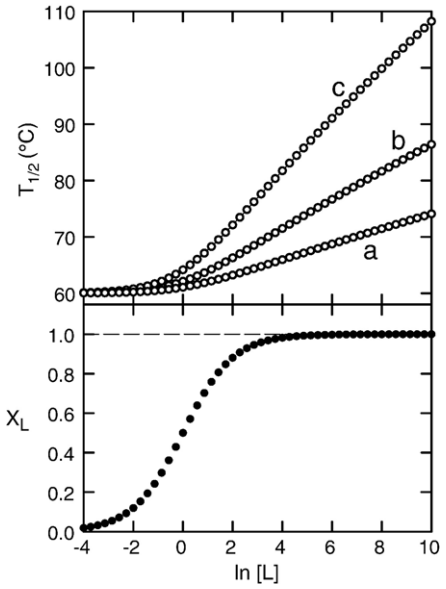

Fig. 1. Upper panel: profiles of denaturation temperature versus logarithm of ligand concentration calculated on the basis of an equilibrium, two-state unfolding model that includes ligand binding to a site in the native state (Eqs. (1) and (4) in the text). For the three profiles shown the denaturation temperature in the absence of ligand is 60 °C and the ligand binding equilibrium constant is taken as unity (in arbitrary units) at all temperatures (i.e., a zero ligand binding enthalpy has been used in the calculation). The profiles differ in the values used for the unfolding enthalpy (a: 600 kJ/mol, b: 300 kJ/mol, c: 150 kJ/mol) and the unfolding heat capacity change (a: 12 $kJ \cdot K^{-1} \cdot mol^{-1}$, b: 6 $kJ \cdot K^{-1} \cdot mol^{-1}$, c: 3 $kJ \cdot K^{-1} \cdot mol^{-1}$). Lower panel: profile of fraction of native protein with bound ligand versus logarithm of ligand concentration corresponding to the denaturation temperature profiles shown in the upper panel. Note that the denaturation temperature increases with ligand concentration, even when the native protein is essentially fully saturated with ligand.

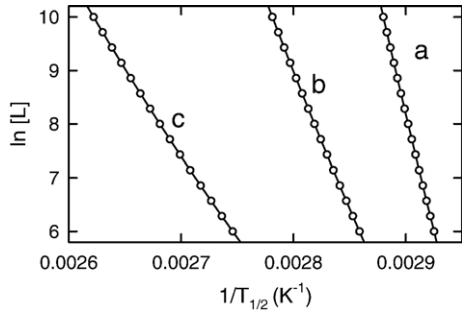

Fig. 2. Sturtevant plots of logarithm of ligand concentration versus the inverse of the absolute denaturation temperature in Kelvin. The data shown correspond to the high ligand concentration range of the theoretical profiles in the upper panel of Fig. 1. The slope of the ln[L] versus $1/T_{1/2}$ plots is related to the unfolding enthalpy through Eq. (16). The plots displayed here show a slight curvature, since a plausible temperature-dependence of the unfolding enthalpies was included in the theoretical simulations. Noise in the data values often prevents the observation of such slight curvatures in Sturtevant plots constructed from actual experimental data.

denaturation always increases with ligand concentration, regardless of the structural consequences (type of conformational change) induced by the binding of the ligand (see Fig. 1 for some illustrative examples). This important point was already noted by Julian Sturtevant about 20 years ago [1,2]. He, of course, did realize that the only way in which a ligand could decrease the denaturation temperature value for a two-state equilibrium unfolding is that it binds to both N and U, being the binding to the unfolded state stronger. If, as depicted in Eqs. (4) and (6), the ligand does not bind to U (that is, the binding site in the native structure is eliminated upon unfolding), $T_{1/2}$ for heat denaturation is bound to increase with ligand concentration. Furthermore, *$T_{1/2}$ will keep increasing with ligand concentration even at ligand concentrations at which the native protein is essentially saturated with ligand* (see Fig. 1 for illustrative examples). This can be clearly seen by substituting $X_L=1$ into Eq. (14) and using Eq. (9). The result is

$$\frac{dT_{1/2}}{d\ln[L]}=\frac{RT_{1/2}^2}{\Delta H_{NL}} \tag{15}$$

and shows that the slope is different from zero even at full saturation of the native protein by the ligand. Julian Sturtevant had the insight to realize that this, apparently counter-intuitive result is actually to be expected from the following facts: (i) under full saturation conditions, unfolding is described by NL↔U+L (Eq. (6)); (ii) within the unfolding transition region, there are significant amounts of both NL and U; (iii) increasing ligand concentration will shift the equilibrium towards NL; (iv) therefore, the larger the ligand concentration, the higher the temperature required to have half of the protein in the unfolded state (i.e., [NL]=[U]). He also showed that the ligand-concentration dependence of the denaturation temperature at saturation ($X_L\cong 1$) can be linearized. Thus, Eq. (15) can be easily written as,

$$\frac{d\ln[L]}{d(1/T_{1/2})}=-\frac{\Delta H_{NL}}{R} \tag{16}$$

and provided that ΔH_{NL} is reasonably constant within the temperature range of interest, a plot of ln[L] versus $1/T_{1/2}$ will be a straight line with an slope equal to $-\Delta H_{NL}/R$. Effects of ligands on the stability of proteins are often described on the basis of these Sturtevant plots (see Fig. 2 for some illustrative examples).

3. A general, partition-function formalism for ligand effects on protein stability

The above two-state analysis can be easily generalized to more complex situations on the basis of a partition-function formalism. Statistical–mechanical analysis of conformational transitions in proteins has a long tradition (see, for instance, Refs. [3,4]). Here, I will use the binding-polynomial approach as described by Wyman and Gill [5]. See Ref. [6] for a different analysis of ligand effects on protein unfolding, which focuses on the characterization of tight binding.

Assume that a given protein state, S (native, unfolded, partially unfolded, etc.), can bind a certain number, n, of ligand molecules. Therefore, the protein state S can exist as $n+1$ different ligation species (with 0, 1, 2, 3, …, n, bound molecules of ligand: S_0, SL, SL_2, SL_3, …, SL_n). The formation of each of these ligation species from the unligated one can be described in terms of the corresponding overall, Adair equilibrium constant:

$$S_0 + iL \leftrightarrow SL_i \tag{17}$$

$$\beta_i=\frac{[SL_i]}{[S_0][L]^i} \tag{18}$$

where, for $i=0$, we obviously have $\beta_0=1$.

We now take the unligated species as reference and assign a statistical weight (defined as a concentration ratio) to each of the ligation species:

$$w_i=\frac{[SL_i]}{[S_0]}=\beta_i[L]^i \tag{19}$$

where we have used Eq. (18). The relevant partition function for this case can be constructed as the sum of all statistical weights:

$$P=w_0+w_1+w_2+\ldots+w_n=\sum_{i=0}^{n} w_i=\sum_{i=0}^{n}\beta_i[L]^i$$
$$=1+\sum_{i-1}^{n}\beta_i[L]^i \tag{20}$$

This type of partition function is meant to describe ligand binding to a given protein state and, in the parlance of the field, is known as a *binding polynomial* [5].

The general idea behind the approach embodied in Eqs. (19) and (20) is that the fraction of protein state S, which occurs as a given ligation species is proportional to the corresponding

statistical weight and the normalizing factor is the binding polynomial (the sum of all weights):

$$f_i = \frac{[SL_i]}{[S_0] + [SL] + [SL_2] + \ldots + [SL_n]} = \frac{w_i}{w_0 + w_1 + w_2 + \ldots + w_n} = \frac{w_i}{P} = \frac{\beta_i [L]^i}{P} \quad (21)$$

and, since $w_0 = 1$, we have that the fraction of state S which is unligated is given by the important relation,

$$f_0 = 1/P \quad (22)$$

It is now straightforward to write expressions for any quantity that can be expressed as an average over the different ligation species. Thus, if the value of a given property (X) is X_0 for the species S with no ligand bound, X_1 for the species with 1 ligand bound, etc., its average value is:

$$X = X_0 f_0 + X_1 f_1 + X_2 f_2 + \ldots + X_n f_n = \sum_{i=0}^{n} X_i f_i = \frac{\sum_{i=0}^{n} X_i w_i}{P} = \frac{\sum_{i=0}^{n} X_i \beta_i [L]^i}{P} \quad (23)$$

where we have used Eq. (21). Actually, Eq. (23) can be used to compute the average number of molecules of ligand bound to protein state S by simply substituting $X_i = i$. The result is:

$$\langle i \rangle = \frac{\sum_{i=0}^{n} i \beta_i [L]^i}{P} \quad (24)$$

and the reader may easily check that the right-hand-side in Eq. (24) is also obtained by differentiating the logarithm of the binding polynomial with respect to the logarithm of ligand concentration. Therefore,

$$\langle i \rangle = \left(\frac{\partial \ln P}{\partial \ln [L]} \right)_T \quad (25)$$

and the binding curve ($\langle i \rangle$ versus ligand concentration) is given by a derivative of the binding polynomial.

Expressions for the average enthalpy of the protein state S with respect to the unligated species can also be found along the same lines:

$$\langle \delta H \rangle = \sum_{i=0}^{n} \delta H_i f_i = \frac{\sum_{i=0}^{n} \delta H_i \beta_i [L]^i}{P} \quad (26)$$

where δH_i is the enthalpy change associated to the formation of species SL_i from the unligated species (i.e., the process shown in Eq. (17)). Note that the average enthalpy $\langle \delta H \rangle$ can be regarded as the enthalpy change associated to the process in which the unligated species binds $\langle i \rangle$ ligands to yield the equilibrium mixture of species at the ligand concentration corresponding to the $\langle i \rangle$ value. The reader may easily verify (by differentiation and use of the van't Hoff equation: $\partial \ln \beta_i / \partial T = \delta H_i / RT^2$) that the average binding enthalpy can also be expressed as a derivative of the binding polynomial:

$$\langle \delta H \rangle = RT^2 \left(\frac{\partial \ln P}{\partial T} \right)_{[L]} \quad (27)$$

One of the advantages of using binding polynomials to describe ligand binding is that simple and convenient expressions for most binding models can be easily constructed using rules that have been described in detail in the literature [5]. For instance, for binding to n identical and independent sites, Eq. (20) can be written as,

$$P = (1 + \kappa [L])^n \quad (28)$$

where κ is the microscopic constant for the binding to a given site. Also, if the sites are identical but not independent and the possible cooperativity in ligand binding can be phenomenologically described in terms of a Hill coefficient (α), the binding polynomial is written as [7]:

$$P = \{1 + (\kappa [L])^{\alpha}\}^{n/\alpha} \quad (29)$$

Many useful examples of the construction of binding polynomials for different binding models can be found in the classic book by Wyman and Gill [5]. It is to be noted, nevertheless, that Eqs. (22), (25) and (27) are general (valid for all binding models) and as such they will be used below.

Assume now a temperature-induced, equilibrium transition between two states of a protein (A and B),

$$A \leftrightarrow B \quad (30)$$

with both states being, in principle, able to bind the ligand. We take B as the higher enthalpy state and, therefore, the conversion from A to B occurs with a positive enthalpy change upon temperature increase. When $[L] = 0$, only the unligated species of A and B (A_0 and B_0) will be populated and the ratio of their concentrations defines an equilibrium constant for the A↔B conversion in the absence of ligand:

$$K_0 = \frac{[B_0]}{[A_0]} \quad (31)$$

In the presence of ligand, there can be ligated species in addition to the unligated ones. We thus use a ratio between the total concentrations of A and B (including, in each case, all the species with different numbers of bound ligands):

$$\rho = \frac{[B]_T}{[A]_T} \quad (32)$$

which plays the role of an apparent (ligand-concentration dependent) equilibrium constant for the A↔B conversion in the presence of ligand. A relationship between K_0 and ρ can be easily found by taking into account that $[B_0]/[B]_T = f_0^B = 1/P^B$ and $[A_0]/[A]_T = f_0^A = 1/P^A$ (see Eq. (22)). The result is,

$$\rho = K_0 \frac{P^B}{P^A} \quad (33)$$

with P^B and P^A being the binding polynomials for states B and A, respectively.

We define the transition temperature ($T_{1/2}$) for the A↔B process as the temperature at which the total concentrations of both states are equal and, consequently, ρ (Eq. (32)) equals unity. Then, the effect of ligand concentration on $T_{1/2}$ is still given by Eq. (13). The two derivatives in the right-hand-side are easily evaluated from Eq. (33), using Eqs. (25) and (27):

$$\left(\frac{\partial \ln\rho}{\partial \ln[L]}\right) = \left(\frac{\partial \ln P^B}{\partial \ln[L]}\right) - \left(\frac{\partial P^A}{\partial \ln[L]}\right) = i^B - i^A = -\Delta i \qquad (34)$$

$$\left(\frac{\partial \ln\rho}{\partial T}\right)_{[L]} = \left(\frac{\partial (\ln K_0 + \ln P^B - \ln P^A)}{\partial T}\right)_{[L]} = \frac{\Delta H_0 + \langle \delta H\rangle^B - \langle \delta H\rangle^A}{RT_{1/2}^2} = \frac{\Delta H}{RT_{1/2}^2} \qquad (35)$$

where we have used superscripts to designate values corresponding to states A and B. In Eq. (34), $\Delta i = \langle i\rangle^A - \langle i\rangle^B$ is the number of ligand molecules released upon the conversion from A to B at a given ligand concentration. In Eq. (35), $\Delta H = \Delta H_0 + \langle \delta H\rangle^B - \langle \delta H\rangle^A$ is the enthalpy change associated to the A to B conversion at a given ligand concentration (note that ΔH_0 is the enthalpy change associated to the conversion process involving unligated species, and $\langle \delta H\rangle^B - \langle \delta H\rangle^A$ takes into account the contribution of ligand binding/release effects to the enthalpy change). Substitution of Eqs. (34) and (35) into Eq. (13) leads to,

$$\frac{dT_{1/2}}{d\ln[L]} = \frac{RT_{1/2}^2 \Delta i}{\Delta H} \qquad (36)$$

which indicates that, qualitatively, the ligand effect on the transition temperature is determined by the sign of Δi. This, if Δi is positive (ligand molecules are released upon the temperature-induced, A to B transition), $T_{1/2}$ will increase with ligand concentration. Conversely, when Δi is negative (there is net uptake of ligand molecules upon the A to B transition), $T_{1/2}$ will decrease with increasing ligand concentration. Clearly, if $\Delta i = 0$, $dT_{1/2}/d\ln[L] = 0$ and the transition temperature is not affected by the ligand. For the simple two-state model, we have analyzed in Section 2 (Eqs. (1), (4) and (6)) that Δi is necessarily positive, since the ligand only binds to the native state and, consequently, $T_{1/2}$ increases with ligand concentration. Actually, the reader may easily verify that Eq. (14) is a particular case of Eq. (36).

Eq. (36) allows us to calculate Δi from experimental $T_{1/2}$ and ΔH values at different ligand concentrations. The simplest scenario would be that the conversion from A to B is observed as a distinct, two-state, equilibrium transition in experimental differential scanning calorimetry (DSC) thermograms (i.e. the low- and high-temperature heat capacity levels of the calorimetric transition correspond to states A and B, respectively); in this case, the required $T_{1/2}$ values could be derived from DSC profiles obtained at different ligand concentrations by standard two-state analysis (ΔH values are simply related to the area under the calorimetric transition after suitable chemical baseline tracing) . It is important to note, however, that the $T_{1/2}$ values can be derived from experimental DSC data without assuming any specific denaturation model; thus, Freire-Biltonen partition functions taking the A and B states as reference could be calculated from [8],

$$\ln Q_B = \int_{T_A}^{T} \frac{\langle \Delta H\rangle_A}{RT^2} dT \qquad (37)$$

$$\ln Q_B = \int_{T_B}^{T} \frac{\langle \Delta H\rangle_B}{RT^2} dT \qquad (38)$$

where $\langle \Delta H\rangle_A$ and $\langle \Delta H\rangle_B$ are the excess enthalpies calculated taking the low-temperature and high-temperature baselines as reference, respectively, and T_A (T_B) is a temperature low (high) enough so that essentially all the protein is in state A (B). These partition functions are related to the concentrations of the reference states through $Q_A = [P]/[A]_T$ and $Q_B = [P]/[B]_T$ [8], where [P] is the total protein concentration (including states A, B and any "intermediate" state significantly populated during the A to B conversion). Once the temperature dependencies of Q_A and Q_B have been obtained, the transition temperature is calculated as the temperature at which $Q_A = Q_B$ (and, therefore, $[A]_T = [B]_T$ and $\rho = 1$). This involved procedure, however, is not required when the transition is symmetric, since, in this case, the Q_A versus T and Q_B versus T profiles will cross at the midpoint of the transition, which will coincide to a good degree of approximation with the temperature corresponding to the maximum of the calorimetric transition. To summarize, provided that an equilibrium, calorimetric transition corresponding to A to B conversion is identified in DSC thermograms, the data required for the application of Eq. (36) can be easily obtained, even if the calorimetric transition involves significantly populated intermediate states [7]. An experimental example of the application of Eq. (36) is given below.

4. An illustrative example: L-phenylalanine binding to human phenylalanine hydroxylase

Mammalian phenylalanine hydroxylase (PAH) catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine, which is the initial and rate-limiting step in the catabolic pathway of L-Phe. PAH and its interaction with L-Phe are of considerable interest, since failure in the hydroxylation of L-Phe caused by mutations in the human PAH gene causes a serious disease: phenylketoenuria. PAH is tetrameric enzyme of 451-residue subunits with a three-domain structure. Each subunit contains a N-terminal regulatory domain, a catalytic domain (containing the active site) and an oligomerization domain, with dimerization and tetramerization motifs [9]. Relevant issues regarding the binding of L-Phe to PAH are whether L-Phe binds to the regulatory domains (in addition to the expected binding to the catalytic domains) and whether

there is communication between the regulatory and catalytic domains.

The above issues were recently addressed on the basis of the effect of L-Phe on the thermal denaturation of PAH as monitored by DSC [7]. Two partially overlapping transitions were observed in DSC thermograms for wild-type human PAH. On the basis of structure-energetics calculations and the comparison with the thermograms for truncated forms of the enzyme, the low-temperature transition could be assigned to the denaturation of the four regulatory domains and the high-temperature transition to the denaturation of two of the catalytic domains. Further denaturation processes at higher temperature could not be interpreted in structural terms, due to the occurrence of irreversible denaturation (aggregation). Although none of the two structurally defined transitions was found to conform to the two-state model, meaningful transition temperatures could obtained along the lines explained in the last paragraph of the preceding section. Plots of transition temperature versus L-Phe concentration are shown in Fig. 3. In both cases (catalytic and regulatory domains), the transition temperature increases with L-Phe concentration at low ligand concentrations. For the higher ligand concentrations, on the other hand, quite different behaviours are observed: the transition temperature for catalytic domain denaturation, increases with ligand concentration and shows no sign of reaching a plateau, while a plateau at high [L-Phe] is clearly observed for the transition temperature values corresponding to regulatory domain denaturation. Application of Eq. (36) to the high [L-Phe] data yields $\Delta i = 1.8 \pm 0.3$ for catalytic domains denaturation and $\Delta i = -0.2 \pm 0.2$ for regulatory domain denaturation. The Δi value (about 2) for catalytic domains simply reflects that, (i) at high L-Phe concentrations, the binding sites in the two domains involved in the calorimetric transition are saturated with ligand; (ii) the binding sites are eliminated upon catalytic domain denaturation; (iii) consequently, two L-Phe molecules are released upon the temperature-induced transition. The Δi value (about zero) for regulatory domain denaturation indicates no net release/uptake of L-Phe at the concentrations at which the binding sites in the catalytic domains are saturated. We must conclude then that binding sites for L-Phe are not eliminated upon regulatory domain denaturation. That is, the analysis of the effect of L-Phe on the thermal denaturation of PAH suggests that, contrary to common belief, there are no binding sites for L-Phe in the regulatory domains of PAH [7].

Once the absence of binding sites in the regulatory domains is accepted, it is clear that the dependence of their transition temperature seen at the lower L-Phe concentrations (Fig. 3) must be ascribed to the interaction between catalytic and regulatory domains. That is, denaturation of the regulatory domains affects the catalytic domains and, as a result, it changes to some extent the affinity of the latter for L-Phe; consequently, at low L-Phe concentration (when the binding sites in the catalytic domains are not fully saturated) some release of L-Phe from the catalytic domains takes place upon regulatory domain denaturation, giving rise to a [L-Phe] dependence of the transition temperature for the regulatory domains (as indicated by Eq. (36)).

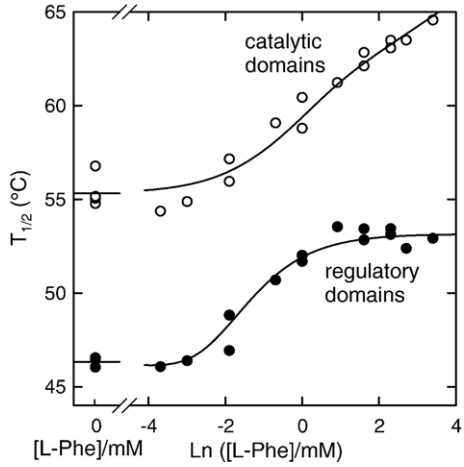

Fig. 3. Plots of experimental transition temperature for the catalytic and regulatory domains of human phenylalanine hydroxylase versus the logarithm of L-phenylalanine concentration. Data derived from experimental DSC profiles and taken from Ref. [7].

5. Concluding remark

The reader may be impressed by the capability of the partition-function-based analysis (embodied in Eq. (36)) to extract relevant information on the interaction of ligands with complex proteins from thermal denaturation studies, as illustrated by the analysis of the binding of L-Phe to PAH described above. However, the general principles involved are actually those uncovered by Julian Sturtevant about 20 years ago. He did realize that ligand effects on equilibrium denaturation temperatures simply reflect the displacement of the folding/unfolding equilibrium by the added ligand and that such displacement is related to the release/uptake of the ligand upon the temperature-induced transition. These are, in fact, the general ideas we have used in our extremely informative analysis (Section 4) of the experimental data (Fig. 3) on L-phenylalanine binding to human phenylalanine hydroxylase.

Acknowledgment

Work in the author's lab was supported by grant BIO2003-02229 from the Spanish Ministry of Education and Science, FEDER funds and grant CVI-771 from the "Junta de Andalucia".

References

[1] H. Fukada, J.M. Sturtevant, F.A. Quiocho, Thermodynamics of the binding of L-arabinose and of D-galactose to the L-arabinose-binding protein of *Escherichia coli*, J. Biol. Chem. 258 (1983) 13193–13198.
[2] S.P. Manly, K.S. Mathews, J.S. Sturtevant, Thermal denaturation of the core protein of the *lac* repressor, Biochemistry 24 (1985) 3842–3846.
[3] J.A. Schellman, The factors affecting the stability of hydrogen-bonded polypeptide structures in solution, J. Phys. Chem. 62 (1958) 1485–1494.

[4] L. Peller, On a model for the helix-random coil transition in polypeptides: I. The model and its thermal behaviour, J. Phys. Chem. 63 (1959) 1194–1206.
[5] J. Wyman, S.J. Gill, Binding and Linkage. The Functional Chemistry of Biological Macromolecules, University Science Books, Mill Valley, California, 1990.
[6] J.F. Brandts, L.-N. Lin, Study of strong to ultratight protein interactions using differential scanning calorimetry, Biochemistry 29 (1990) 6927–6940.
[7] M. Thórólfsson, B. Ibarra-Molero, P. Fojan, S.B. Petersen, J.M. Sanchez-Ruiz, A. Martinez, L-Phenylalanine binding and domain organization in human phenylalanine hydroxylase: a differential scanning calorimetry study, Biochemistry 41 (2002) 7573–7585.
[8] E. Freire, R.L. Biltonen, Statistical mechanical deconvolution of thermal transitions in macromolecules: I. Theory and applications to homogeneous systems, Biopolymers 17 (1978) 463–479.
[9] F. Fusetti, H. Erlandsen, T. Flatmark, R.C. Stevens, Structure of tetrameric human phenylalanine hydroxylase and its implications for phenylketonuria, J. Biol. Chem. 273 (1998) 16962–16967.