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Thermodynamic approach to cold denaturation of proteins [1]

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Abstract

Treatment of protein unfolding in aqueous solutions as a thermodynamic equilibrium involving water molecules provides connections between water activity and low and high temperature unfolding points, here indicated as T_L and T_H.

Given the dome shape of the Gibbs energy difference $\Delta^D_N G$ between native and denatured protein in the (T_L, T_H) range, the corresponding trends of entropy $\Delta^D_N S$ and heat capacity $\Delta^D_N C_p$ variations are consequently drawn; the latter remains positive throughout this range and shows an upward parabolic behaviour with a minimum at some intermediate temperature T_m where the entropy change shows a flexus and becomes zero. The former is negative for $T < T_m$ and becomes positive for $T > T_m$.

Simple expressions are reported for routine estimations of T_L, T_m and the native versus denatured stability, with comparison between calculated and experimental data.

INTRODUCTION

Several authors [1–4] have proposed a two-state equilibrium model for thermodynamic description of the native (N) ⇆ denatured (D) unfolding transition of proteins: any aggregation process is not included since the model refers to diluted aqueous solutions. Thermodynamic parameters $\Delta^D_N H$, $\Delta^D_N C_p$ and $\Delta^D_N S$ experimentally determined at the unfolding point above room temperature T_H are used to extrapolate the $\Delta^D_N G$ trend to much lower temperatures. Because of the formal nature of the mathematical function $\Delta^D_N G(T)$, a further N ⇆ D equilibrium, usually named "cold denaturation", is predicted. The corresponding temperature is here reported as T_L.

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The model does not explicitly involve any thermodynamic and physical constraints. Even so, it shows that ND enthalpy and entropy changes at T_L are negative, whereas those at T_H are positive, though their absolute values are different [3] (i.e. the two processes are not mirror images). This is indeed expected and experimentally proved, as the prevailing forces at T_L are not the same as at T_H.

What is more, the model applies even in various pH and ionic strength conditions, provided that every thermodynamic quantity considered can be treated as a function of these parameters [5]. Experimental difficulties have so far limited the number of proteins for which low T unfolding is demonstrated, since cold unfolding usually occurs only a few degrees above the freezing point of the solution [4]. When low T unfolding has been observed, it has been found that there is good agreement between observed and predicted T_L values.

Although the surprising efficacy of the model has been qualitatively attributed to enthalpy- and entropy-driven processes involving solvation water [3,4], the solvent is by no means included in the stoichiometry of the ND equilibrium. Cold and heat denaturations are therefore implicitly supposed to occur with identical stoichiometry. In other words, if a chemically more reliable picture has to be considered, for example

$$N \cdot nH_2O \leftrightarrows D \cdot dH_2O + (n-d)H_2O \tag{1}$$

the number of interacting water molecules per protein molecule (n and d) would be the same at T_H and T_L, as well as the number and the kind of all bonds broken and formed across the ND transition. This does not coincide with the expected modifications of the structure, e.g. those due to the increased strength of polar interactions and weakening of hydrophobic forces [3,4], and consequent change of the number of solvation water molecules per protein molecule when T decreases. The physical reliability of the model has therefore to be formally justified.

THERMODYNAMIC APPROACH

A straightforward application of the classic procedures to the chemical equilibrium (1) starts from the definition of $\Delta^D_N G$, namely

$$\Delta^D_N G = \mu_D - \mu_N + (n-d)\mu(H_2O) \tag{2}$$

At T_H and T_L this reduces to

$$\mu_D - \mu_N = (d-n)\mu(H_2O)$$

or, in a simpler shorthand notation

$$\Delta\mu = \delta\mu_w \tag{3}$$

where the subscript w stands for water and $\delta = (d-n)$.

It must be pointed out that this equality does not imply that $\Delta\mu$, δ and, of course, μ_w have the same value at T_L as at T_H. The temperature dependence of μ_w can be expressed as

$$\mu_w(T) = \mu_w(T_H) - \int_{T_H}^{T} S_w \, dT$$

$$= \left[\frac{\Delta\mu}{\delta}\right]_{T=T_H} - \int_{T_H}^{T} S_w \, dT$$

$$= \left[\frac{\Delta\mu}{\delta}\right]_{T=T_L} - \int_{T_L}^{T} S_w \, dT$$

where the equilibrium conditions at T_H and T_L have been employed. These expressions lead to

$$\left[\frac{\Delta\mu}{\delta}\right]_{T=T_H} - \left[\frac{\Delta\mu}{\delta}\right]_{T=T_L} = \int_{T_H}^{T_L} S_w \, dT = \mu_w(T_H) - \mu_w(T_L) \tag{4}$$

which also comes directly from eqn. (3). Thus the non-symmetry between low and high T unfolding could be ascribed to the different values of the chemical potential of water.

The mathematical form of eqn. (4) suggests that the ratio $\Delta\mu/\delta$ can be treated as a continuous function of T, which would more adequately represent the thermodynamic folded versus unfolded stability of a protein in aqueous solution. When T does not coincide with T_L or T_H, one can write, according to eqn. (2)

$$\frac{\Delta\mu}{\delta} = \mu_w + \frac{\Delta_N^D G}{\delta}$$

which can be differentiated

$$d\left(\frac{\Delta\mu}{\delta}\right) = -S_w \, dT + d\left(\frac{\Delta_N^D G}{\delta}\right)$$

and compared with eqn. (4). This enables us to state that

$$\int_{T_L}^{T_H}\left[\frac{d}{dT}\left(\frac{\Delta_N^D G}{\delta}\right)\right] dT = 0 \tag{5}$$

The function $\Delta_N^D G/\delta$ is zero at T_H and T_L: to satisfy eqn. (5), its derivative must change sign at some intermediate temperature. It is easy to show that

$$\frac{d}{dT}\left(\frac{\Delta_N^D G}{\delta}\right) = \frac{\Delta_N^D G}{\delta} \cdot \left[\frac{1}{T} - \frac{d}{dT}(\ln \delta)\right] - \frac{\Delta_N^D H}{T \cdot \delta} \tag{6}$$

At either unfolding point T_d (namely, T_H and T_L) one has

$$\frac{d}{dT}\left(\frac{\Delta_N^D G}{\delta}\right)_{T=T_d} = -\left(\frac{\Delta_N^D S}{\delta}\right)_{T=T_d} \tag{7}$$

Because $\delta > 0$ is expected, the sign of this derivative will be opposite to that of the corresponding entropy change (negative at T_L and positive at T_H). The trend of the function $\Delta_N^D G/\delta$ would therefore be similar to that of $\Delta_N^D G(T)$.

This behaviour can be most simply accounted for by assuming δ independent of T; however, in view of the expected modification of the solvent power of water within the (T_H, T_L) range, it seems more reasonable that both $\Delta_N^D G$ and δ change with T. The possibility that $\Delta_N^D G$ and δ may simultaneously tend to zero in the vicinity of either T_d must also be ruled out, since it would imply, against the experimental evidence [3], that water and its activity are devoided of a role in the unfolding transition. If δ is tentatively assumed to increase with T according to a straight line in the (T_L, T_H) range (which is at least qualitatively consistent with the larger solvent power of water at lower T), eqn. (6) becomes

$$\frac{d}{dT}\left(\frac{\Delta_N^D G}{\delta}\right) = -\frac{\Delta_N^D H}{T \cdot \delta}$$

which is in agreement with eqn. (7) and leads one to expect a $\Delta_N^D G/\delta$ maximum at the same temperature T_0 where $\Delta_N^D H$ vanishes (see below).

Equation (3) can be used to write

$$\ln\left(\frac{a_D}{a_N}\right) = -\frac{\Delta_N^D G^\circ}{RT} + \delta \cdot \ln a_w \tag{8}$$

where a stands for activity and $\Delta_N^D G^\circ$ is the Gibbs energy change in conditions of infinite dilution. If T_H and T_L are the unfolding temperatures in the absence of any extra ingredient, except water, and at low protein concentration, then $\ln a_w$ can be ignored

$$\ln\left(\frac{a_D}{a_N}\right) = -\frac{\Delta_N^D G^\circ}{RT}$$

as for a simple ND two-compound equilibrium, although $\Delta_N^D G^\circ$ here includes $\delta \cdot \mu_w^-$.

If T_H and T_L are such that $a_D/a_N = 1$, $\Delta_N^D G^\circ(T_H) = \Delta_N^D G^\circ(T_L) = 0$. However, when $a_w < 1$, the activity ratio a_D/a_N approaches unity at different temperatures, namely T_H' and T_L', which must satisfy the condition

$$\Delta_N^D G^\circ(T_d') - \delta \cdot RT_d' \ln a_w = \Delta_N^D G^\circ(T_H) = \Delta_N^D G^\circ(T_L) = 0$$

where T'_d stands for T'_H and T'_L. Since δ is expected to be positive, one is inclined to guess that $\Delta^D_N G°(T'_d) < 0$. Due to the dome shape of the function $\Delta^D_N G°(T)$ (see below), this means that $T'_H > T_H$ and $T'_L < T_L$, i.e. a decrease of a_w widens the dome and pushes apart the two intercepts where $\Delta^D_N G° = 0$. This explains why polyols and sugars added to protein solutions can act as protectors against both low and high T unfolding, as shown experimentally (ref. 5 and references cited therein).

Equation (8) allows more quantitative conclusions to be drawn

$$\frac{T_d - T'_d}{T'_d} = \delta \cdot \frac{R \ln a_w}{\Delta^D_N S°(T_d)} \tag{9}$$

where the standard entropy change is assumed to be independent of the temperature in the expected narrow ranges (T_d, T'_d). Since $\Delta^D_N S°$ is positive and negative for high and low T unfolding [3,4], respectively, the directions of the shifts of the transition points are confirmed; moreover, if ($\delta \cdot R \ln a_w$) is assumed to have almost the same value at either point, the reduced shift $(T_d - T'_d)/T'_d$ is expected to be different for low and high T unfolding, because of the different $\Delta^D_N S°(T_d)$ values.

The denaturation effect observed in a mixed solvent [3,5,9,10] such as water/methanol, still waits for a thermodynamic interpretation: total or partial replacement of solvation water with methanol could modify both native and denatured protein. Solvated chemical species involved would then change according to the composition. Protein unfolding therefore appears to be rather different from a simple two-state equilibrium. For a given chemical environment (i.e. pH, protein concentration, water activity, etc.), the native state would be progressively modified in a T scan according to adjustments of the fine balance between hydrophobic and polar interactions, with simultaneous changes of its solvation degree. This is consistent with the results of circular dichroism investigations showing gradual protein conformation changes within the (T_L, T_H) range where the protein remains biochemically active, although with variation of its enzymatic efficacy. The unfolding process may thus be supposed to be the eventual step of a gradual modification of the protein structure: the width of the calorimetric signal is indeed quite large and the process does not take the form of an abrupt transition, even in the vicinity of T_H or T_L. However, the progressive slight modifications occurring in the (T_L, T_H) range would be the cause of the temperature dependence of $\Delta^D_N C_p$ (see below).

Since cold unfolding is generally reversible on rewarming [3], one is inclined to argue that water molecules which solvate the internal polar groups, or rearrange their own structure around the hydrophobic chains [3,4,6], should nonetheless keep the protein subunits rather close to one another; when T increases, the solvent power of water decreases while

hydrophobic interactions tend to prevail. At T_L, the remaining "internal" solvation water would be eventually squeezed away and the native structure restored.

The simple two-compound model, therefore, only applies to very diluted solutions, though the formal treatment of the process is still the same for larger concentrations, since the condition $a_w < 1$ affects both low and high T unfolding points in the same way (although to a different extent). The model can thence be used to readily guess whether or not unfolding will occur at a given subzero temperature and tentatively evaluate the stability of the native versus denatured state at various intermediate temperatures. It may therefore be of interest to suggest some formal expressions which, although equivalent to those proposed by others [2,3], seem more adequate for routine calculations.

EXPRESSIONS FOR ROUTINE CALCULATIONS

The $\Delta^D_N G(T)$ for the ND equilibrium can be expressed as

$$\Delta^D_N G(T) = \Delta^D_N G(T_H) - \int_{T_H}^{T} \Delta^D_N S \, dT \tag{10}$$

Since $\Delta^D_N G(T_H) = 0$, this reduces to

$$\Delta^D_N G(T) = (T_H - T) \cdot \Delta^D_N S(T_H) - \int_{T_H}^{T} dT \int_{T_H}^{T'} \Delta^D_N C_p \frac{dT'}{T'} \tag{11}$$

allowing prediction of a curvilinear trend of $\Delta^D_N G(T)$ with a maximum at some lower temperature T_m, where $\Delta^D_N S(T_m) = 0$. Accordingly, one can simplify eqn. (11) to

$$\Delta^D_N G(T) = - \int_{T_H}^{T} dT \int_{T_m}^{T'} \Delta^D_N C_p \frac{dT'}{T'} \tag{12}$$

Since the Gibbs energies of both native and unfolded protein are descending functions of T (the entropy of a compound is always positive), which intersect twice with each other, the $\Delta^D_N G(T)$ function must have a dome shape, like that reported in Fig. 1.

Qualitative prediction of the trend of both $\Delta^D_N S(T)$ and $\Delta^D_N C_p(T)$ is possible as

$$\frac{d\ \Delta^D_N G(T)}{dT} = -\Delta^D_N S$$

and

$$\frac{d^2\ \Delta^D_N G(T)}{dT^2} = -\frac{\Delta^D_N C_p}{T}$$

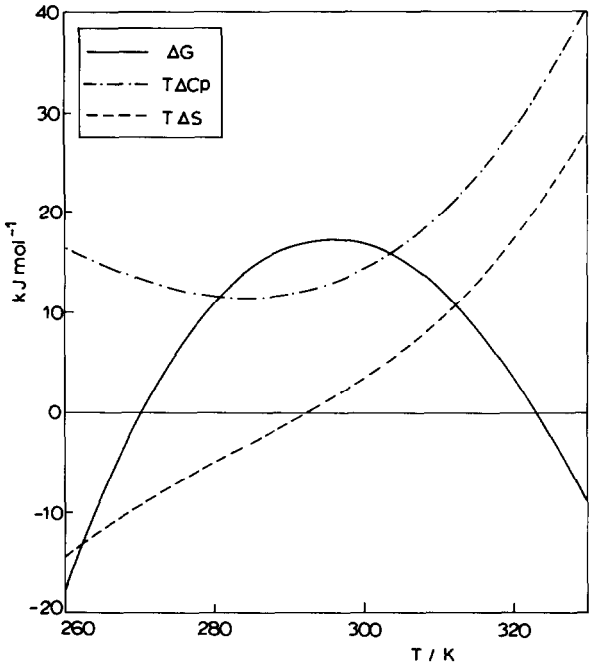

Fig. 1. Trend of the Gibbs energy, entropy and molar heat capacity differences between native and denatured state of a protein. $T\Delta C_p$ and $T\Delta S$ are scaled down by a factor 100 and 10, respectively, to be presented together with ΔG.

The entropy change must be positive and negative at T_L and T_H, respectively (as found experimentally), and zero at the temperature T_m of the dome maximum: $\Delta^D_N S(T)$ therefore is always ascending, with a flexus at T_m (see Fig. 1).

The enthalpy change will also show different signs at T_L and T_H, being zero at some intermediate temperature, $T_0 \leqslant T_m$.

The heat capacity change is positive at either T_d and goes through a minimum at T_m. This means that $\Delta^D_N C_p$ cannot become negative, as untenably suggested [3], and must depend on T, with a trend of the kind reported in Fig. 1.

In a recent paper Privalov et al. [7] reported a $\Delta^D_N C_p$ versus T trend with a maximum, since they observed that the heat capacity of the unfolded protein increases with T up to a plateau (i.e. with a downward curvature), whereas the C_p of the native conformation seems to increase linearly with T, at least within the narrow T range investigated. This trend however concerns a temperature range above T_H and would depend on the decrease of the hydration of non-polar groups which seems to vanish at about 140°C for any protein [6]. This aspect is out of the scope of the two-state model for the unfolding process considered in this paper and, in any case, cannot be directly used to improve the interpretation of the low T unfolding which is still rather qualitative.

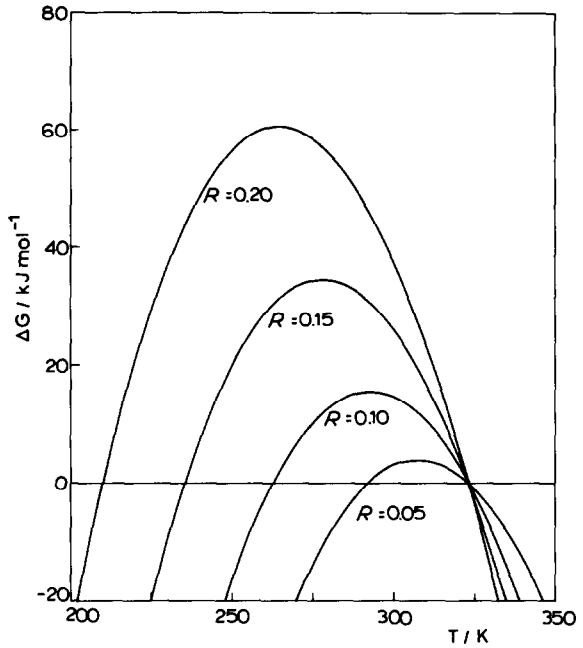

Fig. 2. Gibbs energy differences calculated for various $R = \Delta^{D}_{N}S(T_H)/\Delta^{D}_{N}C_p(T_H)$ at the higher temperature unfolding point, T_H. The values $T_H = 323.2$ K and $\Delta^{D}_{N}C_p = 10$ kJ mol^{-1} K^{-1} have been used.

The $\Delta^{D}_{N}C_p$ trend of Fig. 1 could nonetheless represent the low T extension of those proposed in ref. 7 for the range above T_H where hydration of hydrophobic groups becomes less effective. Improvements of the thermodynamic model presented in this paper should therefore account for the behaviour of $\Delta^{D}_{N}C_p$ throughout the whole temperature range, i.e. from T_L to 140°C, by explicitly including hydration of hydrophobic groups and the consequent variation of δ with T. If, as a rough approximation, which nonetheless matches the experimental uncertainties, $\Delta^{D}_{N}C_p$ is assumed to be independent of T, T_m and T_0 can be evaluated when the ratio $R = \Delta^{D}_{N}S(T_H)/\Delta^{D}_{N}C_p$ and the temperature T_H are known

$$T_m = T_H \exp(-R)$$

$$T_0 = T_H \cdot (1 - R)$$

One can accordingly rewrite eqn. (10) in the form

$$\Delta^{D}_{N}G(T) = \Delta^{D}_{N}C_p \cdot \left[T \ln\frac{T_H}{T} - (1 - R) \cdot (T_H - T) \right] \tag{13}$$

$\Delta^{D}_{N}G(T)$ is then a skewed parabola (the rising branch is steeper) with maximum at $T = T_m$ and intercepts at $T = T_H$ and $T = T_L$ (Fig. 2). It must be noted that the skewing is practically negligible for $R \leqslant 0.1$, whereas it

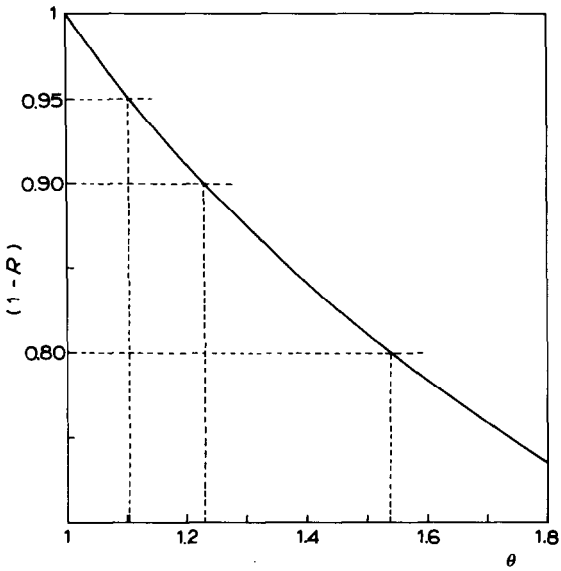

Fig. 3. $\theta = T_H / T_L$ values singled out as roots of the equation $F(\theta) = \ln \theta/(\theta - 1) = (1 - R)$ for three R values.

becomes apparent for $R \geqslant 0.2$. T_L is therefore the value that satisfies the condition

$$\frac{T_L}{T_H - T_L} \ln \frac{T_H}{T_L} = (1 - R)$$

$$\frac{\ln \theta}{(\theta - 1)} = (1 - R) \tag{14}$$

as shown in Fig. 3, with $\theta = T_H/T_L$.

The literature data [8] give an R range of 0.05–0.25; thus, for $T_H = 323.15$ K, and $\Delta_N^D C_p = 10$ kJ mol^{-1} K^{-1} at T_H, one may evaluate the T_m, T_L, and maximum stability of the native state, $\Delta_N^D G(T_m)$, of a given protein (see Table 1). Occurrence of reversible cold unfolding for proteins with $R > 0.15$ is however ruled out, because of the unavoidable ice nucleation at $T < 233$

TABLE 1

Comparison between calculated T_m, T_L and $\Delta_N^D G(T_m)$ for various R values

R	T_m (K)	T_L (K)	$\Delta_N^D G(T_m)$ (kJ mol^{-1})
0.05	307.3	291.4	4.0
0.10	292.40	262.72	15.6
0.15	278.14	235.45	34.6
0.20	264.57	210.04	60.5
0.25	251.67	186.40	93.1

TABLE 2

Comparison between observed and calculated (subscript c) values of T_m and T_L (K) for some proteins in aqueous solution

Protein	T_H	R	T_m	$T_{m,c}$	T_L	$T_{L,c}$
Chymotrypsinogen [10]	313	0.08	288	289	262	263
Metmyoglobin [11]						
pH 3.83	331	0.06	312	311	295	292
pH 3.90	335	0.07	312	312	292	289
Phage T4 lysozyme [12]	301	0.05	286	286	270	272

K [9]. Comparison between calculated and observed T_L values is possible in few cases, some of which are reported in Table 2.

If $\Delta^D_N C_p$ is assumed to depend on T according to a parabola

$$\Delta^D_N C_p = \Delta^D_N C_p(T_H) + bT + cT^2 + dT^3$$

$\Delta^D_N G(T)$ becomes a little more skewed, without significant modifications (typically 1–2 K) of the values obtained above, because of the low values of the coefficients b, c and d. Two major considerations apply with regard to the thermodynamic stability thus evaluated: (a) the maximum thermodynamic stability would seem to correspond to few hydrogen bonds per protein molecule; (b) physiological temperatures do not generally imply the maximum thermodynamic stability of the native state (as defined in a two-state ND model); this means that kinetic and steric requirements, rather than thermodynamic stability, impose the conformational state of these molecules in living organisms.

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