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Denaturation of proteins: electrostatic effects *vs.* hydration

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The unfolding transition of proteins in aqueous solution containing various salts or uncharged solutes is a classical subject of biophysics. In many cases, this transition is a well-defined two-stage equilibrium process which can be described by a free energy of transition ΔG_u and a transition temperature T_m. For a long time, it has been known that solutes can change T_m profoundly. Here we present a phenomenological model that describes the change of T_m with the solute concentration c_s in terms of two effects: (i) the change of the number of correlated counterions Δn_{ci} and (ii) the change of hydration expressed through the parameter Δw and its dependence on temperature expressed through the parameter $d\Delta c_p/dc_s$. Proteins always carry charges and Δn_{ci} describes the uptake or release of counterions during the transition. Likewise, the parameter Δw measures the uptake or release of water during the transition. The transition takes place in a reservoir with a given salt concentration c_s that defines also the activity of water. The parameter Δn_{ci} is a measure for the gain or loss of free energy because of the release or uptake of ions and is related to purely entropic effects that scale with ln c_s. Δw describes the effect on ΔG_u through the loss or uptake of water molecules and contains enthalpic as well as entropic effects that scale with c_s. It is related to the enthalpy of transition ΔH_u through a Maxwell relation: the dependence of ΔH_u on c_s is proportional to the dependence of Δw on temperature. While ionic effects embodied in Δn_{ci} are independent of the kind of salt, the hydration effects described through Δw are directly related to Hofmeister effects of the various salt ions. A comparison with literature data underscores the general validity of the model.

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Introduction

The denaturation of proteins by a globule to coil transition is a classical subject of biophysics.[1] The thermal denaturation in which the protein goes from natural folded state to a random coil in aqueous solution occurs with raising temperature. Cold denaturation,[2] which has been known for a long time, is the transition to denatured state taking place with decreasing temperature. It is well-established that for many proteins chain denaturation is a two state transition[3–6] in which the globular and the denatured form of the protein are well-defined thermodynamic states in equilibrium with each other. Hence, an equilibrium constant K_u can be defined between the globular and denatured state which allows us to treat the denaturation as a fully thermodynamic problem relating the melting temperature T_m to the transition enthalpy ΔH_u and the transition entropy ΔS_u.[7]

A fundamental problem in the field is the change T_m of a given protein with solutes in the aqueous phase. Up to now, there have been an enormous number of experimental studies that started out in the sixties of the last century.[8] There are many investigations that study the change of T_m in the presence of various salts and non-charged solutes which can stabilize or destabilize the globular state.[4,6,7,9–20] This effect is of obvious biological importance and can be traced back to hydration effects embodied in the Hofmeister series.[21–24] The collapse transition of poly(*n*-isopropylacrylamide) (PNIPAM) in aqueous solution is another well-studied and fundamental problem where a coiled polymer undergoes a transition from the coiled to the globular state with raising temperature. Here too there is a large number of fundamental and detailed studies on this transition in solutions of various ions.[23,25–30] Taken together the folding/unfolding transition of proteins and polymers in general is problem of fundamental importance.

Early studies of protein denaturation clearly revealed the central role of charge–charge interaction.[1] The unfolding of the globular protein exposes charged groups to water and this interaction leads to an important contribution to the free energy of unfolding that scales with the logarithm of the salt concentration in solution.[1] This term is due to the release or uptake of ions during unfolding and play an important role both for unfolding of proteins as well as for denaturing of DNA in presence of various salts (see the discussion in ref. 31). A similar process takes place when polyelectrolytes form a complex with a protein (counterion release force; see the discussions in ref. 1

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and 32–34 and further citations given there). Here a wealth of experimental data demonstrates that this effect is purely entropic and therefore independent of temperature.[32,35–37]

The unfolding of proteins also exposes hydrophobic amino acids to water. As mentioned above, hydration therefore plays an important role which has been the subject of exhaustive investigations by Record and coworkers in the frame of the solute partitioning model (SPM).[22,31,38–40] This model treats the partitioning of the solute ions or solutes between the hydrate and the bulk water. Kosmotropic ions are depleted from the hydrate water whereas chaotropic ions are enriched in this phase. Moreover, these investigations have clearly revealed that effects due to the partitioning of solutes scale linearly with salt concentration which is in full agreement with the analysis by Schellman using Kirkwood–Buff integrals.[4,5] Thus, for many kosmotropic salts in the Hofmeister series, a linear relation between the free energy and salt concentration is found ("*m*-value"; see the discussion in ref. 29). In many cases the *m*-value is found to be independent of temperature. Based on these considerations, Chen and Schellman developed a phenomenological model that is based on a *m*-values that do not depend on temperature[6,41] ("linear model"; *cf.* ref. 18). A fact overlooked in later expositions of this theory is the linear dependence of the specific heat Δc_p on salt concentration. Chen and Schellman could demonstrate that this dependence is a direct consequence of the assumption of a constant *m*-value.[6] The notion of a *m*-value independent of temperature, however, is a stringent condition that may not be fulfilled for a given system.[42] Hence, a general model should avoid this prerequisite.

Surveying the literature on denaturation of proteins, it becomes clear that exchange of water and counterions during unfolding present two important factors that determines the stability of proteins in aqueous solution to a large extend. Both are modified by the added solute. Hence, a quantitative treatment of the effect of ions and water is a necessary prerequisite for a quantitative evaluation of data related to the unfolding of proteins in presence of various solutes. In a recent paper we have presented a unified approach for the free energy of complex formation between proteins and polyelectrolytes that comprises both effects.[34] Temperature T and salt concentration c_s were identified as the decisive variables and a closed expression for the free energy $\Delta G_b(T,c_s)$ of complex formation could be derived. In this model counterion release was characterized by Δn_{ci} denoting the net number of released ions during binding whereas hydration was described in terms of the parameter Δw defined already in early expositions of the problem[1,43,44] and used frequently to describe the effect of hydration on complex formation.[44–49] Central for the development of this model is the fact that mixed derivatives of the binding enthalpy $\Delta H_b(T,c_s)$ with regard to T and c_s must be the same. Hence, this Maxwell-relation leads to prediction that the dependence of $\Delta H_b(T,c_s)$ on c_s gives directly the dependence of Δw on temperature. The model thus derived is capable of describing the weak dependence of $\Delta G_b(T,c_s)$ on temperature which in turn leads to a strong compensation of enthalpy and entropy.[34] Moreover, the values obtained for Δn_{ci} and Δw obtained by the present model for the denaturation of a given protein can directly be compared to data deriving from studies of complex formation of polyelectrolytes with proteins.[33,46,47,50,51]

Based on this model we here present a phenomenological approach to unfolding transitions of proteins that are partially charged. A closed expression for the free energy of unfolding will be presented that contains both the effect of electrostatics as well as of hydration. The consequences of the model for data evaluation will be discussed and exemplified using recent experimental data.[16,18] The entire discussion presented here aims at a systematic analysis of experimental data obtained on polymeric unfolding transitions of various systems in aqueous phase.

Theory

We consider the transition of a single chain of a polypeptide from a folded to an unfolded state in sufficiently dilute solution. In each stage of this transition the unfolded state is in equilibrium with the still folded part of the chain. This two-state mechanism is well-established for a great number of systems (see the discussion of this point in ref. 1, 16 and 18). Experimentally, the unfolding transition can be monitored *e.g.* by measurements of the circular dichroism leading to a fraction α of unfolded protein. The equilibrium constant K_u for the process of unfolding is related to α by

$$\alpha = \frac{K_u}{1 + K_u} \tag{1}$$

whereas the free energy of unfolding ΔG_u is related to K_u through

$$\Delta G_u = -RT \ln K_u \tag{2}$$

The basic thermodynamic analysis ΔG_u was already discussed a long time ago by Record, Anderson, and Lohman.[1] In general, the change of the equilibrium constant K_u with the activity $a_\pm$ of an added salt is given by

$$\mathrm{d}\ln K_u = -\left(\Delta n_{ci} - \frac{pm}{55.6}\Delta w\right)\mathrm{d}\ln a_\pm + \mathrm{d}\ln\frac{\gamma_f}{\gamma_u} \tag{3}$$

where Δn_{ci} denotes the total number of released or taken-up ions during the process of unfolding. The parameter Δw treats the release or uptake of water in the course of the unfolding transition while $p = 2$ for monovalent salt with molality m. By definition, Δw is independent of salt concentration. The factor 55.6 is the molality of water and the parameters γ_f and γ_u are the activity coefficients of the chain in the folded and unfolded state, respectively. Note that this equation with necessary adaptions has been the basis of our recent discussion of complex formation of polyelectrolytes with proteins.[34] In the following, the same approximations will be made: (i) the change of the activity coefficients γ_f and γ_u with the activity $a_\pm$ of the added salt give a small but non-negligible contribution of the term Δn_{ci} (see the discussion in ref. 1), (ii) the mean activity coefficient of the salt ions will be set to unity, and (iii) the molality m of the salt will be equated to its concentration c_s. With these approximations the justification of which will be discussed below eqn (3) becomes

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$$\mathrm{d}\ln K_\mathrm{u} = -\Delta n_\mathrm{ci}\,\mathrm{d}\ln c_\mathrm{s} + \frac{2}{55.6}\Delta w \mathrm{d}c_\mathrm{s} \tag{4}$$

Hence, the salt concentration c_s is the variable on which the subsequent thermodynamic analysis is based. With the standard thermodynamic relation $(\partial \ln K_\mathrm{b}/\partial T)_{c_\mathrm{s}} = \Delta H_\mathrm{b}/RT^2$ we obtain the differential of $\ln K_\mathrm{u}$ for monovalent ions

$$\mathrm{d}\ln K_\mathrm{u} = \frac{\Delta H_\mathrm{u}}{RT_\mathrm{m}^2}\mathrm{d}T - \Delta n_\mathrm{ci}\mathrm{d}\ln c_\mathrm{s} + \frac{2}{55.6}\Delta w \mathrm{d}c_\mathrm{s} \tag{5}$$

where ΔH_u denotes the enthalpy change at the unfolding transition and T_m the respective temperature of unfolding. Thus, in the following the unfolding transition will be treated as the function of the two decisive variables, namely temperature T_m and salt concentration c_s.

There is abundant experimental evidence that the parameter Δn_ci is independent of temperature.[32,34,35,37,52–54] It is therefore safe to disregard the dependence of this parameter on T_m. With this assumption and

$$\left(\frac{\partial \ln K_\mathrm{u}}{\partial c_\mathrm{s}}\right)_{T_\mathrm{m}} = -\frac{\Delta n_\mathrm{ci}}{c_\mathrm{s}} + \frac{2}{55.6}\Delta w \tag{6}$$

we obtain the Maxwell-relation[34]

$$\frac{1}{RT_\mathrm{m}^2}\frac{\partial \Delta H_\mathrm{u}}{\partial c_\mathrm{s}} = \frac{2}{55.6}\frac{\mathrm{d}\Delta w}{\mathrm{d}T_\mathrm{m}} \tag{7}$$

This relation demonstrates that the salt dependence of transition enthalpy is directly related to the dependence of the parameter Δw on temperature. As already lined out previously,[34] this relation can now be used to calculate Δw as the function of temperature. In general, the transition enthalpy ΔH_u as the function of the melting temperature T_m and c_s can be rendered as[34]

$$\Delta H_\mathrm{u}(T_\mathrm{m}, c_\mathrm{s}) = \Delta H_\mathrm{u}\left(T_\mathrm{m}^0, c_\mathrm{s} = 0\right) + \left(\Delta c_{\mathrm{p},0} + c_\mathrm{s}\frac{\mathrm{d}c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}\right)\left(T_\mathrm{m} - T_\mathrm{m}^0\right) \tag{8}$$

Here, the quantity $\Delta c_{\mathrm{p},0}$ denotes the change of the specific heat in absence of added salt whereas the coefficient $\mathrm{d}c_\mathrm{p}/\mathrm{d}c_\mathrm{s}$ describes the change of the specific heat with salt or solute concentration.[34] T_m^0 denotes the melting temperature for salt-free solutions. Together with eqn (7), this relations leads to

$$\frac{1}{RT_\mathrm{m}^2}\frac{\partial \Delta H_\mathrm{u}}{\partial c_\mathrm{s}} = \frac{1}{RT_\mathrm{m}^2}\frac{\mathrm{d}\Delta c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}\left(T_\mathrm{m} - T_\mathrm{m}^0\right) = \frac{2}{55.6}\frac{\mathrm{d}\Delta w}{\mathrm{d}T_\mathrm{m}} \tag{9}$$

Integration leads to[34]

$$\Delta w = \Delta w\left(T_\mathrm{m}^0\right) + \frac{\frac{\mathrm{d}\Delta c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}}{0.036R}\left(\ln\frac{T_\mathrm{m}}{T_\mathrm{m}^0} + \frac{T_\mathrm{m}^0}{T_\mathrm{m}} - 1\right) \tag{10}$$

where the quantity $\Delta w(T_\mathrm{m}^0)$ denotes the magnitude of Δw at T_m^0 in salt-free solution.

As already discussed previously,[34] Δw can be interpreted in terms of the solute partitioning model as follows. Both the polyelectrolyte as well as the protein are hydrated in aqueous solution. During the unfolding a certain number Δn_w of water molecules of both reactants is taken up or released. Furthermore, it is assumed that there is a partitioning of the ions between the bulk solution and the hydration water on the surface of the protein described by the partition coefficient $K_{\mathrm{p},+} = (m_+^\mathrm{loc}/m_+^\mathrm{bulk})$ for the cations where m_+^loc denotes the molality of the cations in the hydrated shell whereas m_+^bulk is the respective quantity in bulk. The partition coefficient $K_{\mathrm{p},-}$ of the anions is defined in the same way. With these definitions, Δw can be rendered by[34]

$$\Delta w \cong \frac{1}{2}\left(K_{\mathrm{p},+} + K_{\mathrm{p},-} - 2\right)\Delta n_\mathrm{w} \tag{11}$$

Evidently, the quantity Δw measures the effect of water release on the free energy of unfolding and should not be confused with the total number Δn_w taken up or released during unfolding. For an equal distribution of the ions between the hydrate and the bulk phase, this contribution will vanish.

In the following, we first consider uncharged systems, that is, $\Delta n_\mathrm{ci} = 0$. Integration of eqn (6) at constant temperature then leads to

$$\ln K_\mathrm{u} = \ln K_\mathrm{u}^0 + 0.036\Delta w c_\mathrm{s} \tag{12}$$

where K_u^0 is the equilibrium constant in salt-free solution. Therefore

$$\Delta G_\mathrm{u} = \Delta G_\mathrm{u}^0 - 0.036RT_\mathrm{m}\Delta w c_\mathrm{s} \tag{13}$$

Here, ΔG_u^0 denotes the free energy of unfolding at $c_\mathrm{s} = 0$. Hence, the dependence of ΔG_u on c_s can be written down as

$$\Delta G_\mathrm{u} = \Delta G_\mathrm{u}^0 - 0.036RT_\mathrm{m}\Delta w\left(T_\mathrm{m}^0\right)c_\mathrm{s} + \left(T_\mathrm{m} - T_\mathrm{m}^0 - T_\mathrm{m}\ln\frac{T_\mathrm{m}}{T_\mathrm{m}^0}\right)\frac{\mathrm{d}c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}c_\mathrm{s} \tag{14}$$

In many cases the difference $T_\mathrm{m} - T_\mathrm{m}^0$ does not exceed 10 degrees so that the last term in eqn (14) can be expanded to yield (see the derivation of eqn (11) of ref. 55)

$$\Delta G_\mathrm{u} \cong \Delta G_\mathrm{u}^0 - \left[0.036RT_\mathrm{m}\Delta w\left(T_\mathrm{m}^0\right) + \frac{\mathrm{d}c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}\frac{\left(T_\mathrm{m} - T_\mathrm{m}^0\right)^2}{2T_\mathrm{m}^0}\right]c_\mathrm{s} \tag{15}$$

Eqn (14) may be used to calculate the m-value defined as the derivative of the free energy with regard to solute concentration at constant temperature

$$m = -\left(\frac{\partial G_\mathrm{u}}{\partial c_\mathrm{s}}\right)_{T_\mathrm{m}} = 0.036RT_\mathrm{m}\Delta w\left(T_\mathrm{m}^0\right) + \frac{\mathrm{d}c_\mathrm{p}}{\mathrm{d}c_\mathrm{s}}\frac{\left(T_\mathrm{m} - T_\mathrm{m}^0\right)^2}{2T_\mathrm{m}^0} \tag{16}$$

This expression shows that m is given by a constant plus a term that depends quadratically on $T_\mathrm{m} - T_\mathrm{m}^0$. For small temperature differences the second term will be small and the m-value is a constant in good approximation. However, it

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should be noted that m is in general a quantity that depends explicitly on temperature.

Eqn (14) and (15) contain only the dependence of the free energy on c_s. The quantity ΔG_u^0 for salt- or solute-free solutions can be derived following the prescription of Chen and Schellman:[6] the specific heat $\Delta c_{p,0}$ measured in solute-free systems can be regarded as a constant throughout the rather small temperature range under consideration here. Thus, for solute-free systems we obtain

$$\Delta G_u^0 = \Delta H_u^0 - T_m \Delta S_u^0 = \Delta H_u^0 \left(1 - \frac{T_m}{T_m^0}\right) \tag{17}$$

and

$$\Delta H_u^0(T_m) + \Delta c_{p,0}(T_m - T_m^0) \tag{18}$$

$$\Delta S_u(T_m) = \Delta S_u^0(T_m^0) + \Delta c_{p,0} \ln \frac{T_m}{T_m^0} \tag{19}$$

which gives

$$\Delta G_u^0 = \Delta H_u^0 \left(1 - \frac{T_m}{T_m^0}\right) + \Delta c_{p,0}\left(T_m - T_m^0 - T_m \ln \frac{T_m}{T_m^0}\right) \tag{20}$$

Combination with eqn (14) then leads to

$$\Delta G_u = \Delta H_u^0 \left(1 - \frac{T_m}{T_m^0}\right) - 0.036 R T_m \Delta w(T_m^0) c_s + \left(\Delta c_{p,0} + \frac{dc_p}{dc_s} c_s\right) \times \left(T_m - T_m^0 - T_m \ln \frac{T_m}{T_m^0}\right) \tag{21}$$

For $T_m - T_m^0 \leq 10$ K this expression can be approximated by

$$\Delta G_u = \Delta H_u^0 \left(1 - \frac{T_m}{T_m^0}\right) - 0.036 R T_m \Delta w(T_m^0) c_s - \left(\Delta c_{p,0} + \frac{dc_p}{dc_s} c_s\right) \frac{(T_m - T_m^0)^2}{2T_m^0} \tag{22}$$

Eqn (21) and (22) are the final result for the free energy of unfolding for uncharged systems.

For partially charged proteins eqn (5) shows that a term scaling with $\ln c_s$ must be added to eqn (21).[1] Here it must be kept in mind that there is always a small but finite salt concentration $c_{s,0}$ so that the integration of eqn (5) must start at this concentration. Keeping in mind that Δn_{ci} does not depend on temperature we immediately obtain from eqn (22)

$$\Delta G_u = \Delta H_u^0 \left(1 - \frac{T_m}{T_m^0}\right) - 0.036 R T_m \Delta w(T_m^0)(c_s - c_s^0) - \left[\Delta c_{p,0} + \frac{dc_p}{dc_s}(c_s - c_s^0)\right] \frac{(T_m - T_m^0)^2}{2T_m^0} + \Delta n_{ci} R T_m \ln \frac{c_s}{c_s^0} \tag{23}$$

In many cases the concentration c_s^0 is small and can be disregarded in eqn (23) except for the last term, of course. Eqn (23) also shows that for small concentrations c_s^0 the free energy of unfolding may contain an appreciable contribution originating from the release or uptake of ions during denaturation. Hence, ΔG_u will be dominated by the last term for small c_s. The respective transition enthalpy is given by eqn (8) where c_s is replaced by $c_s - c_{s,0}$. The transition entropy follows as

$$\Delta S_u = \Delta S_u^0 + 0.036 R \Delta w(T_m^0)(c_s - c_s^0) + \left[\Delta c_{p,0} + \frac{dc_p}{dc_s}(c_s - c_s^0)\right] \ln \frac{T_m}{T_m^0} - \Delta n_{ci} R \ln \frac{c_s}{c_s^0} \tag{24}$$

In many cases it is only possible to deduct the change of the free energy of unfolding with increasing solute concentration. Thus, we require the quantity $\Delta\Delta G_u$ which gives the change of ΔG_u with c_s calculated for the transition temperature T_m^0 in solute-free solution:

$$\begin{aligned}\Delta\Delta G_u &= \Delta G_u(T_m^0, c_s) - \Delta G_u(T_m^0, c_s^0) \\ &= -0.036 R T_m^0 \Delta w(T_m^0)(c_s - c_s^0) - \frac{dc_p}{dc_s}(c_s - c_s^0)\frac{(T_m^0 - T_m)^2}{2T_m} \\ &\quad + \Delta n_{ci} R T_m^0 \ln \frac{c_s}{c_s^0}\end{aligned} \tag{25}$$

It is interesting to compare eqn (21) and (23) to phenomenological approach of Chen and Schellman[6] (*cf.* also ref. 41). The generalized van't Hoff equation used by these authors is based on eqn (17)–(19). Moreover, the dependence of the free energy of unfolding is assumed to be linear in c_s as derived above in eqn (13):

$$\Delta G_u(c_s) = \Delta G_u^0 - R T_m \Delta\beta_{23} c_s \tag{26}$$

Thus, the coefficient $\Delta\beta_{23}$ is identical to $0.036\Delta w$ in eqn (13). In the linear model of Chen and Schellman,[6] this linear dependence has be deduced from experiments whereas the above considerations leading to eqn (13) demonstrate that this relation is a direct consequence of eqn (1). Based on these premises Chen and Schellman formulate $\ln K_u$ as follows in the present notation as:[6]

$$-R T_m \ln K_u = \Delta H_u(c_s, T_m) - T_m \Delta S_u(c_s, T_m) + \Delta c_p(c_s)\left(T_m - T_m^0 - T_m \ln \frac{T_m}{T_m^0}\right) \tag{27}$$

where all thermodynamic quantities ΔH_u, ΔS_u, are explicit function of solute concentration and temperature whereas Δc_p is only a function of c_s. All parameters will be treated as adjustable parameters for each c_s in a comparison with experimental data. The present approach, on the other hand, reveals the interrelation between the various quantities and the concentration of solute which is based on the Maxwell-relation eqn (7).

The experimental data are described in terms of 3 adjustable parameters: (i) $\Delta w(T_m^0)$ which is closely related to the classical m-value through eqn (15); (ii) the specific heat $\Delta c_{p,0}$ in absence of

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solutes; and (iii) the parameter dc_p/dc_s describing the dependence of Δc_p on c_s. This parameter has been introduced by Chen and Schellman as well (the parameter $\Delta\overline{c_{p23}}$ in eqn (8) and (9) of ref. 6) but not used further. Its application to complex formation of polyelectrolytes with proteins has been discussed recently.[34] The first two parameters are directly measurable and have an obvious physical meaning. The newly introduced parameter dc_p/dc_s describes the dependence of hydration effects on temperature.

A comprehensive phenomenological analysis of the denaturation temperature for uncharged polymers was presented some time ago by Heyda and Dzubiella.[29] Here, the hydration effects are described in terms of the preferential interaction parameter $\Delta\Gamma_{23}$. If this parameter does not depend on c_s, it follows directly that

$$m = kT\Delta\Gamma'_{23}$$

where $\Delta\Gamma'_{23}$ is defined as the preferential interaction parameter independent of c_s. The analysis of the changes effected by kosmotropic salts showed indeed that this equation provides a very good approximation of the experimental data obtained for the collapse transition of PNIPAM-chains in aqueous solution.[26,29] Thus, these data could be compared directly to the prediction of the SPM with moderate success (*cf.* Table 3 of ref. 29). Moreover, Heyda and Dzubiella could estimate the entropic limit of the preferential interaction parameter $\Delta\Gamma'_{23}$ resulting for a total exclusion of the kosmotropic ions from the surface of the unfolded protein. In this case $\Delta\Gamma'_{23} \cong -\Delta V$ with ΔV being the change of the volume inaccessible for kosmotropic ions upon unfolding the protein. This parameter can be estimated from the change of the solvent accessible surface area (SASA) effected by unfolding and a length parameter l ($\sim$ 0.1 nm) describing the thickness of the layer inaccessible for the ions. The estimate of the m-value derived from this calculation compares favorably with the measured values (*cf.* Table 3 of ref. 29). In this limit, the m-value (see eqn (16)) becomes independent of temperature and the $K_{p,\pm}$ as defined through eqn (11) are much smaller than unity. If, on the other hand, the $K_{p,\pm}$ are approximately unity, the m-value will be small but exhibit a considerable dependence on temperature (*cf.* eqn (11)). In this situation the dependence of the free energy of unfolding should depend quadratically on ΔT_m which has been found previously for the complex formation of polyelectrolytes with proteins.[34] It should be kept in mind, that these considerations disregard the counterion release term in eqn (23). The m-value observed for charged systems where $\Delta n_{ci} \neq 0$, will differ considerably and the predictions of the SPM are related only to the parameter Δw as defined through eqn (10).

In principle, eqn (23) and eqn (26) define stability curves as defined by Becktel and Schellman[3] inasmuch as they describe the free energy ΔG_u as the function of temperature and salt concentration. If $\Delta c_{p,0}$ may be regarded as constant throughout a temperature range of sufficient width, the present approach could be used to construct $\Delta G_u(T,c_s)$ for all pertinent temperatures ranging from cold to thermal denaturation. Given the fact, however, that $\Delta c_{p,0}$ depends on temperature,[7] such stability curves should be regarded with caution.

Results and discussion

Basic predictions of the model

The basis of the present model is eqn (5) which is general except for the neglect of the activity coefficients of the solute. Previous discussions, however, have shown that this approximation is inconsequential and will only change slightly the resulting parameters.[4,33,34] Eqn (5) or its integrated from has been used very often to analyze the release of water upon complex formation of highly charged macromolecules as *e.g.* DNA with various proteins.[33,46,47,50,51] It is thus interesting to compare its magnitude for complex formation with values deriving from protein unfolding. Evidently, the parameter Δw introduced by this equation does not give the number of released water molecules defined as Δn_w but measures the thermodynamic effect of this release (see the discussion of eqn (11) above).[34]

A next prerequisite is the independence of Δn_{ci} on temperature. As already mentioned above, this fact is well-borne out of a large bulk of experimental data and can safely be assumed here as well (see *e.g.* the discussion by Privalov *et al.*[32] and in ref. 34, 37 and 52–54). This fact allows us to use the Maxwell-relation eqn (7) for the next step in which the salt dependence of the unfolding enthalpy ΔH_u is related to the dependence of the parameterΔw on temperature given through eqn (9). Hence, if ΔH_u turns out to depend on the concentration c_s of the solute, it necessarily follows that Δw is not a constant but depends on temperature. This fact is one of the central points inasmuch it shows that in this case the m-value given here by eqn (16) contains a term depending quadratically on the difference $T_m - T_m^0$.

The above model hence makes the following predictions that can compared directly to experiments:

(1) In a first step of the analysis of experimental data, dependence of ΔH_u on salt concentration c_s can be checked. Eqn (8) demonstrates that this quantity is a function of temperature and salt concentration c_s. Moreover, the dependence of ΔH_u on salt concentration c_s gives the dependence of the quantity Δw on temperature as shown by the Maxwell-relation in eqn (7) which in turn leads to the dependence of the m-value on temperature eqn (16). Evidently, if ΔH_u is found to depend on salt concentration, there must be a finite dependence of m on temperature as well (eqn (16)). If, on the other hand, the dependence of ΔH_u on salt concentration c_s is small, the parameter $dc_p/dc_s \cong 0$ and the terms in eqn (23) and (25) depend only on T_m, that is, the quadratic term can be dismissed. Hence, the evaluation of experimental data can begin by a critical check of $\Delta H_u(T,c_s)$.

(2) The term scaling with ln c_s will profoundly change the dependence of the free energy on salt concentration and this dependence will be most marked for small c_s (*cf.* eqn (6)). The dependence of T_m on c_s will therefore be non-linear at small c_s if Δn_{ci} assumes a finite value. Since the effect embodied in this parameter is of entirely entropic origin, the non-linear dependence on c_s thus effected should be independent of the nature of the added salt of same valency, that is, T_m should be a universal function of c_s for small c_s. Hofmeister effects are

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expected to come into play only for higher salt concentrations where $\Delta\Delta G_u$ scales linearly with c_s. Hence, T_m is expected to be independent on the nature of the salt ions if the salt concentration is small. The observation of this effect, however, requires a small $c_{s,0}$ and precise measurements at concentrations only slightly larger than $c_{s,0}$. Evidently, the ionic effect embodied in Δn_{ci} and the change of T_m by hydration may cancel each other. Thus, if $\Delta n_{ci} < 0$ as well as $\Delta w < 0$, eqn (23) demonstrates that can lead to $\Delta T_m = 0$ for a finite salt concentration. This problem has already been discussed by Chudoba *et al.*[30] and is seen directly in the study of the unfolding of RNase A.[16] Similar observations have also been made for thermophilic proteins.[56,57] The present theory allows us to model this effect in terms of the parameters Δn_{ci} and Δw.

(3) If the term quadratic in eqn (23) and (25) can be disregarded, that is, for small ΔT, the combination of both expressions shows that in this case

$$\Delta\Delta G_u \approx \Delta H_u^0 \frac{\Delta T}{T_m} \tag{28}$$

which predicts that $\Delta\Delta G_u/\Delta H_u^0 \approx \Delta T/T_m$ should be an universal function. Hence, for small ΔT, this equation may be used to check the internal consistency of data (*cf.* the discussion of this point by Senske *et al.*[16]).

Evaluation of data

Thus, the evaluation of the experimental data should proceed in the following steps: The unfolding is usually determined by microcalorimetric studies in which the heat change during this process is measured precisely. These measurements yield the heat of transition $\Delta H_u(c_s)$ at different concentrations of the solute c_s and the melting temperature T_m at the respective salt concentration c_s (see *e.g.* the discussion in ref. 15 and 18). In the following, the comprehensive set of data of Francisco *et al.*[18] on the unfolding of ribonuclease A in presence of sodium salts will be used to exemplify the steps of evaluation. Here, the unfolding of RNase A has been observed at a pH of 4 in 10 mM acetate buffer. Therefore, the concentration $c_{s,0} = 0.01$ M in the subsequent analysis.

As outlined above, the analysis may start by the check of the dependence of ΔH_u on c_s (see Table 1 of ref. 18). Fig. 1a displays $\Delta H_u(c_s)$ for a typical kosmotropic salt as NaCl as well as for NaSCN which provides a good example for a chaotropic system. The enthalpy of denaturation in presence of NaCl hardly depends on salt concentration whereas a marked dependence is found for NaSCN. This test splits up the experimental data sets into two classes:

(1) Small ΔT_m; kosmotropic ions: the small dependence of ΔH_u on c_s suggests that the coefficient $d\Delta c_p/dc_s$ in eqn (15), (23) and (25) can be safely neglected and the only relevant parameters are Δn_{ci} and $\Delta w(T_m^0)$. Moreover, the changes $\Delta T = T_m - T_m^0$ are rather small so the term quadratic in ΔT in eqn (23) can hardly be determined. However, this does not imply that this term is zero for kosmotropic salts in general.

(2) Large ΔT; chaotropic ions: for NaSCN there is a marked dependence of $\Delta H_u(c_s)$ on salt concentration which

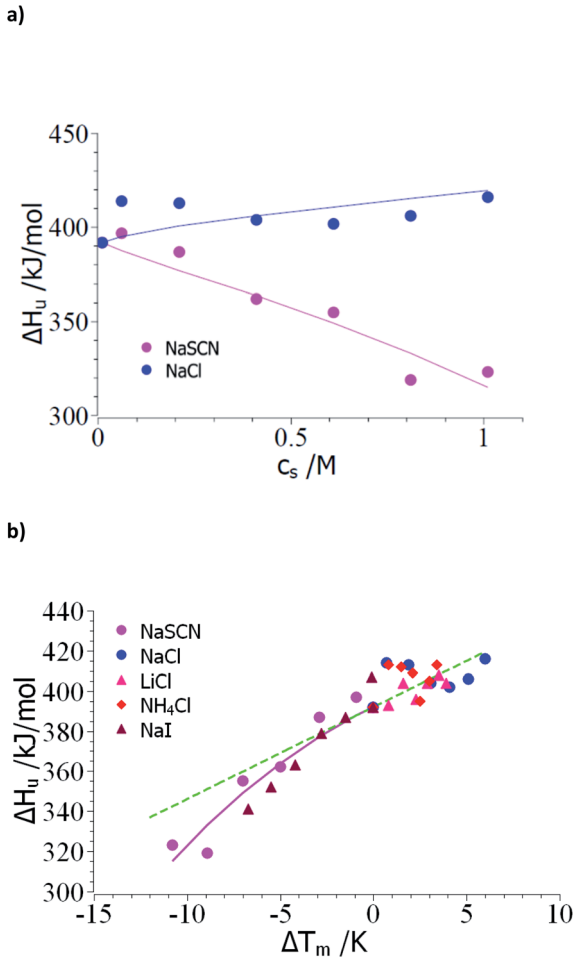

Fig. 1 Evaluation of the measured transition enthalpy ΔH_u by eqn (8). (a) ΔH_u as the function of salt concentration c_s. The marks show the experimental data for the unfolding of ribonuclease in presence of the salts indicated in the graph. These data have been taken from Table 1 of Francisco *et al.*[18] (b) ΔH_u as the function of $\Delta T_m = T_m - T_m^0$. The solid lines indicate the fit of eqn (8) whereas the green dashed line indicates the transition enthalpy calculated by eqn (8) with the average value $\Delta c_{p,0} \cong 4.6$ kJ (K^{-1} mol^{-1}) and $d\Delta c_p/dc_s = 0$. See text for further explanation.

immediately demonstrates that $d\Delta c_p/dc_s$ assumes a finite value and the *m*-value (eqn (16)) in turn depends on temperature. Moreover, the observed ΔT is much larger than in case of the kosmotropic ions. Hence, fits must take into account all terms in eqn (25).

Case (1): small ΔT_m; kosmotropic ions: Fig. 1b gathers all data of the enthalpy ΔH_u as the function of the difference $T_m - T_m^0$. The error of these numbers is of appreciable magnitude and only allows us to obtain an estimate for $\Delta c_{p,0}$ for which an evaluation for the data of all kosmotropic ions (NaCl, NH_4Cl, LiCl) gives an estimate $\Delta c_{p,0} \cong 4.6$ kJ (K^{-1} mol^{-1}) which compares well literature (see ref. 7 and 58). Hence, the subsequent evaluation is based on $d\Delta c_p/dc_s = 0$.

Fig. 2 displays a comparison of the experimental transition temperatures T_m as the function of salt concentration obtained by numerical solution of eqn (23) for $\Delta G_u = 0$. Here the data

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$T_m(c_s)$ obtained for a given salt are fitted to eqn (23) with neglect of the term quadratic in ΔT using the MathLab routine cftool (MATLAB (2021b). Natick, Massachusetts: The MathWorks Inc.). All calculations have been done using the value of the transition enthalpy in salt-free systems $\Delta H_u^0 = 392$ kJ mol^{-1} and the transition temperature $T_m^0 = 326.8$ K given by Francisco *et al.*[18] As mentioned above, the buffer added to all solutions leads to a $c_{s,0} = 0.01$ M.[18] The solid lines in Fig. 2 display the respective fits whereas Table 1 gathers the respective fit parameters. A single value of parameter Δn_{ci} turned out to describe ΔG_u for all systems under consideration here in agreement with the above general considerations. This fact has already been observed by Francisco *et al.*[18] and the presence analysis compares well with eqn (21) of ref. 18 inasmuch T_m can be described by the combination of a linear and a logarithmic term (see eqn (23)). Pegram *et al.* also found that a single parameter was sufficient to describe the dependence of the unfolding of DNA as well as for the DNA-binding domain of the lac repressor at small salt concentrations.[31] Hence, an important prediction of the present model is fully corroborated by the experimental data and the parameter $\Delta w(T_m^0)$ can be compared to data obtained for complex formation of polyelectrolytes with proteins.

The parameter Δn_{ci} is positive for all kosmotropic salt analyzed herein. This finding points to the fact that a small but finite number of ions attached closely to the surface of the protein is released during the unfolding transition. With increasing c_s these ions are released into a reservoir with increasing activity which requires additional free energy during the unfolding transition. Hence, this effect stabilizes the folded state and leads to a higher transition temperature.

The parameter $\Delta w(T_m^0)$ is negative which means that the water molecules needed for the hydration of the unfolded protein must have a higher activity as the bulk water since

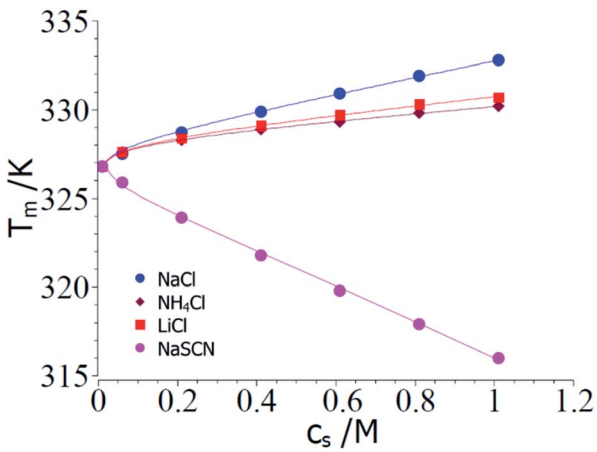

Fig. 2 Comparison of theory and experimental data taken from the denaturation of RNase A for the 3 kosmotropic salt NaCl, NH_4Cl, LiCl and for the chaotropic salt NaSCN.[18] The points show the transition temperatures taken in presence of different salts as indicated in the graph (see Table 1 of ref. 18). The solid lines mark the calculated transition temperatures T_m calculated from the fit parameters Δn_{ci} and $\Delta w(T_m^0)$ (*cf.* Table 1). See text for further explanation.

Table 1 Summary of the parameters deriving from the fits of $\Delta\Delta G_u$[a]

System	Δn_{ci}	$\Delta w(T_m^0)$	$d\Delta c_p/dc_s$
NaCl	0.17	−50.4	0
LiCl	0.17	−26.2	0
NH_4Cl	0.17	−19.4	0
NaSCN	−0.17	103	2.5

[a] Δn_{ci}: number of ions released or taken up during unfolding (eqn (3) and (4)); Δw: effect of water release or uptake (eqn (3) and (4)); $d\Delta c_p/dc_s$: parameter describing the dependence of Δw on temperature (eqn (8) and (10)).

addition of these salts increases the magnitude of ΔG_u. Hence, free energy is needed to transport water from a state of lower activity in bulk to a state of higher activity in the hydrate shell upon unfolding of the protein. This effect is due to a partial depletion of these kosmotropic ions from the hydrate shell of the protein and leads to a stabilization of the folded state. The magnitude of $\Delta w(T_m^0)$ found here is in the same range as found previously for complex formation of proteins with DNA.[47]

It should be noted that the present analysis not only treats ΔG_u but also ΔH_u at the same time. Thus, the independence of the *m*-value of temperature follows here from an analysis of the latter quantity. Only this analysis allows us to disregard the term in eqn (25) that depends quadratically on ΔT^2.

Case (2): large ΔT; chaotropic ions: in the following, the evaluation of the respective parameters will be shown using the data for NaSCN (Table 1 of ref. 18). Fig. 1b shows experimental $\Delta H_u(c_s)$ as the function of ΔT_m whereas the solid lines displays the fit of these data according to eqn (8). This fit can be stabilized by using the experimental value $\Delta H_u(c_s = 0) = 392$ kJ mol^{-1} and the specific heat $\Delta c_{p,0} = 4.6$ kJ (K^{-1} mol^{-1}) estimated from the analysis of the kosmotropic systems shown in Fig. 1a. For NaSCN we obtain for the parameter $d\Delta c_p/dc_s$ a value of *ca.* 2.5 kJ (K^{-1} mol^{-1} M^{-1}). Evidently, the small range of data and the finite accuracy of the data allows for an estimate of these quantities only. However, since these parameters present only corrections in eqn (25) and (23) and not leading terms, this error is inconsequential for the purpose at hand.

In the next step, the parameters $\Delta c_{p,0} = 4.6$ kJ (K^{-1} mol^{-1}) and $d\Delta c_p/dc_s = 2.5$ kJ (K^{-1} mol^{-1} M^{-1}) are introduced into eqn (23) and the values of Δn_{ci} and $\Delta w(T_m^0)$ are derived from a numerical solution of this equation for $\Delta G_u = 0$. Input parameters are the measured T_m measured for different NaSCN-concentrations marked by points in Fig. 2. Table 1 again gathers the data obtained from this fit whereas the solid lines in Fig. 2 displays T_m calculated with the parameters $\Delta c_{p,0} = 4.6$ kJ (K^{-1} mol^{-1}), $d\Delta c_p/dc_s = 2.5$ kJ (K^{-1} mol^{-1} M^{-1}) and the values of Δn_{ci} and $\Delta w(T_m^0)$. Again, a full description of the experimental transition temperatures is achieved. For the chaotropic salt NaSCN the parameter $\Delta w(T_m^0)$ assumes a positive value which is directly related to the fact that SCN^--ions are adsorbed on the unfolded protein chain thus lowering the activity of the hydrate water molecules. Hence, free energy is gained when hydrating the unfolded chain by bulk water having a higher activity. The parameter Δn_{ci} now has assumed a negative value. This finding

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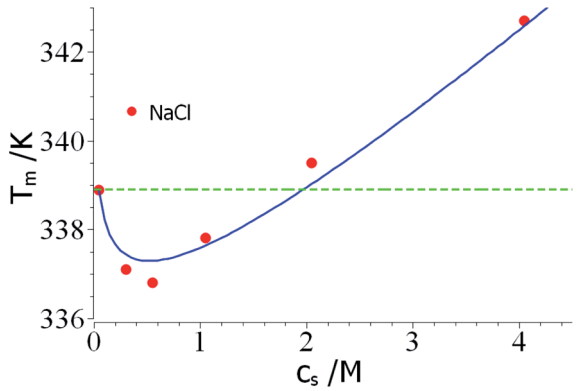

Fig. 3 Reversal of T_m through a competition of counterion release and preferential hydration. The data marked by red points have been measured by Senske *et al.* for the unfolding of RNase A at pH = 5 in presence of an increasing concentration of NaCl.[16] The solid line marks the fit by theory with the parameters $\Delta n_{ci} = -0.60$ and $\Delta w = -31.7$ (eqn (23), the term quadratic in ΔT has been neglected). The green dashed line marks the temperature T_m^0. See text for further explanation.

points to a much stronger interaction of such chaotropic ions with the unfolded protein chains. Thus, Fang and Furo could show that chaotropic ions can associate to PNIPAM chains with a Langmuir-type association behavior while NaCl is only weakly adsorbed.[59] This effect measured through careful measurements of the electrophoretic mobility was found strongest for SCN^--ions. Hence, adsorption of chaotropic ions can diminish or even reverse the effective charge of unfolded proteins. However, further investigations of T_m at very low ion concentrations are needed to clarify this problem.

As mentioned above, the combination of a negative Δn_{ci} with a negative Δw value should lead to a non-monotonic dependence of T_m on salt concentration. This effect is seen in a careful study of the unfolding of RNase A in NaCl solutions by Senske *et al.*[16] These data have been taken using a 50 mM citrate buffer at pH = 5 and are hence not directly comparable to the data of Francisco *et al.*[18] discussed above. Fig. 3 displays the data obtained for solutions with varying concentration of NaCl. Since the range of temperature is rather small, the term quadratic in ΔT in eqn (23) can be disregarded. The fit of the data is shown by the solid line in Fig. 3 and leads to $\Delta n_{ci} = -0.60$ and $\Delta w = -31.7$. At small salt concentrations, the logarithmic term in eqn (23) dominates the transition temperature. In this regime, it stabilizes the unfolded state which takes up ions from solution more easily at higher salt concentration. At higher salt concentration, the term linear in salt concentration in eqn (23) takes over and the unfolded state is now destabilized leading to a higher T_m again.

Conclusions

A phenomenological model describing the unfolding transition of proteins has been presented. Within this model, the change of T_m with the solute concentration c_s is captured by two effects: (i) the change of the number of correlated counterions Δn_{ci} during the unfolding transition, and (ii) the change of hydration expressed through the parameter Δw. The latter parameter is not directly the number of water molecules released or taken up during transition but described the change of the free energy by the release or uptake of water (see the discussion of eqn (11)). The model can be cast in terms of the closed expression eqn (23) giving the free energy of unfolding in terms of the salt/solute concentration c_s. The enthalpy ΔH_u can directly be related to the parameter Δw by the Maxwell-relation eqn (7) leading to eqn (8) in which a new parameter $d\Delta c_p/dc_s$ describes the direct dependence of ΔH_u on salt concentration. The model allows us to discuss the classical *m*-value in terms of these parameters (eqn (16)) and predicts that *m* is depending on temperature if the parameter $d\Delta c_p/dc_s$ assumes a finite value. A first comparison with experimental data taken from literature shows the general validity of the model.

Conflicts of interest

There are no conflicts to declare.

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