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THE EFFECT OF *pH* ON THERMAL STABILITY OF GLOBULAR PROTEINS
A critical insight

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Abstract

In this study we try to re-analyze the *pH* dependence of thermal stability of small globular proteins. From the thermodynamic point of view a long series of calorimetric and spectroscopic investigations has shown that the decreased stability in very acidic conditions can be ascribed to entropic effects. The same conclusion is reached, from a microscopic point of view, by assuming that a binding of protons on equal and noninteracting sites takes place as a consequence of unfolding process. By linking the conformational unfolding equilibrium to the proton binding equilibrium, a model is developed that is able to describe the dependence on the *pH* of the thermal denaturation processes of small globular proteins. The application of the model to hen lysozyme and T4 lysozyme correctly accounts for the experimental results.

Keywords: calorimetry, globular proteins, *pH* dependence of thermal stability, spectroscopic investigations, thermodynamics

Introduction

It is well known that all the physico-chemical properties of globular proteins, and in particular the catalytic efficiency of enzymes, are strongly *pH* dependent. Performing spectroscopic or calorimetric measurements it has been established that the unfolding temperature considerably varies at changing the *pH* of solution for all the proteins [1–5]. Usually, at decreasing the *pH* from neutrality, the thermal stability diminishes and in very acidic conditions the native structure is just denatured at room temperature. The traditional explanation of this behaviour relies on the classical electrostatics and follows the lines of the

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theoretical treatment of Linderstrom-Lang [6]. At decreasing the *pH* of the solution, the net charge on the folded form increases, due to the protonation of some acidic groups and this causes an enhancement of the electrostatic repulsion which tends to destabilize the native structure, because the charge density is greater for the native state than for the denatured one. Thus, in very acidic conditions, the unfolding process results thermodynamically favoured, because it gives rise to a state with a lower electrostatic free energy. Similar arguments account for the stability decrease observed in highly basic solutions due to repulsion of negative charges. The theory predicts maximum stability near the isoelectric point of the protein, where the net charge is zero. However a number of proteins, with acidic or basic isoelectric points, show their maximum thermodynamic stability still near to neutrality. This observation suggests that, in addition to overall charge, other considerations are important in determining the contribution of ionizable groups to the overall folding energy of globular proteins. For example Dill and Stigter show, in a theoretical study, that when there are buried, nontitratable ionic groups, the electrostatic contribution becomes asymmetric and the maximum stability of the protein is not at the isoelectric point [7]. Moreover, in a recent paper, Dahlquist and coworkers [8], have pointed out that the presence, in the native structure, of a salt bridge between the side chains of histidine 31 and aspartic acid 70 with pK_a values very different from those in the unfolded state, can account for the maximum of thermodynamic stability near *pH* 5.5 of bacteriophage T4 lysozyme, whose isoelectric point is above *pH* 10.

Most thermodynamic studies on the influence of *pH* on stability of native conformation have been devoted to the investigation of temperature-induced denaturation at different *pH* values. In this manner it is possible to determine how protein stability depends on solution *pH*. The election technique for these purposes is the differential scanning calorimetry, DSC, because it provides a direct thermodynamic method for evaluating the stability of native conformation [9]. Indeed in a DSC measurement the heat capacity of protein solution is recorded as function of temperature. Being the heat capacity the second temperature derivative of the Gibbs energy, every of variations reflects on stability of native conformation. It is worthnoting that throughout this work we will always refer to proteins whose denaturation process is well represented by a two-state transition. Because in a two-state transition the number of moles does not change and the activity coefficients in very dilute solutions can be assumed as constant in the whole investigated temperature range, we believe right to consider as standard the changes of thermodynamic functions associated to the denaturation process of globular proteins [10].

The aim of this paper is to combine the well established general thermodynamic features with a reasonable average microscopic model of the effect of *pH*,

to gain a better rationalization of the results derived from the experimental DSC measurements. Usually, in very acidic conditions, the decreased thermodynamic stability is ascribed to an uptake of protons by some protein groups. We have developed a thermodynamic model, by linking the conformational unfolding equilibrium to the proton binding on equal and noninteracting sites, that is able to characterize the dependence of the excess heat capacity function (i.e. the physico-chemical observable of DSC measurements), from both the temperature and *pH*. This analysis correctly accounts for the thermodynamic results determined for most globular proteins. Finally the proposed model is applied to the thermal unfolding process of two small globular proteins, hen egg white lysozyme and bacteriophage T4 lysozyme, both of which showing a strong *pH*-dependence of denaturation temperature.

Thermodynamic description

From differential scanning calorimetric measurements it has been possible to derive a very significant and general information. Privalov and coworkers have shown for a series of small globular proteins that plotting the values of the denaturation enthalpy change at the denaturation temperature, $\Delta_d H^\circ(T_d)$, *vs.* the values of the denaturation temperature, T_d, determined for the same protein at changing the *pH* of the solution, a straight line is obtained [9]. Therefore the net standard heat capacity change, $\Delta_d C_p^\circ$, determined form the slope of these plots, results *pH* independent too, and constant with respect to temperature. Thus, the physically correct manner for evaluating $\Delta_d C_p^\circ$ is to calculate the slope of a plot of $\Delta_d H^\circ(T_d)$ *vs.* T_d for the investigated protein. Taking in mind these findings, it is possible to define the denaturation enthalpy function according to the following relation:

$$\Delta_d H^\circ(T) = \Delta_d H^\circ(T_R) + \Delta_d C_p^\circ(T - T_R) \quad (1)$$

where T_R is a selected reference temperature. The Eq. (1) represents a straight line, describing the behaviour of the protein whatever are the *pH* values. This experimental evidence demonstrates that the standard enthalpy change associated to the unfolding process, $\Delta_d H^\circ(T)$, is a universal function of state for a defined protein, because it results *pH* independent and dependent only on temperature. The apparent *pH* dependence of $\Delta_d H^\circ(T_d)$ is the result of the denaturation temperature T_d changes at changing *pH*. However, at the temperature T_d the standard Gibbs energy change $\Delta_d G^\circ$ vanishes:

$$\Delta_d G^\circ(T_d) = \Delta_d H^\circ(T_d) - T_d \cdot \Delta_d S^\circ(T_d) = 0 \quad (2)$$

as consequence the standard denaturation entropy, $\Delta_d S^\circ(T)$, results a *pH*-dependent function. Indeed the function $\Delta_d S^\circ(T)$ is given by the following relation:

$$\Delta_d S^\circ(T) = \Delta_d S^\circ(T_R) + \Delta_d C_p^\circ \cdot \ln(T / T_R) \quad (3)$$

where T_R is a selected reference temperature and the term $\Delta_d S^\circ(T_R)$ is given by:

$$\Delta_d S^\circ(T_R) = [\Delta_d H^\circ(T_d) / T_d] + \Delta_d C_p^\circ \cdot \ln(T_R / T_d) \quad (4)$$

This expression, clearly, shows the dependence of the standard entropy function on the denaturation temperature and therefore on the *pH*. Even if the value of T_R is held fixed, the value of T_d changes at changing the *pH* of the solution and the Eq. (3) represents a different function for each *pH* value, namely it does not give a single curve, but a family of curves, representative of the protein behaviour. In Fig. 1 are reported the functions $\Delta_d H^\circ(T)$ and $T \cdot \Delta_d S^\circ(T)$ of a two-state transition at two *pH* values, assuming that the effect of *pH* can be described according to the previous analysis (i.e. $\Delta_d H^\circ(T_d)$ is a linear function of T_d). The values of thermodynamic parameters used for drawing this figure are collected in Table 1. They have been selected for demonstration purposes to obtain a better separation between the curves. As expected the function $\Delta_d H^\circ(T)$ does not change at changing the *pH* and the temperature where it vanishes, T_H, results a universal temperature for a protein. Instead the function $T \cdot \Delta_d S^\circ(T)$ strongly depends on the solution *pH*, because it depends on the denaturation temperature value. From a different point of view, one can state that the denaturation temperature variation at changing the solution *pH* is due to a net variation of the standard denaturation entropy. The *pH* dependence of thermal stability is so

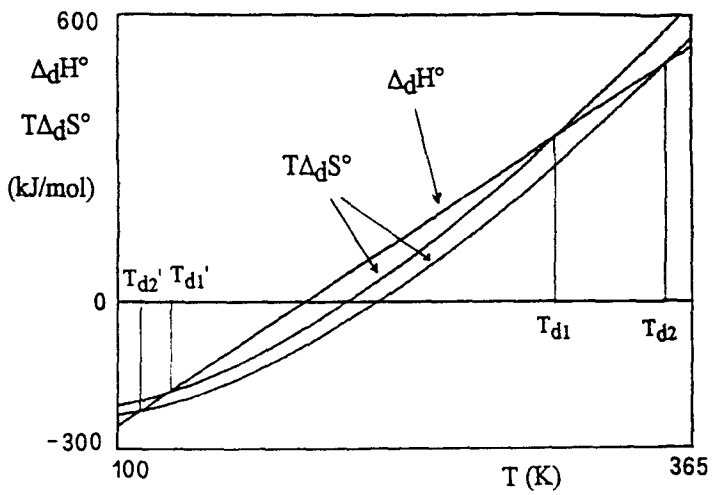

Fig. 1 Plot of the thermodynamic functions $\Delta_d H^\circ$ and $T\Delta_d S^\circ$ for two different *pH* values, according to the general behaviour of small globular proteins

caused by entropic effects. Clearly, the temperature where the function $\Delta_d S^\circ(T)$ vanishes, T_S, does not result a universal temperature for a protein. Finally, in the Fig. 2 some stability curves (i.e. $\Delta_d G^\circ$ *vs.* T) are shown, at different *pH* values, for a model protein whose denaturation process follows the described behaviour. It must be noted that the thermodynamic stability, measured as the value of $\Delta_d G^\circ$ at $T_{max} \equiv T_S$, lowers at decreasing the *pH* of solution.

Table 1 Values of thermodynamic parameters used for drawing the Fig. 1, that represents the typical behaviour of the functions $\Delta_d H^\circ$ and $T\Delta_d S^\circ$ for small globular proteins. The values have been only selected for display purposes to obtain a better separation of the curves

pH	T_d/°C	$\Delta_d H^\circ$ / kJ·mol^{-1}	$\Delta_d C_p^\circ$ / kJ·K^{-1}·mol^{-1}
2.0	30.0	350	3.0
6.0	80.0	500	3.0

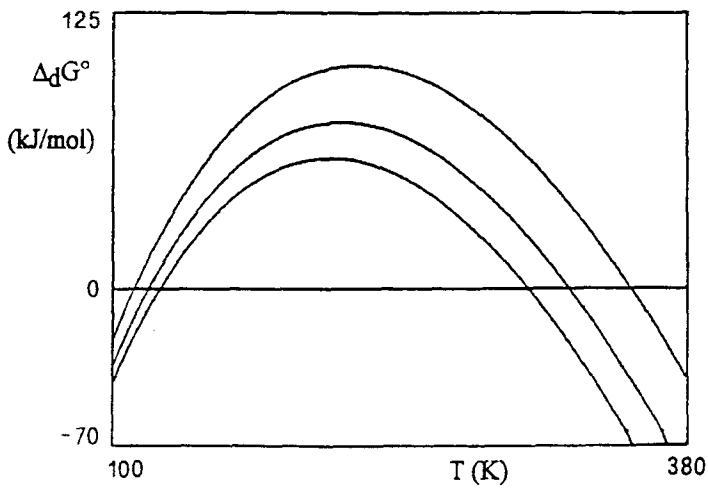

Fig. 2 A series of stability curves, $\Delta_d G^\circ$ *vs.* T, at different *pH* values, for a model globular protein, whose behaviour follows the features outlined in the text (see Thermodynamic description)

The preferential binding model

The decrease of protein thermodynamic stability at decreasing the *pH* of the solution suggests that a preferential binding of a certain number of protons on the unfolded state of the macromolecule takes place. This idea seems reasonable from the microscopic point of view because it corresponds to suppose that during the unfolding process a number of groups capable to bind protons are ex-

posed to the solvent. We want to show how this interpretation can be translated in the mathematical formulae able to describe the experimental results.

The effect of *pH* on the standard denaturation Gibbs energy $\Delta_d G^\circ$ has been theoretically analyzed by Hermans and Scheraga [11] and can be expressed with the following relation:

$$\Delta_d G^\circ = \Delta_c G^\circ - RT \ln\left(\sum^D / \sum^N\right) \quad (5)$$

where the term $\Delta_c G^\circ$ represents the conformational Gibbs energy change occurring during the denaturation, without considering variations in the ionization state of protein; Σ^D and Σ^N are the binding polynomials describing the denaturated and native state, respectively. Formally, the *pH* dependence of the unfolding equilibrium arises from the different values of the proton binding polynomials in the two states at various proton concentrations. Assuming that protons bind on identical and independent sites, the expressions of the binding polynomials take the form [12]:

$$\sum^D = (1 + K_{b,D}[H^+])^{n_D} \quad (6)$$

$$\sum^N = (1 + K_{b,N}[H^+])^{n_N} \quad (7)$$

where $[H^+]$ represents the molar concentrational of protons, $K_{b,D}$ and $K_{b,N}$ are the association constants of the protons on the sites of denaturated and native state, and n_D and n_N represent the number of binding sites on denaturated and native state, respectively. Assuming: $K_{b,D} = K_{b,N}$, as a reasonable basis, because identical are the chemical groups to which binds a proton, the Eq. (5) becomes:

$$\Delta_d G^\circ = \Delta_c G^\circ - \Delta nRT \ln(1 + K_b[H^+]) \quad (8)$$

where $\Delta n = n_D - n_N$ represents the number of proton binding sites that become accessible as a consequence of unfolding process (for instance, due to the exposure of previously buried ionic groups or breaking of salt bridges). The Gibbs energy change associated to the proton preferential binding is given by:

$$\Delta\Delta_d G^\circ = - \Delta nRT \ln(1 + K_b[H^+]) \quad (9)$$

It is physically significant to determine the enthalpic and entropic contribution in order to clarify the origin of this free energy change. From classical equilibrium thermodynamics relations, we obtain:

$$\Delta\Delta_d H^\circ = \Delta n \Delta_b H[(K_b[H^+]) / (1 + K_b[H^+]) \quad (10)$$

$$\Delta\Delta_d S^\circ = \Delta nR \ln(1 + K_b[H^+]) + (\Delta n / T)\Delta_b H[(K_b[H^+]) / (1 + K_b[H^+])] \quad (11)$$

where $\Delta_b H$ represents the enthalpy change associated to the binding equilibrium. The Eq. (9) can be then obtained by combining the Eqs (10) and (11).

This analysis demonstrates that the destabilizing effect of the proton preferential binding on the unfolded form is of entropic origin. In other words, describing the process with a model that hypothesizes the proton binding on identical and independent sites, the Gibbs energy change $\Delta\Delta_d G^\circ$ is due to a net entropic variation, because the enthalpic variation is completely compensated by the second term on the rightside of entropic contribution (Eq. 11). This finding is in very good agreement with the experimental data which show, as summarized above, that the overall entropy change associated to the denaturation process is strongly *pH* dependent.

Actually, the model points out that even the enthalpy function changes at varying the *pH* of the solution, but its change is perfectly balanced by an opposite entropic term. Thus, the whole *pH* dependence of protein thermodynamic stability has to be ascribed to a net entropy change. This entropic contribution to the Gibbs energy change due to the proton binding, arises from the configurational disorder associated to occupied and unoccupied sites. The disorder is larger for the unfolded state because the number of binding sites is greater than in native state. Clearly, the above interpretation is strictly correct only if the hypothesis of noninteracting and equal binding sites is valid. It is noteworthy, however, that, in a series of theoretical investigations, Stigter and Dill have found that a significant fraction of the electrostatic Gibbs energy for the unfolding process is predicted to arise from the entropy of proton binding, rather than simply from the charge energetics [7, 13–15]. It must be remembered that, performing DSC measurements, it is impossible to obtain a microscopic picture of the investigated system, but only average thermodynamic values. Because the protonation enthalpy of acidic groups is very low with respect to the denaturation enthalpy, whereas the entropy change due to the proton binding is significant, it has been experimentally determined that $\Delta_d H^\circ$ is *pH* independent and function of temperature alone, instead $\Delta_d S^\circ$ results strongly dependent on proton molar concentration, $[H^+]$.

On these bases it is possible to derive an analytical expression of the excess heat capacity function $<\Delta C_p(T)>$, in which appears an explicit dependence from the proton molar concentration, $[H^+]$. In the hypothesis that the process is a two-state transition $N <=> D$, the canonical partition function of the system, assuming the folded form as reference, is given by [16]:

$$Q(T) = 1 + K(T) = 1 + K^\circ(1 + K_b[H^+])^{\Delta n} \qquad (12)$$

where $K(T)$ represents the overall equilibrium constant of the denaturation process (i.e. unfolding plus binding); K° is the constant of the purely conformational equilibrium and depends on temperature according to this relation:

$$K^\circ(T) = \exp\{ - (\Delta_c H^\circ / R)(1 / T - 1 / T_c) - (\Delta_c C_p^\circ / R)[1 - T_c / T - \ln(T/T_c)]\} \quad (13)$$

where $\Delta_c H^\circ$, $\Delta_c C_p^\circ$ *e* T_c are the enthalpy, heat capacity change and the transition temperature associated to the purely conformational equilibrium. Instead K_b is the constant of the binding equilibrium and is given by:

$$K_b(T) = K_b^\circ \exp(- \Delta_b H / R)(1 / T - 1 / T_c) \quad (14)$$

where $K_b^\circ = K_b(T_c)$ and $\Delta_b H$ is assumed temperature independent. The population fractions of native and denatured state are, respectively:

$$f_N = 1 / Q \qquad f_D = \{K^\circ(1 + K_b[H^+])^{\Delta n}\} / Q \quad (15)$$

The excess enthalpy function is calculated from the partition function according to the general statistical thermodynamic equation:

$$<\Delta H(T)> = RT^2[\partial \ln Q / \partial T] \quad (16)$$

and it assumes the following form:

$$<\Delta H> = [\Delta_c H^\circ(T_c) + \Delta_c C_p^\circ(T - T_c) + \Delta n \Delta_b H(K_b[H^+]) / (1 + K_b[H^+])] \cdot f_D \quad (17)$$

The mean square excess enthalpy function is given in turn by:

$$<\Delta H^2> = [\Delta_c H^\circ(T_c) + \Delta_c C_p^\circ(T - T_c) + \Delta n \Delta_b H(K_b[H^+]) / (1 + K_b[H^+])]^2 \cdot f_D \quad (18)$$

Finally, the excess heat capacity function is determined by means of a fundamental relation of statistical thermodynamics:

$$<\Delta C_p(T)> = [(<\Delta H^2> - <\Delta H>^2) / RT^2] + f_D \Delta_c C_p^\circ \quad (19)$$

In this manner we obtained an analytical expression of the excess heat capacity, the observable of DSC experiments, where the unfolding conformational equilibrium has been linked to the proton preferential binding. Really, the Eq. (19) allows to define a heat capacity surface that shows the dependence of $<\Delta C_p>$ from both the temperature and *pH* of solution, for a complete characterization of the protein thermal stability [17, 18]. It must be stressed that a single set of thermodynamic parameters (i.e. T_c, $\Delta_c H^\circ(T_c)$, $\Delta_c C_p^\circ$, K_b°, $\Delta_b H$ and Δn), is sufficient for an overall description of the heat capacity surface $<\Delta C_p>$ *vs.T vs. pH.*

Application of the model and discussion

We want to show that the excess heat capacity function, defined by Eq. (19), is able to correctly account for the experimental effect of lowering the *pH* of solution on DSC profiles of small globular proteins. For this purpose we selected two studied and well characterized proteins: the hen egg white lysozyme and the bacteriophage T4 lysozyme. These proteins show a markedly *pH*-dependence of their thermal stability in the range 2.0–4.5 and 2.0–5.5, respectively. Further detailed calorimetric and spectroscopic investigations have unequivocally established that their thermal denaturation process is a two-state transition and there is no evidence of acidic denaturation at room temperature down to at least *pH* = 2.0 [19–24]. This point is remarkable, because we have assumed as reference the protein native state at whatever *pH*.

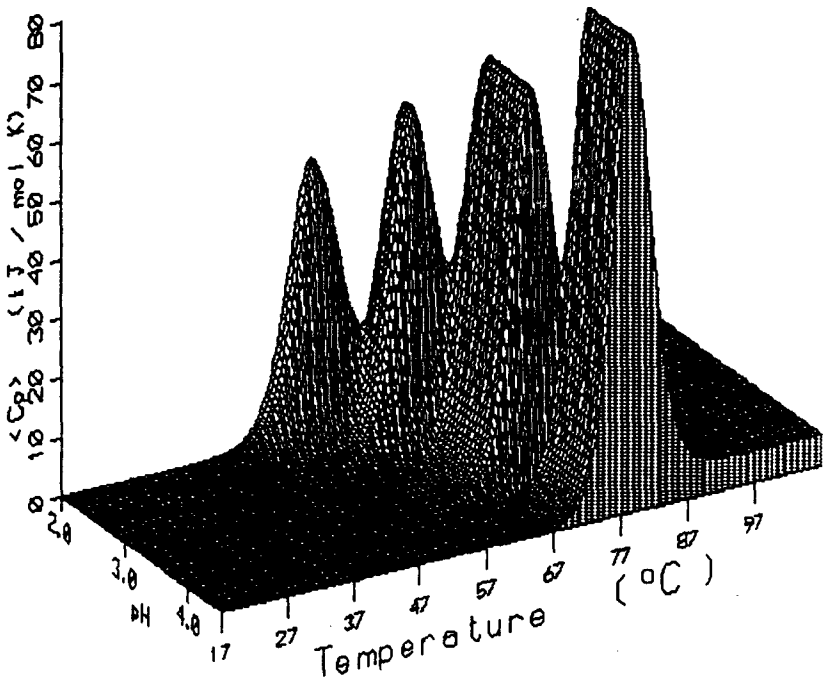

Fig. 3 Excess heat capacity surface, $<\Delta C_p>$ *vs. T vs. pH*, constructed with the DSC curves, simulated using the Eq. (19), that are the best fit of experimental DSC profiles of hen lysozyme

In the Fig. 3 are reported the DSC curves, simulated using the Eq. (19), that represent the best fit of experimental DSC curves of hen egg white lysozyme in the same *pH* conditions and ionic strength 0.1 *M* NaCl [19]. The values of thermodynamic parameters used for this simulation are: T_c = 78.8°C,

$\Delta_c H^\circ(T_c)$ = 569.0 kJ/mol, $\Delta_c C_p^\circ$ = 5.45 kJ/K·mol, K_b° = 1290 M^{-1}, $\Delta_b H$ = −9.2 kJ/mol, Δn = 4.5 and proton molar concentrations corresponding to the *pH* values of solution.

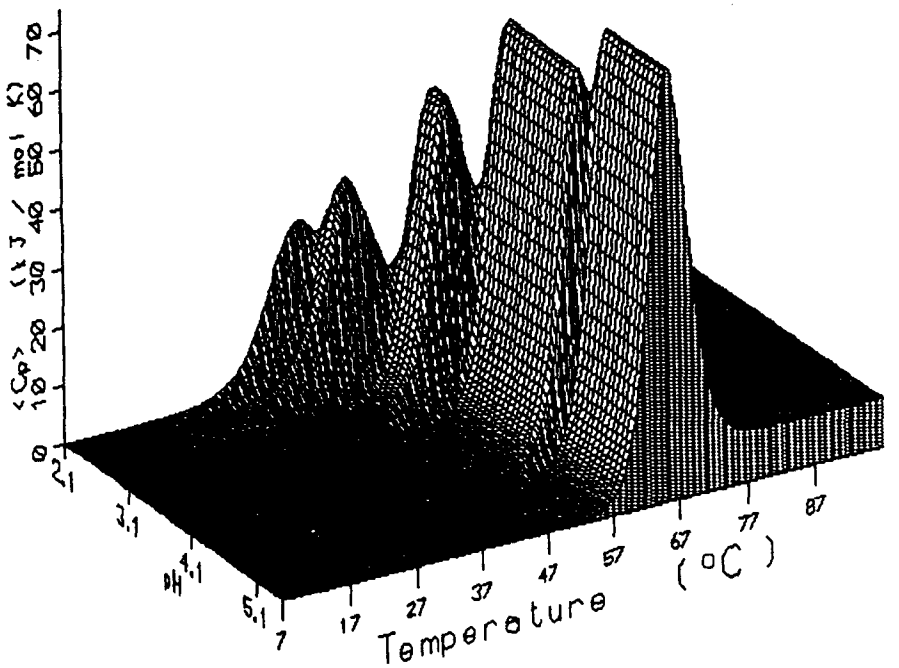

Fig. 4 Excess heat capacity surface, $<\Delta C_p>$ *vs. T vs. pH*, constructed with the DSC curves, simulated using the Eq. (19), that are the best fit of the experimental thermodynamic values of T4 lysozyme

In the Fig. 4 are reported DSC curves, simulated using the Eq. (19), that represent the best fit of the experimental values determined by Schellman's group [22–24] through a van't Hoff analysis of the circular dicroism measurements of T4 lysozyme denaturation, in the same *pH* conditions and ionic strength 0.2 *M* NaCl. The values of thermodynamic parameters used for this simulation are: T_c = 65.7°C, $\Delta_c H^\circ(T_c)$ = 512.8 kJ/mol, $\Delta_c C_p^\circ$ = 8.50 kJ/K·mol, K_b° = 1300 M^{-1}, $\Delta_b H$ = −9.5 kJ/mol, Δn = 4 and proton molar concentrations corresponding to the *pH* values of solution.

It is significant that the determined values of binding enthalpy and association constant are nearly the same for both the proteins (i.e. K_b(25°C) = 2275 M^{-1} for hen lysozyme and K_b(25°C) = 2060 M^{-1} for T4 lysozyme), suggesting that the fitting procedure does not give rise to artifacts. Further, it is worthnoting that the ionization enthalpy of carboxylic groups, which are presumably responsible for proton uptake, is of the same order of magnitude of the determined binding enthalpy values. It must be stressed that the values of Δn do

not necessarily must find a strict correspondence in the amino acid sequence of proteins. Indeed the model does not consider the structural details of a protein, but only tries to put certain aspects of *pH* influence in an interesting light. For this reason the values of Δn must be considered of heuristic nature. In any case it has been firmly established that the number of protons transferred during a two-state denaturation process can be calculated with the following relation [25]:

$$\Delta n = -[\Delta_d H^\circ(T_d) / 2.3R \cdot T_d^2] \cdot [dT_d / dpH] \quad (20)$$

By applying this equation to their experimental results Privalov and coworkers found for Δn a value of 3.0–3.5, depending on the *pH*, for hen lysozyme [20]; while Schellman and coworkers determined a value of 2.8–3.0 for T4 lysozyme [22]. Then the values of Δn obtained according to our analysis are not far away from those determined with Eq. (20).

The agreement between simulated and experimental curves is satisfactory and so we can state that the model, even though very simple, gives a realistic explanation of the phenomenon. In Tables 2 and 3 are reported for each *pH* the values of T_d experimentally determined, and the values of T_d and $\Delta_d H^\circ(T_d)$ for hen lysozyme and T4 lysozyme, respectively, obtained from the analysis of the single simulated curves, performed by Theseus program [26]. Plotting the values of $\Delta_d H^\circ(T_d)$ *vs.* the values of T_d a linear trend is observed for both the proteins. Performing a least squares regression, we have determined a linear

Table 2 Values of thermodynamic parameters obtained from the analysis of simulated DSC curves of hen lysozyme. In the second column are reported the experimental values of denaturation temperature at ionic strength of 0.1 *M* NaCl [19]

pH	$T_{d,exp}$/°C	$T_{d,sim}$/°C	$\Delta_d H^\circ_{sim}$ / kJ·mol^{-1}
2.0	55.0	54.85	396.6
2.5	64.5	64.65	455.5
3.0	71.9	72.05	506.0
4.5	78.6	78.60	565.6

Table 3 Values of thermodynamic parameters obtained from the analysis of simulated DSC curves of T4 lysozyme. In the second column are reported the experimental values of denaturation temperature at ionic strength of 0.2 *M* NaCl [22, 23]

pH	$T_{d,exp}$/°C	$T_{d,sim}$/°C	$\Delta_d H^\circ_{sim}$ / kJ·mol^{-1}
2.1	42.5	42.65	276.0
2.3	47.6	47.55	323.0
2.8	56.9	57.05	412.0
3.5	63.0	63.05	479.1
5.5	65.5	65.50	512.4

correlation coefficient of 0.996 and a slope of 7.0±0.45 kJ/K·mol in the case of hen lysozyme; and a linear correlation coefficient of 0.998 and a slope of 10.2±0.35 kJ/K·mol in the case of T4 lysozyme. The slope of these plots, as known, corresponds to the net heat capacity change associated to the denaturation process and the determined values are in very good agreement with the experimental ones, $\Delta_d C_p^\circ = 6.7$ kJ/K·mol for hen lysozyme [27], and $\Delta_d C_p^\circ = 9.5$ kJ/K·mol for T4 lysozyme [22]. Finally, the linear relationship between $\Delta_d H^\circ(T_d)$ and T_d, derived from the analysis of simulated curves for both the proteins, strictly corresponds to the experimental findings (i.e. $\Delta_d H^\circ$ is *pH* independent and function of temperature alone).

The fundamental hypotheses of the developed model seem very drastic and not corresponding to the physical reality. For instance, because the proton binding sites are electrically charged, they interact between each other through Coulomb's long range forces and thus the independence of the binding sites is not strictly valid. However, these hypotheses lead to a great mathematical simplification of the problem, making possible a direct analytical solution and a reasonable explanation of experimental results. Moreover, assuming that the sites are noninteracting and the intrinsic binding constants are equal for both the native and denaturated states, the net contribution, arising from the groups which are exposed to the solvent in both these states, is zero. In this manner the model allows to take in explicit consideration only the contribution thermodynamic stability due to the groups, fully buried inside the protein core, that during the unfolding process become exposed to the solvent contact. Clearly, there is no direct connection between the phenomenological proton binding constant of the model and the acidic dissociation constant of a carboxylic group, because the uptake of protons is caused by the exposure to the solvent of previously buried groups (i.e. by a change of the environment experienced by a group) and does not concern a simple acid-base equilibrium.

In conclusion, it has been pointed out that the *pH* dependence of protein thermodynamic stability is of entropic origin. Further, the obtained results show that a very simple model, based on the hypothesis that, during the unfolding process a certain number of protons binds preferentially on identical and independent sites of denaturated state, can account for the major features of the *pH* influence on the thermal denaturation process of small globular proteins and then on their thermodynamic stability.

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Zusammenfassung — Vorliegend versuchen wir eine Reanalyse der thermischen Stabilität von kleinen Globularproteinen. Unter thermodynamischen Aspekten zeigte eine lange Reihe von kalorimetrischen und spektroskopischen Untersuchungen, daß die verminderte Stabilität in sehr saurem Milieu Entropie-Effekten zugeschrieben werden kann. Zu der gleichen Schlußfolgerung gelangt man unter mikroskopischen Gesichtspunkten unter der Annahme, daß an entsprechenden Stellen, die keine Wechselwirkung eingehen, infolge eines Entfaltungsprozesses die Bindung von Protonen stattfindet. Durch Verbindung des Konformations-Entfaltungsgleichgewichtes mit dem Protonenbindungsgleichgewicht wurde ein Modell entwickelt, welches sich zur Beschreibung der *pH*-Abhängigkeit des thermischen Denaturationsprozesses von kleinen Globularproteinen eignet. Die Anwendung des Modelles an Hühner-Lysozym und T4 Lysozym erklärt korrekt die experimentellen Ergebnisse.