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THERMODYNAMIC INVESTIGATIONS OF PROTEINS

I. Standard functions for proteins with lysozyme as an example

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A direct method is proposed for obtaining thermodynamic standard functions for native and denatured proteins using experimental data from scanning calorimetry, isothermal calorimetry and potentiometric titrations. The possibility of this approach is demonstrated on the example of lysozyme in the range of pH 1.5–7.0 and temperature 0–100°C. Tests for the validity of the obtained functions of enthalpy and entropy are presented in the form of cyclic processes using experimental data obtained from thermodynamically different pathways. The Gibbs function is checked by comparison with results of an independent method. The methodic problems in determining and checking standard functions for proteins are discussed in detail.

1. Introduction

1.1. General

Many problems concerning the physics of conformational transitions of proteins, the stability of the spatial structure of biopolymers and their participance in intermolecular interactions are too complicated to be treated thermodynamically. Proteins, in particular can exist in different states of biological significance such as in various conformational states, in ligand bonded forms, etc., and each of these states can depend on various states determining various parameters like temperature, pH, activity of low and high molecular admixtures, etc. A description of biochemical systems in terms of thermodynamic functions requires of course an adequate comprehensive treatment.

Many efforts have been undertaken to determine the changes in enthalpy, entropy and Gibbs energy especially upon unfolding of proteins [1–4]. Unfortunately the thermodynamic parameters obtained under quite different conditions of temperature, pH and denaturant are difficult to compare and to use in practice. These difficulties result from limitations in knowledge of the functional dependences of ΔH, ΔS and ΔG on external variables (c.f., for example, the data summarized for lysozyme [5]). Furthermore the most widely used, indirect methods for determining thermodynamic parameters (by means of the temperature dependence of thermodynamic equilibrium states) include several principal a priori assumptions referring to distinct states and absence of intermediates. However there is some doubt about the validity of these assumptions.

These general problems in the thermodynamics of biomacromolecules compelled an elaboration of experimental procedures to obtain thermodynamic standard functions of *single states* of proteins in a *wide parameter range without any extra assumptions*. Based on modern instrumentation evidence is given in this paper for the existence of distinct thermodynamic states using native and unfolded lysozyme as an example.

1.2. Principles of the determination of thermodynamic standard functions of proteins

Infinitesimal changes of the enthalpy can be decomposed in different terms representing variable parameters which influence the state of proteins: temperature, pH,

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denaturant concentration, etc. (for simplicity other parameters are not considered):

$$dH = \left(\frac{\delta H}{\delta T}\right)_{pH,c} dT + \left(\frac{\delta H}{\delta pH}\right)_{T,c} d\,pH + \left(\frac{\delta H}{\delta c}\right)_{T,pH} dc. \quad (1)$$

In this paper only temperature and pH parameters are dealt with. The influence of the denaturant concentration will be discussed in the following paper [6].

Taking the state of native protein at $pH^0 = 7.0$, $T^0 = 25°C$ and the ionic strength $I^0 = 0.1$ as standard, we have for the thermodynamic functions of native protein:

$$H^{0,N}(T, pH) = \int_{T^0}^{T} \left(\frac{\delta H}{\delta T}\right)_{pH} dT + \int_{pH^0}^{pH} \left(\frac{\delta H}{\delta pH}\right)_{T} d\,pH$$

$$= \int_{T^0}^{T} [C_p]^N_{pH}\, dT + (H^N_{pH} - H^N_{pH^0})_{T^0}$$

$$= \int_{T^0}^{T} [C_p]^N_{pH}\, dT + H^{0,N}(pH). \quad (2)$$

The temperature dependent term in eq. (2) contains the partial heat capacity of the native protein in solution at given pH, $[C_p]^N_{pH}$, which is itself a temperature dependent function. This function is determinable experimentally even in diluted solutions using scanning microcalorimetry. The pH-dependent term $H^{0,N}(pH)$ describes the molar heat evolved on titration of native protein with initial $pH^0 = 7.0$ at 25°C. Such titrations can be performed using isothermal microcalorimetry.

Similarly, for the standard entropy we obtain:

$$S^{0,N}(T, pH) = \int_{T^0}^{T} \left(\frac{\delta S}{\delta T}\right)_{pH} dT + \int_{pH^0}^{pH} \left(\frac{\delta S}{\delta pH}\right)_{T} d\,pH$$

$$= \int_{T^0}^{T} \frac{[C_p]^N_{pH}}{T}\, dT + S^{0,N}(pH). \quad (3)$$

Eq. (3) contains also the temperature dependent function of the partial heat capacity $[C_p]^N_{pH}$. The pH-dependent term of the standard entropy $S^{0,N}(pH)$ cannot be obtained directly, but can be calculated if the standard enthalpy $H^{0,N}(pH)$ and the standard Gibbs function $G^{0,N}(pH)$ are known for the given standard temperature of 25°C. The latter function can be obtained from potentiometric titrations [see subsection 3.2, eqs. (9) and (10)]. Using these functions we have for the pH-dependent term of the standard entropy:

$$S^{0,N}_{pH} = \frac{H^{0,N}_{pH} - G^{0,N}_{pH}}{298.15}. \quad (4)$$

Finally, for the Gibbs energy of native protein we have:

$$G^{0,N}(T, pH) = H^{0,N}(T, pH) - TS^{0,N}(T, pH). \quad (5)$$

Corresponding functions for the denatured state can be calculated using the standard functions of the native state and the denaturational changes in enthalpy, entropy and Gibbs energy. As $(\Delta G_d)_{T_d} = 0$ at the temperature of denaturation (T_d), we have $\Delta S_d = \Delta H_d/T_d$ for the denaturational change of entropy, ΔS_d. Thus for the denatured state:

$$H^D(T, pH) = \int_{T^0}^{T_d} [C_p]^N_{pH}\, dT + [\Delta H_d]_{T_d} + \int_{T_d}^{T} [C_p]^D_{pH}\, dT + H^{0,N}(pH) \quad (6)$$

$$= [\Delta H_d]_{T_d} - \int_{T}^{T_d} (\Delta [C_p]_d)_{pH}\, dT + H^{0,N}(T, pH),$$

$$S^D(T, pH) = \frac{[\Delta H_d]_{T_d}}{T_d} - \int_{T}^{T_d} (\Delta [C_p]_d)_{pH}\, d\ln T + S^{0,N}(T, pH), \quad (7)$$

$$G^D(T, pH) = H^D(T, pH) - TS^D(T, pH). \quad (8)$$

Thus, all the data needed for establishing the complete thermodynamic standard fucntions can be obtained by a combination of the following experimental methods: scanning microcalorimetry, isothermal calorimetry and potentiometric titration. The methodic problems of this approach using lysozyme as the object are considered in this paper.

2. Materials and methods

Commercial hen egg white lysozyme (Reanal Co., Hungary) was twice recrystallized and checked as described elsewhere [7]. Stock solutions in 0.1 M NaCl were extensively dialyzed at 4°C against 0.1 M NaCl adjusted with HCl to pH 4.8 to remove inorganic impurities influencing the results of calorimetric and potentiometric titrations. Protein concentrations were

measured with a Hitachi 124 spectrophotometer taking the optical density of 1% solutions at pH 7 and 280 nm as 26.9 [8]. The molecular weight was taken as 14.307 daltons. All solutions were prepared from analytical grade reagents and glass bidistilled water.

pH measurements and potentiometric titrations were performed with a Model 262 pH-meter (USSR) using electrodes type ESL 43-07 and ESL 63-07 and thermostated cells. Calibrations were done with four standard buffer solutions in accordance with ref. [9].

Scanning calorimetric measurements were carried out with an automated differential calorimeter [10] at a heating rate of 1 deg/min and at protein concentrations from 0.1 to 0.5%.

For isothermal calorimetric measurements an LKB 10700-1 flow calorimeter was used in combination with a thermostated flow cell for pH measurements at the outlet tube of the calorimeter. The temperature deviation of the pH measuring cell was less than ±0.1°C from the calorimeter working temperature. The latter was checked with a Hewlett-Packard quarts thermometer inside the calorimeter thermostat. The electrical calibration of the calorimeter was checked by sucrose dilution heat measurements and the heat of neutralization of 0.01 M HCl with carbonate free NaOH.

Isothermal calorimetric measurements were carried out in the usual manner (see, for example, refs. [11, 12]) using 0.1 M NaCl solutions with an initial 0.2–2.0% concentration of protein. No influence of the lysozyme concentration and flow rate on the result was found. The pH change was registered at the calorimeter outlet simultaneously with the heat effect.

In titrations with alkaline solutions CO_2-free KOH prepared according to Kolthoff was used. The amount of protons in the reaction was calculated from potentiometric protein titration curves, the heat of neutralization being taken as $\Delta H = -13.34$ kcal/mol [13].

Potentiometric titrations of lysozyme at a constant temperature were performed in a thermostated 10.0 ml vessel by a micrometer syringe using 0.5–1.0% protein solution in 0.1 M NaCl with initial pH 4.80 ± 0.02 at 25°C. CO_2 was excluded in all experiments with pH higher than 5. Several titrations were made in each case.

Measurements of lysozyme protonization changes ($\Delta\nu$ values) at elevated temperatures were made by the pH-stat method; 10 ml protein solutions (0.2–0.8%) in 0.1 M NaCl were adjusted to the predetermined pH values at 25°C. The temperature was then raised in steps of 5°C (1°C in the range of transition). After each step, readjustment of the pH to the initial value was performed by titration. Correction values were determined in parallel experiments under the same conditions, but in solutions without protein.

3. Experimental

3.1. Determination of the temperature dependent terms

Scanning calorimetry of protein solutions allows one to determine the heat of conformational transitions and to measure partial heat capacities as a function of temperature before and after transition. It has been shown [4,7] that the heat capacities of native and denatured protein can be expressed by pH-independent, linear functions of temperature differing by $\Delta[C_p]_d$ values (see fig. 1). The denaturational change of heat capacity $\Delta[C_p]_d$ was found to be independent of the pH and the denaturation temperature (T_d) within the experimental error. The following data (taken from refs. [4, 7]) were used for the calculation of the temperature-dependent terms in the standard functions of lysozyme:

a) Partial heat capacity of native lysozyme in solution at 25°C and constant pressure, $[C_p]^N_{25°C} = 4.57_8$ kcal mol^{-1}K^{-1} (pH independent).
b) Temperature coefficient of partial heat capacity of native lysozyme in solution, $d[C_p]^N/dT = 0.028_6$ kcal mol^{-1} K^{-2} (pH independent).
c) Change in heat capacity due to denaturation, $\Delta[C_p]_d = 1.57$ kcal mol^{-1} K^{-1} (pH and temperature independent).

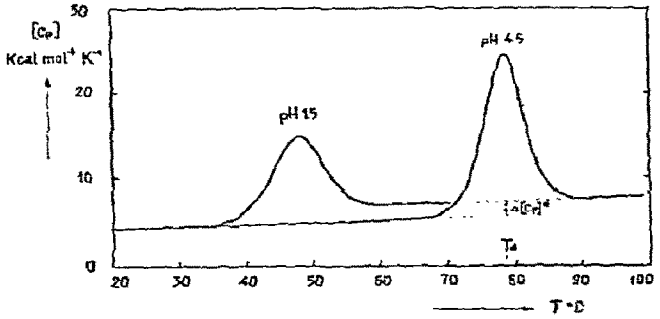

Fig. 1. Temperature dependence of partial heat capacity of lysozyme at different pH of solution (1.5 and 4.5).

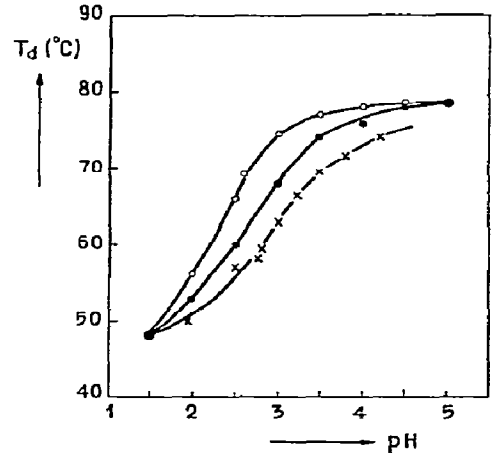

Fig. 2. Denaturation (half conversion) temperature versus pH at different experimental conditions. Curve a) o–o 0.04 M buffer solution, curve b) •–• 0.04 M buffer plus 0.1 M NaCl, curve c) x –x 0.1 M NaCl without buffer.

d) Molar heat of denaturation as a function of T_d (°C), $[\Delta H_d]_{T_d} = 17.10 + 1.5737\,T_d$ (kcal mol^{-1}).
e) The pH dependence of the denaturation temperature T_d (at which half the reaction is observed) is given for various experimental conditions in fig. 2 representing the influence of the ionic strength and buffer content. Curve (b) in fig. 2 is close to standard conditions and was used in all calculations except experiments performed without buffer (subsections 3.3 and 3.4 of this paper).

Using these basic data we can obtain the temperature dependent terms of the thermodynamic functions of the native and the denatured state of lysozyme separately as mentioned above [eqs. (2)–(8)].

3.2. Determination of the pH-dependent terms

The problem arises now to determine the terms $H^{0,N}(\mathrm{pH})$ and $S^{0,N}(\mathrm{pH})$ in eqs. (2)–(8). They represent the change in enthalpy and entropy respectively for a molar protein solution when the pH is changed from $\mathrm{pH}^0 = 7.0$ to any given value at $T^0 = 25$°C.

In the range of our measurements the lowest conversion temperature in unfolding of lysozyme is 48°C at pH 1.5 (figs. 1 and 2). The high thermal stability of lysozyme enables experimental investigation of native lysozyme in the whole pH range at 25°C, i.e. determination of the functions $H^{0,N}(\mathrm{pH})$ and $G^{0,N}(\mathrm{pH})$ without the influence of denaturation.

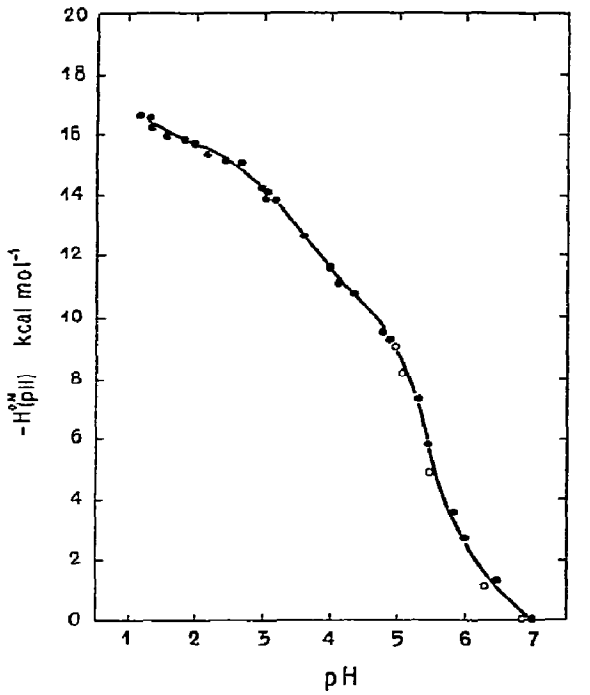

Fig. 3. pH dependence of lysozyme enthalpy at 25°C, measured by calorimetry (solid circles: initial pH 4.80 ± 0.02; open circles: initial pH 7.00 ± 0.02).

The pH dependence of the standard enthalpy of native lysozyme was measured by isothermal calorimetric titrations at an ionic strength of $I^0 = 0.1$ and at $T^0 = 25$°C. The results are given in fig. 3. The curve is standardized to $H^{0,N}_{pH7} = 0$ (standard state) and corresponds to the negative integral heat of ionization of individual groups responsible for proton dissociation in the investigated pH range.

Determination of $G^{0,N}(\mathrm{pH})$ is possible by direct use of titration curves [14,15]. From the general equation

$$G(x) = G(x_0) + \int_{x_0}^{x} (\delta G/\delta x)\,\mathrm{d}x\,, \qquad (9a)$$

follows the equation for the multiple equilibrium of protein ionization:

$$G(\alpha) = \text{const.} + \int_{\alpha_0}^{\alpha} \mu(\alpha)\,\mathrm{d}\alpha$$

$$= G(\alpha_0) - 2.303\,\mathrm{RT} \int_{\alpha_0}^{\alpha} \mathrm{pH}\,(\alpha)\,\mathrm{d}\alpha\,. \qquad (9b)$$

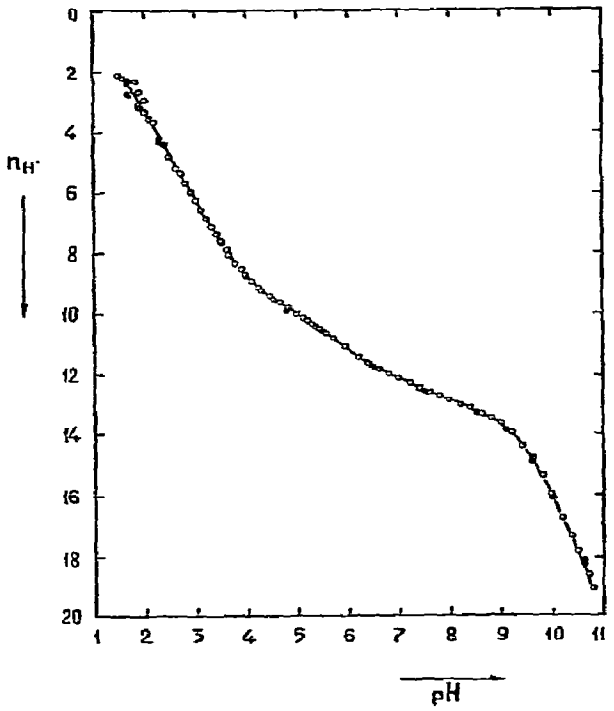

Fig. 4. Net proton uptake of lysozyme in potentiometric titration (solid circles: back titration).

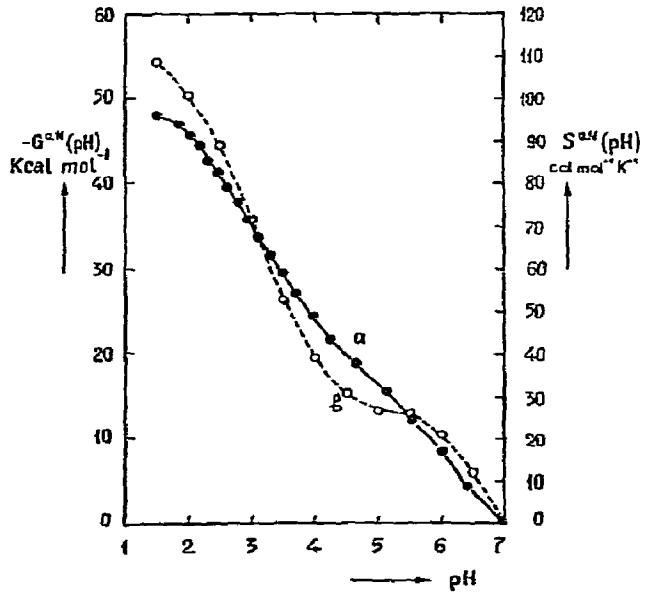

Fig. 5. pH dependence of the standard free energy [curve (a)] and pH dependence of the standard entropy of lysozyme [curve (b)].

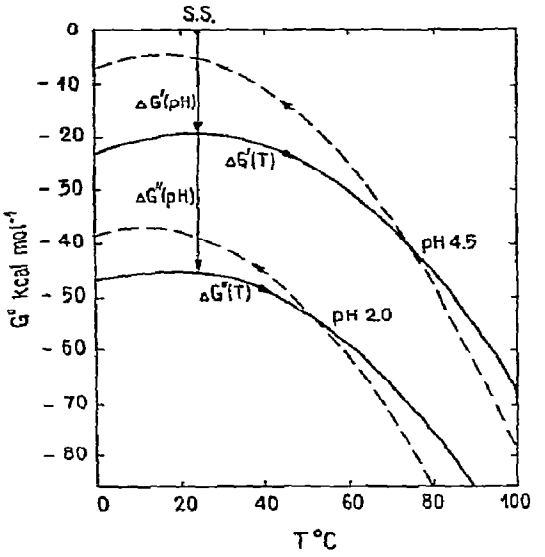

Fig. 6. Free-energy change of lysozyme in different treatments: $\Delta G'(pH)$, at change of pH from the standard state (S.S.) to 4.5 at 25°C; $\Delta G''(pH)$, at change from 4.5 to 2.0 at 25°C; $\Delta G'(T)$, at change of temperature from 25°C at pH 4.5; $\Delta G''(T)$, at change of temperature from 25°C at pH 2.0. Dotted lines denote the change for the denatured state.

Instead of the degree of ionization (α) we can introduce the relative number of protons (ν) which is measurable by potentiometric titrations. At standard conditions ($\nu_0 \equiv 0$ at 25°C and pH 7.0) the integration constant, $G^0(\nu_0)$, is equal to zero and we obtain the Gibbs function $G^{0,N}(\nu)$:

$$G^{0,N}(\nu) = -2.303\,RT \int_{\nu_0}^{\nu} pH\,(\nu)\,d\nu\,. \qquad (10)$$

Transformation of $G^{0,N}(\nu)$ into $G^{0,N}(pH)$ can be done without difficulty using the experimental titration curve.

The results of the potentiometric titrations under given conditions ($I^0 = 0.1$, $T^0 = 25°C$) are represented in fig. 4. They are very close to those of Sakakibara et al. [16]. The pH function of the standard Gibbs energy of native lysozyme obtained using eq. (10) is given in fig. 5, curve (a). Moreover, knowledge of $G^{0,N}(pH)$ and $H^{0,N}(pH)$ allows to obtain $S^{0,N}(pH)$ according to eq. (4) [see fig. 5, curve (b)].

Using the results of scanning calorimetry, isothermal calorimetry and potentiometric titration we can obtain a picture of the Gibbs function of native and denatured lysozyme at any pH and temperature (fig. 6). The arrows

show the pathways for obtaining these functions by moving from the standard state (S.S.) and changing the pH [$\Delta G'$(pH) and $\Delta G''$(pH) in fig. 6] or the temperature [$\Delta G'(T)$ and $\Delta G''(T)$ in fig. 6]. Dotted lines denote the denatured state.

3.3. Control of validity of the thermodynamic state functions

A test of validity of the obtained state functions can be carried out by comparing the thermodynamic functions obtained by independent methods (see below) or by using different pathways according to the scheme in fig. 7.

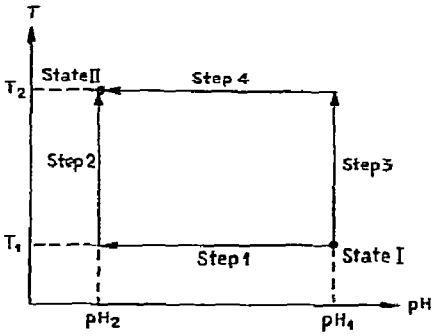

Fig. 7. Scheme of different pathways from state I (native to) state II (denatured) for lysozyme.

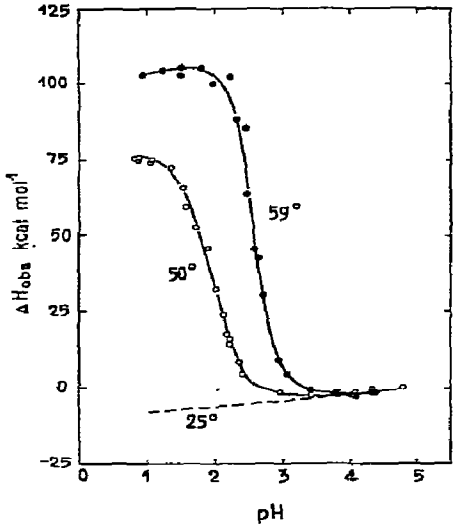

Fig. 8. Heat of transfer of lysozyme from pH 4.8 to a given pH at constant temperature.

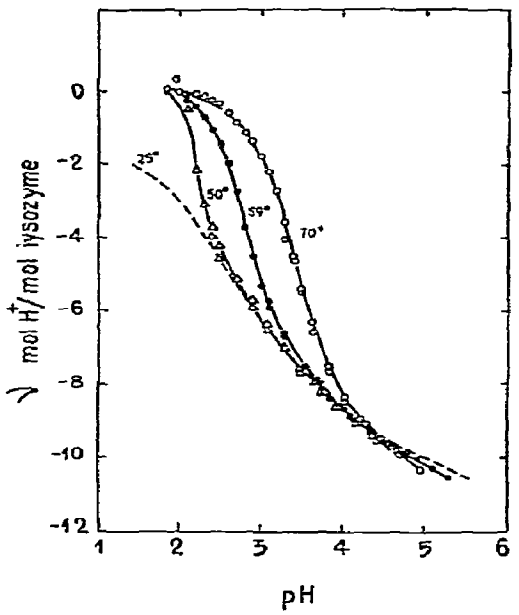

Fig. 9. Protonation of lysozyme versus pH at different temperatures.

The usual experimental procedure in denaturation studies commences from the native state of proteins (state I) expressed in terms of pH_1 and T_1. Adjustment of pH_2 at constant temperature (step 1) is accompanied by ionization effects and gives a second native state. With a rise in temperature (step 2), the denatured state II of protein can be reached (expressed in terms of pH_2 and T_2). An independent way to reach state II lies in firstly adjusting the temperature (step 3) and then the pH (step 4).

The curves in fig. 8 indicate the heat measured by isothermal calorimetry on transfer of protein from pH 4.80 ± 0.02 to a given pH at constant temperature (50° and 59.1°C). The corresponding enthalpy change of native lysozyme at 25° (see also fig. 3) is given for comparison. Free-energy changes for step 4 were determined by eq. (10) using the results of potentiometric titration at high temperature (fig. 9). The changes in enthalpy and entropy for each step of the given cycle are summarized in table 1. They indicate agreement within the experimental error with functions obtained by moving along different pathways to the same point.

Table 1
Comparison of thermodynamic values for lysozyme denaturation obtained using independent pathways (T_1 = 25.00 ± 0.05°C, pH_1 = 4.80 ± 0.02, pH_2 = 1.50 ± 0.02).

Step (see fig. 7)	Thermodynamic expression	Methods used	Results for T_2 = 50°C (kcal mol^{-1})	Results for T_2 = 59.1°C (kcal mol^{-1})
1	$H^{0,N}_{T_1,pH_2} - H^{0,N}_{T_1,pH_1}$	Isothermal calorimetry at 25°C	−6.7	−6.7
2	$H^{D}_{T_2,pH_2} - H^{0,N}_{T_1,pH_2}$	Scanning calorimetry (ΔH_d, C_p)	218.9	289.2
sum (1+2)	$H^{D}_{T_2,pH_2} - H^{0,N}_{T_1,pH_1}$		212.2	282.5
3	$H^{0,N}_{T_2,pH_1} - H^{0,N}_{T_1,pH_1}$	Scanning calorimetry (C_p)	123.8	178.8
4	$H^{D}_{T_2,pH_2} - H^{0,N}_{T_2,pH_1}$	Isothermal calorimetry at T_2	81.5	103.7
sum (3+4)	$H^{D}_{T_2,pH_2} - H^{0,N}_{T_1,pH_1}$		205.3	282.5
Deviation			3.3%	—

Step	Thermodynamic expression	Methods used	Results for T_2 = 50°C (cal mol^{-1} K^{-1})	Results for T_2 = 59.1°C (cal mol^{-1} K^{-1})
1	$S^{0,N}_{T_1,pH_2} - S^{0,N}_{T_1,pH_1}$	Isothermal calorimetry and potentiometric titrations at 25°C	78.2	78.2
2	$S^{D}_{T_2,pH_2} - S^{0,N}_{T_1,pH_2}$	Scanning calorimetry (ΔS_d, C_p)	694.2	908.2
sum (1+2)	$S^{D}_{T_2,pH_2} - S^{0,N}_{T_1,pH_1}$		772.4	986.4
3	$S^{0,N}_{T_2,pH_1} - S^{0,N}_{T_1,pH_1}$	Scanning calorimetry (C_p)	397.9	563.8
4	$S^{D}_{T_2,pH_2} - S^{0,N}_{T_2,pH_1}$	Isothermal calorimetry and potentiometric titration at T_2	379.2	454.0
sum (3+4)	$S^{D}_{T_2,pH_2} - S^{0,N}_{T_1,pH_1}$		777.1	1017.8
Deviation			0.6%	3.1%

3.4. Determination of $\Delta G_d(pH, T)$ in an independent way

The pH-dependent equilibrium of denaturation can be expressed by the general equation (see [17,18]):

$$\left(\frac{\partial \ln K}{\partial \ln a_H}\right)_T = Z_I - Z_{II} . \quad (11)$$

Denoting the differences in protonation stages (Z) of the denatured and native forms as $\Delta\nu_d$ it follows that

$$\frac{1}{RT}\left(\frac{\partial \Delta G_d}{\partial \ln a_H}\right)_T = [\Delta\nu_d]_T , \quad (12)$$

and thus

$$\Delta G_d(\ln a_H) = \Delta G^0_d + RT \int_{\ln a^0_H}^{\ln a_H} \Delta\nu_d(\ln a_H)\, d\ln a_H . \quad (13)$$

Having in mind that ΔG^0_d is zero at proton activity $a_H(\frac{1}{2})$ of the midpoint of transition we obtain:

$$\Delta G_d(\ln a_H) = RT \int_{\ln a_H(\frac{1}{2})}^{\ln a_H} \Delta\nu_d(\ln a_H)\, d\ln a_H . \quad (14)$$

Here $\Delta\nu_d$ can be determined in several ways (see also [19]):

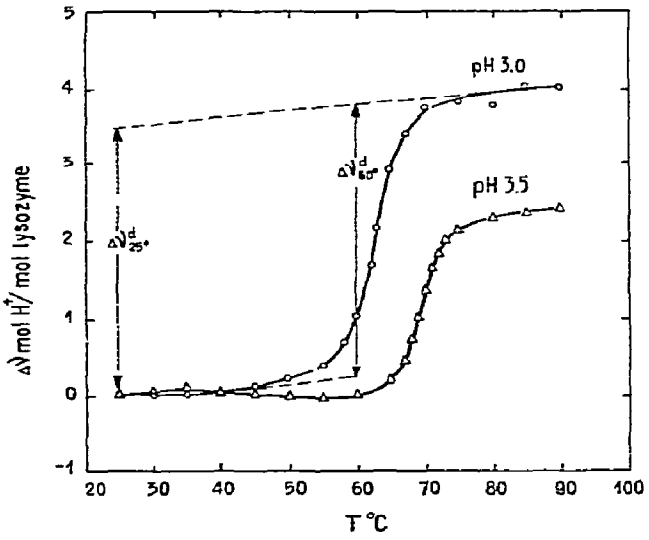

Fig. 10. Proton uptake of lysozyme versus temperature at different pH of solutions.

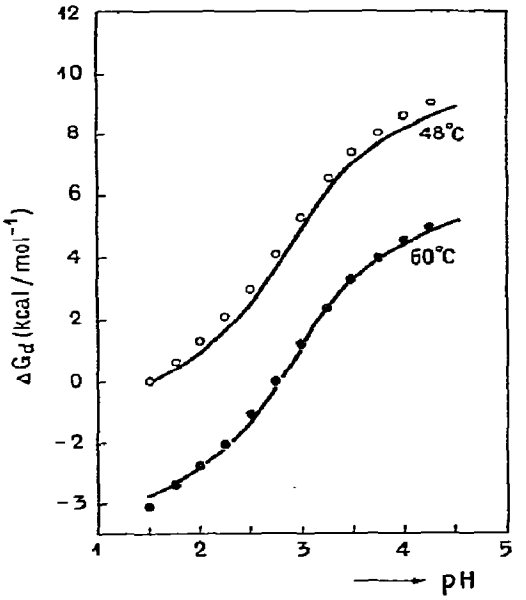

Fig. 12. Gibbs free energy of denaturation versus pH. Results obtained from eq. (14) are represented by circles. Results obtained by eqs. (2)–(8) are represented by lines.

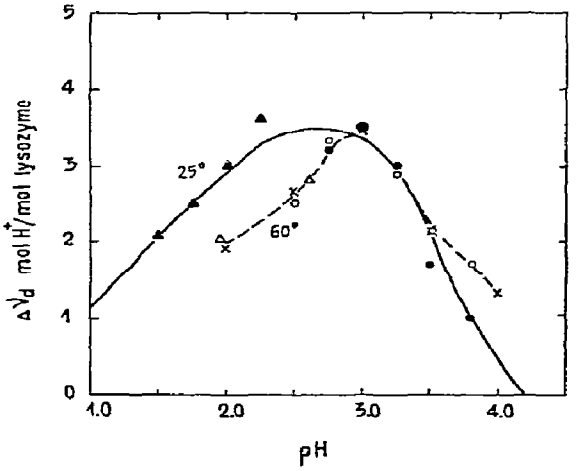

Fig. 11. Change of protonation at unfolding of lysozyme with change of pH at different temperatures. The symbols used correspond to the methods described in subsection 3.4 as follows: △, method: a), temp.: 60°C; ×, method: c), temp.: 60°C; ○, method: titration (compare fig. 9), temp.: 60°C; ▲, method: b), temp.: 25°C; ●, method: titration, temp.: 25°C.

a) By use of eq. (11) with the assumption that the dependence of $\Delta\nu_d$ on $\ln a_H$ is small and that influences of hydration [18] can be neglected.

b) By use of measured or calculated potentiometric titration curves for both the native and denatured protein at the same temperature.

c) By use of the pH-dependence of scanning calorimetric data [14,20].

All these methods suffer experimental and principal difficulties. Therefore we used a fourth, direct difference titration method.

Unbuffered protein solutions of determined pH were heated in steps and the temperature-induced pH changes were compensated by titration. Typical examples of measured curves are given in fig. 10 representing the temperature dependence of the net proton uptake of lysozyme.

The results for $\Delta\nu_d$ obtained by various methods are summarized in fig. 11. The differences in the protonized state of native and denatured lysozyme were found to be pH and temperature dependent.

In fig. 12 the denaturational Gibbs energy changes obtained by eq. (14) are compared with those calculated by means of eqs. (2)–(8). The data used in eqs. (2)–(8) have already been given in subsection 3.1. Since the experiments for the determination of $\Delta\nu_d$ were performed in the absence of a buffer, the corresponding pH-dependence of T_d [curve (c) in fig. 2] was used in this case in contrast with all other calculations.

As seen in fig. 12 the results of both independent approaches are in agreement. The maximum observed difference in ΔG_d is of the order of 0.5 kcal mol^{-1} and probably pertains to uncertainties in the determination of $\Delta \nu_d$ (about ± 0.2 mole protons per mole lysozyme). The determination of ΔG_d of lysozyme by eq. (14) at temperatures lower than 48°C is impossible due to its high thermal stability. The use of eq. (14) is limited by the lowest half conversion temperature in the range of investigations (see above) since the integration constant in eq. (13) is unknown.

It should be added that the temperature dependence of the proton uptake of lysozyme at denaturation (as shown in fig. 10) treated by the Van't Hoff equation also enables the determination of the denaturation enthalpies. The results will be given in the following paper ([6], fig. 8) in a review of the denaturation enthalpies of lysozyme obtained by vairous experimental approaches.

4. Discussion

The approach suggested in this paper corresponds to the usual practice in chemical thermodynamics, i.e. the thermodynamic functions for given states of a protein are formulated separately. This gives the advantage of extending the description to any possible additional states adding more partial differential terms [eq. (1)] corresponding to the additional variable parameters. It is important that the terms in the integrals of eqs. (2)–(8) are determinable *directly* on the basis of modern instrumentation. The thermodynamic parameters of transition between different states (in this paper the denaturation parameters ΔG_d, ΔH_d and ΔS_d) are given by algebraic differences between the corresponding standard functions at given conditions (pH, temperature, etc.).

It is essential to note that the need of any extra assumptions in the proposed evaluation of the thermodynamic functions is excluded whereas the determination of the enthalpy changes from the temperature dependence of the equilibrium constants includes the assumption of a simple transition between the distinct thermodynamic states. Thus, the calculated enthalpy changes will be correct only if the two-state model is valid. Therefore the more recently developed microcalorimetric methods are not only more favourable in obtaining the thermodynamic parameters but also crucial for the validity of such models [4].

A similar problem is connected with the determination of the pH-dependence of the Gibbs function of native protein, i.e., $G^{0,N}$(pH). Alternatively to eq. (10) the Gibbs function can be calculated using ionization data of individual groups. Such data are widely available from analyses of titration experiments with the help of the Linderstrom–Lang equation. On the other hand, there are also serious arguments against the simplifications made in the Linderstrom–Lang model when it is used in calculations of the Gibbs energy [21, 22]. Therefore, the use of eq. (10) which is independent of any model seems to be more advantageous than the Linderstrom–Lang treatment.

As shown in subsections 3.1, 3.2 and figs. 3–6 the methodic problems in calculating thermodynamic standard functions can be solved. Another problem is the reproducibility of the thermodynamic parameters of the investigated process (here the reversible unfolding) using thermodynamically independent approaches. This concludes the question of whether the considered states of lysozyme, the "native" and "denatured", are valid from the point of view of thermodynamics. This is an important point in the approach used since integration of the partial differential functions [as eq. (1)] is possible only if the state is unambiguously defined. But the definition of the thermodynamic states of proteins, both native and denatured, is still unclear. This affects also the problem of the self-organization of the native protein structure since theoretical considerations of protein folding mechanisms usually commence from the denatured state.

The results presented here show that the denaturation of lysozyme by temperature-induced transitions (scanning calorimetry) as well as by pH-induced transitions (isothermal calorimetry, potentiometric titrations) is accompanied by adequate enthalpy and entropy changes (fig. 7, table 1). It has been shown that there are unique enthalpy and entropy changes if the initial and the final states are unambiguously defined. This is a crucial test for the validity of the thermodynamic standard functions given above.

The most important function determining the stability of the structure of proteins is the denaturational change in the Gibbs energy. ΔG_d can be determined as a pH and temperature dependent function by eqs. (2)–(8). The basic data for this evaluation are the de-

naturational enthalpy, ΔH_d, and the change in heat capacity, $\Delta[C_p]_d$, which are directly determinable by calorimetric measurements. An independent approach to obtain ΔG_d according to eq. (14) is based on the pH-dependence of protonation differences of both states, i.e., this approach is based on independent experimental parameters and on another principle in evaluation. Therefore the coincidence between the results of both approaches (fig. 12) does not only evidence the correctness of the data and functions used but also serves as a further argument for the existence of a reversible transition between true thermodynamic states.

Thus, the methodic suppositions for obtaining and checking thermodynamic standard functions of proteins are given.

There is some disagreement in the literature on the characterization of the thermodynamic states of denatured proteins (especially of lysozyme) after heat denaturation and denaturation in guanidine hydrochloride [24–26]. Before giving a general picture of the thermodynamic state functions for protein [27], we shall consider the denatured states of protein in the accompanying paper based on comparative calorimetric investigations [6].

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