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THERMODYNAMIC INVESTIGATIONS OF PROTEINS.
III. Thermodynamic description of lysozyme

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Standard functions of enthalpy, entropy and the Gibbs energy of native and denatured lysozyme in the range of 0–100°C and pH 1.5–7.0 are represented in three-dimensional projections. The denaturational Gibbs energy change reaches 16 kcal mol^{-1} at conditions of maximal protein stability (0°C, pH 4.5–7.0) and equals 14.5 kcal mol^{-1} at 25°C and neutral pH. This result was found to be in agreement with the data reported from guanidine hydrochloride denaturation studies.

Partial thermodynamic functions of the conformational and ionizational changes of the protein are obtained from entropy and Gibbs-energy changes in denaturation. The conformational partial entropy and Gibbs-energy change are found to be independent of pH. The pH-dependent partial ionizational entropy and Gibbs-energy changes are induced by normalization of the ionization behaviour of buried groups and cause a decrease of protein stability.

1. Introduction

In the preceding reports of this series [1,2] we showed the possibilities of evaluating real thermodynamical functions of proteins at all obtainable external conditions (pH, temperature and denaturant concentrations) without any extra a priori assumptions from data obtained by direct experimental measurements using microcalorimetric and titration techniques.

In this paper we shall first demonstrate these functions for a definite globular protein, and, secondly, how these functions could be used for protein state analysis, particularly for analyses of the denatured state. For this demonstration we shall take here the same protein as we did previously, i.e. lysozyme. Some thermodynamic functions of lysozyme under conditions of maximal thermostability have been published earlier [3]. But for a more detailed consideration of the problem of protein stability and its physical states a much more detailed picture is needed. In this paper we shall give standard thermodynamical functions of lysozyme in all the obtainable temperature and acid pH regions. As for denaturants, we previously showed that the protein state in GuHCl is thermodynamically indistinguishable from that obtained on temperature and pH denaturation. But since the last two influences are physically and biologically much more important we shall focus attention on these parameters.

2. Materials and methods

The results reported in this paper are based on the investigations described in detail in the first paper of this series [1]. In scanning calorimetric experiments [1,3–5] 0.04 M glycine or acetate buffer containing 0.1 M l^{-1} NaCl was used. At pH 5–7 a growing tendency toward aggregation of unfolded lysozyme was observed but there was no alteration of denaturation temperature. The denaturational enthalpy change in this region was taken as pH-independent.

Isothermal calorimetric investigations and potentiometric titrations were performed in 0.1 M NaCl without buffers. For the procedure see refs. [1,2].

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3. Results and discussion

3.1. Standard functions for native and denatured lysozyme

In ref. [1] the standard functions of enthalpy, $H^{0,N}(T, \mathrm{pH})$, entropy, $S^{0,N}(T, \mathrm{pH})$, and Gibbs energy, $G^{0,N}(T, \mathrm{pH})$, for native lysozyme and the corresponding functions for denatured lysozyme, $H^{D}(T, \mathrm{pH})$, $S^{D}(T, \mathrm{pH})$ and $G^{D}(T, \mathrm{pH})$, were obtained as temperature- and pH-dependent. The standard state for native protein was chosen in such a way that at $\mathrm{pH}^0 = 7.0$ and $T^0 = 25°\mathrm{C}$ $H^{0,N} \equiv 0$, $S^{0,N} \equiv 0$ and $G^{0,N} \equiv 0$.

As can be seen from the basic equations these standard functions are composed of temperature- and pH-dependent terms as follows:

$$H^{0,N}(T, \mathrm{pH}) = \int_{T^0}^{T} [C_p]^N \, \mathrm{d}T + H^{0,N}(\mathrm{pH}), \qquad (1a)$$

$$S^{0,N}(T, \mathrm{pH}) = \int_{T^0}^{T} [C_p]^N \, \mathrm{d} \ln T + S^{0,N}(T, \mathrm{pH}), \qquad (1b)$$

$$G^{0,N}(T, \mathrm{pH}) = H^{0,N}(T, \mathrm{pH}) - TS^{0,N}(T, \mathrm{pH}). \qquad (1c)$$

The temperature dependent terms in eq. (1) are determined by integration of the partial molar heat capacity of the native protein $[C_p]^N$ taking the integration constant at $\mathrm{pH}^0 = 7.0$ and $T^0 = 25°\mathrm{C}$ equal to zero. $[C_p]^N$ is itself a temperature dependent function which can be obtained directly by scanning calorimetry measurements and can be approximated by a linear equation (with the temperature, t, in degrees Celsius) [1,3,4]:

$$[C_p]^N = a + bt = 3.863 + 0.0286t. \qquad (1d)$$

The terms $H^{0,N}(\mathrm{pH})$, $S^{0,N}(\mathrm{pH})$ and $G^{0,N}(\mathrm{pH})$ can be determined by calorimetric and potentiometric titrations. On the basis of the functions (1a–c) it follows for the denatured state:

$$H^{D}(T, \mathrm{pH}) = H^{0,N}(T, \mathrm{pH}) + [\Delta H_d]_{T_d} - \int_{T}^{T_d} \Delta [C_p]_d \, \mathrm{d}T, \qquad (2a)$$

$$S^{D}(T, \mathrm{pH}) = S^{0,N}(T, \mathrm{pH}) + \frac{[\Delta H_d]_{T_d}}{T_d} - \int_{T}^{T_d} \Delta [C_p]_d \, \mathrm{d} \ln T, \qquad (2b)$$

$$G^{D}(T, \mathrm{pH}) = H^{D}(T, \mathrm{pH}) - TS^{D}(T, \mathrm{pH}). \qquad (2c)$$

The denaturational enthalpy and entropy changes ΔH_d and ΔS_d at half conversion temperature T_d and the heat-capacity change of the protein $\Delta [C_p]_d$ are known for lysozyme from scanning calorimetric investigations [1,3,4]. It should be noted that the approach used and the methods for the determination of needed data are free from any extra assumptions. The standard conditions are formulated in such a manner that the arithmetic difference of the standard functions of the denatured and native state at a given temperature and pH directly corresponds to the denaturational enthalpy, the entropy or the Gibbs-energy changes.

The functions (1a–c) and (2a–c) have been plotted in the range of 0–100°C and pH 1.5–7.0 in fig. 1–4. The most striking characteristics of the enthalpy and entropy functions are the strong temperature dependences, whereas the pH-dependence in the region of investigation is hardly perceptible. The denaturational enthalpy change

$$\Delta H_d(T, \mathrm{pH}) = H^{D}(T, \mathrm{pH}) - H^{0,N}(T, \mathrm{pH}),$$

is a pH-independent, linear temperature function. The denaturational entropy change, $\Delta S_d(T, \mathrm{pH})$, represents a non-linear increasing function of the temperature. At constant temperature ΔS_d decreases with decreasing pH values.

The standard Gibbs function for native lysozyme is represented by a complicated surface (fig. 3). Starting from the standard state at pH 7.0 and 25°C, the Gibbs function slopes down non-linearly in all directions. The temperature dependence at constant pH corresponds to an almost parabolic curve with maximal values near 20–25°C.

The corresponding Gibbs function of denatured lysozyme (fig. 4) depicts a similar plane, but the maximal Gibbs energy is higher and the slope in temperature and pH directions is steeper.

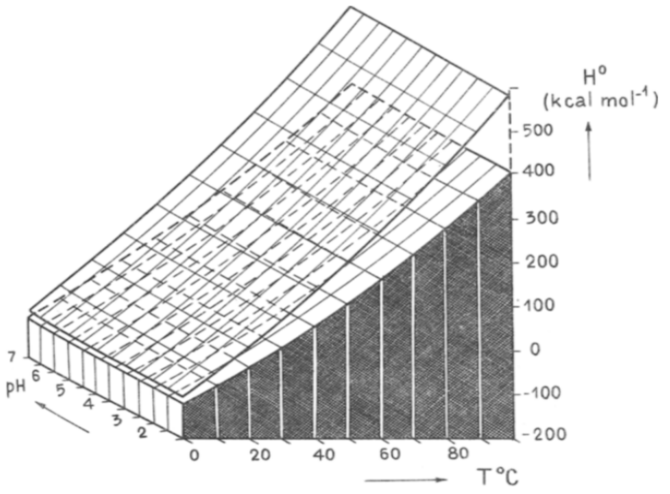

Fig. 1. Standard enthalphy of native lysozyme ($H^{0,N}$) and enthalpy of denatured lysozyme (H^{D}, upper surface) versus temperature and pH.

The most important function for the problem of the stabilization of the protein structure is the Gibbs-energy change on denaturation, $\Delta G_d(T, \text{pH})$, shown in fig. 5. The range of domination of the native form is limited by the straight line $\Delta G_d = 0$ (half reaction) and demonstrates the high thermal stability of lysozyme even at low pH values. The maximal stability of

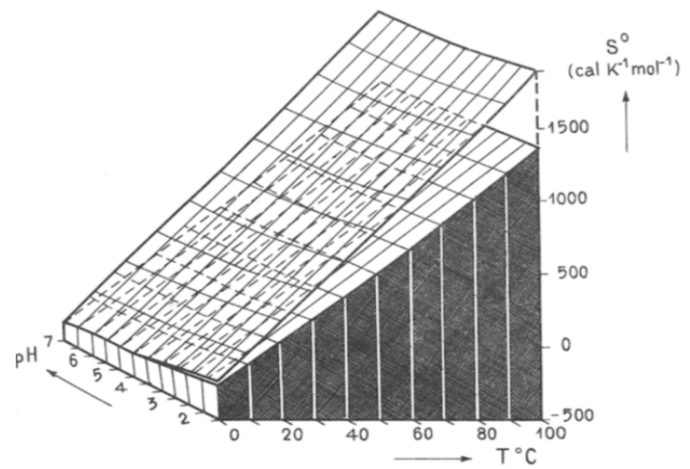

Fig. 2. Standard entropy of native lysozyme ($S^{0,N}$) and entropy of denatured lysozyme (S^{D}, upper surface) versus temperature and pH.

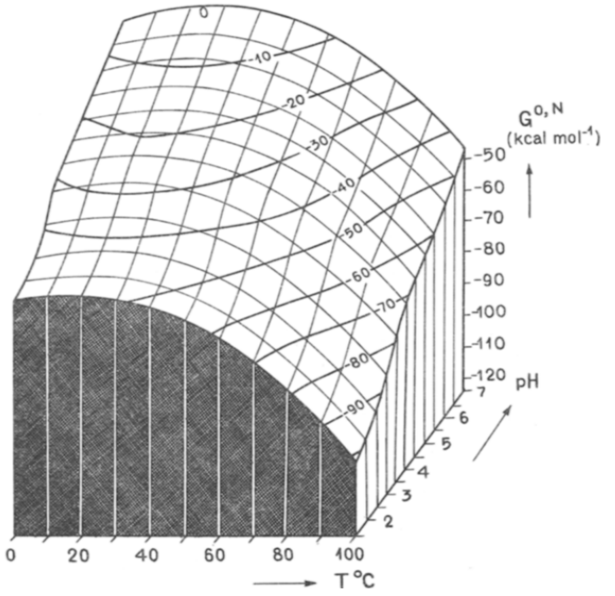

Fig. 3. Standard Gibbs function $G^{0,N}$ of native lysozyme versus temperature and pH.

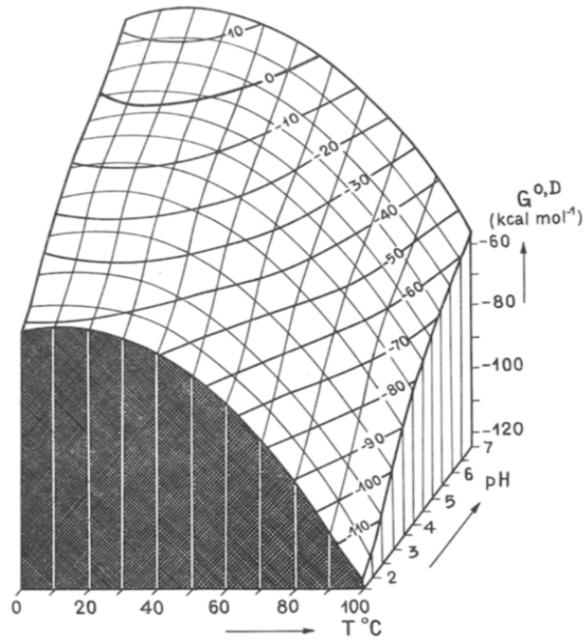

Fig. 4. Gibbs function G^{D} of denatured lysozyme versus temperature and pH.

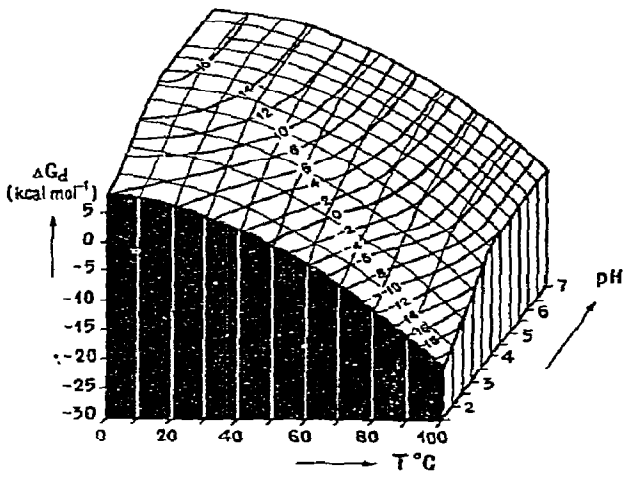

Fig. 5. Denaturational Gibbs energy change ΔG_d versus temperature and pH.

lysozyme lies near pH 4.5–7.0 and $T = 0°C$ where ΔG_d reaches 16 kcal mol^{-1}.

The Gibbs energy of stabilization of several proteins was recently independently determined from heat-denaturation studies [3] and from GuHCl-denaturation experiments [6–10]; the results were found to be generally in good agreement (table 1). The value ΔG_d = 14.2 kcal mol^{-1} for lysozyme at 25°C and neutral pH was reported from GuHCl denaturation. The present paper gives 14.5 kcal under the same conditions. From this coincidence we have, in addition to ref. [2], further evidence that the denatured states of lysozyme in GuHCl and at high temperatures are indistinguishable from the thermodynamic point of view if corrections for preferential binding have been made.

3.2. Partial thermodynamic functions

Denaturation studies are usually performed on dilute protein solutions containing a buffer for compensation of pH changes due to unfolding. Therefore the thermodynamic data obtained in reality are composed as follows

$$\Delta H_{T_d}^{app} \approx \Delta H_{T_d}^{prot} + (\Delta\nu\,\Delta H_i)_{T_d}^{buffer}, \quad (3a)$$

$$\Delta S_{T_d}^{app} \approx \Delta S_{T_d}^{prot} + (\Delta\nu\,\Delta S_i)_{T_d}^{buffer}. \quad (3b)$$

The apparent thermodynamic parameters of denaturation ΔH^{app} and ΔS^{app}, at the temperature T_d describe the sum of the net enthalpy or entropy change of the protein (ΔH^{prot} or ΔS^{prot}) and the molar ionization enthalpy or entropy of the buffer (ΔH_i or ΔS_i) multiplied by the amount of transferred protons, $\Delta\nu$. In eqs. (3a) and (3b) the terms $\Delta H_{T_d}^{prot}$ and $\Delta S_{T_d}^{prot}$ can be additionally divided into the partial enthalpy or entropy of the conformational transition and the partial enthalpy or entropy of ionization of the protein on denaturation. The latter term represents the "normalization" of the ionization behaviour of buried groups in unfolding the protein structure.

Table 1
Gibbs energy of stabilization of protein structure obtained by GuHCl denaturation and heat denaturation (ΔG_d in kcal mol^{-1}).

	GuHCl denaturation			Heat denaturation		
	pH	(ΔG_d) 25°C	Ref.	pH	(ΔG_d) 25°C	Ref.
Bovine pancreatic ribonuclease A	6.6 6	9.7 ± 1.7 13.0	[10] [6]	5.5	10.6 ± 0.6	[3]
Hen egg white lysozyme	7	14.2	[7]	5–7	14.5 ± 0.8	[1,3]
Bovine pancreatic α-chymotrypsin	4.3 (?)	8.3 ± 0.4 12.3	[10] [9]	4	11.6 ± 0.5	[3]
Bovine heart ferricytochrome c	6.5	15.4	[9]	4.8	9.0 ± 0.6	[3]
Sperm whale ferrimyoglobin*	7	14.3	[8]	10	12.0 ± 0.8	[3]

* Cyanide complex.

Table 2
Thermodynamic data of ionization of amino acids at 25°C.

Amino acid residue	Amount in lysozyme	pK	ΔG_i (kcal mol^{-1})	ΔH_i (kcal mol^{-1})	ΔS_i (cal mol^{-1}K^{-1})	Ref.
α-carboxyl group	1	3.4	4.63	0.4	−14.2	[14,15]
Asp side chain	8	3.91	5.34	1.11	−14.2	[16,17]
Glu side chain	2	4.28	5.84	0.38	−18.3	[17]
His	1	6.0	8.18	6.90	− 4.3	[16,18]
α-amino group	1	7.5	10.22	10.5	0.1	[14,15]
Tyr	3	10.09	13.75	6.0	−26.0	[16]
Lys side chain	6	10.79	14.71	12.88	− 6.1	[16,19]
Arg side chain	11	12.48	17.0	12.4	−15.5	[18]

Thus we get from eqs. (3a) and (3b):

$$\Delta H^{app}_{T_d} = (\Delta H_{conf} + \Delta H_i)^{prot}_{T_d} + (\Delta\nu\,\Delta H_i)^{buffer}_{T_d}, \quad (4a)$$

$$\Delta S^{app}_{T_d} = (\Delta S_{conf} + \Delta S_i)^{prot}_{T_d} + (\Delta\nu\,\Delta S_i)^{buffer}_{T_d}. \quad (4b)$$

In the case of lysozyme at pH > 4.5 the denaturation temperature T_d is independent of pH (see fig. 2 in ref. [1]) indicating the absence of ionization processes according to eq. (5) [11]:

$$\Delta\nu = -\frac{[\Delta H_d]_{T_d}}{2.3RT_d^2}\,\frac{dT_d}{d\,pH}. \quad (5)$$

If $dT_d/d\,pH = 0$, the number of protons transferred on denaturation $\Delta\nu$ is equal to zero. In this special case the terms ΔH^{app} and ΔS^{app} in eqs. (3) and (4) are equal to the partial conformational enthalpy or entropy changes, ΔH_{conf} or ΔS_{conf}.

In the pH range from 1.5 to 4.5, $\Delta\nu$ differs from zero [1] and the question arises here to what extent the enthalpy and entropy of normalization of ionization of the protein on unfolding are compensated by the ionization of the buffer compound. To solve this problem the ionization behaviour of random coiled lysozyme can be modelled by the corresponding sum of amino acids (table 2) using:

$$Z^D(pH) = \sum_{i=1}^{i=n} \alpha_i(pH)\,\Delta Z_i, \quad (6a)$$

where Z means the Gibbs function, entropy or enthalpy. The pH-dependence of the ionization of the ith group (α_i) is given by its ionization constant K_i and proton activity a_{H^+}:

$$\alpha_i(a_{H^+}) = \frac{K_i/a_{H^+}}{1 + K_i/a_{H^+}}. \quad (6b)$$

Calculation of $Z^D(pH)$ commences from a high proton activity corresponding to the state of protein which is completely saturated by protons. It is advantageous for the purpose of comparison with standard functions of native lysozyme to normalize these functions to $Z^{0,D} = 0$ at $pH^0 = 7.0$ and $T^0 = 25°C$, i.e., to have the same standard state for native and unfolded lysozyme: $Z^{0,D} = Z^{0,N} = 0$. This differs from the above statement of standard functions discussed above with the standard state $Z^D = Z^{0,N} + [\Delta Z_d]_{25°C}$. In other words, in all the following considerations which we for simplicity perform only at 25°C, the denaturational changes in enthalpy, entropy and Gibbs energy $[\Delta Z_d]_{25°C,\,pH\,7.0}$ are eliminated.*

The differences in the enthalpy functions for native and (approximated by amino acids) denatured lysozyme (see also [14]) do not exceed 3.5 kcal mol^{-1} in the pH range of 1.5–4.5 (table 4, column 1). The corresponding enthalpy changes of the buffer compound $(\Delta\nu\,\Delta H_i)^{buffer}$ (table 4, column 2) are of the same order of magnitude, and the differences remain within the limits of error in the determination of ΔH^{app}. Therefore the values of ΔH^{app} in eq. (4a) can be interpreted as conformational enthalpy changes (ΔH_{conf}) on unfolding.

The entropy changes on denaturation of lysozyme are more complicated. In contrast with ΔH^{app}, the apparent entropy changes (ΔS^{app}) are pH-dependent (table 4, column 4). The corrections for the entropy of ionization of the buffer are more significant than the corrections for the enthalpy of ionization and

* The standard functions of unfolded lysozyme modelled by amino acids according to eqs. (6a), (6b) and the data given in table 3 are marked by primes: $H^{0,D'}$, $S^{0,D'}$ and $G^{0,D'}$.

Table 3
Thermodynamic standard functions of native and denatured lysozyme *. pH dependence at 25°C.

Column	1.	2.	3.	4.	5.	6.	7.	8.
pH	$G^{0,N}$	$H^{0,N}$	$S^{0,N}$	$G^{0,D'}$	$H^{0,D'}$	$S^{0,D'}$	$G^{0,D''}$	$S^{0,D''}$
7.0	0	0	0	0	0	0	0	0
6.5	− 4.32	− 0.8	11.8	− 2.90	− 2.63	0.9	− 4.46	6.1
6.0	− 8.82	− 2.6	20.9	− 6.04	− 5.12	3.1	− 8.36	10.9
5.5	−13.06	− 5.35	25.9	− 9.19	− 7.24	6.6	−11.07	9.5
5.0	−16.53	− 8.6	26.6	−14.28	− 8.95	17.9	−13.17	14.2
4.5	−19.57	−10.3	31.1	−23.05	−10.72	41.4	−16.61	19.8
4.0	−23.32	−11.7	39.0	−37.49	−13.30	81.2	−24.40	37.2
3.5	−28.61	−13.0	52.4	−52.59	−16.01	122.8	−37.93	73.6
3.0	−35.46	−14.25	71.2	−62.22	−17.72	149.3	−51.63	113.8
2.5	−41.62	−15.15	88.8	−66.50	−18.46	161.2	−59.59	138.0
2.0	−45.72	−15.7	100.7	−68.05	−18.72	165.5	−62.82	148.0
1.5	−48.50	−16.2	108.4	−68.56	−18.80	167.0	−63.94	151.5
1.0	−50.60	−17.0	112.8	−68.72	−18.84	167.4	−64.30	152.6

* Enthalpy and Gibbs energy in kcal mol^{-1}, entropy in cal $mol^{-1}K^{-1}$.

reach about 25 cal $mol^{-1}K^{-1}$ at 25°C (table 4, column 3). The partial entropy change of the protein, $\Delta S^{prot} = (\Delta S_{conf} + \Delta S_i)^{prot}$, at a given temperature shows a qualitatively different pH-dependence from ΔS^{app} (fig. 6, curves 1 and 2) and passes through a maximum at pH 2.5 where the amount of protons transferred on denaturation ($\Delta\nu$) is maximal.

The partial entropy change of the protein (ΔS^{prot}) is composed of the conformational entropy (ΔS_{conf}) and the partial entropy of normalization of the ionization behaviour of ionizable groups, $(\Delta S_i)^{prot}$. The latter term is equal to the difference between the standard entropy of denatured and native protein, i.e. without consideration of the conformational entropy change ($\Delta S_{conf} = \Delta S^{app}$ at pH > 4.5, see above). As the denaturational entropy change according to the definition of the standard state is already eliminated we obtain:

$$\Delta S_i^0(\mathrm{pH}) = S^{0,D}(\mathrm{pH}) - S^{0,N}(\mathrm{pH}). \quad (7)$$

The term $S^{0,D}(\mathrm{pH})$ can be obtained from modelled functions according to eqs. (6a) and (6b) and also from experimental results. We will begin with the latter possibility. If the potentiometric titration curve of native lysozyme and the difference in protonation ($\Delta\nu$) of transition into the denatured state are known

Table 4
Partial enthalpy and entropy changes at denaturation of lysozyme *. pH dependence at 25°C.

Column	1.	2.	3.	4.	5.	6.	7.
pH	$H^{0,D'}-H^{0,N}$	$(\Delta\nu\,\Delta H_i)$ of buffer	$(\Delta\nu\,\Delta S_i)$ of buffer	ΔS^{app}	ΔS^{prot}	$S^{0,D'}-S^{0,N}$	$S^{0,D''}-S^{0,N}$
1.5				171.5		58.6	43.1
2.0	−3.0	1.9	−15.1	167.0	182.1	64.3	47.3
2.5	−3.3	3.3	−25.6	160.0	185.6	72.4	49.2
3.0	−3.5	3.3	−25.6	151.7	177.3	78.1	42.6
3.5	−3.0	1.9	−15.1	145.4	160.5	70.4	21.2
4.0	−1.6	0	− 8.8	143.0	151.8	42.2	− 1.8
4.5	−0.4	0	0	141.0	141.0	10.3	−11.3
5.0	−0.4	0	0	140.4	140.4	8.7	−12.4

* Enthalpy in kcal mol^{-1}, entropy in cal $mol^{-1}K^{-1}$.

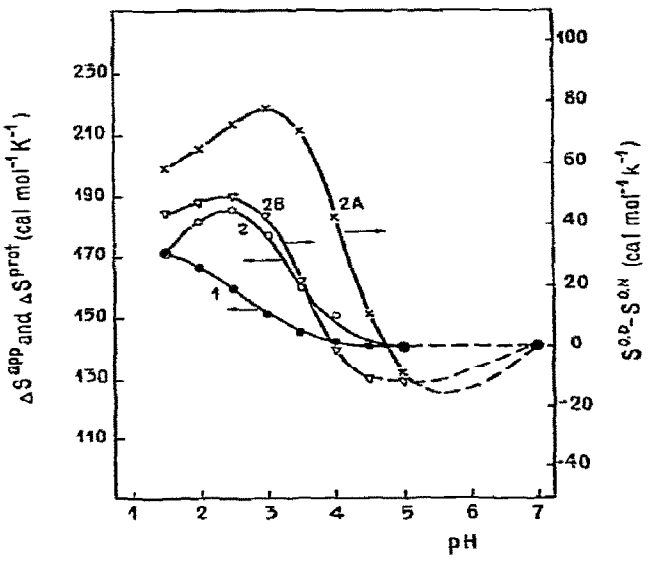

Fig. 6. Entropy changes at denaturation versus pH at 25°C. Curve 1: apparent entropy of denaturation (ΔS^{app}, left scale). Curve 2: partial entropy change ΔS^{prot} (left scale). Curve 2A and 2B: calculated entropy changes at "normalization" of ionization ΔS_i (right scale, see also text).

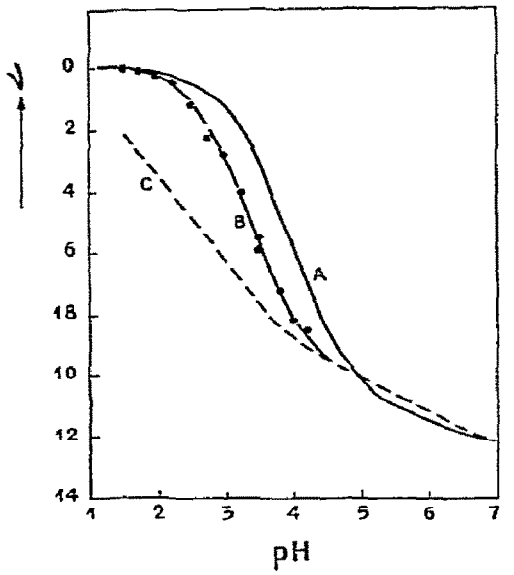

Fig. 7. Protonation of lysozyme versus pH at 25°C. Curve A: native lysozyme. Curve B: denatured lysozyme, constructed by experimental $\nu_{native} + \Delta\nu_d$. Curve C: denatured lysozyme, calculated using ionization data from table 2.

(see [1]) then the titration curve of denatured lysozyme at 25°C can be constructed (fig. 7, curve B). From this curve the pH dependence of the standard Gibbs function can be obtained using eq. (10) in ref. [1]*. Furthermore, using the ionization enthalpies as given in table 3, column 5, the pH-dependence of the standard entropy $S^{0,D''}$ (pH) (table 3, column 8) can be calculated. The partial entropy of normalization of ionization on unfolding of lysozyme, ΔS_i^0(pH), according to eq. (8) is given in table 4, column 7, and fig. 6, curve 2B. This curve is within the experimental error of $\Delta\nu$ determination identical with the pH-characteristics of ΔS^{prot}. Therefore the conclusion can be drawn that the conformational entropy change of lysozyme is pH-independent in the whole investigated pH-range, whereas the pH-dependence of ΔS^{prot} is induced by the normalization of ionization manifested only in the term $(\Delta S_i)^{prot}$.

It is obvious that the pH-dependent term $(\Delta S_i)^{prot}$ also induces the pH-dependence of the partial Gibbs

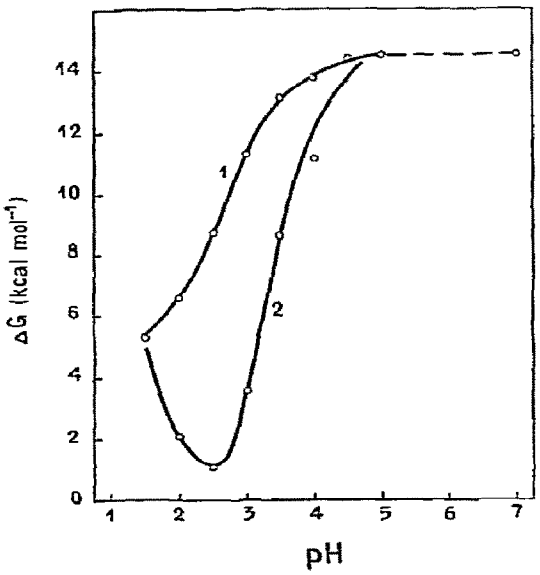

Fig. 8. Gibbs energy changes on denaturation versus pH at 25°C. Curve 1: apparent Gibbs energy of denaturation (ΔG_d). Curve 2: partial Gibbs energy change ΔG^{prot}.

* For this equation the symbols $S^{0,D''}$(pH) and $G^{0,D''}$(pH) are used in contrast with the theoretical functions based on amino acid composition.

energy, $(\Delta G_i)^{prot}$, whereas the partial conformational Gibbs-energy change ΔG_{conf}, remains pH-independent (see fig. 8). But it should be mentioned that the partial Gibbs-energy change, $(\Delta G_i)^{prot}$, cannot be considered as a criterion of protein stability.* As it is the Gibbs-energy change for the whole protein–solution system including the source of protons (or OH^- ions) taken up by the protein in unfolding that is essential in protein denaturation.

Returning to the partial entropy change, the term $S^{0,D}(pH)$ in eq. (7) can be alternatively calculated on the basis of amino acid composition of lysozyme corresponding to eqs. (6a) and (6b) using the basic data given in table 2 (results in table 3, columns 4–6). The obtained partial entropy function $\Delta S_i^{0'}(pH)$ is shown in fig. 6, curve 2A. The shape of this curve is similar to those of curves 2 and 2B, but the entropy changes described by curve 2A are significantly higher than the experimentally observed ones. The difference between the experimental (curve 2 and 2B) and "theoretical" entropy changes (curve 2A) is quite large, but there is no unambiguous explanation for this phenomenon since we do not know how adequately the sum of the entropies of ionization of free amino acids represents the ionization of groups in a polypeptide chain. It is known that the pK values of ionization of carboxyl groups can be decreased by the neighbourhood of peptide groups [16] and furthermore can be influenced by stereochemical effects.* As there is an identity in the ionization enthalpy of a given carboxyl group in a protein, a polypeptide and an amino acid in first approximation, then the observed pK changes must be caused by different ionization entropies. Therefore the standard entropy function calculated on the basis of single amino acids can be overestimated in comparison with the coiled state of a polypeptide, but we do not know to what extent.

The other reason that the "theoretical" entropy function is not reached by the experimental one is the possible presence of electrostatic interactions when cross linkings prevent ionizable groups to be at a maximal distance as in linear polypeptide chains. This assumption is supported by the low increase in viscosity at denaturation as discussed in [2]. Evidence for the presence of electrostatic interaction forces in GuHCl-denatured lysozyme was provided by potentiometric titration studies ($\omega = 0.04$ [20]). The same electrostatic interaction factor was found in our experiments without GuHCl (analysis of curve B, fig. 7). Therefore the entropy function of random coiled lysozyme without electrostatic interactions can be expected to be higher than the experimental curve of $\Delta S_i(pH)$ (fig. 6, curve 2B) but lower than the overestimated curve 2A in fig. 6.

* Unfolding of lysozyme at low pH values is accompanied by proton uptake. In the protein–water subsystem this process is impossible without pH-changes, but these are prevented by considerable Gibbs-energy changes on ionization.

* An example of this is the ionization of tetraalanine (Ala–Ala–Ala–Ala) in different configurations [16]: LLLL, $pK_{carboxyl} = 3.42$; LLDL, $pK_{carboxyl} = 3.24$; LDLL, $pK_{carboxyl} = 3.22$; DLLL, $pK_{carboxyl} = 3.42$.

4. Conclusion

In this series of papers we have presented an experimental approach for the determination of the thermodynamic properties of biopolymers in solution and have given a thermodynamic description of a typical globular protein in a wide scale of external conditions. However, there is every reason to presume that the picture obtained by us for lysozyme is very typical for small compact globular proteins. Perhaps the most remarkable thermodynamic property of these macromolecular systems is the presence of two surfaces describing their state in the phase space. One of the surfaces corresponds to the native state and the other one to the denatured state while thermodynamically stable intermediate states are absent.

It is surprising that in spite of a qualitative difference between two experimentally observable macroscopic states, they differ little in Gibbs energy. Consequently, the energy expenditures on transition from one state to another under the accessible conditions are rather small.

It can be seen that the mentioned thermodynamic properties are not trivial for macromolecular systems, even for globular macromolecules. Attempts to artificially create systems with such thermodynamic properties have up to the present been unsuccessful notwithstanding the fact that in some cases the capability of polypeptides to globularization was achieved. This permits one to presume that the mentioned thermodynamic properties are special properties of globular proteins and that they are necessary to provide some

general biological requirements for such systems. We can only express some ideas on the necessity of these requirements: a) for the effective and reliable functioning of protein its structure must be well defined over a wide range of conditions. A system which may be damaged even by a slight action cannot be reliable. The reserve of protein stability ensuring constant relative disposition of all its elements must considerably exceed RT, i.e. must be not less than a few kcal/mol. b) The system must not be too rigid. A certain mobility is necessary not only to ensure a relative displacement of the structure elements in the process of protein functioning, but also for its sufficiently easy dismantlement without considerable energy expenditures. Since the main exchangeable energy unit in biology is of the order of 5–10 kcal mol^{-1}, the upper limit of the system stability must be of the same order.

Up to now there has been no clear indication as to how the mentioned thermodynamic properties are achieved in globular proteins. The elucidation of this mechanism seems to be one of the most important problems of present day protein physics.

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Appendix: Thermodynamic standard functions of lysozyme

$H^{0,N}(T,pH)$ (kcal mol^{-1})

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	−105.2	−107.8	−113.8	−116.9	−119.4	−120.9
10	− 65.2	− 67.8	− 73.8	− 76.9	− 79.5	− 80.9
20	− 22.5	− 25.1	− 31.1	− 34.2	− 36.7	− 38.2
25	0	− 2.6	− 8.6	− 11.7	− 14.2	− 15.7
30	23.3	20.7	14.7	11.6	9.1	7.6
40	72.1	69.5	63.5	60.4	57.9	56.4
50	123.8	121.2	115.2	112.1	109.5	108.1
60	178.3	175.7	169.7	166.6	164.0	162.6
70	235.6	233.0	227.0	223.9	221.4	219.9
80	295.9	293.3	287.3	284.2	281.6	280.2
90	359.0	356.4	350.4	347.3	344.7	343.3
100	424.9	422.3	416.3	413.2	410.7	409.2

$S^{0,N}(T,pH)$ (cal $mol^{-1}K^{-1}$)

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	−368.7	−347.8	−342.1	−329.7	−297.6	−268.0
10	−224.9	−204.0	−198.3	−185.9	−153.8	−124.2
20	− 76.1	− 55.2	− 49.5	− 37.1	− 4.9	24.6
25	0	20.9	26.6	39.0	71.2	100.7
30	77.5	98.4	104.1	116.5	148.7	178.2
40	235.7	256.6	262.3	274.7	306.9	336.4
50	397.9	418.8	424.5	436.9	469.1	498.6
60	563.8	584.7	590.4	602.8	635.0	664.5
70	733.3	754.2	759.9	772.3	804.5	834.0
80	906.1	927.0	932.7	945.1	977.3	1006.8
90	1082.1	1103.0	1108.6	1121.1	1153.3	1182.8
100	1261.0	1281.9	1287.6	1300.0	1332.2	1361.7

$G^{0,N}(T,pH)$ (kcal mol^{-1})

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	− 4.49	−12.81	−20.36	−26.84	−38.17	−47.67
10	− 1.59	−10.12	−17.71	−24.34	−35.98	−45.78
20	− 0.16	− 8.89	−16.54	−23.28	−35.27	−45.35
25	0	− 8.82	−16.53	−23.32	−35.47	−45.72
30	− 0.16	− 9.09	−16.81	−23.68	−35.98	−46.36
40	− 1.67	−10.83	−18.60	−25.60	−38.21	−48.89
50	− 4.76	−14.12	−21.95	−29.06	−42.01	−52.98
60	− 9.49	−19.04	−26.94	−34.17	−47.44	−58.70
70	−15.88	−25.65	−33.59	−40.96	−54.55	−66.11
80	−23.98	−33.97	−41.96	−49.45	−63.37	−75.23
90	−33.82	−44.02	−52.08	−59.69	−73.92	−86.07
100	−45.45	−55.86	−63.97	−71.71	−86.26	−98.70

$H^{0,D}(T,pH)$ (kcal mol^{-1})

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	− 88.1	− 90.7	− 96.7	− 99.8	−102.3	−103.8
10	− 32.4	− 35.0	− 41.0	− 44.1	− 46.7	− 48.1
20	26.1	23.5	17.5	14.4	11.9	10.4
25	56.4	53.8	47.8	44.7	42.2	40.7
30	87.6	85.0	79.0	75.9	73.4	71.9
40	152.2	149.6	143.6	140.5	139.0	136.5
50	219.6	217.0	211.0	207.9	205.3	203.9
60	289.8	287.2	281.2	278.1	275.5	274.1
70	362.9	360.3	354.3	351.2	348.7	347.2
80	438.9	436.3	430.3	427.2	424.6	423.2
90	517.7	515.1	509.1	506.0	503.4	502.0
100	599.4	596.8	590.8	587.7	585.2	583.7

$S^{0,D}(T,\mathrm{pH})$ (cal $\mathrm{mol^{-1}K^{-1}}$)

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	−366.2	−345.3	−339.6	−324.6	−283.8	−238.9
10	−165.8	−144.9	−139.2	−124.2	− 83.4	− 38.5
20	37.7	58.6	64.3	79.3	120.2	164.9
25	140.4	161.3	167.0	182.0	222.9	267.7
30	244.1	265.0	270.7	285.7	326.6	371.3
40	453.4	474.3	480.0	495.0	535.9	580.7
50	665.1	686.0	691.7	706.7	747.6	792.4
60	879.0	899.9	905.6	920.6	961.5	1006.2
70	1095.1	1116.0	1121.7	1136.7	1177.6	1222.3
80	1313.1	1334.0	1339.7	1354.7	1395.6	1440.3
90	1533.1	1554.0	1559.6	1574.7	1615.6	1660.3
100	1754.8	1775.7	1781.4	1796.4	1836.8	1882.0

$G^{0,D}(T,\mathrm{pH})$ (kcal $\mathrm{mol^{-1}}$)

	pH					
T(°C)	7.0	6.0	5.0	4.0	3.0	2.0
0	11.9	3.6	− 4.0	−11.2	−24.8	−38.6
10	14.5	6.0	− 1.6	− 9.0	−23.1	−37.2
20	15.1	6.3	− 1.3	− 8.8	−23.3	−37.9
25	14.5	5.7	− 2.0	− 9.5	−24.2	−39.1
30	13.7	4.7	− 3.0	−10.7	−25.6	−40.6
40	10.3	1.2	− 6.6	−14.4	−29.7	−45.2
50	4.8	− 4.6	−12.4	−20.4	−36.2	−52.0
60	− 3.0	−12.5	−20.4	−28.5	−44.7	−61.0
70	−12.7	−22.5	−30.4	−38.7	−55.2	−72.1
80	−24.6	−34.6	−42.6	−51.0	−68.0	−85.2
90	−38.7	−48.9	−57.0	−65.6	−83.1	−100.7
100	−55.3	−65.7	−73.8	−82.3	−99.9	−118.3

References

[1] W. Pfeil and P.L. Privalov, Biophys. Chem. 3 (1975) 23.
[2] W. Pfeil and P.L. Privalov, Biophys. Chem. 3 (1975) 33.
[3] P.L. Privalov and N.N. Khechinashvili, J. Mol. Biol. 86 (1974) 665.
[4] N.N. Khechinashvili, P.L. Privalov and E.I. Tiktopulo, FEBS Letters 30 (1973) 57.
[5] P.L. Privalov, V.V. Plotnikov and V.V. Filimonov, Chem. Thermodynamics 7 (1975) 41.
[6] K.C. Aune and C. Tanford, Biochemistry 8 (1969) 4586.
[7] A. Salahuddin and C. Tanford, Biochemistry 9 (1970) 1342.
[8] D. Puett, J. Biol. Chem. 248 (1973) 4623.
[9] J.A. Knapp and C.N. Pace, Biochemistry 13 (1974) 1289.
[10] R.F. Greene, Jr. and C.N. Pace, J. Biol. Chem. 249 (1974) 5388.
[11] P.L. Privalov, O.B. Ptitsyn and T.M. Birshtein, Biopolymers 8 (1969) 559
[12] R.E. Canfield, J. Biol. Chem. 238 (1963) 2698.
[13] A.R. Rees and R.E. Offord, Biochem. J. 130 (1972) 965.
[14] C. Bjurulf, Eur. J. Biochem. 30 (1972) 33.
[15] J. Steinhardt and J.A. Reynolds, Multiple equilibria in proteins (Acad. Press, New York, London, 1969) p. 178.
[16] J.T. Edsall and J. Wyman, Biophysical Chemistry, Vol. I (Acad. Press, New York, 1958) p. 452.
[17] J.W. Larson and L.G. Hepler, in: Solute-solvent interactions, eds. J.F. Coetze and C.D. Ritchie (Marcel Dekker Inc., New York, London, 1969) p. 1.
[18] R.M. Izatt and J.J. Christensen, in: Handbook of Biochemistry (Cleveland, Rubber Co., 1968).
[19] M.A. Marino and R.L. Berger, Analyt. Biochem. 43 (1971) 188.
[20] R. Sakakibara and K. Hamaguchi, J. Biochem. (Tokyo) 64 (1968) 613.