PEPSINOGEN DENATURATION IS NOT A TWO-STATE TRANSITION

Pedro L. MATEO and Peter L. PRIVALOV*

Departamento de Química Física, Facultad de Ciencias, Universidad de Granada, Spain and *Institute of Protein Research, USSR Academy of Sciences, 142292 Poustchino, Moscow Region, USSR*

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1. Introduction

The knowledge of the mode of the denaturation reaction of proteins is important not only to understand their mechanism of folding, but also to obtain information on their structural organization. From this point of view pepsinogen is one of the most interesting objects, since it is sufficiently large (M_r 40 000) and its denaturation is highly reversible, which is a rare situation among proteins. It has been proposed in kinetic studies of pepsinogen denaturation that this process is a two-state transition [1,2], i.e., that the structure of this protein represents a single cooperative system. On this basis the thermodynamic parameters of stabilization of the native structure of pepsinogen have been obtained from equilibrium measurements of denaturation [3].

Here, we present the results of a calorimetric study of the thermodynamics of pepsinogen denaturation which definitely show that this process is not a two-state transition. It is highly probable that a pepsinogen molecule consists of two quasi-independent and thermodynamically rather identical cooperative blocks. The thermodynamic similarity of these two blocks seems to be the main reason that their existence has not been revealed by kinetic studies.

2. Materials and methods

Grade I swine stomach pepsinogen, chromatographically free of pepsin activity, was purchased from Sigma (lot 98C-0451), as a lyophilized powder, and used without further purification. Protein solutions were prepared by dissolving the lyophilized powder in buffer solutions and, prior to calorimetric experiments, they were dialyzed against buffer solutions in a cold room for 24 h. Buffers used were sodium cacodylate, and sodium phosphate, both of them at 5×10^{-3} M.

Protein concentration was spectrophotometrically determined at 278 nm using a molar extinction coefficient $E = 5.17 \times 10^4$ l . cm^{-1} . M^{-1} [4]. Concentration of pepsinogen solutions ranged from 0.60–2.10 mg/ml for all measurements. Calorimetric experiments were carried out in the differential adiabatic scanning microcalorimeter DASM-1M [5] at a heating rate of 2 K/min. Reversibility of the thermal unfolding process was checked by reheating the protein solution in the calorimeter immediately after the cooling from the first run. Two of these consecutive heating runs are shown in fig.1 as original recordings for pepsinogen solution at pH 6.0. Calorimetric or real enthalpies for the denaturational transition ($\Delta_d H^{cal}$) were obtained from the area under the peak using an appropriate calibration mark, while effective, or van't Hoff, enthalpies were calculated from the expression:

$$\Delta_d H^{v'tH.} = 4\,RT_d^2\,\frac{\Delta C_p^{max}}{Q_d}$$

where ΔC_p^{max} is the height of the heat capacity peak at the middle of the transition (T_d), and Q_d is the total heat of denaturation for that experiment [6]. The increase of partial heat capacity of the protein on denaturation ($\Delta_d C_p$) was determined as in [6,7]. For all these calculations M_r 39 687 was used [8,9].

3. Results and discussion

Thermal denaturation of pepsinogen proceeds with an extensive heat absorption and results in an essential heat capacity increase $\Delta_d C_p$ (fig.1). The process, judg-

* To whom correspondence should be addressed

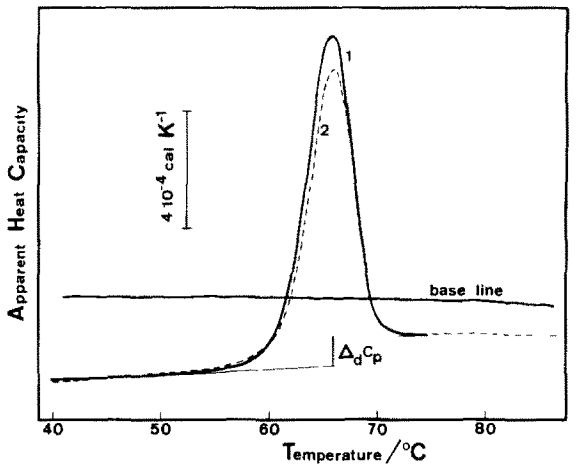

Fig.1. Original calorimetric recording of heat absorption of two consecutive runs of pepsinogen solution ((1) first heating; (2) reheating); protein, 0.82 mg/ml; buffer, 5 mM sodium cacodylate (pH 6.0).

Table 1
Thermodynamic characteristics of thermal denaturation of pepsinogen

pH	Solvent	T_d (°C)	$\Delta_d H^{cal}$ (kcal/mol)	$\Delta_d H^{v't H.}$ (kcal/mol)	R
6.0	Buffer	66.0	270.8	174.0	1.6
6.5	Buffer	64.5	255.6	131.0	2.0
7.1	Buffer	57.5	233.1	130.1	1.8
8.0	Buffer	51.1	181.9	122.3	1.5
7.1	Buffer + 0.1 M NaCl	56.3	194.1	143.0	1.4
6.5	Buffer + 1 M urea	61.0	240.0	125.2	1.9
7.1	Buffer + 1 M urea	54.5	195.8	119.6	1.7

R stands for the ratio $\Delta_d H^{cal}/\Delta_d H^{v't H.}$. Buffers used were 5 mM sodium phosphate for pH 6.5, 7.1 and 8.0, and 5 mM cacodylate for pH 6.0

ing by the perfect reproducibility of the heat absorption peak, is highly reversible. It was found that specific parameters characterizing this process do not show any dependence on protein concentration. Therefore it can be treated as an equilibrium monomolecular reaction. Assuming that this process can be regarded as a two-state transition, one can estimate from its sharpness (i.e., from the relative height of the peak) the van't Hoff enthalpy of denaturation $\Delta_d H^{v't H.}$ If the assumption made on the two-state nature of pepsinogen denaturation is valid, this van't Hoff enthalpy must be equal to the real or calorimetric enthalpy $\Delta_d H^{cal}$ which is estimated from the area of the heat absorption peak.

The values of the calorimetric and van't Hoff enthalpies of pepsinogen denaturation estimated for various conditions are listed in table 1. As can be seen, there is a drastic difference between both these enthalpies at whatever condition studied. In salt-free solutions at about neutral pH the ratio $\Delta_d H^{cal}/\Delta_d H^{v't H}$ amounts to 2.0, while in the presence of salt and/or pH values apart from neutrality it is somewhat lower but still higher than 1.0. This ratio is also very close to 2.0 in solutions containing urea. Therefore it can be concluded that denaturation of pepsinogen is, in no case, a two-state transition process.

Fig.2 presents a plot of calorimetrically obtained enthalpy values of pepsinogen denaturation in different solvent conditions versus transition temperature T_d. As it is clear from fig.2, the slope of this linear dependence is very close to the $\Delta_d C_p$ mean value found by direct calorimetric measurements of the heat capacity change at denaturation (fig.2, —) which is equal to (6.1 ± 0.3) kcal . K^{-1} . mol^{-1}. It follows that the enthalpy of pepsinogen denaturation does not depend noticeably on the pH and ionic strength of the solution but is a linear function of temperature, as it is in the case of other globular proteins [6]. Therefore enthalpy and entropy values of pepsinogen transition into denatured state at any temperature for any set of conditions can be easily estimated from the calorimetrically measured denaturational enthalpies using the equations:

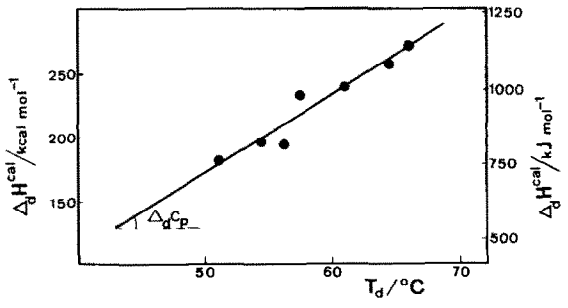

Fig.2. Calorimetric enthalpy of pepsinogen denaturation *vs* transition temperature. The slope of the straight line corresponds to the increase of the partial heat capacity on denaturation ($\Delta_d C_p$).

$$\Delta H(T) = \Delta_d H - \int_T^{T_d} \Delta_d C_p \, dT$$

and

$$\Delta S(T) = \Delta_d H/T_d - \int_T^{T_d} \Delta_d C_p d \ln T$$

By these equations we obtain for the standard temperature 25°C (298 K) and pH 8.0 in salt-free solutions 22.7 kcal/mol and 49 cal . K^{-1} . mol^{-1} for the enthalpy and entropy of pepsinogen transition into the denatured state. These values are in drastic conflict with 31.5 kcal/mol and 105 cal . K^{-1} . mol^{-1} found from the equilibrium studies of pepsinogen denaturation by urea [3]. These differences cannot be explained by the assumption of incomplete unfolding of pepsinogen at thermal denaturation, since the denaturational heat capacity change, which can be regarded as the indicator of the extent of exposure of internal hydrophobic groups in water at unfolding, is higher for the thermal denaturation (6.1 kcal . K^{-1} . mol^{-1}) than that found for urea denaturation in equilibrium studies (5.215 kcal . K^{-1} . mol^{-1}). Therefore the discrepancy found can be explained only by the incorrectness of the basic assumption in equilibrium studies of a two-state nature for pepsinogen denaturation. This assumption was based on the results of kinetic studies of pepsinogen denaturation [1,2]. The incapability of kinetic methods of resolving stages in pepsinogen denaturation seems to be the result of a thermodynamic similarity of those stages.

From the enthalpy and entropy values at 25°C one can estimate the Gibbs' free energy value of pepsinogen denaturation at 25°C bearing in mind that:

$$\Delta G(T_o) = \Delta H(T_o) - T_o \Delta S(T_o)$$

For pH 8.0 in salt-free solution ΔG (25°C) equals 8.1 kcal/mol. As it is evident, this energy corresponds to the work required for the disruption of the whole molecule of pepsinogen at 25°C, but since denaturation of this protein is not of a two-state type, this value cannot be regarded as a measure of stability of its native state. If the deviation from the two-state behaviour is caused by the existence of several cooperative units in the molecule, the stability of the native state would then be expressed by the stability of the least stable unit. Assuming that pepsinogen molecule consists of two thermodynamically identical cooperative units, we would obtain 4 kcal for its stability in the above conditions. The same calculation at pH 6.0, salt-free solution, gives 8.5 kcal/mol for pepsinogen stability (ΔG(25°C) = 17 kcal/mol), which corresponds to the highest value at 25°C, from the studied conditions. This stability value is close to those found for maximum stability of other compact globular proteins [6].

The assumption on the existence of two cooperative units in a pepsinogen molecule is not as unjustified as it might seem. Indeed, the ratio $\Delta_d H^{cal}/\Delta_d H^{v'tH}$ for such a combined system consisting of two independent cooperative units with identical stability, as evident, must be equal to 2.0 and should decrease with an increase of interaction between the units. This situation has already been found in the case of papain which is known to consist of two very alike and independent parts [10]. On the other hand, although the three-dimensional structure of pepsinogen is not yet known, it cannot be expected to be qualitatively different from that of pepsin which has been shown to be composed of two similar and distinct parts [11]. The main thermodynamic difference between pepsinogen and pepsin is that the parts of pepsin are not of equal stability and they unfold at different temperature ranges (fig.3). A detailed comparative analysis on the thermodynamic properties of these two molecules will soon be submitted for publication.

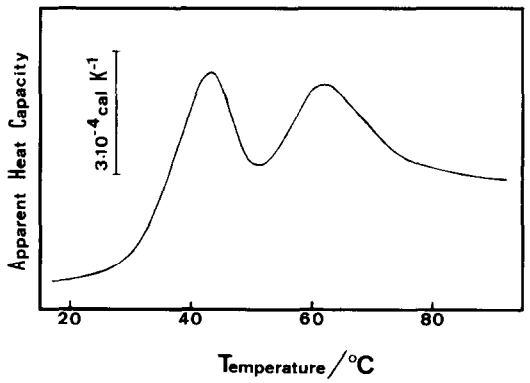

Fig.3. Calorimetric recording of heat absorption at denaturation of pepsin solution; protein, 1.42 mg/ml; solvent, 5 mM; sodium phosphate, 0.1 M NaCl (pH 6.5).

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