KINETIC STUDY OF THE *p*H INFLUENCE ON BSA THERMAL DENATURATION

V.E. Sahini*, G. Csire, C. Popa-Țilimpea and Alina Tîrşoagă

The kinetics of BSA thermal denaturation was studied in buffer solutions at *p*H=2; 7.8 and 10 using a dilatometric method. In order to compare the rate constant values (k) of this process, the plot of ΔV versus temperature was used in order to obtain k (considering some approximations) at a given temperature: $k = \frac{\Delta V}{A-a}$. In this equation, ΔV is the volume difference between the BSA solution and the buffer used as a solvent, A is the area of the $\Delta V = f(T)$ plot obtained for a given step of the considered thermal denaturation process and a is the part of this surface corresponding to a given temperature T. In this study some conformational changes induced by thermal denaturation of BSA macromolecule were evidenced, the temperatures of transition as well as the $\Delta G^{\neq}$ values for the first transition being presented and discussed.

Introduction

Complete denaturation of a protein consists in an irreversible loss of its secondary structure and also of its biological activity [1]. The unfolding process of the macromolecule is the first step in the main modification (non-covalent, cooperative or reversible one) of its native structure. The denaturation can be described using two kinds of micro-states: some of them are unfolded states characterised by a strong influence of the solvent and the other one is a folded state characterised by a higher degree order of the macromolecules [2]. For the discussed cases, the random coil structure is not taken into consideration.

In a relatively simple structural approach of the thermal denaturation of the bovine serum albumin (BSA), it has been recognised that the α –helical structure is disrupted and the β-structure is formed [3]. The aggregation property of this substance is correlated with the occurrence and increase of the β-structure fraction, as well as with the presence of free S-H group of the cysteine-34. Data from Fourier transform-infrared spectra are in agreement with the mechanism of additional disordered structures formed and also with a substantial remaining part of helical structure [4].

Raman spectroscopy proved that the conformational change is reversible below 50 ^{0}C only and partial reversible at higher temperatures [5]. The results of circular dichroism spectra are important in obtaining the change of relative proportion of BSA α–helix, β-structure and random coil [6].

The aim of the present paper is the application of the differential thermal analysis [7] to the study of the thermal denaturation of BSA in quasi-neutral (*p*H=7.8), acidic (*p*H=2) and basic (*p*H = 10) buffer solution using a dilatometric method.

* Dept. of Phys. Chem. Univ. of Bucharest, Bd. Elisabeta 4 – 12, 70346, Bucharest, Romania

Experimental Part

The fraction V of BSA (Sigma) was used without purification.

In order to dilatometrically study the BSA thermal denaturation, a differential thermal analysis procedure [8] has been used, the change taking place with volume variation. Using the $\Delta V = f(T)$ plots, the kinetic parameters of the denaturation transition can be determined. These obtained curves were analysed, in terms of slope, height and surface area.

The ΔV values were obtained by using four glass-flask finished to the top through soldered pipettes, graduated to 10 μl; a graduated microscope was used and the errors of the volume values were of 0.5 μl.

One cell contains the solution of BSA, the second one the solvent (buffer solution); the other two cells containing water, were used one as a thermometer for the determination of the average temperature in the dilatometric cell and the second (using a digital thermometer in the center of the cell) to obtain the temperature in the "coldest" point during the heating.

The solutions are assumed to be diluted enough to consider them as having the water heat capacity.

The bath-water temperature was constantly increased. In order to obtain higher temperatures, two electric hot plates were placed under the bath water. In order to assure a uniform temperature of it, two magnetic stirrers were used. The bath temperature was indicated by using a mercury thermometer ($\Delta T = \pm 0.5\ ^0$C) and at equal time the increase of the volume due to thermal dilatation was determined. The difference of the volumes (ΔV) was read in function of time or bath temperature, which was heated at the constant rate of 1 K/min. The thermal denaturation phenomenon takes place with a very small rate at the beginning of the heating (20^0C). The determination of the ΔV was continued up to 70^0C, when the denaturation process practically finished and the coagulation can be observed. By plotting ΔV versus time / temperature, a curve was obtained after smoothing operations [9] of the ΔV values, for each pH-value the results being presented in Fig. 1.

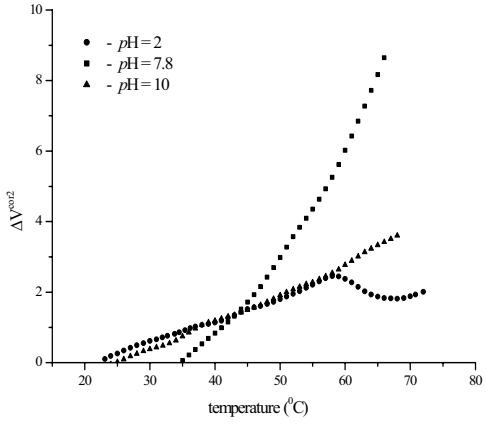

Fig. 1: *Representation of* $\Delta V = f(T)$*;* pH *influence.*

The general theory proposed by Borchardt and Daniels [10], based on the calorimetric equation, as well as the classical chemical kinetics laws, allow us to calculate the rate constant in each point of the DTA plot. By using in DTA the volume variation instead of the temperature one (ΔT), the list of the ten elementary assumptions elaborated by Borchardt and Daniels in their paper is modified. The following assumptions are considered as still valid: (i) the rate constant of the process is small at the beginning of the experiment; (ii) the pressure shall remain constant during the denaturation process; (iii) the Arrhenius law is valid and we admit that the activation energy of the investigated step is constant.

The thermal denaturation process is developing in more successive steps [11] until the protein coagulation occurs. Then, in this paper, a kinetic analysis procedure was used, by considering separately each denaturation step. The volume change can be measured and it can characterise the step development, being directly proportional with the number of molecules denatured in a small interval of time. The heat transfer from bath to solutions occurs both by conduction and convection and the considered temperature inside the flasks is the value read from the water flask, used as a thermometer.

The denaturation process is presumed to be a first order reaction. The obtained data lead, as in many others cases, to a probably reversible mechanism with many steps defined species corresponding to a slow and fast refolding mechanism respectively. For example, one proposes the following mechanism for the pH=7.8 case:

$$I \underset{}{\overset{slow}{\rightleftharpoons}} U_1 \overset{fast}{\rightleftharpoons} U_2 \Longrightarrow N$$

where I corresponds to initial state and N corresponds to final one.

In order to compare kinetically the three studied processes of thermal denaturation, the rate constants were computed, using the modified form of Borchardt and Daniels's relation.

$$k = \frac{\Delta V}{A - a} \tag{1}$$

where ΔV is the volume difference between the BSA and the buffer solutions, A is the area of the $\Delta V = f(T)$ plot obtained for each considered step of thermal denaturation process and a is the part of this surface corresponding to a certain temperature T. In this kinetics approach each denaturation step was studied separately. One considers that the ΔV versus T representation presents a decreasing part symmetrical to the increasing experimental side. The A value used in equation (1) is the surface defined by considering a base line ΔV = const., as it is shown in the Fig. 2.

The modification of the slope of $\Delta V = f(T)$ plot was considered as defining the change from a stage to another one of the denaturation process.

Over the smoothed data points, peak function was fitted and used for further. The value of the fitting function and the corresponding area for each temperature domain were computed. These values allow determining the reaction rate constant k (1) and they were smoothed twice, using the same procedure described above.

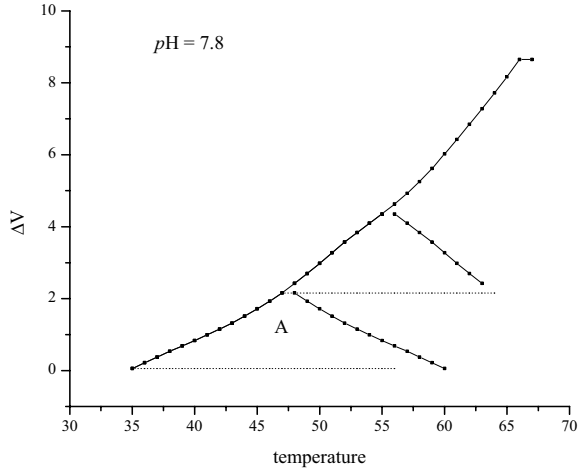

Fig. 2: *Representation of the ΔV=f (T);*
graphical description according to each denaturation step.

Results and Discussion

In this study some conformational changes induced by thermal denaturation of BSA macromolecule were evidenced, the temperatures of transition being presented in Table 1.

Table 1. The *p*H influence on the temperature of transitions

No	*p*H	Temperatures of transition (°C)		
1	2	40	47	58
2	7.8	43	57	
3	10	33	41	59

The first two transitions represent a reversible defolding and the third one corresponds to an irreversible denaturation process. At a *p*H near to the physiological value, the resistance of BSA macromolecule to the thermal perturbation appears to be higher comparing with the acidic and particularly with the basic solutions [12].

The unfolding protein temperature is the most important phenomenon searched for all along of our dilatometric studies. In order to compare our experimental data with other ones from biophysics, the following conclusions may be proposed. Working in physiological conditions (*p*H = 7.8) the first transition occurs at 43^0C, very near of the temperature range ($36 \div 42^0$C) of the homeotherms within the Animals Kingdom, until the native conformation of the albumin is kept.

The acidic medium (*p*H = 2) has a relatively weak influence on the thermal denaturation, the first transition occurring at 40^0C, that is below the body temperature for some

homeotherm species. Thus, the acidic medium produces a conformational modification and, probably, looses some biological properties even in the vital field of temperature.

As it has been mentioned in the literature, the basic medium is more harmful, the unfolding temperature of the first step being lowered to 33^0C, below physiological temperature of all homeotherms.

For a first order reaction, the Gibbs free energy was calculated, using the following equation:

$$\Delta G^{\neq} = 2.303RT \log \frac{K_B}{h} + \log T - \log k \tag{2}$$

In order to analyse the first step of thermal denaturation, the following hypothesis should be considered: according to DTA, the molecular structure stability can be correlated with the expression $\frac{\Delta G^{\neq}}{2\Delta t}$ for the temperature range of the first defolding step.

One considers the temperature range described in our work and the corresponding limits of the $\Delta G^{\neq}$ values are shown in Table 2.

Table 2. The *p*H influence on the $\sum\Delta G^{\neq}/2\Delta t$ value of the first denaturation step

No	*p*H	t_i(^{0}C)	t_f(^{0}C)	$\Delta G_i^{\neq}$(kJ/moll)	$\Delta G_f^{\neq}$ (kJ/mol)	$\frac{\Sigma\Delta G^{\neq}}{2\Delta t}$ (kJ/mol degree)
1	2	34	46	63	69	5.5
2	7.8	22	29.5	58	73	8.7
3	10	25	40	41	60	3.4

One obtains the order of the BSA macromolecules stability for the three *p*H values as 7.8>2> 10, corresponding to our previous comment of these data.

The second transition presumes subsequently unfoldings, occurring at temperatures between $41\div57^0$C. These conformational modifications are reversible transitions, in some cases prior to the transition that enables the irreversible denaturation. In this case, the perturbation effect of acidity and basicity can also be observed, namely the second unfolding occurs at 57^0C, in the quasineutral medium (near physiological value), the acid medium drops it with 10 degrees (at 47^0C) while the basic one drops it with 20 degree (at 41^0C).

The unfolding temperature for the third transition in the basic medium is near to the value in the acidic one, the excessive pH having in both cases a severe influence on the unfolding at high temperature as a step of the coagulation process.

Conclusion

The differential thermal analysis was applied to investigate the kinetics of the conformational change of bovine serum albumin by heat treatment in the buffer solutions with *p*H = 2; 7.8 and 10. The structural change of BSA in acidic solution is found to be

reversible in a larger temperature range, probably because the interaction between macromolecular domains might disappear due to the acidic expansion. In order to compare the *p*H effects upon the thermal denaturation of BSA the temperatures of transition step (with the approximations made in this paper) were obtained. The order of unfolding transition is found to prevail the pH=7.8 as the most stable one followed by *p*H = 2 and 10. The *p*H-effect was also evidenced from the values of numerical first criteria $\frac{\Sigma\Delta G^{\neq}}{2\Delta t}$ for the first unfolding step only.

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