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STUDIES ON THE KINETICS OF PROTEIN DENATURATION UNDER HIGH PRESSURE*

BY KEIZO SUZUKI

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The characteristics in the protein denaturation under pressure obtained by the author up to date are summarized.

Thermodynamic functions in the activation process of denaturation are calculated from the relations of absolute reaction rates using the results of ovalbumin[5] and carbonylhemoglobin[7] to discuss the mechanism of protein denaturation under pressure. Considering the signs of the values of $\Delta H^{\neq}$, $\Delta S^{\neq}$ and $\Delta V^{\neq}$, the results are to be classified into three regions; I: $<30°C$, $>4000\,kg/cm^2$ ($\Delta H^{\neq}<0$, $\Delta S^{\neq}<0$, $\Delta V^{\neq}<0$), II: $>40°C$, $>3000\,kg/cm^2$ ($\Delta H^{\neq}>0$, $\Delta S^{\neq}\gtrless 0$, $\Delta V^{\neq}<0$), and III: $>60°C$, $<3000\,kg/cm^2$ ($\Delta H^{\neq}>0$, $\Delta S^{\neq}>0$, $\Delta V^{\neq}>0$).

In the region III where thermal denaturation is retarded by pressure, the behaviors are to be understood if it is taken into consideration that a disorderliness of configuration by the thermal vibration is retarded by pressure. In the region I where pressure denaturation only occurs, the following mechanism is proposed,

$$P+nH_2O \underset{(1)}{\rightleftarrows} P(H_2O)_n \underset{(2)}{\rightarrow} P_D,$$

where $P(H_2O)_n$ is the protein hydrate which always exists in the equilibrium relation of (1) under high pressure and is constructed with the rigid structure; P_D is the denatured protein. The rate determining step is the process of (2). The formation of $P(H_2O)_n$ is exothermic and accompanied with the volume decrease. And it is discussed with what mechanism much more plenty of water is adhered to protein and then the volume decreases. It is supposed that in the region III both denaturations by pressure and heat occur at the same time.

The denaturation of protein by pressure has been generally well known since the finding of the perfect coagulation of egg white by a pressure of 7000 atm in 30 minutes[1]. The studies of denaturation under high pressure are, however, rare and almost qualitative compared with those by heat, acid, urea and so on, so that there is no theory for the influence of hydrostatic pressure on the mechanism of denaturation.

It seems that there are three common interesting points on the protein denaturation. They are: (1) Although the denaturation phenomena are very complex and characteristic, they should be derived from the changes of structure of proteins. Therefore, the studies of denaturation will give the important key to make clear the structure of protein. (2) This phenomena are probably in the course of the process that the living matter goes to the nonliving matter, and therefore much attentions will be paid from the wide survey of biology. (3) The well-known facts that

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1) P. W. Bridgman, *J. Biol. Chem.*, **19**, 511 (1914)

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

the temperature coefficients of the rate of thermal denaturation are generally very large compared with those of the usual chemical reactions, for example in ovalbumin the rate increases by a factor of several hundreds with the temperature rise of 10°C, that the temperature coefficient is negative in the rate of urea denaturation, and that the activation entropy is negative in the acid denaturation of hemoglobin, and so on, are very interesting from the stand point of chemical kinetics[2]. The above mentioned interests on the denaturation phenomena will be common to the studies of the denaturation under pressure, and especially the third interest from the kinetics became the decisive motive to take up this research under pressure, because kinetical treatments are seldom in the field of high pressure.

From such considerations, the author has performed the studies of ovalbumin[3~5] which is the most typical globular protein, and of hemoglobin[6,7] which is the representative of chromoprotein. Though some studies[8~13] of these proteins under pressure have been reported, more abundant and more quantitative informations in the wide ranges of pressure and temperature are necessary to discuss the mechanism of pressure denaturation and especially the relations between such two opposing roles of pressure that the denaturation is caused by very high pressure, while thermal denaturation is retarded by comparatively lower pressure. From such a stand point, especially the more detailed researches have been performed at the wide conditions of normal pressure ~ 9000kg/cm² and 10~75°C in the previous papers[5,7].

As the measure to examine the denaturation process, the turbidity method[3], the analysis of SH-group[14,15], and so on were investigated. And the most conventional coagulation method, though it was primitive and macroscopic, was selected as the most useful measure under wide experimental conditions. That is, the process of denaturation was examined by the colorimetric measurement of the remaining protein (considered to be native) in the supernatant which was obtained by separating the coagulation formed at the isoelectric point. But it seems to be necessary to examine the denaturation process in use of physical and chemical methods as many as possible, and so such a research should be taken up in a future work.

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15) K. Suzuki and K. Kitamura, *Abstracts of the 30th Annual Meeting of the Japanese Biochemical Society* (1957)

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

The characteristic points obtained by the author up to date are summarized as follows. The rate of denaturation is essentially of the first order with regard to the protein concentration through the whole range of experiment. In Fig. 1 the points with a given rate constant (0.1

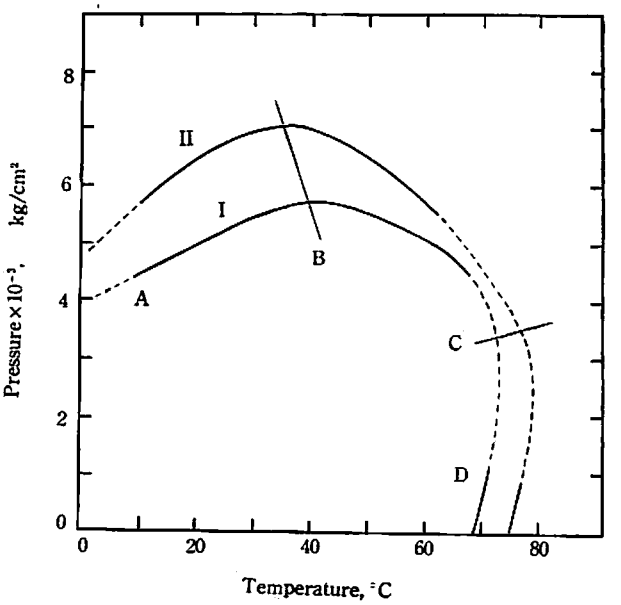

Fig. 1 Variations of rate constants of protein denaturation in relation to temperature and pressure

rate constant: 0.1 min^{-1} on each line

line I : ovalbumin at pH 4.8

line II : carbonylhemoglobin at pH 6.8

min^{-1}) are plotted against temperature and pressure in both cases of albumin and hemoglobin. It is found from this figure that the results resemble each other in both proteins. That is, the figures are composed of three parts: AB, BC and CD respectively. It is to be especially noted that the rate of denaturation has the negative temperature coefficient in the part of AB.

The behaviors of hemoglobin are remarkably different from albumin in the reversibility of coagulation. That is, in ovalbumin the coagulated protein caused by pressure does not change in its nature after releasing pressure and standing for some time, while in hemoglobin the coagulation once formed becomes soluble again. Such a difference may offer any key to discuss the structures of both proteins.

In this paper, the thermodynamic functions are calculated from the relations of absolute reaction rates using the results of ovalbumin[5] and carbonylhemoglobin[7] about which the most quantitative experiments have been carried out in the wide range. And then the mechanism of protein denaturation under pressure will be considered, and the conclusion of this research will be given.

Calculations of Thermodynamic Functions

Substituting the results of ovalbumin[5] and carbonylhemoglobin[7] obtained in the previous reports in the following relations deduced from the equation of absolute reaction rates, the thermodynamic functions in the activation process of denaturation ΔF^*, ΔH^*, ΔS^* and ΔV^* are calculated.

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

Where $\Delta F^{\ddagger}$ is the free energy increase when the activated complex is formed from the native protein; $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ are the analogous increases in heat, entropy and volume.

$$\Delta F^{\ddagger} = \ln\frac{kT}{hk'}, \qquad \Delta H^{\ddagger} = E - RT,$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta F^{\ddagger}}{T} \quad \text{and} \quad \Delta V^{\ddagger} = -RT\frac{d\ln k'}{dP},$$

where k' is the rate constant of the first order; k the Boltzman constant; h the Planck constant; T the absolute temperature; E the apparent activation energy; R the gas constant and P the magnitude of pressure. Some of the results obtained are given in Tables 1 and 2. From these tables, it is found that their characteristic points are nearly the same in both cases of albumin and hemoglobin, though there are some differences that the temperature dependence of the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ below 40°C is rather large in hemoglobin, being not found almost in albumin*. That is, considering the signs of the values of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$, results are to be classified into three regions as shown in Table 3. It is to be noted from these tables that $\Delta H^{\ddagger}$ changes the sign around 40°C, and $\Delta V^{\ddagger}$ has the negative value except the case where the retardation in thermal denaturation occurs. $\Delta S^{\ddagger}$ in the region II seems to change the sign according to the conditions, namely it is supposed $\Delta S^{\ddagger}$ may tend to become negative at the higher pressure and the lower temperature.

Table 1 Kinetics of denaturation of ovalbumin under pressure (pH 4.8)

P kg/cm²	Temperature °C	k' sec⁻¹	$\Delta F^{\ddagger}$ kcal/mole	$\Delta H^{\ddagger}$ kcal/mole	$\Delta S^{\ddagger}$ cal/deg·mole	$\Delta V^{\ddagger}$ cc/mole
4500	10	2.0×10^{-3}	20	−25	−157	−92
	20	4.6×10^{-4}	22	−25	−158	−83
	30	7.7×10^{-5}	24	−25	−161	−81
	50	7.6×10^{-5}	25	32	23	−77
	65	4.5×10^{-4}	25	32	21	−55
	70	1.6×10^{-3}	25	32	22	−38
5000	20	2.9×10^{-3}	21	−26	−158	−83
	30	3.8×10^{-3}	23	−26	−161	−81
	40	1.8×10^{-4}	24			
	50	4.0×10^{-4}	24	30	19	−77
	65	1.7×10^{-3}	24	30	18	−55
	70	2.8×10^{-3}	24	30	18	−38
0	70	2.9×10^{-3}	24	101	221	24
800		1.5×10^{-3}	25	86	179	24
2000		8.4×10^{-4}	25	82	166	
3000		6.3×10^{-4}	25	68	126	
4000		8.0×10^{-4}	25			
4500		1.6×10^{-3}	25	32	22	−38
5000		2.8×10^{-3}	24	30	18	−38

* This difference is due to the fact that the apparent activation energy, E below 40°C depends on temperature in hemoglobin, while does not in albumin as reported in the previous papers[5,7].

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

Table 2 Kinetics of denaturation of carbonylhemoglobin under pressure (pH 6.8)

P kg/cm²	Temperature °C	k' sec^{-1}	$\Delta F^{\ddagger}$ kcal/mole	$\Delta H^{\ddagger}$ kcal/mole	$\Delta S^{\ddagger}$ cal/deg·mole	$\Delta V^{\ddagger}$ cc/mole
6000	10	4.5×10^{-3}	20	−29	−173	−99
	15	6.9×10^{-4}	21	−20	−144	−85
	20	3.9×10^{-4}	22	−17	−133	−68
	25	2.7×10^{-4}	22	−12	−116	−68
	50	8.1×10^{-4}	24	25	4	−47
	60	2.5×10^{-3}	24	25	4	−44
6500	15	3.9×10^{-3}	20	−29	−171	−85
	20	1.5×10^{-3}	21	−21	−144	−68
	25	9.4×10^{-4}	22	−21	−144	−68
	50	1.9×10^{-3}	23	23	0	−47
	60	5.3×10^{-3}	23	23	0	−44
0	72.5	7.0×10^{-4}	26	80	156	17
1000		5.0×10^{-4}	26	73	136	17
2000		3.8×10^{-4}	26	70	128	
3000		5.8×10^{-4}	25	56	88	
4000		1.4×10^{-3}	25	42	49	−25
5000		3.3×10^{-3}	24	37	38	−25

Table 3 Classification into three regions based on the signs of the thermodynamic functions

	Temperature °C	Pressure kg/cm²	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta V^{\ddagger}$
I	<30	>4000	−	−	−
II	>40	>3000	+	±	−
III	>60	<3000	+	+	+

Considerations

The rate of denaturation is of the first order with respect to the protein concentration through all over the regions, and so it is found that the intramolecular process governs the rate determining step.

In the region III (>60°C, <3000 kg/cm² as shown in Table 3) thermal denaturation is retarded by pressure. The values of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ are all positive and the very large value of $\Delta S^{\ddagger}$ at the atmospheric pressure decreases with the rise of pressure in this region. It is generally considered to explain the extraordinary large value of $\Delta S^{\ddagger}$ in thermal denaturation that the disorganization of protein molecule or the unfolding of peptide chains occurs because of the breaking of the subsidiary bridge caused by the thermal vibration. Therefore, the behaviors in the region III are to be understood if it is taken into consideration that such a disorderliness of configuration is retarded by pressure. Dilatometer measurements by Heymann[16] indicated an expansion of 80.3 cc per mole (at 80°C) in the thermal denaturation of ovalbumin at the isoelectric point, and so it

16) E. Heymann, *Biochem. J.*, **30**, 127 (1936)

is also estimated from the principle of Le Chaterier that pressure behaves to check the thermal denaturation. And it is interesting to make a comparison between the values of $\Delta V^{\ddagger}$: 24 cc per mole (at 70°C) obtained in Table 1 and the above 80.3 cc per mole.

In the region I (<30°C, >4000 kg/cm² as shown in Table 3) where pressure denaturation only occurs, it is noteworthy that all the values of $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ become negative. Now, the following mechanism on pressure denaturation is proposed, considering the values of these thermodynamic functions and the facts that the rate is of the first order with regard to the protein concentration, and so on;

$$\mathrm{P}+n\mathrm{H_2O} \underset{(1)}{\rightleftarrows} \mathrm{P(H_2O)}_n \underset{(2)}{\longrightarrow} \mathrm{P_D}, \qquad m\mathrm{P_D} \underset{(3)}{\longrightarrow} (\mathrm{P_D})_m,$$

where P is the native protein; $\mathrm{P_D}$ is the denatured protein; $(\mathrm{P_D})_m$ is the coagulated protein; n is the number of the water molecule combined with protein; m is the number of the denatured protein which forms coagulation; $\mathrm{P(H_2O)}_n$ is the protein hydrate which exists always in the equilibrium relation of (1) under high pressure and is constructed with the rigid structure having much more water molecules adhered (or frozen) to protein. The rate determining step is the process of (2). As the formation of $\mathrm{P(H_2O)}_n$ is exothermic and accompanied with the volume decrease, the equilibrium of (1) tends to move much more toward the right hand if the temperature is lowered and the pressure increases. Accordingly, the forming rate of denatured protein becomes fast at such conditions. (3) is the associating process of denatured protein.

Now, it is deduced in the following way that the rate of denaturation is of the first order with respect to the protein concentration:

$$\frac{d[\mathrm{P_D}]}{dt} = k'_2[\mathrm{P(H_2O)}_n] = k'_2K[\mathrm{H_2O}]^n[\mathrm{P}] = k'[\mathrm{P}], \qquad k' = k'_2K[\mathrm{H_2O}]^n = \text{constant},$$

where k_2' is the rate constant in (2); K is the equilibrium constant in (1); k' is the over-all rate constant which is obtained actually from the experiment.

The problem that $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta V^{\ddagger}$ which are deduced from the rate constant k' are negative will be considered. Now, it is irrational in itself that activation enthalpy and activation entropy become negative in an elementary process. Therefore, $\Delta H_2^{\ddagger}$ and $\Delta S_2^{\ddagger}$ of $\mathrm{P(H_2O)}_n$ in the process of only (2) should take positive values respectively. And it is reasonable to assume that the activation volume, $\Delta V_2^{\ddagger}$ takes also positive value, because the denatured protein would be in the unfolding state. These problems are to be explained in the following way. It is readily known that the relations of $\Delta H_2^{\ddagger}=\Delta H^{\ddagger}-\Delta H$, $\Delta S_2^{\ddagger}=\Delta S^{\ddagger}-\Delta S$ and $\Delta V_2^{\ddagger}=\Delta V^{\ddagger}-\Delta V$ are established, where ΔH, ΔS and ΔV are quantities in (1) respectively. Accordingly, $\Delta H_2^{\ddagger}$ and $\Delta S_2^{\ddagger}$ may be positive, even if the formation of $\mathrm{P(H_2O)}_n$ is exothermic (moreover, $|\Delta H|>|\Delta H^{\ddagger}|$) and $\mathrm{P(H_2O)}_n$ is in the lower state of entropy (moreover, $|\Delta S|>|\Delta S^{\ddagger}|$), because of the rigidness of the structure, according to the denaturation mechanism above described. And it will be reasonable to assume such properties of $\mathrm{P(H_2O)}_n$.

In the case of $\Delta V_2^{\ddagger}$ the same elucidation will be applied from the mechanism that the process of (1) is accompanied with the volume decrease. It is clear that the volume usually

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

decreases in the solvation, but in this case it is necessary to consider with what mechanism much more plenty of water is adhered to protein and then the volume decreases. The first is such a case that owing to the increase of ionization of the dissociating groups in protein caused by pressure, water molecules are attracted to the ionizing groups by the electrostriction, and then the total volume decreases. Though the volume decrease is about 10cc per mole of ionizing group[17], it seems this volume change becomes very large in macromolecules such as proteins, for this volume change is additive. Therefore the increase of pressure favors the process of ionization, and large influences of pressure are expected on the volume contraction of the system.

As the second mechanism, the functions of the water molecule modified by pressure[17*,18] are to be considered. Owing to its characteristic actions, the water molecule modified by pressure behaves in the analogous way to urea molecule in the urea denaturation in which it is supposed that a great number of urea are adhered to protein[11,19], and therefore the total volume decreases, because much more water molecules are adhered (or frozen) to the protein molecule.

It seems to be difficult to decide uniquely which mechanism is more favorable, and which mechanism is more predominant if both mechanisms may participate in the process at the same time**. It is, however, supposed that the former mechanism may be predominant at relatively higher temperature, and the latter at relatively lower temperature. But from the experimental results obtained by Drickamer *et al*[18***] on self-diffusion in water and the necessity that $P(H_2O)_n$ must be more thoroughly lower state in enthalpy and entropy to explain the negative values of ΔH^* and ΔS^* as above mentioned, the latter mechanism in which is considered the functions of the water molecule modified by pressure seems to be more favorable than the former.

It is, however, common to both mechanisms to have to acknowledge the existence of the singular protein hydrate in the intermediate process to denaturation, though the structures will be not necessarily the same.

It is supposed that in the region III (>60°C, >3000kg/cm² as shown in Table 3) both denaturations by pressure and heat occur at the same time.

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* *cf.* p. 304

** The process seems to be complex from the fact that the relations between the logarithm of rate constant and the reciprocal of the absolute temperature do not follow a linear relationship as shown clearly in carbonylhemoglobin[7].

*** It has been found from the experimental results of self-diffusion in water at 25°C that the tetrahedrally coordinated structure of the usual water is broken down with the increase of pressure, and a stabilized new structure is formed around 4000atm. It is very interesting to note that the pressure denaturation begins at the same pressure region.

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18) R. B. Cuddeback, R. C. Koeller and H. G. Drickamer, *J. Chem. Phys.*, 21, 589 (1953)

19) F. G. Hopkins, *Nature*, **126**, 328, 383 (1930)

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Laboratory of Physical Chemistry
Faculty of Science and Engineering
Ritumeikan University
Kyoto, Japan

$$-\frac{dx}{dt} = \frac{k_2[E]_0}{1 + K_m/x}$$

$$-\frac{dt}{dx} = \frac{1}{k_2[E]_0} + \frac{K_m}{k_2[E]_0 x}$$

$$-dt = \frac{dx}{k_2[E]_0} + \frac{K_m}{k_2[E]_0}\frac{dx}{x}$$

$$t = \frac{x_0 - x}{k_2[E]_0} + \frac{K_m}{k_2[E]_0}\ln\frac{x_0}{x}$$