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Kinetics of the Denaturation of Ovalbumin and S-Ovalbumin by Alcohols

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The kinetics of denaturation of ovalbumin and S-ovalbumin by butyl alcohol isomers (butyl alcohol, isobutyl alcohol, *s*-butyl alcohol, and *t*-butyl alcohol) have been studied. The reactions were characterized as first order with respect to the protein and 11th order for the alcohols, irrespective of the kind of protein or alcohol. The rate constant was, however, significantly reduced by the branching of the alcohol structure and by the conversion of native ovalbumin to S-ovalbumin. The measurements were also performed on ovalbumin and S-ovalbumin whose SH groups were blocked from SH⇌S–S exchange reactions by modification with iodoacetamides. By the conversion to S-ovalbumin, the modified protein also exhibited a considerable resistivity against denaturation, suggesting that SH⇌S–S exchange reactions play no essential role in the formation of S-ovalbumin. On the basis of these results as well as determined activation parameters, the mechanism of the denaturation is discussed in the light of a combination of the hydrophobic interactions, between hydrophobic residues of the proteins and the alcohol molecules, and the fluctuations of the protein structures.

S-ovalbumin (stable ovalbumin) which is produced by a long-time storage of shell eggs[1] or by holding isolated ovalbumin in a warm and alkaline solution[2] is known to exhibit a considerable resistivity against various denaturation conditions, such as heat and urea.[2,3] Many attempts have been made in order to find some structural differences between ovalbumin and S-ovalbumin, which are very similar to each other.[2] (a) SH⇌S–S exchange reactions,[2-4] (b) an increase in the antiparallel β-sheet geometry with the expense of the same amount of the α-helical conformation,[5] (c) liberation of carboxyl groups,[6] (d) a reduced radias,[7] and (e) an increase in the surface hydrophobicity[7] have been suggested in explanation of the increased resistivity. However, it is still unclear that which of these plays an essential role for the conversion to S-ovalbumin.

Effects of alcohols on protein denaturation and as protein denaturants have extensively been studied for many proteins.[8-15] The main result of these studies is that the effectiveness of the alcohols increases with increase in the alkyl residue content while it reduces for branched alcohols. The result has been attributed to hydrophobic interactions between hydrophobic residues of the proteins and the alcohol molecules. On the other hand, it has also been pointed out that the effectiveness of the alcohols is highly correlated with their ability to disrupt water structure.[16,17] Furthermore, Bull and Breese[18] have recently studied the kinetics of the denaturation of many proteins by alcohols and reported an enhanced effect for alcohols having longer alkyl chains. However, the details of the kinetic aspects and the mechanism of the denaturation are still not fully elucidated.

The present paper describes the kinetics of the denaturation of ovalbumin and S-ovalbumin by butyl alcohol isomers and presents a possible denaturation mechanism as well as suggesting the difference between these proteins.

Experimental

Materials. The ovalbumin was prepared from fresh egg white by a method, essentially same with that of Kekwick and Cannan.[19] It was recrystallized three times from a $(NH_4)_2SO_4$ solution at pH=4.7 and stored at 4 °C. Before using, the preparation was exhaustively dialyzed against distilled water. The S-ovalbumin was prepared from the ovalbumin by the method of Smith and Back.[2]

The SH-blocked ovalbumin was prepared by the following method.[20] A stock solution of iodoacetamide was added dropwise to an aliquot of the dialyzed ovalbumin solution with stirring so as to make a final solution containing 10 moles of iodoacetamide per mole of SH group. The pH of the solution was then adjusted to 8.0 using a 0.2 M† NaOH solution. The solution was then kept at room temperature (about 18 °C) for 20 h, after which it was exhaustively dialyzed against water. This procedure was carried out in the dark. The SH group content of the preparation was determined according to the method of Boyer,[21,22] and it was found that two of the three SH groups which are exposed in the native ovalbumin[23] were blocked by the above method. For the conversion to the modified S-ovalbumin, this sample was also treated by the same method[2] as cited above.

The concentrations of the ovalbumin and S-ovalbumin solutions were determined spectrophotometrically, using the optical density $E^{1\%}_{1\,cm}=7.12$[24,25] at 280 nm.

p-Chloromercuribenzoic acid (guaranteed grade) was purchased from Katayama Chemicals. All other chemicals used were of reagent grade. Iodoacetamide, alcohols, sodium acetate and acetic acid were purchased from Wako Pure Chemical Industries and ammonium sulfate was from Katayama Chemicals.

Methods. The following procedure of the present kinetic measurements is similar to that of Bull and Breese.[18] All sample solutions were prepared in 0.2 M acetate buffer of pH=4.7. A 0.5 ml of ovalbumin solution (5×10^{-4} M) thermostated at 30 °C was rapidly mixed with 2 ml of an aqueous alcohol solution of an appropreate concentration,

† 1 M=1 mol dm^{-3}.

which was also thermostated at 30 °C: time=zero. It was found that the denatured protein began to precipitate in the course of time. After a time passage, the solution was diluted with 30 ml of the acetate buffer solution in order to stop the denaturation, and was kept at room temperature for 2 h for the full precipitation of the denatured protein. The solution was then centrifuged at 11000 rpm for 30 min, and an aliquot of the supernatant solution was subjected to the determination of the concentration of the remaining native protein. Blank measurements were also performed in the absence of the denaturants.

Analysis of Kinetic Data. Kinetics of the denaturation of the proteins by alcohols may be described by the equation:

$$P + m\,R\text{-}OH \longrightarrow D \qquad (1)$$

where R-OH, P, and D denote the alcohol molecule, the native protein, and the denatured one respectively and where m is the number of alcohol molecules required to denature one molecule of the protein. The rate of denaturation at any time can then be written by:

$$\frac{d\,[D]}{d\,t} = k\,[P]\,[R\text{-}OH]^m \qquad (2)$$

where [D], [P], and [R-OH] are the molar concentrations of the denatured and native proteins and alcohol, respectively and where k is the rate constant of the denaturation. The initial rate becomes in the logarithmic form as:

$$\log\left[\frac{d\,[D]}{d\,t}\right]_0 = m \log[R\text{-}OH]_0 + \log[P]_0 + \log k \qquad (3)$$

where the subscript 0 means the quantity at time zero: for instance $[P]_0$ is the initial concentration of the protein. According to this equation, m (the order of reaction) and k can be determined from the slope and the intercept of the plot of $\log[d[D]/dt]_0$–$\log[P]_0$ vs. $\log[R\text{-}OH]_0$, respectively.

Results and Discussion

Kinetic Parameters. Figure 1 shows the time courses of the denaturation of ovalbumin and S-ovalbumin in the presence of *s*-butyl alcohol. As can be seen in this figure, S-ovalbumin is highly resistant to denaturation as compared to ovalbumin. In order to ensure the order of reaction with respect to the protein, the dependence of the initial rate of the denaturation on the concentration of ovalbumin was measured (see Eq. 3). The result shown in Fig. 2 yields the slope of 1.09, confirming that the reaction is first order with respect to the protein.

Figure 3 shows the plots of $\log[d[D]/dt]_0$–$\log[P]_0$ vs. $\log[R\text{-}OH]_0$ for the denaturation of the four kinds of proteins by the butyl alcohol isomers. In accordance with Eq. 3, each plot exhibits a linear dependence; m and k determined from these plots are summarized in Table 1. The solubility of isobutyl alcohol is so small that the determination of the denaturation curve by the low alcohol concentration required an extremely long time (1 or 2 days) and the data were not collected.

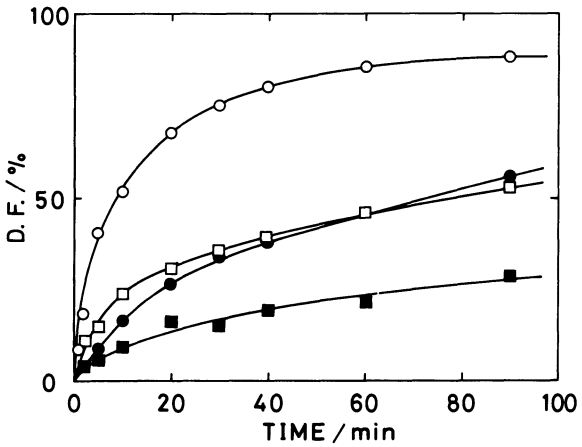

Fig. 1. Typical time courses of the denaturation of ovalbumin and S-ovalbumin by *s*-butyl alcohol. The ordinate shows the denatured fraction (D.F.) of the proteins. The initial concentrations of the alcohol are 1.1 M (○) and 0.95 M (●) for ovalbumin and 1.2 M (□) and 1.1 M (■) for S-ovalbumin.

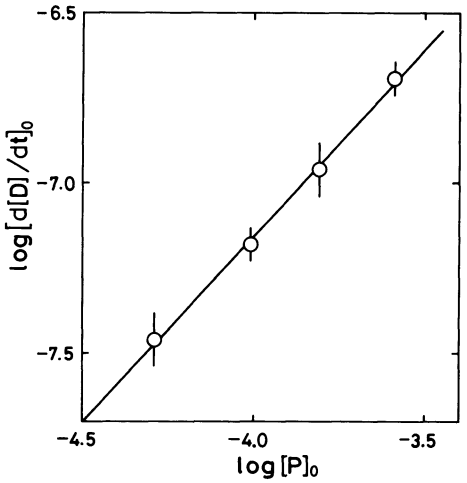

Fig. 2. Plot of $\log[d[D]/dt]_0$ vs. $\log[P]_0$ for ovalbumin in the presence of 1 M *s*-butyl alcohol. The solid line represents a least-square fit to the data, the slope of which is 1.09.

The following points can be seen from Table 1. At first, the order of reaction with respect to alcohols (m) is, within the experimental error, 11th, being irrespective of the kinds of protein or alcohol structure. The value of m for butyl alcohol is relatively in agreement with that reported by Bull and Breese.[18] They also reported that the order of the denaturation reaction by streight-chain alcohols decreases with the increase in the hydrocarbon content. Considering this together with the present results, it is suggestive for the denaturation mechanism that the number of alcohol molecules required for the denaturation depends on their hydrocarbon content

but is independent of the degree of branching.

Secondarily, k is, on the other hand, highly dependent on the structure of the denaturants and on the state of the protein. It progressively decreases with increase in the degree of branching of the denaturant's alkyl chain. Furthermore, it reduces by a factor from 3 to 10 on the conversion from ovalbumin to S-ovalbumin, that is, S-ovalbumin is highly resistant to the denaturation as compared to the native ovalbumin.

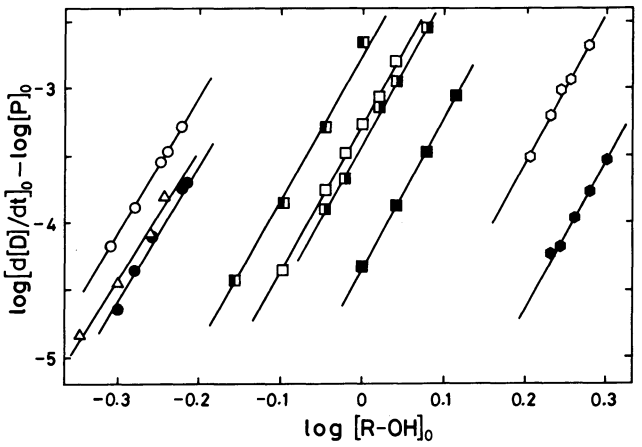

Fig. 3. Plot of $\log[d[D]/dt]_0 - \log[P]_0$ vs. $\log[R\text{-}OH]_0$ for the denaturation of the proteins by butyl alcohol isomers. The denaturants are differentiated by the symbols as circle (butyl alcohol), triangle (isobutyl alcohol), square (s-butyl alcohol), and hexagon (t-butyl alcohol). Open symbols denote ovalbumin and filled ones S-ovalbumn. Half filled symbols ◧ and ◨ denote SH-modified native ovalbumin and S-ovalbumin produced from the SH-modified ovalumin respectively.

Table 1. Rate Constants and Orders of Reaction of the Denaturation by Butyl Alcohol Isomers at 30°C[a)]

Alcohol	Ovalbumin	$k/M^{-m}\,S^{-1}$	m
n-BuOH	n-	2.14×10^{-1}	11.7
	S-	6.46×10^{-2}	11.5
i-BuOH	n-	2.95×10^{-2}	9.6
	S-	—	—
s-BuOH	n-	5.38×10^{-4}	11.1
	S-	4.68×10^{-5}	10.8
	n-mod.-	1.93×10^{-3}	11.3
	S-mod.-	3.89×10^{-4}	11.1
t-BuOH	n-	1.83×10^{-6}	11.0
	S-	2.36×10^{-7}	10.3

a) Abbreviations: n-BuOH=butyl alcohol, i-BuOH=isobutyl alcohol, s-BuOH=s-butyl alcohol, t-BuOH=t-butyl alcohol, n-=native ovalbumin, S-=S-ovalbumin, n-mod.-=SH-modified native ovalbumin, S-mod.-=S-ovalbumin produced from the SH-modified native ovalbumin. The error of m was estimated as ±1.

Finally, the magnitude of k for S-mod. -ovalbumin is about one fifth of that for n-mod. -ovalbumin, showing that a considerable resistivity is achieved for S-mod. -ovalbumin. Although only two of the three SH groups, which are exposed in native ovalbumin,[23)] were assured to be blocked by iodoacetamide molecules, it is reasonable to assume that all of the easily exchangeable SH groups were blocked by the present procedure. Therefore, it may be concluded that SH⇌S-S exchange reactions do not play any essential role in the conversion of ovalbumin to S-ovalbumin.

Activation Parameters. From the temperature dependence of k, the activation parameters of the denaturation of ovalbumin and S-ovalbumin by s-butyl alcohol were determined in the usual manner.[26)] Figure 4 shows the plot of $\ln[k/T]$ vs. T^{-1}. The slope and the intercept of the plot provide the activation enthalpy (ΔH^*) and the activation entropy

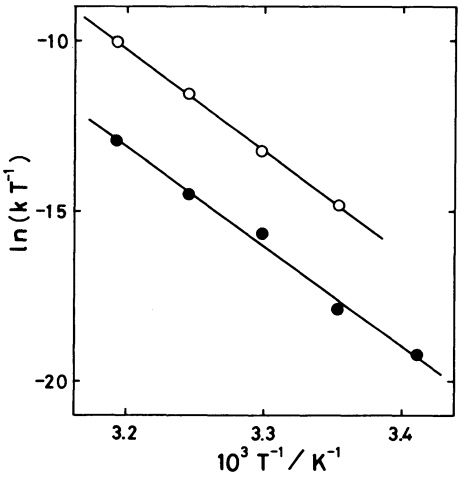

Fig. 4. Plots of $\ln(kT^{-1})$ vs. T^{-1} for the denaturation of ovalbumin and S-ovalbumin by s-butyl alcohol. Open circles denote ovalbumin and filled ones S-ovalbumin. The solid lines represent least-square fits to the data.

Table 2. Activation Parameters of the Denaturation by s-Butyl Alcohol[a)]

Protein	ΔG^*	ΔH^*	ΔS^*	$\Delta\Delta G^*$	$\Delta\Delta H^*$	$\Delta\Delta S^*$
	kJ mol^{-1}	kJ mol^{-1}	J K^{-1} mol^{-1}	kJ mol^{-1}	kJ mol^{-1}	J K^{-1} mol^{-1}
Ovalbumin	93	248	511			
S-ovalbumin	100	243	472	7	−5	−39

a) ΔΔ means the difference between the activation parameter for S-ovalbumin and that for native ovalbumin. The errors of ΔH^* and ΔS^* were estimated as 2—5% of those values.

(ΔS^*) of the denaturation respectively. The results are summarized in Table 2.

Denaturation Mechanism. The effects of alcohols on the protein denaturation and as the denaturants have been found to become pronounced with increasing alkyl residue content of the alcohols and to reduce with the increase in the degree of branching. They have been interpreted in terms of hydrophobic interactions:[8-15] bound alcohol molecules penetrate into the protein molecule, and interact with hydrophobic residues by replacing the native hydrophobic bonds between these residues and thereby destabilize the protein structures. These result have been derived from static information such as the effects on the transition temperature for thermal denaturation and on the denaturation midpoint, i.e., the concentration of a denaturant required to produce 50% change in the particular observable. The mechanism is, however, not sufficient to account for the present data for the effects of the branching of alcohol molecules on the kinetic parameters (Table 1).

It has been well-known that the structure of a globular protein is not rigid but fluctuates about its most stable conformation. The fluctuations may accompany an exposure of a part of hydrophobic regions to the solvent by openings of channels or crevices, the degree of which must necessarily depend on the degree of the spatial extent of the fluctuations. The so called "breathing"[27,28] motions may be involved in these fluctuations. A large fluctuation is expected to occur only rarely while small ones occur frequently. It is reasonable to assume that the alcohol molecules approach to the hydrophobic region by taking, among the possible conformations, the most fine-line-shape of the hydrophobic portion and by orienting along the channels or the crevices. Therefore, straight-chain molecules can easily approach the hydrophobic region through the frequently opening narrow channels or crevices. Thereby hydrophobic interactions between alcohol molecules and the hydrophobic residues will soon be achieved, resulting in the subsequent denaturation. This leads to large values of k for normal alcohols. On the other hand, branched alcohol molecules which have large diameters must, at the surface of the protein, wait for a wide channel or a wide crevice to open. Small values of k are, therefore, expected for the branched molecules. This mechanism seems to interpret well the observed correlation between the degree of branching of the alcohol molecules and the order of the value of k for these molecules, i. e., butyl alcohol > isobutyl alcohol > *s*-butyl alcohol > *t*-butyl alcohol (Table 1).

An alternative explanation for the effects of alcohols as protein denaturants is based on their ability to destabilize the structure of water.[16,17] Protein molecules, stabilized by hydration around their exposed hydrophobic residues, could be destabilized by the collapse of the hydrated water structure. It is likely that the collapse of the water structure enhances the fluctuations of the protein structure, and hence makes the alcohol molecules easily accesible to the hydrophobic region. The resultant hydrophobic interactions between alcohol molecules and the hydrophobic residues further destabilize the protein structure and amplify the structural fluctuations. Like this, the hydrophobic interactions and the collapse of the hydrated water structure may act cooperatively on the protein denaturation. Both the collapse of the water structure and the enhanced fluctuations will result in a large activation entropy. These considerations are consistent with the result of Segawa and Kume[29] that isopropyl alcohol enhances the fluctuations of the activated state of the thermal denaturation of lysozyme. A schematic illustration of the denaturation mechanism is shown in Fig. 5. Although it is well-known that alcohol molecules have, in general, the effect of increasing α-helix content of many proteins, the role of this fact on the potein denaturation is still unclear.[16] For the sake of simplicity, such a fact is not illustrated in Fig. 5.

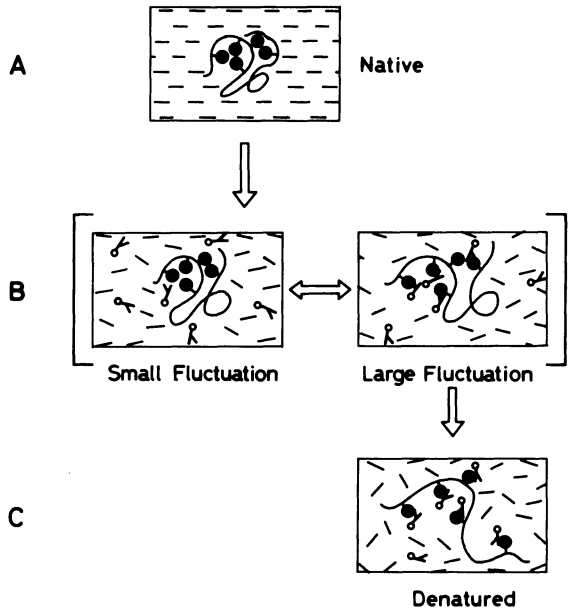

Fig. 5. A schematic illustration of the possible denaturation mechanism by alcohols. An addition of alcohol molecules (O—<) to the protein solution (A) destabilizes the structure of water, and hence enhances the fluctuations of the protein structure (B). When a large fluctuation takes place, alcohol molecules can approach so closely to the hydrophobic region of the protein that resultant hydrophobic interactions, between hydrophobic side chains (filled circles) and hydrophobic portions of alcohols, break the native hydrophobic bonds and thereby lead to the full denaturation of the protein structure (C).

According to the above mechanism, it may be concluded that S-ovalbumin is in a state in which the structural fluctuations are to some extent depressed as compared to the native ovalbumin. Nakamura and Ishimaru[7] reported that the Stokes radius of S-ovalbumin (25.5 Å) is smaller than that of ovalbumin (27.3 Å). The compact form of S-ovalbumin seem to be responsible for the depressed fluctuations. It can also be seen from Table 2 that the positive value of $\Delta\Delta G^*$ (7 kJ mol^{-1}) arises from the entropy term ($-T\Delta\Delta S^*$=12 kJ mol^{-1} at 30 °C) rather than the enthalpy term ($\Delta\Delta H^*$=−5 kJ mol^{-1}). This implies that the stability of S-ovalbumin is achieved by changes in the state of the structural fluctuations and/or in the hydration state of the protein rather than by any other strong bond formations. The increase in the surface hydrophobicity[7] and the change of a part (3—4%) of the protein from the α-helix to the antiparallel β-sheet conformation[5] in the conversion from ovalbumin to S-ovalbumin may also be closely related to the above changes. The fact that the number of alcohol molecules, required for the denaturation, decreases with the increase in the hydrocarbon content of the denaturant[18] but does not depend on the degree of branching for both of the ovalbumin and S-ovalbumin (Table 1) suggests that a definite amount of hydrophobic bonds must be broken in the activation process and it is not changed by the conversion of ovalbumin to S-ovalbumin. This is also supported by the small value of $\Delta\Delta H^*$ in Table 2.

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