Effect of Hydration on the Thermal Stability of Protein as Measured by Differential Scanning Calorimetry. Lysozyme-D_2O System

Yukihisa FUJITA* and Yukinao NODA

Department of Chemistry, Hyogo College of Medicine, Mukogawa, Nishinomiya, Hyogo 663

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The thermal denaturation of the deuterated lysozyme has been investigated by differential scanning calorimetry in the range of deuterium oxide (D_2O) content from 0.03 to 1.6 g of D_2O per g of protein. At D_2O contents above 0.55 g/g, the temperature, T_d, and enthalpy change, ΔH_d, of denaturation were almost independent of the degree of hydration. At lower D_2O contents, however, both T_d and ΔH_d showed marked dependence on the degree of hydration. The values of T_d increased with a decrease in the D_2O content. Whereas the values of ΔH_d decreased with a decrease in the D_2O content in the same region. The degree of hydration dependency of ΔH_d exhibited a break at approximately 170 mol/mol, which indicates that at least two types of hydration contributes to the thermal stability of the protein. The conformational enthalpy change of the protein and the enthalpy change of the hydration layer have also been estimated from the degree of hydration dependency of ΔH_d. The results have been compared with those of the lysozyme-H_2O system.

There has been a great deal of experimental evidence which has established that the interaction of water with proteins has an important role in determining the structure and biological function of the protein. This role, however, is poorly understood. The physical properties of water in the protein solution have been extensively investigated by several techniques, such as dielectric relaxation measurement,[1] NMR spectroscopy,[2] and calorimetry.[3] The studies have revealed that the properties of the interacting water are evidently different from those of pure water *e.g.*, lower mobility and reduced freezing point. It has been also observed that the hydration values in globular proteins do not differ greatly, being approximately 0.3 g of water per g of protein.[4]

In a previous paper,[5] the effect of hydration on the thermal stability of lysozyme was investigated by differential scanning calorimetry (DSC). It was observed that the essential hydration for stabilizing the spatial structure of lysozyme in water was completed at about 0.75 g/g. Below this water content, both the temperature and enthalpy change of denaturation showed a marked dependence on the degree of hydration. It was further suggested that at least two types of hydration contributed to the thermal stability of the protein, the threshold water content being approximately 0.33 g/g.

The present work was undertaken to investigate the effect of hydration of deuterium oxide (D_2O) on the thermal stability of deuterated lysozyme by DSC and to compare the results with those of the lysozyme-H_2O system.

Experimental

Materials. The hen egg-white lysozyme used in the present study was a recrystallized ($\times 6$) sample from Seikagaku Kogyo Co. The deuterium oxide (D_2O, purity 99.8%) was purchased from Aldrich Chemical Co.

Methods. Prior to calorimetric measurement, the lysozyme sample was deuterated as follows: the lysozyme was dissolved in D_2O(approximately 3% concentration), shaken for 50 h at 313 K, and lyophilized. After reaction, the extent of hydrogen-deuterium exchange was estimated by IR absorption according to the methods described by Blout *et al.*[6] and Nakanishi *et al.*[7] The amount of undeuterated peptide group was taken as proportional to the ratio of the absorbance of the amide II (at 1540 cm^{-1}) to that of the amide I (at 1650 cm^{-1}). In the absorbance measurement, the baseline for the amide I band was drawn parallel to the 100% transmittance at 1800 cm^{-1}, and the baseline of the amide II band taken as the absorption of the completely deuterated lysozyme. Complete deuteration was achieved by heating a solution of the exchanging protein at 353 K. From the IR measurements, approximately 83% of the total hydrogen atoms of the peptide exchanged, this amount corresponding to 106 peptide groups per molecule. It is a valid assumption that the hydrogen atoms of the protein side chain more rapidly exchange compared with those of the peptide group. In the lysozyme molecule there are in all 260 exchangeable hydrogen atoms.[8] The degree of deuteration which accounted for the number of deuterium atoms in the side chain was 92%, therefore, the molecular weight of the deuterated lysozyme has been taken as 14500.

The D_2O content of the sample was adjusted by conditioning in constant humidity apparatus at the appropriate relative humidity for 7 days. Higher D_2O contents were adjusted either by directly adding D_2O or by placing the sample in the saturated vapor at 293 K for an appropriate period. The relative humidity was maintained by a saturated aqueous solution in contact with an excess of solute at 293 K.[9]

The thermal denaturation of lysozyme was measured with a Rigaku Denki standard-type differential scanning calorimeter. For calorimetric measurements, the heating rate was 2.5 K/min.

The exact dry weight and D_2O content of sample were determined gravimetrically by drying the punctured sample pan at 378 K *in vacuo* for 24 h.

Results and Discussion

The thermal denaturation of deuterated lysozyme has been measured in the D_2O content range from 0.03 to 1.6 g/g. The temperature, T_d, and the enthalpy change, ΔH_d, of denaturation have been estimated from the temperature of the peak and the peak-area of the thermogram obtained, and plotted as a function of the D_2O content as shown in Figs. 1 and 2, respectively.

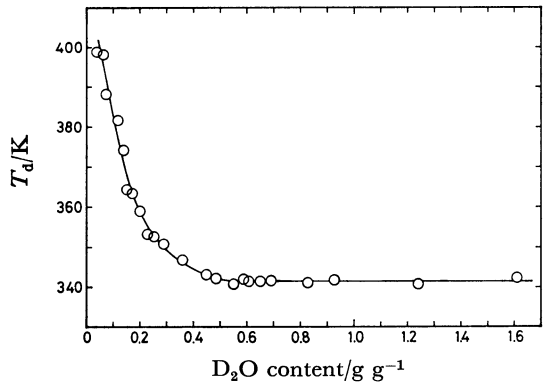

Fig. 1. The temperature of denaturation, T_d, of deuterated lysozyme as a function of the D_2O content.

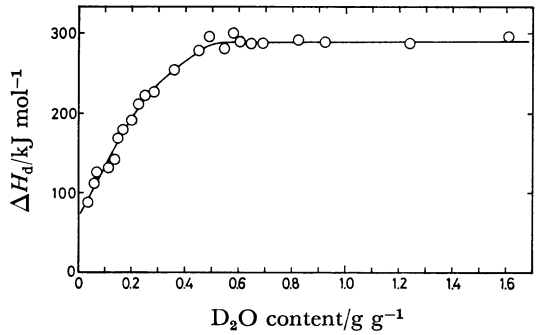

Fig. 2. The enthalpy change of denaturation, ΔH_d, deuterated lysozyme as a function of the D_2O content.

The data shows a marked dependency on the D_2O content, and the behavior has a resemblance to that for the lysozyme–H_2O system.

Both T_d and ΔH_d were slightly dependent on the D_2O content above 0.55 g/g which was a lower value than that reported for the lysozyme–H_2O system. The mean values in this region were 341.4 K and 298 kJ/mol, values which are almost identical not only with those for the lysozyme–H_2O system above 0.75 g/g, but also with those for lysozyme in aqueous solution.[10,11] The thermal denaturation of several proteins has been investigated in both H_2O and D_2O solutions and differences in the thermal stability of proteins in H_2O and D_2O solutions have been reported.[12,13] Nakanishi *et al.*[14] have, however, reported from UV measurements that the temperature of denaturation of lysozyme in D_2O solution was identical with that in H_2O solution. From NMR spectroscopic studies, McDonald *et al.*[15] also observed that the extent of denaturation of lysozyme in D_2O exhibited the same temperature dependency as that in H_2O. It appears reasonable to assume that a similar conformational change which takes place in solution occurs in the solid state containing a lot of water and that the conformational change of lysozyme by thermal denaturation makes little difference in H_2O and D_2O. In addition, the indication is that the hydration, an essential process for stabilizing the spatial structure of lysozyme in water, is completed at about 0.55 g/g for the lysozyme–D_2O system, which is a smaller value than that reported for the lysozyme–H_2O system, namely 0.75 g/g.

Below a D_2O content of 0.55 g/g, the T_d increased gradually with decrease in the D_2O content. The increase became much more marked at D_2O contents lower than 0.2 g/g. The ΔH_d, on the other hand, decreased with decrease in the D_2O content in the same region. In order to analyze the experimental data in this region of D_2O content it has been assumed that the observed enthalpy change of denaturation, ΔH_d, may be expressed as follows:

$$\Delta H_d = \Delta H_{conf} + n_{hyd}\Delta H_{hyd}$$

where ΔH_{conf} is the enthalpy change due to conformational changes in the protein, n_{hyd} is the degree of hydration, expressed by the number of mol of water adsorbed per mol of protein, and ΔH_{hyd} is the enthalpy change of the hydration layer, expressed on the basis of one mol of water per mol of protein. A plot of ΔH_d against n_{hyd} is shown in Fig. 3 together with that for the lysozyme–H_2O system. As may be seen, the relationship may be represented by two segments of a straight line, with the break occurring at 170 mol/mol which is a lower value than that for the lysozyme–H_2O system, namely 260 mol/mol. This indicates that at least two types of hydration exist and contribute to the thermal stability of the protein.

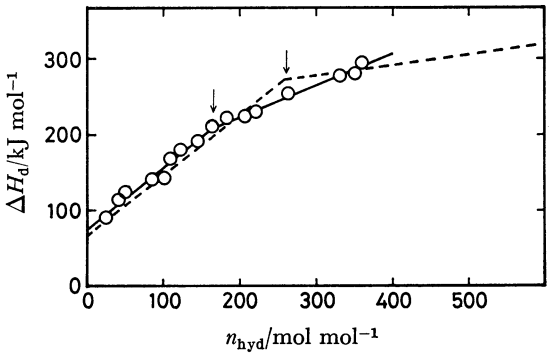

Fig. 3. The plots of the enthalpy change of denaturation, ΔH_d, of lysozyme against the degree of hydration, n_{hyd}, for lysozyme–D_2O system(solid line) and lysozyme–H_2O system (broken line).

Calorimetric studies of frozen protein solutions have revealed that water in protein solutions may be classified into four different states.[16,17] The four states are described as (1) non-freezable water, (2) freezable water with both heat and temperature of fusion different from the values for bulk water, (3) freezable water with the heat of fusion of bulk water, but a temperature of fusion lower than that of bulk water, and (4) bulk water. From recent NMR studies of aqueous protein solutions, Grosch and Noack[18] have suggested that at least three types of water which may be distinguished: (a) "rotationally bound" water, the rotational motion of which is considerably hindered by strong interactions with the protein, (b) "translationally hindered" water, the translational diffusion of which is hindered by interaction with the protein surface, and (c) "free bulk" water, the motion of which is not appreciably altered by interaction with the protein.

It has been assumed here that the hydration may be classified into two types as follows: (1) primary hydration *i.e.*, below n_{hyd} of 170 mol/mol for the lysozyme–D_2O system and 260 mol/mol for the lysozyme–H_2O system, and (2) secondary hydration *i.e.*, the range of n_{hyd} from 170 to 400 mol/mol for the lysozyme–D_2O system and from 260 to 600 mol/mol for the lysozyme–H_2O system. The values of ΔH_{hyd} and ΔH_{conf} have been estimated from the slopes and the intercepts, respectively, and are summarized in Table 1.

Table 1. The enthalpy change due to conformational changes of the protein, ΔH_{conf}, and the enthalpy change of hydration layer, ΔH_{hyd}, in primary and secondary hydration

Hydration	Water content	n_{hyd}	ΔH_{conf}	ΔH_{hyd}
	g g^{-1}	mol mol^{-1}	kJ mol^{-1}	kJ mol^{-1}
Lysozyme–D_2O system				
primary	0.23	170	72	0.83
secondary	0.55	400	140	0.43
Lysozyme–H_2O system				
primary	0.33	260	66	0.80
secondary	0.75	600	240	0.14

Primary hydration was completed at about 170 mol/mol for the lysozyme–D_2O system and 260 mol/mol for the lysozyme–H_2O system. The values of ΔH_{hyd} in the primary hydration region for both systems were considerably larger compared with those in the secondary hydration region. This indicating that the water molecules in this region contribute significantly to the stability of the protein compared with those in the secondary hydration region. The values of 170 mol/mol and 260 mol/mol correspond approximately to 1.0 and 1.5 molecules of water per hydrogen-bonding site, polar amino acid residue and peptide group, on the lysozyme molecule, respectively.[8] In this region, the water molecules are assumed to be tightly bound to the hydrogen-bonding sites, probably corresponding to the "rotationally bound" water molecules or state (1) in the calorimetric studies. It appears probable that the water molecules are selectively arranged in the vicinity of the polar regions of the protein by hydrogen bonds and form part of a first hydration monolayer.

The values of ΔH_{hyd} were almost identical for D_2O and H_2O in the primary hydration region. It is suggested that the primary hydration for D_2O contributes to the thermal stability of lysozyme in a similar manner to that for H_2O: the strength of interaction between the protein and D_2O are the almost identical to that between the protein and H_2O. The amount of primary hydration for the lysozyme–D_2O system, however, was considerably smaller than that for the lysozyme–H_2O system.

Secondary hydration was completed at about 400 mol/mol for the lysozyme–D_2O system and 600 mol/mol for the lysozyme–H_2O system. These values are comparable to approximately 3 and 5 molecules of water per amino acid residue on the lysozyme molecule, respectively. In the secondary hydration region, it is probable that the water molecules interact with the surface of the protein by repulsive hydrophobic interactions with the nonpolar parts of the protein and by hydrogen bonds on the some polar groups of the protein, correspond to the "translationally hindered" water molecules or states (2) and (3) in the calorimetric studies. It has also been assumed that the water molecules form parts of second and higher hydration layers which ambivalently interact not only with the water molecules in the primary hydration region, but with the normal bulk water.

The differences in ΔH_{hyd} between the lysozyme–D_2O system and the lysozyme–H_2O system in the secondary hydration region were considerable by a factor of approximately 3. It is suggested that the contribution of secondary hydration to the thermal stability of lysozyme is larger for D_2O than for H_2O. In this region, the D_2O molecules may interact more strongly with the protein compared with the H_2O molecules. The amount of secondary hydration for the lysozyme–D_2O system was smaller than that for the lysozyme–H_2O system. The differences in degree of hydration between D_2O and H_2O are too large to explain purely in terms of molecular size. Nemethy and Scheraga[19] compared the structures of H_2O and D_2O and noted that the hydrogen bond was stronger in D_2O than in H_2O and, therefore, more structural order existed in D_2O than in H_2O. One of the reasons for the lower degree of hydration and the higher value of ΔH_{hyd} in the secondary hydration region for the lysozyme–D_2O system may arise from the stronger intermolecular interaction for D_2O. It appears probable that the increase in strength of the hydrophobic interaction of the protein results from the increasing strength of interaction between the D_2O molecules. Secondary hydration may be affected markedly by the strength of the intermolecular interactions.

In primary hydration, the value of ΔH_{conf} for the lysozyme–D_2O system was slightly larger than that for the lysozyme–H_2O system. The ΔH_{conf} in this region may be associated with the intramolecular interactions, probably hydrogen bonds, in the protein molecule. Presumably, the small difference in ΔH_{conf} reflects the increase in strength of the intramolecular hydrogen bond of the protein by deuteration. A more accurate measurement, however, is necessary in order to discuss the structure of the protein in detail from the value of ΔH_{conf}. The variation of ΔH_{conf} in primary and secondary hydration regions suggests that the exhaustive removal of the water molecules associated with the protein brings about changes in the structure of the protein, as reported by Hanafusa.[20]

The higher contribution of hydration to the stabilization of the protein in the secondary hydration region may compensate for the lower degree of hydration in the lysozyme–D_2O system compared with the lysozyme–H_2O system. The thermal stabilities of lysozyme in H_2O and in D_2O are, therefore, almost identical.

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