

Temperature-induced Structural Changes in Chicken Egg White Ovomucoid

Tsukasa MATSUDA, Kenji WATANABE and Yasushi SATO

*Department of Food Science and Technology, Faculty of Agriculture,
Nagoya University, Nagoya 464, Japan*

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Temperature-induced structural changes in chicken egg white ovomucoid were investigated by circular dichroism (CD) and ultraviolet absorption (UV). A major unfolding transition was brought about between 60 and 90°C with a rise in temperature, as monitored by variations in CD at 222 nm and UV at 287 nm. Thermal transition was reversible when there was no prolonged exposure to the denaturing condition. The Van't Hoff plot for the major unfolding process of the ovomucoid yielded the following thermodynamic parameters at pH 7.0 between 60 and 90°C: $\Delta H_{\text{VH}} = 73 \text{ kcal} \cdot \text{mol}^{-1}$ at 74°C and $\Delta S_{\text{VH}} = 209 \text{ cal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ at 74°C, on the assumption of a two-state transition. The equilibrium CD spectra at various temperatures suggested that the secondary structure change caused by heating from 20 to 60°C was qualitatively different from that from 60 to 90°C. By prolonged exposure to high temperature of 90°C, the reversible denatured state was slowly transformed into an irreversible denatured state.

Ovomucoid is a glycoprotein with a trypsin-inhibitory activity which accounts for about 10% of the protein in the egg white of several avian species.¹⁾ The native conformation of ovomucoid is resistant to extreme pH and temperature, and to treatment with concentrated urea solution, trichloroacetic acid-acetone and 9 M urea at 80°C.^{2,3)} Earlier studies also showed that ovomucoid could be heated at 100°C for a long period with little change in its physico-chemical properties.⁴⁾ This phenomenon is usually considered to partly depend on the presence of 9 disulfide bonds in ovomucoid. Thus, the thermodenaturation of ovomucoid has been studied by many researchers,^{2~6)} but details of its thermodenaturation process have yet to be elucidated. It is of interest to determine how the ovomucoid molecule might unfold with a rise in temperature, in order to understand the thermodenaturation mechanism of the ovomucoid and to characterize the thermodenatured protein.

The present paper describes the temperature-induced changes of the chicken ovomucoid conformation which was monitored

by CD at 222 nm, UV at 287 nm, and the equilibrium CD spectra at various temperatures.

MATERIALS AND METHODS

Ovomucoid used in this study was isolated from chicken egg white,⁷⁾ and its homogeneity was confirmed as described in a previous paper.⁸⁾ The carbohydrate moiety of the ovomucoid was isolated after extensive pronase and endo- β -N-acetylglucosaminidase H digestion.⁸⁾ Reagents were of analytical grade and used without further purification. Guanidine hydrochloride was of ultrapure grade.

The conditions for CD measurements except the temperature were based on the method described previously.⁸⁾ In the case of a sample containing 6 M guanidine hydrochloride, a reference without protein in the same solution was used as the control. The measurements of UV were made on a Shimadzu double beam spectrophotometer model UV-200S.

The denaturation experiments were conducted according to two typical methods.

Thermal transition experiments. To monitor the changes in the conformation of ovomucoid the changes in the CD at 222 nm and the UV at 287 nm were measured. Ovomucoid solution (2~3 mg in 2 ml of phosphate buffer, pH 7.0, $I=0.1$) in a stoppered cuvette was placed in a thermostatically controlled cell holder, and the temperature was varied in steps of 1~4°C. At each temperature the CD and the UV were measured with time until

constant values were observed. This usually took 2~5 min. Moreover, the ovomucoid solutions, heat-treated as described above, were cooled in steps of 1~5°C, and the CD and the UV were measured in a similar manner to that above to test the reversibility of the thermal transition.

Equilibrium CD spectra measurements. Ovomucoid solution (300~600 μg in 2 ml of phosphate buffer, pH 7.0, $I=0.1$) and its carbohydrate moiety solution (500~600 μg in *N*-acetylglucosamine in 2 ml of the same buffer) were placed in constant temperature baths and maintained at the desired temperature for 3 min. The solutions were then transferred to cuvettes thermostatically maintained at the same temperature as that of the preincubation, and the CD at 222 nm was measured. After equilibrium had been reached, the CD spectra between 200~240 nm were measured at each temperature (60, 70, 80 and 90°C).

During optical measurements the temperature of the solution was controlled by water through the cell-holder jacket from a HAAKE FE2 constant temperature bath and monitored continuously by means of a thermocouple connected to a digital thermometer.

RESULTS

The variation in the CD at 222 nm brought about by varying the temperature of the ovomucoid solution is shown in Fig. 1. The transition was effected between 60 and 90°C and had a midpoint at near 74°C. The negative ellipticity at 222 nm decreased slowly in a linear manner with a rise in temperature from 20 to 60°C, and it varied rapidly from -8400

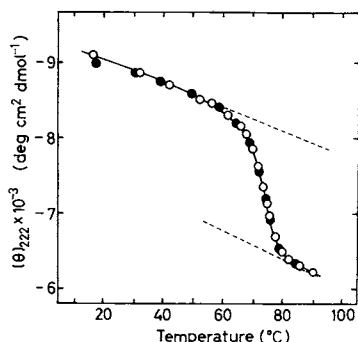


FIG. 1. Temperature-induced Structural Change of Ovomucoid Monitored by Variation in CD at 222 nm.

Experimental values were obtained by heating (—○—) or cooling down (—●—) protein solutions and keeping them at the temperature indicated until constant values were observed.

to -6200 as the temperature was increased from 60 to 90°C. These changes were reversible below 90°C if there was no prolonged exposure to the denaturing condition.

The variation in the difference spectral intensity of the UV at 287 nm caused by varying the temperature of the ovomucoid solution is shown in Fig. 2. The transition was almost reversibly brought about between 60 and 90°C and had a midpoint at near 74°C in a similar manner to that in the CD at 222 nm (see Fig. 1).

For the unfolding process a two-state transition between the two conformations, N and D, below and above the transition temperature range, respectively, was assumed. Based on this assumption an apparent equilibrium constant can be calculated with the CD at 222 nm and the UV at 287 nm at temperature T , respectively;

$$K' = \{X(T) - X_N(T)\} / \{X_D(T) - X(T)\}$$

The values of pure states X_N and X_D at each temperature have been obtained by extrapolating the values from the pre- and post-transition regions as indicated by dashed straight lines in Figs. 1 and 2. As shown in Fig. 3, the Van't Hoff plot of $\ln K'$ versus the reciprocal temperature $1/T$ yields a straight line in either case. The two straight lines coincided with each other and provided the following values for the thermodynamic parameters for the unfolding process at pH 7.0: $\Delta H_{\text{VH}} = 73 \text{ kcal} \cdot \text{mol}^{-1}$ at 74°C; $\Delta S_{\text{VH}} = 209$

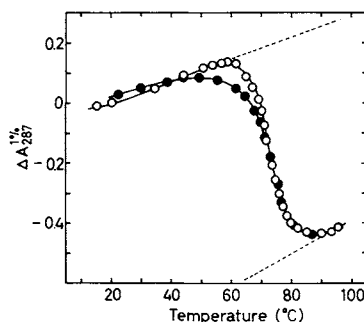


FIG. 2. Temperature-induced Structural Change of Ovomucoid Monitored by Variation in UV at 287 nm.

Conditions and symbols are the same as in Fig. 1.

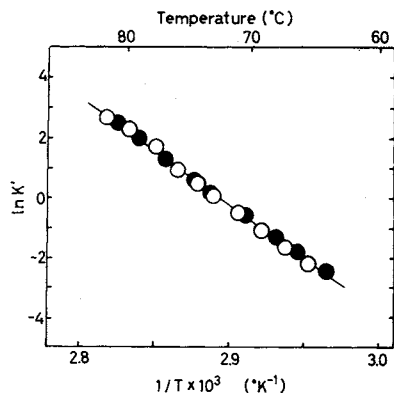


FIG. 3. Van't Hoff Plot of the Apparent Equilibrium Constants Calculated from the Data of CD at 222 nm in Fig. 1 (—○—) and of UV at 287 nm in Fig. 2 (—●—).

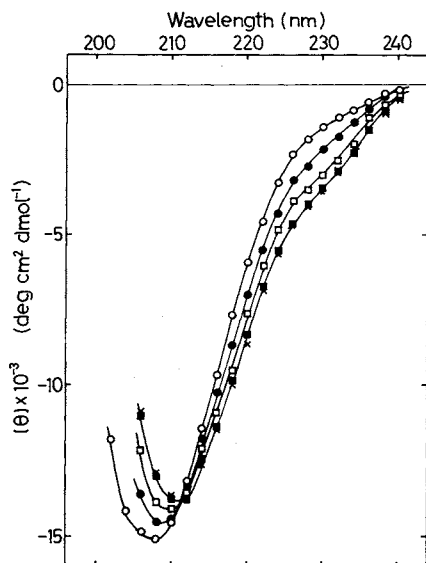


FIG. 4. Equilibrium CD Spectra of the Carbohydrate Moiety of Ovomuroid at Various Temperatures.

The spectra were measured at 20 (—○—), 60 (—●—), 70 (—□—), 80 (—■—) and 90°C (—×—) after equilibrium of the CD at 222 nm had been reached. The data are expressed as the ellipticity on *N*-acetylglucosamine in the carbohydrate moiety.

$\text{cal} \cdot \text{deg}^{-1} \cdot \text{mol}^{-1}$ at 74°C.

We have already reported that the carbohydrate moiety in ovomucoid contributes to the CD spectra in the far ultraviolet region.⁸⁾ Hence, the equilibrium CD spectra of the carbohydrate moiety in ovomucoid were measured at various temperatures. As shown in

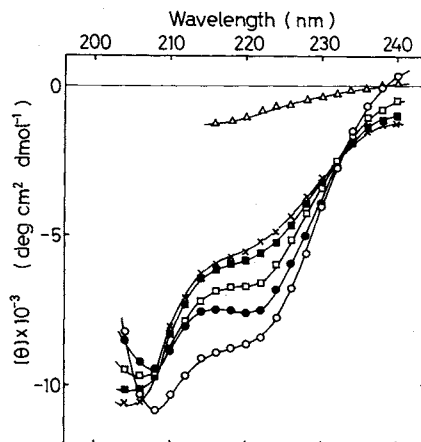


FIG. 5. Equilibrium CD Spectra of Ovomuroid at Various Temperatures and at 20°C in 6M Guanidine Hydrochloride.

The spectra were measured at 20 (—○—), 60 (—●—), 70 (—□—), 80 (—■—) and 90°C (—×—) after equilibrium of the CD at 222 nm had been reached, and at 20°C in 6M guanidine hydrochloride after 24 hr (—△—).

Fig. 4 the spectrum varied depending upon the temperature as reported for glycophorin⁹⁾ and the variations were reversible between 20 and 90°C. In order to observe the net changes of the polypeptide chain in ovomucoid, all of the equilibrium CD spectra of ovomucoid were corrected by subtracting the spectra of the carbohydrate moiety under the same conditions as for corresponding samples.⁸⁾

The equilibrium CD spectra of ovomucoid at various temperatures and at 20°C in 6M guanidine hydrochloride are shown in Fig. 5. The spectrum at 20°C in the far ultraviolet region had one negative maximum at about 208 nm and one shoulder at about 222 nm, the intensities of which varied as the ovomucoid solution was heated from 20 to 60°C. The ellipticity between 204 and 230 nm decreased, and one negative minimum newly appeared at 216 nm. The major changes in the CD spectra occurred in the temperature range of 60 to 90°C, where the ellipticity at 222 nm decreased noticeably, and the maximum initially at 208 nm was shifted to a shorter wavelength. In this temperature range the spectra had shapes and intensities intermediate between those shown at 60 and 90°C. An example is the

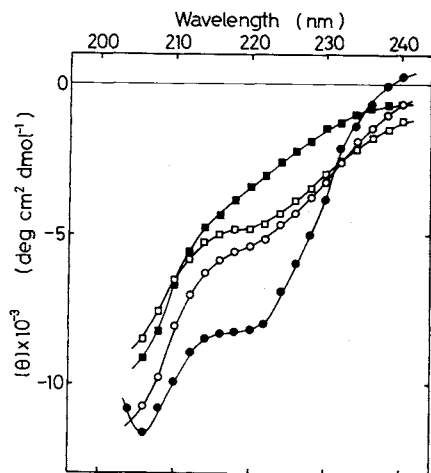


FIG. 6. Effect of Prolonged Heating on the Equilibrium CD Spectrum of Ovomucoid and Its Reversibility Obtained on Cooling.

Measurements were obtained for the spectra when the ovomucoid was kept for 20 min (—○—) at 90°C and then cooled to 20°C (—●—), and when kept for 5 hr (—□—) at 90°C and then cooled to 20°C (—■—). The solutions were cooled to 20°C within 4 min, and the spectra were measured again after equilibrium had been reached at 20°C.

spectrum at 70°C shown in Fig. 5. The spectrum at 90°C still retained a greater negative ellipticity than that in 6 M guanidine hydrochloride.

As depicted in Fig. 6 the equilibrium spectrum at 90°C, which was maintained for 20 min at 90°C, was varied slowly by further maintenance at that temperature. This resulted in a decrease of negative ellipticity at the shorter wavelength after 5 hr. The spectra of ovomucoid solutions cooled to 20°C after heating for 20 min and 5 hr at 90°C are also shown in Fig. 6. The changes of the spectrum caused by heating for 20 min at 90°C were almost reversible, whereas the decrease of the negative ellipticity on heating for 5 hr at that temperature could not be reversed; in fact, it was further decreased at the longer wavelength by cooling.

DISCUSSION

The thermodenaturation of a protein has

been studied with small compact globular proteins.¹⁰⁻¹²⁾ These studies have shown that the dependence on temperature of the observed UV and CD could be generally represented by a sum of simpler functions—one sigmoidal function and two very smooth functions below and above the sigmoidal one.¹³⁾ Equilibrium thermodynamics are in principle applicable to the study of protein denaturation, when it is shown that renaturation of even completely unfolded proteins is a reversible, thermodynamically driven process,¹⁴⁾ and the two-state models are typical in thermodenaturation of small compact globular proteins.¹³⁾ The UV and CD variations of ovomucoid depending on temperature could also be represented by the above described functions.

Ovomucoid, as well as other small globular proteins, appeared to unfold in an all or none manner as the temperature rose from 60 to 90°C. The thermal transition curves observed on measuring two independent properties (namely, CD and UV) proceeded reversibly by means of the same process. The slight deviation of the refolding curve of the UV at 287 nm from the unfolding one was probably due to the prolonged exposure to the denaturing condition at above 90°C. Though the carbohydrate moiety in the ovomucoid contributed to the CD spectra, its contribution at 222 nm seemed to be negligible in the analysis of the thermal transition process of the ovomucoid because the ellipticity at 222 nm derived from the carbohydrate moiety was probably no more than 1% of that derived from the polypeptide chain in ovomucoid as described previously.⁸⁾ Thus, the coincidence of the two straight lines from the Van't Hoff plot suggested that the thermal transition of the ovomucoid might be approximated by a two-state model between 60 and 90°C. In that coincidence, however, there is some uncertainty because the apparent equilibrium constant depends to some extent on how the values from the pre- and post-transition regions are extrapolated. Therefore, the simple two-state assumption for the

thermal transition of ovomucoid must be treated with caution. The Van't Hoff enthalpy of ovomucoid, which was not so different from that of lysozyme ($73 \text{ kcal} \cdot \text{mol}^{-1}$)¹¹ and ribonuclease ($80 \text{ kcal} \cdot \text{mol}^{-1}$),¹² suggested that the ovomucoid thermodenaturation process might be similar to that of lysozyme and ribonuclease. Nevertheless, in order to clarify whether the thermal transition of ovomucoid can be well expressed by equilibrium thermodynamics or not, more extensive data are required.

The minor changes of UV and CD below the sigmoidal curve of the major transition, which were called the pre-denaturational changes,¹³ suggested that the native conformation of ovomucoid was not so rigid; rather, it varied according to the temperature. Moreover, the equilibrium CD spectrum at 60°C indicated that a small amount of the ordered secondary structure was disorganized with a rise in temperature from 20 to 60°C .

We have already reported on the secondary structure of polypeptide chain in the ovomucoid,⁸ and predicted that the ovomucoid contained every kind of structural element of α -helix, β -structure and β -turn, in which the β -structure content was predominant. On the basis of the reference CD spectra of the secondary structure reported by Greenfield and Fasman¹⁵ and Chang *et al.*,¹⁶ it was suggested that the decrease of negative ellipticity at about 215 nm caused by heating from 20 to 60°C could be ascribed to the diminution of the β -structure content and, moreover, the noticeable decrease of negative ellipticity at about 222 nm caused by heating from 60 to 90°C could be ascribed to the diminution of the α -helix content. Though thermodenatured proteins may contain regions of unique structure which can not be analyzed by such standard CD curves as described above, it can be said that the secondary structure change caused by heating from 20 to 60°C is qualitatively different from that from 60 to 90°C .

The reversible denatured state appeared to be slowly transformed into an irreversible denatured state on the prolonged exposure to

the high temperature of 90°C . The irreversible disorganization of ovomucoid caused by maintaining it at 90°C for 5 hr may be due to the exchange reactions of disulfide bonds exposed by unfolding of ovomucoid molecules.¹⁷ Donovan and Beardslee have reported that, in differential scanning calorimetric studies on the heat denaturation of ovomucoid, a pronounced tailing was observed in the thermogram,¹⁸ and attributed it to a heterogeneity of ovomucoid with respect to denaturation temperature. However, it is not likely that ovomucoid has such heterogeneity because its amino acid sequence, which has been recently determined by Kato *et al.*,¹⁹ showed no heterogeneity. That tailing may be ascribable to the irreversible transition which was observed at 90°C in this experiment. The enthalpy of thermodenaturation of ovomucoid determined by the differential scanning calorimetric study was $152 \text{ kcal} \cdot \text{mol}^{-1}$,¹⁸ which was much larger than the value ($73 \text{ kcal} \cdot \text{mol}^{-1}$) provided by the Van't Hoff plot in this study. This disagreement suggests that the thermodenaturation process of the ovomucoid does not consist of the major unfolding process between 60 and 90°C alone.

Although the denatured state kept for 5 hr at 90°C may not necessarily be the final one, it can be said that the ovomucoid was not completely unfolded in the irreversible, as well as the reversible, thermodenatured state as indicated by the comparison of their CD spectra with that in 6 M guanidine hydrochloride, where the ovomucoid was regarded as in the state of a crosslinked random coil.

Ovomucoid has been regarded as one of the proteins whose conformation could not be easily destroyed even at high temperature.⁴ Though it is true that the midpoint of thermal transition of ovomucoid is at a relatively higher temperature, 74°C , than that of ribonuclease,^{12,13} the high heat stability of the ovomucoid should be ascribed to the rapid equilibrium of the thermal transition which results in the high reversibility of its thermodenaturation.

There have been many discussions on heat

stability of proteins, and it was suggested that special features such as covalent cross linkages,²⁰⁾ the carbohydrate moiety,²¹⁾ hydrogen and hydrophobic bonds²²⁾ are required in order for a protein to be heat stable. It is still uncertain why the ovomucoid shows a characteristic behavior on thermodenaturation as described above. Studies to elucidate the unusual thermostability of ovomucoid with regard to the disulfide bond, the carbohydrate moiety and the three separated domains are now in progress.

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