Note

State of Water in Maillard-reacted Ovalbumin as Studied by Broad-line Pulsed NMR

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It has been known that carbohydrate moieties in glycoprotein^{1,2)} and lactosylated enzyme³⁾ contribute to the stabilization of their protein moieties against heat and other denaturing conditions. The conformational stability of ovalbumin reacted with glucose in the Maillard reaction has also been demonstrated in our previous studies.^{4~6)} Such a stabilization of protein-sugar conjugates has been reported to be associated with noncovalent interaction and cross-linking between carbohydrate and protein, and the degree of hydration.^{1,3,6)} This study describes the state of water in Maillard-reacted ovalbumin as studied by broad-line pulsed NMR in order to get information on the conformational characteristics from the aspect of hydration.

Freeze-dried ovalbumin containing D-glucose corresponding to 30% of the dry weight was stored for 4 days at 50°C and 65% relative humidity, and the monomer type of the Maillard-reacted complex (mOVG) was separated from the polymer one by Sephadex G-150 column chromatography after removing the unreacted ovalbumin by mild heat-treatment, dialyzed and freeze-dried as described previously.^{4~7)} The mOVG which showed only one band on polyacrylamide gel electrophoresis, was estimated to be partially denatured from circular dichroism (CD) spectra (denaturation ratio calculated from α -helix content: 18%) even though 62% of ϵ -amino groups were blocked, and could be recognized to be a product in the initial stage of the Maillard reaction from the quantity of lysine present as fructoselysine (6.2 to 20 residues of total lysine).⁶⁾

Measurements of unfrozen water (UFW) content and spin-spin relaxation time T_2 as a function of temperature were carried out using Bruker minispec p20 broad-line pulsed NMR spectrometer (20 MHz for observation of ^1H) equipped with a minicomputer system (HP 1000).⁸⁾ A

sample in a 7 mm OD glass tube was cooled or warmed at the rate of about 3°C/min using nitrogen gas and liquid nitrogen. The sample temperature was measured with a chromel-constantan thermocouple inserted in the sample tube. The UFW content was determined by extrapolating the liquid portion of the free induction decay curve after 90° pulse and T_2 was calculated from its slope. These procedures were done at every pulse with the minicomputer and the results were plotted as a function of temperature on a semi-logarithmic graph by graphic plotter. The samples of OV and mOVG were dissolved in distilled water at 40% concentration of freeze-dried materials. Concentrations with respect to protein solutions were 36% for the former and 26% for the latter.

UFW contents as a function of temperature in aqueous solutions of OV and mOVG in the freezing and thawing processes are shown in Fig. 1a and b. UFW contents with the thawing process at -30°C per gram of dry OV and mOVG were 0.318 g and 0.308 g, respectively. One gram of mOVG contained 0.85 g of protein and 0.15 g of hexose, of which the main carbohydrates were glucose and fructose. It has been shown that both free glucose⁸⁾ and fructose⁹⁾ could hold 0.2 g of bound water per gram of samples with a broad-line pulsed NMR spectrometer. And it has also been pointed out that the denatured form of protein was more heavily hydrated than the native conformation, and native ovalbumin held 0.323 g of bound water against 0.332 g for the denatured one.¹⁰⁾ Judging from these

FIG. 1. UFW Contents as a Function of Temperature in Aqueous Solutions of OV (a) and mOVG (b) in the Freezing (—) and Thawing (-----) Processes.

FIG. 2. Spin-Spin Relaxation Time T_2 as a Function of Temperature of UFW in Aqueous Solutions of OV (a) and mOVG (b) with the Thawing Process.

reported values, the attachment of hexose to OV might not result in the increase of bound water in the carbohydrate-protein complex, although mOVG in a slightly denatured form might hold additional bound water. This estimation agreed with the result that the UFW content of mOVG was slightly less than that of OV as described above. Thus, the difference of hydration between OV and mOVG can not be explained by the UFW content.

The freezing curves in Fig. 1 show the difference between OV and mOVG in weakly bound water from 0 to -20°C (this was regarded as weakly bound water by Nagashima and Suzuki¹¹), for the mOVG curve suddenly dropped at 0°C (Fig. 1b), but that of OV dropped gradually from 0 to -20°C (Fig. 1a). These results indicate that OV has some weakly bound water, whereas mOVG has almost none.

Spin-spin relaxation time T_2 as a function of temperature of UFW in aqueous solutions of OV and mOVG with the thawing process is shown in Fig. 2. The T_2 values of OV and mOVG at -30°C were 0.49 and 0.23 msec, respectively. The molecular mobility of unfrozen water in

mOVG was found to be depressed more than in OV. This would depend on the facts that mOVG had a different conformation from OV to hold tightly bound water, and the possibility that bound water in mOVG might be interposed between protein and bound carbohydrate.

Not only tightly bound water but also weakly bound water has been reported to affect protein thermodenaturation.^{12,13} However, more studies are needed to clarify the contribution of bound water, especially that tightly bound water expressed by T_2 , to the conformational stability of mOVG.

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