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A method to study protein denaturation by measurements of apparent molar volumes

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Abstract

Protein denaturation was studied by measurements of apparent molar volumes of bovine serum albumin (BSA)–water–urea systems at 25°C. The viscosity measurements carried out on aqueous BSA solutions at various fixed concentrations as a function of temperature have shown that the denaturation process can best be followed at 60 g kg^{-1} BSA solution. The apparent molar volumes of urea in water and in 60 g kg^{-1} BSA solution were measured at 25°C and the differences between the two were interpreted in terms of structural changes taking place during denaturation of BSA by urea. A simple method was proposed to calculate the volume change per mole of protein upon denaturation. The sample calculation for BSA has shown that there is a volume contraction of 1854 cm^3 per mol BSA when the urea concentration reaches 13 M.

INTRODUCTION

The denaturation of proteins is important for biochemists and food scientists. Various methods to study the denaturation include optical rotatory dispersion, viscometry, dielectric dispersion and dilatometry, as reviewed by Joly [1] and by Tanford [2]. Most investigations in this area involve examination of the protein structure when it is in native form and when it is under the effect of a denaturing factor such as temperature or a denaturing agent.

It is well known that the determination of apparent molar volumes and specific volumes reveals important information about solute–solvent and solute–solute interactions [3–7]. Although volume determination is an accurate and sensitive method that has been used successfully in solution studies of amino acids [8, 9] and low molecular weight peptides [10], there are rather few applications on protein solutions. The work of Prakash et al. [11] on the interactions of eight proteins with solvent components in 8 M urea by apparent partial specific volumes, the work of Dayhoff et al. [12] on partial specific volumes of three proteins, and the work of Kim and Kauzmann [13] on the concentration dependence of specific volumes of three protein solutions are some examples in this area, but none of them

involve denaturation studies. The purpose of the present study was to examine protein denaturation by measurements of apparent molar volumes of protein–water–denaturing agent systems. Bovine serum albumin (BSA) was chosen as the protein and urea was selected as the denaturing agent. Viscosity measurements were used to find the optimum BSA concentration for studies of protein denaturation by urea.

EXPERIMENTAL

Materials

BSA was obtained from Sigma Chem. Co. (A-7906) and used without further purification. It was kept in a desiccator at about 0°C when not in use. Urea, obtained from a local chemical company with a purity of 99%, was recrystallized from 700 ml l^{-1} ethanol solution and dried in a vacuum at room temperature. The water used in all experiments was doubly distilled.

Density and kinematic viscosity determinations

The well-known buoyancy balance method was used for density determinations. The apparatus used and the details of the procedure followed were described in refs. 8 and 14.

The viscosity measurements were made with Cannon-Fenske glass capillary viscometers obtained from Herzog-Lauda Co. as certified. The kinematic viscosity was determined from the measured flow time. The details of the measurement of flow time were described in refs. 8 and 14.

Treatment of data

The measured densities were used to calculate apparent molar volumes using the equation [3]

$$V_\phi = \frac{1000(d_\circ - d)}{mdd_\circ} + \frac{M}{d} \tag{1}$$

where V_ϕ is the apparent molar volume, m is the molality, M is the molecular weight of solute, and $d_\circ$ and d are the densities of solvent and solute, respectively. It was found that the reproducibility of the density measurements to ± 3 in the sixth decimal place allowed an uncertainty of $\pm 0.3\ cm^3\ mol^{-1}$ in V_ϕ at 0.01 M. This uncertainty decreases to $\pm 0.03\ cm^3\ mol^{-1}$ at 0.1 M.

The kinematic viscosities were calculated from the measured flow times by

$$\eta_{kin} = kt \tag{2}$$

where t is the measured flow time and k is the viscometer constant which was provided for every viscometer by the company and checked with a few liquids of known viscosity. When the flow time is in seconds, η_{kin} is determined in units of $10^{-6}\,m^2\,s^{-1}$. It was found that the reproducibility of flow time measurements to ± 0.5 s allowed an uncertainty of $\pm 5 \times 10^{-11}\,m^2\,s^{-1}$ in kinematic viscosity.

RESULTS AND DISCUSSION

In order to find the optimum BSA concentration in aqueous solution at which the denaturation process can be clearly followed, the kinematic viscosity of BSA in water at concentrations of 10, 30, 40, 50, 60 and 70 g kg^{-1} was measured as a function of temperature and the results are given in Fig. 1. Kinematic viscosity decreases with temperature up to 55°C at all BSA concentrations studied. However, above 55°C there is a sudden increase in kinematic viscosity for BSA solutions above 30 g kg^{-1}. This must be due to some structural changes (denaturation) in BSA. When the denaturation process is to be examined by the viscosity method, the specific

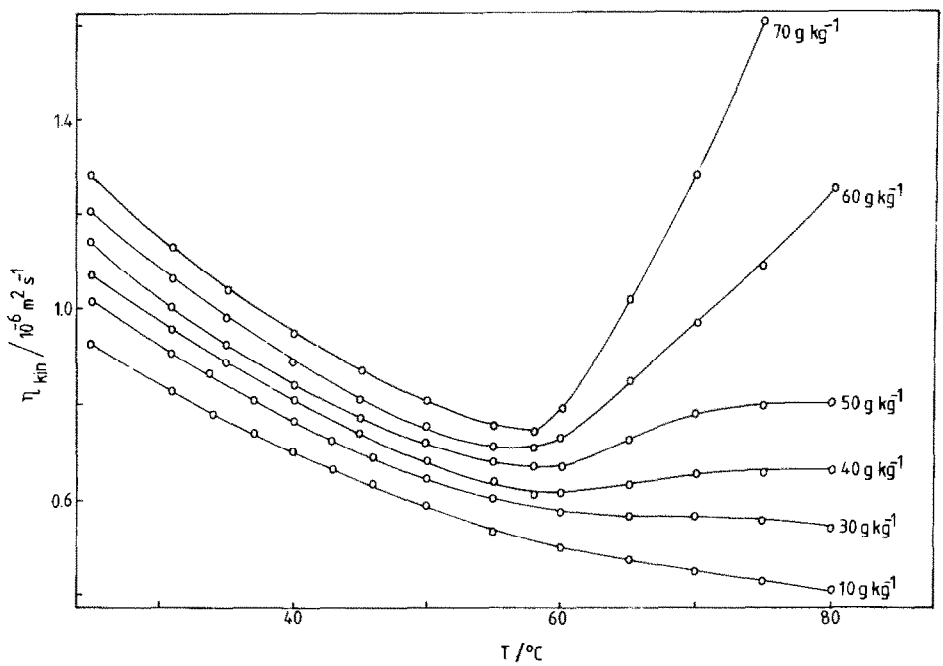

Fig. 1. Kinematic viscosity of BSA in water plotted against temperature at various concentrations.

and then the intrinsic viscosities are determined and further interpretations are made. Such studies are numerous in the literature [1], including one from our laboratory [15]. Because the main purpose of the present work was to demonstrate the applicability of the volume method to denaturation studies, from the viscosity results presented in Fig. 1, it was concluded that the structural changes at 55°C are most striking when the BSA concentration is 60 g kg^{-1} or higher. Due to possible aggregation or gel formation problems at higher BSA concentrations, 60 g kg^{-1} was chosen as optimum for volume studies.

In order to follow the structural changes in proteins by volume measurements, the V_ϕ values of urea, the denaturing agent, in water and in 60 g kg^{-1} BSA solution were determined as a function of molality at 25°C. The results are given in Fig. 2. It can be seen from this figure that at low molalities, the V_ϕ values of urea in water are very close to those in 60 g kg^{-1} BSA. However, a marked difference starts at about 3 M and increases with molality, reaching a value of as much as 0.2 cm^3 mol^{-1} at 13 m. It has been calculated that the experimental error limit for V_ϕ is less than ±0.01 cm^3 mol^{-1} at this molality. Therefore, such a large difference in V_ϕ is

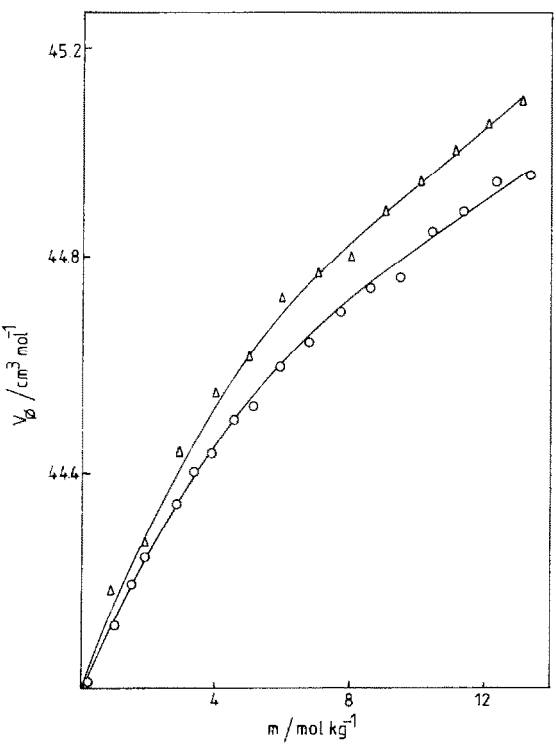

Fig. 2. Apparent molar volume of urea in water (△) and in 60 g kg^{-1} BSA solution (○) plotted against molality at 25°C.

a clear indication of structural changes in BSA, such as intramolecular hydrogen bond breakages, intermolecular hydrogen bond formations and disruption of hydrophobic interactions [16]. The contribution of these processes to volume changes can be explained in the following way. First of all, some "holes" exist in the native structure of a globular protein such as BSA. Upon denaturation, those holes can be filled with solvent or denaturant molecules. This process results in negative volume contribution. Secondly, exposure of hydrophobic groups to solvent is accompanied by a volume contraction due to ordering of solvent molecules around these groups. A third contribution to volume change comes from breakage of intramolecular hydrogen bonds and formation of intermolecular hydrogen bonds. This contribution is expected to be small because one hydrogen bond is being broken while another is being formed.

A simple calculation can be made to estimate the volume contraction per mole of BSA upon denaturation. The difference in V_ϕ of urea in water and in 60 g kg^{-1} BSA solution δV_ϕ can be assumed to be mainly due to structural changes in BSA and can be converted to apparent volume change per mole of BSA upon denaturation ΔV_ϕ by the equation

$$\Delta V_\phi = \frac{\delta V_\phi W_U M_{BSA}}{W_{BSA} M_U} \tag{3}$$

where W_U and W_{BSA} are the weights of urea and BSA, respectively, present in the solution at any molality, and M_U and M_{BSA} are the molecular weights of urea and BSA, respectively. At 13 M urea where the denaturation process is expected to be most complete, δV_ϕ was measured as 0.137 cm^3 mol^{-1}, and the weights of urea and BSA present in solution are calculated as 117 and 9.5 g, respectively. M_U is 60.06 g mol^{-1} and M_{BSA} is about 66.000 g mol^{-1}. When all these values are substituted into eqn. (3), ΔV_ϕ is calculated as 1854 cm^3 (mol BSA)$^{-1}$. Tanford [2] has reported that the volume contraction upon denaturation for ribonuclease is about 200–500 cm^3 mol^{-1}, and much larger for serum albumin. Therefore, the value calculated above is in agreement with this conclusion.

CONCLUSIONS

The denaturation of proteins can be successfully studied by measurements of apparent molar volumes. The method is simple, sensitive and accurate. It does not require sophisticated instruments but provides important and useful structural information about the denaturation of proteins. Here, the denaturation of BSA by urea was taken as an example system. This system can be changed by using other proteins and other denaturing agents. The effects of other denaturing factors such as temperature can also be studied by apparent molar volume measurements.

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