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Effects of alcohol on the solubility and structure of native and disulfide-modified bovine serum albumin

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ABSTRACT

Differential precipitation of human plasma by ethanol is one of the most important processes for purifying therapeutic proteins, including human serum albumin. Better understanding of the effects of ethanol on the structure and stability of proteins is critical for effective and safe application of ethanol-induced protein precipitation. Here, we examined the effects of ethanol on the structure and solubility of bovine serum albumin (BSA) and SH-modified BSA. Ethanol caused BSA denaturation in a bimodal fashion, i.e., reduction of α-helix at low concentration and subsequent induction of the α-helical structure at higher concentration. In contrast, the solubility of BSA decreased monotonically. The secondary structure of SH-modified BSA was different from that of native BSA. Ethanol resulted in enhanced secondary structures of SH-modified BSA and decreased solubility monotonically. These results suggest the favorable interaction of ethanol with hydrophobic residues, leading to protein denaturation, but the unfavorable interaction with charged residues, leading to a reduction of protein solubility.

1. Introduction

Ethanol is a protein precipitant, and plasma fractionation by ethanol-induced differential protein precipitation is one of the most critical technologies developed to produce life-saving biopharmaceuticals from pooled blood [1–5]. Human serum albumin (HSA) is one of the plasma-derived products, and is a key protein used as plasma expander for patients who have lost blood due to injuries or ischaemic stroke [6–8]. It is assumed that aqueous ethanol solution does not cause irreversible denaturation of plasma proteins, including HSA, during the fractionation processes. Ethanol is a protein destabilizer, and can cause protein denaturation at elevated temperatures, at which the temperature itself has no denaturing effects, or at higher ethanol concentrations [9–12]. Thus, care must be exercised when using ethanol as a precipitant.

We have previously characterized the structure and solubility of lysozyme as a function of ethanol concentration [9]. Ethanol had little effect on the structure of lysozyme below 60% but caused increasing structural changes above 60%, indicating favorable interactions of ethanol with the denatured structures. The solubility decreased, however, monotonically with ethanol concentration, indicating that ethanol can result in reduction of solubility regardless of the structure: i.e., ethanol reduced the solubility of both the native and denatured structures. It is thus clear that the favorable interaction of ethanol, which is the driving force for denaturation, does not lead to increased solubility of the denatured state. In addition, ethanol was shown to induce both α-helix and β-sheet and to decrease the solubility of the reduced lysozyme. Here, we studied the effects of ethanol on the structure and solubility of bovine serum albumin (BSA) in view of the commercial use of the solvent such as protein precipitation during purification and crystallization.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), (3-bromopropyl)trimethylammonium bromide (TAP bromide) and N-ethylmaleimide (NEM) were from Sigma Chemical, Co. (St. Louis, MO). Sodium hydroxide, dithiothreitol (DTT), hydrochloric acid, guanidine hydrochloride (GdnHCl), and monoiodoacetic acid were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tris(hydroxymethyl)aminomethane was from Nacalai Tesque Inc. (Kyoto, Japan). Ethanol was from Kanto Chemical Co., Inc. (Tokyo, Japan). All chemicals used were of reagent grade and were used as received.

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2.2. Preparation of reduced BSA

Reduced BSA was prepared as reported previously [9,13]. Briefly, BSA was dissolved at 20 mg/mL in water containing 6 M GdnHCl, 1 mM EDTA, and 40 mM DTT. The mixture was incubated at 37 °C for 3 h, sufficient for complete reduction.

2.3. Preparation of SH-modified BSA

Three SH-modifying agents, i.e., TAP bromide, NEM, and iodoacetic acid, were used to generate TAP-BSA, NEM-BSA, and reduced carboxyamidomethylated-BSA (RCM-BSA), respectively. TAP-BSA and RCM-BSA were prepared as follows. The reduced BSA, prepared as described above, was mixed with TAP bromide and iodoacetic acid dissolved in 1 N NaOH at final reagent concentrations of 100 mM TAP bromide or iodoacetic acid, 20 mg/mL BSA, 6 M GdnHCl, 40 mM DTT, and 1 mM EDTA and incubated at room temperature for 3 h in the dark. These SH-modified BSA (i.e., TAP-BSA and RCM-BSA) were dialyzed against 10 mM HCl for 1 day and then lyophilized. NEM-BSA was prepared by incubating 20 mg/mL BSA at room temperature for 3 h in the dark with 50 mM NEM, 6 M GdnHCl, 40 mM DTT, and 1 mM EDTA. The reaction mixture was dialyzed against 10 mM HCl for 1 day and then lyophilized.

2.4. Circular dichroism measurement

Far-UV circular dichroism (CD) measurement was performed using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The CD data were expressed as the mean residue ellipticity, calculated based on the protein concentration, the path length of the cell, and the mean residue weight. Temperature was controlled with a Peltier cell holder and a temperature programmer. Ethanol used here was of high purity (99.5%) and did not interfere with far-UV CD measurements. As ethanol is hygroscopic, aqueous ethanol solutions were stored in tightly sealed vessels. The BSA concentration used for CD measurements was 0.005 mg/mL throughout the widely different ethanol concentration range, with a 1 cm path length cell. Note that such low protein concentrations and long path length were required for measurements at high ethanol concentrations, at which BSA has limited solubility. The protein solutions were prepared in water or ethanol/water mixture and hence were not buffered to avoid precipitation of electrolytes at high ethanol concentrations. Although these conditions cause pH variation, the same variation should occur in both CD and solubility measurements and should therefore not affect the conclusions about the relation between the structural changes and solubility. BSA was incubated with water or ethanol/water mixtures at room temperature for at least 1 h to ensure equilibrium.

2.5. Solubility measurement

Solubility of BSA or SH-modified BSA was measured by dispersing excess protein powder in water or aqueous ethanol solution and incubating the suspension at 25 °C for 3 days, sufficient for complete equilibration. The suspension was centrifuged at 14000 × *g* for 30 min and the absorbance at 280 nm of the supernatant was determined, after appropriate dilution, on a Jasco V-630 UV–vis spectrophotometer. The extinction coefficient used for native BSA and SH-modified BSA was 0.63 mL mg^{-1} cm^{-1}. Three independent measurements were performed for solubility determination.

2.6. Dynamic light scattering

Aggregation of SH-modified BSA was examined by dynamic light scattering (DLS) on a light scattering photometer (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, UK), equipped with a 4 mW He–Ne ion laser. The solution containing 0.5 mg/mL BSA or SH-modified BSA in water was used for the DLS measurements.

3. Results

3.1. Structural changes of native BSA in ethanol solutions

BSA is an α-helical protein and has been studied extensively as a model protein for protein stability, folding, and denaturation [14–17]. Here, we determined the structure of BSA by CD at 25 °C as a function of ethanol concentration. Note that all the experiments were performed in pure water, i.e., in the absence of electrolytes, to avoid precipitation of salts at higher ethanol concentrations: CD measurements were performed at 0.005 mg/mL throughout, so that the protein was soluble even at the highest ethanol concentration (99%). Fig. 1A shows the far-UV CD spectra of BSA from 0% to 50% ethanol. The spectrum of BSA in water has a typical α-helical form with characteristic double minima at 209 and 222 nm, corresponding to ~65% α-helix. No significant changes in the spectra were observed between 0% and 20% ethanol, indicating no apparent effects of such low ethanol concentrations on the secondary structure of BSA. The spectrum of BSA began to change at 30% ethanol, with a slight increase in the ellipticity at 209 nm; the ellipticity at 222 nm appeared to be retained. A further increase in the ellipticity at 209 nm along with a simultaneous increase in the ellipticity at 222 nm was evident at 40%. A marked change was observed at 50% ethanol (see the top curve in Fig. 1A). The 209 nm peak disappeared and the 222 nm peak or shoulder shifted to ca. 225 nm, indicating large structural changes at this ethanol concentration. The structures of BSA above 50% ethanol are shown in Fig. 1B. There were small, but significant changes between 50% and 60% ethanol. The ellipticity at 209 nm increased slightly, while that at 225 nm decreased. Nearly parallel changes were observed upon increasing ethanol concentration from 60% to 70% and 80%, with both the ellipticities at 209 and 225 nm decreasing similarly. Marked changes in spectral shape were observed at 90% and 99% ethanol. The characteristics of α-helical structures were apparent, in particular at 99% ethanol. In fact, the spectral shapes were similar at 0% and 99% ethanol. However, it should be emphasized that the folding in water is completely different from that in 99% ethanol and that the α-helix present in 99% ethanol could be on different sequences from that present in the native structure. There was no clear indication of isoelliptic wavelength at which all spectra overlap with each other; thus, the transition does not appear to be of two states. Taken together, Fig. 1A shows reduction in α-helix with increasing ethanol concentration from 0% to 50%, and Fig. 1B shows induction of α-helix with increasing ethanol concentration from 50% to 99%. This bimodal transition can be clearly seen in Fig. 1C, which plots the ellipticities at 209 and 222 nm as a function of ethanol concentration. It is clear that the structural changes of BSA by ethanol are complex functions of ethanol concentration, accompanied by reduction and induction of α-helix and probably formation of β-sheet structures at intermediate ethanol concentrations (50–60%).

3.2. Structure of reduced BSA in water

Organic solvents are generally α-helical inducers [9,18–20]. However, ethanol induced no additional α-helix over the amount presented in the native protein, as even 99% ethanol resulted in a spectrum close to the native spectrum (see Fig. 1A and B). Such inability of ethanol to enhance α-helical structure may be due to disulfide bonds in BSA that put constraint on the freedom to adjust the secondary structure: note that BSA has 17 disulfide bonds. Accordingly, reduction of disulfide bonds should enhance the exposure of the internal residues and solvation of hydrophobic side