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Research paper

Structural study of lysozyme in two ionic liquids at cryogenic temperature

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ABSTRACT

We have investigated the structure and activity of chicken egg-white lysozyme in aqueous solutions of two typical ionic liquids, 1-butyl-3-methylimidazolium chloride and ethylammonium nitrate, at cryogenic temperature. An increase in structural disorder due to the unfolding and a decrease in the α-helical structure of lysozyme were noticeable upon glass formation. However, a decrease in the structural stability after cooling was less than that before cooling. The secondary and tertiary structures showed good reversibility upon cooling to 77 K and then reverting back to ambient temperature. We discussed an influence of a cooling upon the structure in aqueous ionic liquid solutions.

1. Introduction

Ionic liquids (ILs) are molten salts comprising a cation and an anion and exhibit unique physical and chemical properties such as negligible vapor pressure, nonflammability, high chemical/thermal stability, high conductivity, and high solubility in other liquids [1]. During the last decade, ILs have demonstrated promise for applications in many areas including biological/biochemical sciences. One feature of ILs that attracted our interest is that certain ILs stabilize the native structures of proteins while maintaining their bioactivities [2,3]. For example, Fujita and Ohno proposed the mixtures of ILs and water, which they termed hydrated ILs, as novel solvents for proteins [4–6]. They reported that the hydrated ILs retain the basic properties of pure ILs, while the inclusion of a small amount of water in the ILs considerably improves protein solubility. Regarding the effects of ILs on proteins, maintaining the biological activity and/or function of a biomolecule is essential for applications in media other than physiological buffer systems, because proteins are sometimes unstable when handled in vitro and stabilizing agents are necessary to ensure their long-term stability [5]. Thus, if a biomolecule can be introduced into an appropriately biocompatible IL, the technological usefulness of the biomolecule may be greatly improved.

Another related stability issue is the refolding of proteins in ILs. Recently, we reported the structural modification of chicken egg-white lysozyme (38% α-helix and 10% β-sheet) as a model protein in aqueous 1-butyl-3-methylimidazolium nitrate (abbreviated as [bmim][NO_3]) solutions over a broad range of IL concentrations (x; 0–30 mol%IL) [7]. Interestingly, the addition of [bmim][NO_3] with $x > 20$ induced the refolding of unfolded lysozyme (i.e., the reformation of the α-helical structure). Similarly, Lange et al. [8] demonstrated that the addition of imidazolium-based ILs (up to 4 M) to the renaturation buffer caused high protein renaturation without aggregation, whereas its addition to the folded protein decreased the structural stability. However, the stabilities of most biomolecules at ambient temperature in these conditions are still not sufficient for many practical applications which realize the long shelf life.

Alternatively, subjecting proteins to low temperature might be a promising method to stabilize and preserve proteins in aqueous media. However, two underlying issues should be considered before subjecting proteins to low temperature [9,10]: (1) ice nucleation and (2) structural changes to the protein. Since ice formation plays a major damaging role during cryopreservation, the possibility of achieving low-temperature storage while avoiding ice formation is mandatory. The first problem can be avoided by the addition of a freezing-protection agent (i.e., a cryoprotectant; CPA) such as dimethylsulfoxide (DMSO), sugar, or salt. In fact, DMSO has been widely used as an additive in the cryopreservation of living cells and tissues [11]. However, in relation to the second problem, CPA results in the conflicting effects of protection and toxicity because exposure to high salt concentrations during freezing is known to damage proteins; normally, vitrification of the sample solution

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requires high CPA chemical concentrations not normally encountered by living organisms. Earlier, Fahy [12] reviewed the toxicities of agents such as DMSO. Pronounced osmotic and chemical toxicity is likely unless we control the exposure to CPA. ILs may provide good alternative candidates because ILs are "salts" that consist of only cations and anions. Our past results and studies on low temperature suggest that IL–water mixtures might be useful for the freezing preservation of aqueous protein solutions [13,14]. Here, in this relation, using structure–activity relationship to assess the environmental risks of ILs, Jastorff et al. [15] developed a set of toxicity and ecotoxicity test systems. One can say that ILs with short alkyl chains should be preferred [16], if few data are taken into account.

In this study, we investigated the effects of aqueous mixtures of two typical ILs on the secondary and tertiary structures of cryopreserved lysozyme along with the activity combined use of low temperature treatment to 77 K to facilitate future advances in cryobiology and search for a potential as CPA. The basic information gained from this study will also greatly enhance our current biological and chemical knowledge of ILs such as CPA.

2. Materials and methods

2.1. Samples

As sample ILs, we selected the most popular ILs, imidazolium-based and ammonium-based ILs; 1-butyl-3-methylimidazolium chloride (hereafter abbreviated as [bmim][Cl]; Kanto Chemical Co.) and ethylammonium nitrate (abbreviated as EAN; Iolitec GmbH) were used without further purification. Many studies have been performed using lysozyme as a model protein [17,18]. Chicken egg-white lysozyme (Wako Pure Chemical Co.) and *Micrococcus lysodeikticus* (Sigma-Aldrich) were thus used in this study without further purification. Bovine pancreatic trypsin inhibitor (BPTI; Sigma-Aldrich), bovine pancreatic ribonuclease A (Sigma-Aldrich), and horse heart cytochrome *c* (Sigma-Aldrich) were used to determine the [bmim][Cl]–protein interaction site. All the mixtures containing different concentrations of ILs were prepared by mixing the required amount of IL and D_2O (99.9%, Aldrich Co.) in x (mol%IL). The solubility of [bmim][Cl] in water limited the concentration of this IL to $x > 30$. All samples were prepared in a dry box to avoid contamination by atmospheric H_2O and CO_2. Throughout the measurements, the protein concentration in the solution was adjusted to 20 mg/ml.

The entire sample solution was immersed directly into liquid nitrogen and kept at 77 K for ~30 min. Before and after removing the sample from the liquid nitrogen, the absorption spectra were recorded. The retention of protein structure after cooling to low temperature (i.e., 77 K) was also confirmed by comparing the FTIR second-derivative spectrum at room temperature before and after the temperature excursion.

2.2. FTIR and near-UVCD spectral measurements

Fourier transform infrared (FTIR) spectra were recorded using a Nicolet 6700 FTIR spectrometer equipped with an MCT liquid nitrogen-cooled detector. Typically, 512 interferograms were collected to obtain a spectrum with a resolution of 4 cm^{-1}. Near-ultraviolet circular dichroism (UVCD) spectra were measured over the wavelength range of 250–300 nm on a JASCO J-820 spectropolarimeter (scan rate = 20 nm/min with a 0.1-nm step; five accumulations). The obtained spectra were converted to mean molar ellipticity units using $[\theta] = \theta_{obs}/(10ncl)$, where θ_{obs} is the observed ellipticity, l is the path length, c is the protein concentration, and n is the number of residues. The sample was loaded into a cell with CaF_2 windows and a 100-μm Teflon spacer for FTIR and near-UVCD spectral measurements. All exchangeable backbone amide protons were deuterated by incubating the protein in a D_2O solution for 1 day. The completion of the hydrogen–deuterium exchange was confirmed by the cessation of shifts in the amide II band. Solvent spectra were also measured under the same conditions used for the protein solution measurements; the solvent spectra were then subtracted from the protein solution spectra.

2.3. Activity test

We tested lysozyme activity in ILs according to the published procedure [19,20], which is based on the decrease in the turbidity due to the bacteriolysis for *Micrococcus lysodeikticus* bacteria by lysozyme, in 0.1 M phosphate buffer (pD 7) equilibrated at 298 K for 30 min. Lysozyme was added to the stirred assay mixture, and the activities were obtained by measuring the decrease in absorbance at 450 nm using Nicolet GENESYS 10S UV–vis spectroscopy (Thermo SCIENTIFIC). The decrease in absorbance at 450 nm was measured from 0 to 60 s at 2-s intervals. When the decrease in absorbance at 450 nm in aqueous solutions without ILs was 100%, the lysozyme activity in the aqueous solution of ILs was evaluated.

3. Results and discussion

3.1. Structural changes of lysozyme in aqueous IL solutions at cryogenic temperature

Fig. 1 shows representative changes in the (a) FTIR (secondary structure) and (b) near-UVCD (tertiary structure) spectra of lysozyme in aqueous [bmim][Cl] solutions at several [bmim][Cl] concentrations (x) after cooling. Both spectra significantly changed with increasing x. Peaks at ~1618 cm^{-1} and ~1690 cm^{-1}, which arose from the intermolecular β-sheet structure and indicated protein aggregation [22], these peaks were not observed throughout the studied range of x before and after cooling. Similar spectral changes were also observed in the aqueous EAN solutions. A decrease in absorbance at 1654 cm^{-1} ($Abs_{1654cm^{-1}}$) and the disappearance of positive CD intensity at 292 nm ($[\theta]_{292}$) were observed with increasing x before and after cooling (Fig. 1(c) and (d)), indicating the disruption of the secondary and tertiary structures of lysozyme. The changes in $Abs_{1654cm^{-1}}$ and $[\theta]_{292}$ in aqueous [bmim][Cl] solutions were different from those in aqueous EAN solutions. The values of $Abs_{1654cm^{-1}}$ and $[\theta]_{292}$ in aqueous [bmim][Cl] solutions decreased smoothly with increasing x. In contrast, $Abs_{1654cm^{-1}}$ and $[\theta]_{292}$ in aqueous EAN solutions plateaued at approximate values of x of 20 for $Abs_{1654cm^{-1}}$ and 10 for $[\theta]_{292}$ before and after cooling, and further increases in x caused $Abs_{1654cm^{-1}}$ and $[\theta]_{292}$ to decrease.

To determine the midpoint transition concentrations for the unfolding of the secondary structure ($[x]_{1/2}^{unfolding}$) and the disruption of the tertiary structure ($[x]_{1/2}^{disruption}$), the experimental data were fitted (Fig. 1(c) and (d)) to the following sigmoidal curve [23,24], assuming a two-state mechanism for the change in the measured values (A) of $Abs_{1654cm^{-1}}$ and $[\theta]_{292}$:

$$A = A_0 + \frac{a}{1 + \exp\left(\frac{x - x_0}{b}\right)}$$

where A and A_0 are the measured and initial $Abs_{1654cm^{-1}}$ (or $[\theta]_{292}$) values, and x and x_0 are the measured and initial ILs concentrations (mol%IL), respectively. The values of $[x]_{1/2}^{unfolding}$ for the secondary structure determined using this equation were 10.3 ± 3.32 mol%IL before cooling (9.81 ± 3.21 mol%IL after cooling) for [bmim][Cl]