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Structure-based analysis reveals hydration changes induced by arginine hydrochloride

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ABSTRACT

Arginine hydrochloride has been used to suppress protein aggregation during refolding and in various other applications. We investigated the structure of hen egg-white lysozyme (HEL) and solvent molecules in arginine hydrochloride solution by X-ray crystallography. Neither the backbone nor side-chain structure of HEL was altered by the presence of arginine hydrochloride. In addition, no stably bound arginine molecules were observed. The number of hydration water molecules, however, changed with the arginine hydrochloride concentration. We suggest that arginine hydrochloride suppresses protein aggregation by altering the hydration structure and the transient binding of arginine molecules that could not be observed.

1. Introduction

Solvents play a major role in refolding of proteins [1–6]; arginine hydrochloride has been one of the frequently used co-solvents [7–13]. Arginine hydrochloride is considered to improve the refolding efficiency of recombinant proteins by its ability to suppress aggregation of the proteins [14–16]. Moreover, arginine hydrochloride is now used in various other applications, e.g., solubilization of proteins from loose "flocculate-type" inclusion bodies [17,18], milder elution of antibodies from protein-A affinity resins [19,20], and improved separation and recovery of proteins in various chromatographies [13,21–24]. Thermodynamic interactions of proteins with solvent components, both water and arginine in this case, determines the effects of the solvent additives on proteins. Although small arginine binding has been suggested from thermodynamic measurements (e.g. equilibrium dialysis), the physical state of both arginine and water binding has never been experimentally determined.

Recently, a great deal of attention has been given to the structure of water and the assistive at high additive concentrations [25]. X-ray crystallography is useful for high-resolution structural analysis and for characterizing the interactions between water and proteins [26–30]. We investigated arginine binding and hydration and their effects on the structures of hen egg-white lysozyme (HEL). This is the first report examining the physical state of protein solution in aqueous arginine solution.

2. Materials and methods

2.1. Materials

Hen egg-white lysozyme was purchased from Seikagaku Corp. (Tokyo, Japan). Crystallization buffer was purchased from Hampton Research (Aliso Viejo, CA, USA). All other reagents were of biochemical research grade.

2.2. Crystallization of HEL in presence of arginine hydrochloride, NaCl, betaine, and sucrose at various concentrations

Hen egg-white lysozyme was dissolved with arginine hydrochloride at 25, 50, 100, 125, 150, 200, 250, 375, 500, 750, and 1000 mM in 20 mM Tris–HCl buffer (pH 6.9) and diluted to 20 mg mL^{-1}. The HEL solution was dialyzed against the buffer for 12 h at 4 °C and then subjected to crystallization by the oil-batch method [31] to prevent the arginine hydrochloride concentration from being changed by vapor diffusion. Two microliters of HEL was mixed with an equal

Abbreviations: HEL, hen egg-white lysozyme; B-factor, relative temperature; DMSO, dimethyl sulfoxide; GdnHCl, guanidine HCl.

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volume of 100 mM HEPES (pH 7.5) in 1.4 M sodium citrate on 72-microwell plates, and then covered with 10 μl of mineral oil. As a reference, crystals of HEL were also prepared in solutions containing NaCl at different concentrations.

2.3. Diffraction data collection and processing

The high-resolution diffraction data were collected on a beamline BL6A, NW12, or BL17A diffractometer (Photon Factory, Tsukuba, Japan) under cryogenic conditions. Each crystal was soaked for 1 min in a buffer containing 25% glycerol in addition to the crystallization reagents and arginine hydrochloride, and then mounted on a goniometer. The diffraction data were indexed, integrated, scaled, and merged by using the HKL2000 software package [32]. All crystals used were isomorphous crystals whose space group was $P4_32_12$, with unit cell parameters $a=b=c.a.$ 77 Å and $c=37$ Å. The data collection statistics are shown in Supplementary Tables 1 and 2.

2.4. Structure solution and refinement

The structures in each condition were refined from the crystal structure of HEL (PDB ID: 1BWI), which is an isomorphous crystal, as described above. The complete atomic model with a total of 129 residues, including side chains, was rebuilt manually with the molecular graphics program XtalView [33]. Positional and individual temperature factor (B-factor) refinement was carried out with the program REFMAC5 [34]. To monitor the refinement, a 5% subset of all reflections, which was unified like those in the initial mode, was set aside for calculation of the free R factor (Rfree). After iterative cycles of refinement and manual model fitting, water molecules were located by using an Fo–Fc map. At an electron density that was obviously redundant despite location of the water molecules, sodium or chloride ions were placed instead of the water molecules on the basis of the magnitude of the redundancy map and the surrounding conditions. The stereochemical quality of the final refined models was analyzed with the program PROCHECK [35]. The parameters of the data collection and reduction are shown in Supplementary Tables 1 and 2.

3. Results

With a hope to observe both arginine binding and hydration, we crystallized HEL in a solution containing arginine hydrochloride at various concentrations. The oil-batch method was used for crystallization to keep the concentration of arginine hydrochloride constant. Diffraction-quality crystals were obtained in the identical crystallization buffer, regardless of the arginine hydrochloride concentration. Under all conditions, high-resolution diffraction data were collected, and the structures of HEL containing arginine hydrochloride at various concentrations were determined at resolutions higher than 1.65 Å. We examined the backbone and side chain structure of lysozyme in the presence of arginine hydrochloride at various concentrations (Fig. 1). Neither the backbone nor the side-chain structure of HEL was altered by the presence of arginine hydrochloride. Moreover, the relative values of the B-factor of the residues of HEL in the presence of arginine hydrochloride at various concentrations did not change (Fig. 2).

Then we investigated the solvent molecules around HEL in the crystal structure. Even at high concentrations of arginine hydrochloride, no bound arginine molecule was observed around HEL. We

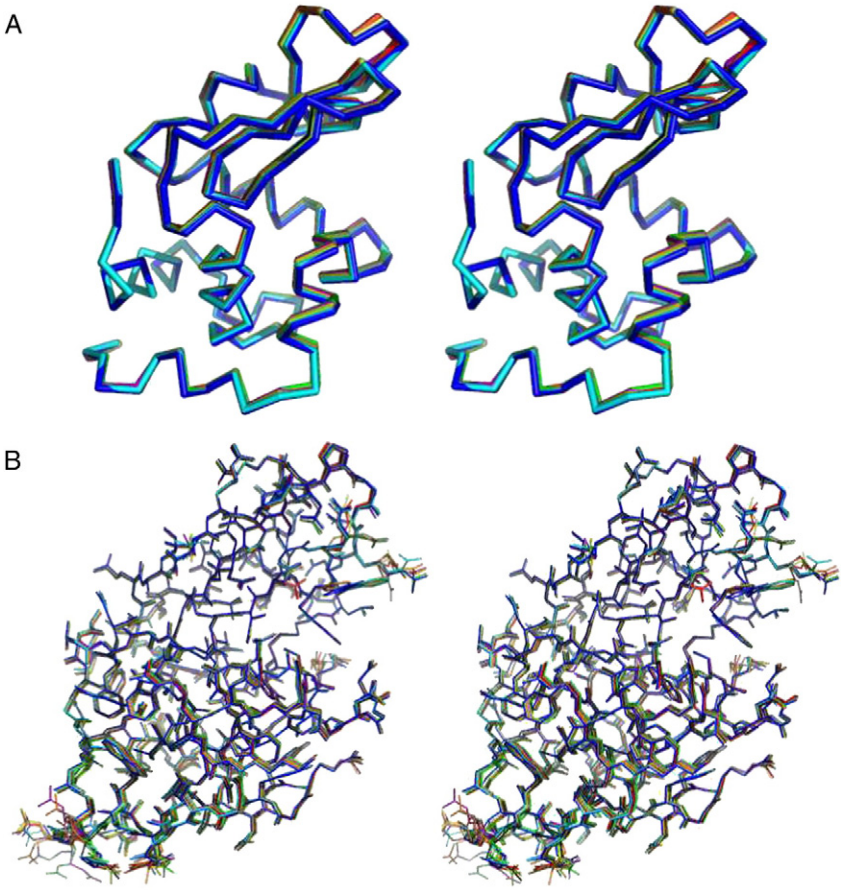

Fig. 1. Crystal structure of HEL. Structures in the presence of 0, 25, 50, 100, 125, 150, 200, 250, 375, 500, 750, and 1000 mM arginine hydrochloride are drawn in blue, cyan, gray, purple, light green, green, yellow, orange, magenta, black, brown, and red, respectively. (A) Backbone structure of HEL in the presence of arginine hydrochloride at the concentrations listed above. (B) Crystal structure of HEL shown with side chains.

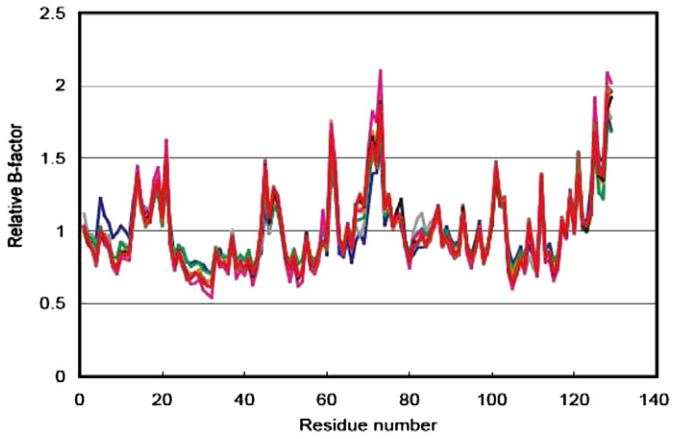

Fig. 2. Superimposed plots of relative temperature factor (B-factor) values of lysozyme residues in the presence of arginine hydrochloride at various concentrations. Colors corresponding to each arginine hydrochloride concentration are identical to those in Fig. 1.

examined the hydration water molecules, sodium ions, and chloride ions around HEL in the crystal structure (Fig. 3). The number of hydration water molecules around HEL obviously changed with increasing arginine hydrochloride concentration (Fig. 4A). At first, the number of hydration water molecules increased in the presence of arginine hydrochloride at very low concentration. Then, as the arginine hydrochloride concentration increased further, the number of hydration water molecules decreased, subsequently increased, and then decreased again. Additionally, a sodium ion was observed at the interface of two HEL domains in the presence of arginine hydrochloride at concentrations above 50 mM, although it was not observed in the absence of arginine hydrochloride (Fig. 3).

To evaluate the degree of hydration derived from the presence of arginine hydrochloride, we crystallized HEL in the presence of glycine betaine and sucrose, which are known to be preferentially excluded from the biopolymer surface, and subsequently preferentially hydrated the biopolymer [36–38]. In addition, crystal structures in sodium chloride at various concentrations were determined to elucidate the effect of chloride ions, because the number of chloride ions also increased with increasing arginine hydrochloride concentration. Crystallization was performed in an identical manner to that in the presence of arginine hydrochloride, and diffraction-quality crystals were obtained. The high-resolution structure of HEL was determined in the presence of glycine betaine, sucrose, and NaCl. Analysis of the crystal structure revealed that the numbers of hydration water molecules in the presence of 1 M glycine betaine and 1 M sucrose were 144 and 173, respectively. Analysis of the number of water molecules around HEL in the presence of sodium chloride at various concentrations (Fig. 4B) showed that as the sodium chloride concentration increased, the number of hydration water molecules increased, at first sharply and then gently.

4. Discussion

Two striking observations were made in this study on protein structure and binding of arginine in aqueous arginine solutions; i.e., the absence of structure changes and different hydration pattern between arginine and NaCl. First, no structure changes even at the highest arginine concentration were observed, consistent with the observed marginal effects of arginine on the stability of native state [14,15,39–41] (Figs. 1 and 2). To our surprise, however, even local structures at protein surface have not been altered by arginine. This is the first observation that arginine does not alter protein structure at atomic level. Second, hydration pattern of HEL as a function of arginine concentration was totally different from NaCl. NaCl is a protein-structure stabilizer and has not been used as a co-solvent that suppresses protein aggregation [42]. HEL showed a monotone increase in hydration with NaCl concentration (Fig. 4B). This result is consistent with the observed little thermodynamic binding of NaCl, which has been correlated with its protein-stabilizing or salting-out effects [43]. The importance of the observed monotone increase in hydration of HEL in NaCl solution will be discussed in depth elsewhere. A simple comparison of hydration pattern for NaCl and arginine hydrochloride points to the uniqueness of arginine, although the level of hydration water is relatively similar between different additives, i.e., ~140–160 (0.17–0.20 g water per g protein). Two other structure-stabilizing additive, betaine glycine and sucrose, showed a similar level of water binding to NaCl and arginine in 1 M solution, meaning that the only data at 1 M cannot distinguish arginine from other additives.

Thermodynamic measurements (e.g., equilibrium dialysis) have suggested small binding of arginine with ribonucles, lysozyme and BSA [39]. As the thermodynamic interaction cannot distinguish arginine binding from decreased hydration (both can cause apparent arginine binding in equilibrium dialysis), the proposed small arginine binding may reflect the observed variation in hydration as a function of arginine concentration, as no physical arginine binding was observed. However, crystal structure will not reveal transient binding of arginine. Such arginine binding may be responsible for the observed small thermodynamic arginine binding. We would need additional information on such binding to fully correlate the physical binding of water and arginine with the thermodynamic solvent interaction data. Nevertheless, it is clear that there is no strong arginine binding. The effects of arginine are most likely due to both the complex hydration changes and the unstable transient arginine binding, which both contribute to the thermodynamic interactions. However, since it is also suggested that arginine hydrochloride mainly acts on partially denatured proteins [15], we may need more investigations of arginine binding by using more unstable proteins.

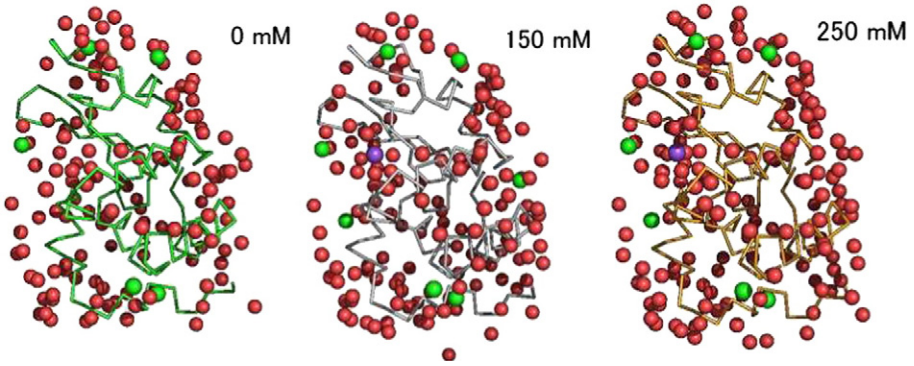

Fig. 3. Crystal structures of HEL, with molecules around HEL, in the presence of arginine hydrochloride. Hydration water molecules, sodium ions, and chloride ions are drawn in red, purple, and green, respectively.

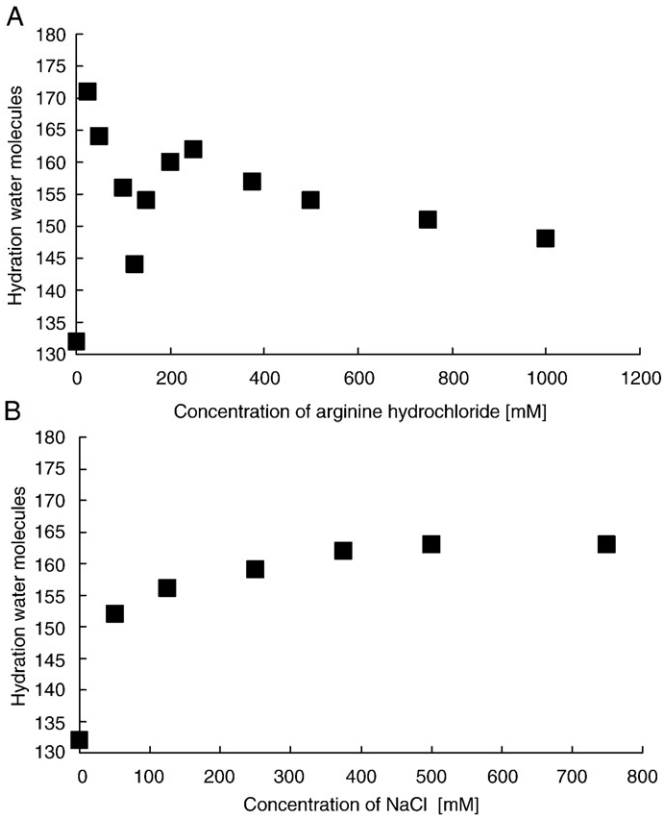

Fig. 4. Number of hydration water molecules in the presence of arginine hydrochloride (A) or sodium chloride (B) at various concentrations.

The number of hydration water molecules changed with increasing arginine hydrochloride concentration, as described above (Fig. 4A). The number of hydration water molecules observed in the crystal structure (0.17–0.20 g/g) was fewer than that calculated from equilibrium dialysis experiments (0.3–0.4 g/g) [39,44], possibly because only stabilized water molecules are observed in a crystal structure. The latter experiments detect transient bindings of both water and arginine. The increase in the number of hydration water molecules around HEL with increasing arginine hydrochloride concentration could be described as: (1) an initial drastic increase in the presence of arginine hydrochloride at concentrations below 25 mM, (2) then a mild decrease over a wide range of arginine hydrochloride concentration, and finally (3) a secondary increase at around 200 mM. These results indicate that arginine hydrochloride both increases and decreases the number of hydration water molecules, depending on its concentration. As there is no stably bound arginine nor effects of arginine on protein structure, it is more likely that other factors, such as the potential transient binding of arginine, are involved in the observed effects of arginine on changes in hydration. The isoelectric point of HEL is about 11; i.e., HEL has many positively charged residues at the experimental pH of 7.5, at which arginine also has a positive charge. Consequently, arginine is excluded from the area around HEL by the electrostatic repulsion, which may contribute to the initial drastic increase in the number of hydration water molecules. It is not clear, however, how such arginine exclusion leads to the increase in stably bound water molecules. Above 25 mM, hydration decreased up to 125 mM arginine concentration and then again increased. These hydration changes may reflect changes in arginine binding, which were too unstable to be observed. Additional work that can measure those unstable, transient bindings would complement the analysis described here. In addition, we crystallized HEL in the presence of 1.4 M Na-citrate and its structure is determined in the presence of 25% glycerol. Although the concentrations of these agents remained at a constant concentration, both agents likely also affect hydration and the overall high ionic strength might counteract a mildly denaturing effect of arginine. Additional study in the conditions with low ionic strength would verify the hydration changes induced by arginine hydrochloride.

Lastly, a sodium ion was observed at the interface of two HEL domains in the presence of above 50 mM arginine hydrochloride. This sodium ion was not observed in the crystal structure without arginine hydrochloride, but it has been observed in the same position in the crystal structure of HEL in the presence of GdnHCl [45]. Although GdnHCl is a denaturant and arginine hydrochloride is not, arginine has a guanidium group like GdnHCl. Therefore, the appearance of sodium ions in the crystal structure with increasing arginine hydrochloride concentration may be due to the unstable transient binding of arginine hydrochloride and its effects on proteins.

In summary, we investigated the effects of arginine hydrochloride on HEL by crystallography. Neither the backbone nor side-chain structure of HEL was altered by the presence of arginine hydrochloride. The B-factor of the side chains did not change either. However, sodium ions, which were not observed in lysozyme in the absence of the additive, were observed in the presence of arginine hydrochloride at concentrations above 50 mM. In addition, the number of hydration water molecules changed with the arginine hydrochloride concentration. It is plausible that these changes in hydration condition contribute to the ability of arginine hydrochloride to suppress protein aggregation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2008.07.009.

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