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The protective effects of osmolytes on arginine kinase unfolding and aggregation

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Abstract

Osmolytes are a series of different kinds of small molecules that can maintain the correct conformation of protein by acting as molecular chaperons. In this study, the protective effects of four compatible osmolytes, *i.e.*, proline, sucrose, DMSO and glycerol, were studied during arginine kinase (EC 2.7.3.3) unfolding and aggregation. The results showed that all the osmolytes applied in this study obviously prevented AK unfolding and inactivation that was due to a GdnHCl denaturant by reducing the inactivation rate constants (k_i), increasing the transition free energy changes ($\Delta\Delta G_i$) and increasing the value for the midpoint of denaturation (C_m). Furthermore, the osmolytes remarkably prevented AK aggregation in a concentration-dependent manner during AK refolding. Our results strongly indicated that osmolytes were not only metabolism substrates, but they were also important compounds with significant physiological protective functions for proteins, especially in some extremely harsh environments.

Keywords: Osmolytes; Arginine kinase; Unfolding; Aggregation; Transition free energy

1. Introduction

Almost every functional protein is the product of folding a disordered polypeptide into a specific three-dimensional conformation. During the process of protein folding, various small compounds called "chemical chaperones" play important roles in forming the correct protein conformation and protecting it against thermal denaturation and aggregation [1–4]. These compounds including a variety of polyols, sugars, polysaccharides, organic solvents, some amino acids and their derivatives seem to improve the ability of cells to adapt to different metabolic insults due to the compounds' stabilizing effects on the protein conformation [5–8]. Most of such compounds have no substrate specificity, but some of them specifically stabilize certain proteins [9]. Several recent reports about the function of osmolytes in the unfolding process of some proteins both *in vivo* and *in vitro* have been published [10,11].

Besides protecting the conformation of proteins, osmolytes are known to stabilize proteins against aggregation. Protein aggregation is a frequently observed phenomenon during protein unfolding or refolding, which was recognized mainly as the result of nonspecific interactions between the hydrophobic regions of the polypeptide chains [12]. Because preventing aggregation is important during protein purification, new approaches based on "artificial chaperones" have been introduced to prevent aggregation by aiding protein folding [13]. Some osmolytes are known to stabilize proteins against aggregation; these are recognized as protein folding helpers [14] and they are classified as 'compatible osmolytes', including polyols and free amino acids, and the 'counteracting osmolytes' such as glycine betaine and trimethylamine *N*-oxide (TMAO)

Abbreviations: AK, arginine kinase; GdnHCl, guanidine hydrochloride; DMSO, dimethysulfoxide; ANS, 8-anilino-1-naphthalenesulfonic acid; UV, ultraviolet; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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[15]. Compatible osmolytes protect proteins that are subjected to threatening conditions such as extreme temperature fluctuations, excessive dryness or high salt environments [16], while the counteracting osmolytes protect cellular proteins against urea inactivation [10]. Compatible and counteracting osmolytes may have different mechanisms for protecting proteins because of the variety of the relevant environmental stresses [17].

AK (ATP: L-arginine phosphotransferase EC 2.7.3.3), is a member of what appears to be a highly conserved family of phosphotransferases that reversibly catalyze the transfer of phosphate from phosphoarginine to ADP, yielding ATP as follows:

arginine + MgATP → *N*-phospho-L-arginine+MgADP

AK is found in a wide variety of invertebrates where it serves a function analogous to that of creatine kinase in vertebrates. Phosphoarginine plays a critical role as an energy reserve because it can be changed to ATP when energy is needed [18]. AK is an important phosphagen kinase that is directly associated with muscle contraction, ATP regeneration and energy transportation in the cellular energy metabolism of invertebrates [19,20]. Recently, the 3D structure of AK has been studied by Zhou et al., and the results showed that AK shared same subunit topology as CK. A small β-helical N-terminal domain is followed by a larger C-terminal domain that is similar to the C-terminal domain of glutamine synthetase. Evidences further suggested that residues 309–318 would get much more close to the substrate while it bind to the enzyme active site and induce the conformational change [21].

AK from locust is a kind of monomeric enzyme with the molecular weight of 40 kDa and an isoelectric point of 6.3 [22]. In this study, we focused on analyzing the protective effects of compatible osmolytes on AK, and we provided insight into the role of chemical chaperones that act against guanidine denaturation. The results of our study showed that proline, sucrose, glycerol and DMSO not only prevented AK from inactivation and unfolding, but they also inhibited aggregation due to their stabilizing the AK conformation. Our study also provided a useful strategy to help induce correct protein folding *in vitro* by adding some osmolytes into the refolding buffer.

2. Materials and methods

2.1. Materials

ATP, proline, glycerol, sucrose, DMSO and ANS was obtained from Amresco. GdnHCl, L-arginine, glycine and 2-mercaptoethanol were purchased from Sigma. Sephacryl S-200 HR and DEAE Sepharose CL-6B were obtained from Amersham. All the other agents were local analytical grade products.

2.2. Purification of AK

AK was isolated from the leg muscle of the locust *Migratoria manilensis* by sephacryl S-200 HR gel filtration chromatography and DEAE Sepharose CL-6B fast flow chromatography [22]. The purified AK was homogenous, as was determined by SDS-PAGE.

2.3. Enzymatic activity assay

The enzyme concentrations were determined by the Bradford method. AK was denatured by using 1.0 M GdnHCl that contained various osmolytes. The enzymatic activities were measured by employing a direct continuous pH-spectrophotometric assay method [23].

2.4. Fluorescence measurements

AK was denatured by placing it a series of GdnHCl solutions that contain various concentrations of sucrose, proline, DMSO or glycerol for 2 h. An excitation wavelength of 295 nm was used for determining AK's intrinsic fluorescence intensity, and the emission spectra were recorded from 310 to 400 nm. For the binding studies using the hydrophobic dye ANS, AK was incubated with a 40-folded molar excess of ANS in the dark. The ANS fluorescence emission spectra were recorded after 30 min at wavelengths from 400 to 600 nm. The samples were excited at 380 nm. The fluorescence spectra were measured with an F-2500 spectrophotometer (Hitachi) with using a 1-cm path-length cuvette. The final enzyme concentration was 2.1 μM.

2.5. Aggregation studies

The GdnHCl-denatured AK was diluted 30-folded with standard buffer (0.1 M glycine/1 mM EDTA/1 mM 2-mercaptoethanol buffer, pH 8.4) that contained various concentrations of osmolytes at 25 °C. The aggregation was monitored by measuring the turbidity at 400 nm with a U/V spectrophotometer (Thermo Spectronic).

3. Results

3.1. Inactivation of AK in GdnHCl in the presence of various osmolytes

The AK enzymatic activities were measured over time after AK was denatured in 1.0 M GdnHCl that contained the different osmolytes. The native AK activity at the same concentration was taken as 100%. Fig. 1 shows the protective effects by investigating the kinetics of AK inactivation: in the presence of osmolytes, the residual activities of the GdnHCl-denatured AK were higher than that of the control (without osmolytes); with the increasing concentrations of osmolytes, the rate of AK inactivation gradually became slower. These four osmolytes that were used at the concentrations applied in this study did not modulate the native AK activities (data not shown). Fig. 2 shows the semilogarithmic plot of the kinetic course of inactivation of GdnHCl-denatured AK in the absence and presence of different concentrations of osmolytes. The inactivation rates constants (k_i) were calculated from the data of the semilogarithmic plot, and the transition free energies ($\Delta\Delta G_i$) were calculated through the formula: $\Delta\Delta G_i = -RT\ln(k_{i,osmolytes}/k_{i,none})$ [24]. The data listed in Table 1 indicated that as the concentrations of osmolytes were increased, k_i was correspondingly reduced and $\Delta\Delta G_i$ was correspondingly increased.