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Effects of osmolytes on arginine kinase from *Euphausia superba*: A study on thermal denaturation and aggregation

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ABSTRACT

Investigations of energy-related enzymatic properties may provide valuable information about the mechanisms that are involved in the adaptation to extreme climatic environments. The protective effects of osmolytes on the thermal denaturation and aggregation of arginine kinase from *E. superba* (ESAK) was investigated. When the concentration of glycine, proline and glycerol increased, the relative activation was significantly enhanced, while the aggregation of ESAK during thermal denaturation was decreased. Spectrofluorometry results showed that the presence of these three osmolytes significantly decreased the tertiary structural changes of ESAK and that thermal denaturation directly induced ESAK aggregation. The results demonstrated that glycine, proline and glycerol not only prevented ESAK from inactivation and unfolding but also inhibited aggregation by stabilizing the ESAK conformation. We measured the ORF gene sequence of ESAK by RACE, and built the 3D structure of ESAK and osmolytes by homology models. The results showed that the docking energy was relatively low and that the clustering groups were spread to the surface of ESAK, indicating that osmolytes directly protect the surface of the protein. Our study provides important insight into the protective effects of osmolytes on ESAK folding.

1. Introduction

Antarctic krill (*Euphausia superba* Dana) is a crustacean that lives in the cold environment of the Southern Ocean and has a particularly active way of life. *E. superba* has been shown to express high growth rates with a sufficient food supply and is capable of high swim and escape velocities [1,2]. This energy-demanding lifestyle and the accompanying exceptionally high metabolic rates gave rise to questions about the types of physiological adaptations that enable these animals to inhabit the cold oceans to compensate for the rate-limiting, extremely low temperatures [3]. In recent years, several studies have been performed on the temperature-dependent regulation of metabolic pathways in Antarctic krill [4,5]. Such regulations in long- and short-term adaptations are likely to be related to alterations in the catalytic site, which consequently affect the enzymatic level. Thus, the investigation of enzymatic properties may provide valuable information on mechanisms that are involved in the adaptation of invertebrates to extreme climatic environments.

We focused our work on important enzymes involved in energy metabolism in Antarctic krill such as arginine kinase (ATP: L-arginine phosphotransferase, EC 2.7.3.3, AK). As a member of the phosphagen kinase family, AK is a key enzyme for cellular energy metabolism in invertebrates. AK catalyzes the reversible phosphoryl transfer from phosphocreatine to adenosine triphosphate (ATP). AK has a direct relationship with intracellular energy movement, muscle contraction and ATP regeneration [6–10]. AK is critically involved in energy metabolism in invertebrate cells of species such as insects, shrimps, crabs, and mollusks and is highly expressed in

Abbreviations: ESAK, arginine kinase from *Euphausia superba*; ANS, 1-anilinonaphthalene-8-sulfonate; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide.

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invertebrate muscle tissues that require large amounts of energy [11–13].

Accordingly, the active and functional regulation of AK in Antarctic krill will influence the krill's physiological activity and adaptability to the environment. However, by living in extreme environments, many organisms protect themselves by producing intracellular low-molecular weight organic compounds in response to environmental stress, known as osmolytes [14,15]. Some osmolytes, such as proline, glycerol, sucrose and DMSO, have shown protective effects on AK [16]. However, the detailed characteristics of *E. superba* arginine kinase (ESAK) have not been well investigated so far. ESAK could be an attractive model for studying protein thermal denaturation and aggregation as it correlates with its physiological function.

In this study, we investigated the effects of temperature on ESAK and the protective effects of osmolytes on ESAK during the thermal denaturation and aggregation process. In addition, the intrinsic and ANS-binding fluorescence spectra of unfolding ESAK were measured. Homology modeling and computational docking simulations were also performed to understand the role of the osmolytes at the atomic level based on the previous strategy reported by our group [17]. Our results provide insight into the protective effects of different osmolytes on the ESAK denaturation and suggest mechanism for preventing inactivation and aggregation during temperature change.

2. Materials and methods

2.1. Materials

ATP, arginine, magnesium acetate, thymol blue, glycine, proline, glycerol and ANS were purchased from Sigma–Aldrich (St. Louis, USA). Other chemicals were locally obtained and were of the highest analytical grade.

2.2. ESAK purification and identification

ESAK was fractionated from muscle samples with 30–50% saturation of $(NH_4)_2SO_4$. Then it was purified by Hiprep 26/60 Sephacryl S-200 HR gel filtration chromatography, Blue Sepharose 6 FF affinity chromatography, and finally dialyzed with 20 mM Tris-acetic acid buffer (pH 8.0). Purified ESAK was shown to be homologous in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. ESAK activity assay

The activities of ESAK during thermal denaturation in both the absence and presence of osmolytes were measured following proton generation during the reaction of ATP and arginine with thymol blue at 575 nm and 20 °C, as previously described [18,19]. The substrate comprised 5.7 mM arginine, 5 mM ATP, 6.6 mM magnesium acetate, and 0.015% thymol blue, pH 8.0. The reaction volume was 1 mL, and 10 μL of enzyme solution was added to the substrate system to measure the enzyme activity. The final concentration of ESAK was 3 μg/ml. All experiments were conducted using a Shimadzu UV-1800 spectrophotometer at 20 °C. In all experiments, blanks were subtracted to correct for the absorbance of the buffer components. The ESAK was incubated at different temperatures for 2 h and assayed at 20 °C in both the absence and presence of osmolytes. Denaturation rates were determined by a semi-logarithmic plot.

2.4. Kinetic analysis

For the evaluation of the inactivation kinetics and rate constants, the transition free energy was calculated based on methods described in previous reports with slight modifications [20,21]. The transition free energy change per second is given by $\Delta\Delta G^\circ = -RT\ln k$. The data were calculated from the semi-logarithmic plots, and k is the time constant for the major phase of the inactivation reaction.

2.5. Intrinsic and ANS-binding fluorescence spectroscopy

The fluorescence emission spectra of ESAK were measured based on the previous report [17]: briefly, an excitation wavelength of 280 nm was applied with the emission wavelengths ranged between 300 and 400 nm for ESAK samples. 40 μM ANS was used to label hydrophobic surface of ESAK and for the ANS-binding fluorescence, an excitation wavelength of 380 nm was used with the emission wavelength ranged from 400 to 600 nm.

2.6. Aggregation measurements

Aggregation of ESAK induced by temperature was followed by recording the absorbance at a wavelength of 400 nm based on the previous report [17].

For the kinetic analysis of ESAK aggregation, the following equations, which were outlined in previous reports [21,22], were applied:

$$\Delta AG = AG_\infty - AG_t \quad (1)$$

where AG_∞ is the absorbance at the end of the aggregation reaction before reaching the precipitation state and AG_t is the absorbance at time t during aggregation. The experimental data were fitted to first-order expressions as:

$$\Delta AG = \exp\left(\frac{-k_{AG}}{t}\right) \quad (2)$$

$$\Delta AG = P_1 \exp\left(\frac{-k_{AG1}}{t}\right) + P_2 \exp\left(\frac{-k_{AG2}}{t}\right) + P_3 \exp\left(\frac{-k_{AG3}}{t}\right) \quad (3)$$

where k_{AG} is the rate constant for a monophasic reaction (Eq. (2)). P_1 to P_3 indicate the fractions reacting with the rate constants k_{AG1}, k_{AG2} and k_{AG3}, respectively. The change of transition free energy during aggregation in the presence of an additive is expressed as:

$$\Delta\Delta G_{AG} = RT\ln\left(\frac{k_{AG,\text{none}}}{k_{AG,\text{additive}}}\right) \quad (4)$$

2.7. cDNA cloning of ESAK and RACE

Total RNA was isolated from the body muscle of the krill using Trizol reagent (Invitrogen, Japan) following the manufacturer's protocol, resuspended in DEPC-treated water, and stored at −80 °C. The integrity was assessed by analysis on a 1.5% agarose gel, and concentrations were determined by a spectrophotometer. cDNA was synthesized from 2 μg of total RNA by M-MuLV reverse transcriptase (Takara, Japan) at 42 °C for 1 h with Oligo dT primer following the protocol of the manufacturer.

Oligonucleotide primers for the amplification of ESAK cDNA were designed based on the transcriptome library sequenced by Illumina's Solexa sequencing technology. Initially, PCR was performed using the prepared cDNA as a template, with the primers of AKF1 and AKR1 (Supplementary Table 1) to obtain partial fragments of the AK gene from *E. superba*. The PCR products were separated on a 1.2% agarose gel and were then purified using a PCR purification kit. The purified PCR product was ligated with