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The effect of Zn^{2+} on *Euphausia superba* arginine kinase: Unfolding and aggregation studies

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ABSTRACT

Arginine kinase plays an important role in the cellular energy metabolism of invertebrates. We investigated the effects of Zn^{2+} on the enzymatic activity and unfolding and aggregation of *Euphausia superba* arginine kinase (ESAK). Zn^{2+} inhibited the activity of ESAK (IC_{50} = 0.027 ± 0.002 mM) following first-order kinetics consistent with the transition from a mono-phasic to a bi-phasic reaction. Double-reciprocal Lineweaver–Burk plots indicated that Zn^{2+} induced non-competitive inhibition of arginine and ATP. Circular dichroism spectra and spectrofluorometry results showed that Zn^{2+} induced secondary and tertiary structural changes in ESAK with exposure of hydrophobic surfaces and directly induced ESAK aggregation. The addition of osmolytes such as glycine and proline successfully blocked ESAK aggregation, recovering the conformation and activity of ESAK. Our study demonstrates the effect of Zn^{2+} on ESAK enzymatic function and folding and unfolding mechanisms, and might provide important insights into other metabolic enzymes of invertebrates in extreme climatic marine environments.

1. Introduction

Phosphagen kinases are a group of highly conserved enzymes that catalyze the reversible transfer of phosphoryl groups from phosphagens, for example the generation of creatine phosphate catalyzed by creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2, CK) and of phosphoarginine catalyzed by arginine kinase (ATP: arginine phosphotransferase, EC 2.7.3.3, AK). Arginine kinase in invertebrates plays a central role in both temporal and spatial ATP buffering in cells with high and fluctuating energy requirements (such as muscle and nerves) by catalyzing magnesium-dependent reversible phosphorylation between L-arginine and ATP [1]. Most AKs are monomers with an average molecular mass of approximately 40 kDa [2], although marine echinoderms possess a dimeric AK with a molecular weight of 80 kDa [3]. All AKs share the same functional the N-terminal domain of ATP: guanido phosphotransferase as a guanidine substrate specificity domain (GS domain) and an ATP-gua Ptrans domain responsible for ATP binding [4,5]. Homologous amino acid sequence alignments of arginine kinase suggest that members of the arginine enzyme family evolved from a common ancestor.

The evolution of marine invertebrate metabolism patterns generally leads to further adaptation to the aquatic environment. Some marine invertebrates utilize inorganic metal ions and amino acids as osmotic solutes to maintain cell volume to adapt to the ionic strength of seawater [6]. The increasing pollution of heavy metal ions in seawater, however, often leads to intracellular accumulation of these ions in marine organisms. Thus, it is necessary to investigate the effects of metal ions and amino acids on AK structure and function. It has previously been reported that metal ions, especially divalent metal ions, can affect the structure and stability of some proteins [7,8].

Zn^{2+} is the second most abundant transition metal in organisms after iron and is the only metal present in all enzyme classes [9,10]. It is an essential component of most enzymes and plays important roles in metabolism and disease [11–15]. Zn^{2+} may contribute to

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

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the regulation of many biological processes, and adequate Zn^{2+} is necessary for maintaining good health [16]. For this reason, Zn^{2+} was previously thought to be non-toxic [17–19]; however, excessive Zn^{2+} can be toxic to organisms in different ways [20]. There is evidence that Zn^{2+} can induce hydrophobic exposure, unfolding, and aggregation in a broad variety of proteins, including creatine kinase, GroEL and aminoacylase [21–23]. Concentrations of Zn^{2+} in the tissues of aquatic organisms, including marine invertebrates, are usually far in excess of that required for normal metabolism. Much of the excess Zn^{2+} is bound to macromolecules or present as insoluble metal inclusions in tissues [24]. For this reason, it is necessary to study the effect of Zn^{2+} on proteins in aquatic organisms.

To date, several AKs from different kinds of shrimp have been purified and characterized. According to previous reports, AK in shrimps is monomeric [25,26]. The Antarctic krill *Euphausia superba* Dana is a crustacean that is highly abundant in oceans of the southern hemisphere. Investigation of energy-related enzymatic properties of Antarctic krill might provide valuable information on mechanisms involved in adaptation to extreme climatic marine environments. In this work, we investigated the effects of Zn^{2+} on arginine kinase from *E. superba* with respect to functional and structural changes. This study provides insight into unfolding responses of ESAK in response to Zn^{2+} and might reveal the functional role of osmolytes such as glycine and proline to prevent aggregation.

2. Materials and methods

2.1. Materials

ATP, arginine, magnesium acetate, thymol blue, zinc acetate dihydrate, glycine, proline, and ANS were purchased from Sigma–Aldrich. All other chemicals were locally obtained and of the highest analytical grade.

2.2. ESAK purification

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

2.3. ESAK activity assay

AK activity was measured by following proton generation during the reaction of ATP and arginine with thymol blue at 20 °C, as previously described [27,28]. The substrate mix was composed of 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. Absorption was recorded at 575 nm using a Shimadzu UV-1800 spectrophotometer.

2.4. Kinetic analysis

For evaluation of the inactivation kinetics and rate constants, the transition free energy was calculated based on methods described in a previous report with slight modifications [29]. The transition free energy change per second is given by $\Delta\Delta G^{\circ} = -RT\ln k$. The data were calculated from semi-logarithmic plots, and k is the time constant for the major phase of the inactivation reaction.

For general analysis of non-competitive inhibition, the Lineweaver–Burk equation can be written in double reciprocal form:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad (1)$$

Secondary plots can be constructed from

$$Y\text{-intercept} = \frac{1}{V_{max}} + \frac{1}{K_i V_{max}}[I] \quad (2)$$

The K_i, K_m, and V_{max} values can be derived from these two equations. The secondary replot of Y-intercept vs. [I] is linearly fitted assuming a single inhibition site or a single class of inhibition sites.

2.5. Protein unfolding and refolding

For the unfolding experiment, ESAK was dissolved in buffer with different concentrations of Zn^{2+} for 2 h at 20 °C. The refolding experiment was carried out by addition of different concentrations of osmolytes to ESAK that was previously treated with 1 mM Zn^{2+}. The circular dichroism (CD) spectra were measured using a JASCO J-815 spectropolarimeter and a 1-cm path-length cuvette. Wavelengths were 200–250 nm. Fluorescence emission spectra were measured with a F-4500 spectrofluorometer (Hitachi, Japan) and a 1-cm path-length cuvette. The excitation and emission wavelengths were 280 nm and 300–400 nm, respectively. ANS was used to probe the hydrophobic surface, and a 50-fold excess concentration of ANS was added to the samples for 30 min in the dark. Fluorescence was measured using a 1-cm path-length cuvette and excitation and emission wavelengths of 380 nm and 400–600 nm, respectively. All spectra were collected at 20 °C in 20 mM Tris–acetic acid buffer (pH 8.0).

Based on the previous report [30], the α-helix content of ESAK was estimated from the mean residue ellipticity ($[\theta]$, $^{\circ}$ cm^2/dmol) at a wavelength of 222 nm by CD spectra, using Eqs. (7) and (8).

$$[\theta]_{222} = \frac{\theta_\lambda \times M_{RW}}{10 \times C \times L} \quad (7)$$

$$\alpha\text{-helix content}\,(\%) = -\frac{[\theta]_{222} + 4000}{29,000} \quad (8)$$

In the equation, θ_λ is the ellipticity got from the CD spectra; M_{RW} is the mean residue molecular weight, and is about 118.7 for ESAK; C is the concentration of ESAK, and L is the path-length.

2.6. AK aggregation measurement induced by Zn^{2+}

Aggregation of AK induced by Zn^{2+} was followed by recording absorbance at a wavelength of 400 nm in a Shimadzu UV-1800 spectrophotometer using a 1-cm path-length cuvette. To measure the effect of Zn^{2+}, the aggregation time course was measured at different Zn^{2+} concentrations. The final enzyme concentration was 8.0 μM and the recording time was 7000 s.

For kinetic analysis of AK aggregation, the following equations, which are outlined in previous reports [31,32], were applied:

$$\Delta AG = AG_\infty - AG_t \quad (3)$$

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

$$\Delta AG = \exp\left(-\frac{k_{AG}}{t}\right) \quad (4)$$

$$\Delta AG = P_1 \exp\left(-\frac{k_{AG1}}{t}\right) + P_2 \exp\left(-\frac{k_{AG2}}{t}\right) + P_3 \exp\left(-\frac{k_{AG3}}{t}\right) \quad (5)$$

where k_{AG} is the rate constant for a monophasic reaction (Eq. (4)). 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:

$$\Delta\Delta G_{AG} = RT\ln\left(\frac{k_{AG,none}}{k_{AG,additive}}\right) \quad (6)$$

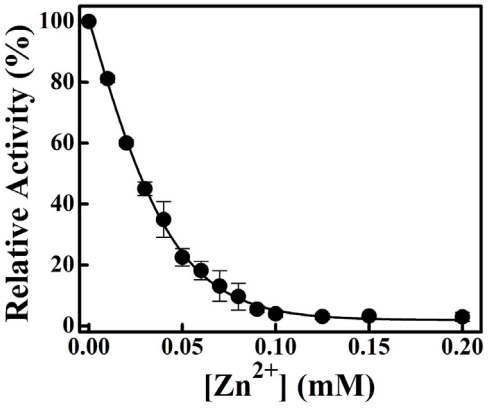

Fig. 1. The effect of Zn^{2+} on the activity of ESAK. Data are presented as means ($n = 3$). ESAK was incubated with different Zn^{2+} concentrations at 20 °C for 2 h in 20 mM Tris–acetic acid buffer (pH 8.0) and then added to the assay system at corresponding Zn^{2+} concentrations. The final concentration of the enzyme was 2.0 μM.