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Purification, characterization, and unfolding studies of arginine kinase from Antarctic krill

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ABSTRACT

The regulation of enzymatic activity and unfolding studies of arginine kinase (AK) from various invertebrates have been the focus of investigation. To gain insight into the structural and folding mechanisms of AK from *Euphausia superba* (ESAK), we purified ESAK from muscle properly. The enzyme behaved as a monomeric protein with a molecular mass of about 40 kDa and had pH and temperature optima of 8.0 and 30 °C, respectively. The K_m^{Arg} and K_m^{ATP} for the synthesis of phosphoarginine were 0.30 and 0.47 mM, respectively, and k_{cat}/K_m^{Arg} was 282.7 s^{-1}/mM. A study of the inhibition kinetics of structural unfolding in the denaturant sodium dodecyl sulfate (SDS) was conducted. The results showed that ESAK was almost completely inactivated by 1.0 mM SDS. The kinetics analyzed via time-interval measurements revealed that the inactivation was a first-order reaction, with the kinetic processes shifting from a monophase to biphase as SDS concentrations increased. Measurements of intrinsic and 1-anilinonaphthalene-8-sulfonate-binding fluorescence showed that SDS concentrations lower than 5 mM did not induce conspicuous changes in tertiary structures, while higher concentrations of SDS exposed hydrophobic surfaces and induced conformational changes. These results confirmed that the active region of AK is more flexible than the overall enzyme molecule.

1. Introduction

Phosphagen kinases are a type of phosphoryl transfer enzyme that plays a central role in energy metabolism in animals. They catalyze the reversible transfer of a phosphoryl group between ATP and guanidino compounds such as creatine, glycocyamine, taurocyamine, opheline, lombricine or arginine, whose phosphorylated forms are collectively called phosphagens [1,2]. In vertebrates, the unique phosphagen system consists of creatine and its corresponding creatine kinase (ATP: *N*-creatine phosphotransferase, EC 2.7.3.2, CK), while arginine kinase (ATP: L-arginine phosphotransferase, EC 2.7.3.3, AK) is the most widely distributed phosphagen kinase in invertebrates. AK catalyzes the reversible transfer of a phosphoryl group from Mg^{2+}ATP to arginine, leading to the formation of phosphoarginine (PArg) and Mg^{2+}ADP [3]. 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 [4,5].

The Antarctic krill *Euphausia superba* is a crustacean present in great amounts in the oceans of the southern hemisphere. It is unique among other crustaceans living in the cold environment due to its particularly active lifestyle. Antarctic krill has been characterized as expressing high growth rates with sufficient food supplies and being capable of fast swimming and escape velocities [6–8]. This energy-demanding lifestyle requires an exceptionally high metabolic rate. This gives rise to questions about the type of physiological adaptations that might compensate for the rate-limiting extreme low temperatures experienced in the cold oceans. Such regulations are likely to be related to enzyme levels and functions. Consequently, the investigation of energy-related enzymatic properties in *E. superba* may provide valuable information on mechanisms that are involved in the adaptation of invertebrates to extreme climatic environments.

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

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AK plays a key role in cellular energy metabolism as an ATP buffer in invertebrates, and it is an ideal paradigm for the study of classical enzymology of bimolecular reactions [9]. To date, several AKs from different types of shrimp have been purified and characterized. In this study, we describe the purification and kinetic properties of AK from Antarctic krill. Using sodium dodecyl sulfate (SDS), we studied the inactivation and conformational changes of muscle AK purified from *E. superba*, in order to analyze the unfolding processes of AK. This study was undertaken to extend investigation to the structure, conformation and function of muscle AK from another krill living in a cold environment.

2. Materials and methods

2.1. Materials

Arginine, ATP, magnesium acetate, thymol blue, SDS, and 1-anilinonaphthalene-8-sulfonate (ANS) were all purchased from Sigma. Hiprep 26/60 Sephacryl S-200 HR and Blue Sepharose 6 FF were obtained from GE Healthcare Life Sciences. All other reagents were local products of pure analytical grade quality.

2.2. ESAK purification

All procedures were performed at 0–4 °C. The body muscle of the krill was homogenized with a buffer system [20 mM Tris–acetate, pH 8.0, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mM ethylenediaminetetraacetic acid]. Centrifugation was conducted at 18,000 × *g* for 20 min. The supernatant was collected as crude enzyme and fractionated with 30–50% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in a minimum volume of the same buffer and applied to a Hiprep 26/60 Sephacryl S-200 HR column equilibrated with the same buffer with a flow speed of 2 ml/min. The fraction possessing AK activity was pooled and applied to a Blue Sepharose 6 FF column (2.5 cm × 15 cm) equilibrated with 20 mM Tris–acetate buffer containing 0.5 mM PMSF at pH 8.0. The column was washed with the same buffer and then eluted with 1.5 M KCl at a flow rate of 2 ml/min. AKTA™ *prime Plus* purification system was used to run the columns. Finally, the active sample tubes were collected and dialyzed with 20 mM Tris–acetate buffer at pH 8.0. The purified AK was stored on ice or at −80 °C until use.

2.3. ESAK activity assay

AK activity was measured using a modification of previous procedures [10]. The reaction mixture consisted of 10.34 mM Arg, 2.07 mM ATP-Na, and 3.10 mM magnesium acetate dissolved in 100 mM Tris–acetate at pH 8.0. The reaction was carried out at 20 °C for 1 min by adding 10 μl of enzyme sample to 290 μl of the reaction mixture. The reaction was stopped by the addition of 250 μl of 2.5% trifluoroacetic acid. The mixture was heated for 1 min at 100 °C to fully hydrolyze the formed PArg and was then plunged into an ice bath for 1 min and finally incubated at 20 °C for 5 min. The inorganic phosphate level was determined with a phosphate determination reagent (PDR). The PDR was freshly prepared by mixing 1 ml of 0.2 M bismuth nitrate dissolved in 6 M nitric acid, 1 ml 0.14 M ammonium molybdate, 0.5 ml of 1% ascorbic acid and 2 ml of distilled water. Next, 450 μl of PDR was added to the equilibrated solution for 3 min for color development, and the solution absorbance was measured at 660 nm with a Shimadzu UV-1800 spectrophotometer. The control was prepared in parallel with the same conditions except that the 10-μl enzyme sample was replaced with buffer. For the accurate analysis of the phosphate released and the PArg formed, a standard curve was constructed using the same procedure, except that 10-μl standard solutions of various concentrations of KH_2PO_4 were added instead of the enzyme sample. A unit of AK is the amount of enzyme that catalyzes the formation of 1 μmol inorganic phosphate per min. The protein concentration was determined from the absorbance at 280 nm using an absorbance of 0.67 in 1-cm cuvettes corresponding to 1 mg of protein per ml [11]. Certain kinetic parameters were determined using different concentrations of substrates in the assay mixture. ESAK and SDS were dissolved in 20 mM Tris–acetate buffer (pH 8.0). All the measurements were repeated 3 times.

2.4. Intrinsic and ANS-binding fluorescence measurements

The intrinsic fluorescence emission spectra were measured with an F-4500 fluorescence spectrophotometer using a 1-cm path-length cuvette. An excitation wavelength of 280 nm was used for the tryptophan fluorescence measurement, and the emission wavelengths ranged between 300 and 400 nm. To probe the hydrophobic surface changes, the fluorescence spectra changes were studied by labeling with 40 μM ANS for 30 min prior to measurement. An excitation wavelength of 390 nm was used for the ANS-binding fluorescence, and the emission wavelength ranged from 400 to 600 nm.

3. Results

3.1. Purification of AK from E. superba

The enzyme was purified by Sephacryl S-200 HR gel filtration chromatography and Blue Sepharose 6 FF affinity chromatography (Fig. 1). The parameters of the purification procedure are summarized in Table 1. A purification of approximately 5.0-fold with a yield of 45.7% activity was achieved. The molecular weight was obtained by SDS polyacrylamide gel electrophoresis and estimated to be 39.4 kDa, based on calibration with standard reference proteins of known molecular weights.

3.2. Kinetic constants

Kinetic evaluations of the forward reactions catalyzed by AK with L-arginine and ATP were conducted. From Lineweaver–Burk plots, the kinetic parameters revealed apparent K_m values of 0.29 and 0.45 mM for L-arginine and ATP, respectively (Fig. 2). The kinetic parameters K_m^{Arg}, K_m^{ATP}, k_{cat}, k_{cat}/K_m^{Arg} and V_{max} of ESAK for the forward reaction were also tested and are shown in Table 2.

Table 1
Purification steps and recovery of AK from muscles of *Euphausia superba*.

Step	Total protein (mg)	AK activity (U)	Specific activity (U/mg)	Purification ratio	Relative yield (%)
Crude extraction	66.3	23,760	358	1	100
$(NH_4)_2SO_4$ salt fractionation	236	16,640	701	2.0	70
Sephacryl S-200 column	16.7	12,652	757	1.1	76
Blue Sepharose 6 FF column	6.1	10,873	1780	2.4	86