Biochimie 94 (2012) 566–573

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

α-Crystallin protects human arginosuccinate lyase activity under freeze–thaw conditions

Yi-Yu Wei[a], Chih-Wei Huang[b], Wei-Yuan Chou[a], Hwei-Jen Lee[a,*]

[a] Department of Biochemistry, National Defense Medical Center, No. 161, Sec. 6, MinChuan E. Rd., Neihu, Taipei, Taiwan
[b] Department of Pharmacy Practice, Tri-Service General Hospital, No. 325, Sec. 2, Chenggong Rd., Neihu, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 1 June 2011
Accepted 8 September 2011
Available online 25 September 2011

Keywords:
Argininosuccinate lyase
Cold labile
Freeze–thawing
Ice-induced inactivation
α-Crystallin
Cryoprotection

ABSTRACT

Argininosuccinate lyase (ASL) catalyzes the conversion of argininosuccinate into arginine and fumarate, a key step in the biosynthesis of urea and arginine. ASL is a tetrameric enzyme but it dissociates into inactive dimers under low temperature conditions. This study investigates the inactivation process under low temperature conditions. Inactivation was caused by dissociation of tetrameric ASL into dimers, with increased exposure of hydrophobic areas without disturbance of the secondary structure or the micro-environment surrounding the key tryptophan residues. Most activity was retained when temperatures were changed at a rate of >1 °C/min, whilst freezing or thawing more slowly resulted in greater loss of activity. Inactivation was reduced by inclusion of α-crystallin, a structural protein found in ocular lenses and a member of the small heat-shock protein family, by stabilization of the ASL quaternary structure. In addition, α-crystallin was able to restore the function of ASL that had been inactivated by slow freezing and thawing. The effect of α-crystallin was similar to that of bovine serum albumin, suggesting that both proteins exerted their effects by hydrophobic interactions. α-Crystallin therefore acts as a cryo-preservative that protects ASL activity during freezing and thawing.

1. Introduction

Argininosuccinate lyase (ASL) (EC 4.3.2.1) catalyzes the conversion of argininosuccinate into arginine and fumarate, a key step in the biosynthesis of urea in the livers of ureotelic species and arginine in all organisms [1]. In humans, deficiency of this enzyme results in the clinical condition argininosuccinic aciduria [2,3]. ASL belongs to a super-family of metabolic enzymes that include class II fumarase [4], aspartase [5], adenylosuccinate lyase [6] and 3-carboxyl-*cis*,*cis*-muconate lactonizing enzyme [7]. They have low overall sequence homology but possess three highly conserved regions. In contrast, avian δ-crystallin, which has over 70% amino acid identity with ASL, is assumed to have been recruited from the metabolic enzyme to function as a structural protein in taxon-specific eye lens [8,9]. ASL is a homotetrameric protein with a molecular weight about 200 kDa. The overall topology of terameric ASL consists of two pairs of closely associated dimers stacked perpendicularly to each other. The determined structure of ASL revealed that the conserved regions, which are located in different monomers, are assembled to form a functional active site [10,11]. Hydrophobic interactions predominantly stabilize the stable quaternary structure of ASL. Previous studies have reported that ASL was dissociated into catalytically inactive dimers at cold temperatures. The loss of activity was accelerated in Tris buffer and prevented by phosphate buffer or substrate [12]. Interactions between the dimers were disrupted by high concentrations of guanidine hydrochloride or urea, and by sodium dodecyl sulfate [13,14].

Freeze–thawing is a stress condition that can potentially damage the stability of proteins [15,16]. This is related to the formation of ice crystals that may lead to surface-induced denaturation of proteins at the ice–water interface, increasing concentrations of buffer salts, and the changes in pH [17]. The extent of damage by freeze–thawing depends on the conformational stability of proteins. Hydrophobic interactions that stabilize proteins are thought to be weakened under low temperature conditions [18,19]. Multi-subunit proteins such as aldolase, lactate dehydrogenase, liver alcohol dehydrogenase, and yeast alcohol dehydrogenase were found to be more susceptible than monomeric proteins to denaturation under freezing conditions due to their tendency to undergo subunit dissociation [16,20,21]. A wide variety

Abbreviations: ASL, Argininosuccinate lyase; ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, Circular dichroism; λ_{max}, Maximum emission wavelength; PBS, Phosphate buffered saline.

* Corresponding author. Tel.: +886 2 87910832; fax: +886 2 87923106.
E-mail address: hjlee@mail.ndmctsgh.edu.tw (H.-J. Lee).

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doi:10.1016/j.biochi.2011.09.006

of compounds, including sugars, polyols, amino acids and certain salts, are effective at minimizing protein denaturation during freeze–thawing [22]. The mechanism of protection is thought to be their preferential exclusion from the hydrate surface of protein. Several compounds are effective as cryo-protectants but can act as protein denaturants at higher temperatures [23].

α-Crystallin is a major structural protein of the ocular lens, which belongs to the family of small heat-shock proteins [24–26]. It accounts for about 35% of protein in mammalian lens and plays a key role in maintaining the transparency and refraction properties [27,28]. α-Crystallin is a heterogeneous oligomeric protein with an average molecular weight about 800 kDa. It consists of two subunits, αA- and αB-crystallin, which are present at a molar ratio of 3:1 in mammalian lenses [8,29]. This structural protein can function as a molecular chaperone that recognizes protein substrates under stress conditions [30,31], and it is efficient at suppressing the heat-induced aggregation of β- and γ-crystallins. It is also effective at preventing aggregation of proteins including those proteins denatured by guanidine hydrochloride or urea undergoing refolding [31–33]. α-Crystallin is a polydispersed and dynamic oligomer which binds to proteins in unstable conformations and prevents their aggregation and amyloid fibril formation [30,31,34,35].

α-Crystallin is an effective chaperone when protecting proteins from denaturation by heat-shock, but the protective effects under low-temperature denaturing conditions have been rarely studied. Cold-induced cataract is commonly occurs in the eye lens of many species except for Antarctic notothenid fishes. No cataracts were observed in the lenses of these species when cooled to −12 °C, indicating the presence of highly cold-stable lens proteins [36]. α-Crystallin was reported to preserve transparency at low temperature but does not prevent cold aggregation of γ-crystallin [37]. In this study, the stability of ASL under a variety of freezing-thaw conditions and the protective effects of α-crystallin were investigated. α-Crystallin is able to stabilize the quaternary structure of ASL and protect it from cold-induced inactivation.

2. Materials and methods

2.1. Materials

Restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). dNTPs, DNA molecular ladders and protein molecular mass markers was obtained from Protech (Taipei, Taiwan). 1-Anilinonaphthalene-8-sulfonic acid (ANS) was from Molecular Probes (Eugene, OR). All chemicals were of analytic grade or higher and were purchased from the Sigma–Aldrich (St. Louis, MO) or J. T. Baker (Phillipsburg, NJ) unless otherwise stated. Chromatography systems and columns were supplied by GE Healthcare (Uppsala, Sweden).

2.2. Protein production and purification

The vector of pET-ASL was transformed into *Escherichia coli* BL21 (DE3) cells and was grown in 2YT broth containing 70 μg/ml of kanamycin at 37 °C, until the $OD_{600\ nm}$ was greater than 0.6. Expression was induced with 0.4 mM isopropyl-β-*d*-thio-galactopyranoside (IPTG) for another 16 h at 27 °C. Cells were harvested and kept frozen at −80 °C until used.

The frozen cells were resuspended in 50 mM Tris–HCl buffer (pH 7.5) and lysed by sonication. The lysate was centrifuged at 15,000 g for 45 min at 4 °C. The supernatant was filtered and then loaded onto a Q-Sepharose anion exchange column (Hiprep 16/10 Q XL, GE Healthcare) equilibrated in the same buffer. The column was washed with the column buffer to remove unbound proteins and eluted with a linear gradient of 0–0.4 M NaCl in the same buffer. Recombinant ASL was eluted at approx. 0.2 M NaCl. The eluted protein was pooled and then treated with ammonium sulfate to 1.2 M. After filtration, the sample was loaded onto a hydrophobic interaction column (Source™ 15PHE) equilibrated 50 mM Tris–HCl, 10% (v/v) glycerol, pH 7.5 supplemented with 1.2 M ammonium sulfate. The column was washed with the column buffer and eluted with a gradient to 50 mM Tris–HCl, 10% (v/v) glycerol, pH 7.5. The retained proteins were eluted at ~0.3 M ammonium sulfate. Fractions were pooled and loaded onto S-300 Sephacryl column (26 mm × 85 cm) pre-equilibrated in PBS (Phosphate Buffered Saline) buffer (0.137 M NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.5). The highest purity fractions was pooled and concentrated to about 5 mg/ml.

Porcine lens α-crystallin was purified from fresh porcine eye balls. The homogenized supernatant was filtered and loaded onto S-300 Sephacryl column equilibrated with PBS buffer. The retained proteins were then analyzed by SDS-polyacrylamide gel electrophoresis. The fractions of α-crystallin were pooled and kept frozen at −80 °C. The concentration of proteins was determined by the method of Bradford using BSA as the standard [38].

2.3. Freezing and thawing experiment

All experiments were carried out at a cryo-microscope system including an Olympus System Microscope (Model BX50) (Olympus Optical Co., Ltd., Japan) and a Linkam BCS 196 cryostage (Linkam Scientific, Tadworth, Surrey, U.K.). The cryostage consists of a silver block in a heating cast and a tube for introducing liquid nitrogen by a cooling pump (Linkam LNP). The freezing and thawing temperature on cryostage was precisely controlled by operation of a temperature profile on Link 2.0 Programmer Control software.

System set up for freezing and thawing experiments follows the protocol as described previously [39]. The samples of ASL (0.4 mg/ml) alone or in mixture with 4-fold concentration of α-crystallin were incubated at room temperature for 1 h and then about 55 μl was loaded on to a quartz crucible and topped by a cover slip. The samples were cooled from room temperature to −1 °C, at a cooling rate of 30 °C/min. The sample was held for 2 min and seeded (by using glass capillary, 0.3 mm) at −1 °C. Then the cooling rates were change to appropriate rate between −1 °C and −30 °C. It was also held for 2 min at −30 °C. Finally, the sample was warmed at an appropriate rate from −30 °C to 10 °C. It was held for 1 min at 10 °C before activity assay.

2.4. Enzyme activity assay

Enzyme activity was measured at 25 °C by monitoring the absorption of fumarate at 240 nm in a Perkin-Elmer Lambda 40 spectrophotometer. The reaction mixture contained 1 mM sodium argininosuccinate in 50 mM Tris–HCl buffer (pH 7.5). Assays were performed at least triplicate and a molar absorption coefficient of $2.44 \times 10^3\ M^{-1}cm^{-1}$ was used for all calculations [14]. The activity is normalized as percentage relative activity by dividing the specific activity of ASL with and without freezing treatment in the absence or presence of α-crystallin.

The chaperone activity of α-crystallin was determined by monitoring the aggregate formation of ASL at 55 °C through measurements of light scattering at 360 nm in a Perkin-Elmer Lambda 40 spectrophotometer equipped with a Peltier temperature controller accessory.

2.5. Circular dichroism studies

Circular dichroism (CD) spectra were measured using a Jasco J-815 spectropolarimeter equipped with a Peltier temperature controller accessory. Experiments were performed in PBS buffer