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Original Contribution

Identification and characterization of a copper-binding site in αA-crystallin

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ABSTRACT

Previous studies have shown that both αA- and αB-crystallins bind Cu^{2+}, suppress the formation of Cu^{2+}-mediated active oxygen species, and protect ascorbic acid from oxidation by Cu^{2+}. αA- and αB-crystallins are small heat shock proteins with molecular chaperone activity. In this study we show that the mini-αA-crystallin, a peptide consisting of residues 71–88 of αA-crystallin, prevents copper-induced oxidation of ascorbic acid. Evaluation of binding of copper to mini-αA-crystallin showed that each molecule of mini-αA-crystallin binds one copper molecule. Isothermal titration calorimetry and nanospray mass spectrometry revealed dissociation constants of 10.72 and 9.9 μM, respectively. 1,1′-Bis(4-anilino) naphthalene-5,5′-disulfonic acid interaction with mini-αA-crystallin was reduced after binding of Cu^{2+}, suggesting that the same amino acids interact with these two ligands. Circular dichroism spectrometry showed that copper binding to mini-αA-crystallin peptide affects its secondary structure. Substitution of the His residue in mini-αA-crystallin with Ala abolished the redox-suppression activity of the peptide. During the Cu^{2+}-induced ascorbic acid oxidation assay, a deletion mutant, αAΔ70–77, showed about 75% loss of ascorbic acid protection compared to the wild-type αA-crystallin. This difference indicates that the 70–77 region is the primary Cu^{2+}-binding site(s) in human native full-size αA-crystallin. The role of the chaperone site in Cu^{2+} binding in native αA-crystallin was confirmed by the significant loss of chaperone activity by the peptide after Cu^{2+} binding.

αA-Crystallin is one of the abundant proteins in the mammalian lens and belongs to the family of small heat shock proteins (sHSP) [1]. In addition to its refractive role, αA-crystallin, like other sHSP members, exhibits a chaperone-like function that is believed to be involved in maintaining lens transparency [2–5]. Studies of the critical residues responsible for the chaperone-like activity of αA-crystallin have revealed that R116 [6], R49 [7], R21 [8], and F71 [9,10] are critical for chaperone function. Other studies have reported that the C-terminal region [11–13] and the SRLFDQFFG sequence motif [14] are critical for chaperone-like function. There is a general agreement that the hydrophobic regions play a role in the chaperone-like activity of αA-crystallin [15]. Our hydrophobic site-specific reagent (1,1′-bis (4-anilino)naphthalene-5,5′-disulfonic acid; bis-ANS) binding study suggested that the chaperone site in human αA-crystallin is residues 70–88, which was subsequently confirmed by the demonstration of inhibited aggregation activity in a synthetic peptide consisting of αA70–88 residues (KFVIFLDVKHFSPEDLTVK) [16]. We called this peptide "mini-chaperone" or "mini-αA-crystallin" [16]. The mini-αA-crystallin sequence is highly conserved among several sHSPs. During homology modeling studies, this region aligns to the β3 and β4 region in the α-crystallin domain of sHSP16.5 [17]. Mini-αA peptide functions like a molecular chaperone by preventing the aggregation and precipitation of denaturing substrate proteins caused by oxidative, thermal, and chemical denaturing agents [16,18,19].

Copper is present in micromolar concentration (3–10 μM) in lens tissue and it is mostly bound to the lens protein [20–23]. Ortwerth and James [22] reported that lens proteins tightly bind Cu^{2+} ions and suppress Cu^{2+}-mediated generation of reactive oxygen species, as well as the oxidation of ascorbic acid. This was further confirmed in a study that showed that both αA- and αB-crystallins are involved in redox silencing [24]. Under some conditions Cu^{2+} interaction with α-crystallin was found to modulate the chaperone activity [25–27]. However, none of the studies carried out thus far have pinpointed the specific Cu^{2+}-binding sequence in human αA-crystallin. This study was undertaken to determine whether mini-αA-crystallin binds Cu^{2+} and inhibits copper-induced oxidation of ascorbic acid, like native α-crystallin or its subunits. We show that the αA-crystallin chaperone site is also a Cu^{2+}-binding site in αA-crystallin and the αA70–88 sequence is sufficient to suppress Cu^{2+}-induced oxidation of ascorbic acid.

Materials and methods

Reagents

Mini-αA-crystallin (DFVIFLDVKHFSPEDLTVK) and the alanine analog (DFVIFLDVKAFSPEDLTVK) were supplied by GenScript Corp.

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(Piscataway, NJ, USA). The purity of the peptides was >95% as determined by high-performance liquid chromatography (HPLC) and mass spectroscopy. Dry peptides were weighed on a microbalance and dissolved in HPLC-grade water, and fractions of solutions were used to determine the concentration of peptides by amino acid analysis. Copper sulfate solution from a Pierce protein assay kit was used as the source of Cu^{2+}. The actual contents of copper and peptide were determined by flame photometry and amino acid analysis at the Experimental Station Chemical Laboratories, University of Missouri, Columbia.

Ascorbic acid oxidation

A 100 mM stock solution of ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA) was prepared in HPLC-grade water. For the oxidation experiments, 500 μM ascorbic acid was prepared in Chelex-treated phosphate buffer (50 mM, pH7.2) in the presence and absence of Cu^{2+}. The assays were carried out at 25 °C. In other experiments, ascorbic acid (500 μM) and Cu^{2+} (4 μM) were incubated (25 °C) in the presence and absence of mini-αA-crystallin. The absorbance of ascorbic acid was measured at 260 nm, using a 1-cm cell path in a spectrophotometer, as described earlier [22].

Circular dichroism spectroscopy

Far-ultraviolet (UV) circular dichroism (CD) spectra of the peptide in the presence and absence of 1 μM copper were recorded using a Jasco J-815 spectropolarimeter (Easton, MD, USA). Mini-αA-crystallin (0.1 mg/ml) was prepared in 10 mM phosphate buffer, pH 7.2. Far-UV CD spectra were recorded at 25 °C. All of the reported spectra were the cumulative averages of six scans after subtraction of the buffer blank.

Fluorescence spectroscopy

The relative hydrophobicity of mini-αA-crystallin was measured using the hydrophobic dye bis-ANS (Molecular Probes, Eugene, OR, USA). A stock solution of the dye (14.8 μM) was prepared in 95% ethanol. Peptides were titrated with increasing concentrations of copper in the range of 0–100 μM. Ten microliters of bis-ANS stock solution was added to 15 μM mini-αA in 1 ml of 50 mM PO_4 buffer at pH 7.2. The mixture was incubated at 37 °C for 20 min. The interaction of bis-ANS with mini-αA-crystallin in the absence and presence of copper was examined by recording the emission spectra between 450 and 600 nm. The samples were excited at 385 nm in a Jasco spectrofluorimeter FP-750. Emission at 490 nm was used to calculate $F_0 - F/F_0$ values. Analysis of the saturation binding curve at one site was performed using the equation $f = b_{max} \times \mathrm{abs}(x)/(K_D + \mathrm{abs}(x))$, as described earlier [28], where b_{max} is the maximum binding (maximum extent of quenching), x is the Cu^{2+} concentration, and f is the fluorescence quenching at a given concentration of Cu^{2+}. K_D value was obtained from a semilog plot.

Isothermal titration calorimetric assay

Isothermal titration calorimetry was performed in a VP-ITC (Microcal, Northampton, MA, USA). Mini-αA-crystallin was prepared in 10 mM cholamine chloride buffer containing 100 mM NaCl (pH 7.4). Copper sulfate, 1.0 mM, was prepared in the same buffer. For each titration, the sample cell (1.41 ml) contained mini-αA, and the buret contained the Cu^{2+} solution. After thermal equilibration, injections of titrant, 10 μl, were made at 4-min intervals. The titration protocol included a 2-μl preinjection, the heat from which was neglected during the subsequent analysis. The data were analyzed with a single-site model, using the Origin-based software supplied with the instrument.

Analysis of mini-αA-crystallin–copper binding by mass spectrometry

Peptide and Cu^{2+} were prepared in 5 mM ammonium acetate buffer (pH 6.3). The final reaction solution was prepared using the same buffer at 10 μM concentration of mini-αA-crystallin and various concentrations of Cu^{2+}. The pH was recorded after the addition of Cu^{2+} ion and mini-αA-crystallin. The samples were analyzed in the positive ion mode by static nanospray mass spectrometry on an Agilent 6520 QTOF mass spectrometer at the University of Missouri Proteomics Center. The resulting spectra were subjected to the Agilent resolved isotope deconvolution software program (Agilent, Santa Clara, CA, USA).

Effects of Cu^{2+} on mini-αA-crystallin chaperone-like activity

To test whether copper binding to mini-αA-crystallin modulates chaperone-like activity, mini-αA-crystallin was saturated with 1 mM $CuSO_4$ solution, and the peptide–Cu^{2+} complex was isolated by Sephadex-G20 gel-filtration chromatography. Protein aggregation assays were performed using 75 μg of citrate synthase (CS) in 1 ml of 40 mM Hepes–KOH buffer (pH 7.0) in the presence of the mini-αA-crystallin–Cu^{2+} complex (50 μg) or mini-αA-crystallin alone (50 μg). The extent of aggregation of CS was monitored at 360 nm up to 1 h, as described earlier [10].

αAΔ70–77 region in copper binding

To test whether this region is the only copper-binding region in αA-crystallin, a deletion mutant αAΔ70–77 was created by site-directed mutagenesis, and the recombinant protein was purified [10]. Purified protein was used in the copper-induced ascorbic acid oxidation assay, as described above. Oxidation assays were carried out using 100 μg of recombinant protein αAΔ70–77 or wild-type αA-crystallin in the presence and absence of Cu^{2+} (5 μM).

Results

Inhibition of Cu^{2+}-mediated ascorbic acid oxidation by mini-αA-crystallin

Ascorbic acid is readily oxidized in the presence of Cu^{2+} ion (Fig. 1A). In the presence of air/oxygen and Cu^{2+}, ascorbic acid is converted to dehydroascorbic acid, with the generation of reduced copper ion. Our baseline experiment of the rate of ascorbic acid oxidation at increasing concentrations of Cu^{2+} revealed that in about 30 min, 500 μM ascorbic acid was completely oxidized by 4 μM Cu^{2+} (Fig. 1B). In another experiment, the effects of Cu^{2+} (4 μM) on the oxidation of ascorbic acid (500 μM) were determined in the presence of various concentrations of mini-αA-crystallin (Fig. 2A). The amount of ascorbic acid remaining in the reaction mixture that contained both Cu^{2+} and mini-αA-crystallin showed a sigmoidal profile (Fig. 2A),

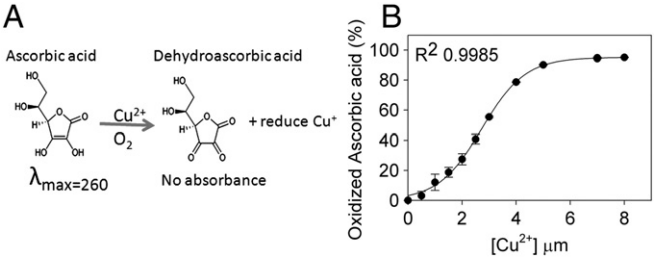

Fig. 1. (A) Schematic representation of the oxidation pathway of ascorbic acid. (B) Standard curve of Cu^{2+}-induced oxidation of ascorbic acid. Cu^{2+}-induced oxidation of ascorbic acid was determined as described under Materials and methods by following 260 nm absorbance.