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Reactive cysteine residues in the oxidative dimerization and Cu^{2+} induced aggregation of human γD-crystallin: Implications for age-related cataract

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ABSTRACT

Cysteine (Cys) residues are major causes of crystallin disulfide formation and aggregation in aging and cataractous human lenses. We recently found that disulfide linkages are highly and partly conserved in β- and γ-crystallins, respectively, in human age-related nuclear cataract and glutathione depleted LEGSKO mouse lenses, and could be mimicked by in vitro oxidation. Here we determined which Cys residues are involved in disulfide-mediated crosslinking of recombinant human γD-crystallin (hγD). In vitro diamide oxidation revealed dimer formation by SDS-PAGE and LC-MS analysis with Cys 111-111 and C111-C19 as intermolecular disulfides and Cys 111-109 as intramolecular sites. Mutation of Cys111 to alanine completely abolished dimerization. Addition of αB-crystallin was unable to protect Cys 111 from dimerization. However, Cu^{2+}-induced hγD-crystallin aggregation was suppressed up to 50% and 80% by mutants C109A and C111A, respectively, as well as by total glutathionylation. In contrast to our recently published results using ICAT-labeling method, manual mining of the same database confirmed the specific involvement of Cys111 in disulfides with no free Cys111 detectable in γD-crystallin from old and cataractous human lenses. Surface accessibility studies show that Cys111 in hγD is the most exposed Cys residue (29%), explaining thereby its high propensity toward oxidation and polymerization in the aging lens.

1. Introduction

The transparency of the lens depends on a combination of homeostatic processes that minimize physical and chemical damage to crystallins, which are prone to their accumulation because they do not turn over. These include e.g. truncation, glycation, oxidation and disulfide bond formation, isomerization, racemization, deamidation, and phosphorylation [1]. Oxidation is one of the major post-translational modifications that is strongly associated with aggregation involving disulfide and non-disulfide bonds. The association between high molecular weight (HMW) aggregate and cataract is well established [2]. Oxidation extensively impacts on the sulfur-containing amino acids, cysteine and methionine [3,4]. Nuclear cataracts are associated with conformational changes and exposure of buried Cys residues thus increasing vulnerability to oxidizing species. As a result, total protein thiols decrease and protein disulfides increase [5,6]. This affects particularly the cysteine-rich γ-crystallins that easily form disulfide-bonded aggregates [7]. Among these, human γD-crystallin is one of the most abundant and significant γ-crystallins of the lens nucleus [8]. One example of a critical cysteine residue is the point mutant R14C which results in hereditary juvenile cataract involving non-native cross-links with Cys 110 (now Cys111) [9].

To understand the role of cysteines in cataractogenesis, we recently determined disulfide cross-link formation sites by H_2O_2 oxidation in total mouse lens homogenate compared to those found in old and cataractous human lens and glutathione depleted LEGSKO mouse lens, an animal model for age-related nuclear cataract (ARNC) [7]. Results showed that β-crystallin disulfide linkages are highly conserved in nuclear cataracts and LEGSKO mouse lenses and reproducible by in vitro oxidation of young mice lens by H_2O_2. However, some of the γ-crystallin disulfide formation sites present in human cataracts, such as 33 and 42, were not mimicked by in vitro oxidation, apparently

Abbreviations: Cys, cysteine; WT, wild type; hγD, human gamma D crystallin; HMW, high molecular weight aggregate; ARNC, age-related nuclear cataract; IPTG-isopropyl, 1-thio-β-D-galactopyranoside; EDTA, ethylenediamine tetraacetic acid; PMSF, phenyl methanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD, circular dichroism; UV, ultraviolet

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necessitating prior conformational changes.

The study below was initiated with the goal of identifying the in vitro oxidation sites of cysteine residues in recombinant human γD-crystallin. Below we report the identification of Cys 111 as the major residue responsible for disulfide formation in protein dimers as well as for Cu^{2+}-induced aggregation of hγD-crystallin.

2. Materials and methods

2.1. Human γD-crystallin and αB-crystallin recombinant protein expression

Human γD-crystallin in the pET 3d expression vector, a kind gift from Dr. Noriko Fujii, was transformed into *E. coli* BL21 (DE3) pLysS cells. Recombinant protein was induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) with a final concentration of 0.3 mM. The cells were collected 5 h after IPTG induction, and lysed via repeated thaw and sonication cycle (six rounds of 8 pulses per minute) in 20 mM Tris-HCl, pH 8 containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM phenyl methanesulfonyl fluoride (PMSF). The cell lysate was centrifuged at 20,000 × rpm for 20 min, and the supernatant was subjected to ion exchange column chromatography on a Q-Sepharose XL column (Amersham Biosciences, Piscataway, NJ) followed by a cation exchange column chromatography on a Toyopearl GigaCap S-650M column (TOSOH, Tokyo, Japan) in a HPLC system (WATERS, Milford, MA).

Human αB-crystallin construct with His-tag C-terminal was a kind gift from Dr. Mark Petrash. Recombinant αB-crystallin was also produced in BL21 (DE3) pLysS *E. coli* system induced by 1 mM IPTG. The recombinant αB-crystallin protein was purified via Ni-NTA affinity column (GE Health Care, Chicago, IL) following the manufacturer's protocol. The αB-crystallin was eluted by 250 mM imidazole. The purity of the recombinant protein was verified by a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after Coomassie blue stain. Both purified human γD and αB-crystallin were dialyzed against 50 mM Chelex-treated phosphate buffer (pH 7.4) and stored in −80 °C until use.

2.2. In-gel digestion and mass spectrometry analysis

Human γD WT and diamide oxidized (see below) crystallin samples, before cysteine alkylation was used for in-gel digestions. Briefly, samples were first separated by 12% SDS-PAGE, and the monomer and dimer band were excised and destained with 50% acetonitrile in 100 mM ammonium bicarbonate followed by 100% acetonitrile. The dehydrated gel pieces were dried in a SpeedVac centrifuge, and then digested for 5 h in 50 mM ammonium bicarbonate containing 1% (WT/WT) sequencing grade modified trypsin (Promega, Madison, WI) and then with 1% (WT/WT) Asp-N (Roche, Germany) digestion overnight digestion at 37 °C. The resulting proteolytic peptides were extracted from the gel with 50% acetonitrile in 5% formic acid, the peptides were then dried and reconstituted in 0.1% formic acid for LC-MS/MS analysis.

The digests were analyzed by LC-MS/MS using Orbitrap Elite Hybrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA), equipped with a Waters nanoAcquity UPLC system (Waters, Taunton, MA). The spectra were acquired in the positive ionization mode by data-dependent methods consisting of a full MS scan at 120,000 resolution and MS/MS scans of the twenty most abundant precursor ions in ions trap by collision-induced dissociation at normalized collision energy of 35%. A dynamic exclusion function was applied with a repeat count of 2, repeat duration of 30 s, exclusion duration of 45 s, and exclusion size list of 500. The obtained data were submitted for customized γD-crystallin database search using MassMatrix [10]. Carbamidomethylation of Cys and oxidation of Met was selected as variable modifications. The mass tolerance was set as 10 ppm for precursor ions and 0.8 Da for product ions. The candidates of disulfide bonds formation suggested by the software were further verified by manual interpretation of the MS/MS spectra.

2.3. Site-directed mutagenesis

The γD-crystallin cysteine to alanine mutants, C19A, C33A, C42A, C79A, C109A, C111A, C19-111A C19-42A, C109-111A, C19-33-42A and C19-33-42-111A, were generated by site-directed mutagenesis using the Quick Change II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. The primer pairs used for mutagenesis are listed in Table S1. All mutagenesis sites were validated by DNA sequencing analysis.

2.4. Human γD crystallin cysteine residue oxidation

Human wild type (WT) and mutant recombinant γD-crystallin at the concentration of 1 mg/ml in 50 mM Chelex-treated phosphate buffer (pH 7.4) was incubated with freshly prepared 10 μM diamide at 37 °C for 3 h. The reaction was terminated by additional 30 min incubation with 50 μM (five equivalent) of L-cysteine to block excess unreacted diamide. The reaction mixture was concentrated using 3kDA Amicon filters, and free cysteines were alkylated with 100 mM iodoacetamide in 200 mM Tris-buffer (pH 8.5), 5 mM EDTA, 4 M urea buffer at 37 °C for 30 min. Disulfide bond formation was monitored by 12% SDS-PAGE.

In other experiments, 1:1 and 1:3 (WT/WT) mixtures of hγD and αB crystallin in 50 mM Chelex treated potassium phosphate buffer were incubated 60 min at 37 °C and the mixture was oxidized with 10 μM diamide and processed as described above. The formation of γD-crystallin dimer was determined by 12% SDS-PAGE and also further confirmed by western blot analysis using polyclonal human γD crystallin antibody (Sigma, St. Louis, MO).

2.5. Biophysical characterization

2.5.1. Secondary and tertiary structure analysis

A circular dichroism (CD) spectropolarimeter (Jasco 810, Kyoto, Japan) was used to record the CD spectra of the γD crystallin and mutants. Far-ultraviolet (UV) CD measurements were performed between wavelengths of 195 to 250 nm at room temperature. Scans were performed using a cylindrical quartz cuvette with a 1 mm path length. Protein samples of 0.1 mg/ml were prepared in 50 mM potassium phosphate buffer (pH 7.4). Spectra represented are the average of five scans after subtracting blank [11].

Near-ultraviolet (UV) CD measurements were performed between wavelengths of 240 to 320 nm at room temperature. Scans were performed using a cylindrical quartz cuvette with a 10 mm path length. Protein samples of 1 mg/ml were prepared in 50 mM potassium phosphate buffer (pH 7.4). Spectra represented are the average of five scans. The buffer signal was subtracted and smoothed [12].

2.5.2. Determination of tryptophan fluorescence

The intrinsic tryptophan fluorescence spectra of γD-crystallin mutants were recorded with an Agilent fluorescence spectrophotometer (Santa Clara, CA). The excitation was set as 295 nm and emission was recorded from 300 nm to 400 nm. Protein samples at the concentration of 0.1 mg/ml in 50 mM potassium phosphate buffer (pH 7.4) were used for the measurement as described in a previous study [11].

2.5.3. Equilibrium unfolding

Human γD-crystallin WT and mutants (1 mg/ml) were diluted into serial concentrations of guanidinium chloride and incubated overnight at 37 °C. The intrinsic tryptophan fluorescence spectra were recorded with Tecan fluorimeter (Tecan Trading AG, Switzerland), and the ratio of fluorescence intensities at 360 and 320 nm was used as a marker of the protein folding state. These wavelengths were chosen because they represent the emission peaks of unfolded and natively folded γD,