**Evaluation of structure, chaperone-like activity and protective ability of peroxynitrite
modified human α -Crystallin subunits against copper-mediated ascorbic acid oxidation**

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Abstract

The copper-catalyzed oxidation of ascorbic acid (ASA) to dehydroascorbate (DHA) and hydrogen peroxide plays a central role in pathology of cataract diseases during ageing and in diabetic patients. In the current study, the structural feature, chaperone-like activity and protective ability of peroxynitrite (PON) modified α A- and α B-Crystallin (Cry) against copper-mediated ASA oxidation were studied using different spectroscopic measurements and gel mobility shift assay. Upon PON modification, additional to protein structural alteration, the contents of nitrotyrosine, nitrotryptophan, dityrosine and carbonyl groups were significantly increased. Moreover, α B-Cry demonstrates significantly larger capacity for PON modification than α A-Cry. Also, based on the extent of PON modification, these proteins may display an improved chaperone-like activity and enhanced protective ability against copper-mediated ASA oxidation. In the presence of copper ions, chaperone-like activity of both native and PON-modified α -Cry subunits were appreciably improved. Additionally, binding of copper ions to native and PON-modified proteins results in the significant reduction of their solvent exposed hydrophobic patches. Overall, the increase in chaperone-like activity/ASA protective ability of PON-modified α -Cry and additional enhancement of its chaperoning action with copper ions appear to be an important defense mechanism offered by this protein.

Keywords: α -Crystallin; Copper ion; Peroxynitrite; Ascorbic acid, Chaperone activity.

1. Introduction

As highly abundant chaperone-like proteins present in eye lens, α A- and α B-Crystallin (Cry) rescue misfolded proteins from irreversible aggregation in the lenticular tissues [1]. These highly stable and long-lived proteins belong to the small heat shock protein family and perform both structural and refractive functions [2]. They exist in both homo- and hetero-oligomers with a dynamic quaternary structure which is important for their chaperone-like activity [3-5]. While α A-Cry is principally expressed in eye lens, the expression of α B-Cry has been detected in several non-lenticular tissues including heart, muscle, kidney and brain [6]. The upregulation of α B-Cry has been reported in the different pathological states such as ischemic heart, multiple sclerosis, Alzheimer, Parkinson and Alexander's disease [7]. Additionally, this protein has been suggested to be an important therapeutic target in cancer pathology [8,9]. Due to their limited turnover, across the life span, various chemical modifications are accumulated on α A- and α B-Cry which eventually alter their structures and functions.

As the main components of eye antioxidant defense system, ascorbic acid (ASA) and glutathione (GSH) play important function to protect lens crystallins against oxidative damages [10,11]. However, in the presence of increased lenticular level of copper ions, as indicated with age and in the cataractous lenses, a significant portion of ASA is converted into dehydroascorbate (DHA) and hydrogen peroxide (H_2O_2) (**Scheme 1**) [12-16]. Also, under GSH deficiency or copper ion excess, the rapid formation of DHA, possessing highly reactive carbonyl groups, may result in the fast ascorbylation of lens crystallins. This modification alters the short-range interactions between different classes of lens crystallins, leading to protein aggregation, light scattering and eventually to the senile cataract formation [17]. On the other hand, the reaction of lens proteins with H_2O_2 , generating either during ASA oxidation or from

superoxide radical, may be responsible for the deleterious alteration of their structures [17,18]. Additionally, the modification of lens proteins with the hydroxyl radical derived from H₂O₂ via copper ion catalyzed Fenton reaction may play a role in the development of the nuclear cataract (Scheme 1) [19,20].

► Scheme 1 to be inserted here ◀

As indicated in **Scheme 1**, during hyperglycemia in dietetic patients and under inflammatory conditions, a significant amount of super oxide and nitric oxide (NO[•]) can be produced in eye lenses. Nitric oxide is produced by the activity of two constitutive nitric oxide synthases (nNOS and eNOS) and one inducible enzyme counterpart (iNOS), and these three NOS isoforms have been identified in different parts of the eye [21]. Also, there are several oxidases in body, acting to form superoxide anion from molecular oxygen. In addition, xanthine oxidase (XOD), NADPH oxidase and uncoupled eNOS have been recently identified to participate in the lenticular generation of superoxide anion [22]. The rapid reaction between these two radicals, super oxide and nitric oxide, results in generation of peroxynitrite (PON) which is a powerful oxidative and nitrating agent [23]. As reported previously, PON can damage a broad range of various biomolecules including proteins [24]. The PON-induced modification and aggregation of lens crystallins has been speculated in the etiology of the cataract diseases [25]. In addition, PON reduces the lenticular level of GSH which subsequently can alter the equilibrium of ASA oxidation in the favor of DHA formation [26, 27].

In the lenticular tissues, metallothioneins serve for detoxifying copper ions [28]. Recently, a similar function has been attributed to α -Cry by which prevents the copper-mediated ASA oxidation [29, 30]. The copper sequestration ability, known as redox silencing activity, of α -Cry seems to be an important defense mechanism provided by this chaperone molecule. In

addition, copper ions are capable to stimulate the expression of α -Cry gene [31] (**Scheme 1**). Moreover, interaction of copper ions with α -Cry is important in modulation of both structure and chaperone-like activity of this protein [29, 32]. In the present study, we have addressed the protective action of native and PON-modified α A- and α B-Cry in a copper-mediated ASA oxidation system. Also, structure and chaperone functional aspect of these recombinant proteins and their PON-modified counterparts were evaluated in the presence of copper ions.

2. Materials and methods

2.1. Materials

Bovine pancreatic insulin, diethylaminoethyl (DEAE)-cellulose, Q-Sepharose, Sephacryl S-300 HR, isopropyl- β -D-1-thiogalactopyranoside (IPTG), 2,4-dinitrophenyl hydrazine (DNPH), 1-anilino-8-naphthalene sulfonate (ANS), thioflavin T (ThT) and other chemicals were purchased from Sigma-Aldrich chemical company.

2.2. Methods

2.2.1. Expression and purification of recombinant α A- and α B-Cry

The cDNA of human recombinant α A- and α B-Cry subunits which cloned into the bacterial vector pET-28 (b+) was expressed in *Escherichia coli* BL21 (DE3). The expression was induced by 0.25 mM IPTG when the optical density at 600 nm of the cell culture reached 0.6. The incubation was done in LB broth at 37 °C and the cells were harvested for 16 h after induction. Then bacterial cell pellets were centrifuged at 5000 $\times$ g for 20 min at 4 °C. The resulting pellets were resuspended in 25 mM Tris buffer, pH 7.2, containing 5 mM EDTA, 10 mM β -mercaptoethanol (β -ME), 100 mM NaCl and 0.01% NaN₃ (lysis buffer) and lysed by

sonication. The bacterial lysates were centrifuged at $8600 \times g$ for 40 min at 4°C and the supernatant dialyzed against 50 mM sodium phosphate buffer, pH 6.5. Then the protein sample was loaded on a DEAE-cellulose (0.8×15 cm) anion exchange column which pre-equilibrated with the same buffer at 4°C . The protein fractions were collected at a flow rate of 1 mL min^{-1} in the presence of 0.05–0.4 M sodium phosphate buffer with a fraction size of 2 mL. The protein concentration was determined with Bradford assay and protein purity was assessed by SDS-PAGE (12% acrylamide). The highly purified fractions were collected and dialyzed overnight at 4°C in 25 mM Tris buffer, pH 8.0, containing 0.5 mM EDTA, 10 mM β -ME and 0.01% NaN_3 . The dialyzed sample was then applied onto a Q-Sepharose (12.5×0.5 cm) anion exchange column which pre-equilibrated with the same buffer at 4°C . The protein fractions were collected at a flow rate of 1 mL min^{-1} with a fraction size of 2 mL. The bound proteins were eluted with a 0–0.5 M NaCl gradient. The protein fractions were pooled and dialyzed against 25 mM Tris buffer, pH 8.0, containing 0.1 M NaCl, 0.5 mM EDTA, 10 mM β -ME and 0.01% NaN_3 . The concentrated protein samples were then applied onto a Sephacryl S-300 HR gel filtration column (1.5×100 cm) that pre-equilibrated with the same buffer (4°C , flow rate 0.25 mL min^{-1} , fraction size 2 mL) [33–35]. The purity of the recombinant αA - and αB -Cry were confirmed with SDS-PAGE (12% gel). At the end, the highly purified protein fractions were collected and dialyzed against double distilled water and stored at -20°C until further use.

2.2.2. PON-modification of recombinant αA - and αB -Cry

Synthesis of PON was performed by reacting ice-cold solution of sodium nitrite (0.6 M) with H_2O_2 (0.7 M) in acidic solution (0.6 M HCl) and rapidly quenching the reaction in NaOH (1.5 M), as described previously [36]. Then, the reaction mixture was frozen at -20°C and the

upper layer was collected as the concentrated PON. Before each use, the concentration of stored PON was determined using molar extinction coefficient of $1670 \text{ M}^{-1} \text{ cm}^{-1}$ at 302 nm. Then, α A- and α B-Cry (2 mg mL^{-1}) were individually incubated in the absence and presence of 1, 7 and 35 mM PON at room temperature for 30 min. The experiment was done in 50 mM sodium phosphate buffer, pH 7.4, containing 10 mM HCO_3^- (Buffer A). At the end of incubation, the solutions were individually dialyzed against double distilled water to remove excess PON by using an appropriate dialysis tube (cutoff of 10,000 Da). The formation of nitrotyrosine by PON was examined using absorption spectrophotometry at 428 nm (ϵ at 428 nm: $4200 \text{ M}^{-1} \text{ cm}^{-1}$). Also, the absorbance of nitrotryptophan at 400 nm (ϵ at 400 nm: $5200 \text{ M}^{-1} \text{ cm}^{-1}$) and dityrosine at 330 nm (ϵ at 330 nm: $4000 \text{ M}^{-1} \text{ cm}^{-1}$) were measured.

2.2.3. Carbonyl assay

Carbonyl contents of native and PON-modified α A- and α B-Cry were determined after the reaction between this reactive group and DNPH, according to previously published protocol [25]. The final absorbance was read at 360 nm and the carbonyl content was determined using a molar extinction coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol mg}^{-1} \text{ protein}$ [37].

2.2.4. Protective ability of α -Cry samples in Cu^{2+} -mediated ASA oxidation system

The solutions containing $300 \text{ }\mu\text{M}$ ASA were incubated with a fixed concentration of either native or PON-modified α A- and α B-Cry ($60 \text{ }\mu\text{g mL}^{-1}$) and increasing concentrations of copper ions ($0\text{--}30 \text{ }\mu\text{M}$). The incubations were done at $25 \text{ }^\circ\text{C}$ for 30 min in 50 mM sodium phosphate buffer, pH 7.2 (Buffer B). Then, the absorbance of ASA was recorded at 260 nm as a function of time, during 20 min at $25 \text{ }^\circ\text{C}$ with a T90⁺ UV-Vis spectrophotometer instrument (PG

Instrument Ltd., UK) equipped with Peltier Temperature Controller (Model PCT-2). The percentage of ASA oxidation was quantified using the following equation:

$$\%Oxidation = \left(1 - \frac{A}{A_0}\right) \times 100 \quad (1)$$

where A_0 and A respectively stand for the optical density of ASA at 260 nm in the absence and presence of copper ions. Also, the protective ability of native and PON-modified α A- and α B-Cry against copper-mediated ASA oxidation was evaluated with a fixed concentration of copper ions (4 μ M) and increasing concentrations of each protein (0–100 μ g mL⁻¹). The experiment was done in a similar manner as mentioned above. The protective ability of these proteins was quantified using the following equation:

$$\%Protection = \left(\frac{A}{A_0}\right) \times 100 \quad (2)$$

In this equation, A_0 and A respectively represent the values of optical density of ASA at 260 nm in the absence and presence of protein.

2.2.5. The assessment of chaperone-like activity

The chaperone-like activity (anti-aggregation ability) of native and PON-modified α -Cry subunits was measured with bovine pancreatic insulin as a target protein [38]. Aggregation of insulin (0.3 mg mL⁻¹) in Buffer B was induced with 20 mM dithiothreitol (DTT) at 40 °C in the absence and the presence of native and PON-modified α A- and α B-Cry (0.05, 0.1 and 0.2 mg mL⁻¹). The progress of aggregation was recorded by light scattering at 360 nm. Also, the anti-aggregation ability of protein samples in the absence and the presence of Cu²⁺ (15 μ M) was

recorded. Additionally, the chaperone-like activity was expressed in the terms of the percentage of protection using following equation:

$$\%Protection = \left(1 - Ar / Ar_0\right) \times 100 \quad (3)$$

In this equation, Ar_0 and Ar are the area under the curves of optical density versus time (after 30 min of the incubation) in the absence and presence of chaperone, respectively. The area under aggregation curves reflects the combined action of three important parameters which are normally used for the quantification of chaperone-like activity. These parameters include A_{lim} (the limiting value of absorbance at $t \rightarrow \infty$), the product $k_1 A_{lim}$ (the initial rate of aggregation, where k_1 is the rate constant of the first order [39]) and t_{onset} (the lag phase before initiation of aggregation).

2.2.6. The fluorescence measurements

Intrinsic fluorescence study

Dityrosine formation was confirmed by recording the fluorescence spectra after excitation of native and PON-modified αA - and αB -Cry (0.15 mg mL^{-1}) in Buffer A, at 320 nm. The slit bandwidths of excitation and emission channel were 10 and 20 nm, respectively. Also, Tyr and Trp fluorescence spectra of the protein samples (0.15 mg mL^{-1}) were monitored after excitation at 280 nm and 295 nm, respectively [40,41]. The slit bandwidths were fixed at 10 nm in both channels. The fluorescence measurements were done at 25 °C in buffer B and the protein samples were titrated with increasing concentrations of Cu^{2+} (0–300 μM). The dependence of fluorescence intensity of different α -Cry samples on the concentration of Cu^{2+} -ions was assessed

at 337 nm in the absence and presence of different concentrations of Cu^{2+} . Approaches to analysis of fluorescence titration data are given in Supplementary data [42].

ANS fluorescence study

The native and PON-modified αA - and αB -Cry (0.15 mg mL^{-1}) were incubated in Buffer B, with $100 \text{ }\mu\text{M}$ ANS at $37 \text{ }^{\circ}\text{C}$ for 20 min. The ANS fluorescence spectra were collected by titrating of the mixture with increasing concentration of Cu^{2+} ($0\text{--}30 \text{ }\mu\text{M}$). The ANS fluorescence was recorded between $400\text{--}600 \text{ nm}$ with excitation wavelength at 365 nm and slit bandwidths of 10 nm in both channels [43,30].

Thioflavin T fluorescence study

The native and PON-modified αA - and αB -Cry (1 mg mL^{-1}) were incubated in Buffer B at $60 \text{ }^{\circ}\text{C}$ for 2 hours in the absence and presence of $15 \text{ }\mu\text{M}$ Cu^{2+} . At the end, the protein samples were diluted to a final concentration of 0.15 mg mL^{-1} in the same buffer and incubated with $20 \text{ }\mu\text{M}$ ThT solution for 5 min at room temperature. The protein samples were excited at 440 nm and the ThT fluorescence emission spectra were collected between 450 and 600 nm . Also, the slit bandwidths in both channels were fixed at 10 nm [43].

2.2.7. Far UV-CD measurements

The far UV-CD spectra of native and PON-modified αA - and αB -Cry (0.2 mg mL^{-1}) in Buffer B were recorded with a CD spectrophotometer instrument (Aviv, Model 215, USA) at $25 \text{ }^{\circ}\text{C}$ in a path length quartz cell from 190 to 260 nm . The experiments were done in the absence and presence of $50 \text{ }\mu\text{M}$ Cu^{2+} . The results were converted to molar ellipticity $[\theta]$ and expressed as

deg cm² dmol⁻¹ based on a mean amino acid residue weight (MRW). The molar ellipticity was calculated using the following equation:

$$[\theta] = \left(\frac{100MRW}{Cnl} \theta_{\lambda} \right) \quad (4)$$

where θ_{λ} is the observed ellipticity in milli degrees at a given wavelength, n is the number of amino acid residues (173 and 175 for α A- and α B-Cry, respectively), l is the path length of the cell (cm) and C is the protein concentration (mM) [44]. Also, the CD spectrum of Buffer B was subtracted from each recorded spectrum of protein sample.

2.2.8. Gel electrophoresis

To detect the formation of high molecular weight (HMW) protein aggregates, the native and PON-modified α A-Cry subunits were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing (sample buffer without β -ME) and reducing (with β -ME) conditions on a 12% polyacrylamide separating gel. Also, 10 μ g of each protein sample were loaded into wells of gel and the protein bands were visualized by the Coomassie Brilliant Blue (CBB) staining method [45].

2.2.9. DLS measurements

Dynamic light scattering (DLS) experiments were carried out with a Nanotracs Wave (Microtrac) dynamic light scattering instrument at 25 °C with protein concentration of 2 mg mL⁻¹ using a laser wavelength of 780 nm and a scattering angle of 90°. DLS data were analyzed by Microtrac FLEX operating software [46].

2.2.10. Protein assay and statistical analysis

The concentrations of proteins (insulin, α A- and α B-Cry) were measured from their corresponding UV absorption spectra on a T90⁺ UV-Vis spectrophotometer instrument. The concentration of insulin was determined using an extinction coefficient of 1.08 for 1 mg mL⁻¹ at 276 nm. Also, the extinction coefficients of 0.725 and 0.693 at 280 nm were used, respectively, for 1 mg mL⁻¹ of α A- and α B-Cry [29, 47].

To characterize the degree of agreement between the experimental data and calculated values, we used the coefficient of determination R^2 (without considering the statistical weight of the measurement results) [48]. Also, origin Pro 8.0 SR0 software (OriginLab Corporation, USA) was used for the calculations.

3. Results and Discussion

3.1. The analysis of PON modification of human recombinant α A- and α B-Cry

Since PON generation in eye ball seems to be an important molecular event, underlying pathomechanism of cataract diseases, in the current study both human α A- and α B-Cry were individually subjected to modification by this highly reactive oxidative agent. To achieve a diverse extent of PON modification, the protein samples were individually incubated with different concentrations of PON (0, 1, 7 and 35 mM) for 30 min at room temperature. The PON-modified proteins were subsequently analyzed by electrophoretic mobility shift assay (EMSA) and spectroscopic assessments (**Figs. 1 and 2**). As shown in **Fig. 1**, upon PON modification of the recombinant α -Cry subunits, the new bands corresponding to HMW protein aggregates were

appeared. The formation of new protein bands was accompanied with a significant reduction in the intensity of native/monomeric form of these proteins. **Fig. 1a** and **1c** respectively indicate the EMSA of α A- and α B-Cry under both non-reducing and reducing conditions. As shown, no significant variation on the basis of either pattern or intensity of the protein bands can be seen between the protein samples which subjected to the reducing and non-reducing electrophoresis. These results can be explained with the involvement of dityrosine cross-linking in structure of the modified proteins, forming HMW protein aggregates. Additionally, based on results of EMSA, α B-Cry demonstrates more intense HMW bands than α A-Cry protein counterpart. Cross-linking of lens α B-Cry which alters its structure and interaction has been already implicated in pathomechanism of cataract disorders [49,50]. Additionally, ROS-induced formation of dityrosine cross-links has been suggested to play a significant role in the pathogenesis of Alzheimer's disease [40,50]. According to the intensity of HMW protein bands it can be suggested that α B-Cry is potentially more vulnerable to PON modification than α A-Cry.

► **Fig. 1 to be inserted here** ◀

The carbonyl content (carbonyl stress) is the most commonly used biomarker of protein oxidation which increases when protein exposed to the oxidative agents such as PON and also during non-enzymatic glycation under chronic hyperglycemia [51,52]. The carbonyl content of α A- and α B-Cry was examined in the presence of DNPH (**Figs. 1b** and **1d**). Native forms of α A- and α B-Cry demonstrate carbonyl contents of 1.5 ± 0.0 and 2.6 ± 0.4 nmol mg⁻¹ proteins, respectively. Upon modification with 7 mM PON, the carbonyl content of α A- and α B-Cry would increase to 4.2 ± 0.1 and 9.0 ± 0.4 , nmol mg⁻¹ proteins, respectively. The significant

enhancement in carbonyl content of PON-modified α -Cry suggests that this oxidative agent causes extensive oxidation of the recombinant proteins which may subsequently alter their structural properties. Additionally, in the presence of 7 mM PON, the carbonyl contents in α A- and α B-Cry increase to 2.8 and 3.4 folds of their initial values in the native protein counterparts. In the same way, the extent of modification of α B-Cry in the presence of 1 and 35 mM PON is appreciably larger than α A-Cry. The protein carbonyls can result from either direct oxidation of various amino acids such as lysine, arginine, histidine, proline, glutamic acid and threonine or oxidative cleavage of the protein backbone [51,53]. These results suggest a marked increase in the carbonyl contents of α B-Cry after PON modification which can be explained with the numbers and/or accessibility of the target residues in this protein. The increases in the carbonyl content of these proteins may also enhance their susceptibility for subsequent structural alteration and cross-linking. The chemical modification of human recombinant α -Cry was further evaluated by Trp, Tyr and dityrosine fluorescence assessments. In their primary structures, α A- and α B-Cry contain 1 and 2 Trp, as well as 6 and 2 Tyr, respectively [54, 55]. Upon modification with 7 mM PON, Trp fluorescence emission intensities of α A- and α B-Cry were significantly reduced compared to their native protein counterparts (**Fig. 2a**). The reduction in fluorescence emission intensity can be due to the conversion of Trp residues to the nitrotryptophan and/or protein structural alteration upon PON modification. Moreover, the extent of reduction was larger in the case of α B-Cry compared to α A-protein counterpart. The conversion of Tyr to dityrosine upon PON modification was confirmed with the excitation of Tyr residues at 280 nm (**Fig. 2b**) and dityrosines at 320 nm (**Fig. 2c**). As a result of PON modification, a significant reduction in Tyr fluorescence (**Fig. 2b**) and a major increment in fluorescence spectra of dityrosine (**Fig. 2c**) of recombinant α A/ α B-Cry were observed. These

findings suggest nitration of Tyr residues and a significant conversion of these residues to dityrosine. Also, the obtained results are in well agreement with those of EMSA which suggested the existence of dityrosine protein cross-linking in the PON-modified samples of α A- and α B-Cry.

► Fig. 2 to be inserted here ◀

The structural changes and solvent accessible hydrophobic surface in PON-modified and native proteins were studied by ANS binding analysis (**Fig.2d**). As shown, the increased ANS fluorescence intensity of PON-modified α A-Cry suggests the enhancement of protein hydrophobic surfaces. On the contrary, the ANS fluorescence intensity of PON-modified α B-Cry was significantly reduced compared to the native protein counterpart, showing reduction of the protein surface hydrophobic patches. The results indicate that α A- and α B-Cry may adopt different folding variants upon PON modification which are important in the terms of their ability to perform their intrinsic functions in the eye lens. The ThT dye which is often used as a diagnostic of amyloid structure was also used to investigate possible formation of amyloid entities by the PON-modified recombinant proteins. As shown in **Fig. 2e**, ThT fluorescence intensity of both PON-modified α A- and α B-Cry were reduced compared to the native protein samples which may indicate the absence of amyloid entities. As shown in **Fig. 2f**, the absorbance profile of PON-modified proteins shows a new peak with the maximum intensity at 428 nm, suggesting the formation of new chromophore, 3-nitrotyrosine. The results of deconvolution are not presented in the paper because this deconvolution is a routine procedure [56].

Also, the generation of yellow color in samples of PON-modified proteins, with absorption peak at 428 nm, indicates the formation of nitrotyrosine. In addition, the content of

nitrotyrosine, nitrotryptophan and dityrosine in PON-modified α A-Cry sample was calculated spectrophotometrically to be 10.2 ± 1.9 , 9.9 ± 3.2 , 12.7 ± 1.7 nmol mg⁻¹ proteins, respectively. Accordingly, the contents of these chromophores in PON-modified α B-Cry were 19.3 ± 2.1 , 11.3 ± 1.5 , 15.0 ± 2.0 nmol mg⁻¹ proteins. Overall, the result of EMSA and spectroscopic measurement (**Figs. 1 and 2**) demonstrate that PON can induce a collection of significant modifications in the recombinant proteins, specifically Tyr and Trp nitration, dityrosine cross-linking and significant increase in the protein carbonyl contents. Additionally, our results suggest an important level of PON modification of the recombinant proteins and the modification extent was significantly larger in the case of α B-Cry compared to α A-protein counterpart.

3.2. The PON-modified α -Cry subunits improve their protective ability against copper-mediated ASA oxidation

Oxidative stress has been implicated in the etiology of a large number of human long-term degenerative diseases, including cataract. Additionally, there is a growing body of evidences pointing out that ASA oxidation can contribute to the development of cataract formation [14,17]. The copper-mediated oxidation of ASA has been already reported in the lenticular tissues [57]. Therefore, we have studied the effects of Cu²⁺ on oxidation of ASA (300 μ M) in the presence of different concentrations of native and PON-modified recombinant α -Cry subunits. The copper-mediated ASA oxidation was monitored as a function of time by recording the optical density change at 260 nm (see Fig.1 in Ref [42]). The experiments were done with the protein samples which have been previously incubated with different PON concentrations as 0, 1, 7 and 35 mM for 30 min at room temperature. The ASA oxidation was monitored in the

presence of a fixed concentration of each α -Cry subunit ($60 \mu\text{g mL}^{-1}$) and different concentrations of copper ions for 20 min (see **Fig. 1** in Ref. [42]). Using Eq. (1), the absorbance values at $t = 10$ min were used to plot the percentage of ASA oxidation versus concentration of copper ion. Then, the resulted curves were fitted according to a sigmoid equation and indicated as **Fig. 3**.

► **Fig. 3 to be inserted here** ◄

While upper panel (**Figs. 3a, b and c**) indicates the percentage of ASA oxidation in the presence of α A-Cry, the corresponding data in the lower panel (**Figs. 3a', b' and c'**) were obtained in the presence of α B-Cry. As indicated in these figures, the percentage of ASA oxidation is significantly lower in the presence of PON-modified α -Cry subunits compared to their native protein counterparts. These results suggest that PON modification of recombinant human α -Cry subunits may enhance their ASA protection abilities in a copper-mediated oxidation system. However, as shown in **Fig. 3**, the maximum quantity of protection was observed for the moderately modified α -Cry subunits which have been incubated with 7 mM PON. It seems that the extents of PON modification might be an important parameter, contributing in their protective ability against copper induced-ASA oxidation. Also, in this study the experiments were repeated in the presence of a fixed concentration of copper ion ($4 \mu\text{M}$) and increasing concentrations of each α -Cry subunit (see **Fig. 2** in Ref. [42]). Using equation 2, the absorbance values at $t = 10$ min were used to plot the percentage of protection against protein concentration and the resulted curves were fitted according to a sigmoid equation, as indicated in **Fig. 4**.

► **Fig. 4 to be inserted here** ◄

The upper and lower panels respectively indicate the protection ability of α A-Cry and α B-Cry against copper-mediated ASA oxidation. Both proteins demonstrate a dose-dependent protection activity against ASA oxidation (**Fig. 4**). Additionally, the PON-modified α A-Cry and α B-Cry indicate significantly larger protection than their native protein counterparts. The results of this study provide further indications, confirming that PON-modified α -Cry subunits are more efficient against Cu^{2+} -mediated ASA oxidation than native protein counterparts. Also, the different protection abilities of α A- and α B-Cry can be explained with the extent of PON modification which results in formation of dissimilar folding variants, displaying different functional properties.

3.3. Evaluation of chaperone-like activity of PON-modified α -Cry subunits in the presence of copper ions

Since α -Cry plays an important role in the maintenance of lens transparency, chaperone-like activity (anti-aggregation ability) of the PON-modified α -Cry subunits and their native protein counterparts was evaluated in the presence of bovine pancreatic insulin. The chaperone-like activities of native and modified α -Cry samples which have been already incubated with different concentrations of PON were examined and compared (**Fig. 5**). The aggregation of insulin was registered in a chemical-induced aggregation system with 20 mM DTT at 40 °C. Also, the chaperone-like activity of these proteins was expressed in the terms of their protection ability according to Eq. (3). As shown in **Fig. 5**, both native and PON-modified recombinant α -Cry subunits demonstrate a concentration-dependent chaperone-like activity.

► Fig. 5 to be inserted here ◀

As shown in **Fig. 5**, the moderately-modified α A-Cry, treated with 7 mM PON, displays significantly larger anti-aggregation activity than its native protein counterpart. Also, incubation with 1 and 35 mM PON, giving respectively low and extensively modified α A-Cry samples which indicate weaker chaperone-like activity than native protein. It seems that level of PON modification is critical for the enhancing of biological activity of this protein. Also, the results of chaperone-like activity of α B-Cry (native and PON-modified forms) are shown in **Fig. 5**. As shown in this figure, both native and PON-modified α B-Cry indicated concentration dependent chaperone-like activity. Moreover, in contrast to the results obtained for α A-Cry, the chaperone-like activity of PON-modified α B-Cry was significantly attenuated after modification with different concentrations of PON. Although, the role of surface hydrophobicity in chaperone-like activity of α -Cry is still a controversial matter, the chaperone-like ability of PON-modified α A- and α B-Cry might be correlated with the extent of their exposed hydrophobic surface which is important for the interaction with a client protein. As shown in **Fig. 2** and **Fig. 5**, the PON-modified α A- and α B-Cry display respectively higher and lower hydrophobic surfaces and chaperone-like activities than their native protein counterparts. Our results are not entirely compatible with those of previous study in which the chaperone-like activity of bovine α -Cry remained unchanged upon PON modification [58]. This discrepancy can be likely explained with different target residues in bovine and human α -Cry which are available for PON modification. Subsequently, the different degrees of PON modification induce different folding states in these proteins which explain their dissimilar chaperone activities.

Additionally, in the current study, the impact of copper ions on chaperone-like activity of native and PON-modified α -Cry subunits were investigated (**Fig. 6**). The α -Cry subunits which have been incubated with different concentrations of PON were used to examine the impact of copper ions on their chaperone-like activities. Similar to the native proteins, the chaperone-like activity of PON-modified α -Cry subunits was increased in the presence of copper ions.

► **Fig. 6 to be inserted here** ◄

However, the consequence of interaction of Cu^{2+} with α A- and α B-Cry and their corresponding modified forms are drastically different. Overall, chaperone-like activity of recombinant α -Cry subunits and their PON-modified forms toward the aggregation of insulin was significantly increased in the presence of copper ions.

3.4. Size distribution analysis of α -Cry upon PON modification and in the presence of copper ions

α -Cry populates a range of interconverting oligomeric states which may influence the chaperoning action of this protein. The size distribution analysis was performed for α A- and α B-Cry after PON modification, and as a result of their interaction with copper ions (**Fig. 7**). Modification of these proteins with 1 and 7 mM PON leads to only slight changes in their size distributions. However, in the presence of 35 mM PON a marked increment in the size distribution of both proteins (**Fig. 7**) and a significant reduction in their chaperone-like activities were observed (**Fig. 6**).

► **Fig. 7 to be inserted here** ◄

Also, the size distributions of both native and PON-modified proteins were assessed after interaction with copper ions (insets of **Fig.7**). According to our results, after interaction with this transition metal, the main trend is a shift toward the lower size ranges. Therefore, the chaperone-like activity of these proteins might be inversely correlated with their oligomeric size distributions. The chaperone activity of α -Cry and other small heat shock proteins is linked to rate of a constant subunit exchange between their dimeric and oligomeric forms. While dimeric forms are considered to be chaperone active state, the oligomeric forms of this protein largely fail to perform the efficient chaperoning function [59, 60]. Previous study indicated that formation of HMW species by α -Cry affects the dynamic structure of this protein and decreases the rate of its subunit exchange [61]. Also the important role of subunit mobility in the chaperoning mechanism of α B-Cry has been already documented [62]. The enhancement of chaperone activity of this protein after moderate PON modification (**Fig. 5**) and during interaction with copper ions (**Fig. 6**) can be explained with the reduction in the oligomeric size distribution and subsequent increment in the subunit exchange [61]. This condition likely increases the quantity of chaperone active dimeric forms, reflecting with the enhancement in chaperoning function of this protein.

3.5. Structural analysis of PON-modified α -Cry upon interaction with copper ions

Previous studies have shown that both α A- and α B-Cry interact with copper ion and such interaction is important in modulating their structure and chaperone-like function [29,32]. Additionally, the concentration of copper ions has been indicated to elevate significantly in the cataractous lenses [63,64]. We have investigated the effect of Cu^{2+} on the structure of native and the moderately PON-modified α A- and α B-Cry. As shown in **Fig. 8**, titration of lens crystallins with the increasing concentrations of Cu^{2+} clues to quenching of their Trp fluorescence. The

dependences of fluorescence intensity of native and PON-modified α A- and α B-Cry on the different concentrations of Cu^{2+} -ions are presented as insets in panels' a-d of **Fig. 8**. When analyzing the titration data, it was assumed that native and PON-modified α A-Cry contained two equivalent Cu^{2+} -binding sites per subunit with the dissociation constants of 9.7 ± 0.7 and 17 ± 2 μM , respectively, whereas native and PON-modified α B-Cry contains two non-equivalent Cu^{2+} -binding sites per subunit with the dissociation constants of 0.94 ± 0.19 and 36 ± 3 μM (native α B-Cry) and 4.3 ± 0.9 and 70 ± 8 μM (PON-modified α B-Cry) (see supplementary data [42]). The comparison of the dissociation constants shows that modification results in the decrease in the affinity of α A-Cry and α B-Cry to Cu^{2+} -ions. α A-Cry and α B-Cry contain, respectively, 7 and 9 His residues which are potentially anchor sites for copper binding. It seems that reduced copper affinity of these proteins is interrelated with their degree of PON modification and consistent with their biological activities such as chaperone-like activity and protective ability against copper-mediated ASA oxidation.

► **Fig. 8 to be inserted here** ◀

Also, Trp fluorescence study is indicative of the structural alteration of native and PON-modified proteins. As shown in **Fig. 8**, Trp fluorescence emission intensity of both PON-modified and native proteins are significantly reduced in the presence of increasing concentrations of copper ion, suggesting movement of these residues to more polar microenvironment. In fact, the results suggest structural alteration of both native and PON-modified α -Cry upon interaction with the copper ions. Additionally, based on variation in the extent of Trp fluorescence intensities, it can be suggested that copper-induced emission properties were more pronounced in the case of native α A/ α B-Cry than their PON-modified protein counterparts. The obtained results can be

explained with the modification of Trp residues to nitrotryptophan which alters their emission characteristic as indicated in **Fig. 2**. To gain further insights into the structural alteration of the recombinant α -Cry subunits upon PON modification and during interaction with copper ions, ANS fluorescence analysis was performed. ANS binds to the external hydrophobic patches of proteins and due to such binding the fluorescence emission intensity increases with concomitant blue shift of the emission maximum [65]. As shown in **Fig. 9**, the binding of copper ions to native and moderately PON-modified α A- and α B-Cry results in the reduction of ANS binding to these proteins, suggesting that such binding reduces the protein's external hydrophobic patches. Upon binding to the copper ions, the reduction of hydrophobic surfaces was less pronounced in the case of α A-Cry compared to α B-Cry. This result may explain different chaperone-like activities of α A- and α B-Cry in the presence of increasing concentrations of copper ions (**Fig. 6**). Additionally, native recombinant α -Cry is more susceptible to copper-induced structural changes than their PON-modified forms. This finding may also suggest that copper ions induce different folding variants in these proteins.

► **Fig. 9 to be inserted here** ◄

Also, far UV-CD spectra were collected in the range of 200-260 nm with the aim to analyze changes in the secondary structure contents of α A- and α B-Cry upon PON modification and as a result of interaction with copper ions (**Fig. 10**).

► **Fig. 10 to be inserted here** ◄

As shown in this figure, PON modification induces significant changes in the secondary structure contents of these recombinant proteins. As a result of PON modification, a significant transitional switch from β -sheet to other structures was observed for both α A- and α B-Cry (**Fig.**

10). Additionally, in the presence of Cu^{2+} , both native and PON-modified proteins demonstrate minor alteration in the secondary structure contents. The effect of copper ions on secondary structures was slightly higher in the case of PON-modified proteins compared to their corresponding native forms (**Fig. 10**). In conclusion, while PON modification exerts a drastic effect on the secondary structure content of $\alpha\text{A}/\alpha\text{B-Cry}$, only slight changes were observed as a result of interaction of copper ions with these proteins.

3.6. The reduction of ThT fluorescence intensity of $\alpha\text{-Cry}$ in the presence of Cu^{2+}

Previous study indicated that thermal stress can initiate the pathway of amyloidogenesis in lens crystallins [66]. In the current study, recombinant human $\alpha\text{A-}$ and $\alpha\text{B-Cry}$, as well as their PON-modified forms were subjected to thermal stress at 60 °C for 2 hours in the absence and presence of 15 μM Cu^{2+} (**Fig. 11**). The ThT fluorescence experiment which has been already applied under amyloidogenic conditions to diagnose the existence of protein amyloid-like entities was used in this study. As a result of thermal stress, native forms of these proteins demonstrate a significant increment in ThT fluorescence emissions compared to their non-heated protein sample. These observations can be likely explained with the formation of amyloid-like entities in the presence of thermal stress.

► **Fig. 11 to be inserted here** ◀

However, in the presence of copper ions the intensity of ThT fluorescence indicates a significant reduction compared to the non-heated protein sample. This effect can be explained with the specific impact of copper ions on structure of these proteins (**Fig. 10**). On the other hand, the PON-modified $\alpha\text{A}/\alpha\text{B-Cry}$ demonstrated significantly lower fluorescence intensities compared to their native protein counterparts (**Fig. 8**). Moreover, a similar trend was observed

for ThT fluorescence emission intensities of PON-modified recombinant α A/ α B-Cry under thermal stress. Additionally, the impact of thermal stress on ThT fluorescence of PON-modified Cry was significantly lower than what observed for their native protein counterparts. These results may notify the important role of the specific amino acid residues which can be modified by PON in the amyloidogenic pathway of α A/ α B-Cry under thermal stress. Moreover, in the presence of copper ions, a slight increment and a reduction in the ThT fluorescence intensities were observed for PON-modified α A- and α B-Cry, respectively. According to the ThT fluorescence results, thermal stress may induce amyloidogenic pathway in both native and PON-modified α A- and α B-Cry and this pathway can be altered in the presence of copper ions.

Conclusions

Similar to nitric oxide, PON has been linked to both beneficial and deleterious effects. While low levels of this compound contributes in modulation of various intracellular signaling pathways and reduces the intraocular pressure, high level of PON exerts its damaging effects by altering protein structure and function via irreversible nitration of specific amino acid residues. As human recombinant α A- and α B-Cry were treated with different concentrations of PON, α B-Cry demonstrated significantly higher level of modification. Depending on the extent of PON modification, these proteins (α A/ α B-Cry) can achieve specific folding variants which may indicate improved chaperone-like activity and enhanced protective ability against copper-mediated ASA oxidation. Additionally, in the presence of copper ions both native and their PON-modified forms displayed enhanced chaperoning activity. The enhanced chaperoning action might be explained with the impact of copper ions on the oligomeric size distribution of these proteins. Increasing of chaperone-like activity/ASA protective ability of PON-modified α -

Cry and further enhancement of its chaperoning function in the presence of copper ions seems to be an important defense mechanism provided by this protein. These findings are particularly important in patients with diabetes mellitus and during ageing which are accompanied with significant elevation of copper ions and oxidative stress in the lenticular tissues. Additionally, the increment of biological activity of α -Cry after PON modification may suggest a new beneficial effect of PON, particularly in the ocular system.

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Figure legends

Scheme 1. The schematic representation of the role of copper ions in expression of α -Cry gene and oxidation of ascorbic acid.

A) Copper ions induce the expression of α -Cry gene. They catalyze the oxidation of ASA in the lenticular tissues. Also, the copper sequestration feature of α -Cry prevents the oxidation of ASA in eye lens, protecting eye ball from the deleterious properties of copper ion elevation in patients with diabetes mellitus and during ageing. B) The impact of PON on copper-mediated oxidation of lenticular ASA. The high levels of lenticular copper ions during ageing and in patients with diabetes mellitus accelerate the conversion of ASA into DHA and hydrogen peroxide which subsequently modify lens crystallins. Also, hydroxyl radical which is derived from H_2O_2 via copper ion catalyzed Fenton reaction can actively participate in modification of lens crystallins and indicates a role in development of the nuclear cataract. DHA can be converted back to ASA with reduced form of glutathione. Under pathological conditions such as diabetes and inflammation when the rate of PON generation is significantly elevated in eye ball, this oxidative agent may also participate in modification of lens crystallins which is one of the significant underlying mechanisms of the cataract development.

Fig.1. The mobility shift assay and carbonyl content analysis of native and PON-modified α A- and α B-Cry.

The SDS-PAGE analysis of α A- (a) and α B- (c) Cry (2 mg mL^{-1}) which incubated with different concentrations of PON at room temperature for 30 min. At the end of incubations $10 \text{ }\mu\text{g}$ of each protein sample was loaded onto the SDS-PAGE well under non-reducing and reducing conditions. While the electrophoresis was performed on a 12% SDS-PAGE gel, visualization of the protein bands was achieved using CBB staining protocol. The carbonyl content of native and PON-modified α A- (b) and α B-Cry (d) were studied. The bars indicate average carbonyl content ($\pm$ SD) of three independent assays. The carbonyl content was expressed as nmol mg^{-1} protein.

Fig. 2. Evaluation of the structural properties of native and PON-modified α A- and α B-Cry.

Different protein samples (2 mg mL^{-1}) incubated with 7 mM PON at room temperature for 30 min were used for the structural analysis. Native (thick line) and PON-modified α A/ α B-Cry (dotted line) were subjected to different spectroscopic assessments. Also, Blue and Red respectively stand for α A- and α B-Cry. a) Trp fluorescence analysis, excitation at 295 nm ; b) Tyr polarity assessment, excitation at 280 nm ; c) dityrosine fluorescence measurement, excitation at 320 nm ; d) ANS fluorescence study, excitation at 365 nm and e) ThT fluorescence measurement, excitation at 440 nm . Also, dashed line stands for the fluorescence emissions of ANS and ThT in the absence of protein. f) Absorption spectra of native and PON-modified proteins for nitrotyrosine diagnosis at 420 nm . Prior to the measurements, the modified proteins were centrifuged at $13,500 \times g$ for 30 min to remove any existed protein aggregates. All the measurements were done in Buffer A.

Fig. 3. Evaluation of copper mediated-ASA oxidation in the presence of native and PON-modified α -Cry subunits and increasing concentration of copper ions.

The protein samples which have been already incubated with different PON concentrations as 0, 1, 7 and 35 mM at room temperature for 30 min were used for the assessment of their protection abilities against ASA oxidation. The experiments were done in Buffer B (at 25 °C) in the presence of a fixed concentration of each α -Cry sample (60 $\mu\text{g mL}^{-1}$) and increasing concentrations of copper ions (0–30 μM) followed by absorbance of ASA (300 μM) at 260 nm for 20 min. In accordance with Eq. (1), the optical density at $t = 10$ min was used to plot the percentage of ASA oxidation against increasing concentration of copper ions.

Fig. 4. Evaluation of the protective ability against copper-mediated ASA oxidation in the presence of increasing concentrations of native and PON-modified α -Cry subunits.

The protein samples which have been previously incubated (at room temperature for 30 min) with different PON concentrations as 0, 1, 7 and 35 mM were used for the assessment of their protection abilities against ASA oxidation. The experiments were done in Buffer B (at 25 °C) in the presence of a fixed concentration of each α -Cry sample (60 $\mu\text{g mL}^{-1}$) and increasing concentrations of copper ions (0–30 μM) by monitoring the absorbance of ASA (300 μM) at 260 nm for 20 min. In accordance with Eq. (2), the optical density at $t = 10$ min was used to plot the percentage of protection against increasing concentrations of each protein. Also a, b and c are referred to α A-Cry and a', b' and c' stand for α B-Cry.

Fig. 5. Chaperone-like activity of native and PON-modified α A- and α B-Cry.

The protein samples which have been previously incubated at room temperature for 30 min, with different PON concentrations as 0, 1, 7 and 35 mM were used for the assessment of their chaperone-like activities. The aggregation of bovine pancreatic insulin (0.3 mg mL^{-1}) was induced in the presence of DTT (20 mM) in Buffer B at 40°C . The progress of aggregation was followed by monitoring the increment of light scattering at 360 nm in the presence of different concentrations of the chaperone as 0, 0.05, 0.1 and 0.2 mg mL^{-1} . Using Eq. (3), the chaperone-like activity was expressed in the terms of the percentage of protection which is also indicated in this figure.

Fig. 6. Effect of copper ions on the chaperone-like activity of native and PON-modified α A- and α B-Cry.

The protein samples which have been previously incubated at room temperature for 30 min with different PON concentrations as 0, 1, 7 and 35 mM were used for the assessment of their chaperone-like activities. The experiments were done in the absence and presence of $15 \text{ }\mu\text{M}$ Cu^{2+} and 0.1 mg mL^{-1} of each α -Cry chaperone. The aggregation of bovine pancreatic insulin (0.3 mg mL^{-1}) was induced in the presence of DTT (20 mM) in Buffer B at 40°C . The progress of aggregation was followed by monitoring the increment of light scattering at 360 nm. Also, Eq. (3) was used with the aim to express the chaperone-like activity in the terms of the percentage of protection.

Fig.7. DLS analysis of the protein size distribution.

DLS experiment was done using different α -Cry samples in buffer B. The plots indicate the relative volumes of oligomers with different sizes. The Insets indicate relative volumes of the protein oligomers in the presence of copper ions ($15 \text{ }\mu\text{M}$). Also, the horizontal axis shows the

distribution range of particle size in diameter (nm). The average size diameters of different samples are indicated in the corresponding graphs.

Fig. 8. Quenching study of Trp fluorescence of native and PON-modified α A- and α B-Cry.

Trp fluorescence spectra of native (**a, c**) and PON-modified (**b, d**) α A- (**a, b**) and α B- (**c, d**) Cry (0.15 mg mL⁻¹) were collected. The experiments were done in Buffer B at 25 °C in the absence and presence of different concentrations of Cu²⁺ (0–300 μ M). The Trp fluorescence emission spectra of each sample were collected between 300 and 500 nm with an excitation wavelength of 295 nm. Insets show the dependence of fluorescence intensity of α A-Cry (panel **a**), PON- α A-Cry (panel **b**), α B-Cry (panel **c**) and PON-modified α B-Cry (panel **d**) on the different concentrations of Cu²⁺ ions at 337 nm.

Fig. 9. ANS fluorescence study of native and PON-modified α A- and α B-Cry.

Native (**a, c**) and PON-modified (**b, d**) α A- (**a, b**) and α B- (**c, d**) Cry (0.15 mg mL⁻¹) were incubated in Buffer B with 100 μ M ANS at 37 °C for 20 min. Then, the ANS fluorescence spectra were collected by titrating the mixture with increasing concentration of Cu²⁺ (0–30 μ M) between 400–600 nm with an excitation wavelength of 365 nm.

Fig. 10. Far UV-CD analysis of native and PON-modified α A- and α B-Cry.

Far UV-CD spectra of native (thick and dotted lines) and PON-modified (dashed and dashed & dotted lines) α A- (**a**) and α B- (**b**) Cry (0.2 mg mL⁻¹) in Buffer B at 25 °C. The experiments were done in the absence and presence of 50 μ M Cu²⁺.

Fig. 11. ThT fluorescence assay of native and PON-modified α A- and α B-Cry.

Native **(a, c)** and PON-modified forms **(b, d)** of α A- **(a, b)** and α B- **(c, d)** Cry (1 mg mL^{-1}) were incubated in Buffer B at 60°C for 2 hours in the absence and presence of $15 \text{ }\mu\text{M Cu}^{2+}$. At the end, the protein samples were diluted to a final concentration of 0.15 mg mL^{-1} in the same buffer and ThT fluorescence was measured in the presence of $20 \text{ }\mu\text{M ThT}$. Also, the protein samples were excited at 440 nm and the ThT fluorescence emission spectra were collected between 450 and 600 nm .

Graphical abstract

Evaluation of structure, chaperone-like activity and protective ability of peroxynitrite modified human α -crystallin subunits against copper-mediated ascorbic acid oxidation.

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Scheme 1.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11