Conformational stability of bovine α -crystallin

Evidence for a destabilizing effect of ascorbate

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Short-term incubation of bovine α -crystallin with ascorbate alters the protein conformational stability. The denaturation curves with urea and guanidinium-chloride show different patterns, suggesting a deviation from a two-state mechanism owing to the presence of one or more intermediates in the unfolding of ascorbate-modified α -crystallin. Furthermore, the latter protein profiles are shifted to lower denaturant concentrations indicating a destabilizing action of ascorbate, which is capable of facilitating protein dissociation into subunits as demonstrated by gel filtration with 1.5 M-urea. The decrease in conformational stability cannot be ascribed to any major structural alteration, but rather to localized changes in the protein molecule. In fact, no difference between native and ascorbate-treated α -crystallin can be detected by amino acid analysis but perturbation of the tryptophan and tyrosine environment is indicated by alterations in intrinsic fluorescence. Furthermore, turbidity and light-scattering measurements suggest an involvement of the lysine side chains, since aggregatability patterns with acetylsalicylic acid are significantly altered. The ascorbate-destabilizing effect on the conformational stability of α -crystallin, probably exerted through oxidative modification of amino acid residues and/or the formation of covalent adducts, provokes unfavourable steric interactions between residues along the polypeptide chains, thus favouring aggregation and insolubilization of crystallins which can lead to cataract formation, as also demonstrated by proteolytic digestion patterns which show a lower rate of degradation of the ascorbate-modified α -crystallin.

INTRODUCTION

The lens of the vertebrate eye is a suitable model for the study of conformational alterations at the molecular level, since there is virtually no turnover and most changes observed in protein structure or conformation are due to post-translational modifications [1,2]. The unusually high ascorbic acid concentration in the lens [2,3] led to the consideration that it may also modulate some metabolic reactions non-enzymically, i.e. as a reducing agent [3]. However, the ascorbate system (ascorbate/oxygen/metals) has already been shown to inactivate enzymes [4–6] and to induce conformational and functional changes [7] in proteins through metal-catalysed oxidative reactions which produce oxygen radical species and mediate post-translational modifications [8,9]. These alterations have been found to occur at different rates [10]. Furthermore, it has been proved that ascorbate can undergo a Maillard reaction with lens protein amino groups, forming covalent adducts [11,12] and inducing non-disulphide covalent cross-links and the cleavage of crystallins [13–15].

If the role of ascorbate in chemical modification of lens protein is not in doubt, what, in our opinion, has not yet been studied extensively is the relationship between ascorbate-induced modification and the resulting changes in protein structural properties. Since there is growing interest in determining how small structural changes can alter conformational stability [16], we carried out a short-term incubation of α -crystallin with ascorbate to study the early steps of post-translational modification and to gain information about changes in the conformational stability of the lens protein.

EXPERIMENTAL

Chemicals

L(+)-Ascorbic acid, Tris and KCl were from Merck (Darmstadt, Germany). Electrophoresis-grade acrylamide was from Bio-Rad (Richmond, CA, U.S.A.). KI was from Carlo Erba (Milan, Italy). Urea, dithiothreitol and acetylsalicylic acid were from Sigma (St. Louis, MO, U.S.A.). Type XIV proteinase from *Streptomyces griseus* (Pronase E) was from Calbiochem (San Diego, CA, U.S.A.). High-purity guanidinium chloride (GdmCl) was from Pierce (Rockford, IL, U.S.A.). Sodium borohydride (99%) was from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were of the best quality commercially available. All solutions were prepared with highly purified water (resistivity = 18 Mohm·cm) obtained through a Milli-Q water-purification system (Millipore Corp., Bedford, MA, U.S.A.).

α -Crystallin purification

Calf lenses were homogenized in 10 mM-Tris/HCl buffer, pH 7.4, and centrifuged at 15000 *g* for 30 min to remove insoluble material. The supernatant, containing water-soluble proteins, was applied to a column (2.6 cm × 90 cm) of Sephacryl S200 SF (Pharmacia, Uppsala, Sweden), equilibrated with the same buffer. The eluting flow rate was 20 ml/h. Fractions of the first peak were pooled together and further chromatographed on a column (2.6 cm × 50 cm) of Bio-Gel A-5 (Bio-Rad) equilibrated with the same buffer, to remove the high-*M_r* proteins. The eluting flow rate was 15 ml/h. α -Crystallin fractions were singly pooled and thoroughly mixed. All experiments were performed at 4 °C.

Abbreviation used: GdmCl, guanidinium chloride.

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Purity was checked by SDS/PAGE, and two typical bands, representing the two subunits of α -crystallin [1], were obtained. Protein concentration was determined by the method of Bradford [17].

Ascorbate treatment

α -Crystallin was allowed to react with ascorbate in 50 mM-Tris/HCl buffer, pH 7.4, as described by Levine [18] as follows: α -crystallin (1.3 mg/ml) was incubated in aerobic conditions, at 37 °C, in the dark, with 100 mM-ascorbate, along with the respective control sample (protein with buffer alone) for 6 h, after which ascorbate was removed by passage through a Sephadex G-25 column (Pharmacia) and by ultrafiltration through a Diaflo YM10 membrane (Amicon, Beverly, MA, U.S.A.). Ascorbic acid was measured in the eluate as absorbance at 265 nm. Control and ascorbate-treated α -crystallin preparations were then divided into portions, stored at -30 °C and thawed just before use. Iron and copper contamination in the 50 mM-Tris/HCl buffer was $\leq 0.3 \mu\text{M}$ and $\leq 0.1 \mu\text{M}$ respectively, as determined by atomic absorption measurements (Perkin-Elmer model 4000; Beaconsfield, Bucks., U.K.).

Denaturation studies

Conformational stability was studied under the influence of urea, GdmCl and alkaline pH. Protein solution (0.14 mg/ml) was added to increasing urea or GdmCl concentrations or pH values, then left at 20 °C for 20 h before measurement, by the methods of Liang & Pelletier [19] and Siezen & Bindels [20]. Unfolding curves were determined by measuring the chosen spectral property as a function of denaturant concentration. Intrinsic fluorescence measurements were performed at 25 °C, with excitation wavelength at 295 nm and monitoring the fluorescence yield and the shift in emission maximum. Difference absorption measurements were evaluated by following the change in absorbance at 287 nm. Refolding was performed by dilution at 25 °C. Dithiothreitol (1 mM) was present in all the unfolding-refolding studies.

The fraction of unfolded protein, F_u , was calculated using the equation:

$$F_u = (Y_t - Y_{\text{obs}}) / (Y_t - Y_u)$$

where Y_{obs} is the observed variable parameter, e.g. intrinsic fluorescence or difference absorbance, and Y_t and Y_u are the values of the variable parameter characteristic of the folded and unfolded conformations. Values of Y_t and Y_u in the transition region were obtained by extrapolation of the linear portions of the pre- and post-transition regions of the unfolding curve respectively [21,22].

Spectroscopy

The fluorescence and polarization studies were performed on a LS5 Luminometer (Perkin-Elmer), a ratio-recording luminescence spectrometer equipped with a jacketed cell holder connected to a Multitemp II constant-temperature bath (LKB, Bromma, Sweden) and an automatic polarization control unit plus data station with software.

Fluorescence quenching of native and ascorbate-treated α -crystallin, dissolved in 50 mM-Tris/HCl buffer, pH 7.4, at 25 °C, was accomplished by progressive addition of small portions of acrylamide or iodide dissolved in the same buffer. The excitation wavelength was at 300 nm, the emission wavelength was at 340 nm.

The light-scattering determination at a 90 ° angle was carried out after filtration with 0.45 μm pore-size Millipore membranes;

the light-scattering intensity of different protein solutions was examined at 362 nm by the procedure of Hand & Somero [23].

Different absorption measurements were obtained on an HP 8450 A, a diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA, U.S.A.). The degree of turbidity was determined by measuring the absorbance at 450 nm, by the method of Maiti *et al.* [24].

Thermal denaturation experiments were done as previously described [5]. The proteins underwent melting curves with absorbance monitored at 280 nm; the heating rate was 1.0 °C/min. Hyperchromicity was calculated as the fractional increase in absorbance.

Proteolysis study

Proteolysis was studied by incubating, for various time-periods, native and ascorbate-treated α -crystallin with type XIV proteinase (Pronase E), which is a non-specific proteolytic enzyme. Protein solution (2 mg/ml in 50 mM-Tris/HCl buffer, pH 7.4) was incubated with 10 μg of Pronase E for various time-periods. At the end of the incubation, protein solutions were diluted in denaturing buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.25% Bromophenol Blue in Tris/HCl buffer, pH 6.8) and heated at once at 95 °C for 4 min, to inactivate the proteinase completely. The degradation was studied by SDS/PAGE performed on a Mini-PROTEAN II dual slab cell (Bio-Rad) with precast Ready gel, as described by Laemmli [25].

Evaluation of extent of oxidation

Determination of the protein carbonyl content after treatment with sodium borohydride was performed as described by Lenz *et al.* [26].

The bityrosine content of a protein solution containing about 2 mg/ml was estimated at 325–335 nm excitation and 400–420 nm emission, as described by Davies *et al.* [27].

Protein solubility

Protein solubility was estimated in high-salt buffer by the method of Davies & Delsignore [28]. The loss of soluble protein was assessed in 50 mM-sodium succinate buffer, pH 4.0, containing 3.0 M-KCl. Protein solutions were diluted with buffer and left for 180 min on ice. The solutions were centrifuged (2500 g) for 10 min and the remaining soluble protein was measured [17].

Electrophoretic study

SDS/PAGE was performed on the above-mentioned Mini-PROTEAN II dual slab cell (Bio-Rad) with precast Ready gel [25]. Isoelectric focusing, in 6 M-urea, was performed on an LKB Multiphor system with horizontal slab gels cast according to the LKB protocol with pH 3–10 Ampholines.

Chromatography

High-performance gel-permeation chromatography was carried out with a TSK-3000 SW column using an LKB h.p.l.c. system. Samples were eluted at 0.25 ml/min in 50 mM-Tris/HCl buffer, pH 7.4. Gel chromatography was performed on a Superose 12 HR/13 column (Pharmacia) equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, containing 1.5 M-urea.

Amino acid analysis

Native and ascorbate-treated α -crystallin were hydrolysed with azeotropic HCl in the gas phase using a PicoTag Workstation (Waters, Milford, MA, U.S.A.) and analysed as phenylthiocarbamyl derivatives using a PicoTag column (Waters) as described elsewhere [29].

Fig. 1. GdmCl and urea denaturation curves of native (○, ●) and ascorbate-treated (△, ▲) α -crystallin in terms of the fraction of unfolded protein (F_u)

Intrinsic fluorescence (open symbols); difference absorbance at 287 nm (closed symbols). Each curve represents an average of three experimental results. Experiments were performed as described in the Experimental section.

Fig. 2. Facilitating action of ascorbate on subunit dissociation at 1.5 M-urea; gel-permeation profiles of native and ascorbate-treated α -crystallin

Protein solutions (0.14 mg/ml) were added to 1.5 M-urea, then left at 20 °C for 20 h before being applied to a Superose 12 HR/13 column equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, containing the same urea concentration of the sample. Flow rate was 0.25 ml/min. ———, Native α -crystallin; —, ascorbate-treated α -crystallin.

RESULTS AND DISCUSSION

GdmCl and urea were used to unveil changes in protein conformation when α -crystallin is modified by ascorbate [30]. Since this protein is oligomeric and behaves as a complex system, only the qualitative evaluation of conformational stability of

native versus ascorbate-treated α -crystallin after chemical denaturation appears appropriate [31].

The GdmCl and urea unfolding profiles for native and ascorbate-treated α -crystallin, in terms of the fraction of unfolded protein (F_u) [21,22], are shown in Fig. 1. According to what is called the multiple-variable test [32], the plots of F_u versus denaturant should be coincident no matter which physical technique is used to follow unfolding. The presence of stable intermediates leads to non-coincidence of these plots [33,34] as in our case: in fact, the plots do not overlap for the native α -crystallin and this is even more so for the ascorbate-treated protein, so confirming experimental data of van den Oetelaar & Hoenders [35] and Siezen & Bindels [20]. Moreover, the plots of ascorbate-treated α -crystallin are shifted to lower denaturant concentrations, indicating a partial unfolding even at very low denaturant concentrations, and they show a hyperbolic unfolding behaviour, suggesting a decreased conformational stability.

Furthermore, in terms of the fraction of unfolded protein (F_u), fluorescence and difference absorbance data are indicative of a facilitating action of ascorbate on subunit dissociation, which is corroborated by the gel-filtration profiles at 1.5 M-urea shown in Fig. 2. Whereas the native protein is eluted as a single peak, the ascorbate-treated α -crystallin presents three distinct peaks, which indicate dissociation into subunits at lower denaturant concentrations.

As shown in Fig. 3, the ascorbate-treated α -crystallin produces emission-maximum profiles that are steeper and shifted to lower denaturant concentrations whereas the hyperbolic decrease in fluorescence yield is indicative of a less co-operative transition, subsequent to a conformational alteration induced by ascorbate on the α -crystallin molecule [36], which causes a stepwise exposure of aromatic residues, probably through the formation of intermediates or separate folding domains [21,22].

The destabilizing action of ascorbate is corroborated by the effects of alkaline pH on α -crystallin structure, as shown in Fig. 4. The pH profiles of emission maximum and fluorescence yield appear to almost overlap for native and ascorbate-modified α -crystallin. The largest differences can be observed above pH 10, and are probably due to alteration of tyrosine environment with enhancement of tryptophan-ionized tyrosine non-radiative energy transfer or increase in tyrosine deprotonation rate [37–39]. Involvement of tyrosine residues is moreover indicated by a slight difference in the emission spectra observed after differential excitation (at 280 and 295 nm) of native and ascorbate-treated protein (results not shown).

Since protein unfolding/denaturation can enhance proteolytic susceptibility as a result of increased accessibility of peptide bonds to proteinase [40], we have examined whether the susceptibility of α -crystallin to proteolytic degradation is modified after incubation with ascorbate. Fig. 5 shows that the ascorbate-treated protein has a lower rate of degradation than the native α -crystallin when incubated with type XIV proteinase (Pronase E) and then is less prone to proteolysis.

As we recently reported, the ascorbate system (ascorbate/oxygen/trace metals) is able to induce conformational and functional changes in enzymes and carrier proteins through metal-catalysed oxidative reactions which produce oxygen radical species [4,5,7]. The modification found in the lens that most strongly implicates metal-catalysed oxidation is the introduction of carbonyl groups into proteins [15,41]. We found that ascorbate-treated α -crystallin has almost twice as much carbonyl content as the native protein: 2.96 (± 0.12) versus 1.60 (± 0.16) nmol of carbonyl/mg of protein ($\pm$ S.E.M.).

Since we have found that ascorbate-modified alkaline phosphatase is more sensitive to heat than the native enzyme [5], thermal denaturation experiments with both native and

Fig. 3. Change in fluorescence yield (open symbols) and tryptophan emission maximum (closed symbols) of native ($\circ$, $\bullet$) and ascorbate-treated ($\triangle$, $\blacktriangle$) α -crystallin as a function of GdmCl and urea concentrations

Each curve represents an average of three experimental results. Fluorescence yield of native and ascorbate-treated protein (12% less than native) is expressed as percentage of fluorescence emission at 0 M-denaturant. Experiments were performed as described in the Experimental section.

Fig. 4. Change in (a) tryptophan emission maximum (closed symbols) and (b) fluorescence yield (open symbols) of native ($\circ$, $\bullet$) and ascorbate-treated ($\triangle$, $\blacktriangle$) α -crystallin as a function of increasing alkaline pH

Each curve represents an average of three experimental results. Experiments were performed as described in the Experimental section.

Fig. 5. SDS/PAGE patterns of α -crystallin incubated with proteinase type XIV (Pronase E)

The samples were loaded on a precast Mini-PROTEAN II Ready Gel (4–20% polyacrylamide/2.6% cross-linker/Tris/HCl) (Bio-Rad). The gel was stained with Coomassie Blue R-250. Further details are given in the Experimental section. Lane 1, protein markers (Pharmacia); lanes 2, 4 and 5, ascorbate-treated α -crystallin; lanes 3, 6 and 7, native α -crystallin. Incubation time: lanes 2 and 3, 0 min; lanes 4 and 6, 30 min; lanes 5 and 7, 60 min.

ascorbate-treated α -crystallin were performed. The data on native α -crystallin, reported in Table 1, are consistent with the differential scanning calorimeter thermograms recently reported by Steadman *et al.* [42]. The modified protein displays slightly lower T_m and T_D values which are indicative of a minor conformational stability. Our results are interesting in view of the evolutionary relationship and structural homology between α -crystallin and the small heat-shock proteins [43,44]. The similarity in amino acid sequence of the two proteins (over 40%) could mean that a particular domain may serve to facilitate aggregation [43].

Bityrosine formation, which is also considered a 'marker' for protein modification according to Davies *et al.* [27], was,

Table 1. Thermal stability of native and ascorbate-treated α -crystallin

Protein concentration was 0.15 mg/ml. T_m is the transition temperature between folded and unfolded states of the protein and T_D is the precipitation temperature of denatured protein. Experiments were performed as described in the Experimental section. Each value represents the mean ($\pm$ S.D.) of three separate experiments.

α -Crystallin	T_m ($^{\circ}$ C)	T_D ($^{\circ}$ C)
Native	73 ($\pm$ 1)	76 ($\pm$ 1)
Ascorbate-treated	70 ($\pm$ 1)	73 ($\pm$ 1)

Table 2. Fluorescence quenching by acrylamide and iodide

Stern-Volmer constants, K_{SV} , and accessibility fractions, f_a , for tryptophan residues in native and ascorbate-treated α -crystallin. Results are means of three separate experiments performed in triplicate ($\pm$ S.D.). K_{SV} was measured as described by Eftink & Ghiron [50]; f_a was measured as described by Lehrer [51].

α -Crystallin	K_{SV} (M^{-1})		f_a	
	Acrylamide	KI	Acrylamide	KI
Native	2.3 ($\pm$ 0.1)	1.3 ($\pm$ 0.1)	0.5 ($\pm$ 0.1)	0.3 ($\pm$ 0.1)
Ascorbate-treated	2.4 ($\pm$ 0.1)	1.2 ($\pm$ 0.1)	0.9 ($\pm$ 0.1)	0.2 ($\pm$ 0.1)

however, negligible, as was any other kind of non-tryptophan fluorescence.

No significant modification after ascorbate treatment was observed in the solubility of α -crystallin in high-salt buffer, measured as described by Davies & Delsignore [28].

H.p.l.c. profiles, SDS/PAGE and isoelectric focusing pattern of native and ascorbate-modified α -crystallin are identical (results not shown), indicating that mobility and size of the protein are not modified. This does not, however, exclude the modification of a small number of residues which would not influence the overall size and charge properties of the protein [45]. Similarly, amino acid analysis did not show any difference between native and ascorbate-treated α -crystallin (results not shown). This is generally the case when only a single or very few residues are modified [46].

In spite of the lack of gross structural alterations, conformational stability of α -crystallin already appears to be modified in the early phases of the incubation with ascorbate. This led us to hypothesize that small and localized conformational changes in the chemical structure of the lens protein could be responsible for the altered mechanism of unfolding, similarly to the recent reports of Prinsze *et al.* on the pronounced influence of very mild oxidation on some properties of different proteins [47].

In fact, a fluorescence deactivation of 12% with no evident shift in the emission wavelength peak (results not shown), together with the hyperbolic decrease in fluorescence quantum yield demonstrated in Fig. 3, suggest a perturbation of the tryptophan microenvironment in the ascorbate-treated protein, since excitation at 295 nm eliminates the tyrosine component of fluorescence and decreases the probability of intertryptophan energy transfer [39,48].

The enhancement of the degree of fluorescence polarization (with excitation at 295 nm and emission at 340 nm) from 0.170 ($\pm$ 0.002) for the native to 0.198 ($\pm$ 0.003) for the ascorbate-treated α -crystallin is indicative of a decrease in fluorophore mobility or rather an enhancement of structural rigidity of the molecular regions containing the tryptophan residues.

Fig. 6. (a) Turbidity and (b) light-scattering intensity (in arbitrary units) measurements of different concentrations of α -crystallin

Each concentration was incubated for 24 h with acetylsalicylic acid (10 mM) at 37 $^{\circ}$ C and then heated at 100 $^{\circ}$ C for 5 min in 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 M-KCl, 1 mM-2-mercaptoethanol, 0.8 mM-EDTA and 3 mM-sodium azide. Experiments were done in triplicate at 37 $^{\circ}$ C. Further details are described in the Experimental section. $\circ$, Native; $\bullet$, native + acetylsalicylic acid; $\triangle$, ascorbate-treated; $\blacktriangle$, ascorbate-treated + acetylsalicylic acid.

Since rigidity and tryptophan burial are closely related [49], fluorescence-quenching studies with common quenchers such as acrylamide and KI have been performed [50–52].

An unchanged quenching constant (K_{SV}) with an increased accessibility fraction (f_a) for acrylamide (Table 2) account for a major accessibility to the quencher of the inner tryptophan residues in the ascorbate-modified protein, whereas no modification of the tryptophan residues located close to the protein surface is indicated by the unchanged K_{SV} and f_a values for the polar quencher, KI.

Also the ϵ -amino groups of lysine are affected by ascorbate action, since the *N*-terminal amino groups of α -crystallin are acetylated [53]. Taking account of the hypothesis of Stevens *et al.* [54] on the importance of free amino groups in maintaining the state of crystallins, the two proteins (native and ascorbate-modified) were incubated for 24 h with acetylsalicylic acid, which is known to bind covalently to lysine [53] and to be a powerful anti-aggregating agent [53], then were heated at 100 $^{\circ}$ C for 5 min, and the resulting turbidity of the solution was measured (Fig. 6a). Ascorbate treatment alters the reactivity of lysine residues of α -crystallin which are the targets of acetylation by acetylsalicylic acid, resulting in their being unable to prevent the heat-induced aggregation of the protein. Light-scattering experiments (Fig. 6b) confirmed the turbidimetric titrations. This altered aggregability

pattern could account for an increased facility of the modified α -crystallin to form aggregates which are important in the development of cataractogenic process. Ortwerth & Olesen [11] demonstrated that a prolonged (6 days) incubation of α -crystallin with ascorbate even causes a decrease in lysine content.

In conclusion, our results indicate that small and localized alterations, probably exerted through oxidative modification of amino acid residues [8,9,13–15] and/or the formation of covalent adducts [55–57], take place even in the early phases of incubation of α -crystallin with ascorbate, causing a decrease in conformational stability and decreased susceptibility to proteolysis of the lens protein. The destabilizing action of ascorbate could be ascribed to conformational rearrangement of the peptide backbone with consequent alteration in the reactivity and accessibility of specific amino acid residues. These localized modifications would affect both the interactions of α -crystallin with the surrounding environment and the interactions of subunits with each other. In fact, the N-terminus of the A₂ subunit, which contains the single tryptophan and four tyrosine residues [58,59], is supposed to be important in α -crystallin aggregation and in providing hydrophobic intermolecular contact sites [60]; on the other side, the C-termini of both A and B chains, which contain several lysine residues, are primarily surface-exposed [61].

Our data are of particular interest in connection with the hypothesis forwarded by Liang *et al.* [62] that, with aging, α -crystallin undergoes a change in tertiary structure with a partial unfolding of the protein and an alteration in the micro-environment and reactivity of the amino acid residues that are responsible for intermolecular aggregation and cross-linking of aged lens proteins.

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