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Calcium-induced decrease of the thermal stability and chaperone activity of α-crystallin

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Abstract

α-Crystallin, one of the major proteins in the vertebrate eye lens, acts as a molecular chaperone, like the small heat-shock proteins, by protecting other proteins from denaturing under stress or high temperature conditions. α-Crystallin aggregation is involved in lens opacification, and high [Ca^{2+}] has been associated with cataract formation, suggesting a role for this cation in the pathological process. We have investigated the effect of Ca^{2+} on the thermal stability of α-crystallin by UV and Fourier-transform infrared (FTIR) spectroscopies. In both cases, a Ca^{2+}-induced decrease in the midpoint of the thermal transition is detected. The presence of high [Ca^{2+}] results also in a marked decrease of its chaperone activity in an insulin-aggregation assay. Furthermore, high Ca^{2+} concentration decreases Cys reactivity towards a sulfhydryl reagent. The results obtained from the spectroscopic analysis, and confirmed by circular dichroism (CD) measurements, indicate that Ca^{2+} decreases both secondary and tertiary–quaternary structure stability of α-crystallin. This process is accompanied by partial unfolding of the protein and a clear decrease in its chaperone activity. It is concluded that Ca^{2+} alters the structural stability of α-crystallin, resulting in impaired chaperone function and a lower protective ability towards other lens proteins. Thus, α-crystallin aggregation facilitated by Ca^{2+} would play a role in the progressive loss of transparency of the eye lens in the cataractogenic process.

Keywords: UV–Vis spectroscopy; Fourth derivative spectroscopy; Fourier-transform infrared spectroscopy; Circular dichroism spectroscopy; Chaperone activity; Cataract formation

1. Introduction

Crystallins are a major class of proteins in the eye lens [1]. The crystallin family is composed of α-crystallins and β/γ-crystallins in vertebrates [2]. Proper supramolecular assembly of these proteins is critical to maintain lens transparency [3]. α-Crystallin is a multimeric protein composed of two subunits, αA and αB, which are associated to form high molecular weight oligomers of approximately 800 kDa [4]. Both subunits have been cloned and isolated from different mammalian species including human [5]. The two polypeptide chains are expressed from single copy genes with 55% identity [2] and about 60% homology at the amino acid sequence level [6]. α-Crystallins are highly conserved proteins through evolution, and they appear to be related to the small heat-shock proteins of *Drosophila melanogaster* [7].

α-Crystallin was proposed to be heat-stable on the basis of circular dichroism (CD) and fluorescence spectroscopic studies [8]. In vitro, it can function as a molecular chaperone to prevent thermal aggregation of a number of enzymes and other lens structural proteins such as β- and γ-crystallins [9–11]. The protection mechanism may involve preferential binding of the other proteins to α-crystallin complex via hydrophobic surface interactions [12–14], with a kinetic competition between aggregation and interaction of unfolding proteins with α-crystallin [15]. α-Crystallins gradually become high molecular weight aggregates with aging and cataract formation and eventually lead to the formation of insoluble protein [16]. Structural modifications, in the aggregation process, involve partial unfolding with subsequent exposure of hydrophobic surfaces, which promote hydrophobic interaction. In aged and cataractous lenses, aggregation and insolubilisation take place

Abbreviations: UV–Vis spectroscopy, ultraviolet–visible spectroscopy; FTIR spectroscopy, Fourier-transform infrared spectroscopy; CD spectroscopy, circular dichroism spectroscopy; Tris, tris(hydroxymethyl) aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HEPES, 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid

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because of the high concentration of partially unfolded protein.

Ca^{2+}-induced aggregation of lens α-crystallin was initially studied by sedimentation analysis [17] and the possible role for Ca^{2+} in the cataractogenesis process was discussed [18]. As high as 64 mM Ca^{2+} was found in cataractous lenses [19]. In human lenses with dense, highly localised opacities, Ca^{2+} distribution is not uniform and is highest in regions that scatter most light. It has been proposed that α-crystallin and certain water-insoluble species are largely responsible for this scattering and may contain Ca^{2+}-binding sites [20]. Other crystallins, like β- and avian core protein δ-crystallins, are known to be calcium-binding proteins [21,22]. Recently, it was found that γ-crystallin from bovine lenses shows significant calcium-binding ability [23], the greek key βγ-crystallin fold being the calcium-binding motif. Although this structure is absent in α-crystallin [23], it is of interest to study the effect of Ca^{2+} on α-crystallin properties because this ion has been proposed to have a role in the cataractogenic process. In vivo, importance of Ca^{2+} has also been stressed by the report of Ca^{2+}-induced opacification in the intact rat lens [24]. Here we show, for the first time, that Ca^{2+} decreases the thermal stability of α-crystallin by promoting partial unfolding of the protein, and that as a consequence, its function is altered, resulting in a decreased ability to act as a molecular chaperone. It is proposed that the deleterious effect of high Ca^{2+} concentration upon the chaperone function of α-crystallin may play an important role in the molecular mechanism of cataract formation and development.

2. Materials and methods

2.1. Sample preparation

All chemicals used were of reagent grade. α-Crystallin from bovine eye lens was obtained from Sigma Chemical Co.(St. Louis, MO, USA) and used without further purification. Purity of α-crystallin samples was checked by SDS-PAGE (only two bands detected with Coomassie blue staining corresponding to αA and αB monomers), and the oligomeric status of the protein was verified by means of size exclusion chromatography (data not shown). A stock solution of α-crystallin was prepared in 50 mM Tris–HCl buffer (pH 7.4), and split into several samples. To these solutions, small aliquots of a concentrated $CaCl_2$ solution were added to obtain the desired $CaCl_2$ concentration, and these were incubated at room temperature (25–30 °C) for 1 h. The protein concentration for the experiments ranged between 0.4 and 0.7 mg/ml, except for Fourier-transform infrared (FTIR) spectroscopy, for which α-crystallin was prepared in D_2O solution at a concentration of 12.5 mg/ml in 30 mM HEPES buffer with 100 mM NaCl, pH 7.2 (uncorrected pH).

2.2. UV–visible spectroscopy

UV–Vis absorption spectra were recorded on a Cary 1E spectrophotometer (Varian, Australia) equipped with thermostatted cell holders. Data acquisition was performed by using the following parameters: data points each 0.2 nm, time constant of 1 s and a band width of 2 nm. Thermal studies were carried out by increasing the temperature at a rate of 2.5 °C/min. Fourth derivative spectra were obtained from four absorption spectra coadded to improve the signal-to-noise ratio, by using the Savitzsky–Golay algorithm and 300 points with the Grams/32 software (Galactic Industries).

2.3. CD measurements

CD measurements were carried out in a Jasco-700 CD spectropolarimeter at 25 °C. The reported CD spectra are the average of five scans, smoothed by polynomial curve fitting (J-700 for Windows Standard Analysis, Version 1.0; Jasco Corp.). The CD data were expressed as molar ellipticity in deg. cm^2/dmol, with 114 as residue molecular weight [5,10]. Protein concentration was 1.0 mg/ml with 0.2 mm light path for far-UV CD measurements, and with 10 mm light path for near-UV CD measurements.

2.4. FTIR spectroscopy

FTIR spectroscopy was performed using a Mattson Polaris infrared spectrometer equipped with a MCT high-sensitivity detector. The sample compartment was continuously purged with dry air with a dew point better than −80 °C. A mechanical shuttle was used, which allowed obtaining blocks of sample and background spectra, thus compensating for the residual water vapour in the sample chamber. Temperature heating rate was 1 °C/min (with a 5-min period between spectra for temperature stabilisation). Each spectra, obtained from 500 coadded scans with a spectral resolution of 2 cm^{-1}, was manually corrected for D_2O absorption until a flat baseline in the 1700–1800 cm^{-1} region was obtained.

2.5. Chaperone activity experiments

The chaperone activity of α-crystallin was determined by DTT-induced aggregation of insulin B chain [13]. Briefly, 0.1 mg of insulin was reduced with 20 mM DTT in the absence or presence of 0.2 mg of α-crystallin in a final volume of 0.5 ml of 50 mM Tris pH 7.2 containing either no Ca^{2+} or different [Ca^{2+}] (5 or 25 mM Ca^{2+}). UV–Vis absorption spectra of these samples were recorded using the same instrumental parameters as stated above (see UV–Vis absorption spectrophotometry section) between 220 and 550 nm. Insulin aggregation kinetics, as relative scattering at 360 nm, was derived from the recorded UV–absorption spectra. Assays were performed at the temperature of 20 °C. Four

different experimental conditions were compared in this assay: insulin alone, insulin alone with Ca^{2+}, insulin with α-crystallin, insulin and α-crystallin with Ca^{2+}.

2.6. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) reactivity of α-crystallin

Free thiol concentration was measured by using DTNB (Ellman's reagent) [25]. α-Crystallin (0.4 mg/ml) was pre-incubated 60 min after Ca^{2+} addition (in the 0–50 mM range) at room temperature, and subsequently incubated with 100 μM DTNB for 30 min. UV–Vis absorption spectra were recorded and changes at 412 nm were analysed.

3. Results

3.1. UV absorption spectra of α-crystallin: effect of Ca^{2+} addition

UV absorption spectra show Ca^{2+}-induced α-crystallin aggregation as measured by the increase in absorbance at 360 nm (Fig. 1a). The presence of certain absorption in the high-wavelength UV region, extending to the visible region, is highly characteristic of α-crystallin solutions. This can be basically attributed to light scattering of high-molecular mass species present to a certain extent in α-crystallin solutions, which are highly prone to aggregation [26,27].

The light scattering of α-crystallin solution in the presence of Ca^{2+} could be explained in a typical manner as an electrostatic interaction of the protein with its environment. The metal cation selectivity for an anionic ligating site of the protein is usually explained with the so-called Eisenman series [28,29]. We observe that α-crystallin solution in the presence of Ca^{2+} shows more light scattering than α-crystallin in the presence of Na^{+} (Fig. 1b), while Mg^{2+} has an intermediate behaviour (data not shown). So the anionic sites of interaction of the cation with α-crystallin have a field strength stronger with Ca^{2+} and other 2A cations, which are preferred to the monovalent 1A cations. Thus, electrostatic interaction may be mainly responsible for the Ca^{2+}/α-crystallin interaction, and a differential effect can be detected with regard to other metal ions.

3.2. Thermal stability of α-crystallin in the presence of Ca^{2+}

Fig. 2 shows the heat denaturation curves for α-crystallin, in the absence and in the presence of different Ca^{2+} concentrations, obtained from absorbance at 360 nm, reflecting protein aggregation. A decrease in the transition midpoint with increasing [Ca^{2+}], from 76 °C (no Ca^{2+}) to 64 °C (100 mM Ca^{2+}) can be detected. A linear dependence of the transition temperature upon Ca^{2+} increase can be observed from 2 to 50 mM, but a deviation from linearity is apparent at the highest concentration of 100 mM (Fig. 2, inset).

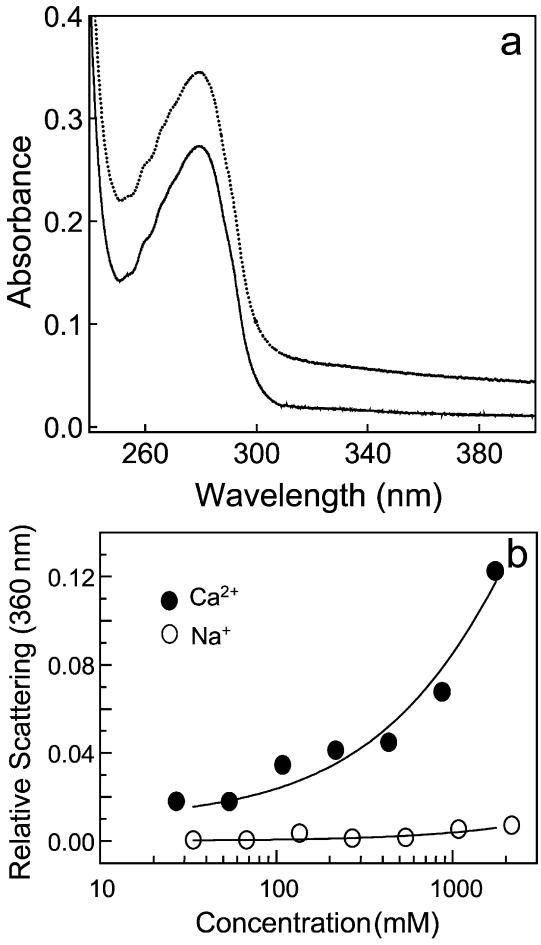

Fig. 1. (a) UV–visible absorption spectra of α-crystallin (0.4 mg/ml) both in the absence (—) and in the presence (· · ·) of 100 mM Ca^{2+}. (b) Light scattering changes as a function of cation addition to the samples: Ca^{2+} (●) and Na^{+} (○). Experiments were performed in 50 mM Tris–HCl buffer (pH 7.4). α-Crystallin aggregation was monitored by light scattering at 360 nm for α-crystallin samples with increasing addition of metal ion salts. Temperature, 20 °C.

3.3. CD spectra of α-crystallin

CD spectra of α-crystallin in the near-UV region shows a dramatic loss of tertiary structure at 65 °C, when compared to 25 °C, in the presence of Ca^{2+} while in the control sample the structure is still stable at this temperature (Fig. 3a,b). The tertiary structure stability was followed by the spectral change at 272 in the near-UV CD spectra (Fig. 3c). α-Crystallin, in the absence of Ca^{2+}, keeps its tertiary structure in the temperature range from 25 to 65 °C. In the case of the Ca^{2+}-containing sample, the tertiary structure change confirms the results obtained from UV–Vis spectroscopy measurements and also would reflect the formation of protein aggregates. This change in tertiary structure stability could be related to the loss of chaperone activity of α-crystallin in the presence of Ca^{2+} (see below).

Far-UV CD spectra in the absence and in the presence of Ca^{2+} have also been obtained at different temperatures (Fig. 4a,b). Native α-crystallin has a predominant β-sheet struc-

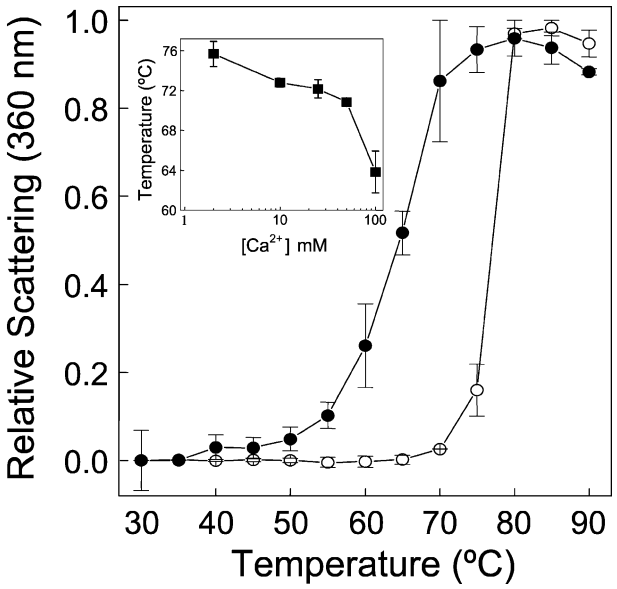

Fig. 2. Heat denaturation profiles of α-crystallin (0.4 mg/ml) in the absence (○) and in the presence (●) of 100 mM Ca^{2+}. Inset: plot of the thermal transition, temperature versus [Ca^{2+}]. Sample preparation was identical to that for the samples in Fig. 1. UV spectra were recorded in cycles with increasing temperature at a rate of 2.5 °C/min (for each cycle a spectrum was recorded at a given temperature). Inset—T_m values were graphically calculated over the heat denaturation profiles from three independent experiments. The plot is linear between 2 and 50 mM Ca^{2+} (r=0.99).

ture and has a secondary structure resistant at temperatures above 65 °C. In contrast, there are some changes in the CD spectra in the presence of Ca^{2+} which could be interpreted as reflecting a somewhat changed conformation of the protein. We have followed the spectral signal at 225 nm, which shows a significant change at 65 °C in the presence of Ca^{2+} reflecting protein aggregation, while the control sample without added Ca^{2+} remains basically unchanged (Fig. 4c). In our case, there is a change in secondary structure, while the loss of some secondary structure had been correlated directly with the heat stability of α-crystallin [30]. The spectral changes analysed both in the far- and in the near-UV indicate that the conformation of α-crystallin in the presence of Ca^{2+} is different to that of urea-denatured samples (Figs. 3 and 4). CD spectra thus provide evidence of a decrease of the thermal stability of both secondary and tertiary structures, confirming the results obtained by the other spectroscopic techniques used in the present work.

3.4. Secondary structure stability by FTIR spectroscopy

Fig. 5a shows FTIR spectra of α-crystallin from 30 to 90 °C with no added Ca^{2+}. The infrared absorption spectrum of α-crystallin at 30 °C is very similar to that reported by Surewicz and Olesen [31], with the presence of an amide-I component at 1631 cm^{-1} highly characteristic of β-sheet secondary structure. Upon heating to 90 °C, a gradual increase in the component bands at 1647 and 1620 cm^{-1} is observed as well as a decrease in the intensity of the band

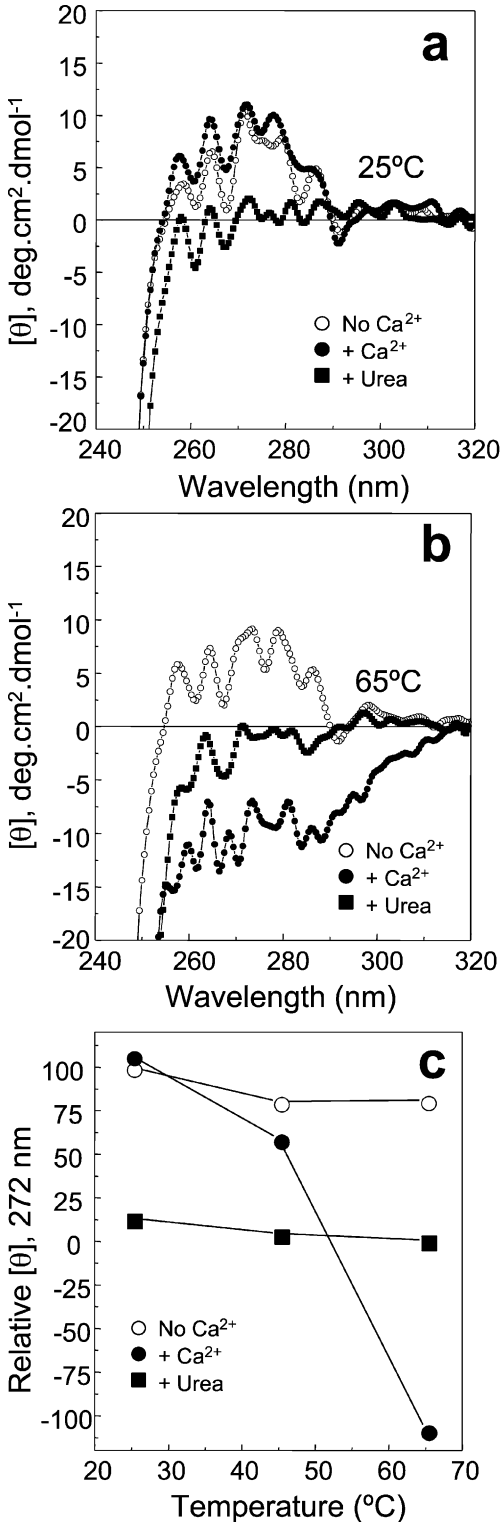

Fig. 3. Near-UV CD spectra of α-crystallin at 25 °C (a) and 65 °C (b). Change in ellipticity at 272 nm versus temperature (c). Samples were prepared in 50 mM Tris–HCl buffer, pH 7.4 (○), or containing the addition of either 100 mM Ca^{2+} (●), or 8 M urea (■). The tertiary structure thermal stability was represented by changes in the molar ellipticity [θ] at 272 nm relative to the obtained value for α-crystallin in Tris–HCl buffer (no Ca^{2+}, no urea) at 25 °C.

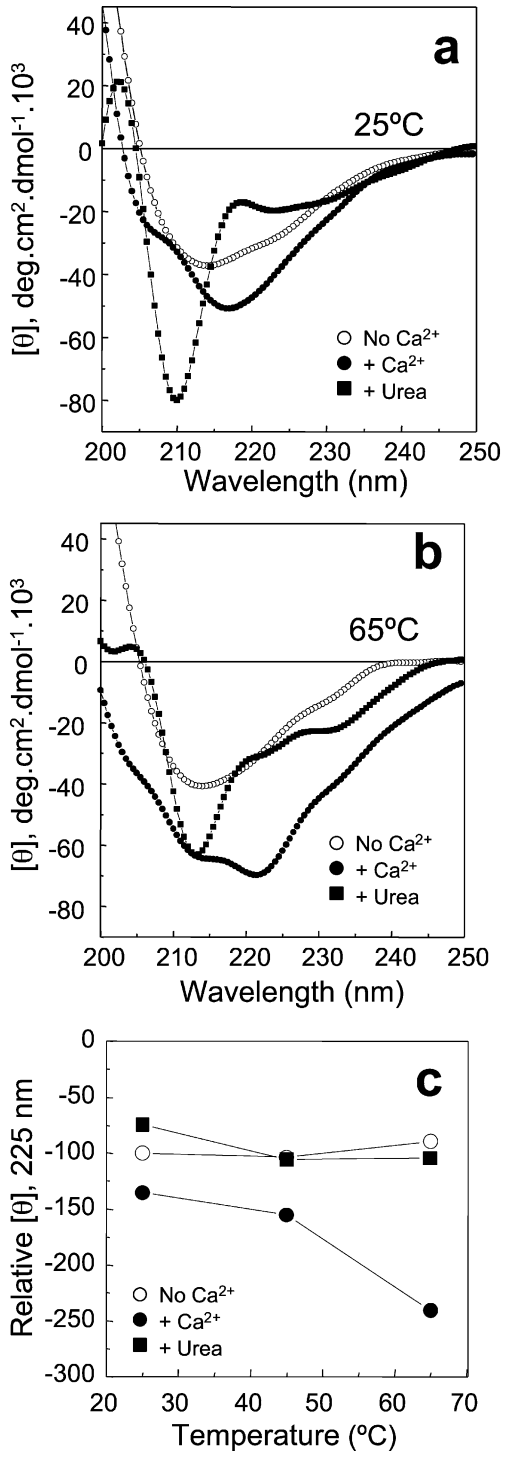

Fig. 4. Far-UV CD spectra of α-crystallin at 25 °C (a) and 65 °C (b). Change in ellipticity at 225 nm versus temperature (c). Samples were prepared in 50 mM Tris–HCl buffer, pH 7.4 (○), or containing the addition of either 100 mM Ca^{2+} (●), or 8 M urea (■). The secondary structure thermal stability was represented by changes in the molar ellipticity [θ] at 225 nm relative to the obtained value for α-crystallin in Tris–HCl buffer (no Ca^{2+}, no urea) at 25 °C.

at 1631 cm^{-1} (Fig. 5a). Fig. 5b shows the thermal transition profiles obtained from the plot of the I_{1631}/I_{1647} ratio from 30 to 90 °C, both for α-crystallin in the absence and in the presence of 100 mM Ca^{2+}. A clear decrease of the midpoint of the thermal transition temperature can be observed from 62 °C (no Ca^{2+}) to 57 °C (100 mM Ca^{2+}).

3.5. Fourth derivative UV spectra of α-crystallin

Fourth derivative spectrum of α-crystallin shows resolved peaks at 248, 253, 260 and 267 nm corresponding to Phe residues, at 285.2 nm corresponding to Tyr residues, and at 292.4 nm corresponding to both Tyr and Trp residues (Fig. 6a). The aromatic amino acid composition of α-crystallin including both αA and αB subunits is 27 Phe, 8 Tyr and 3 Trp [26]. The high Phe content is already detected in the UV-absorption spectrum as a fine electronic structure, under the global envelope, in the region between 250 and 265 nm. In spite of the lower ε (molar extinction coefficient) of Phe, when compared to Tyr and Trp, the high relative abundance of these residues strengthens its contribution to

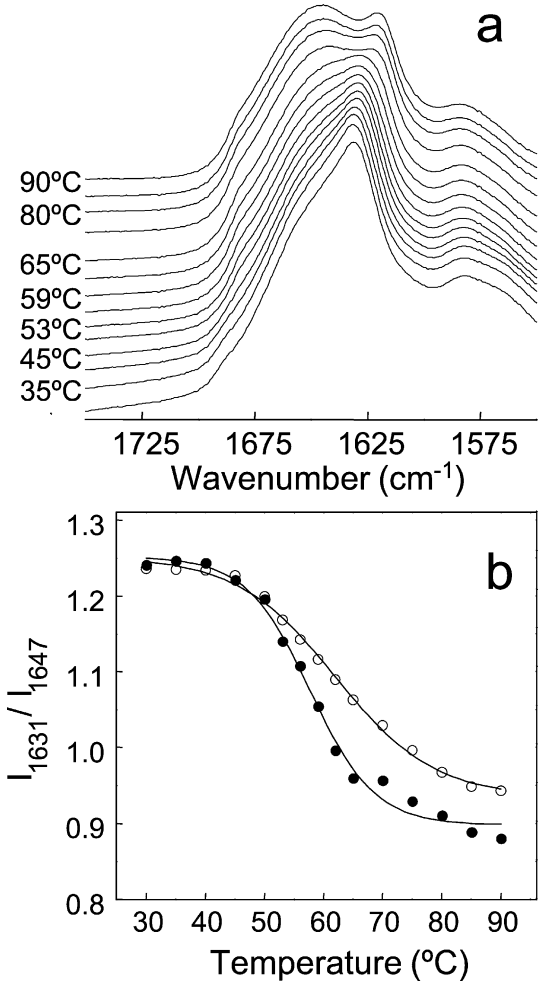

Fig. 5. (a) Absorbance Fourier-transform infrared spectra of α-crystallin in the amide-I region at different temperatures in the 30–90 °C range. (b) Ratio of α-crystallin amide-I band intensities at 1631/1647 cm^{-1} as a function of temperature, no Ca^{2+} (○) and 100 mM Ca^{2+} (●). α-Crystallin was prepared in D_2O solution at a concentration of 12.5 mg/ml in 30 mM Hepes buffer containing 100 mM NaCl (pH 7.2, uncorrected).

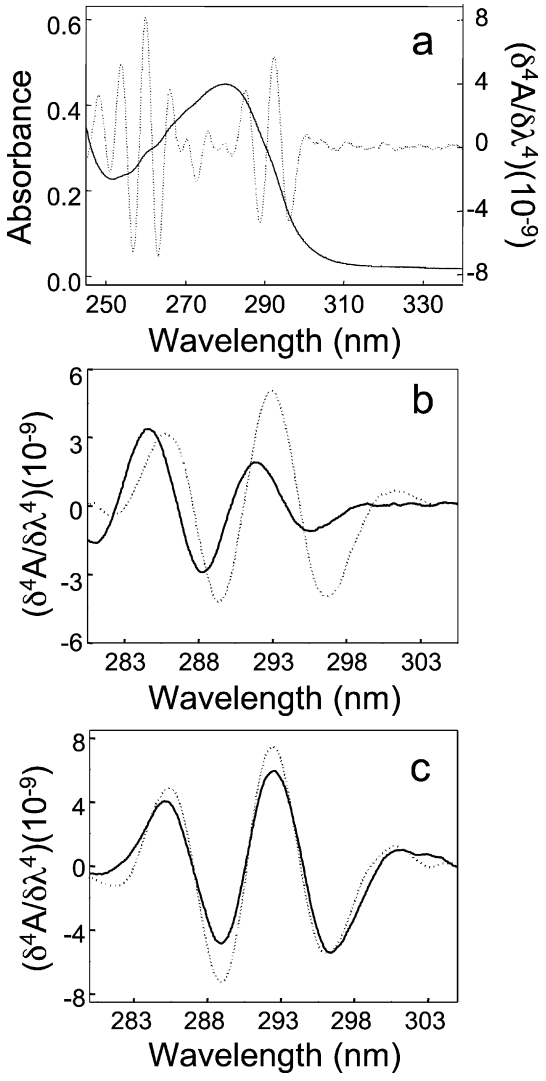

Fig. 6. (a) UV absorbance (—) and fourth derivative (· · ·) spectra of α-crystallin. (b) Fourth derivative UV spectra of α-crystallin in the absence (· · ·) and in the presence (—) of 8 M urea. (c) Fourth derivative UV spectra of α-crystallin in the absence of added Ca^{2+} (—) and in the presence of 25 mM Ca^{2+} (· · ·). α-Crystallin was at a final concentration of 0.7 mg/ml in 50 mM Tris–HCl buffer (pH 7.4). UV–Vis spectra were acquired at 20 °C. Fourth derivative spectra were obtained from four coadded UV absorption spectra by using the Savitzsky–Golay algorithm.

the fourth derivative spectrum. The location of the longest-wavelength minimum at 296.3 nm can be considered as reflecting a highly hydrophobic environment for Trp residues in α-crystallin, when compared with previously reported studies [32]. The wavelength of peaks corresponding to Tyr and Phe reflect also a highly hydrophobic environment for these residues [32,33]. The ratio of the amplitudes of the peaks at 292.4 and at 285.2 nm is 1.2, confirming the proposed hydrophobicity of the Tyr and Trp environment [32,33].

Upon urea addition to α-crystallin solution, a blue-shift to shorter wavelength of the minimum at 296.3–294.7 nm and of that at 288.8–287.6 nm, and blue-shifts of the maxima at 282.4 and 285.2 nm to 281.3 and 284.0 nm, respectively, can be detected (Fig. 6b). The blue-shift of the Trp 296.3 nm minimum, as well as those for Tyr peaks, can be interpreted as an increased aqueous character for Trp and Tyr residues but still reflecting a highly nonpolar environment. Ca^{2+} also induces a blue-shift of the longest-wavelength minimum from 296.3 nm to 295.6 nm (Fig. 6c), reflecting a change in the Trp environment to a slightly more polar condition. Sample changes in the amplitude of the fourth derivative peaks can also be detected.

3.6. Chaperone activity of α-crystallin in the presence of Ca^{2+}

Chaperone activity of native α-crystallin has been evaluated both in the absence and in the presence of Ca^{2+}. This activity has been determined at two different [Ca^{2+}] (5 and 25 mM). Fig. 7 shows the obtained results from a DTT–insulin aggregation assay. The reduction of the insulin interchain disulfides leads to aggregation of the B chain while the A chain remains in solution [34]. The aggregation can be monitored by measuring the apparent absorbance due to light scattering at 360 nm as shown in Fig. 7 (curve 4). The ability of α-crystallin to protect insulin B chain from aggregation was tested by reducing insulin to a final insulin/α-crystallin weight ratio of 1:2. This ratio was sufficient for complete protection of the insulin B chain from aggregation (Fig. 7, curve 1). Ca^{2+} presence inhibits the protective effect of α-crystallin in the insulin B chain aggregation process, the loss of chaperone activity being increased at higher [Ca^{2+}], from 5 to 25 mM (Fig. 7, curves 2 and 3,

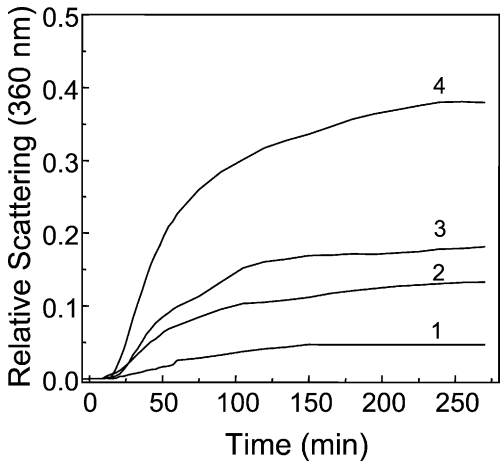

Fig. 7. Chaperone activity of α-crystallin in the absence and presence of Ca^{2+}. The incubation mixture contained, in a final volume of 0.5 ml of 50 mM Tris–HCl (pH 7.2), 0.1 mg of insulin and 20 mM DTT with the following additions: (curve 1) 0.2 mg of α-crystallin; (curve 2) 0.2 mg of α-crystallin and 5 mM Ca^{2+}; (curve 3) 0.2 mg of α-crystallin and 25 mM Ca^{2+}; (curve 4) control with no α-crystallin, no Ca^{2+} added. Kinetics of insulin aggregation at different conditions was followed by light scattering at 360 nm. Data were acquired at 20 °C. The maximal error in the experimental data is 5%.

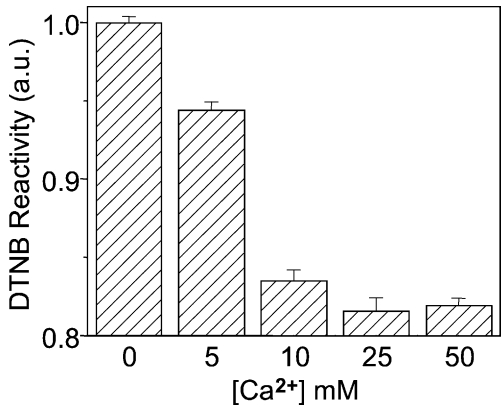

Fig. 8. Relative thiol reactivity of α-crystallin towards DTNB as a function of [Ca^{2+}]. α-Crystallin samples were prepared in the same way as those in Fig. 1 and treated with 100 μM DTNB reagent in the presence of different [Ca^{2+}]. DTNB reactivity was measured by the absorbance increase at 412 nm. UV–Vis spectra were acquired at 20 °C. The values are mean ± S.D. calculated from three independent assays.

respectively). The aggregation of insulin B chain alone in the presence of Ca^{2+} was investigated in a control experiment, and under this condition, the aggregation of insulin was slightly inhibited in a chaperone-like manner (data not shown). Taking this observation into account, the loss of chaperone activity of α-crystallin in the presence of Ca^{2+} could be even more severe than that derived from the spectroscopic data, indicating that the decrease in chaperone activity induced by calcium ions is mainly due to their binding to α-crystallin and not to insulin.

3.7. Reactivity of α-crystallin towards DTNB in the presence of Ca^{2+}

In the absence of Ca^{2+}, α-crystallin shows reactivity towards DTNB, indicating a high free sulfhydryl content in agreement with previous studies [35,36]. In the presence of Ca^{2+}, we find a reduction of the reactivity towards DTNB corresponding to about 15–20% of the accessible Cys groups in the native protein without Ca^{2+} (Fig. 8). This decrease can result from a decrease in free thiol accessibility possibly due to disulfide bond formation upon partial unfolding of the protein.

4. Discussion

We have carried out the study of the effect of Ca^{2+} on the structure and chaperone activity of α-crystallin. Previous studies have indicated that the supramolecular status and state of oligomerization of α-crystallin may depend on the experimental conditions [37], and that the aggregation of α-crystallin can be induced by Ca^{2+} [17,18]. In our case, UV-absorption spectra obtained upon increasing [Ca^{2+}], to concentrations similar to those reported in some cataractogenic processes [20,24], reflect an aggregation characterised by increased extinction at all wavelengths in the 240–400 nm region. This aggregation process was followed by the change in relative scattering at 360 nm by means of UV–Vis spectroscopy. The light scattering increase upon metal ion addition indicates that the interaction mechanism is likely to be mainly of an electrostatic nature but we find a differential effect between Ca^{2+} and Na^{+}, suggesting differential binding to anionic sites in the protein. In our experiments, we have also checked the pH of the salt-containing samples because it is known that the structure and activity of α-crystallin are sensitive to these changes [30,37]. We find only slight changes in the pH of the Ca^{2+}-containing samples (data not shown), indicating that the observed behaviour may not be due to changes in the apparent isoelectric point of the protein.

It was also of interest to investigate the thermal stability of α-crystallin in solution because chaperone activity is known to be influenced by temperature, and that the physiological process of crystallin opacification may be related to this activity loss. Although initially α-crystallin was described as a protein with a high thermal stability, on the basis of CD and fluorescence spectroscopic measurements [9], more recently this notion has been changed in view of calorimetric [38] and FTIR spectroscopic studies [31]. These latter studies reported a thermal transition with a midpoint at about 60 °C, and a complete loss of the native secondary structure from the infrared spectroscopic measurements [31]. We have used UV–Vis spectroscopy to investigate the effect of Ca^{2+} on the thermal stability of α-crystallin. A lowering of the midpoint of the transition upon increasing Ca^{2+} concentration can be detected, this thermal behaviour showing a linear relationship between Ca^{2+} concentration and the midpoint of the thermal transition. This result is interpreted as an increased thermally induced aggregation propensity of α-crystallin as a result of Ca^{2+} addition to the sample. It is known that α-crystallin has itself a strong tendency towards aggregation on thermal denaturation [38–40], and this fact is consistent with the view that α-crystallin in the presence of Ca^{2+} has a more unfolded conformation at high temperatures, like those used in our thermal assay.

The effect of Ca^{2+} on the thermal transition detected previously by FTIR spectroscopy [31] was also studied. The I_{1631}/I_{1647} ratio of the amide-I band in the absence and presence of 100 mM Ca^{2+} indicate that there are differences in the secondary structure at high temperature in the presence of Ca^{2+}. A clear decrease of 5 °C in the thermal transition midpoint is observed, reflecting a destabilising effect of Ca^{2+} on the secondary structure of α-crystallin. CD measurements, in the far-UV and near-UV regions, also indicate that Ca^{2+} decreases the thermal stability of α-crystallin. These results confirm the observed decrease in secondary and tertiary structure stability detected by means of the UV–Vis and FTIR spectroscopies used in the present study. The precise temperature decrease value for the different transitions depends on the spectroscopic technique used

because the conditions are slightly different. For example, for FTIR measurements, the protein concentration of the sample is much higher than that used for CD spectroscopic measurements.

We have used fourth derivative spectrophotometry to investigate the environment of aromatic residues in α-crystallin both in the absence and in the presence of Ca^{2+}. Fourth derivative spectroscopy allows the partial separation of the different electronic components corresponding to Phe, Tyr and Trp residues [32,33]. The fourth derivative represents the best compromise between the best resolution and the best signal-to-noise ratio [32,33]. Fourth derivative spectra have the additional advantage, common to all even-number derivatives that the maxima of the fourth derivative peaks correspond to the same wavelengths as those in the original absorption spectrum. This technique has been applied to the study of conformational changes both in native and recombinant proteins [41,42]. Careful analysis of the wavelength of the different maxima and minima on the fourth derivative spectra for the three aromatic amino acids (Phe, Tyr and Trp) indicate that all of them are probably in a highly hydrophobic environment, in a nonaqueous region, confirming the known proposal of α-crystallin as a typical model of a hydrophobic protein. Other proteins with lower relative Phe content, like cytochrome *c* or bacteriorhodopsin, show less-resolved features in their corresponding fourth derivative spectra [32]. In particular, we have found that Tyr and Trp are in a highly hydrophobic environment, thus confirming the proposed hydrophobic environment for both residues in α-crystallin. These results are in agreement with previous fluorescence spectroscopic studies, which reported that Trp and Tyr aromatic residues in α-crystallin are in a region of the structure of the protein highly shielded from the solvent [43].

Addition of urea to 8 M has been reported to induce complete dissociation of the oligomeric complex into its different subunits, a process accompanied by gross disruption of the secondary and tertiary structures [37]. It has been reported that the protein unfolds in 2.5 M guanidine hydrochloride or 4 M urea [44,45]. In the present study, 8 M urea addition to α-crystallin sample results in significant changes in the absorption spectrum with important hyperchromism in the 290–330 nm region (not shown). Main changes in α-crystallin fourth derivative spectra correspond to changes in the wavelength and in the amplitude of the fourth derivative peaks. The observed blue-shifts for the Tyr and Trp bands can be interpreted as a change in the polarity of their microenvironment towards a more hydrophilic condition. Specifically, the blue-shift of the Trp 296.3-nm minimum can be interpreted as a somewhat increased aqueous character for the Trp residues but still reflect a highly nonpolar environment. The effect of Ca^{2+} on the fourth derivative spectra has been also analysed (Fig. 6c). The main difference regarding wavelength location is a blue-shift of the longest-wavelength minimum from 296.3 to 295.6 nm reflecting a change in the Trp environment to a slightly more polar condition. Sample changes in the amplitude of the fourth derivative peaks are also observed. These results reflect that the environment of the aromatic residues has changed upon Ca^{2+} addition to the sample, although this change is smaller than that observed in the case of the denaturing agent urea. Trp residues are essential for the function of α-crystallin as a molecular chaperone [46], and the environment of Trp residues can be affected by Ca^{2+} in some cases [47]. Conformational changes in α-crystallin, occurring in the aggregation process in cataract formation, and associated to the loss of its function as a molecular chaperone, could be related to changes in the environment of Trp residues like the ones induced by Ca^{2+} reported in the present study. This does not exclude, however, chemical and structural changes at other locations, and involving other amino acid residues, like for example the single Cys at position 131 in the αA subunit (see discussion below on DTNB reactivity of the Ca^{2+}-containing samples).

An important point of the present study was to assess the presumed influence of Ca^{2+} on the chaperone activity of α-crystallin. The function of α-crystallin as a molecular chaperone has been clearly established [9–12]. This activity requires that the integrity of the tertiary structure of the protein is maintained [30]. Our CD results indicate that Ca^{2+} has a destabilizing effect on the tertiary structure of α-crystallin, and this would explain the consequent decrease of the chaperone activity of the protein. The influence of Ca^{2+} upon the chaperone activity of another reduced 'substrate', alcohol dehydrogenase used in an analogous aggregation assay, was shown to be slightly inhibited by 5 mM Ca^{2+} [48]. High accumulation of Ca^{2+} in cataractogenic lenses has been reported, suggesting a physiopathological role for Ca^{2+} in the eye lens opacification process [19]. In vivo, importance of Ca^{2+} has also been stressed by the report of calcium-induced opacification in the intact rat lens [24]. Our results indicate an important decrease in α-crystallin chaperone activity in the presence of Ca^{2+}. This effect could be explained by a conformational change involving exposure of hydrophobic domains (with a concomitant exposure of Tyr and Trp residues; resulting in blue shifts in their corresponding derivative spectra) during the α-crystallin aggregation process in the presence of Ca^{2+}, and that this conformational change affects the ability of α-crystallin to bind other proteins. The conformational changes induced by Ca^{2+} can result in the production of a partially misfolded α-crystallin, and the loss in its chaperone activity (which can presumably protect eye-lens proteins) may be a major contributing factor to the opacification of mammalian lenses in the cataractogenic process.

An additional interesting aspect that has been studied is the effect of Ca^{2+} upon the reactivity of α-crystallin towards DTNB. This reactivity can provide an estimation of the free sulfhydryl groups present in the sample under different conditions. We find that in the absence of Ca^{2+}, α-crystallin shows reactivity towards DTNB indicating a high

free sulfhydryl content in agreement with previous studies [35,36]. In the presence of Ca^{2+}, we find a reduction of the reactivity towards DTNB, which could be interpreted as a decrease in free thiol groups accessibility due to a conformational change of the protein. A more likely explanation would be that there is a partial unfolding with exposure of some free sulfhydryl groups that can form disulfide bonds, resulting in decreased reactivity towards DTNB.

Disulfide bond formation in high molecular weight α-crystallin aggregates has been associated with human cataract for a long time [36]. Recently, there have been reports on the oxidation of cysteine residues from αA-crystallin during cataractogenesis of the human lens, with increased intramolecular disulfide bonding [49,50]. Our results have been obtained with bovine α-crystallin. It should be noted that bovine αA-crystallin [51] and αB-crystallin [52] have very similar sequences to the human αA-crystallin and αB-crystallin polypeptide chains [6]. For example, the number of aromatic amino acid residues is the same in the bovine and in the human proteins. However, in the case of Cys residues, human αA-crystallin has two Cys at positions 131 and 142, while the bovine protein has only the one at 131 position. Different location and physical reduced or oxidised status was proposed for bovine and human Cys residues [39]. Furthermore, most of the studies carried out on cataractous lenses are carried out with human lenses. This observation has to be taken into account when analysing our DTNB reactivity results. In our case, there is only one possible Cys group, that at position 131 in αA-crystallin (αB-crystallin has no Cys residues) involved in the formation of disulfide bonding and this has to be intermolecular. In any case, we observe a decrease of the DTNB probably due to disulfide bond formation of Cys-131 of different αA-crystallin molecules. A complete understanding of the process and especially whether the accessibility of the Cys residues, in our experiments in the presence of Ca^{2+}, can be related to the decrease of the chaperone activity will require further study.

In summary, we have shown that Ca^{2+} reduces the stability of both secondary, tertiary and quaternary structures of α-crystallin, as seen from the decrease in the midpoints of the thermal transitions observed by means of FTIR, CD and UV spectroscopies. A decrease in the molecular chaperone function is also clearly detected in the presence of Ca^{2+}. The decreased chaperone activity is also accompanied by a reduction in DTNB reactivity (possibly reflecting disulfide bond formation) and a partial unfolding of the polypeptide chain. Although it is not clear at the present moment which would be the specific molecular mechanism by which Ca^{2+} could be involved in cataract formation, our results suggest that, in addition to age-related and photooxidation modifications, the decrease in protective capacity (i.e. reduced chaperone activity) may also be responsible for the pathophysiology of the condition. As pointed out before, as high as 64 mM Ca^{2+} has been found in cataractous lenses [19] and a role has been proposed for Ca^{2+} in the cataractogenic process [24]. The observed decrease of the chaperone activity of α-crystallin in the presence of Ca^{2+} could mean a decreased interaction with the other lens crystallins in the cataractogenic lens, thus facilitating the formation of macromolecular aggregates responsible for eye lens opacification. Further studies are needed to unravel the details of the conformational changes in α-crystallin induced by Ca^{2+}.

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