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Calcium Ion Binding to δ- and to β-Crystallins

THE PRESENCE OF THE "EF-HAND" MOTIF IN δ-CRYSTALLIN THAT AIDS IN CALCIUM ION BINDING*

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Abnormal levels of endogenous calcium ions are known to induce eye lens opacity, and a variety of causative factors has been proposed, including calcium-mediated aggregation and precipitation of the lens proteins crystallins. We have specifically looked in some detail at the interaction of Ca^{2+} with various crystallins and its consequences. Lenses incubated in solutions containing 10 mM Ca^{2+} or 5 mM Tb^{3+} opacified. Fluorescence titration of crystallins with $TbCl_3$ revealed that this ion binds to δ- and β-crystallins in solution. Equilibrium dialysis showed that four Ca^{2+} ions bind to one δ-crystallin tetramer with an affinity of 4.3×10^3 M^{-1}. Analysis of the amino acid sequence of δ-crystallin reveals the presence of a calmodulin-type "helix-loop-helix" or "EF-hand" calcium ion binding conformational motif in the region comprising residues 300–350. This is a novel feature of the molecule not reported so far. No other crystallins appear to have this motif. β-Crystallin also binds four Ca^{2+} ions/aggregate unit of mass 160 kDa, with an affinity of 2.6×10^3 M^{-1}, presumably in the midregion of the molecule that is rich in anionic and polar residues. Circular dichroism spectroscopy shows that the binding of calcium ion leads to subtle conformational changes in the molecules, notably in the tertiary structure.

The role of calcium ions in the physiology of cataract formation has been a subject of interest and investigation. The aqueous humor calcium ion concentration in a human eye with a normal transparent lens ranges from 0.5 to 2.0 mM, while it is far larger (0.1–64 mM) in subjects with cataractous lenses. Abnormal levels of Ca^{2+} appear to induce lenticular opacity. As a recent review (1) highlights, the effects of endogenous calcium on lens transparency involve several factors: alteration in the permeability of substances across membrane channels, change in the organization of membrane proteins in the gap junctions (2), precipitation of calcium oxalate and phosphate (3), and aggregation of the lens proteins crystallins at high Ca^{2+} concentrations (4–6). In addition, several other physiological and biochemical processes in the eye lens, *e.g.* protein biosynthesis and metabolism (7, 8), membrane transport and ATPase activity (9–11), proteinase-mediated hydrolysis of lens filaments and of α-crystallin (12, 13), and Ca^{2+}-activated transglutaminase-mediated dimerization of β-crystallin (14) are also dependent on Ca^{2+} ion concentration. The cytotoxic effects of internal calcium on lens physiology have recently been reviewed (15).

We have focused our attention in this paper on calcium ion binding to the lens proteins α-, β-, γ-, and δ-crystallins. The first three are in all vertebrate lenses, though in varying proportions depending on the species, while δ-crystallin is the core protein of avian and reptilian lenses (16). Our study expands and elaborates in some detail some features of such ion binding to crystallins. The early report of Adams in 1929 showed that α- and β-crystallins flocculate in 2.5% $CaCl_2$ solutions (17), and later papers reported the Ca^{2+}-induced aggregation of α-crystallin fractions (4–6). We have analyzed the amino acid sequences of the various crystallins for the presence of calcium ion binding motifs or sequences in them. We find in this analysis that δ-crystallin possesses one calmodulin-type "helix-loop-helix" or "EF-hand" motif that binds calcium ions (18–20) in its primary structure. β-B1b crystallin appears to have regions in its sequence with a propensity for binding this ion. We have assessed the Ca^{2+} binding tendency of these proteins by interacting them first with the trivalent lanthanide ion Tb^{3+} which is a luminescent probe that mimics calcium ion (21). Tb^{3+} ion binds to β- and to δ-crystallins and displays an enhanced fluorescence quantum yield thereupon, while its interaction with α- or γ-crystallins is less pronounced under the same conditions. Next we show that radioactive $^{45}CaCl_2$ binds to β- and to δ-crystallins with significant affinity. Upon binding, calcium ion induces conformational changes in these two molecules, as evidenced by absorption, CD, and fluorescence spectral changes in the protein. We also observed a slight alteration in the rate of photodamage to β- and δ-crystallins in the presence of calcium, probably reflecting conformational effects. It is of interest in this connection to note that the crystal form of δ-crystallin is altered in the presence of calcium ions (22).

MATERIALS AND METHODS

Crystallins—α-, β-, and γ-crystallins were purified from rat lenses by gel filtration methods described earlier (23). δ-Crystallin was, however, purified from 3-day-old chick lenses by Sephacryl S-200 chromatography in pH 9.1 Tris buffer followed by elution in a thiol affinity column from which it elutes in preference to the other crystallins that are retained.

In our present studies we did not observe any significant differences between β-heavy or β_H- and β-light or β_L-crystallins (24) with regard to calcium or terbium binding. Hence we present mostly the results with β_H-crystallin as representative of all β-crystallins.

Equilibrium Dialysis—The detailed methodology of equilibrium dialysis has been described by Potter *et al.* (25). All buffers used for the dialysis experiments were passed through a Chelex 100 column, and all glassware was soaked in EDTA solutions and washed with deionized water prior to use. Protein samples (300 μl, 0.5–2.0 mg/ml)

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in 0.2 M KCl, 20 mM EGTA,[1] 0.02 M imidazole buffer, pH 7.1, were introduced on one side, and the buffer containing known amounts of $CaCl_2$ and ^{45}Ca (specific activity, 98 mCi/g) was introduced on the other side of a dialysis membrane (pretreated sequentially with 0.2% $NaHCO_3$ and EDTA solutions) in a semi-micro dialysis cell. Dialysis was done at ambient temperature, about 25 °C, under constant shaking for 40–50 h, after which 20-μl aliquots were removed from each compartment and the radioactivity measured by scintillation counting. The distribution of radioactive Ca^{2+} between the two compartments was determined from the average of triplicate measurements.

Fluorescence Spectra—All fluorescence spectra were measured using a Hitachi model F-4000 instrument with a built-in computer and in the Correct Spectrum mode. The spectral bandwidths were 3 nm, and the excitation beam shutters were kept closed except during scans, so as to minimize photodamage to the proteins. The quantum yield measurements were done using quinine sulfate as the standard.

Photodamage—The damage occurring to the protein upon irradiation with 295-nm radiation was measured by recording the changes in the intensity of tryptophan emission with time, using the fluorimeter lamp as the irradiation source. The excitation and emission bandwidths were kept at 20 and 1.5 nm, respectively, and emission spectra were recorded as described earlier (23).

CD Spectra—These were recorded in a Jasco J-20 instrument at room temperature. A mean residue molecular weight of 110 was used in the calculation of ellipticity values. The proteins were dissolved in 10 mM Tris·HCl buffer containing EGTA, pH 7, with and without added calcium ions.

Ultraviolet Difference Spectra—Protein solutions of equal concentration in the above pH 7 buffer containing EGTA, with and without Ca^{2+}, were kept in the sample and reference compartments, respectively, and spectra were recorded in the 230–340-nm region in a Hitachi model 330 spectrophotometer. Protein concentrations were determined using the standard Lowry method.

RESULTS AND DISCUSSION

Fig. 1 shows that isolated intact rat lenses opacify upon incubating them in pH 7 Tris buffer containing 10 mM $CaCl_2$. While this is in accord with earlier reports (26), we also found that chick lenses, which contain δ-crystallin as the nuclear protein, also opacify under these conditions. As noted earlier (26), this effect is specific to Ca^{2+}, since incubation with Na^+ or Mg^{2+} ions did not cause opacification. However, we found that incubation with 5 mM $TbCl_3$ induces lens opacity (see Fig. 1); terbium and similar trivalent lanthanum ions are known to be mimics of calcium ion and have been used as spectroscopic probes to monitor Ca^{2+} binding to peptides and nucleotides since they display greatly enhanced luminescence upon binding to these species (21).

The opacification produced in the lens by $CaCl_2$ or $TbCl_3$ could be due to aggregation of the crystallin molecules upon salt binding or due to other factors such as membrane disruption, binding to membrane-associated proteins, salting out, and so on. In an effort to delineate the cause, we next studied the interaction of $TbCl_3$ with isolated crystallins in solution by following the changes that occur in the fluorescence intensity of Tb^{3+} at 488 and 545 nm when titrated with aqueous solutions of α-, β-, and γ-crystallins. Of the three, β-crystallin interacts best with the lanthanide ion and increases its fluorescence. α-Crystallin increases Tb^{3+} fluorescence less efficiently, while γ-crystallin appears to interact with the ion the least of all. We next compared the terbium binding affinity of β-crystallin with that of calmodulin, a standard calcium-binding protein. One molecule of the latter was found to bind four terbium ions, and the dissociation constant, K_d, was found to be about 0.8 μM under the chosen conditions of the experiment. These results are in general agreement with the earlier report of Wallace *et al.* (27), though they found stronger affinity under their chosen conditions. In comparison, β-crystallin was found in our experiments to bind Tb^{3+} ion somewhat weaker, with a K_d value of 2.2 μM. We tried δ-crystallin next and found that it also binds Tb^{3+} ions with about the same affinity as β-crystallin does. In all these cases, the metal ion binds proximally to the aromatic fluorophores Trp (and Tyr) of the protein, since fluorescence energy transfer from these residues to the metal ion was observed. Excitation at 285 nm, the absorption band maximum of the protein aromatic residues, resulted in terbium emission in the visible region. Such energy transfer results indicate that the metal ion is placed at a distance of about 5 Å from the donor fluorophore in the protein (21, 27).

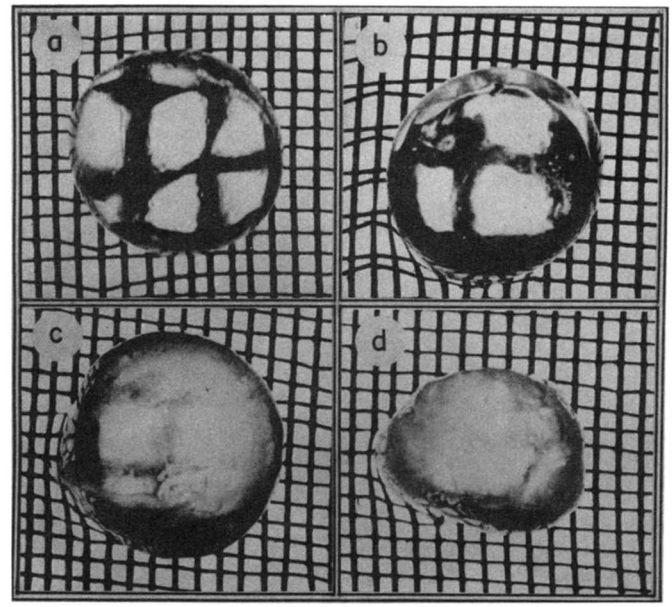

FIG. 1. **Photographs of rat lens incubated for 4 h at 298 K in: (*a*) 10 mM Tris·HCl, pH 7 buffer; (*b*) the above buffer containing 140 mM NaCl; (*c*) the above buffer containing 10 mM $CaCl_2$; and (*d*) the above buffer containing 5 mM $TbCl_3$.** After incubation, these lenses were placed on a wire mesh and photographed.

Based on these results, we next studied calcium ion binding by the equilibrium dialysis method using radioactive $^{45}CaCl_2$ and individual crystallins in solution. α- and γ-crystallins was found not to bind any calcium ions, while β- and δ-crystallins did. β_H-crystallin, with an aggregate molecular mass of 160 kDa, was found to bind about four calcium ions and the dissociation constant calculated at 0.38 mM. In comparison, δ-crystallin, with its tetrameric mass of 200 kDa, was also seen to bind four Ca^{2+} ions but with a dissociation constant of 0.23 mM, and calmodulin also bound four calcium ions with a K_d value of 4 μM. These values were calculated with the generally accepted assumption that the binding sites are equivalent and independent. Some comments regarding the use of Tb^{3+} and other lanthanide ions as Ca^{2+} mimics are in order in this context. One is that the higher affinity of the proteins for Tb^{3+} than for Ca^{2+} is usual and reflects the higher charge density of the lanthanide ion. Second is the tendency of these ions to form weak "outer sphere complexes" utilizing their d and f electronic levels, which the calcium ion cannot do. Thus, while Ca^{2+} binding is invariably mimicked by the lanthanide ions, the reverse is not always true. Even substances that do not bind calcium might display weak complexation towards lanthanons (21, 28). This might explain why α-crystallin, which does not appear to bind Ca^{2+} with any avidity, is able to enhance the fluorescence of added $TbCl_3$.

[1] The abbreviation used is: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

In an effort to understand why β- and δ-crystallins bind calcium ions and α- and γ-crystallins do not, we next analyzed the amino acid sequences of various crystallin molecules in order to detect any regions, motifs, or sequences that display affinity to the Ca^{2+} ion. Calcium ion-binding proteins are known to often contain consensus sequences and conformational motifs that are responsible for the complexation. The calmodulin family is characterized by the EF-hand or the helix-loop-helix motif (18–20). The loop region is a sequence of about 12 residues with alternating amino acids which contain anionic or electronegative groups in their side chains that coordinate octahedrally with the calcium ion. This ion binding region is flanked by two amphipathic α-helical segments (the E- and F-helical regions) which stabilize the binding site. Calmodulin contains four such EF-hand motifs in its primary structure, where the four Ca^{2+} ions bind.

When we analyzed the sequences of the various crystallins for the presence of such EF-hand motifs, we found to our surprise that the region comprising residues 300–350 in chicken δ_2-crystallin might well qualify as a candidate for this helix-loop-helix motif. The 18-residue sequence 300–317 contains only helicogenic residues and has been suggested to adopt the α-helical fold (29). In Fig. 2, we show a helical wheel or the Edmundson projection diagram (30, 31) of this fragment in the α-helical form. It is worthy of note that the hydrophilic residues are naturally clustered together, while the hydrophobic residues are segregated into another cluster, making the helix an amphipathic one. In the sequence of chicken δ_1-crystallin, a closely related variant protein, the amino acid replacements in this region are the conformationally and polarity wise conservative ones of Ile for Val in position 310 and again for Leu-317 of the sequence (32). The residues Pro in position 318 and Ser in 319 are both nonhelicogenic and would form the start of the loop region. The 13-residue sequence 318–330 contains several nonhelicogenic residues and, interestingly enough, contains in alternating positions residues with side chains that are capable of cation binding, namely Thr, Asn, Asp, Gln, Asp, and Glu. Here again, the replacement of Tyr-321 by Phe and Asn-322 by Ser in α_1-crystallin (32) is innocuous in terms of ion binding ability. The sequence 331–350 contains many helicogenic residues and is predicted to be in the α-helical conformation (29). As Fig. 2 reveals, the striking feature here too is the segregation of the hydrophobic and hydrophilic residues to produce an amphipathic helix. Again, the residue replacements seen in α_1-crystallin (Leu for Phe-333 and Ala for Val-345) are functionally acceptable ones. In sum, the region 300–350 in α-crystallin possesses all the features described for the EF-hand motif of the calmodulin family of calcium-binding proteins.

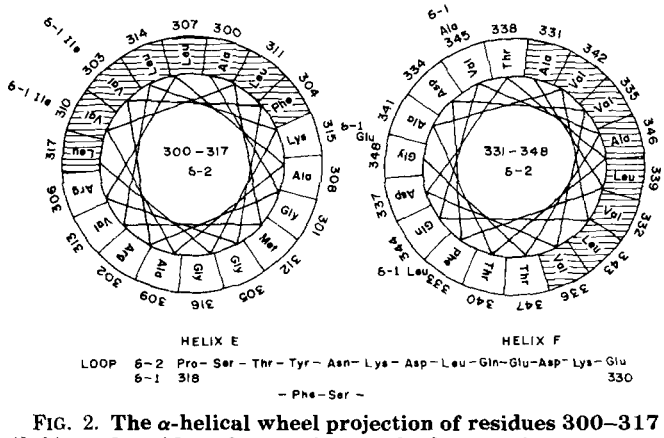

FIG. 2. **The α-helical wheel projection of residues 300–317 (*left*), and residues 331–350, and the loop region sequence 318–330 of chick δ_2-crystallin.** The *shaded parts* of the helical wheels represent the hydrophobic regions. The residue changes seen in chick lens δ_1-crystallin are also shown at the respective positions.

We have also analyzed the amino acid sequences of the α-, β-, and γ-crystallin families, as also of the duck lens ε-crystallin (33), the frog lens ρ-crystallin (34), and the guinea pig lens ζ-crystallin (35), and could not detect the presence of the EF-hand motif in any of these. This is not surprising in the case of α-, β-, γ-, ε-, and ρ-crystallins which do not adopt the α-helical conformation to any major extent, unlike δ-crystallin. ζ-crystallin is related to alcohol/polyol dehydrogenases but devoid of the zinc ion binding domain present in the latter,[2] and in this analysis it did not reveal the Ca^{2+} binding motif of the calmodulin class. We also looked for the presence in these molecules of other calcium binding sequences, *e.g.* the lipocortin consensus sequence (37) and the clotting factors sequence (38), but failed to find any of these two. However, the 100-residue sequence in the middle region of the β-crystallin chains, namely the sequence comprising residues 118–224 of β-B1b crystallin (39), is notably rich in anionic and polar amino acids that can bind Ca^{2+}, in particular the regions 118–131 (YPRWDTWTSSYRSD) and 182–206 (SITVSSGT WVGYQYPGYRGYQYLLE). It is interesting to note that the majority of the Tyr (Y) and Trp (W) residues of β-B1b crystallin are located in and near these two regions. This might be the reason behind the fluorescence energy transfer observed from the protein to the Tb^{3+} ion. The nature and type of the calcium binding site in β-crystallin needs to be investigated further, particularly since this protein binds Ca^{2+} and Tb^{3+} with about the same affinity as δ-crystallin does, even though it lacks the EF-hand motif. That β-crystallins might belong to a single superfamily that also includes the calcium binding protein S of the bacterium *Myxococcus xanthus* has already been noted (16). The details of this protein S type calcium binding site need to be studied, particularly since γ-crystallins, which also belong to the same family, do not bind calcium or terbium ions. We could not detect potential ion-complexing regions in the primary structures of γ-crystallins or even in those of α-, ε-, ρ-, or ζ-crystallins. It is thus likely that the Ca^{2+}-induced aggregation of α-crystallin reported earlier (4–6) involves nonspecific binding.

We next monitored the conformational changes induced by the addition of calcium ions to solutions of these proteins. Fig. 3 shows that the fluorescence spectral band of β-crystallin is red-shifted by about 10 nm, and the intensity increased by about 10% upon the addition of $CaCl_2$ at a mole ratio of 4 or more. These changes are indicative of a greater degree of exposure of the aromatic side chains (largely of Trp, as judged by the emission band maximum values) to the aqueous medium upon ion binding. The fluorescence spectrum of δ-crystallin is of particular interest since, unlike the other crystallins, it shows an unusually blue-shifted emission band, a doublet with the more intense band at 315 nm and the other at 325 nm (see Fig. 4). The band positions and the relative intensities here are insensitive to changes in the excitation wavelength in the region 260–295 nm. These results suggest the primary fluorophore in δ-crystallin to be Trp, rather than Tyr or Phe, and that the microenvironment of the fluorophore is quite apolar or hydrophobic (40). Titration with calcium ions does not cause any band shift in this case but reduces the intensity of emission. The relative intensity ratio of the two bands, however, depends on the excitation wavelength. Upon exciting at 290 nm or above, the intensity of the 315-nm band is higher than that of the 325-nm band, whereas the

[2] T. Borras, personal communication.

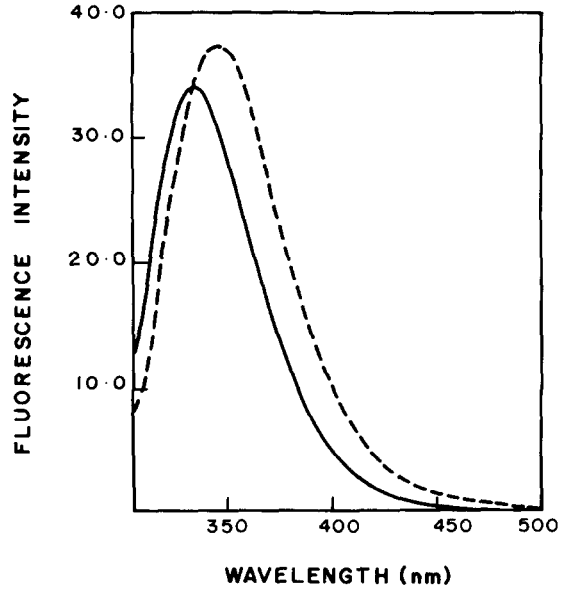

FIG. 3. **Corrected fluorescence spectrum of rat lens β-crystallin in 10 mM Tris·HCl, pH 7 buffer in the absence (——) and presence (– – –) of 10 mM $CaCl_2$.** Excitation was at 280 nm, and the spectral bandpass values were 3 nm.

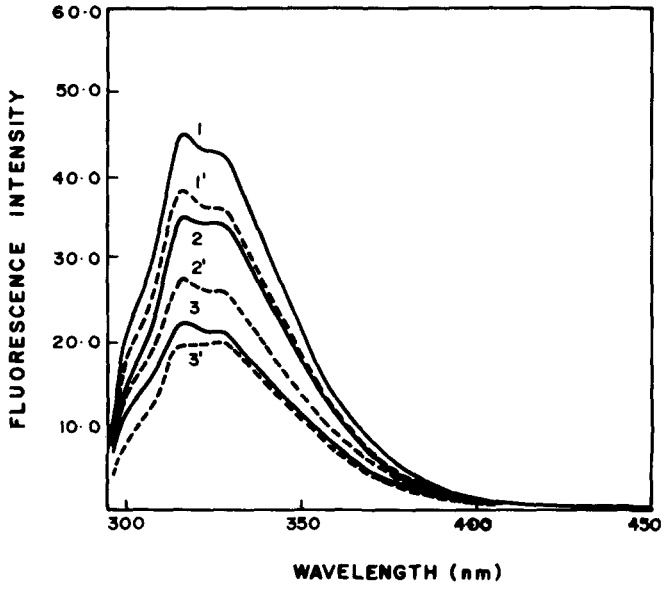

FIG. 4. **Corrected fluorescence spectrum of chick lens δ-crystallin in 10 mM Tris·HCl buffer, pH 7, in the absence (——) and presence (– – –) of 10 mM $CaCl_2$.** *Curves 1, 2,* and *3* are for the calcium-free protein but excited at 270, 280, and 290 nm, respectively. *Curves 1′, 2′,* and *3′* are for the protein solution containing calcium and excited at 270, 280, and 290 nm, respectively.

TABLE I

Fluorescence changes upon calcium ion binding to β- and δ-crystallins

	β-Crystallin		δ-Crystallin	
	Free	+10 mM Ca^{2+}	Free	+10 mM Ca^{2+}
Emission maximum, in nm	334	344	315, 325	315, 325
Emission quantum yield	0.057	0.065	0.109	0.086
Emission anisotropy	0.066	0.060	0.142	0.170

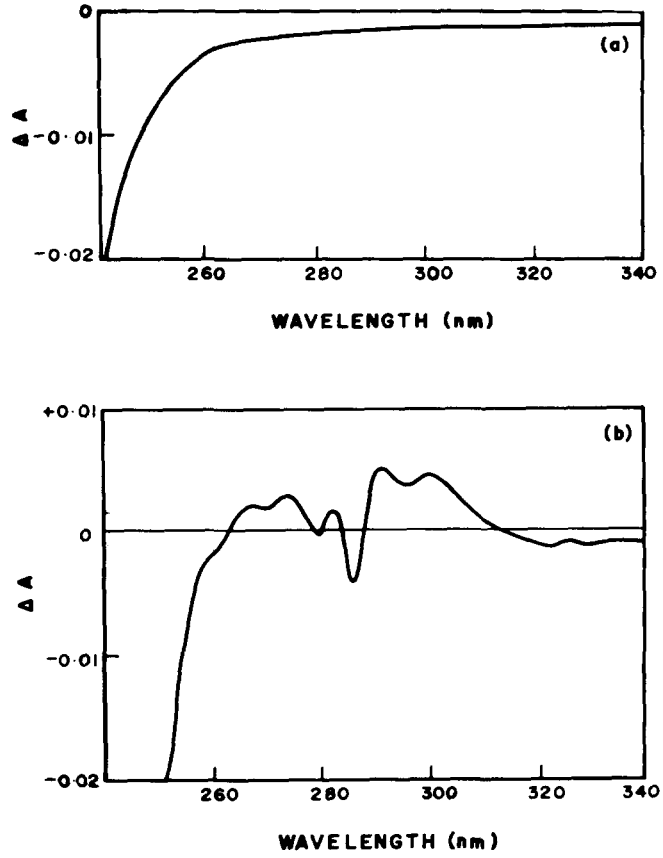

FIG. 5. **Ultraviolet difference spectra of (*a*) β-crystallin and (*b*) δ-crystallin.** The protein solution in 10 mM Tris·HCl buffer, pH 7, containing EGTA was kept in the same compartment, while that containing 10 mM $CaCl_2$ was in the reference compartment.

opposite behavior is noticed upon excitation at 280 nm or below. It appears likely that the 315-nm band has, as is to be expected, a higher contribution from the emission of Tyr (and Phe) residues while the 325-nm band is largely due to Trp. Table I lists the fluorescence band positions, quantum yields, and the emission anisotropy values of β- and δ-crystallins in the presence and absence of $CaCl_2$. The quantum yield and band position data suggest that the Trp residues in β-crystallin are exposed to the solvent when the protein binds calcium ions, but their mobility is not significantly altered since the anisotropy value is unchanged. In contrast, calcium ion binding to δ-crystallin leads to a greater degree of burial of the aromatic residues into a more hydrophobic environment and also a reduction in their freedom of motion.

That the tertiary structural environment around the aromatic residues is altered differently in β- and in δ-crystallins is also suggested by ultraviolet difference spectral changes. The difference spectrum of β-crystallin, in the presence and absence of Ca^{2+}, shown in Fig. 5, does not display any perturbation in the 260–300-nm region upon ion binding. With δ-crystallin, on the other hand, maxima are seen at 298 and 292 nm (the region of the ^1L_b band of Trp (41)) and minima at 285, 278 (both of which are in the ^1L_b band of Tyr (41)), 274, and 268 nm (^1L_b of Tyr and ^1L_a of Trp (41)). Since the ^1L_a and ^1L_b bands display different medium effects (41), it appears that the Tyr and Trp residues of δ-crystallin are differently affected by calcium binding. This becomes even more suggestive when we follow the changes in the CD spectra of the two crystallins upon calcium ion addition. Figs. 6 and 7 show these changes in β- and in δ-crystallins, respectively. The ellipticity values of the 294-, 272-, and 260-nm bands are reduced

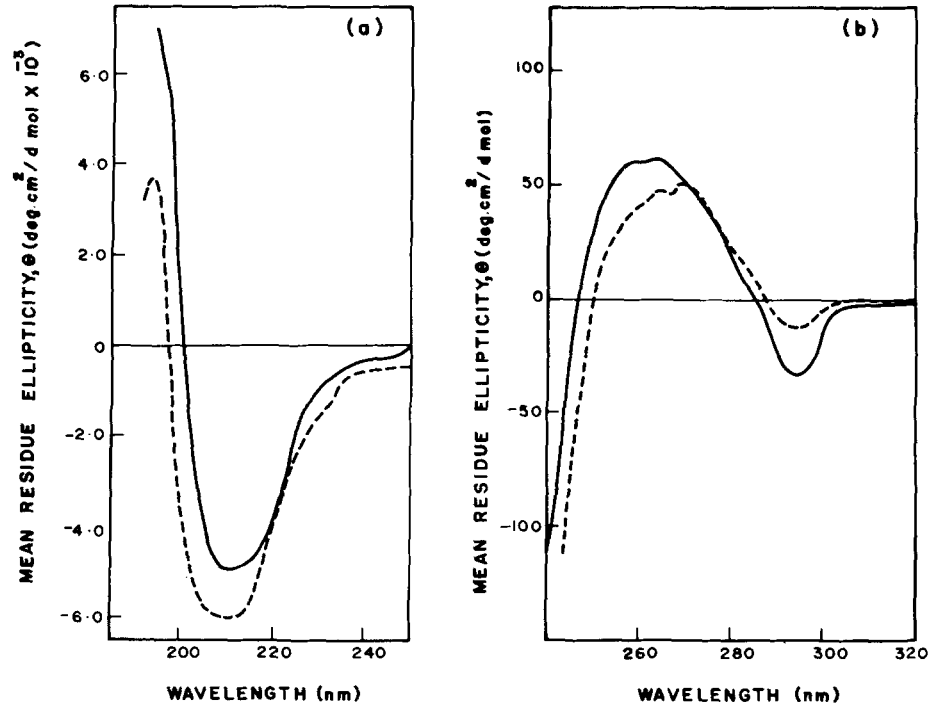

FIG. 6. **Circular dichroism spectra of native β-crystallin in 10 mM Tris·HCl, pH 7 buffer in the absence (——) and presence (– – –) of 10 mM $CaCl_2$.** *Panel a* focuses on the far ultraviolet region while *b* concentrates on the near ultraviolet region.

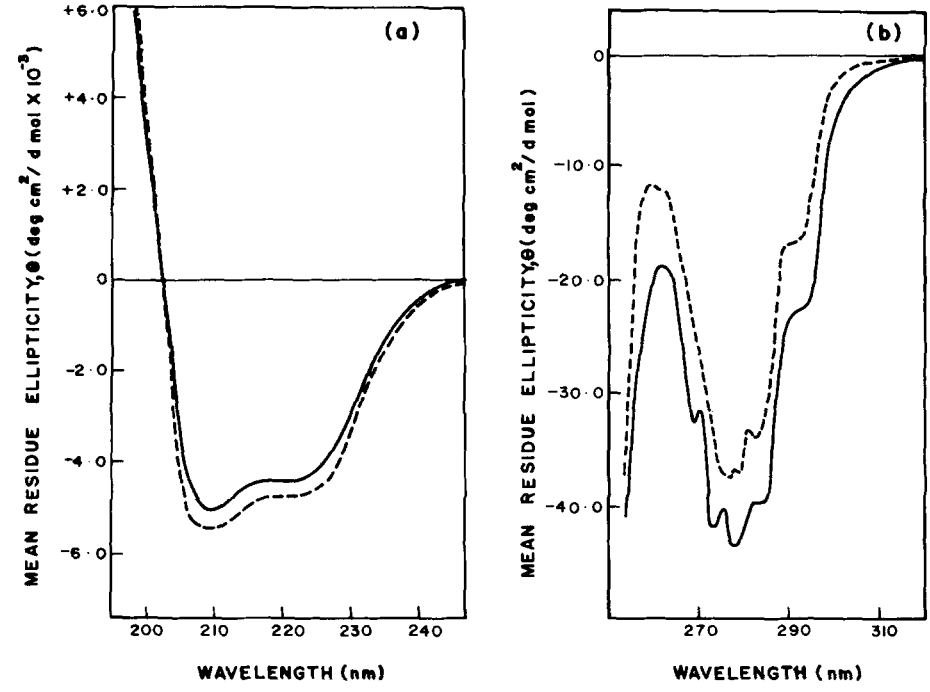

FIG. 7. **Circular dichroism spectra of native δ-crystallin in pH 7 buffer in the absence and presence of 10 mM $CaCl_2$.** Conditions and other details were the same as in Fig. 6.

considerably with $CaCl_2$, suggesting increased freedom of motion of the aromatic side chains in β-crystallin. The red shifting of the latter two bands might also indicate a more polar environment upon ion binding. The far ultraviolet CD spectrum of β-crystallin is also altered with calcium; the ellipticity of the negative band near 215 nm is increased while the positive band near 195 nm is attenuated, suggesting a decrease in the secondary structural order and increase in the extent of unordered conformation (42). Here again, δ-crystallin behaves differently. The freedom of motion of the aromatic side chains is not affected as much in the avian protein upon ion binding and corroborates the fluorescence spectral results. The α-helical conformation that δ-crystallin adopts is only marginally affected by the cation, again in contrast to β-crystallin.

There is now growing evidence (43, 44)[3] that photodamage of proteins in general, and crystallins in particular, may be modulated by the microenvironment of the labile residues (Trp, Tyr, His, Cys). In light of the finding that calcium ion binding alters the environment around the aromatic residues and the conformations of β- and δ-crystallins, we studied the effect of added $CaCl_2$ on the rate of photodamage of these molecules by following the loss of Trp fluorescence intensity with time of irradiation with 295-nm light. As Fig. 8 shows,

[3] S. Chenchal Rao, Ch. Mohan Rao, and D. Balasubramanian, manuscript in preparation.

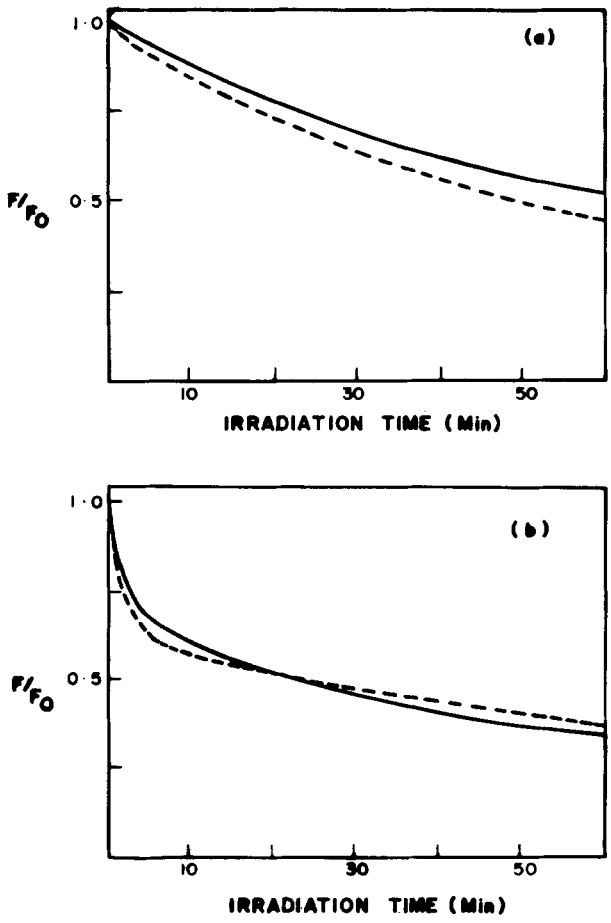

FIG. 8. **The variation in the intensity of Trp fluorescence of (*a*) β-crystallin and of (*b*) δ-crystallin solutions in 10 mM Tris·HCl buffer, pH 7, as a function of the time of irradiation with 295-nm light from the spectrofluorimeter lamp.** The excitation bandpass was kept at 20 nm during the irradiation. Curves in the absence (——) and presence (– – –) of calcium are shown in each case.

this rate is increased noticeably in the case of β-crystallin in the presence of calcium. As noted above, calcium binding exposes the aromatic residues of this protein to a more polar environment; the rate of Trp photooxidation has been reported to be higher when the residue is in a polar environment than otherwise (44). The behavior of δ-crystallin, shown in Fig. 8, is consistent with this point, since calcium binding here alters the environment around the aromatic residues in the opposite fashion.

These results demonstrate a hitherto unrecognized feature of β- and of δ-crystallins, namely that these are capable of binding calcium ions with defined stoichiometry and moderate affinity. The significance of this property on lenticular processes and cataract in particular is yet to be understood. Until now, the effect of Ca^{2+} has been thought to involve largely the membrane-associated proteins of the lens, but the recent paper by Clark *et al.* (36) has shown that even calf lens cortical homogenate devoid of membranes has a decreased transmittance in the presence of 10 mM calcium; interestingly, the nuclear fraction is not affected even up to a concentration of 50 mM calcium. The action of calcium appears to be primarily on the cortical cells of the calf lens. It would be interesting to study the behavior of avian lenses in this connection.

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