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Conformational studies on δ-crystallin, the core protein of the bird eye lens[+]

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Abstract. Proteins that perform other functions elsewhere appear to be recruited for structural purposes in the eye lens. The lens being a tissue with very little metabolic activity and little or no turnover, the lens proteins, crystallins, are long lived. In an effort to understand whether their recruitment might be related to their conformation and structural stability, we have examined these features of the avian lens protein δ-crystallin. The native molecule is a tetramer (molecular mass 200 kDa) that is highly α-helical in conformation, and with an unusually blue tryptophan fluorescence (315, 325 nm), which is only partially quenched by conventional quenchers. We show that the fluorescence doublet arises due to Trp residues that are effectively buried inside the rigid hydrophobic core of the tetrameric aggregate. The protein is heat stable up to 91°C. Guanidinium chloride (GuHCl) effects the complete denaturation of δ-crystallin, whereas heat or urea treatment results in only partial unfolding or dissociation. The initial transition is the disruption of the quaternary structure by perturbing the intersubunit interactions, leading to exposure of hydrophobic contact surfaces (as monitored by extrinsic probe fluorescence). This initial transition is seen upon heating to 60°C as well as in 1 M GuHCl and 4 M urea. We show that in 2·2 M GuHCl the molecule is swollen but is still largely helical with the Trp residues being present in a somewhat more polar environment than in the native molecule. Beyond 4 M GuHCl there is a gradual unfolding of the molecule, which is complete in 6 M GuHCl. This structural robustness of δ-crystallin might be important in its recruitment as the core protein of the avian lens.

Keywords. Eye lens proteins; denaturation intermediates; swollen globule; protein quaternary structure; *S*-class protein fluorescence.

1. Introduction

One of the startling findings in the recent past is that certain proteins that perform designated functions (e.g., as enzymes) in given tissues are recruited to act as the structural proteins of the eye lens, called crystallins (Wistow and Piatigorsky 1988). α-crystallin, which is an abundant structural protein in the crotical region of the lens, belongs to a class of heat shock proteins (de Jong *et al* 1988); recent evidence suggests

[+]Dedicated to Professor C N R Rao on his sixtieth birthday.

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Abbreviations. ANS, 8-anilinonaphthalene-1-sulphonic acid; EDTA, ethylene diamine tetraacetate; GuHCl, guanidine hydrochloride; HPGPC, high performance gel permeation chromatography; OD, optical density; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Tris, *tris*(hydroxymethyl) aminomethane; UV-CD, ultraviolet circular dichroism.

its role as a chaperonin as well (Horwitz 1992). β- and γ-crystallins belong to the superfamily of the protein *S* class (Wistow *et al* 1985; Lubsen *et al* 1988). δ-crystallin, which replaces γ-crystallin as a core protein of the avian lens, is identified as the enzyme argininosuccinate lyase (Piatigorsky *et al* 1988), while ε-crystallin is lactate dehydrogenase (Wistow *et al* 1987), and ζ-crystallin belongs to the class of sorbitol dehydrogenases (Rodokanaki *et al* 1989). It is of interest to investigate whether there are any structural features that these enzymes have, which make them specially suitable for recruitment as lens proteins.

While the components from other tissues turn over with time, the eye lens is noted for its sluggish metabolism, and little or no turnover. Consequently, the crystallins that constitute over 35% (w/v) of the lens stay there for decades, as long-lived molecules. Their structural integrity is thus vitally needed in order to keep the lens transparent over the years. This requires that the crystallins be structurally and conformationally robust, since conformations, intermolecular interactions, and solubility play a crucial role in the biophysics of lens transparency (Tardieu and Delaye 1988). The cortical lens protein α-crystallin appears to fulfil some of these needs; it is a micellar type multimeric aggregate that provides for the short range structural order, and a conformational stability that is not perturbed even upon heating to 90° (Maiti *et al* 1988). Indeed, the other common crystallins, except the γ-crystallin of the mammalian lens nucleus, are also multimeric proteins. Their structural stability is worthy of study in this context. We focus attention in this paper on δ-crystallin which is the core protein of the avian lens.

δ-Crystallin is an aggregate of four subunits, each of molecular mass around 50 kDa. Two variants of δ-crystallin, namely $\delta 1$ and $\delta 2$, are known and their sequences have been determined (Nickerson and Piatigorsky 1984; Nickerson *et al* 1986). The molecule has been identified to be essentially the same as the enzyme argininosuccinate lyase, which has been recruited in the lens of the birds and reptiles as the structural protein of the lens nucleus or core (Piatigorsky and Wistow 1989). It would be of interest to study the conformational aspects of this protein in order to understand whether there are any special features that render it particularly suitable for its choice as a lens protein. Earlier results have shown it to be different conformationally from the mammalian crystallins, in that it is highly α-helical and also that it shows few age dependent conformational changes (Yu *et al* 1977; Horwitz and Piatigorsky 1980). We had also shown earlier that δ-crystallin has the E-F hand (helix-loop-helix) motif for calcium ion binding that is typical of the calmodulin family (Sharma *et al* 1989). It is not clear what role these features play in providing the bird lens with a greater degree of spherical packing, higher accommodative power and long-term stability (Kuck *et al* 1976), in comparison to the mammalian eye lens. We have therefore investigated the conformational features of chick lens δ-crystallin is some detail in this paper.

2. Experimental procedures

2.1 *δ-Crystallin purification*

δ-Crystallin was isolated from lenses of 3-day-old chicks according to the previously described method (Narebor and Slingsby 1985). This procedure gives almost

exclusively δ1-crystallin, which we report on here. The lenses were homogenized in Tris-HCl (0·05 M; *p*H 9·0) buffer containing 0·1 M NaCl, 1 mM EDTA and 0·02% sodium azide. The extract was spun at 10 000 g for 1 h at 4°C and the supernatant was chromatographed on Sephacryl S-200 gel filtration column (4 cm × 140 cm) equilibrated in the same buffer. The *p*H was kept at 9·0 to ensure efficient separation of δ-crystallin from β-crystallin aggregates.

2.2 *Solutions for denaturation*

Both urea and guanidine hydrochloride (GuHCl) of high purity were obtained commercially. These showed only negligible fluorescence at the highest concentrations and were hence used without further purification. Since 8 M urea did not denature the protein completely, GuHCl had to be used in order to effect complete denaturation.

The amount of GuHCl needed for 8·0 M solution was weighed into a standard flask, to which calculated amounts of sodium dihydrogen phosphate and disodium hydrogen phosphate (for 0·1 M solution) were added, dissolved in deionized water and the *p*H adjusted to 7·0. The solution was then passed through 0·22 μm Millipore filter. The molarity was determined from refractive index measurements (using a Schmidt–Haensch – DUR Refractometer thermostated at 25°C), using the equation as described earlier (Pace *et al* 1989). Urea of 9 M concentration was made in a similar manner.

Stock protein solutions were prepared in deionized water to an approximate concentration of 2 mg/ml, passed through 0·22 μm Millipore filters and their absorption spectra measured. These solutions were then diluted tenfold into stock GuHCl and buffer solutions to give protein solutions dissolved in various concentrations of GuHCl and urea, each containing constant amount of protein. Similar solutions were prepared from a stock tryptophan (Trp) solution.

2.3 *Fluorescence spectroscopy*

All fluorescence measurements were done using a Hitachi model F-4000 spectrofluorimeter using a 1·0 cm pathlength quartz cuvette, thermostated at 25 ± 0·5° and corrected for the lamp spectral intensity distribution. The excitation wavelength was 295 nm and emission was scanned in the range 300 to 500 nm, with excitation and emission handwidths of 3 nm. The wavelength 295 nm was chosen specifically in order that Trp residues alone be excited and their emission studied, without interference from the tyrosine (Tyr) residues.

For fluorescence quenching studies, the protein concentrations used were about 0·1 mg/ml in phosphate buffer (0·1 M, *p*H 7·0) and had an OD of less than 0·05 at 295 nm. Potassium iodide, cesium chloride and acrylamide were used as quenchers. The stock concentrations of these were 5 M in the same buffer. The KI solution contained 100 μM sodium thiosulphate, in order to avoid the formation of triiodide. The quenchers were not used beyond 1 M effective concentration in order to avoid denaturing the protein (Eftink and Ghiron 1977). Shifts of Trp fluorescence to longer wavelengths were not observed at 1 M quencher concentration suggesting that protein denaturation has not occurred (Burstein *et al* 1977). Dilution corrections were done in order to account for the addition of quencher solutions. Fluorescence intensities were also corrected for attenuation of excitation wavelength by added acrylamide

using the correction factor previously described (Eftink and Ghiron 1977), namely

$$F_{corr} = F_{meas} \times 10^{0.23}[\text{acrylamide}]/2,$$

where F_{corr} and F_{meas} are corrected and directly measured fluorescence intensities respectively and the brackets denote the molar concentration of acrylamide. The fluorescence data were then plotted according to the Stern–Volmer equation

$$F_0/F = 1 + K_{SV}[Q],$$

and graphically analysed by the modified Stern–Volmer equation (Lakowicz 1983).

$$F_0/\Delta F = 1/f_a K_{SV}[Q] + 1/f_a,$$

where F_0 and F are fluorescence intensities before and after the addition of quencher, $\Delta F = F_0 - F$, $[Q]$ is concentration of the quencher, K_{SV} the Stern–Volumer quenching constant, and f_a the faction of fluorophore residues available to the quencher.

The fluorescence quantum yields (φ) of tryptophan were determined and previously described (Parker and Rees 1960),

$$\text{area}_{(Pr)}/\text{area}_{(R)} = \varphi_{(Pr)/\varphi}(R)_{(R)}OD_{(R)},$$

where the subscripts Pr and R refer to protein and reference substance tryptophan respectively. The area was calculated for the whole corrected emission spectrum from 300 nm to 500 nm, both for protein and tryptophan under similar conditions of buffer and denaturant concentrations. OD is the optical density at 295 nm. φ_R was taken as 0·2 for all calculations. Baseline substraction was done for all spectra, thereby correcting for Raman scattering.

For thermal denaturation studies, the sample was equilibrated in a constant temperature fluorescence cell connected to a water bath. The temperature was measured by a copper–constantin thermocouple (connected to a Bat-10 Physitemp thermometer) and Trp fluorescence spectra were recorded in the region 300 to 500 nm.

The extrinsic fluorescence probe 8-anilinonaphthalene-1-sulphonic acid (ANS) was used to monitor the changes in the accessibility of hydrophobic regions in the protein, both during heat and chemical denaturation of δ-crystallin. ANS was twice recrystallized from methanol and a stock of 25 mM was prepared in methanol. This stock was added to the protein solution to get a final effective concentration of 250 μM ANS. Excitation was done at 370 nm and corrected emission spectra were recorded from 400 nm to 600 nm. The excitation and emission bandpass were 3 nm each. The typical fluorescence spectrum of free ANS diluted into buffer showed an emission peak at about 520 nm. The protein to probe ratio was 100:1. The use of ANS to monitor accessible hydrophobic surfaces in proteins has been earlier described by Cardomone and Puri (1992).

2.4 *Circular dichroism spectroscopy*

CD-spectra were recorded on a Jasco J-20 automatic recording spectropolarimeter or a Jobin-Yvon mark V dichrograph. The instruments were calibrated with *d*-10-camphorsulphonic acid before use. The protein concentrations were determined by amino acid analysis. Cells of 0·02 cm and 0·05 cm pathlengths were used to record

the peptide CD in the far UV range (250–190 nm) and a 5·0 cm pathlength cell was used to record the CD in the aromatic region of 310–250 nm.

For denaturation studies, spectra in the far UV were recorded in the region of 210–230 nm, along with the baselines. The values at 222 nm were used for analysis of denaturation curves. Complete far-UV and near-UV CD of the protein were measured at a few denaturant concentrations.

The ellipticity values [Θ] values were calculated as given below

$$[\Theta] = \Theta_{meas}\, 100\, MRW/(c\, l)$$

where [Θ] is the mean residue ellipticity in deg.cm^2.dmol^{-1}, $[\Theta]_{meas}$ the measured value of ellipticity, MRW the mean residue weight (taken as 115), c the concentration of protein in mg/ml and 1 the pathlength in cm.

2.5 *HPGPC studies*

High performance gel permeation chromatographic studies were done using a TSK G3000SW (7·5 × 600 nm) fitted with a guard column. The column was equilibrated with phosphate buffer (0·1 M, *p*H 7·0) at a flow rate of 0·6 ml/min. For denaturation studies, a stock solution of GuHCl was diluted into phosphate buffer to give the required concentration of GuHCl and at least 6–8 bed volumes of this were passed through the column at 0·6 ml/min before injecting the protein. Protein pre-equilibrated in the buffer/GuHCl by dialysis was used for loading the column. A sample of 50–100 μl was used per injection, and at least 3 injections were made for each buffer condition.

2.6 *Analytical ultracentrifugation*

Analytical ultracentrifugation studies were carried out on a Beckman Spinco model E analytical ultracentrifuge equipped with Schleiren optics and RTIC unit. The AnHTi rotor was run at 68,000 rpm for determination of the $s_{20,w}$ of the native δ-crystallin (8 mg/ml) in phosphate buffer (0·1 M, *p*H 7·0). Sedimentation velocity runs were also done for the protein in 5·4 M urea and 2·2 M GuHCl, where the active protein is expected to be disaggregated. Correction factors for temperature and solvent viscosity were included in the calculations.

3. Results and discussion

3.1 *Native δ-crystallin*

The native molecule of δ-crystallin from chicken lens has been shown to have a molecular weight around 200 kDa by ultracentrifugation (Clayton and Truman 1967), chromatography (Williams and Piatigorsky 1979; Pal *et al* 1980), and HPLC (Williams *et al* 1982). Two heterogenous polypeptides of approximately 48 and 50 kDa could be resolved on SDS-PAGE in the presence of 8 M urea (Piatigorsky *et al* 1977; Raszelbach *et al* 1977). δ-crystallin has no cysteines; thus the native molecule of 200 kDa is a tetrameric aggregate that is held together by non-covalent forces. Our analysis of native δ-crystallin using HPGPC on TSK G3000SW shows the native molecular

weight to be around 200 kDa. The two polypeptides of δ crystallin, δ1 and δ2, are known to be two distinct gene products which share a 91% homology (Nickerson *et al* 1986; Wawrousek and Piatigorsky 1987). We work here largely with δ1-crystallin. Our analysis of the sequence has shown that δ-crystallin has a 13-residue-long non-helicogenic region (residues 318–330) rich in anionic charged amino acids, flanked on either side by helicogenic residues (300–317 and 331–350) that can fold into amphipathic α-helix. This region is typical of the 'E helix–loop–F helix' motif of calcium binding proteins, and has been suggested as the binding site for calcium in δ-crystallin (Sharma *et al* 1989).

We have further noted that calcium binding to δ-crystallin perturbs the vibronic fine structure of the aromatic side chains in δ-crystallin. The aromatic CD spectrum, in the absence of calcium, shows a well-defined structure (figure 1). The minimum at 292 nm is attributed to the ^1L_b band of Trp which also has a contribution at 268 nm, while the three prominent band minima at 274, 278 and 285 nm are assigned as the vibronic bands of the ^1L_b bands of the Tyr chromophore (Strickland 1974).

The far UV CD spectrum of δ-crystallin (figure 1) is characteristic of high α-helical content, in agreement with earlier studies on both δ-crystallin in the lens *in situ* by Raman spectroscopy (Kuck *et al* 1976; Yu *et al* 1977) and in solutions of purified δ-crystallin (Piatigorsky *et al* 1977; Williams *et al* 1982). The α-helical content of embryonic chicken δ-crystallin has been found to be as high as 82% (Horwitz and Piatigorsky 1980).

The fluorescence spectrum of δ-crystallin excited at 280 nm shows a shoulder at 307 nm and a doublet at 315 and 325 nm (figure 2a). Since both Tyr and Trp are excited at this wavelength, the assignment of the emission bands poses a problem. Upon excitation at 295 nm (or at 300 nm), the protein shows a highly blue-shifted fluorescence doublet at 315 and 325 nm, but the shoulder at 307 nm is absent. As the absorption of Tyr at 295 and 300 nm is negligible, the emission doublet is assignable

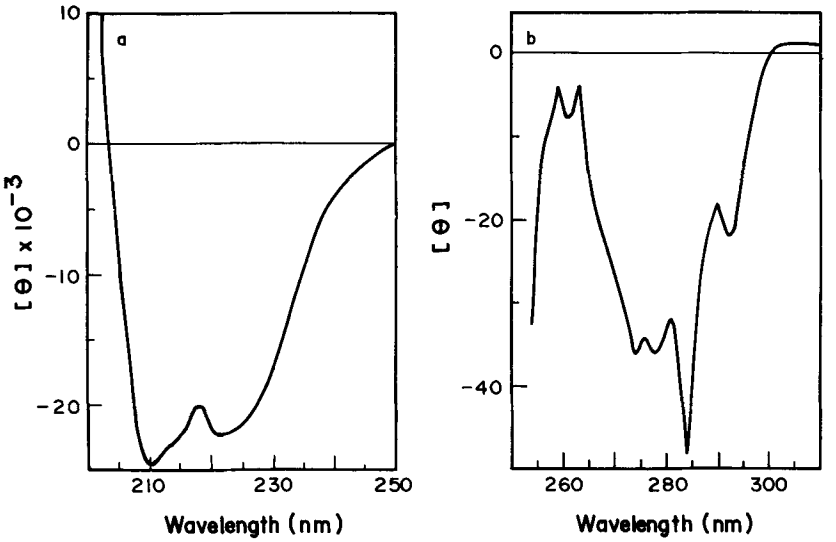

Figure 1. Circular dichroism spectra of δ-crystallin: (**a**) peptide absorption region; (**b**) aromatic absorption region. Protein concentration around 0·8 mg/ml in *p*H 7·0 phosphate buffer. Ellipticity in units of deg.cm^2.dmol^{-1}.

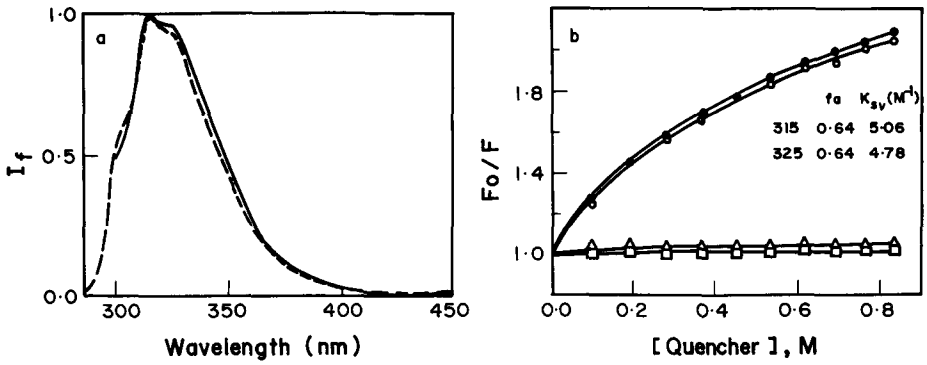

Figure 2. (**a**) Fluorescence emission spectra of native δ-crystallin excited at 280 nm (——) and 295 nm (-----). (**b**) Stern–Volmer plots for quenching the Trp emission doublet in δ-crystallin by (i) acrylamide at 315 nm (●) and at 325 nm (○); (ii) iodide (Δ) and (iii) cesium (□).

to the Trp residues of the protein; hence the 307 nm band should be due to the Tyr residues. Such unusually blue-shifted Trp emission maxima are present only in a few other proteins, belonging to the S-class, such as *E. coli* L-asparaginase and ribonucleases T1 and C2 (with Trp emission maxima at 318, 320 and 320 nm respectively) (Demchenko 1986). We thus classify the Trp environment of δ-crystallin as belonging to the S-class, with a largely non-polar and rigid hydrophobic environment surrounding it (Burstein *et al* 1977). It is noteworthy that amongst the eye lens proteins, δ-crystallin is unique in providing such a highly apolar environment for its Trp residues; γ-crystallin displays its Trp emission maximum at 324 nm while β- and α-crystallins display their emission at higher wavelengths, namely 330–338 nm (Chakrabarti *et al* 1986).

In order to probe the nature of the microenvironment of Trp residues in δ-crystallin, we looked at their accessibility to fluorescence quenchers. The Stern–Volmer plots (figure 2b) show that ionic quenchers like iodide and cesium do not alter the protein fluorescence. This behaviour of Trp residues shows they are accessible for quenching by iodide (Augusteyn *et al* 1988; Phillips and Borkman 1988). Thus the Trp environment in δ-crystallin is non-ionic, buried and not accessible to either iodide or cesium ions. However, acrylamide, which can partition into hydrophobic pockets of proteins, quenches the emission effectively, confirming an apolar microenvironment for Trp, as suggested by the emission maxima themselves. The quenching plots are curved, suggesting that some ground state interaction is likely. Replotting the Stern–Volmer plots by the modified equation gave values for the quenching constant (K_{SV}) and the fractional accessibility (f_a) for quenching by acrylamide. From the viewpoint of accessibility, the Trp environment thus seems to belong to the "single Trp class" (Eftink and Ghiron 1977a). Since the f_a value for acrylamide was 0·64 both in the case of the 315 and 325 nm bands, this suggests that part of the Trp environment is inaccessible to the quencher. It is possible that the Trp contained in this inaccessible region of the protein is involved in intersubunit interactions in the native tetramer. (We note parenthetically that we measured the emission lifetimes of the Trp doublet of the native protein; the decay could be fitted to two lifetimes, 1·3 ns and 4·4 ns, with pre-exponential values of 0·15 and 0·85 respectively. Neither these nor the time

resolved emission spectra that we measured were particularly helpful in analysing and assigning the nature of the emission doublet to any greater extent than was possible from steady state measurements and quenching data.)

3.2 *Thermal stability of the native structure*

We next investigated the structural stability of δ-crystallin by studying its denaturation properties. First we studied the thermal denaturation profile of the protein by monitoring the temperature dependent changes in its fluorescence spectrum. The native protein displays an emission doublet at 315 and 325 nm as seen above, while the denatured protein does so around 350 nm (in GuHCl), as described below.

Figure 3a shows a sharp change in the wavelength of the emission maxima of δ-crystallin upon heating. The 315, 325 nm emission doublet, characteristic of the native protein, is retained until around 60°C, at which point it coalesces and shifts into a single band centred around 330 nm. This value suggests that the protein is not denatured yet, but maintains a tertiary structure in which the Trp residues are in a non-polar environment. Unfortunately the solution becomes turbid and the protein precipitates beyond 60°C, with an apparent T_m of 60·2°C. It is apparent however, that the fluorescence band is still in the 330 nm region even around 91°C. These results suggest that the tertiary structure of δ-crystallin is resistant to heat even around 91°C, unlike γ-crystallin (the core protein of the mammalian lens), which denatures around 72°C (Kono *et al* 1990). The behaviour of δ-crystallin is akin to that of the cortical lens protein α-crystallin, which is also multimeric, and which does

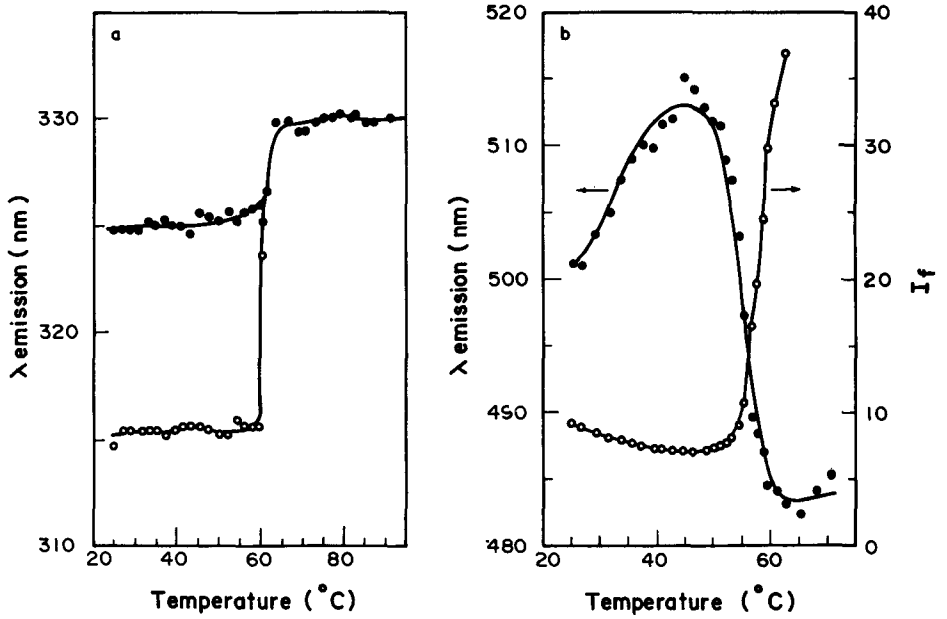

Figure 3. Changes in the fluorescence properties of δ-crystallin upon heating: (**a**) emission maximum of the Trp doublet, 315 nm (○) and 325 nm (●); (**b**) emission maximum (●) and relative intensity (○) of externally added fluorescence probe ANS (250 uM ANS and 0·6 mg/ml protein in *p*H 7·0 phosphate buffer). *I* is intensity in arbitrary units.

not denature even at 95°C (Maiti *et al* 1988). When we analysed the thermal transition around 60°C, using the van't Hoff plots, we could estimate an enthalpy value of 128 kcal mol^{-1} and an entropy of transition value of 384 e.u. Walsh *et al* (1991) had estimated the enthalpy of the first thermal (35–51°C) transition of bovine α-crystallin to be around 91 kcal mol^{-1}, and the second transition (50–73°C) to be around 65 kcal mol^{-1}. It is thus noteworthy that the bird lens consists of two heat-resistant proteins, δ-crystallin which is rich in the nucleus and α-crystallin which is abundant in the cortex.

3.3 Monitoring hydrophobic surfaces using ANS

On the other hand, since there are subtle changes in the fluorescence spectra of δ-crystallin with temperature, we reasoned that heating could probably be affecting the quaternary structure of this protein. One way of monitoring alterations in the quaternary structure is to monitor the concomitant changes in the hydrophobic contacts between the subunits. Such hydrophobic surfaces tend to get exposed upon the dissociation of a multimeric protein. These can be readily monitored by using the extrinsic fluorescence probe ANS, whose emission wavelength and intensity are both notably dependent on the polarity of the environment (Cardamone and Puri 1992). ANS was added to a solution of δ-crystallin, and the emission maximum and intensity of the added ANS were monitored as the solution was heated. We expected that if the quaternary structure of the protein was altered upon heating, ANS could then bind to the hydrophobic surfaces that might get exposed and reflect this in its fluorescence spectra. Figure 3b shows that the emission wavelength of ANS, added to δ-crystallin solution, exhibits an emission maximum at 510 nm at 40°C. After an initial red shift up to 515 nm at 45°C, we see a rapid drop in the band maximum down to 482 nm at 59°C. Similarly we see a decrease in the intensity of fluorescence up to 55°C, followed by a large increase between 55° and 59°C, where the protein is still in solution and not precipitated out. Thus the protein seems to undergo dynamic changes in the quaternary structural organization in the temperature range 45–55°C, leading to exposure of hydrophobic surfaces, where ANS binds and blue-shifts its emission. In this connection it is interesting to note the parallel behaviour of the intrinsic Trp fluorescence of the protein with temperature (figure 3a). Just as the tetrameric structure appears to dissociate, exposing hydrophobic surfaces for ANS binding, the Trp emission doublet collapses and red-shifts to a broad single band centred at 330 nm at 60°C and beyond. This suggests that the characteristic 315, 325 nm Trp doublet, seen in the native protein, arises due to the quaternary structure, which places the Trp residues of δ-crystallin in an unusually apolar environment.

3.4 *Incomplete denaturation in urea*

In order to fully analyse the contribution of the quaternary and tertiary structure to the origin of the Trp emission doublet, we decided to study the complete denaturation profile of δ-crystallin, using chemical denaturants. Figure 4a shows the fluorescence emission spectra of δ-crystallin dissolved in 9 M urea and in 7 M GuHCl. In 9 M urea the doublet characteristic of the native molecule is lost and the Trp emission is red-shifted to around 340 nm. This falls short of the value normally seen in totally

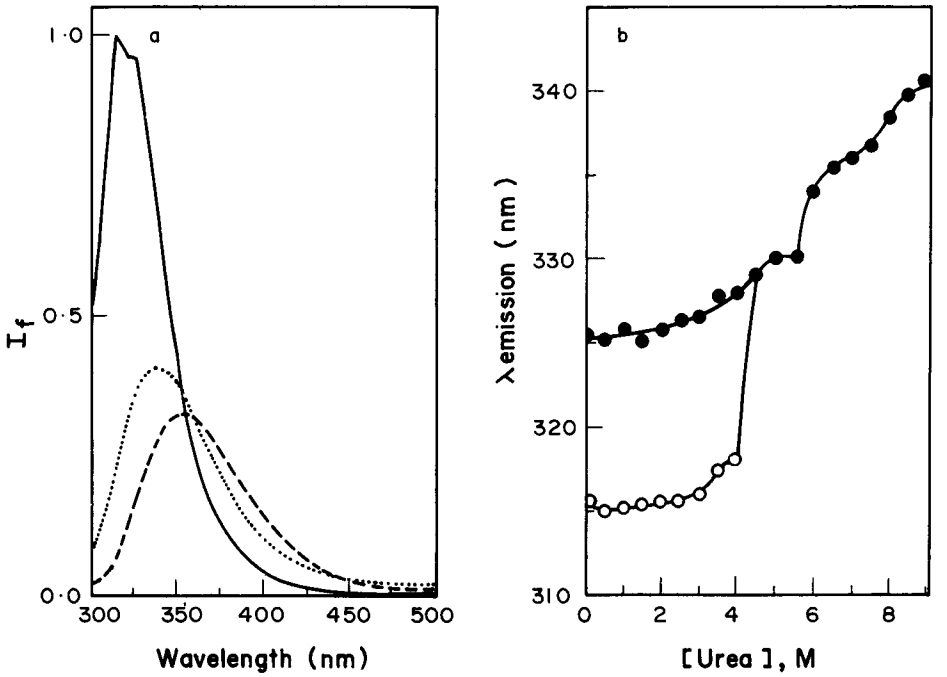

Figure 4. (**a**) Corrected fluorescence spectra of δ-crystallin excited at 295 nm: Native (——); in 9 M urea (·····) and 7 M GuHCl (-----); (**b**) Changes in the emission maximum of the Trp doublet, 315 nm (○) and 325 nm (●), as a function of urea concentration.

denatured proteins (usually 348–352 nm), suggesting that even 9 M urea is not able to denature δ-crystallin completely.

However, GuHCl is more effective in this regard, since the Trp emission of δ-crystallin = 354 nm in 7 M GuHCl is indicative of total denaturation.

Figure 4b shows the changes in the Trp emission wavelength of δ-crystallin with increasing concentrations of urea. The doublet characteristic of the native tetramer collapses and red-shifts into a broad singlet at 330 nm at 4 M urea, and stays so until 5 M urea. This is remarkably similar to the transition that occurs upon heating the protein to 60°C (figure 3), and suggests that the native molecule reaches the same (or a very similar) intermediate stage of folding under these two conditions. However, unlike the thermal behaviour, further addition of urea is able to unfold the protein some more. The Trp emission gradually shifts, with increasing concentrations of urea, to a final value of around 340 nm, which suggests that total denaturation of δ-crystallin is not achieved in urea.

3.5 *Denaturation by guanidinium chloride*

Accordingly we decided to use GuHCl as the denaturant to effect the unfolding of δ-crystallin. Figure 5 shows the GuHCl induced denaturation profiles of δ-crystallin as followed by changes in the ellipticity at 222 nm, and in its Trp emission spectra. Ellipticity changes at 222 nm show that the secondary structure of δ-crystallin undergoes a complex multiphasic transition. The first stage is between 0 and 2 M GuHCl, with the transition occurring a little beyond 1 M GuHCl (see figure 5a,

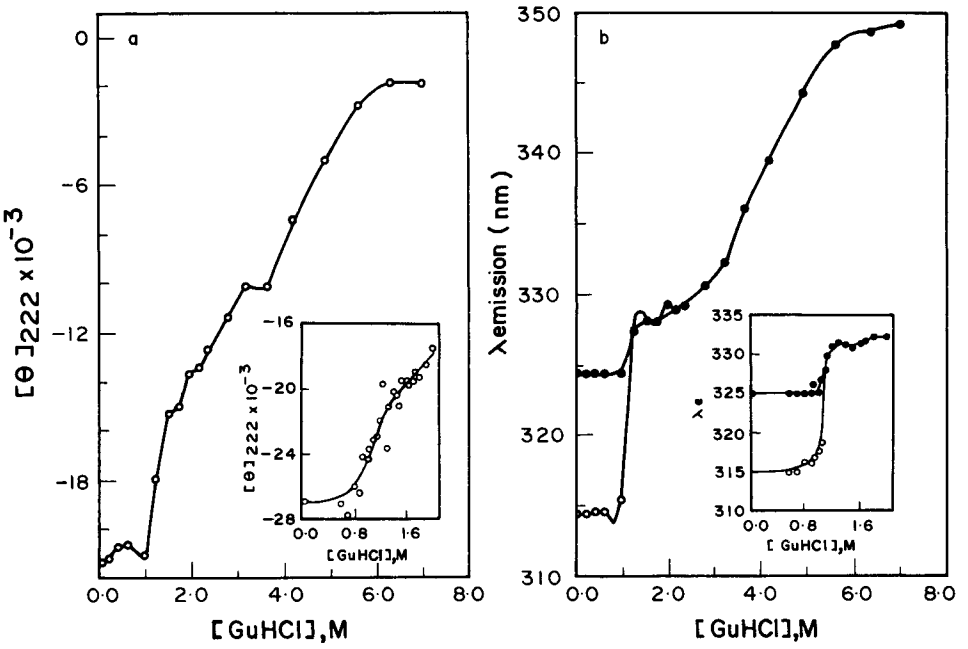

Figure 5. Denaturation of δ-crystallin by GuHCl: (**a**) ellipticity 222 nm and (**b**) emission maximum of the Trp doublet, 315 nm (○) and 325 nm (●). Insets show the initial phase of the transition in the concentration range O to 2 M GuHCl.

inset); this is followed by a gradual loss of secondary structure, resulting in decreased ellipticity, and ending in the completely unfolded state beyond 6 M GuHCl. Fluorescence studies show that the emission doublet is lost at 1·2 M GuHCl (figure 5b and its inset), with the appearance of a composite emission band centred at 330 nm, indicating the movement of buried Trp residues to a slightly more polar environment (We also monitored a concurrent drop in the Trp emission quantum yield from a value of 0·38 in native δ-crystallin to 0·18 for the protein in 1·2 M GuHCl). This initial transition is followed by a more gradual movement of the Trp residues to a more polar environment as the protein gets unfolded at increasing concentrations of GuHCl, suggested by the red-shift in the emission maximum from 328 nm at 1·2 M GuHCl to 348 nm at 6 M GuHCl. There is also a corresponding drop in the quantum yield to Trp, from a value of 0·18 at 1·2 M GuHCl to a value of 0·1 at 6 M GuHCl, consistent with what is expected for completely unfolded proteins (Kronman and Holmes 1971). The denaturation curves show that total unfolding of δ-crystallin is a multiphasic process (Yutani *et al* 1979; Matthews and Crisanti 1981; Saito and Wada 1983).

A few words about the nature of the initial phase of the transition, in the region between 0 and 2 M added GuHCl. At this concentration the fluorescence doublet of the native δ-crystallin is lost, generating a composite band centred at 330 nm, indicating the movement of buried Trp residues to a slightly more polar environment. The CD spectrum of δ-crystallin at 1·6 M GuHCl, illustrated in figure 6, shows only a slight loss of the secondary structure as evidenced by a decrease (about 20%) in

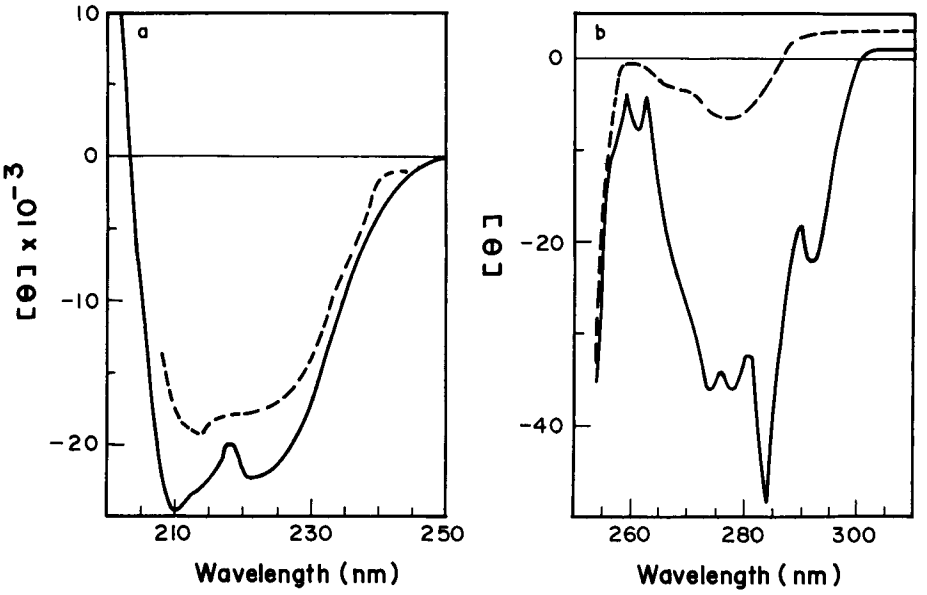

Figure 6. Circular dichroism spectra of δ-crystallin in phosphate buffer (———) and in 1·6 M GuHCl (-----): (**a**) peptide absorption region; (**b**) aromatic absorption region. Ellipticity expressed as deg.cm^2.dmol^{-1}.

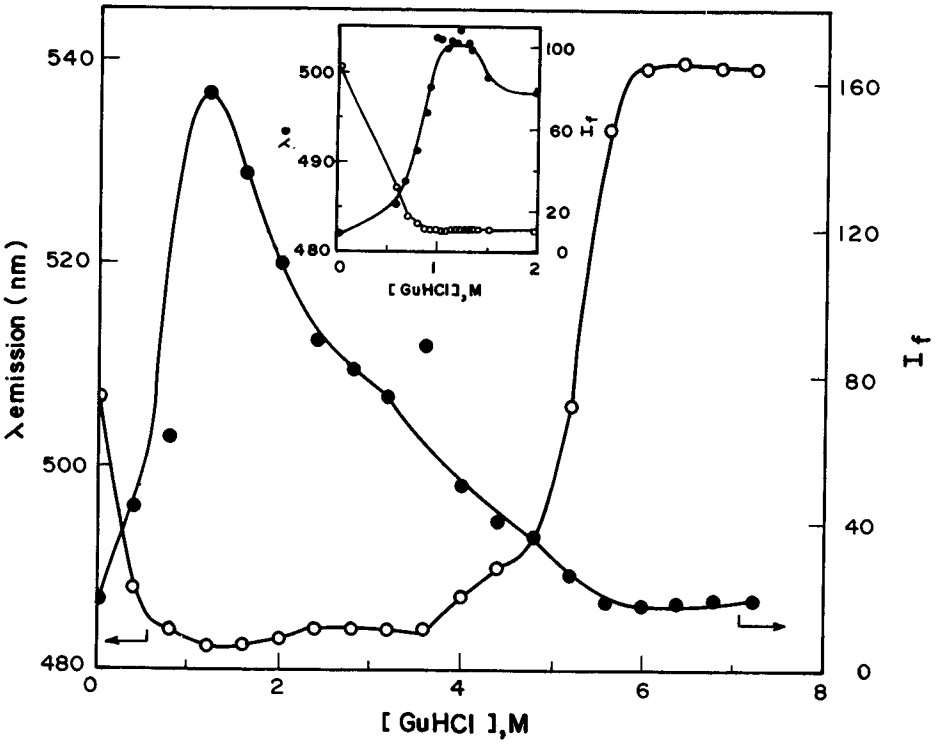

Figure 7. Changes in the fluorescence properties of ANS, added to δ-crystallin at various concentrations of GuHCl. Emission maximum (○) and relative intensity (●). Inset shows the initial phase in the concentration range of O to 2 M GuHCl. *I* is fluorescence intensity in arbitrary units.

the ellipticity in the far-UV region. However, large alterations in the CD spectra of the aromatic side chains can be seen in the near UV-CD spectrum of the protein at 1·6 M GuHCl. These alterations appear to indicate greater chain flexibility around the aromatic side chains at this concentration of the denaturant. However, there is no large change in the polarity of their environment, since the emission maximum value of 330 nm still indicates a substantially apolar microenvironment around the Trp residues.

In order to specifically look at the quaternary structural alterations, we have again utilized the binding of the extrinsic fluorescence probe, ANS, to δ-crystallin at various concentrations of GuHCl. Figure 7 shows that the emission maximum of ANS added to the protein solution shifts from 508 nm in 0 M GuHCl to 482 nm in 1 M GuHCl, indicating the loosening of the subunits and exposure of the hydrophobic contact areas between them. The increased ANS fluorescence intensity, peaking at 1 M GuHCl, also supports such an interpretation.

[It is worth noting here that the intrinsic Trp fluorescence starts showing changes from 0·8 M with an apparent first transition occurring at 1·2 M (figure 5b), whereas the change in the extrinsic probe fluorescence starts right from 0·4 M GuHCl. This minor discrepancy arises because small changes in the quaternary structure may not affect the intrinsic Trp fluorescence as much as it would affect the accessibility of the extrinsic probe to hydrophobic surfaces in the tetrameric aggregate. Incidentally, this demonstrates the sensitivity of ANS as a probe for monitoring quaternary structural alterations in multimeric proteins.]

In the denaturant concentration range between 1 and 4 M GuHCl, there is a drop in the ANS fluorescence intensity, without any major changes in the emission maximum. It is suggested that in this range of GuHCl concentrations, all the available hydrophobic regions are exposed for access by ANS; however the protein may be "swelling" due to a greater loosening of the tertiary and secondary structure. Such a swelling would be expected to increase the reorientational and vibrational relaxations modes of the fluorophore and thus reduce its emission quantum efficiency, though not necessarily the microenvironment polarity, which alters the band maximum value. This interpretation is supported by the fact that there is a gradual red-shift in the Trp emission band from 328 to 338 nm and a similar reduction in the ellipticity at 222 nm, seen in figure 5. Beyond 4 M GuHCl, the emission maximum of the extrinsic probe gradually shifts to that of free ANS, occurring at 540 nm, with a decreased quantum yield and hence lower fluorescence intensity. At these concentrations, the protein, after having lost the quaternary structure, starts to gradually unfold, losing its secondary and tertiary structures as well, thereby allowing the side chains greater mobility and a more polar environment. The apparent free energy of denaturation, expressed as (Pace 1975; Pace *et al* 1989)

$$\Delta(G^{app})_D = \Delta G^{H_2O}{}_D + m[\text{GuHCl}],$$

was estimated to be 2·3 kcal mol^{-1}, with the transition occurring at 3 M GuHCl, with a value of m around 0·73 kcal mol^{-1} M^{-1}. This is distinctly lower when compared with the $(\Delta G^{H_2O})_D$ value of 4·2–8·3 kcal mol^{-1}, seen in bovine-crystallins denatured in GuHCl (Kono *et al* 1990).

3.6 *Gel filtration and sedimentation*

The multiphasic denaturation profile suggests the possibility of intermediates. In order to look for isolable intermediates, particularly at 1·2 M GuHCl, we decided to follow the denaturation, using HPGPC on a TSK G3000SW column. The chromatographic profiles are shown in figure 8. These profiles show the presence of at least two species in all concentrations of GuHCl beyond 0·8 M. The retention times of these two peaks suggest that the molecule is undergoing both dissociation and swelling simultaneously. Between the denaturant concentrations of 1·2 and 2·4 M GuHCl, there are changes in the relative amounts of these two species, with small changes in the retention times, indicating conversion from one state to another, as well as small changes in the effective hydrodynamic radii. This interpretation is supported by the relatively constant emission maximum of ANS at these concentrations of GuHCl. Large changes in the hydrodynamic radii are evident at, and beyond 4 M GuHCl. Once again this change may be attributed to a packing change in the molecule (as evident from monitoring the intrinsic fluorescence). We then isolated the two peaks at 1·2 M GuHCl and looked at their Trp fluorescence. The inset in figure 8 shows that the peak with retention time 26 min (peak 1) shows a skewed 315, 325 nm emission doublet resembling the native protein, whereas the peak eluting at 33 min (peak 2) shows a composite emission maximum at 330 nm. Thus, at this concentration

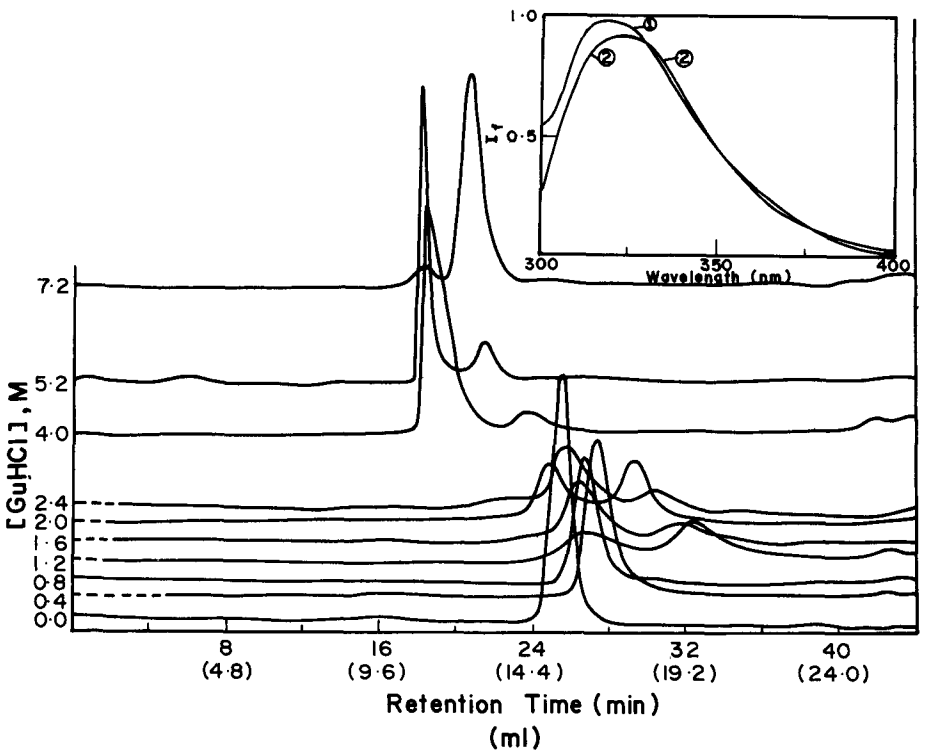

Figure 8. High performance gel permeation chromatograms (HPGPC) of δ-crystallin on TSK G3000SW column equilibrated at different concentrations of GuHCl. Inset shows the corrected fluorescence spectra of the two components isolated in 1·2 M GuHCl. Peak 1 and peak 2 had retention times of 26 and 33 min, respectively.

of GuHCl the 315/325 nm doublet of the native molecule is lost in one of the molecular intermediates. It is hence clear that the association of the subunits into a well-defined geometry generates the blue emission doublet in the native δ-crystallin.

In order to characterize the particle size of the protein in the intermediate denaturant concentrations, we next measured the sedimentation coefficient values ($s_{20,w}$) of δ-crystallin in the native form and when dissolved in 2·2 M GuHCl. Using the velocity sedimentation approach on an analytical ultracentrifuge, we found the $s_{20,w}$ value for the native protein to be 7·8 s, while the value in 2·2 M GuHCl was 3·3 s. These values suggest that at 2·2 M GuHCl, δ-crystallin is dissociated from its native tetrameric state into a smaller particle. (Unfortunately, measurement of $s_{20,w}$ values have proved difficult in urea, because of the high viscosity of the solutions.) It is not certain, however, whether the 3·3 s particle is a dimer or a monomer. Horwitz and coworkers (1977, 1980) have suggested that it is the dimer.

3.7 *"The swollen globule"*

The following dynamic picture emerges from the above results: (a) the denaturation is multiphasic in character; (b) changes in the quaternary structure are evident in the initial phase, both in heating (beyond 60°C) and in chemical denaturation (1 M GuHCl and 4 M urea). It is apparent that the native tetramer of δ-crystallin is held together by polar and hydrophobic interactions between the subunits. Heating is expected to weaken the polar cohesive interactions, while the addition of urea or GuHCl is known to weaken hydrophobic interactions. Dissociation of the tetramer is thus effected by heat as well as these denaturants; (c) this initial transition appears to lead to a "swollen" molecule, wherein there is a significant increase in the freedom of motion of the aromatic residues (as monitored by near-UV-CD) with the Trp residues in an appreciably apolar environment (as monitored by the values of emission maxima). The secondary structural order of this molecular species is lost only slightly, in comparison with the chains in the native conformation. This state may thus perhaps be called a "swollen globule", rather than the "molten globule" where there is complete loss of tertiary structure with no loss of secondary structure (Ptitsyn 1987). Total denaturation of the protein leading to chain unfolding does not occur upon heating the protein up to 91°C in aqueous buffer, or by dissolving it in 9 M urea; it occurs only upon addition of GuHCl at concentrations beyond 6 M.

The structural robustness of δ-crystallin is perhaps important to its recruitment as the core protein of the avian lens. In this regard, its similarity to α-crystallin, which is used as the structural protein in the lens cortex is also noteworthy. Studies on the structural stability of other enzyme crystallins, e.g., the ε-, ρ- and ζ-crystallins would be of value towards an understanding of the conformational basis of their recruitment as structural proteins.

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