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Acid-Induced Dissociation of αA- and αB-Crystallin Homopolymers

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ABSTRACT Homopolymers were constructed from the αA and αB polypeptides isolated from the lens protein α-crystallin. As the pH is lowered from 7.0 to 3.4, these homopolymers dissociate to smaller species with molecular masses ranging from 80 to 250 kDa for the αA and around 140 kDa for the αB dissociation products. The pK_a for this dissociation was 3.8 ± 0.2 for αA and 4.1 ± 0.1 for αB homopolymers. Further decreases in pH, to 2.5, resulted in the presence of only denatured αB polypeptides, whereas the αA dissociation products remained intact.

Fractionation of the acid dissociation products from the αA homopolymer at pH 2.5 yielded stable species with molecular masses of 220 ± 30, 160 ± 20, and 90 ± 10 kDa. The majority of the population at acid pH consisted of the 160 kDa species. Conformational analysis of these species revealed that most of the secondary structure of the original αA homopolymer was retained but that the tertiary structure was perturbed. Fluorescence quenching and energy transfer measurements suggested that the molecule had undergone acid expansion, with the greatest perturbation observed in the smallest particles.

The results from this work suggest that αA homopolymers are heterogeneous populations of aggregates of a "monomeric" molecule with a molecular mass of 160 kDa. This "monomeric" molecule may be formed from the association of two tetrameric units.

INTRODUCTION

α-Crystallin, the major protein of the mammalian lens, is an aggregate of two types of subunits, αA and αB. The amino acid sequences of the αA and αB chains are over 60% homologous and are related to the small heat shock proteins (Ingolia and Craig, 1982; de Jong et al., 1988). While the protein is found predominantly in the lens, homopolymers of both subunits have recently been found in other tissues such as kidney, heart, and striated muscle, but at much lower concentrations than the α-crystallin observed in the lens (Kato et al., 1991; Bhat and Nagineni, 1989).

Attempts to determine the quaternary structure of α-crystallin have been hampered by the polydispersity of the protein and controversy over its molecular weight. The protein is generally isolated as a heterogeneous population of aggregates, most with molecular masses of 800 ± 200 kDa, but populations with average molecular masses ranging from a low of 280 kDa to >10,000 kDa have been isolated (Bloemendal, 1981). The relationship between these forms has not been established. Despite the difficulties in establishing the molecular weight of the protein, several models have been proposed for the quaternary structure. These models can be categorized into two major groups: those where the subunits are in equivalent positions (Augusteyn and Koretz, 1987; Thomson and Augusteyn, 1988a) and those where the subunits are located in three different environments (Bindels et al., 1979; Tardieu et al., 1986; Walsh et al., 1991). None of these models satisfactorily explain all of the properties of the protein.

Several observations suggest that α-crystallin populations may consist of polymers of some smaller "monomeric" form. The molecular weight of the protein has been shown to decrease with increasing temperature (Bindels et al., 1978), low ionic strength (Siezen at al., 1980a), low pH (Augusteyn et al., 1988a), and low concentrations of deaggregating agents (Thomson and Augusteyn, 1984). High concentrations of chaotropic agents result in complete denaturation; reassociation of the polypeptides produces aggregates with molecular masses in the range of 200–300 kDa. This size is very similar to that of newly synthesized α-crystallin (300 kDa) (Asselbergs et al., 1978).

The size of α-crystallin increases with age (Siezen et al., 1980b), but the mechanism involved is not understood. Chainlike structures of small particles have been observed with electron micrographs of α-crystallin and subunit homopolymers (Siezen et al., 1980b; Augusteyn et al., 1989). Light scattering data obtained with homopolymers suggest that these particles are less than 200 kDa (Augusteyn et al., 1989). All of these observations strongly indicate that α-crystallin populations may be composed of mixtures of polymers of some smaller monomeric unit, possibly with a molecular mass below 200 kDa.

We have previously reported that, at low pH, α-crystallin dissociates into particles composed only of αA chains and denatured αB polypeptides (Augusteyn et al., 1988a). Protonation of Asp 127 in the αB subunit was shown to be responsible for the loss of tertiary structure at low pH. The αA dissociation product obtained at pH 2.5 had a $S_{20,w}$ value of 9.4 S, significantly lower than that obtained for α_m-crystallin (12.2 S). However, it was not possible to make conclusions regarding the protein's structure because of the complex behavior of the two subunits. In the present study we have attempted to simplify the interpretations by investigating purified homopolymers constructed from A and B chains. The data should provide more information on the

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"monomeric" molecular weight of the protein and possibly on the subunit arrangement.

MATERIALS AND METHODS

Protein purification

Bovine αA and αB polypeptides were purified by chromatofocusing (Sluyterman and Wijdenes, 1978) and used to construct homopolymers as described previously (Thomson and Augusteyn, 1988b). Purity checks on these proteins were performed as described previously (Thomson and Augusteyn, 1983).

Protein samples (10 mg/ml) were dialyzed against 500 volumes of 0.1 M glycine buffer at the desired pH. In order to avoid the effects of fluctuations in the ionic strength during adjustment of pH with acid, all buffers were prepared from a stock solution of 0.2 M glycine (pH 6.0).

Sedimentation measurements

Sedimentation velocity experiments were performed at 20°C using a Beckman Model E analytical ultracentrifuge. The protocol for the experiments was described in detail previously (Augusteyn et al., 1988a).

Circular dichroism

Circular dichroism spectra in the near- and far-UV regions were obtained with an AVIV circular dichroism spectrometer (model 60 DS; AVIV Associates), as described elsewhere (Augusteyn et al., 1992a). The temperature was maintained at 20°C with a circulating water bath. The data were analyzed with the data analysis system PROSEC on the AVIV spectrometer (Chang et al., 1978).

Aromatic amino acid measurements

Tyrosine emission spectra were obtained from the difference between fluorescence emission spectra (normalized at 380 nm) obtained by exciting at 280 nm (Trp and Tyr) and 295 nm (Trp only) (Parker and Rees, 1960).

Acrylamide quenching of tryptophan fluorescence was studied by observing the changes in intensity of emission at the peak wavelength following titration of protein solutions with small aliquots of 5 M acrylamide as described previously (Augusteyn et al., 1988b). Corrected fluorescence data were analyzed according to the Stern-Volmer equation (Stern and Volmer, 1919) for homogeneous systems. In the case of multiple sites a modified version of the Stern-Volmer equation was used (Stryjewski and Wasylewski, 1986):

$$\frac{F}{F_o} = \sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q])\exp V_i[Q]}$$

where f_i is the fractional contribution of component i to the total fluorescence, K_i and V_i are the dynamic and static quenching constants for fluorescent component i, and Q is the concentration of quencher.

Quantum yields were calculated according to the method of Parker and Rees (1960), using the following equation:

$$\phi_P = \phi_W \frac{A_P}{A_W} \frac{D_W}{D_P}$$

where A is the area under the corrected emission spectrum and D is the absorbance at the exciting wavelength. The subscripts P and W refer to protein and tryptophan, respectively. A value of 0.2 for ϕ_W, reported by Teale and Weber (1957), was used in these calculations.

Sulfhydryl group accessibility

Sulfhydryl groups were labeled by reacting the proteins in the dark with saturating concentrations of 5-[2-((iodoacetyl)aminoethyl]-aminonaphthalene-1-sulfonic acid (1,5-IAEDANS). The reaction was terminated after 24 h by the addition of mercaptoethanol (1 mM final concentration). Excess reagent was removed by desalting on Sephadex G-10 (PD-10 column; Pharmacia) followed by exhaustive dialysis against 0.1 M glycine (pH 7) until the absorbance and fluorescence spectra of the dialysate were the same as that of the buffer. The proteins were then dialyzed against 0.1 M glycine buffer at the appropriate pH.

Quenching of the cysteine-labeled IAEDANS fluorescence (excited at 344 nm) by acrylamide was monitored at 465 nm and performed essentially as described by Augusteyn et al. (1987).

TNS binding

Protein (0.03 mg/ml in 0.1 M glycine at pH 7 or 2.5) was reacted with 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid (2,6-TNS) at 40 mM (final concentration). TNS binding was measured by monitoring fluorescence emission between 400 and 600 nm (excitation 317 nm) with a Perkin-Elmer model LS-5 spectrofluorimeter. All results were corrected for the 1.43-fold increase in quantum yield at pH 2.5.

Fluorescence energy transfer

The efficiency of fluorescence energy transfer from tryptophan to either IAEDANS-labeled cysteine or TNS was estimated from the relative decrease in emission intensity of the donor in the presence of acceptor according to the procedures of Föster (1960) and Lakowitz (1983). The efficiency of energy transfer from Tyr to Trp was calculated from the decrease in intensity of the excitation spectrum of the protein relative to the excitation spectrum of L-Trp in solution. A Föster distance (R_o) of 1.33 nm was used to calculate the average Trp-Tyr distances and a distance of 1.77 nm to calculate the average Trp-Cys distances.

RESULTS

Homopolymers were constructed from pure αA and αB polypeptides as described previously (Thomson and Augusteyn, 1988b). These had sedimentation coefficients of 10–11 S and 13–14 S and molecular masses of 300 ± 50 kDa and 440 ± 80 kDa, respectively. Dialysis of either homopolymer into 0.1 M glycine at pH 7.0 or 8.0 had no measurable effects on its sedimentation coefficient, tryptophan microenvironment, circular dichroism, or sulfhydryl accessibility.

Acid dissociation of homopolymers

αA homopolymer

Fig. 1 *a* shows that as the pH decreased from 7 to 2.5, the sedimentation coefficient of the αA homopolymer decreased from 10.1 to 4.4 S. No data could be obtained between pH 6 and 4 since the protein precipitated around its isoelectric point (pH 5).

At pH 2.5, the sedimentation coefficient exhibited extremely high concentration dependence ($y = -0.24x + 7.3$; $r^2 = 0.993$), much greater than that observed for α-crystallin at pH 8 ($y = -0.0075x + 12.38$; $r^2 = 0.967$) (Thomson and Augusteyn, 1988a). Extrapolation to zero protein concentration yielded a value for $S_{20,w}$ of 7.3. This compares with a value of 9.4 S for the αA particle obtained after dissociation of the αB chains from α-crystallin (Augusteyn et al., 1988a). It was not possible to determine whether the high concentration dependence observed was due to interactions between

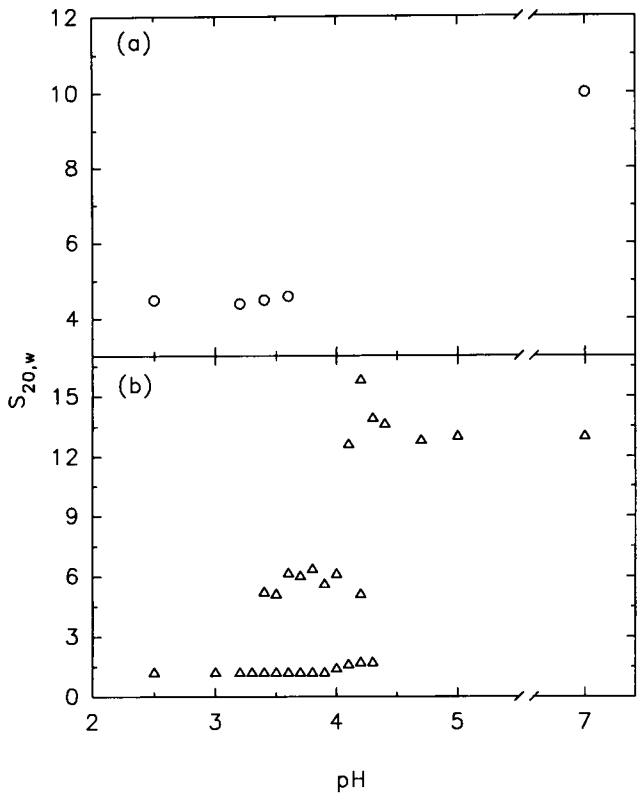

FIGURE 1 The effect of pH on the sedimentation coefficient of the homopolymers constructed from pure αA (*a*, ○) and αB (*b*, △) subunits. Proteins in 0.1 M glycine (pH 7) were dialyzed against the same buffer at the required pH. Sedimentation coefficients were determined at 52,400 rpm at 20°C with a protein concentration of 12 mg/ml.

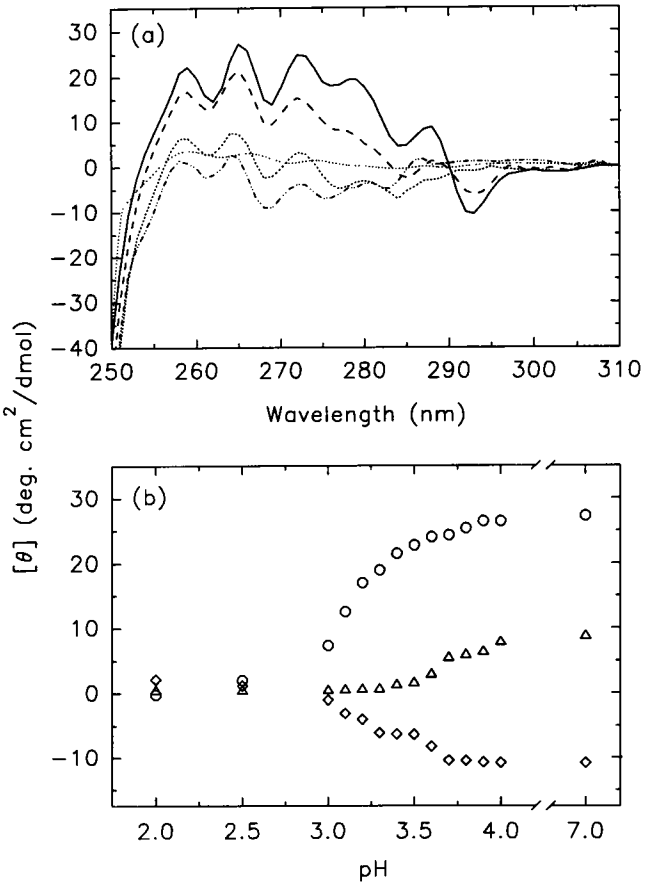

FIGURE 2 (*a*) circular dichroism spectra of αA homopolymers as a function of pH. ——, pH 7; – – – –, pH 3.7; - - - -, pH 3.3; – ·· – ··, pH 2.5 in the Far-UV region. The dotted line represents the denatured protein in the presence of 7 M urea in 0.1 M glycine (pH 2.5). (*b*) relative proportion of secondary structure remaining (○) and the shift in wavelength minimum (△) as a function of pH, in 0.1 M glycine. Data were obtained from spectra like those in *a*.

species, at the low ionic strength of the glycine buffer used, or to particle asymmetry induced by the dissociation. This effect could not be diminished by increasing the ionic strength since the sample precipitated in the presence of salts. Significant dependence of the sedimentation coefficient on angular velocity was also observed. This was found to be due to the heterogeneity of the population. To reduce the heterogeneity, the population was fractionated as described below.

The circular dichroism (CD) spectrum in the far-UV region for the αA homopolymer at pH 7 is consistent with a protein containing substantial β-sheet structure (Fig. 2 *a*). This was virtually unchanged at pH 4. As the pH is decreased from 4 to 2.5 there is a gradual but small loss of ordered structure with the concomitant appearance of random structure, indicated by a shift to lower wavelengths (from 217 to 214 nm) and a decrease in ellipticity around the 200-nm region. However, the proportion of sheet conformation lost was no more than 12% of the total (Fig. 2 *b*). The partial loss of structure appears to coincide with the protonation of an amino acid with a pK_a of about 3.5.

The near-UV CD spectrum (Fig. 3 *a*) of the αA homopolymer showed a loss of ellipticity for the 278-, 288-, and 292-nm bands reflecting a change in the environment of Trp and Tyr residues. The midpoint of the transition coincides with that observed in the far-UV region (i.e., pH 3.5 ± 0.1) (Fig. 3 *b*), indicative of the protonation of an acidic amino acid and accompanying loss of a salt bridge or hydrogen bond breakage.

The effect of lowering the pH on tryptophan fluorescence emission is summarized in Fig. 4. The emission wavelength increased only slightly with decreasing pH, from 333 to 335 nm, indicating that very little structural alteration had taken place. The midpoint of the transition appears to be at pH 3.9 ± 0.1.

αA-Crystallin contains five tyrosine residues, some of which are exposed, and one tryptophan residue, which is buried (Augusteyn et al., 1992b). The efficiency of tyrosine-tryptophan energy transfer for the αA aggregate decreased from 0.43 at pH 7 to 0.23 at pH 2.5. This corresponds to an increase in average Trp-Tyr distance from 1.35 to 1.62 nm.

Since α-crystallin is known to have a hydrophobic surface (Lawson et al., 1981), the effects of pH on the binding of a hydrophobic probe were examined. The addition of TNS at pH 8 results in a biphasic uptake of the probe, with an immediate increase in fluorescence, followed by a slow time-dependent increase, suggesting that there are at least two

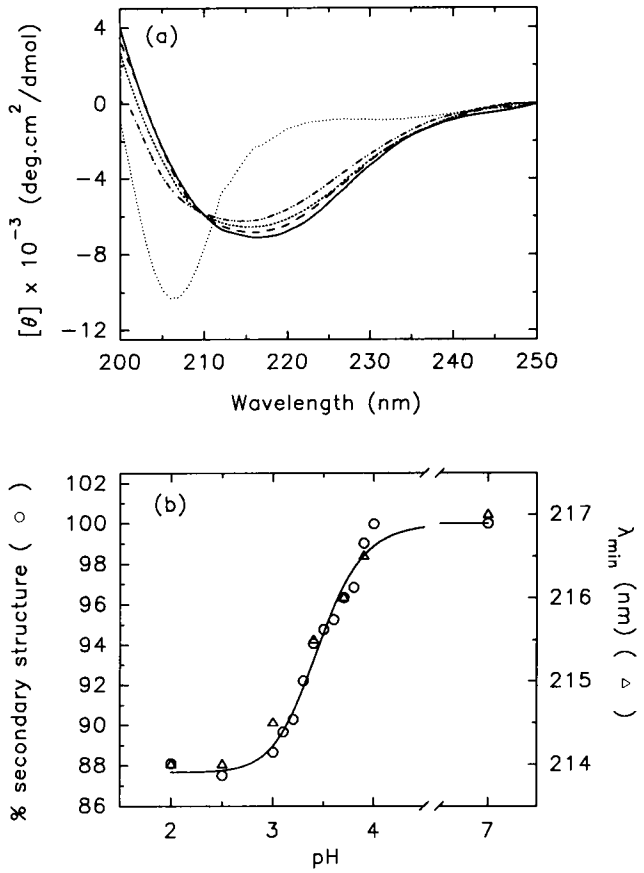

FIGURE 3 (*a*) circular dichroism spectra of αA homopolymers in the near-UV region, in 0.1 M glycine, as a function of pH. ——, pH 7; – – – –, pH 3.4; - - - - -, pH 3.0; – ·· – ··, pH 2.5. The dotted line represents the denatured protein in the presence of 7 M urea and 0.1 M glycine (pH 2.5). (*b*) change in ellipticity of the 292 (○), 287 (△), and the 265 (◇) nm peaks as a function of pH in 0.1 M glycine. Data were obtained from spectra such as those in Fig. 3 *a*.

types of sites to which TNS binds. Lowering the pH resulted in the complete loss of the time-dependent binding (Fig. 5), indicating that the second site is now either no longer capable of binding TNS or has become more readily accessible. The 28% reduction in fluorescence is indicative of a significant loss of binding sites. The wavelength of maximum emission increased only slightly from 422 to 424 nm as the pH was decreased from 7 to 2.5, indicating that there has been no significant change in the probe's microenvironment.

αB homopolymer

As the pH is decreased, the sedimentation coefficient of the αB homopolymer changes from 13–14 S, at pH 7, to about 6 S at pH 3.4 (Fig. 1 *b*). Subunits start to appear at pH values of 4.5 or less. Below pH 3.3 the 6 S species has completely dissociated into subunits. The proportions of the various species are shown in Fig. 6. The data suggest that the 6 S species is a transitory intermediate between the 13–14 S species and denatured subunits.

The wavelength of maximum tryptophan fluorescence from the αB homopolymer increased from 335 nm at pH 7

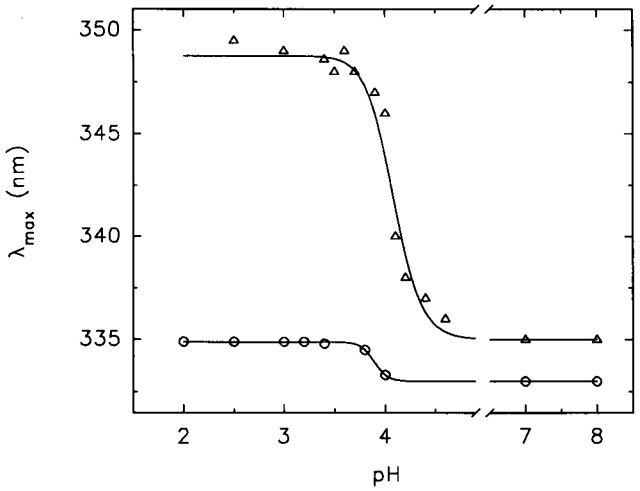

FIGURE 4 The effect of pH on the maximum wavelength of tryptophan fluorescence emission in homopolymers constructed from pure αA (○) and αB (△) polypeptides. Proteins in 0.1 M glycine (pH 7) were dialyzed against the same buffer at the required pH. Fluorescence spectra were recorded at <0.05 mg/ml, over the range 300–400 nm following excitation at 295 nm.

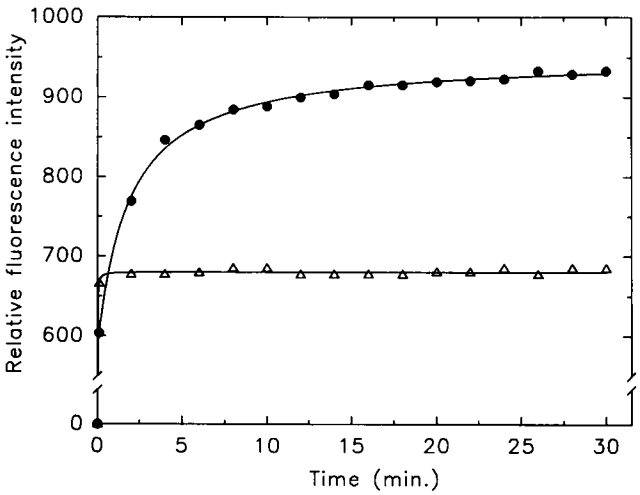

FIGURE 5 Fluorescence intensity of TNS bound to αA, in 0.1 M glycine (pH 7) (●) and pH 2.5 (△) as a function of reaction time. Protein concentration, 0.03 mg/ml; TNS concentration, 40 μM (final concentration). The data shown are corrected for the percentage increase in quantum yield of the TNS at pH 2.5.

to a maximum of 349–350 nm at pH 2.5 (Fig. 4). This is consistent with complete exposure of the tryptophan residues to the solvent and suggests that the 1.2 S subunits observed at the low pH are completely denatured. The midpoint for this transition was observed at pH 4.1 ± 0.1.

Isolation of dissociation products

αA homopolymer

Sedimentation equilibrium analysis of the αA homopolymer at pH 2.5 revealed that it was relatively heterogeneous, with an average molecular mass of 160 ± 60 kDa. In order to

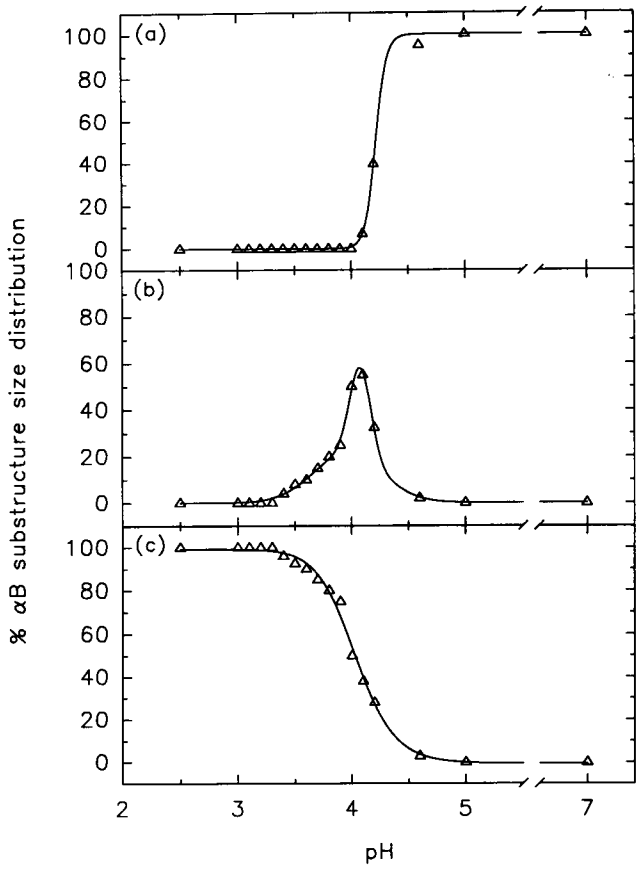

FIGURE 6 The effect of pH on the dissociation of the αB homopolymers. The extent of dissociation of the 12 S species (*a*), 6 S species (*b*), and 1.2 S species (*c*) was calculated from the areas under the peaks in sedimentation velocity experiments at each pH and is expressed as the percentage of total species present.

reduce the heterogeneity, the αA homopolymer was fractionated in 0.1 M glycine (pH 2.5) by gel filtration (Fig. 7 *a*). Sedimentation velocity analysis confirmed the heterogeneity of the sample with sedimentation coefficients across the peak ranging from 8.5 to 6.3 S (at 3 mg/ml).

The peak was divided into three sections (shaded in Fig. 7 *a*), and each was rechromatographed. The elution profiles are shown in Fig. 7 *b*. Three distinct peaks were obtained with their maximum absorbances at elution volumes corresponding to their positions in the original fractionation, indicating that these represent stable dissociation products (Fig. 7 *a*). To minimize contamination, samples were selected from each of the three peaks, as shown in Fig. 7 *b*. These were then subjected to more detailed analysis. No changes were observed in the size and other properties of the peaks over a 2-week period.

Sedimentation equilibrium analyses yielded weight average molecular masses of 220 ± 30, 160 ± 20, and 90 ± 10 kDa, respectively, for the three populations. Further subfractionation of the 90 kDa population did not yield smaller species. From a consideration of these molecular masses and the possible cross-contamination between the species, it may be concluded that these represent dodecamers (240 kDa), octamers (160 kDa), and tetramers (80 kDa), respectively. The major species by far was the octamer.

αB homopolymer

Sedimentation velocity experiments indicated the presence of a transitory 6 S species. Several attempts were made to isolate this by gel chromatography. Fig. 8 shows a typical elution profile obtained in 0.1 M glycine (pH 4). Significant amounts of subunits were present. Sedimentation analysis revealed that the major species has a sedimentation coefficient in the range of 5–6 S. The molecular mass could not be measured by sedimentation equilibrium experiments since the protein aggregated in the centrifuge. An estimate of the molecular mass was obtained from the elution profile, which indicated that the dissociation product had a molecular mass of about 140 kDa. Further characterization of this dissociation product was not possible because of its continual dissociation into denatured subunits and/or aggregation.

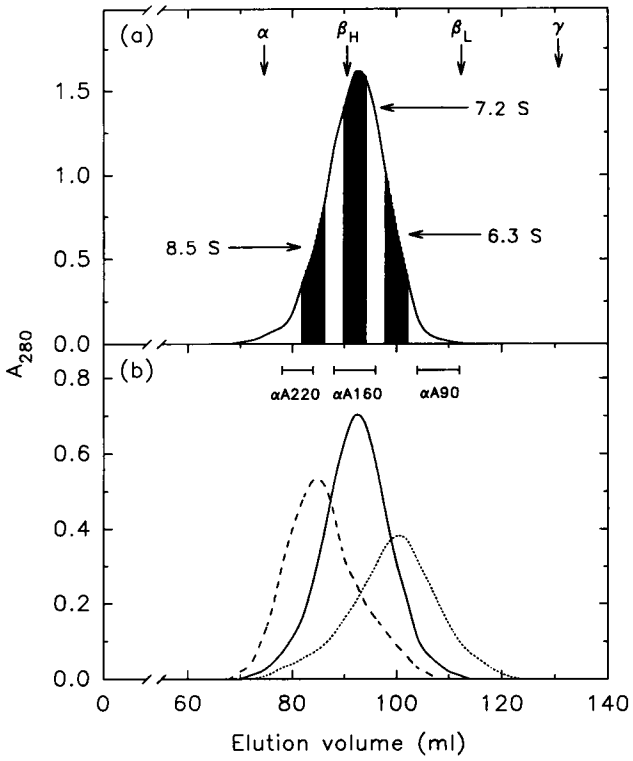

FIGURE 7 (*a*) gel filtration elution profiles of purified αA homopolymer on Sephacryl S300SF (Pharmacia) (1.2 × 180 cm; flow rate, 10 ml/h in 0.1 M glycine, pH 2.5). Sedimentation coefficients, obtained at 3 mg/ml (52400 rpm), for the shaded portion of the curve, are indicated within the figure. The elution positions for lens proteins α- (800 kDa), β_H- (160 kDa), β_L- (60 kDa), and γ- (20 kDa) crystallin, chromatographed at pH 7.0 in 0.1 M glycine, are shown for size comparison. The bar indicates the portion of the protein used for sedimentation analysis. (*b*) rechromatography of the shaded areas in *a* on Sephacryl S300SF (Pharmacia). Experimental conditions are identical to those described in *a*. The bars indicate the fractions that were used for further analysis.

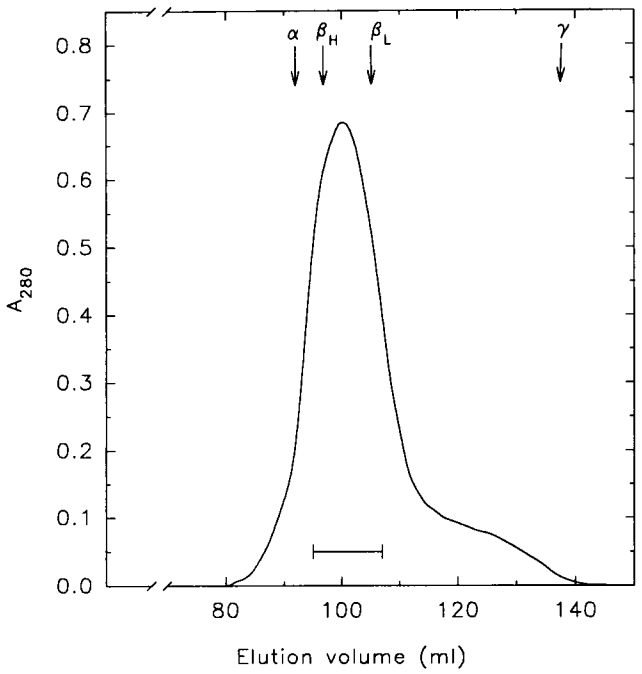

FIGURE 8 Gel filtration elution profile of purified αB homopolymers on Sephacryl S300SF (Pharmacia) (1.4 × 145 cm; flow rate, 20 ml/h) in 0.1 M glycine (pH 4.0). The elution positions for lens proteins α- (800 kDa), β_H- (160 kDa), β_L- (60 kDa), and γ- (20 kDa) crystallin, chromatographed at pH 7.0 in 0.1 M glycine, are shown for size comparison. The bar indicates the portion of the protein used for sedimentation analysis.

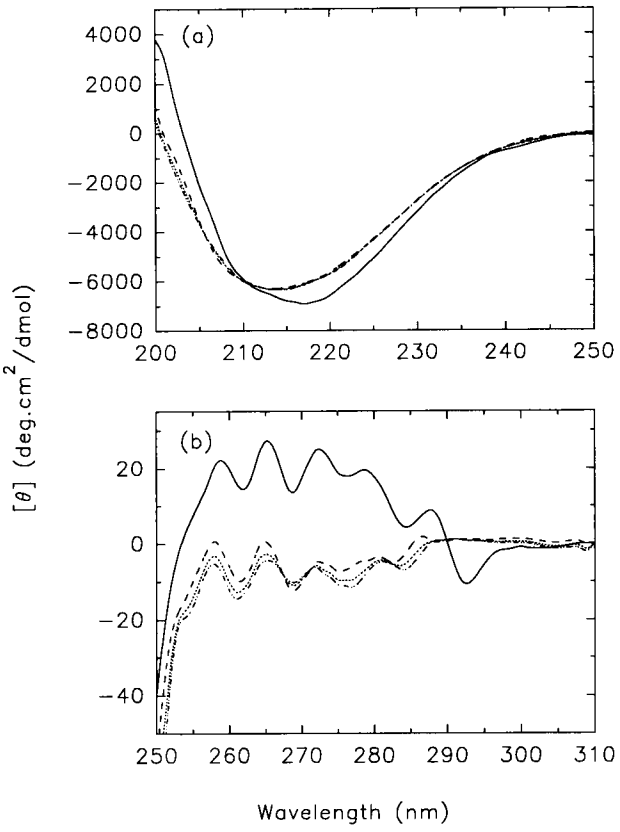

FIGURE 9 (*a*) far-UV circular dichroism spectra, in 0.1 M glycine, of αA homopolymers at pH 7 (——), αA220 at pH 2.5 (– – – –), αA160 at pH 2.5 (- - - -), and αA90 at pH 2.5 (·····). A protein concentration of 1.5 mg/ml and a path length of 0.1 cm were used. (*b*) near-UV circular dichroism spectra, in 0.1 M glycine, of αA homopolymers at pH 7 (——), αA220 at pH 2.5 (– – – –), αA160 at pH 2.5 (- - - -), and αA90 at pH 2.5 (·····). A protein concentration of 1.5 mg/ml and a path length of 0.5 cm were used.

Conformational studies on the dissociation products of the αA homopolymer

Circular dichroism

The far-UV CD spectra are shown in Fig. 9 *a*. All of the dissociation products show a slight loss in secondary structure, relative to the αA homopolymer at pH 7, with a decrease in the λ_{min} from 217 to 213 nm at pH 2.5. No significant differences between the three dissociation species αA220, αA160, and αA90 could be detected.

The near-UV CD spectra are shown in Fig. 9 *b*. They exhibit reduced ellipticity with complete loss of the bands at 278, 288, and 292 nm. The shape of the phenylalanine bands at 258 and 265 nm does not appear to have altered, despite the reduction in ellipticity.

Aromatic amino acid environments

At pH 2.5, the wavelength of maximum tryptophan fluorescence emission for the αA220, αA160, and αA90 species was 336–337 nm, and the quantum yields were in the range of 0.115–0.133 compared with an emission maximum of 333 nm and a quantum yield of 0.12 at pH 7 for the αA homopolymer (Table 1). These values are much lower than that for the exposed tryptophan in the fully denatured αA polypeptide (λ_{max}, 350 nm; ϕ, 0.18). Acrylamide quenching of the fluorescence indicated that, at pH 2.5, tryptophan is more accessible (K_{SV} = 3.1–4.5 M^{-1}) for the dissociation products than at pH 7 (K_{SV} = 1.9 M^{-1}). For the αA particle obtained from α-crystallin at pH 2.5, K_{SV} = 3.5 M^{-1}. The quenching parameters obtained for the proteins at pH 2.5 are still much lower than those obtained when the proteins are denatured in 7 M urea (K_{SV} = 11 M^{-1}) (Table 1), indicating that substantial structure has remained.

TABLE 1 Accessibility parameters of tryptophanyl and tyrosyl residues of α-crystallin, αA homopolymers, and their dissociation products in 0.1 M glycine

	αA*	αA220‡	αA160§	αA90‖	α_c¶
λ_{max} (nm)	333	336	336	337	350
Quantum yield	0.12	0.115	0.129	0.133	0.18
K_{SV} (M^{-1})	1.9	3.1	3.6	4.5	11
V (M^{-1})	0.7	0.7	0.6	0.5	0
Relative tyrosine emission	0.23	0.39	0.41	0.48	1.00

* αA-homopolymer, pH 7.
‡ αA dissociation product; molecular mass, 200 kDa (pH 2.5).
§ αA dissociation product; molecular mass, 160 kDa (pH 2.5).
‖ αA dissociation product; molecular mass, 80 kDa (pH 2.5).
¶ α-crystallin, pH 7 in 7 M urea.

Significant tyrosine emission was observed with the αA homopolymer at pH 7 (Table 1). In the dissociation products, an increase in tyrosine emission was detected with decreasing molecular mass.

Sulfhydryl accessibility

It has been previously reported (Augusteyn et al., 1987) that the single cysteine residue in position 131 of the αA chain is very close to the surface of the protein. The sulfhydryl groups in the αA dissociation products were labeled with 1,5-IAEDANS to monitor their accessibility by fluorescence spectroscopy. Stern-Volmer plots for acrylamide quenching of the probe's fluorescence were nonlinear, suggesting that the probe was in different microenvironments. At pH 8 about 90% of the fluorescence was quenched with a relatively low K_{SV} of 0.84 M^{-1}, suggesting that the labeled cysteine residues are buried (Augusteyn et al., 1987). The remaining fluorescence was quenched with a K_{SV} of 23 M^{-1}. This value is too high to represent any label on the protein but most likely indicates the presence of free IAEDANS in solution. The 220 kDa species had a quenching constant virtually identical to that of the αA homopolymer at pH 8. As the molecular mass of the dissociation products decreased the accessibility of the label to acrylamide increased to 0.92 M^{-1} in the 160 kDa species and to 1.16 M^{-1} in the 90 kDa species (Table 1).

Energy transfer measurements confirm the conclusion that the acid pH has not grossly affected the microenvironment of the cysteine. For the αA homopolymer at pH 8 average Cys-Trp distances of 1.85 nm were calculated. At pH 2.5 the efficiency of transfer decreased by only 13%, indicating negligible changes in the average distance between the tryptophan and cysteine.

DISCUSSION

The work described in this paper examines the effects of low pH on the structure of homopolymers constructed from purified αA and αB chains. At pH 7–8 both αA and αB homopolymers exist as aggregates of 300 ± 50 kDa and 440 ± 80 kDa, respectively. As the pH is lowered the proteins dissociate to form species with molecular masses in the range of 80–250 kDa for the A chains and about 140 kDa for the B chains. While further decreases in pH (below pH 3.4) resulted in the complete dissociation of αB into denatured polypeptides, the αA dissociation products were stable at pH values as low as 2.

Attempts to fractionate the dissociation products of the B chains were hampered by their continual dissociation into subunits and nonspecific aggregation, often leading to precipitation. The precipitation was enhanced in the presence of salt and/or attempts to raise the pH (Augusteyn et al., 1888a). Because of these difficulties, further studies on the αB homopolymer at the low pH were not pursued.

The αA homopolymer was not subject to aggregation at the low pH, and fractionation of its dissociation products yielded relatively homogeneous and stable species with average molecular masses of 220, 160, and 90 kDa. Detailed characterization of these species indicates that there has been relatively little perturbation of the secondary and tertiary structure during the dissociation. The increased accessibility of tryptophan to quenching with acrylamide and the decreased energy transfer between tyrosine and tryptophan are consistent with an acid-induced expansion of the subunits rather than their denaturation. In general, the smaller the aggregates the more perturbed the overall structure.

The conformational data, especially the CD results shown in Fig. 9, may lead to the interpretation that the αA dissociation products are intermediates in the folding pathway of αA homopolymers. Folding studies have shown the existence of intermediate conformational states for various proteins (Kim and Baldwin, 1982). One type of intermediate state is referred to as the *molten globule* (MG) (Kuwajima, 1989). This state is typically formed at low pH either in the presence or absence of salt, depending on the protein under investigation. The previous studies have used monomeric proteins that were all well characterized and often of known crystal structure. These can be categorized into three major groups; those that form MG directly (carbonic anhydrase, α-lactalbumin), those that require salt and low pH to form the MG (β-lactamase), and those that are acid resistant (T4-lysozyme).

In this study a polymeric structure has been subjected to the effects of low pH, conditions analogous to those that result in the formation of MGs. It is not known if polymeric proteins can form molten globules. Our data suggest that the αA homopolymer belongs to the third group since its secondary and tertiary structures were virtually unperturbed. The near-UV CD spectra could be interpreted as indicating that the tertiary structure was altered or lost. However, fluorescence quenching, energy transfer, and maximum emission of tryptophan fluorescence revealed that a more likely explanation for the near-UV results is an acid expansion of the molecule rather than formation of a molten globule or denaturation.

It is essential for the determination of α-crystallin's quaternary structure to obtain a molecular weight for the smallest possible aggregate. Previous investigations of the αA and αB homopolymers have yielded estimates for the minimum molecular mass of 195–237 kDa (Augusteyn et al., 1989). In the present study, the use of acid pH has led to the isolation of species with an even lower molecular mass. While the protein species isolated at the acid pH consisted of a mixture of species with molecular masses ranging from 80 to 250 kDa, the majority of the dissociation product (>75%) had a molecular mass of 160 kDa. The results also indicated the presence of an even smaller species with a minimum molecular mass of 80 kDa. Further subfractionation of this species did not reveal the presence of lower molecular mass species.

Our studies do not yet permit us to conclusively determine which of the two species, the octamer or tetramer, may be the repeating unit. A tetrameric assembly of subunits is favored

by the recent findings of Merck et al. (1992) that a recombinant protein constructed from residues 64–173 of αA-crystallin forms a stable tetramer. However, this construct lacks the NH_2-terminal domain that has been postulated to be responsible for subunit assembly (Augusteyn and Koretz, 1987). The presence of this domain could result in the association of tetramers into octamers. These octamers would display twofold symmetry, and the small amount of tetramer observed in the present work could have been produced by splitting along this plane of symmetry. The greater structural perturbation observed with the tetramer would suggest that it has been destabilized through loss of interactions with another structure. For these reasons, we conclude that the monomeric form of αA-crystallin is an octamer.

Whether the same structure is found in α-crystallin is not clear. Acid dissociation of bovine and kangaroo α-crystallins does not yield either octamers or tetramers. This is not due to the presence of modified chains such as phosphorylated or truncated αA polypeptides since aggregates constructed from these dissociated in the same manner as the αA homopolymer. Further work will be required to determine the relationship between the structures of the homopolymer and native α-crystallins.

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