Structural Organization and Stability of a Thermoresistant Domain Generated by in Vivo Hydrolysis of the α-Crystallin B Chain from Calf Lens†

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Received February 18, 1992; Revised Manuscript Received June 3, 1992

ABSTRACT: A protein fragment (M_r ~9000) isolated from the cortex of nonpathological calf lenses has been structurally characterized. The polypeptide structure was well organized (39% α-helix, 33% β-structure, and 28% remainder) according to the far-ultraviolet circular dichorism. The fluorescence was heterogeneous for the presence of two tryptophan classes. Structure perturbation by pH and denaturant revealed cooperative structural transitions which are characteristic of a globular organization. A single-step unfolding curve induced by Gdn-HCl (midpoint = 1.38 M Gdn-HCl) was monitored by emission maximum shift as well as by far-ultraviolet circular dichroism. This transition was analyzed as a two-state process. The standard free energy of unfolding in the absence of the denaturant, $\Delta G°$ (H_2O), was found to be 10.80 ± 0.25 kJ/mol at 20 °C and pH 7.4. The fragment also shows an unusual thermal resistance. Its structure was unperturbed up to 90 °C according to the fluorescence and dichroism. This last property, its peculiar amino acid composition, and the sequence of a small segment are shared, among crystallins, only with the N-terminal region of the α-crystallin B chain. A search for proteolysis sites along the α-crystallin B chain sequence revealed that it possesses specific points for proteinase attack. These sites are particularly exposed and clustered in a very flexible region in the middle of the protein sequence. They are also well represented in the C-terminal extension of the molecule while a few are buried in the N-terminal region. In conclusion, our findings suggest that the fragment can reasonably be identified as the free N-terminal domain of the α-crystallin B chain generated by in vivo hydrolysis. It folds autonomously both in vitro and in vivo independently of the native C-terminal extension. The structural stability has probably favored its accumulation in the degradation pathway of the α-crystallin B chain. A physiological role for the fragment has also been postulated.

The mammalian lens has a very high protein content. In fact, it represents about 35% on a wet weight basis (Lindley et al., 1985). Most of these proteins are represented by three structural components designated as α-, β-, and γ-crystallins (Bloemendal, 1982; Chiou et al., 1988; Harding & Dilley, 1976; Herbrink & Bloemendal, 1974; Lindley et al., 1985; Van Kamp & Hoenders, 1973) which are believed to contribute significantly to the optical properties of the lens, i.e., refractive index, transparency, flexibility, etc. (Lindley et al., 1985). Crystallins are organized in a highly complex supramolecular system (Lindley et al., 1985). Their role is practically unknown.

The lens has often been depicted as a closed system which accumulates the covalent modifications of its proteins. These covalent (or posttranslational) modifications "may serve as marking steps for protein degradation" (Stadtman, 1990). Since a correlation between the in vivo degradation rate of the enzymes and their thermal stability was reasonably demonstrated by McLendon and Radany (1978), we focused our attention on the end products of the protein degradation process in order to correlate the properties of the most stable and structured fragments with their hydrolytic susceptibilities.

In our laboratory the population of the water-soluble lens crystallins has been separated by chromatography (Bjork, 1964; Van Dam, 1966). A small protein fraction, not previously described, has been found in the low molecular mass region. We here report the structural characterization and the molecular properties of a polypeptide (M_r ~9000) purified from that fraction. We also show evidence that this polypeptide may be a stable fragment generated by in vivo hydrolysis of the α-crystallin B chain. The fragmentation is probably due to the activity of proteolytic enzymes (proteinases) present in the lens. An alternative hypothesis has also been considered.

MATERIALS AND METHODS

Eyes of 4–6-month-old calves were obtained from a local slaughterhouse and the lenses removed within 2 h of death. Lenses were carefully examined for opacities. Only completely clear and nonpathological specimens were selected. Lenses were decapsulated, and the cortical tissue was homogenized in 5 volumes of ice-cold 0.1 M Tris-HCl, 0.15 M KCl, and 0.01 M β-MSH,[1] pH 7.8. The homogenate was centrifuged at 27000*g* for 1 h. Pellet, containing water-insoluble proteins, was discarded, and the supernatant was designated as total cortical extract.

The protocol procedure of crystallin preparation was essentially performed according to Bjork (1964) and Van Dam (1966). The Sepharose CL-6B resin was changed into a Sephadex G-75. This variation improves the resolution at low molecular masses. Aliquots (3–5 mL) of cortical extract were applied to Sephadex G-75 column (3 × 100 cm) to isolate the different classes of crystallins. The suitable fractions were pooled, concentrated by lyophilization, and rechromato-

† This work was supported by the Consiglio Nazionale delle Ricerche (Rome, Italy) "Target Project on Biotechnology and Bioinstrumentation" and by the Italian Ministry of Education (MPI 40%).

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[1] Abbreviations: β-MSH, β-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Gdn-HCl, guanidine hydrochloride.

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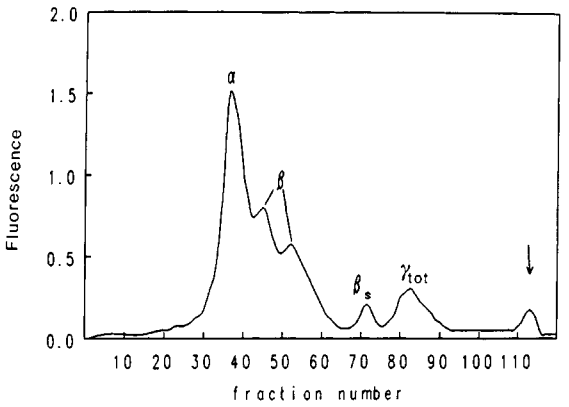

FIGURE 1: Characteristic fractionation pattern of bovine lens cortical extract by Sephadex G-50 gel filtration. Cortical extract (3 mL) was applied to the column and eluted with 0.1 M Tris-HCl, 0.15 M KCl, and 0.01 M β-MSH, pH 7.8, at a flow rate of 13 mL/h. Fractions (5 mL) were collected and detected by fluorescence. The arrow indicates the peak under study.

graphed by FPLC using a Superose 12 (20 mM Tris-HCl and 100 mM KCl, pH 7.8) or on a calibrated Sephadex G-50 column (50 mM NaH_2PO_4 and 0.01 M β-MSH, pH 7.4) to obtain purified protein samples for spectroscopic studies. This step is important to eliminate some minor protein components (see also Figure 2), the presence of which may favor aggregation. Moreover, we have always used fresh prepared polypeptide samples for our studies.

Unless otherwise stated, all the operations were carried out at 4 °C. Care was also taken to perform experiments in the dark in order to avoid undesirable photochemical reactions. Moreover, all solutions were degassed and carefully saturated with nitrogen to minimize tryptophan oxidation phenomena. Although it has been reported that in vivo enzymatic degradation of lens proteins is very slow (Simkin, 1959), experiments were carried out in the presence of proteinase inhibitors (diethylenetriaminepentaacetic acid, ethylenediaminetetracetic acid, or diisopropyl fluorophosphate) (Beswick & Harding, 1984; Wagner et al., 1985).

Molecular mass determination was performed either by gel fitration chromatography on a Sephadex G-50 or by FPLC on a Superose 12 column. Myoglobin (M_r 17 100), cytochrome *c* (M_r 12 000), and aproteinin (M_r 6500) were used as molecular mass markers. The elution buffer was 50 mM NaH_2PO_4 and 0.01 M β-MSH, pH 7.4. The SDS–PAGE in 8 M urea was performed using SDS–Molecular Weight-17 Kit (Sigma) for the molecular mass range 2510–16 950 Da. The procedure for SDS molecular mass determination is a modification of the method of Swank and Munkres (1971).

The polypeptide concentration was determined by the amount of aromatic residues according to Wetlaufer (1962). A molar absorption coefficient at 280 nm of 1.4×10^4 M^{-1} cm^{-1} was calculated. A very similar value (1.6×10^4 M^{-1} cm^{-1}) was determined by the Bradford method (Bradford, 1976).

Absorption spectra were obtained on a Cary 219 double-beam recording spectrophotometer or on a Perkin Elmer Lambda Array 3840 spectrophotometer using 1-cm cuvettes.

Fluorescence measurements were made on a MPF-B 66 Perkin Elmer recording spectrofluorometer equipped with thermostated 1-cm quartz cuvettes. The solvent emission was corrected in all spectra.

Circular dichroism spectra were performed with a Jobin-Yvon Mark III dichrograph in the far-ultraviolet (200–250 nm), using 0.1-cm cuvettes. Far-ultraviolet circular dichroism spectra are presented in terms of mean residue molecular ellipticity [θ] (in deg·cm^2·$dmol^{-1}$), based on a mean residue weight of 115. Secondary structure calculations were made by a computerized program with five different methods of analysis (Menèndez-Arias et al., 1989).

Unfolding–refolding was monitored by fluorescence and far-ultraviolet circular dichroism. Spectroscopic-grade Gdn-HCl was purchased from Schwarz-Mann Biotech. Prior to the measurements the samples were equilibrated for 24 h at 20 °C. Thermal denaturation studies were performed by a circulating water bath. All measurements were repeated three times, and only after the spectra displayed no further changes with time, they were recorded. Sample temperature was determined using a thermocouple inserted into a reference cuvette.

Volume, hydrophobicity, and local flexibility plots of the α-crystallin B chain were performed according to Chothia (1984), Manavalan and Ponnuswamy (1971), and Ragone (Ragone et al., 1989), respectively. Plots were generated by a routine of the program FAST, version 1.1 (Facchiano et al., 1989). A five-residue shifting window was used along the protein sequence. The threshold value (1300) in the flexibility plots corresponds to the product between normalized values of the highest hydrophobicity and the largest volume among turn-inducing residues, according to Ragone et al. (1989).

The amino acid composition of our polypeptide was determined with an LKB-4150 amino acid analyzer. 6 N HCl and 105 °C were used for hydrolysis. Three different hydrolysis times were used for analysis (24, 36, and 75 h) by extrapolating to zero time when necessary (Thr, Ser, etc.). Quantitation of sulfhydryl groups was also attempted by the 5,5′-dithiobis(2-nitrobenzoic acid) method (Ellman, 1959) with negative results. Tryptophan amount was determined from the ultraviolet absorption spectrum by the second derivative method (Balestrieri et al., 1978).

Data analysis was performed by computerized methods.

RESULTS

Purification and Characterization

During chromatographic purification of the lens proteins for physicochemical studies (Pulcini et al., 1989; Stiuso et al., 1988, 1990), a very small protein fraction sometimes appeared in the region of the low molecular masses. Figure 1 shows a typical elution pattern of the soluble lens crystallins from Sephadex G-75. The fraction under investigation is indicated by an arrow. The protein amount was quite low and variable from preparation to preparation. Therefore, numerous cycles of accumulation were carried out. Samples under the fraction maximum were collected, pooled, and concentrated by lyophilization. When a concentrated specimen was rechromatographed by FPLC (Superose 12), various fractions were detected, as shown in Figure 2. The fraction on the front of the solvent probably represents a mixture of protein aggregates. The major fraction is a single narrow symmetrical band. This fraction was also checked for molecular mass and purity. Protein purity was tested by SDS–PAGE, or else on a Sephadex G-50 column (not shown) and on a Superose 12, both calibrated for molecular masses. A single band was constant for each technique with a molecular mass of about 9000 Da (Table I). These results indicate that the band is single and homogeneous.

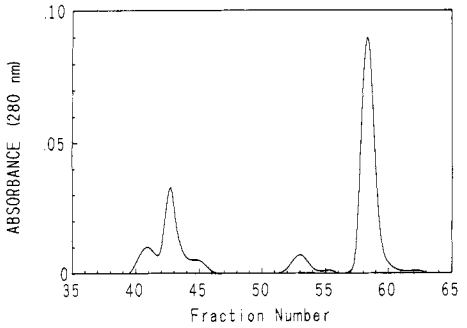

FIGURE 2: FPLC elution pattern by Superose 12 in 20 mM Tris-HCl and 100 mM KCl, pH 7.8.

Table I: Molecular Mass Determination

	Da
Sephadex G-50	9000 ● 1000
FPLC–Superose 12	8000 ± 500
SDS–PAGE, 8 M Urea	8500 ± 1000

Structural Characterization

Since a molecular mass of about 9000 Da corresponds to about 80 residues, we became interested in the structure. A detailed investigation of the polypeptide structural properties was performed by spectroscopic methods.

The absorption spectrum (not shown) is centered at 276 nm. No absorption is evident above 305–310 nm, while a deep minimum is located around 250 nm (Donovan, 1969; Wetlaufer, 1962). A shoulder is also present at 292 nm. This spectral feature is indicative of tryptophan.

The protein fluorescence is essentially due to tryptophanyl residues (Kronman, 1976; Longworth, 1971). This can be strongly influenced by the structural features and polarity of the tryptophan microenvironment (Chen et al., 1969; Lehrer, 1971). The fluorescence spectrum (Figure 3, part A) shows an emission maximum at 337 nm (excitation at 280 nm), suggesting partially buried tryptophan(s). Part B of the same figure shows the excitation spectrum of the protein by emission at 330 nm. The excitation spectrum reflects the absorption spectrum of the protein fluorophores.

The secondary protein structure can be evaluated by far-ultraviolet circular dichroism spectra (Adler et al., 1973). Such analyses are based on the assumption that chiroptical bands represent the sum of contributions from helical, β-sheet, and random conformations (Fasman et al., 1970). The far-ultraviolet circular dichroism spectrum of the polypeptide (Figure 4) shows a negative band centered at about 217 nm. The band was analyzed by a computer fitting method taking into account protein spectra with known conformations (Menèndez-Arias et al., 1989). The results indicate a high level of structural organization, characterized by 39% α-helix, 33% β-sheet, and 28% remainders (rms = 8). This suggests that the purified polypeptide is structurally well organized. The relatively high amount of helical content in respect to the extensive presence of β structure found in various crystallins probably depends on the peculiar morphology of the CD band. In fact, the band is centered at 217–218 nm. This position is intermediate between the β structure (minimum at 215–216 nm) and the strong helical contribution at 222 nm. Therefore, the contributions at 222 and 216 nm have identical weight. This might lead to overestimation of the helical structure. Attempts with other methods gave similar or worse results. The figure also shows the spectrum of the polypeptide denatured by Gdn-HCl. The dichroic signal is lost, and the spectrum has the characteristic morphology of an unordered polypeptide.

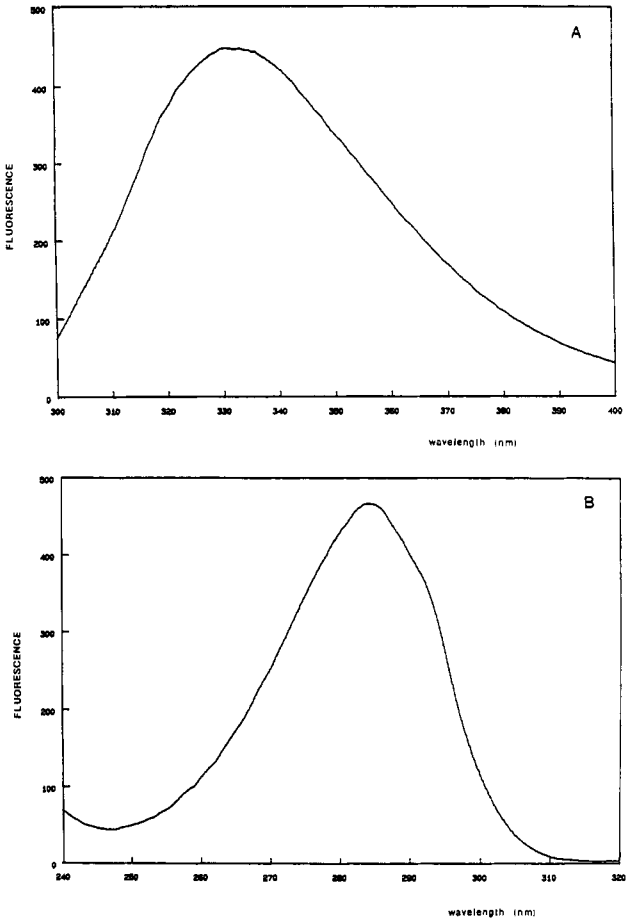

FIGURE 3: Emission (part A) and excitation (part B) fluorescence spectra of the polypeptide in 50 mM phosphate buffer and 100 mM KCl, pH 7.4. Excitation was at 280 nm. Emission was at 330 nm.

Effect of Structure-Perturbing Agents

The conformational stability of this system was investigated by perturbing its structure with various denaturing agents: Gdn-HCl, pH, and temperature, respectively.

Denaturation by Gdn-HCl. It is well-known that at high concentration Gdn-HCl is a strong denaturant which destabilizes the structural organization of macromolecules (Tanford, 1970). The unfolding was monitored by spectroscopic techniques to estimate conformational stability.

Figure 5 shows the fluorescence dependence of the polypeptide on the guanidine concentration. The fluorescence behavior is complex, and an evident sigmoidal denaturation transition was not discernible. The fluorescence increase at low denaturant concentration (between 0 and 0.5 M) reveals quenched (or partially quenched) tryptophans in the protein matrix. Then, the fluorescence drops down between 0.5 and 4 M denaturant. The complex fluorescence behavior suggests tryptophan fluorescence heterogeneity. Figure 6 shows fluorescence and fluorescence difference spectra (see inset of Figure 6) at various denaturant concentrations. The red shift of the fluorescence emission maximum monitors the exposure of buried tryptophan residues to the solvent (Kronman, 1976; Lehrer, 1971). At 3 M Gdn-HCl the fluorescence maximum is centered at 350 nm, indicating a complete unfolding of the protein matrix and a complete exposure of tryptophan(s) to the solvent. Difference spectra clearly indicate that tryptophanyl residues are located in different structural environ-

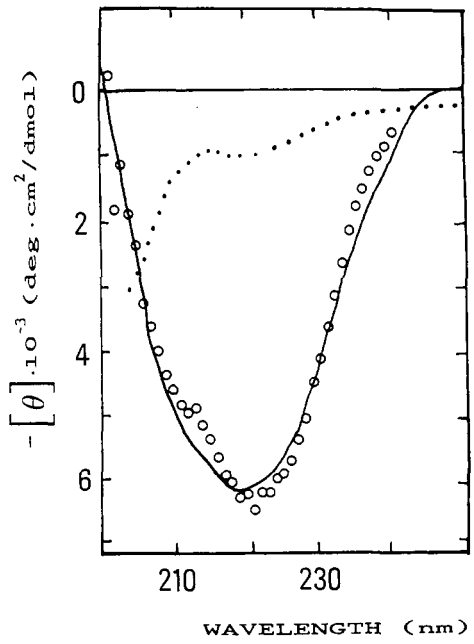

FIGURE 4: Far-ultraviolet circular dichroism spectrum of the polypeptide in 50 mM phosphate buffer and 100 mM KCl, pH 7.4. Unfilled circles represent the spectrum fitted for secondary structure content (Menèndez-Arias et al., 1989). The fit indicates a high level of structural organization, characterized by 39% α-helix, 33% β-sheet, and 28% remainders (rms = 8). The dotted spectrum represents the protein denatured by 4 M Gdn-HCl, pH 7.4, in the same buffer.

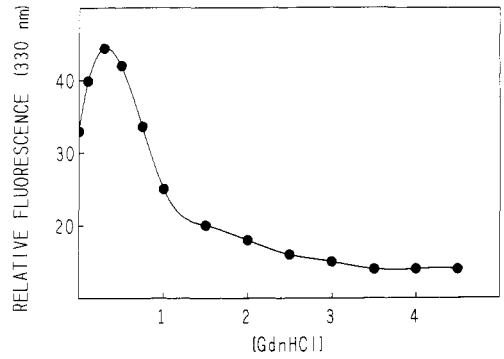

FIGURE 5: Effect of Gdn-HCl concentration on the fluorescence intensity at 330 nm of the polypeptide in 50 mM phosphate buffer and 100 mM KCl, pH 7.4. Excitation was at 280 nm. Emission and excitation slits were 3 and 4 nm, respectively. The error estimates are within the contours of the plotted symbols.

ments and that they can be separated into two emitting classes. The first class includes a buried residue(s) emitting around 330 nm. The second one includes an external tryptophan(s) emitting at 350 nm, which is thus in contact with the solvent. The spectral increase from 0 to 0.5 M Gdn-HCl also reveals that the external tryptophan is partially quenched in the native protein.

Figure 7 shows the dependence of both the fluorescence maximum shift and the far-ultraviolet circular dichroism (220 nm) on the denaturant concentration. Data are normalized and reported as fraction of unfolded form. Experimental points fit the same curve. Therefore, a two-state transition can be assumed (Pace, 1986). A fluorescence maximum shift from 337 to 350 nm was observed. This is indicative of the aromatic fluorophore exposure to the aqueous medium upon increasing Gdn-HCl concentration (Kronman, 1976; Lehrer, 1971). Such a parameter essentially reflects changes in tertiary structure while the far-ultraviolet circular dichroism reflects the unfolding of the secondary structure. The denaturation is reversible, and the curve shows a sigmoidal shape with a

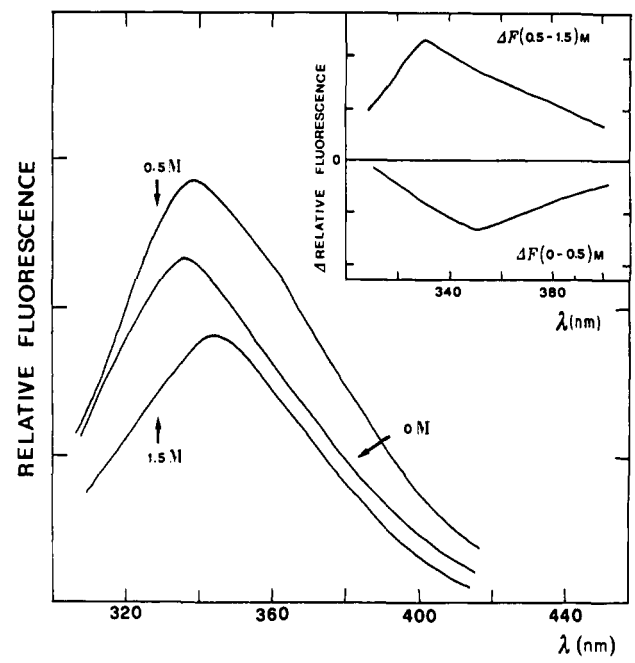

FIGURE 6: Fluorescence emission spectra of the polypeptide at different Gdn-HCl concentrations. The inset shows fluorescence difference spectra at various denaturant concentrations. Experimental conditions as in Figure 5.

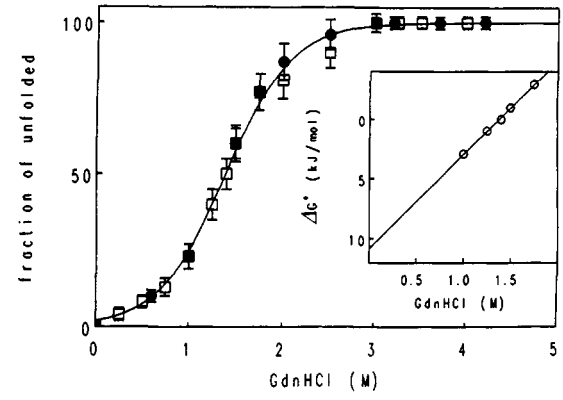

FIGURE 7: Effect of the denaturant (Gdn-HCl) on emission maximum (330 nm) (□) and circular dichroism at 220 nm (forward ■ and reversal ●) of the polypeptide. Data have been normalized and reported as fraction of unfolded. Experimental conditions as in Figure 5. Bars at data points represent the range of error.

midpoint centered at 1.38 M guanidine. Equilibrium unfolding and refolding experiments indicate that the polypeptide retains a well-organized structure which is cooperatively lost due to the effect of denaturants. By assuming a two-state mechanism for unfolding, the fraction of unfolded protein, F_u, has been calculated by the equation:

$$F_u = (Y_f - Y_{obs})/(Y_f - Y_u)$$

where Y_{obs} is the observed variable parameter (maximum shift and circular dichroism), and Y_f and Y_u are the values of Y characteristic of the native and denatured conformations. The standard free energy difference ($\Delta G°$) between the folded and unfolded states has been calculated by the equation:

$$\Delta G° = -RT \ln [F_u/(1 - F_u)]$$

where R is the gas constant and T is the absolute temperature. The values of Y (folded and unfolded) in the transition region were extrapolated from the linear portions of the pre- and posttransition regions of the denaturation curve, respectively (Pace, 1986). $\Delta G°$ was found to vary linearly with Gdn-HCl

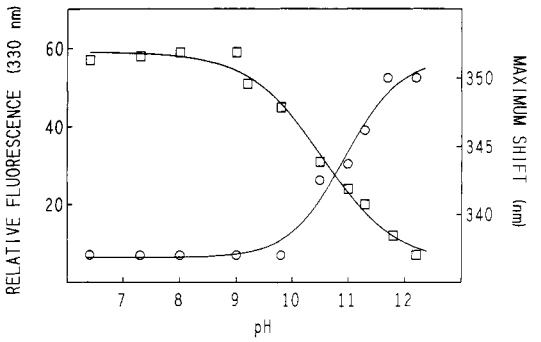

FIGURE 8: Effect of pH on the fluorescence emission intensity (□) at 330 nm and maximum shift (○) of the purified polypeptide in 50 mM phosphate buffer and 100 mM KCl, pH 7.4. Excitation was at 280 nm.

concentration (see inset in Figure 7). A linear extrapolation was used to estimate the structure stability [$\Delta G°(H_2O)$]. A least squares analysis was used to determine the $\Delta G°$ (r = 0.999) in the absence of denaturant (Pace, 1986). A $\Delta G°(H_2O)$ value of 10.80 ± 0.25 kJ/mol was determined for the fragment stability at 25 °C and pH 7.4. This value is consistent with the low molecular mass of the polypeptide.

Effect of pH. The effect of increasing pH values, from 6.5 to 12.2, is shown in Figure 8. The figure reports the pH dependence of both the fluorescence intensity and the maximum shift of the polypeptide. The titration reveals a relevant fluorescence quenching at 330 nm. The large pH interval of this effect suggests two overlapping phenomena. In fact, while the fluorescence quenching starts at pH 9, the red shift of the fluorescence maximum starts only at pH 10. This suggests that the fluorescence quenching reasonably due to the tyrosinate formation overlaps a conformational transition. Both effects end concomitantly at pH 12.2. The fluorescence maximum centered at 350 nm reveals that the tertiary structure is lost at the pH. The transition was not completely reversible. We did not attempt to drop the pH down into the acidic range because of irreversible protein aggregation.

Amino Acid Composition. The aforementioned experimental data demonstrate that a small polypeptide with a molecular mass of about 9000 Da behaves as a well-organized globular system which is able to unfold cooperatively. In consequence, some questions arise about its origin. Is the purified polypeptide a small native protein or a proteolytic fragment? And, in this last case, what is its origin?

To gain greater insight into the structural features and the origin of this polypeptide, we determined its amino acid composition. Table II shows that only 19 amino acids are present. In fact, cysteine is lacking. We have considered a residue composition corresponding to a polypeptide molecular mass of 9000 Da. The considerations that (i) the molecular mass of some crystallins or of their subunits is around 20 000 Da (van der Ouderaa et al., 1973, 1974), (ii) numerous crystallins show the presence of two structural domains in their fold (Blundell et al., 1981; Wistow, 1985), (iii) the absence of cysteine and the presence of two tryptophans are two distinctive features of our polypeptide, and (iv) the polypeptide possesses a molecular mass of about 9000 Da (which means half a subunit) led us to compare the polypeptide amino acid composition with those of other crystallins or their subunits. Comparisons were carried out between the fragment and those crystallin parts of like molecular mass. Therefore, we decided to use both N-terminal and C-terminal extensions of all known crystallins separately. The inspection revealed that the specific composition of the polypeptide is shared only with that of the N-terminal region of the α-crystallin B chain. Twelve amino acids out of twenty showed the same values. It has to be emphasized that differences are generally small in the diverging cases (e.g., His, Ile, Phe, Pro). They could reasonably be ascribed to the heterogeneous polypeptide populations owing to the proteinase's attack on both sides of the protein with a weighted molecular mass of 9000 Da. Moreover, the presence of two tryptophan residues, two rare residues, is almost in agreement with the fluorescence heterogeneity shown by the polypeptide. Table II clearly demonstrates that other crystallins cannot be taken into consideration owing to their marked dissimilarity. The comparison with the C-terminal extensions is not shown because the lack of correspondence was complete. This analysis suggested that the fragment may have originated from the α-crystallin B chain. It might reasonably be identified as part of the N-terminal domain of that protein. We have also performed a global evaluation of the physicochemical features of residues. Table III shows the content of acidic, basic, charged, and hydrophobic residues. Even the net charge is reasonably similar for both peptides. It is also interesting to note the relevant content of hydrophobic residues of the fragment.

We have also attempted several steps of automated Edman analysis. The N-terminal 15 amino acid residues of the polypeptide determined were as follows: Ala-Ile-His-Pro-Trp-Ile-Arg-Arg-Pro-Phe-Phe-Pro-Phe-His-Ser. This sequence corresponds to segment 4–19 in the primary structure of the α-crystallin B chain. We think this result confirms that the polypeptide is the N-terminal part of that crystallin. However, studies are in progress to unravel the remaining part of the sequence. In this context, we were curious to assess the presence of a specific structural feature which is known to be peculiar to the α-crystallin.

Thermal Denaturation. α-Crystallin has been considered a heat-stable protein among crystallins because it does not denature upon heating. In fact, it has been recently found (Maiti et al., 1988) that the α-crystallin from bovine lens is stable at temperatures up to 90 °C. Therefore, we probed the thermal resistance of our fragment to determine whether that specific feature was shared. Strikingly, the fragment was very resistant to thermal denaturation. We found that it retained an unperturbed structure (Figure 9) between 25 and 90 °C on fluorescence and circular dichroism. No evident cooperative transition was seen. The constant fluorescence emission maximum indicated that the tryptophan microenvironments were unperturbed. The decreased fluorescence intensity with temperature rise is due to the thermal deactivation of the excited state of tryptophanyl residues although a slight change of slope near 70 °C suggests that other phenomena might compete with the deactivation. No evident change in the far-ultraviolet circular dichroism was detected. This does not exclude that subtle noncooperative phenomena may be induced by the high temperature. Experiments performed by differential scanning calorimetry (Steadman et al., 1989) revealed the presence of subtle structural alteration of the α-crystallin above 70 °C. Precipitation was detectable by light scattering only at concentrations higher than 2 mg/mL. Although the lower protein concentration of our experiment did not show protein precipitation, the partial recovery of the fluorescence emission at room temperature, after incubation at 80 °C for various lengths of time, revealed that subtle and irreversible noncooperative phenomena kinetically compete with the native protein. The aforementioned

Table II: Amino Acid Composition of the N-Terminal Extensions of Crystallin Subunits (Residues 1–80)[a]

residues	experimental	calculated from sequences									
	α-B	α-B	α-A	β-B3	β-A3-A1	β-B1	β-Bp	γ	γII	γII-I	γs
Ala	5	3	1	8	2	16	8	0	0	0	9
Arg	5	7	6	3	3	2	10	8	8	8	13
Asn	2	2[b]	0	2[b]	4	2[b]	6	4	4	4	6
Asp	5	5[b]	7	0	3	1	10	6	5[b]	6	10
Cys	0	0[b]	0[b]	1	2	1	2	6	5	6	5
Gln	1	1[b]	3	5	7	3	17	6	9	6	8
Glu	4	4[b]	3	10	8	5	15	3	1	3	14
Gly	4	2	4[b]	10	4[b]	10	19	7	7	7	14
His	3	4	2	2	1	0	8	2	3[b]	2	7
Ile	3	4	5	2	7	0	6	3[b]	3[b]	3[b]	7
Leu	10	10[b]	9	6	2	3	10[b]	4	3	3	10[b]
Lys	1	1[b]	3	4	6	5	13	1[b]	1[b]	1[b]	9
Met	2	2[b]	1	1	3	0	2[b]	2[b]	2[b]	3	7
Phe	8	10	11	3	4	3	8[b]	5	5	5	10
Pro	11	9	5	3	6	17	14	4	3	4	7
Ser	13	10	8	6	5	5	17	7	7	7	10
Thr	2	2[b]	3	3	7	1	7	1	1	1	5
Trp	2	2[b]	1	1	2[b]	0	4	2[b]	2[b]	2[b]	4
Tyr	1	1[b]	4	3	2	1[b]	9	8	8	8	12
Val	1	1[b]	4	5	2	4	14	1[b]	3	1[b]	8
Asx	0	0[b]	0[b]	0[b]	0[b]	0[b]	2	0[b]	0[b]	0[b]	0[b]
Glx	0	0[b]	0[b]	2	0[b]	1	0[b]	0[b]	0[b]	0[b]	0[b]
total	83	80	80	80	80	80	80	80	80	80	80

[a] Sequences are from the Protein Information Resource (PIR) Data Bank. [b] Indicates values similar to the experimental ones.

Table III: Properties of the Constituting Amino Acids of the α-Crystallin Fragment 1–83

residue type	calcd[a]	exptl[b]
acidic	9	9
basic	8	6
charged	17	15
net charge	−1	−3
hydrophobic	25	22

[a] Calculated values are from the 1–80 fragment of α-crystallin B chain according to van der Ouderaa et al. (1973). [b] Experimental values obtained by using a fragment molecular mass of 9000 Da.

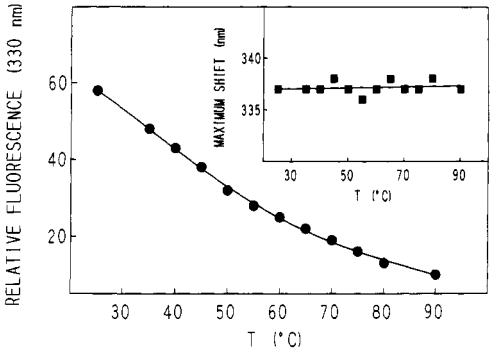

FIGURE 9: Changes of tryptophan fluorescence intensity and emission maximum (see inset) of the purified polypeptide in 50 mM phosphate buffer and 100 mM KCl, pH 7.4, as a function of the temperature. Excitation was at 280 nm.

results suggest that hydrophobic residues play an important role in the determination of the polypeptide thermoresistance. Interactions between apolar residues are thought to be important in determining the three-dimensional structure of a protein. However, the fragment thermoresistance is striking considering that it is stable enough even in the absence of a large part of its native structure.

DISCUSSION

During crystallin purification by chromatography we observed a low molecular mass protein fraction. This was present in very small and variable amounts and was detected by fluorescence in the tail of the elution pattern. The fraction was heterogeneous, and the most abundant component has been purified and structurally characterized.

The main conclusions of our work are the following: (i) we have purified a small soluble polypeptide (M_r 9000) from nonpathological calf lenses, (ii) some specific molecular properties of this polypeptide are shared only with the N-terminal domain of the α-crystallin B chain, and (iii) the polypeptide retains a well-organized structural architecture, i.e., it can be considered a globular protein. In fact, its structure (both α-helix and β-structure are present) is perturbable by Gdn-HCl, showing a cooperative denaturation curve. A complete and reversible unfolding is detectable according to the criteria of the spectroscopic methods. These data demonstrate that the polypeptide can fold autonomously.

To our knowledge, these data show for the first time that the structural domain of a crystallin can exist folded in vivo independently of the native C-terminal extension. This indirectly supports the view that the contribution of interdomain interactions to the folding and stability of each domain is only marginal at least for the α-crystallin. Limited proteolysis experiments on the γB-crystallin have demonstrated that the isolated N-terminal domain can unfold/fold independently of the C-terminal counterpart (Sharma et al., 1990; Rainer et al., 1990). We have investigated the origin of this fragment by comparing some of its specific structural properties with those shown by other known crystallins. Its amino acid composition as well as its peculiar thermoresistance suggests that it can have originated from the fragmented α-crystallin B chain. In addition, its molecular mass, the sequence of the N-terminal 15 residues, and two tryptophanyl residues indicate that we are dealing with the N-terminal domain of that crystallin.

α-Crystallin is the most abundant among lens proteins (Harding & Dilley, 1976). In fact, it represents more than 20% of the total proteins. The α-crystallin structural organization is complex. At least four subunits, i.e., A_1, A_2, B_1, and B_2, concur to form this high molecular mass system (Spector & Katz, 1965). Each subunit has a molecular mass of about

20 000 Da (van der Ouderaa, 1973, 1974), and it is known that they interact in a noncovalent manner (Bloemendal, 1977). Several complex models for the architecture of its quaternary organization have been presented (Bindels et al., 1979; Siezen et al., 1980; Tardieu et al., 1986; Augustein & Koretz, 1987, van den Oetelaar et al., 1990). They show different molecular masses according to the proposed architecture. However, the subunits of the crystallin are very prone to form aggregates, and the protein, upon aging, increases its molecular mass even in the absence of protein synthesis (Siezen et al., 1979; Bindels et al., 1982; Bessems et al., 1983). A subunit exchange among native α-crystallin molecules has been postulated to be under the control of a dynamic quaternary structure that adapts changes to cytoplasmic conditions (van den Oetelaar et al., 1990). The A_2 and B_2 chains are primary gene products (Delcour & Papaconstantinou, 1972), while the A_1 and B_1 chains arise from A_2 and B_2, respectively, as a result of post-translation modifications, deamination (Hoenders & Bloemendal, 1981), or phosphorylation (Chiesa et al., 1987a,b; Spector et al., 1985; Voorter et al., 1986). The B chain is more basic polypeptide than the A chain (Schoenmakers et al., 1969). Both chains are approximately 175 residues long (van der Ouderaa et al., 1973, 1974).

The α-crystallin is the only one among crystallins that does not denature upon heating up to 90 °C (Maiti et al., 1988). We found a similar behavior in our polypeptide. This is not surprising since it was shown that the ubiquitous heat-shock proteins are similar to the α-crystallin (Ingolia & Graig, 1982; Wistow, 1985).

Another consideration deserves some attention. In vivo degradation of the lens proteins has been observed (Simkin, 1959). However, protein turnover has been reported to be very slow in the lens compared to other tissues (Delcour & Papaconstantinou, 1972; Waley, 1964). A variety of proteinases exist in the lens tissue (Hanson & Frohne, 1976; Spector, 1963; Swanson et al., 1978; Blow et al., 1975; Tse et al., 1981), and their activity is controlled by specific inhibitory proteins (Wagner et al., 1985). The proteinase activity is higher in the cortex than in the nucleus (Taylor et al., 1983). In particular, it has been observed that two proteinases, i.e., the neutral proteinase (Waley & van Heyningen, 1962) and the 25K serine proteinase (Srivastava & Ortwerth, 1983), are active on crystallins in vivo. Both proteinases have specific sites of hydrolysis. The neutral proteinase acts on sequence stretches including Leu, Ile, or Val (Waley & van Heyningen, 1962; Wagner et al., 1979) while the 25K serine proteinase hydrolyzes segments including Arg, Lys, or Asn (Srivastava & Ortwerth, 1983). On this ground, we checked the position of these specific sites of hydrolysis in the α-crystallin B chain sequence. The result was compared to the local flexibility of the chain by a flexibility plot, since the most probable sites of enzymatic hydrolysis are located on very flexible and exposed segments (Ragone et al., 1989; Fontana, 1988).

Figure 10 shows the flexibility plot of the B chain according to Ragone et al. (1989). It has been demonstrated that plots generated by this method are comparable to those from X-ray thermal factors at high resolution (Ragone et al., 1989). This approach has already been successfully used by us to study the structural organization of β/γ-crystallins (Stiuso et al., 1990). This figure also reports volume (Chothia, 1984) as well as hydrophobicity (Manavalan & Ponnuswamy, 1971) plots of the same chain. The presence of a flexible segment in the middle of the molecule, featured by small mean volume and low mean hydrophobicity of residues, addressed our

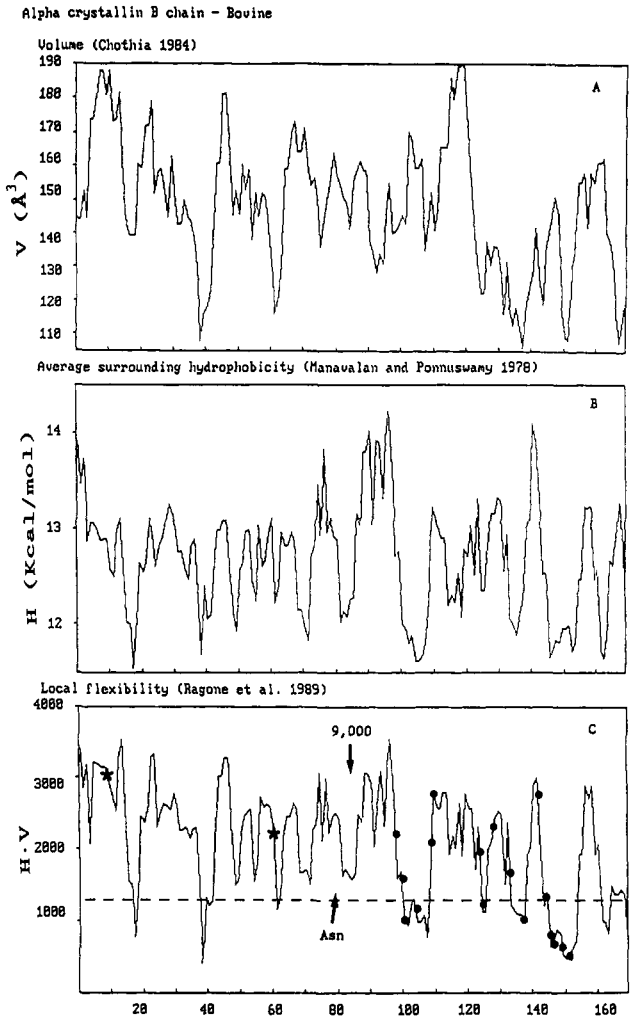

FIGURE 10: (A) Volume, (B) hydrophobicity, and (C) flexibility plots of the α-crystallin B chain. Asterisks indicate tryptophan positions along the sequence. The arrow indicates the sequence position corresponding to M_r 9000. Position of Asn residue 80 is also indicated by an arrow. Possible hydrolysis sites for proteinases are highlighted by filled circles on more flexible segments. The dashed line in (C) indicates the flexibility threshold value as suggested by Ragone et al. (1989).

attention to the similar physicochemical properties shown by the hinge segment present in both β_s- and γB-crystallins (Stiuso et al., 1990). This segment connects the bilobal domain organization shown by those proteins (Blundell et al., 1981; Stiuso et al., 1990).

Notwithstanding that the α-crystallin sequence is not homologous to the β/γ B-crystallin family, Wistow (1985), and Argos and Siezen (1983) predicted a four-motif β-barrel structure (the so-called β/γ fold) for that protein. Accordingly, it is remarkable that our experimental and predictive results reasonably substantiate the view that the α-crystallin B chain would adopt a two-domain organization. In addition, the monolobal state of the domains suggests that the interactions at the interface are not critical for their globular compactness.

The position of specific hydrolysis sites has also been mapped onto the protein sequence. Plots show that the large structural segment between residues 99 and 109 should be very flexible. Other segments (i.e., around residues 18, 38, 62, and 125 and between residues 123 and 125, 134 and 138, 142 and 153, and 161 and 165) are also to be regarded as flexible. Two important considerations are to be borne in mind. First, no specific hydrolysis site is available on flexible segments in the N-

terminal part of the sequence. Sites are embedded in highly hydrophobic and rigid regions characterized by large residues, and thus they are not available to proteinase attack. Second, the large flexible segment in the middle of the molecule is very rich in hydrolytic sites. Therefore, we cannot rule out that structural changes induced by the enzymatic attack at the interface between the domains contribute to weakening of the bilobal structure of the α-crystallin. However, the presence of a cluster of specific hydrolysis sites on the large flexible segment at the sequence position around 100 is consistent with a protein fragment of M_r about 9000 in vivo. The N-terminal part survived in solution owing to its intrinsic compactness and conformational stability. Our experimental results clearly showed a well-organized globular structure, whose hydrolysis sites are located in the hydrophobic core of the protein matrix. Therefore, it is conceivable that they are not available to proteinases, while the C-terminal fragment sites are exposed and located in flexible segments. This domain should be completely destroyed in vivo by enzymes. Probably, more hydrolysis sites become available at the N-terminal tail of the stable N-terminal domain by taking out small fragments.

Experiments of in vitro enzymatic hydrolysis of the α-crystallin are widely described in the literature. These experiments were carried out to probe the α-crystallin structure not only by proteolytic enzymes like trypsin, chymotrypsin, etc. (Siezen & Hoenders, 1979), but also by the neutral proteinase present in the cortex homogenate (Wagner et al., 1982). All these studies have demonstrated that (i) in the lens, proteinases preferentially work on the α-crystallin, (ii) the enzymatic activity of proteinases is remarkable on the α-crystallin B chain, and (iii) hydrolytic enzymes produce fragments in the M_r range 9000–10 000. These observations strongly support our experimental findings. We can also exclude that our polypeptide is an experimental artifact. Experiments were carried out at low temperature in the presence of proteinase inhibitors. This procedure should minimize the hydrolytic effects. Moreover, the slow kinetics of the α-crystallin proteolysis (Simkin, 1959) needs a time longer than our experimental procedure of separation to generate reliable fragments.

In conclusion, the experimental evidence suggests that we are dealing with a structured fragment generated by in vivo hydrolysis of the α-crystallin B chain. This was experimentally detectable because of its structural stability coupled with a strong resistance to the hydrolytic attack. Since previous studies observed that thermal resistance can be related to the rate of protein intracellular turnover (McLendon & Radanay, 1978), the relevant thermal resistance of our fragment suggests a plausible mechanism about its slow degradation in vivo. Specifically, the stability and the organization characterizing the fragment suggest that it is not very vulnerable to the proteolysis in the lens. In fact, the high stability reduces the fraction of unfolded polypeptide, thus limiting its susceptibility to proteolysis. According to this view, correlations between increasing thermal stability and decreasing proteolytic susceptibility in vivo have been reported for a number of proteins (Pakula et al., 1986; Parsell & Sauer, 1989). All these observations open a window on site-directed mutagenesis as a tool to control the thermoresistance of specific proteins, and hence their intracellular turnover. This fragment is one of the most reasonable candidates to be considered as an intermediate of the degradation pathway of α-crystallin because of its long lifetime. Its organized globular matrix and its specific structural features also suggest that we are probably in the presence of a free structural domain generated by in vivo hydrolysis of the α-crystallin B chain.

The focal points to highlight from the aforementioned conclusions are as follows: (i) To the best of our knowledge, this is the first time that a protein domain generated by in vivo hydrolysis has been purified and characterized. It folds autonomously both in vivo and in vitro independently of the native C-terminal extension. (ii) Our results weaken the theory of the lens as a closed protein system which remains unperturbed during its life span. Recent experimental data have demonstrated that the lens can be permeated by peptide fragments up to a M_r of 3500 (Chiou & Chuang, 1988). (iii) The presence of this domain in nonpathological calf lenses might have a functional meaning. In fact, the proteolytic cleavage might modulate interactions among α-crystallin subunits and with other lens crystallins in the still not well-known supramolecular organization of the lens (Wistow & Piatigorsky, 1988). It has been proposed that the α-crystallin subunits undergo several posttranslational modifications like C-terminal clipping (Van Kleef et al., 1975; Van Kleef et al., 1976). It was also observed that these degraded subunits are involved in the supraaggregation of the α-crystallin molecules (Siezen et al., 1979). Therefore, we cannot exclude that the age-related increase of the α-crystallin size may proceed through subunit exchange/uptake of postsyntetically shortened A and/or B chains.

We have also to point out that a spontaneous cleavage with aging has been reported for the αA subunit of the bovine α-crystallin (Voorter et al., 1988; Smith et al., 1991) at residue Asn 101. This same residue undergoes in vivo deamidation, isomerization, and racemization. Accordingly, the presence in the B chain of a unique Asn residue at position 80 does not exclude a nonenzymatic origin of our polypeptide. A more detailed structural investigation of the architecture of the normal lens proteins is necessary to solve this and other intriguing points.

ACKNOWLEDGMENT

We thank Raffaele Viscardi for help in the computer editing of the manuscript.

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