Communication

Conformational Properties of Substrate Proteins Bound to a Molecular Chaperone α-Crystallin*

(Received for publication, February 21, 1996)
Kali P. Das, J. Mark Petrush, and Witold K. Sureauzcz†

From the †Mason Eye Institute and Department of Biochemistry, University of Missouri, Columbia, Missouri 65212 and the ‡Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri 63110

α-Crystallin, the major protein of the ocular lens, acts as a molecular chaperone by suppressing the nonspecific aggregation of damaged proteins. To investigate the mechanism of the interaction between α-crystallin and substrate proteins, we prepared a tryptophan-free mutant of human αA-crystallin and assessed the conformation of thermally destabilized proteins captured by this chaperone using fluorescence spectroscopy. The fluorescence emission characteristics of bound substrates (rhodanese and γ-crystallin) and the results of fluorescence quenching experiments indicate that the proteins captured by α-crystallin are characterized by a very low degree of unfolding. In particular, the structure of rhodanese bound to αA-crystallin appears to be considerably more native-like compared to that of the enzyme bound to the chaperonin GroEL. We postulate that α-crystallin (and likely other small heat shock proteins) recognizes preferentially the aggregation-prone conformers that occur very early on the denaturation pathway. With its ability to capture and stabilize these early non-native structures, α-crystallin appears to be uniquely well suited to chaperone the transparency properties of the ocular lens.

α-Crystallin, the major protein of the vertebrate eye lens, consists of two types of highly homologous 20-kDa subunits, αA and αB. The A and B chains noncovalently self-associate to form a large macromolecular complex of approximately 40 subunits (1, 2). Spectroscopic data provided strong evidence that the secondary structure of α-crystallin is dominated by β-sheets (3, 4). Much less specific information is available regarding the tertiary and quaternary structure of the protein. While a number of structural models have been proposed for α-crystallin oligomers (2), none of them is generally accepted.

Believed for many years to be strictly lens-specific proteins, αB- and αA-crystallin have recently been found in many nonlenticular tissues (5–8). Furthermore, αB-crystallin has been associated with a number of neurodegenerative disorders (5, 6, 8). Another important recent development is the rapidly growing evidence that α-crystallin belongs to a family of small heat shock proteins (shSHPs). This is indicated by extensive structural similarities between α-crystallin and other shSHPs (8, 9) as well as by the recent findings that αB-crystallin is inducible by various stress conditions (10, 11) and that αB- and αA-crystallin are able to confer cellular thermoresistance (12, 13).

Despite the abundance of shSHPs in both eukaryotic and prokaryotic organisms (14), no obvious function has been associated with these ubiquitous proteins. A new light on the potential physiological role of α-crystallin and related shSHPs has been shed by recent findings that these proteins act in vitro as molecular chaperones by preventing the aggregation of other proteins under conditions of thermal stress or other insults (1, 15–20). The chaperone function of α-crystallin and other shSHPs is likely to be of considerable importance in vivo. In the context of eye research, it has been postulated that the ability of α-crystallin to suppress the aggregation of damaged proteins may be critical for maintaining the transparency of the ocular lens and that aging-related deterioration of the chaperone function could contribute to the development of cataracts (1, 2, 15).

Despite the rapidly growing interest in the chaperone function of α-crystallin and related shSHPs, very little is known about the mechanism by which these proteins interact with their substrates. Recent results indicate that, in contrast to other known chaperones, α-crystallin has very low affinity for folding intermediates formed during protein refolding reactions in vitro and that its substrate specificity is limited to non-native structures that occur on the denaturation pathway only (21). However, the critically important information about the specific conformational state(s) of the non-native structures that are recognized by α-crystallin is still missing. In this work, we have cloned and overexpressed αα-crystallin mutant in which the sole Trp residue has been replaced with Phe. Fluorescence spectroscopy experiments with the complexes formed between the Trp-free αα-crystallin and various substrate proteins allowed us, for the first time, to gain a direct insight into the conformation of proteins bound to this chaperone.

MATERIALS AND METHODS

Reagents and Proteins—bis-ANS was purchased from Molecular Probes. Acrylamide, bovine pancreas insulin, and bovine liver rhodanese were obtained from Sigma. The low molecular mass β-(γ-) and γ-crystallin fractions were isolated from young bovine lenses and purified as described previously (21).

Cloning and Overexpression of Human αα-Crystallins—Complementary DNA clones encoding human αα-crystallin were constructed using RNA PCR (Perkin Elmer). First strand cDNA synthesis was carried out on human lens total RNA by priming with a downstream primer/adapter (GGCTGCTATCTAA) designed to anneal in the 3′-untranslated region of the αα-crystallin mRNA. For amplification of target sequences by PCR, an upstream primer (ATGGACGTGACCATCCAG) was deduced from the human crystallin gene sequence deposited by J. aworski and Pietrak, GenBank™ accession number X14789. Both PCR primer/adaptors contained sequences that permitted ligation-free cloning of the PCR product into the pDI DIRECT cloning vector (PCR-Direct, Clontech). Site-directed mutagenesis was carried out using a PCR-based system (Life Technologies, Inc.). Nucleotide changes were confirmed by DNA sequencing.

1 The abbreviations used are: shSP, small heat shock protein; PCR, polymerase chain reaction; bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid, dipotassium salt.

* This work was supported in part by grants from University of Missouri Research Board, Research to Prevent Blindness, Inc., and National Institutes of Health Grants EY 05856, P30 EY02687, and P60 DK20579. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 573-882-8484; Fax: 573-882-8474.
otide sequence analysis of both strands of the wild type and mutant clones confirmed their structures. For overexpression studies, wild type and W9F mutant coding sequences were transferred into the expression plasmid pMON20,400 and cultivated in shake flasks as described previously (22). Crystallins were extracted from host cells essentially as described by Merck et al. (23) and were purified by chromatography on a TSKgel ion exchange column (EM Separations) using a linear gradient (0–0.5 M) of NaCl. Fractions corresponding to a single predominant peak were further purified by gel permeation chromatography on a Sephacryl S-300 column (Pharmacia Biotech Inc.). Virtually all protein from this column was found in fractions eluting at a position similar to that observed for bovine α-crystallin. Purified recombinant α-A-crystallins showed on SDS-gel electrophoresis a single band corresponding to a molecular mass of approximately 20 kDa.

Preparation of the Complexes between α-A-Crystallin and Substrate Proteins—One ml of a solution containing 0.25 mg of α-A-crystallin and a known amount of the substrate protein in 50 mM phosphate buffer, pH 7.2, was incubated for 1 h at a temperature which induces thermal denaturation of a given substrate (55, 60, and 65 °C for rhodanese, βl-crystallin, and γ-crystallin, respectively). While in the absence of the chaperone, the thermally denaturating proteins aggregate, α-crystallin prevents the aggregation process by forming stable, water-soluble complexes with the aggregation-prone proteins (1, 15, 16). After incubation, the samples were cooled down to room temperature and the complexes were separated from the remaining free substrate proteins by repetitive filtration using a 100-kDa cut-off Microcon microconcentrators (Amicon) until no tryptophan fluorescence was detectable in the filtrate. The absence of unbound substrates in the retentate was further verified by size exclusion chromatography on a Sephacryl S-300 column. The molar ratio of α-crystallin to the substrate protein in a given complex was estimated by subtracting the amount of the unbound substrate (as determined spectrophotometrically) from the initial amount of this protein in the incubation mixture.

Tryptophan Fluorescence Measurements and Quenching Experiments—Tryptophan fluorescence spectra were measured on an SLM 8100 spectrofluorometer using the excitation wavelength of 295 nm. Quenching experiments were performed by titrating the solution of free- and W9F α-A-crystallin-bound proteins with freshly prepared 5 mM solutions of acrylamide or potassium iodide. Fluorescence intensities were measured at the wavelength corresponding to the emission maximum of each protein and were corrected for dilution, blanks, and the inner filter effect. The effective quenching constants (a and b) were calculated from the inverse slopes of the F0/F versus 1/Q plots according to the modified Stern-Volmer equation: F/F0 = 1 + [Q]/KQ, where F0 and F are the fluorescence intensities in the absence and presence of the quencher. KQ is the molar concentration of the quencher, and F0 is the initial concentration of the protein-bound dye (24, 25). The ratio of quenchable fluorescence, Fq, was obtained from the ordinate intercept of the linear portion of the F0/F versus 1/Q plot (24).

Fluorescence Measurements with bis-ANS—α-A-Crystallin (0.1 mg/ml) with or without bound substrate was incubated with 20 μM bis-ANS for 1 h at room temperature, and fluorescence emission spectra of protein-bound dye were recorded using the excitation wavelength of 390 nm. Since binding of bis-ANS to α-crystallin at room temperature is dependent on the thermal history (preincubation temperature) of the sample (19), prior to incubation with the probe, control α-crystallin was subjected to the same treatment (i.e. 1 h incubation at elevated temperature) as used to prepare α-crystallin complex with a given substrate protein.

RESULTS

The fluorescence characteristics of tryptophan residues depend strongly on the microenvironment and thus provide a sensitive probe of the conformational state of proteins. Measurements of intrinsic fluorescence properties of substrate proteins have been used previously to study protein interaction with the chaperonin GroEL (26–28) and the heat shock protein DnaJ (29), providing critical information about the conformation of folding intermediates that are captured by these chaperones. Fluorescence studies with proteins bound to α-crystallin are hampered by the fact that, in contrast to tryptophan-free GroEL and DnaJ, α-crystallin itself contains Trp residues whose emission spectrum overlaps with those of substrate proteins. Therefore, in order to facilitate characterization of the conformational states of proteins bound to α-crystallin, we have cloned and overexpressed in Escherichia coli the mutant of αA in which the sole Trp at position 9 was replaced with Phe. As anticipated, the conservative Trp→Phe substitution had a negligible effect on the structural and functional properties of the protein. Thus, the Sephacryl S-300 size exclusion chromatography profiles for the wild type αA-crystallin and the W9F mutant were essentially identical. Furthermore, the mutation fully preserved the chaperone function of the protein as indicated by a very similar ability of both the wild type and Trp-free α-crystallin to suppress the thermal aggregation of rhodanase and γ-crystallin (data not shown).

In general, when the protein unfolds, it exposes buried tryptophans to the aqueous solvent; this results in the shift of the fluorescence emission maximum toward longer wavelength. The fluorescence spectrum of native rhodanese has a maximum at 332 nm (Fig. 1A, trace 1) and is consistent with a largely hydrophobic Trp residue (30). A small (5 nm) red-shift in the fluorescence spectrum indicates that the tertiary structure of the chaperone-bound enzyme is somewhat looser than that of the native form; however, the average environment of Trp in the bound protein appears to be still fairly hydrophobic. Notably, the position of the emission maximum for rhodanase associated with αA-crystallin is much closer to that observed for the native enzyme than for its chemically denatured form. The fluorescence properties of γ-crystallin bound to αA-crystallin were very similar to those of rhodanase. γ-Crystallin contains four Trp residues which are uniformly distributed throughout the molecule. As shown in Fig. 1B, unfolding of bovine γ-crystallin in guanidinium HCl results in a 23 nm red-shift of the fluorescence maximum (from 330 nm in the native form to 353 nm), γ-Crystallin bound to αA-crystallin shows an emission maximum at 337 nm, i.e. red-shifted only by 7 nm from that of its native state. These observations suggest that the conformation in which both proteins are captured by α-crystallin is characterized by a rela-
The fluorescence of bis-ANS is strongly dependent on the polarity of the environment; it is very weak in water and increases dramatically upon binding to hydrophobic sites of proteins. This compound has been widely used as a probe to assess the exposure of hydrophobic surfaces in proteins (19, 30). As shown previously, bis-ANS has considerable affinity for α-crystallin; this indicates the presence of surface-exposed hydrophobic patches (19). In contrast, essentially no binding of the probe could be detected to native rhodanese, γ- and βc-crystallin. However, when the same proteins were bound to the chaperone, the fluorescence of bis-ANS in the presence of the complexes was substantially higher than that for α-crystallin alone. Furthermore, the increase in fluorescence intensity appeared to correlate with the amount of bound substrates (Fig. 3). This strongly indicates that, in contrast to the native states, the conformation of chaperone-associated proteins is characterized by high affinity for the hydrophobic probe.

**DISCUSSION**

Employing site-directed mutagenesis and fluorescence spectroscopy, we have explored the structural properties of non-native proteins that are bound to αA-crystallin. The present data clearly show that the fluorescence characteristics of Trp residues in α-crystallin-bound conformers are much closer to those of native proteins than the fully unfolded ones. This strongly suggests that α-crystallin stabilizes aggregation-prone proteins in a conformation which, although compromised, remains relatively compact and is characterized by a low degree of unfolding.

The specific conformational features of substrate proteins bound to various functionally different chaperones have been the subject of many recent studies (for review see Refs. 31 and 32). For example, DnaK and PapD are believed to bind peptides in an extended conformation. In contrast, substrate proteins bound to the chaperones GroEL and DnaJ appear to be in a partially folded conformation, often characterized as a molten globule state with a largely preserved secondary structure and a collapsed, highly flexible tertiary structure (26–29, 32). It is informative to compare the fluorescence characteristics of the same substrate, rhodanese, bound to α-crystallin and the two latter chaperones. The fluorescence emission maximum for rhodanese bound to GroEL or DnaJ occurs at 342–343 nm (26, 29), i.e., it is red-shifted by 10–11 nm compared to that of the native enzyme. In contrast, λmax maximum for rhodanese associated with α-crystallin occurs at a wavelength as low as 337 nm, indicating a substantially more hydrophobic (native-like) environment of Trp residues as compared with that of rhodanese bound to GroEL or DnaJ. A more compact (less accessible) tertiary structure of the substrate protein associated with α-crystallin than that bound to GroEL is further supported by the quenching data for rhodanese stabilized by these different chaperones (cf. data for GroEL in Refs. 26 and 27 and present results for α-crystallin). A potential complication in the interpretation of fluorescence data for substrate proteins is caused by the possibility that the chaperone itself could affect the environment of tryptophan residues by shielding them from the aqueous environment. This indeed may be a factor for GroEL-
consistent with the putative physiological role of a such a unique substrate specificity would be fully pathway. very early on the denaturation nation-prone conformers that occur numbers a much less significant. case, the role of shielding effects mentioned above would be of substrate proteins on the surface of the chaperone. In this above, one can postulate that a-crystallin preferentially recognizes non-native structures characterized by an increased surface hydrophobicity but a remarkably low degree of unfolding, i.e., the aggregation-prone conformers that occur very early on the denaturation pathway. Such a unique substrate specificity would be fully consistent with the putative physiological role of a-crystallin as a "junior chaperone" specifically designed to suppress irreversible aggregation of proteins under stress conditions. The above function of a-crystallin may be of particular importance in the ocular lens (15). Major physiologically relevant factors known to induce aggregation of lens proteins include ultraviolet radiation and oxidative stress. While the above insults are highly unlikely to cause extensive unfolding of protein molecules, they may induce perturbations in the tertiary structure, leading to an increased surface hydrophobicity and, eventually, protein aggregation (17). With its ability to capture and stabilize these early non-native structures, a-crystallin appears to be uniquely well suited to chaperone the transparency properties of the ocular lens.

Acknowledgments—The skillful assistance of Terry Griest and Manjari Monoharan is gratefully acknowledged.

REFERENCES

1. Horwitz, J. (1993) Invest. Ophthalmol. Vis. Sci. 34, 10–21
2. Groenen, P. J. T. A., Merck, K. B., de Jong, W. W., and Bloemendal, H. (1994) Eur. J. Biochem. 225, 1–19
3. Siezen, R. J., and Argos, P. (1983) Biochim. Biophys. Acta 748, 56–67
4. Surewicz, W. K., and Giesl, P. R. (1985) Biochemistry 24, 9655–9660
5. Iwaki, T., Kume-Iwaki, A., Lien, R. K. H., and Goldman, J. E. (1989) Cell 57, 71–78
6. Liao, J., McDermott, H., Pike, I., Spendlove, I., Landon, M., and Mayer, R. (1992) J. Pathol. 166, 61–68
7. Srinivasan, A. N., Nagineni, C. N., and Bhat, S. P. (1992) J. Biol. Chem. 267, 23337–23341
8. Sax, C. M., and Piatti-Gorsky, J. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. 69, 155–201
9. Merck, K. B., Groenen, P. J. T. A., Voorter, C. E. M., de Haard-Hoekman, W. A., Horwitz, J., Bloemendal, H., and de Jong, W. W. (1993) J. Biol. Chem. 268, 1046–1052
10. Klemenz, R., Frohli, E., Steiger, R. H., Schafer, R., and Aoyama, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3652–3656
11. Dasgupta, S., Hohman, T. C., and Carper, D. (1992) Exp. Eye Res. 54, 451–470
12. Aoyama, A., Frohli, E., Schafer, R., and Klemenz, R. (1993) Mol. Cell. Biol. 13, 1824–1835
13. Van den Isel, R. P. R. A., Overkamp, P., Knauf, U., Gaestel, M., and de Jong, W. W. (1994) FEBS Lett. 355, 54–56
14. Jakob, U., and Buchner, J. (1994) Trends Biochem. Sci. 19, 205–211
15. Horwitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
16. Wang, K., and Spector, A. (1994) J. Biol. Chem. 269, 36765–36770
17. Arakhabhat, Z. T., Huang, Q.-L., Ding, L.-L., Altenbach, C., Steinhoff, H.-J., Horwitz, J., and Hubbell, W. L. (1995) Biochemistry 34, 509–516
18. Das, K. P., and Surewicz, W. K. (1995) FEBS Lett. 369, 321–325
19. Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) J. Biol. Chem. 268, 1517–1520
20. Das, K. P., and Surewicz, W. K. (1995) Biochem. J. 311, 367–370
21. Petras, J. M., Harter, T. M., Devine, C. S., Olin, P. O., Liu, S. Q., and Srivastava, S. K. (1992) J. Biol. Chem. 267, 24833–24840
22. Merck, K. B., de Haard-Hoekman, W. A., Oude Essink, B. B., Bloemendal, H., and de Jong, W. W. (1992) Biochim. Biophys. Acta 1130, 267–276
Conformational Properties of Substrate Proteins Bound to a Molecular Chaperone
-Crystallin
Kali P. Das, J. Mark Petrash and Witold K. Surewicz

J. Biol. Chem. 1996, 271:10449-10452.
doi: 10.1074/jbc.271.18.10449

Access the most updated version of this article at http://www.jbc.org/content/271/18/10449

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 12 of which can be accessed free at
http://www.jbc.org/content/271/18/10449.full.html#ref-list-1