Communication

Insight into the Secondary Structure of Non-native Proteins Bound to a Molecular Chaperone \(\alpha\)-Crystallin

AN ISOTOPE-EDITED INFRARED SPECTROSCOPIC STUDY*

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\(\alpha\)-Crystallin, a major lens protein, acts as a molecular chaperone by preventing the aggregation of proteins damaged by heat and other stress conditions. To characterize the backbone conformation of protein folding intermediates that are recognized by the chaperone, we prepared the uniformly \(^{13}\)C-labeled \(\alpha\)-crystallin. The labeling greatly reduced the overlapping between the conformation-sensitive amide I bands of \(\alpha\)-crystallin and unlabeled substrate proteins. This procedure has allowed us to gain insight into the secondary structure of \(\alpha\)-crystallin-bound species, an understanding which has previously been unattainable. Analysis of the infrared spectra of two substrate proteins (\(\gamma\)- and \(\beta\)-crystallins) indicates that heat-denatured conformers captured by \(\alpha\)-crystallin are characterized by a high proportion of native-like secondary structure. In contrast to the chaperone-bound species, the same proteins subjected to heat treatment in the absence of \(\alpha\)-crystallin preserve very little native secondary structure. These data show that \(\alpha\)-crystallin specifically recognizes very early intermediates on the denaturation pathway of proteins. These aggregation-prone species are characterized by native-like secondary structure but compromised tertiary interactions. The experimental approach described in this study can be further applied to probe the backbone conformation of proteins bound to chaperones other than \(\alpha\)-crystallin.

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The high resolution structure of \(\alpha\)-crystallin is currently unknown. However, recent cryo-electron microscopy data for \(\alpha\B-crystallin indicate that the protein forms spherical particles with a diameter of 8–18 nm and a large central cavity (4). For many years it was believed that the expression of \(\alpha\)-crystallin was restricted to the ocular lens. More recently, relatively large quantities of both \(\alpha\B- and \(\alpha\A-crystallin have been found in various non-lenticular tissues (5–8). Furthermore, \(\alpha\B-crystallin has been associated with neurodegenerative disorders such as Alexander's disease, diffuse Lewy body disease, Creutzfeldt-Jakob disease, and Alzheimer's disease (5, 6, 8). These findings were accompanied by data showing that \(\alpha\)-crystallin belongs to the family of small heat shock proteins (sHSPs)\(^1\) (2, 8, 9). While sHSPs are abundant both in eukaryotic and prokaryotic organisms, no obvious function has been associated with these proteins. An important step toward unraveling the functional role of \(\alpha\)-crystallin and related sHSPs was the discovery that these proteins act as molecular chaperones by preventing the aggregation of other proteins denatured by heat or other stress conditions (10–14). The chaperone function of \(\alpha\)-crystallin is likely to be of considerable importance in vivo. In particular, the above function was suggested to be instrumental in the prevention of cataract formation in the ocular lens (10, 13). In non-lenticular tissues, the role of sHSPs may be to maintain substrate proteins in a folding-competent state (15, 16).

It was shown that \(\alpha\)-crystallin specifically binds aggregation-prone structures that occur on the denaturation pathway of proteins (17). A key to understanding the molecular basis of this apparent "substrate specificity," is to determine the conformational properties of non-native proteins recognized by this chaperone. Although recent fluorescence studies provided some insight into the tertiary structure of these species (18, 19), important information at the level of protein secondary structure is still missing. Determination of the secondary structure of individual components in large protein-protein complexes presents a difficult experimental problem. To circumvent these difficulties, herein, we have applied the strategy of "isotope-edited" infrared spectroscopy. The present data shows that the aggregation prone folding intermediates captured by \(\alpha\)-crystallin are characterized by a high proportion of native secondary structure.

MATERIALS AND METHODS

Proteins—Cloning of human \(\alpha\A-crystallin has been previously described (18, 20). The protein was overexpressed in Escherichia coli strain JM101 essentially as outlined in our previous study (18). To obtain \(^{13}\)C-labeled \(\alpha\A-crystallin, cells were cultured in the presence of MOPS-buffered minimum medium containing a mixture of trace minerals, ammonium chloride, and uniformly \(^{13}\)C-labeled glucose (2 mg per 400 ml of culture) as a sole carbon source. The protein was purified by the standard protocol (18), dialyzed against 10 mM ammonium bicarbonate, and freeze-dried. The oligomerization state, far/near-UV circular dichroism spectra, FTIR spectra, as well as the chaperone activity of \(\alpha\A-crystallin were unaffected by the lyophilization/buffer reconstitution procedure. The low molecular mass \(\beta\) and \(\gamma\)-crystallins were isolated from young bovine lenses and purified as described previously (17).

Preparation of the Complexes between \(^{13}\)C-labeled \(\alpha\A-crystallin and \(\gamma\)-Crystallins—To prepare protein-protein complexes for FTIR measurements, lyophilized \(^{13}\)C-labeled \(\alpha\A-crystallin was dissolved in

\(^1\)The abbreviations used are: sHSP, small heat shock protein; FTIR, Fourier-transform infrared; MOPS, 4-morpholinepropanesulfonic acid.
50 mM phosphate buffer, pH 7, and combined with either γ or βγ-crystallin at a 1:0.8 molar ratio of αA monomer to the substrate protein monomer. The mixtures were then incubated for 30 min at 65 °C. In the absence of the chaperone, the thermally denatured γ- and βγ-crystallins aggregate. However, α-crystallin prevents the aggregation, forming water-soluble complexes with these proteins (10, 11). After incubation, the samples were cooled down to room temperature, and the complexes were separated from the residual free substrate proteins (approximately 18% and 19% for γ and βγ-crystallin, respectively) by filtration on a 100-kDa cut-off Microcon filter as described previously (18). The molar ratio of α-crystallin to the substrate protein in a given complex was estimated by subtracting the amount of the unbound substrate recovered in the filtrate (as determined spectrophotometrically) from the initial amount of this protein in the incubation mixture (18). The absence of unbound substrates in the final samples was verified by size-exclusion chromatography on a Superose 6 HR column (data not shown for brevity). Samples of thermally aggregated γ- and βγ-crystallins were obtained by high temperature (65 °C) incubation of these proteins in the absence of αA-crystallin. The aggregates were collected by low-speed centrifugation and resuspended in the buffer. Because water interferes with infrared spectroscopic measurements, the buffer used in these studies was prepared in 2H2O.

**FTIR Spectroscopy:** Samples of a soluble or aggregated protein were placed between two calcium fluoride windows separated by a 50-μm thick spacer. Infrared spectra were recorded at 25 °C on a Bruker IFS 66 instrument. Typically, 500 interferograms were averaged and Fourier-transformed to give a resolution of 2 cm⁻¹. Before further processing, spectra in the 1500–1800 cm⁻¹ region were corrected for the weak absorption of the 2H2O buffer and, if necessary, for the residual water vapor signal. The technique of Fourier self-deconvolution was used to resolve the overlapping infrared bands (21, 22). The secondary structure content was estimated by curve fitting of original (non-deconvolved) amide I band contours (22).

**RESULTS**

Infrared spectroscopy is an established method for studying the secondary structure of proteins (22–24). However, the application of this technique to determine the backbone conformation of individual components in protein-protein complexes is complicated by the overlapping of the spectroscopic signals from the two macromolecules. To circumvent this fundamental problem and facilitate studies with the chaperone-bound protein, we have prepared a uniformly 13C-labeled αA-crystallin. It was verified that the labeling did not affect the conformation function of the protein as indicated by an essentially identical ability of both the labeled and unlabeled αA-crystallin to suppress the thermal aggregation of γ- and βγ-crystallins. Consistent with the previous data (25, 26), the conformation-sensitive amide I band of unlabeled αA-crystallin has a maximum at 1632 cm⁻¹. Upon labeling, the maximum of this band shifted to 1591 cm⁻¹ (Fig. 1A, trace 1). The observed shift of the amide I band by 41 cm⁻¹ toward lower frequency is consistent with theoretical predictions as well as limited experimental data available for other proteins (27–29). Importantly, 13C-labeling of α-crystallin greatly reduced the overlapping of its amide I band with that of the unlabeled substrate protein. This opened up a possibility of analysis of the amide I bands (and thus the secondary structure) of the chaperone-bound proteins.

To test this approach, we first analyzed the infrared spectrum of an “inert” mixture of 13C-labeled αA-crystallin with an unlabeled γ-crystallin incubated at room temperature. Under these conditions, there is no complex formation between these two proteins (11). As shown in Fig. 1A, the spectrum of the above mixture shows well separated maxima corresponding to 13C-αA-crystallin (1591 cm⁻¹) and γ-crystallin (1637 cm⁻¹). While the high frequency region above approximately 1650 cm⁻¹ of the amide I band of γ-crystallin contains very little contribution from α-crystallin, a considerable spectral overlapping still exists at lower wave numbers. However, this residual overlapping can be easily removed by subtracting the spectrum of 13C-αA-crystallin alone. The well separated 1591 cm⁻¹ band maximum of the latter protein serves as an invaluable marker for determining the subtraction factor (γ-crystallin and most other proteins contain no infrared bands with a maximum at 1591 cm⁻¹). In our experience, the subtraction factor can be reliably determined using the original spectra. However, the procedure is especially accurate when applied to spectra subjected to band-narrowing by Fourier self-deconvolution. As an illustration, Fig. 1B shows the infrared spectrum of γ-crystallin when measured alone (trace 1) and the one obtained upon applying the subtraction procedure to the spectrum of an inert mixture of 13C-αA-crystallin and γ-crystallin (trace 2). The spectra of γ-crystallin represented by traces 1 and 2 are indistinguishable, clearly demonstrating the validity of the experimental approach used in this study.

The amide I band of the native γ-crystallin exhibits a maximum at 1637 cm⁻¹ (Fig. 2, trace 1). As for other proteins, the observed amide I band contour is a composite of overlapping components which represent different elements of protein secondary structure (22–24). These components can be better resolved by Fourier self-deconvolution (21, 22). The deconvolved spectrum of the native γ-crystallin is dominated by a very strong band at 1636 cm⁻¹ (Fig. 3, trace 1). This band is highly characteristic of a β-sheet structure (22–24). All other amide I component bands are much weaker. These bands may be as-

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**Fig. 1. Panel A, infrared spectrum of 13C-labeled αA-crystallin alone (trace 1) and that of an “inert” mixture of 13C-labeled αA-crystallin and unlabeled γ-crystallin (trace 2). The proteins were incubated at room temperature, i.e. under the conditions that do not lead to the formation of the chaperone-substrate complex. Panel B, infrared spectrum of the native γ-crystallin at room temperature (trace 1) and the difference spectrum obtained upon subtracting the spectrum of 13C-labeled αA-crystallin from that of an inert mixture of 13C-αA- and γ-crystallins (trace 2). The concentration of αA- and γ-crystallins was 12 and 9.6 mg/ml, respectively.**

**Fig. 2. Infrared spectra of native γ-crystallin (trace 1), heat-treated γ-crystallin bound to αA-crystallin (trace 2), and γ-crystallin thermally denatured in the absence of the chaperone (trace 3). The concentration of γ-crystallin was 7.9 mg/ml, and the final stoichiometry of γ-crystallin to αA monomer was approximately 0.66:1.**
α-Crystallin as a Molecular Chaperone

The amide I component bands of γ and β1-crystallins and their conformational assignment

| Protein       | β-Sheet | Helix | Turns | Unordered |
|---------------|---------|-------|-------|-----------|
| Native γ-crystallin | 1636 (57%) | 1656 (8%) | 1663 (15%) | 1645 (5%) |
| Chaperone-bound γ-crystallin | 1634 (50%) | 1656 (9%) | 1662 (17%) | 1644 (9%) |
| Native β1-crystallin | 1631 (36%) | 1655 (11%) | 1666 (18%) | 1643 (28%) |
| Chaperone-bound β1-crystallin | 1631 (29%) | 1653 (9%) | 1662 (13%) | 1643 (34%) |

*This band may also contain contributions from turns.*

signaled to α-helix (1656 cm⁻¹) and turns (1665 and 1686 cm⁻¹). The band at 1673 cm⁻¹ likely represents β-sheet structure, although this band may also contain contributions from turns. The fractional areas of these bands, which provide an estimate of protein secondary structure (22, 23), are shown in Table I. Overall, the data indicates that the secondary structure of the protein consists largely of β-strands and little α-helical structure. A very high content of β-sheet structure in γ-crystallin is fully consistent with the crystallographic data (30). Furthermore, the crystal structure of γ-II crystallin also indicates the presence of short α-helical regions (30).

The infrared spectrum of γ-crystallin bound to α-crystallin was obtained by subtracting the signal of 13C-labeled αA-crystallin from the spectrum of the thermally prepared protein complex. To ensure reliable subtraction, a control sample of 13C-labeled αA-crystallin alone was pretreated in the same way as the protein used to prepare the complex. The amide I band contour of the chaperone-bound γ-crystallin (Fig. 2, trace 2) has a maximum at 1637 cm⁻¹, and its overall shape is remarkably similar to that of the native protein. Upon deconvolution (Fig. 3, trace 2), the spectrum shows a dominant β-sheet band at 1634 cm⁻¹. A small shift of this band to lower frequency as compared with the 1636 cm⁻¹ β-sheet band in the spectrum of the native protein likely reflects a higher degree of hydrogen-deuterium exchange in the chaperone-bound protein. Consistent with the visual inspection of the spectra, quantitative analysis indicates that the secondary structure of the chaperone bound γ-crystallin is characterized by only slightly decreased content of β-sheet structure (approximately 61% versus 66% in the native protein), whereas the content of α-helical structure remains essentially unchanged. In contrast to the close similarity of the amide I bands of the native and α-crystallin-bound γ-crystallin, a dramatically different spectrum was observed for γ-crystallin thermally denatured in the absence of the chaperone (Figs. 2 and 3, traces 3). The spectra were deconvolved using the parameters given in the legend to Fig. 3. The concentration of β1-crystallin was 10.4 mg/ml, and the final stoichiometry of β1-crystallin monomer (average molecular mass of 24 kDa) to αA monomer was approximately 0.72:1.

A very similar trend was observed for another lens protein studied, β1-crystallin. This protein is also characterized by a high content of β-structure (25, 32). The deconvoluted spectrum of the native β1-crystallin shows a major β-sheet band at 1631 cm⁻¹ (Fig. 4). This band, together with a weaker β-structure-characteristic band at 1673 cm⁻¹, account for 40% of the total area of the amide I contour (Table I). The prominent 1631 cm⁻¹ band is also seen in the spectrum of the chaperone-bound β1-crystallin, although its integrated intensity is somewhat decreased. Compared with the native protein, the overall content of β-sheet structure in the bound β1-crystallin is reduced from approximately 40 to 36% (Table I). In contrast to this relatively small change, a much more drastic perturbation of the secondary structure was observed for β1-crystallin subjected to heat treatment in the absence of the chaperone. The spectrum of this protein (Fig. 4, trace 3) shows a dominant band at 1618 cm⁻¹ (27% of the total area of amide I band contour) and is very similar to that of the thermally denatured γ-crystallin. The integrated intensity of the native β-sheet band around 1631 cm⁻¹ is reduced to 3%, indicating that thermally aggregated β1-crystallin preserves very little native secondary structure.

**FIG. 3.** Infrared spectra of γ-crystallin after band narrowing by Fourier self-deconvolution using a 15 cm⁻¹ half-width Lorenzian line and a resolution enhancement factor of 2.2. Trace 1, native γ-crystallin; trace 2, heat-treated γ-crystallin bound to αA-crystallin; trace 3, γ-crystallin thermally denatured in the absence of the chaperone.

**FIG. 4.** Infrared spectra of native β1-crystallin (trace 1), heat-treated β1-crystallin bound to αA-crystallin (trace 2), and β1-crystallin thermally denatured in the absence of the chaperone (trace 3). The spectra were deconvolved using the parameters given in the legend to Fig. 3. The concentration of β1-crystallin was 10.4 mg/ml, and the final stoichiometry of β1-crystallin monomer (average molecular mass of 24 kDa) to αA monomer was approximately 0.72:1.

**TABLE I**

| Protein       | β-Sheet | Helix | Turns | Unordered |
|---------------|---------|-------|-------|-----------|
| Native γ-crystallin | 1636 (57%) | 1656 (8%) | 1663 (15%) | 1645 (5%) |
| Chaperone-bound γ-crystallin | 1634 (50%) | 1656 (9%) | 1662 (17%) | 1644 (9%) |
| Native β1-crystallin | 1631 (36%) | 1655 (11%) | 1666 (18%) | 1643 (28%) |
| Chaperone-bound β1-crystallin | 1631 (29%) | 1653 (9%) | 1662 (13%) | 1643 (34%) |

*This band may also contain contributions from turns.*

The family of molecular chaperones includes a number of structurally unrelated proteins that share the common property of being able to recognize and bind non-native conformers of other proteins. By doing so, they prevent unwanted intermolecular interactions and, in certain cases, facilitate the correct folding of substrate proteins (33–36). Some members of the
chaperone family (e.g. HSP60) have ATPase activity and are directly involved in protein folding in vivo. Many others, however, lack ATPase activity and play a less defined auxiliary role in the cell. α-Crystallin and other sHSPs belong to this second group of chaperones. These chaperones appear to act as a passive binder of non-native proteins in response to cellular stress conditions (34).

There has been great interest in characterizing the specific conformational states of non-native proteins recognized by molecular chaperones. For example, DnaK binds polypeptide chains in a completely extended conformation. HSP60, on the other hand, associates with relatively compact protein folding intermediates, whereas HSP90 appears to have dual substrate specificity (33–36). In contrast to these chaperones, very little specific information is available regarding the conformation of sHSPs. The present study provides, for the first time, a direct insight into the secondary structure of non-native proteins bound to α-crystallin. To obtain this information, we have used a strategy of isotope-edited infrared spectroscopy. Uniform $^{13}$C-labeling of the recombinant α-crystallin protein greatly reduced the overlapping between its amide I band and the respective bands of substrate proteins. This allowed infrared spectroscopic analysis of the secondary structure of α-crystallin-bound species. At a qualitative level, the main conclusions of this study were already evident from a simple visual comparison of the spectra of free- and chaperone-bound proteins. These conclusions were further reinforced by a quantitative band-fitting analysis. Although quantitative determination of protein secondary structure by band-fitting of infrared spectra is not without pitfalls and limitations (24), the present data are likely representative of non-native proteins bound to sHSPs. Furthermore, the experimental approach described in this work should prove useful for studying conformational properties of proteins bound to other members of the chaperone family.

It was previously demonstrated that α-crystallin has an enormous capacity for binding non-native proteins on the denaturation pathway, but apparently does not interact with the intermediates that occur on the refolding pathway (17). Furthermore, it was shown that the non-native proteins associated with α-crystallin are characterized by an increased surface hydrophobicity and partially compromised tertiary interactions (18, 19). The present data extends these observations, affording a more detailed insight into the substrate specificity of α-crystallin. In particular, the finding that the conformationally compromised proteins recognized by this chaperone retain a high proportion of native-like secondary structure provides crucial experimental support to the earlier hypothesis (18) that α-crystallin and other sHSPs interact specifically with aggregation-prone folding intermediates that occur very early on the denaturation pathway. The complexes of sHSPs with such intermediates may provide a reservoir for other components of the chaperone machinery to renature the bound proteins (15, 16). In the context of eye research, recent data indicate that the aggregation of lens proteins under physiologically relevant stress conditions such as oxidative stress or ultraviolet radiation is accompanied by only small loss of native secondary structure. Therefore, the substrate specificity of α-crystallin described above is fully consistent with the postulated role of this protein as a chaperone that protects the transparency of the ocular lens.

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