Binding of Destabilized βB2-Crystallin Mutants to α-Crystallin

THE ROLE OF A FOLDING INTERMEDIATE*

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Age-related changes in protein-protein interactions in the lens play a critical role in the temporal evolution of its optical properties. In the relatively non-regenerating environment of the fiber cells, a critical determinant of these interactions is partial or global unfolding as a consequence of post-translational modifications or chemical damage to individual crystallins. One type of attractive force involves the recognition by α-crystallins of modified proteins prone to unfolding and aggregation. In this paper, we explore the energetic threshold and the structural determinants for the formation of a stable complex between α-crystallin and βB2-crystallin as a consequence of destabilizing mutations in the latter. The mutations were designed in the framework of a folding model that proposes the equilibrium population of a monomeric intermediate, Binding to α-crystallin is detected through changes in the emission properties of a bimane fluorescent probe site-specifically introduced at a solvent exposed site in βB2-crystallin. α-Crystallin binds the various βB2-crystallin mutants, although with a significantly lower affinity relative to destabilized T4 lysozyme mutants. The extent of binding, while reflective of the overall destabilization, is determined by the dynamic population of a folding intermediate. The existence of the intermediate is inferred from the biphasic bimane emission unfolding curve of a mutant designed to disrupt interactions at the dimer interface. The results of this paper are consistent with a model in which the interaction of α-crystallins with substrates is not solely triggered by an energetic threshold but also by the population of excited states even under favorable folding conditions. The ability of α-crystallin to detect subtle changes in the population of βB2-crystallin excited states supports a central role for this chaperone in delaying aggregation and scattering in the lens.

In the inner regions of the lens, transparency and refractivity are dependent on the stability, high solubility, and packing of three families of proteins, collectively referred to as crystallins (1–3). One of these families, the α-crystallins, consists of two small heat-shock proteins (sHSP) that can recognize and bind non-native states of proteins. The other two families, the β- and γ-crystallins (4–6), are evolutionarily related structural proteins that have close to 30% sequence identity and similar polypeptide chain folds. The double Greek key motif characteristic of their structures has been found to occur in microbial stress proteins (7, 8). Subunits of β-crystallins assemble into dimers and higher oligomers, whereas γ-crystallins are monomeric.

The β-crystallin family of the vertebrate lens consists of six distinct gene products that segregate into two classes, basic and acidic (9). Despite extensive sequence similarity, one of the hallmarks that distinguish members of the two classes is N- and C-terminal extensions of variable lengths, the age-related truncation of which adds another dimension of molecular diversity (10–13). The structures of βB2-crystallin and extension truncation mutants have been determined to high resolution (5, 14–18). Wild type βB2-crystallin structure reveals an all-β-sheet fold consisting of two domains with identical double Greek key topology. βB2-crystallin homodimers assemble as a result of intermolecular domain pairing. Compelling evidence suggests that, at low concentrations, some β-crystallins may populate a monomeric state in solution (19–21). Two models for the conformation of monomeric β-crystallin have been proposed. The first is a γ-crystallin-like closed conformation with intra-subunit pairing between the N- and C-terminal domains (20), whereas the second has an open conformation with an unfolded N terminus (21, 22).

In the low protein turnover environment of lens fiber cells, the β-crystallins are subject to extensive modifications, either programmed or as a result of oxidative and other types of damage (12, 13, 23–26). Regardless of their origin, these modifications are expected to shift the equilibrium between dimeric, monomeric, and unfolded β-crystallin. The consequences of the altered equilibrium may include changes in solubility of the crystallins and the balance of forces that defines their mutual interactions, both of which may lead to aggregation and subsequent loss of transparency.

One of the hypothesized mechanisms by which the lens delays the onset of scattering is through the chaperone activity of the resident small heat-shock protein, α-crystallin (3, 27, 28). Whether due to age-related modifications or as a consequence of changes in the physico-chemical environment, β- and γ-crystallins that significantly populate non-native states associate with the α-crystallins and are sequestered; hence, their aggregation is suppressed. Although previous studies have demonstrated a reduction in light scattering by heat-denatured β- and γ-crystallins in the presence of α-crystallin (29–33), the energetic and structural aspects of this interaction have not been investigated under conditions that mimic the lens environment. Rarely do site-specific modifications cause complete or global unfolding; therefore, α-crystallins must detect the increased excursions of substrate proteins to non-native states under conditions where the folded state is still the predominantly populated state.

In the context of a mechanistic study of α-crystallin chaperone activity, we have developed an equilibrium binding assay...
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EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—The CDNA of mouse βB2-crystallin (a generous gift from Drs. J. P. Hejtmancik and Y. Sergeev) was cloned between the NdeI and XhoI sites of the plasmid PET-20b(+). The cloned DNA was verified by DNA sequencing and determined to have an identical DNA sequence to that deposited in the GenBankTM under accession number P26775. A cysteine-less βB2-crystallin was generated in which Cys-38 and Cys-67 were mutated to leucine and serine, respectively. Overlap, extension site-directed mutagenesis was performed to generate the PCR fragments G61D/F74A, L165A, and E167L using either a cysteine-less template or a variant containing a putative monomeric intermediate. This paper reports the thermodynamic and solution characteristics of these mutants and the analysis of their interactions with the α-crystallins.

RESULTS

General Methodology—The folding model of βB2-crystallin, proposed by Jaenicke and co-workers (21, 22), involves the population of a partially unfolded monomeric intermediate. Equilibrium sedimentation studies of βB3-crystallin molecular weight were interpreted in terms of a reversible equilibrium involving a monomeric intermediate with a γ-crystallin-like conformation (20). Therefore, the folding equilibrium of β-crystallin can be written as shown in Equation 1,

\[ \text{D} \rightleftharpoons 2\text{I} \rightleftharpoons 2\text{U} \]  

(Eq. 1)

where D is the native dimer, I is the putative monomeric intermediate, and U is the unfolded state. Regardless of its conformation, it is expected that the equilibrium population of I can be enhanced by selective destabilization of the dimer. A direct approach to destabilize D is by disruption of the interactions at the monomer-monomer interface. Two mutants of βB2-crystallin were designed for this purpose. The L165A substitution reduces the buried surface area between the two monomers. The original leucine is involved in Van der Waals (VDW) contacts with Ile-43 and Val-81 from the other subunit. The second mutant, E167L, disrupts an ionic interaction with Arg-79 from the paired domain. Unlike L165A, the mutation does not significantly reduce the molar volume of the side chain.

The third βB2-crystallin mutant was designed to disrupt the packed core of the N-terminal domain. The mutation G61D/F74A simultaneously reduces the buried hydrophobic surface...
area and introduces a negative charge. The two sites are proximal in the folded structure, such that the removal of the bulky phenylalanine side chain allows the accommodation of the new aspartate side chain. A molecular model of the three mutants is shown in Fig. 1.

To detect complex formation, an environmentally sensitive reporter group, such as a spin label or a fluorescence probe, is site-specifically incorporated at a solvent-exposed site in the substrate. This is achieved via site-directed mutagenesis to introduce a unique cysteine followed by reaction with the spectroscopic probe. Because mouse \( \beta B2 \)-crystallin has two endogenous cysteines, a cysteine-less variant, hereafter referred to as WT*, was constructed. Cysteine 38, located in a buried environment, was replaced with a leucine, while cysteine 67, located in a surface loop and having a partially water-exposed side chain, was substituted with a serine. WT*–\( \beta B2 \)-crystallin is expressed in the soluble fraction of \( E. \ coli \) and has an identical elution volume on a Superdex 75 column as the WT. Far- and near-UV CD spectra of the WT* are consistent with a lack of significant effects on the structure due to the cysteine substitutions (data not shown). All three destabilizing mutations were constructed in the WT* background as well as in a variant of WT*, where a unique cysteine is introduced at the solvent exposed site, Asn-66, in a surface loop.

Structure, Stability, and Solubility of the \( \beta B2 \)-Crystallin Mutants—In contrast to WT and WT*, all three \( \beta B2 \)-crystallin mutants were found in inclusion bodies when expressed in \( E. \ coli \). Following isolation of the inclusion bodies, the mutants were refolded with >80% efficiency, purified, and then reacted with monobromobimane as described under “Materials and Methods.” Analytical SEC was used to verify that the mutants assemble into a dimer. Table I shows that at a 2 \( \mu M \) concentration, all three mutants have significant changes in their elution volume, although not to an extent consistent with a shift to a monomer. Similar changes were obtained when the injected sample concentration was 10 \( \mu M \). This may reflect changes in the hydrodynamic radius, the molecular mass, an enhanced interaction with the column matrix, or a shift in the putative dimer/monomer equilibrium. The last interpretation is supported by the fact that the largest shift occurs for the two interface mutants \( \beta B2 \)-E167L and \( \beta B2 \)-L165A.

To determine the consequences of the mutations on the thermodynamic stability of \( \beta B2 \)-crystallin, unfolding curves were constructed from the changes in tryptophan emission intensity as a function of increased urea concentration. As reported previously, fluorescence unfolding curves of WT–\( \beta B2 \)-crystallin do not show a discrete intermediate (21, 22). Nevertheless, the non-coincidence of the curves obtained by two spectral probes, fluorescence and CD, was interpreted as an indication of a multi-state equilibrium (22). Equation 1 predicts that the dimer to intermediate transition is bimolecular and, thus, is concentration dependent. Quantitative analysis of the unfolding curves (41) in Fig. 2 is hindered by the large number of parameters to be determined and the monophasic nature of the corresponding tryptophan unfolding curves that provide little restriction on the values of \( \Delta G \) and \( m \) (the denaturant dependence of \( \Delta G \)) for the intermediate. Therefore, the curves in Fig. 2 were compared using \( C_{1/2} \), the urea concentration at the tran-
When concentrated to more than 2 M at low concentrations for days, they tended to precipitate.

The unfolding curves of the Asn-66BI variant have virtually superimposable unfolding in the Complex with the WT-background reproduced the trends of Fig. 2 and have values similar to those of their bimane-labeled variants.

Measurements were performed using a protein concentration of 2 $\mu$M at 23 °C, pH 8.

The near-UV CD of $\beta$2-crystallin mutants precludes the use of this approach at the required concentrations. An alternative detection method, introduced by Sathish et al. (42), is based on the change in the spectral properties of the fluorescent probe bimane. In general, the emission $\lambda_{\text{max}}$ of this probe in proteins reflects the solvent accessibility at the site of attachment (39, 40). However, upon binding of bimane-labeled T4L to $\alpha$-crystallin, the blue shift in $\lambda_{\text{max}}$ is accompanied by a decrease in the emission intensity. The latter has been shown to arise from a binding-induced transition in the conformation of T4L (42).

The use of bimane-labeled $\beta$2-crystallin allows for a convenient assay to screen for binding. Bimane-labeled $\beta$2-crystallin mutants are incubated with $\alpha$-crystallin and the phosphorylation mimics of $\alpha$B, namely S45D, S45D/S59D (D2), and S19D/S45D/S59D (D3). Previous studies have revealed that the Ser to Asp substitutions result in the activation of two-mode binding manifested by increased extent of binding and higher affinities (38). After incubation, the $\beta$2-mutant/chaperone mixture is loaded on a size-exclusion column. The complex is detected by the presence in the chaperone peak of bimane emission at 470 nm. The stability of the complex on the time scale of SEC suggests a relatively slow off rate. Fig. 4 compares the extent of binding between $\beta$2-L165A and the various $\alpha$B-crystallins at the same molar ratio. The D3 form has the highest extent of binding, as inferred from the drop in the intensity of the $\beta$2-crystallin peak and the concomitant increase in the intensity of the chaperone peak.

Complex formation results in a change in the fluorescence characteristics of the bimane. This is illustrated in Fig. 5 by the emission spectra of $\beta$2-L165A in the free and bound states. The increase in quantum yield upon binding is accompanied by a blue shift in the $\lambda_{\text{max}}$, suggesting transfer to a more buried environment. The sign of the change in the intensity is opposite to that observed upon binding of bimane-labeled T4L to either the high or the low affinity mode of $\alpha$-crystallin (42). To inves-

![Fig. 2. Urea-induced unfolding of $\beta$2-crystallin mutants as reported by tryptophan fluorescence.](image)

![Fig. 3. CD analysis of $\beta$2-crystallin mutants.](image)

![Table 1. Retention times of $\beta$2-crystallin mutants on a Superdex 75 column.](table)

| Mutant          | Retention time (Minutes) |
|-----------------|--------------------------|
| $\beta$2-WT     | 27.1                     |
| $\beta$2-N66BI  | 27.5                     |
| $\beta$2-G61D/F74A/N66BI | 27.4                     |
| $\beta$2-L165A/N66BI | 27.8                     |
| $\beta$2-E167L/N66BI | 28.2                     |
Urea-induced unfolding of βB2-crystallin mutants as reported by the bimane probe. Measurements were performed at 23 °C and pH 8 with a protein concentration of 2 μM.

Fig. 6. Urea-induced unfolding of βB2-crystallin mutants as reported by the bimane probe. Measurements were performed at 23 °C and pH 8 with a protein concentration of 2 μM.

As shown by the unfolding curves of Fig. 6. Furthermore, for βB2-E167L the curve is biphasic, suggesting the presence of an equilibrium intermediate where the bimane emission characteristics are distinct from those of the folded and unfolded states, consistent with the folding model of Equation 1.

Binding Isotherms of βB2-Crystallin Mutants to α-Crystallin—The rather small increase in intensity observed in the presence of αA and αB-crystallin in Fig. 5 indicates that the concentrations used in the binding assay are significantly smaller than the dissociation constant. The limited solubility of βB2-L165A and βB2-E167L prevents the use of the high concentration necessary for quantitative analysis. Among the α-crystallins and their variants, only αB-D2 and αB-D3 bind βB2-L165A to an extent that an isotherm can be constructed. Because this mutant precipitates at 37 °C (in the absence of α-crystallin), binding isotherms were obtained at 23 and 30 °C and the latter is shown in Fig. 7a. Superimposed on the binding isotherm to αB-D3 is a non-linear, least squares fit based on a single mode binding model. A fit using a two-mode model slightly improves χ², but the resulting value of n2 is 0.07, which is significantly different from the value obtained from analysis of T4L binding. Similarly, a single mode binding model provides a satisfactory, although not unique, fit to the binding isotherm of αB-D2 (data not shown). Although the binding of βB2-L165A is expected based on its marginal stability, the resulting value of Kd (Table II), when compared with those obtained with T4L mutants (42), suggests a markedly reduced affinity.

In contrast to βB2-L165A, the stability of βB2-E167L relative to the WT, deduced from the midpoints of the unfolding transitions, suggests that this protein will not significantly bind αB-D2 or αB-D3. Fig. 7b shows that, at 37 °C, this mutant binds with an affinity similar to that of βB2-L165A (Table II), and SEC experiments similar to those reported in Fig. 4 indicate that this mutant binds αB-D1 and both WT α-crystallins at 37 °C (data not shown). In contrast, to obtain measurable binding of βB2-G61D/F74A, a concentration of 10 μM and a large excess of αB-D3 is required (Fig. 7b).

The differential recognition of βB2-E167L relative to βB2-WT and βB2-G61D/F74A is not correlated with changes in overall stability, as defined by the equilibrium population of U, because the Trp unfolding curve of βB2-G61D/F74A is left-shifted relative to that of βB2-E167L. Rather, the bimane unfolding curves (Fig. 6) indicate that binding is triggered by the dynamic population of a folding intermediate. Consistent with this interpretation, the unfolding curves of βB2-E167L and...
The finding that the complex with \( \beta B2 \)-crystallin is stable even in the presence of WT nor the addition of the latter to \( \beta B2 \)-crystallin is critical because, in the complex formation with \( \beta B2 \)-crystallin, the sign of the intensity change due to binding and unfolding is the same.

**DISCUSSION**

That the \( \alpha \)-crystallins bind destabilized \( \beta B2 \)-crystallin mutants lends support to the hypothesis that their chaperone activity plays a critical role in the long-term maintenance of lens transparency. Although previous studies have shown that \( \alpha \)-crystallin can suppress the heat-induced aggregation of \( \beta \)-crystallins (29–32), the novel aspect of this work is that binding occurs to \( \beta B2 \)-crystallin mutants of similar stability to the WT and under equilibrium conditions where the mutants are predominantly in the folded state. Furthermore, the use of site-directed mutants more closely mimics the localized age-related damage that may occur in the lens than the extreme temperatures used to unfold and aggregate the \( \beta \)-crystallins. The finding that the complex with \( \alpha \)-crystallin is stable even in the presence of WT \( \beta B2 \)-crystallin is critical because, in the lens, damaged proteins exist in a background of stable proteins.

**Determiants of \( \beta B2 \)-Crystallin Stability**—Despite the different nature of the two interface mutations manifested by their differential effects on the stability curves, both result in changes in the apparent surface properties of the \( \beta B2 \)-crystallin molecule as inferred from the tendency to aggregate at high concentrations. The change in the surface properties for \( \beta B2 \)-E167L occurs despite the lack of a significant change in its stability. Presumably, residual hydrophobic interactions occur between the introduced Leu and Arg-79 side chains. Because \( \beta B2 \)-L165 and \( \beta B2 \)-E167 are buried at the interface between the subunits, it is unlikely that the mutations cause loss of solubility of the dimer. An alternative interpretation, based on Equation 1, is that the mutations increase the equilibrium population of the monomeric intermediate I by reducing the stability of the dimer. The limited solubility and the tendency to bind \( \alpha \)-crystallin reflect the partially unfolded structure of I. Such a mutation-induced shift in the dimer-monomer equilibrium can account for the shift in the retention times in SEC. Furthermore, it is directly supported by the biphasic unfolding curve of \( \beta B2 \)-E167L reported by the bimane probe (Fig. 6). The lack of the explicit detection of this intermediate in the tryptophan unfolding curve may be due to the existence of five such residues in \( \beta B2 \)-crystallin. Consequently, the population of this intermediate may not affect their environments in a similar fashion.

Despite its location at a solvent-exposed site in a surface loop, the emission intensity of the bimane is sensitive to the conformational state of the \( \beta B2 \)-crystallin molecule. A similar effect is observed in T4L, where global unfolding significantly decreases the bimane emission intensity at a surface-exposed site (42). The mechanistic origin of this effect has not been investigated, although it is likely to involve a change in the average distance between the bimane probe and various Trp and Tyr residues in the folded versus unfolded states. Because Asn-66BI is in close proximity to Tyr-62, the increase in the bimane emission intensity in the intermediate may reflect the partial unfolding of the N terminus. For both T4L and \( \beta B2 \)-crystallin, the sign of the intensity change due to binding and unfolding is the same.

Another remarkable result is the rather limited destabilization observed in \( \beta B2 \)-G61D/F74A. Phe to Ala substitutions in the buried core of T4L as well as the introduction of negative charges result in at least a 3–5 kcal/mol change in the free energy of unfolding (43). The tolerance of \( \beta B2 \)-crystallin to the substitution can also be interpreted within the context of Equation 1. If I has an unfolded N terminus and the transition between D and I occurs under ambient temperatures, then the contribution of this region to the overall stability of the protein...
may not be significant. The core mutations do not affect the solution behavior of ββ2-crystallin.

α-Crystallin/ββ2-Crystallin Interactions—Both α-crystallin subunits bind the ββ2-crystallin mutants. However, despite the large decrease in the stability of ββ2-L165A, the extent of binding to WT αA- and αB-crystallin is not sufficient for quantitative analysis. This suggests that the $K_d$ values associated with binding are in the tens of micromolar range. Furthermore, it suggests a significantly lower affinity than that reported for T4L. If binding was determined by an absolute energetic threshold, the opposite result is predicted. ββ2-crystallin unfolding curves indicate significantly lower overall stability than T4L (34).

The limited solubility of the mutants prevented the use of the higher concentrations needed for the construction of binding isotherms. Therefore, insight into the thermodynamics of binding was gleaned from binding curves to αB-D3 and αB-D2, both of which bind ββ2-crystallin mutants to a larger extent. The use of these variants is solely for the purpose of enhancing binding and is thermodynamically equivalent to increasing the temperature without the complications of aggregation. The conclusions obtained from these curves are applicable to both α-crystallin subunits (38).

Analysis of binding isotherms reveals that the highest affinity of αB-D3 and αB-D2 is for the most destabilized ββ2-crystallin mutant consistent with previous studies on the binding of T4L destabilized mutants (38). However, a novel finding of the present work is that α-crystallin can interact differentially with proteins that have similar overall stability. Whereas αB-D3 binds ββ2-E167L, as shown in Fig. 7, it does not significantly interact with the WT, although the overall stabilities, reflected in Trp unfolding curves, are similar (Fig. 6). An increase in the equilibrium population of an intermediate with the large decrease in the stability of αB-D3 is for the most destabilized B2-crystallin. However, despite the difference in the equilibrium population of U between αB-D3 and αB-D2, the opposite result is predicted. Therefore, insight into the thermodynamics of binding may reflect a relatively low equilibrium population of the extensively unfolded states, even in marginally stable α-crystallin mutants such as ββ2-L165A.

Concluding Remarks—The results of this paper demonstrate that the mechanistic outline of α-crystallin chaperone activity, deduced from studies with T4L (34, 38, 42), is general and applies to the interaction with native lens proteins. The large $K_d$ suggests a lower intrinsic affinity for ββ2-crystallin than for T4L. This is not surprising, considering the need to avoid attractive interactions with native lens proteins. Because the intermediate that triggers binding has also to be populated by the WT, the low affinity may reflect a negative design element to avoid significant binding to α-crystallin. It is noted that macromolecular associations are likely to be enhanced in the crowded molecular environment of the lens, where protein concentrations can reach 500 mg/ml.

The results confirm the previously proposed mechanism of the "sensor" in the α-crystallins (34). Recognition and binding are not triggered by an absolute energetic threshold, but rather by one that is defined relative to the ladder of excited states that a protein can populate. Therefore, the nature of the mutations may be critical for the recognition by the chaperone as exemplified by the mutant ββ2-E167L. The results also provide insight into the determinants of stability in the β-crystallin family as well as the equilibrium that describes their folding reaction.
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