Mechanism of Chaperone Function in Small Heat-shock Proteins

To further develop the mechanistic understanding of small heat-shock protein (sHSP) chaperone activity, we investigate the nature of the intermediate states recognized by α-crystallin and the conformations that are stably bound. The model substrates consist of a set of well-characterized, destabilized T4 Lysozyme (T4L) mutants that have been shown to differentially bind α-crystallin in a manner that reflects their free-energy of unfolding. A new approach for the detection of complex formation is introduced based on the conformational sensitivity of the fluorescent probe bimane, site-specifically introduced in T4L. Emission spectra of bimane-labeled T4L reveal two distinct patterns of intensity changes upon binding that depend on the molar ratio of α-crystallin to T4L. This directly demonstrates the two-mode nature of the binding process by the α-crystallins. Biphasic binding isotherms, obtained and analyzed over a wide range of T4L concentrations, demonstrate a substantially quenched bimane fluorescence in the low affinity-bound T4L that is similar to the quenching level observed due to denaturant unfolding. Furthermore, the pattern of intensity changes that occur upon binding of a T4L variant, bimane-labeled at an alternative solvent-exposed site, establishes a direct correlation between the quenching level observed in binding and unfolding. The results can be interpreted in terms of a model where α-crystallin binds to at least two conformationally distinct non-native states of T4L, one of which is substantially unfolded and is bound with low affinity. A high affinity binding mode to compact states may be relevant to chaperone function in the lens, where protein damage is unlikely to cause global unfolding.

The cellular response to increased temperature and other forms of stress involves the expression of multiple superfamilies of heat-shock proteins (HSPs) (1). Many HSPs are molecular chaperones that recognize and bind protein non-native states thereby suppressing aggregation and facilitating refolding and/or subsequent degradation (2, 3). Because the recognition process, the mechanism of substrate release, and the downstream fate of the bound substrate seem to be distinct in each superfamily, HSPs have evolved distinct roles in the context of the life cycle of proteins as well as during stress (2). Small heat shock proteins (sHSPs) are a ubiquitous superfamily of heat-shock proteins characterized by the relatively small mass of the polypeptide chain and the presence of a conserved protein module in their C termini, the α-crystallin domain (4, 5). They assemble into oligomeric structures with varying degrees of order and substantial divergence in size and symmetry across the evolutionary spectrum. Ten sHSPs have been identified in the human genome (6). Constitutive expression of two sHSPs, the α-crystallins of the vertebrate lens, plays a critical role in the acquisition and maintenance of lens optical properties (7–9). In the heart, α-crystallin and heat-shock protein 27 are involved in stress tolerance (10). Inherited mutations in the α-crystallins have been linked to pathogenic conditions such as cataract and desmin-related myopathy (11, 12).

Recent studies have led to an outline of the mechanism of recognition and binding of non-native protein states by αA- and αB-crystallin (13, 14). Both proteins are “sensors” of protein stability: they are able to distinguish between mutants of T4 lysozyme (T4L) that have similar crystal structures but different free energies of folding. A two-mode binding model has been proposed primarily on the basis of binding isotherms that do not conform to those expected from one set of independent binding sites (13, 14). The term “mode” was employed to highlight that the two sets of binding sites may be overlapping or identical. In this case, the change in the apparent number of binding sites reflects a shift in the equilibrium between multiple forms of the dynamic α-crystallin oligomer. One of the characteristics that distinguish sHSP chaperone function is the hypothesized role of their flexible and dynamic oligomers (15–18). Temperature and phosphorylation-induced activation of binding have been interpreted in terms of the transient dissociation of the oligomeric structure (14, 19–22). Analysis of the thermodynamics of T4L binding by the α-crystallins suggests a conformational specificity for each mode, with the low affinity mode reserved for more globally unfolded states (13). However, direct evidence of conformational heterogeneity was masked by the similar spectroscopic signatures of T4L bound through the two modes.

The basis of the previously reported binding assay was the change in the motional state of a nitroxide spin label introduced in T4L. Upon binding, the state transitions from highly mobile to almost immobile on the time scale of continuous wave electron paramagnetic resonance (EPR). In general, the determinants of the spin label mobility include the backbone configuration at the site of attachment and details of the local structural environment (23). However, steric contacts with the chaperone seem to dominate the spin label mobility in bound T4L leading to a similar motional state in the two modes. Therefore, to explore the nature of the conformational states of
bound T4L and the environments in which they are bound, we have developed a fluorescence-based assay for the detection of complex formation between sHSP and their substrates. A bimane probe is attached at the non-destabilizing cysteine of T4L instead of the spin label, and the changes in its fluorescence properties are detected upon unfolding. The rationale for the choice of the bimane probe is based primarily on the sensitivity of its parameters, such as the quantum yield and the emission wavelength, to the local protein and solvent environments (24, 25). In addition, the bimane group is comparatively small and is less likely than other bulkier fluorophors to significantly complicate the thermodynamics of the problem.

Binding of bimane-labeled T4L mutants results in a change in the fluorescence characteristics, the nature of which depends on the molar ratio of T4L to α-crystallin. This provides direct evidence of two distinct modes of binding. Furthermore, binding through the low affinity mode results in changes in the bimane emission intensity that are similar to those observed during denaturation unfolding. This result indicates that the T4L bound with low affinity is substantially unfolded. The results of this report establish a general, high sensitivity assay that can be used for detection of binding by heat-shock proteins.

EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—The detailed description of the cloning and site-directed mutagenesis of both αB-crystallin and T4L were previously reported (13, 14, 23). In this report, single-site mutants are named by specifying the original residue, the number of the residue followed by the new residue. For bimane-labeled mutants, the suffix “B” is used.

Protein Expression, Purification, and Labeling—The expression and purification of T4L mutants, αB-crystallin, and αB-crystallin phosphorylation mimics were carried out as previously described (13, 14, 23).

Briefly, T4L mutants were expressed in the Escherichia coli strain K38 using permissive induction temperatures (13). Sequential cation exchange and size-exclusion chromatographies were used to purify the mutants to apparent homogeneity. Following elution from the cation-exchange column, the mutants were incubated with a 10-fold molar excess of monobromobimane in a pH 7.6-buffered solution. The reaction (Scheme 1) was allowed to proceed for 2 h at room temperature and then overnight at 4 °C. The reaction mixture was then purified by size-exclusion chromatography (SEC) using a Superdex 75 column equilibrated with the appropriate buffer. Different pH values were obtained by varying the molar ratios of Mes and Tris while maintaining the total ionic strength constant. The labeling efficiency was determined by comparing the absorption at 280 and 380 nm. Consistently, a 280/380 ratio of 10:1 was obtained. This ratio indicates stoichiometric labeling of the introduced cysteine (24). Protein concentrations were determined using an extinction coefficient of 1.228 cm⁻²·mg⁻¹ for T4L and 0.947 cm⁻²·mg⁻¹ for αB-crystallin.

αB-crystallin and its variants were purified using sequential anion exchange and SEC. For the triply phosphorylated analog, S19D/S45D/S59D, a phenyl-Sepharose affinity chromatography step was inserted prior to SEC to further purify the protein as previously described (14).

Denaturant-unfolding Curves—To determine the free energy of unfolding, ΔG_u of select bimane-labeled mutants, unfolding curves were constructed by monitoring tryptophan emission as a function of urea concentration. Bimane emission was also monitored to determine the effect of global unfolding on the fluorescence characteristics. The curves were then fit to a two-state unfolding model using non-linear least squares as described previously (26). Six parameters were used to fit the data: a slope and an intercept for the pretransition and post-transition regions, ΔG_u, and m, the denaturant dependence of the free energy of unfolding, for the transition region.

Fluorescence Spectroscopy—Binding studies of fluorescence-labeled T4L mutants were carried out on a Photon Technology International L-format spectrofluorometer equipped with a sample holder controlled by a peltier heater/cooler. Samples containing a constant concentration of a T4L mutant and varying concentrations of αB-crystallin were incubated at the desired temperature for 2 h. αB-crystallin and T4L solutions were buffered by a combination of Mes and Tris and contained 50 mM NaCl. Fluorescence emission spectra of the samples were recorded between 400 and 500 nm by exciting the bimane molecule at 380 nm.

Analysis of Binding Isotherms—Using the appropriate equations, both simulations and curve fitting were performed using the program Origin (OriginLab Inc.). For non-linear least-square fits, the Levenberg-Marquard method was used.

RESULTS

Equilibrium Binding of T4L to α-Crystallin: General Methodology—The chaperone function of sHSP involves the recognition of non-native states of their substrate. Although such states become abundant following exposure to extreme physicochemical conditions, they are populated under native conditions albeit to a small extent. Presumably, the rare excursions to these partially or globally unfolded states result in transient binding to sHSP. To achieve a stable complex, i.e., a significant population of substrate-chaperone complex, the energetics of binding have to be comparable to the energetics that define the refolding from the binding-competent states to the native state. In vitro, this balance can be manipulated by reducing the stability of the native state of a given protein.

For this purpose, a set of destabilized mutants of T4L, most having a crystal structure similar to that of the WT (27), were constructed. Each has an introduced cysteine at a non-destabilizing site for the attachment of a spectroscopic reporter group. The properties of the reporter group have to be sensitive to the association with the chaperone thus allowing the observation and quantitation of the complex. In this report, the T4L mutants were labeled either at position 151 or at position 116 with the probe monobromobimane. Most of the binding studies were carried out using the triply phosphorylated analog of αB-crystallin, αB-D3 (S19D/S45D/S59D), to achieve the significant binding required for detailed statistical analysis of the data (14). Because both WT αA- and αB-crystallin use two-mode binding (13, 14), the conclusions concerning the conformations of bound T4L apply to both proteins.

Fig. 1 shows the emission spectra of bimane-labeled T4L-L99A/A130S in the absence and presence of αB-D3 at 23 °C, pH 8. Comparison of these spectra reveals significant changes in the fluorescence characteristics of the bimane group that depend on the molar ratio of T4L to αB-D3. In the presence of a large excess of αB-D3, corresponding to high affinity binding, there is a 14-nm shift in the maximum emission wavelength, λmax, and a drop in the fluorescence intensity. In the case of an equimolar ratio, corresponding to binding through both modes of affinities, a substantial drop in the emission intensity is observed. Irrespective of the origin of the quenching of the bimane emission, it must necessarily arise from the population of T4L bound with low affinity, because high affinity binding does not result in extensive quenching. Consequently, the variation in the emission intensity at a single wavelength (460 nm) as a function of the molar ratio between αB-D3 and T4L is biphasic, as shown in Fig. 1b. The wavelength was selected to maximize the changes in the emission intensity across the titration range. A similar biphasic curve is obtained if the wavelength is chosen to be 468 nm, the emission λmax of free T4L. It is noted that the biphasic behavior is observed in the direct binding curve and is not deduced from its reciprocal representation. Given the sensitivity of the bimane to its environment, this result indicates that either there are two physi-
FIG. 1. a, emission spectra of bimane-labeled T4L-L99A/A130S obtained at different molar ratios of αB-D3 to T4L at 23 °C, pH 8. The excitation wavelength was 380 nm. b, binding isotherm of T4L-L99A/A130S constructed by following either the change in emission intensity at 460 nm or the shift in λ_{max} as a function of increased concentration of αB-D3. The T4L concentration was 30 μM.

Cally distinct sets of binding sites and/or that T4L is binding in two distinct conformations that have altered bimane fluorescence.

**Minimalist Two-mode Binding Model for Analysis of the Change in the Fluorescence Intensity**—The equation corresponding to two-mode binding is given by,

\[
\frac{r_i}{r_1} = r_1 + r_2 \tag{1}
\]

where

\[
r_i = n_i L (K_{D_b} + L) \quad (i = 1 \text{ or } 2) \tag{2}
\]

In Equation 2, \(r_i\) is the ratio of bound T4L to total α-crystallin in a given mode of binding, \(L\) is the fraction of free, native state T4L, \(n_i\) is the number of binding sites, and \(K_{D_b}\) is the apparent dissociation constant associated with each binding mode. Because α-crystallin does not bind the native state of T4L, \(K_{D_b}\) is a ratio between the intrinsic dissociation constant and the equilibrium constant that characterizes the interconversion of T4L between the native state and the state recognized by the chaperone. Binding of spin-labeled T4L to α-crystallin results in two distinct spectroscopic observables that arise from the bound and free species. This allows the direct measurement of the fractional population of free T4L as a function of added α-crystallin. In contrast, the various states of bimane-labeled T4L contribute to the same fluorescence envelope. Binding is inferred from changes in the intensity and shifts in the emission λ_{max}. The former quantity, although proportional to the population of the various microstates, cannot be directly interpreted in terms of the bound and free T4L fractions. Therefore, to quantitatively analyze the binding curve of Fig. 1b, Equation 2 has to be rewritten such that \(L\) is numerically determined based on the known concentrations of added T4L and αB-D3, the unknown binding parameters \(n_1, n_2, K_{D_{1}}\), \(K_{D_{2}}\), and the relative fluorescent emission intensities of T4L in each mode, \(F_1\) and \(F_2\), as outlined previously (28).

The changes in the fluorescence intensity at a single wavelength expected for two-mode binding are illustrated in Fig. 2. Panel a shows the change in the binding isotherms for different values of \(K_{D_{2}}\), the emission intensity in the low affinity mode. It is clear that the biphasic shape is the direct result of the different emission intensity in each binding mode. \(K_{D_{2}}\) is a ratio between the intrinsic dissociation constant and the equilibrium constant that characterizes the interconversion of T4L between the native state and the state recognized by the chaperone. Binding of spin-labeled T4L to α-crystallin results in two distinct spectroscopic observables that arise from the bound and free species. This allows the direct measurement of the fractional population of free T4L as a function of added α-crystallin. In contrast, the various states of bimane-labeled T4L contribute to the same fluorescence envelope. Binding is inferred from changes in the intensity and shifts in the emission λ_{max}. The former quantity, although proportional to the population of the various microstates, cannot be directly interpreted in terms of the bound and free T4L fractions. Therefore, to quantitatively analyze the binding curve of Fig. 1b, Equation 2 has to be rewritten such that \(L\) is numerically determined based on the known concentrations of added T4L and αB-D3, the unknown binding parameters \(n_1, n_2, K_{D_{1}}\), \(K_{D_{2}}\), and the relative fluorescent emission intensities of T4L in each mode, \(F_1\) and \(F_2\), as outlined previously (28).

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ble-reciprocal curves (\(1/r\) versus 1/L) obtained using the same thermodynamic parameters and shown in Fig. 2c. For intermediate ratios of \(K_{D_{1}}\) to \(K_{D_{2}}\), the reciprocal representation is linear, which is normally interpreted to imply a single binding mode having a larger apparent dissociation constant. In contrast, the fluorescence binding curve indicates two-mode binding. In fact, the sensitivity of the reciprocal representation to two-mode binding occurs only for \(K_{D_{2}} \gg K_{D_{1}}\). The ability to detect two-mode binding in the intermediate range using the double-reciprocal representation is further reduced by the limited range of \(L\) dictated by the sensitivity consideration of EPR spectroscopy (13, 14).

**Single-mode, High Affinity Binding**—To eliminate binding at the low affinity site, we have previously used either the more stable T4L mutants or pH values that reduce the affinity of the α-crystallins (14). Given the sensitivity range of fluorescence spectroscopy, an alternative approach is to use concentrations of T4L significantly below \(K_{D_{2}}\). Fig. 3a shows the binding isotherm of αB-D3 to T4L-D70N obtained at 10 μM. The superimposed curve is a non-linear least squares fit to a single mode binding. The resulting parameters reported in Table I indicate that the number of high affinity sites, \(n_1\), is 0.25, which is at variance with the results reported previously using EPR detection of binding. The value of 0.5 reported by Koteiche and Mchaourab (14) was based on the binding isotherms of L99A and L99A/A130S. At concentrations of T4L similar to those used in the EPR binding assay, both of these mutants have biphasic isotherms (see below).

Further insight into the origin of the difference in the thermodynamic parameters between the two methods was obtained from the analysis of two binding curves constructed using either bimane-labeled or spin-labeled T4L-D70N at a concentration of 40 μM. The binding curve obtained from EPR spectroscopy results in \(n_1 = 0.33\) and a \(K_{D_{1}}\) of about 13 μM, both of which are close to those obtained from the corresponding 40 μM fluorescence binding isotherm, shown in Fig. 3a. However, the \(K_{D}\) obtained at this T4L concentration is significantly larger than the one obtained at 10 μM. The origin of these variations in both \(n\) and \(K_{D}\) must arise from a small contribution by the low affinity mode at the higher concentration, a contribution not explicitly detectable in the shape of either isotherms due to a large \(K_{D_{2}}\). A possible explanation for the difference in the \(K_{D_{1}}\) for D70N obtained by EPR and fluorescence is the marginally lower stability of D70N labeled with the bimane relative to the spin-labeled version as discussed below.

Another example of the effect of undetected contribution by the low affinity mode is the binding of the T4L mutants to the singly phosphorylated mutants of αB-crystallin (14). High af-

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*H. A. Koteiche and H. S. Mchaourab, unpublished results.*
finity binding was reported to be the predominant mode of binding by the single-phosphorylation analogs of α-crystallin at 23 °C, pH 8. Fig. 3a shows the binding isotherm of L99A/A130S to the α-crystallin mutant S45D (α-B-D1) at 10 μM. The parameters for this panel are identical to those of panel a with $F_2 = 0.2$. c, inverse representation of the binding curves shown in panel b.

Fig. 3. a, effect of increasing T4L concentration on the binding isotherms of T4L-D70N to α-B-D3. The solid lines are non-linear least-square fits obtained using the parameters reported in Table I. b, binding isotherms of α-B-D1 to two T4L mutants with different ΔG_unf. The fits were obtained assuming predominant binding by the high affinity mode. c, enhancement of the low affinity contribution to the binding by α-B-D1 by increasing the T4L concentration to 40 μM.

### Table I

| T4L mutant               | $n_1$ | $K_{D1}$ (μM) | $F_1$ |
|--------------------------|-------|---------------|-------|
| D70N (10 μM)             | 0.26  | 1.14          | 0.82  |
| D70N (40 μM)             | 0.31  | 7.24          | 0.75  |
| D70N (40 μM, EPR)        | 0.32  | 12            |       |

finity binding was reported to be the predominant mode of binding by the single-phosphorylation analogs of α-crystallin at 23 °C, pH 8. Fig. 3b shows the binding isotherm of L99A/A130S to the α-crystallin mutant S45D (α-B-D1) at 10 μM. The initial part of the curve can be fit assuming predominant binding at a single set of sites. However, the slow increase in slope, observed at the high ratios of α-B-D1 to T4L, indicates a minor contribution from the low affinity mode. Consistent with this interpretation, the sloping baseline is less pronounced in the 10 μM isotherm of T4L-L99A, which has a larger ΔG_unf (Fig. 3b) suggesting diminished binding through the low affinity mode. Furthermore, Fig. 3c shows that increasing the concentration of T4L-L99A/A130S to 40 μM, similar to the concentration used for EPR detection, enhances binding at the low affinity mode and results in a biphasic isotherm. It is noted that, although the shape of the isotherm indicates two-mode binding, the...
contribution of the low affinity mode is not enough to allow a unique fit. Taken together, these results indicate that for certain mutants the contribution by the low affinity mode persists even at low concentrations. This contribution is at the origin of the $n = 0.5$ obtained for the D1 variants by Koteiche and Mchaourab (14).

**Activation of Two-mode Binding Detected by Bimane Fluorescence**—Fig. 4 shows the binding isotherms of T4L-L99A/A130S and L99A/F153A to aB-D3 at pH 7.2, 23 °C. Both isotherms display the biphase changes in fluorescence intensity indicative of two-mode binding. T4L-L99A/F153A is the most destabilized mutant in the set presented in this report with a $\Delta G_{\text{uns}}$ of about 4.5 kcal/mol (14). The results of the non-linear least square fits in Table II confirm the expected higher affinity of aB-D3 to T4L-L99A/F153A relative to T4L-L99A/A130S. To optimize the extent of binding for each mutant, the binding curve for T4L-L99A/F153A was obtained at 10 μM, whereas for T4L-L99A/A130S was obtained at 40 μM.

Fig. 5 reproduces the pH activation of binding previously reported (14). Qualitatively, an increase in the $K_D$ of both modes at the lower pH is manifested by the shallower and right-shifted dip. The binding parameters, reported in Table II, demonstrate the significant decrease in binding affinity at pH 7.2. The change in the $K_D$ has a contribution from the increased stability of L99A/A130S at pH 7.2 relative to pH 8 as well as a decrease in the intrinsic affinity of aB-D3. Comparison of the binding curves of T4L-L99A/A130S to aB-D3 and aB-D1 (Fig. 3c), qualitatively confirms that the fully phosphorylated form has a higher affinity in both modes for the non-native states of T4L.

Similarly, the extent of binding of aB-D3 to T4L-D70N at 37 °C is higher than the extent of binding to T4L-L99A/A130S at 23 °C, as shown by the characteristics of the binding isotherms in Fig. 6. At the respective temperatures, the two mutants have similar values of $\Delta G_{\text{uns}}$ (14). Yet, significantly smaller $K_{D_{\text{low}}}$ are obtained at 37 °C (Tables II and III). Thus, the increased affinity must result from changes in the binding modes of the chaperone. This has been proposed to be a consequence of changes in the dynamics of the oligomeric structure (14).

**The Conformations of Bound T4L**—As described above, the two distinct fluorescence characteristics of the bimane-labeled T4L provide direct evidence of the presence of two modes of binding. These two modes can arise from two distinct sets of binding sites and/or the binding of two different conformations of T4L. To gain insight into the conformation of bound T4L, we investigated the origin of the substantial quenching of the bimane fluorescence in the low affinity mode. If the low affinity mode preferentially binds highly unfolded states (13), then quenching of the bimane fluorescence might reflect the intrin-

![Image](image-url)
The N116C mutation was introduced into the L99A mutation background, and the mutant reacted with the bimane label. Fig. 8a superimposes the changes in the emission intensity of the bimane upon unfolding to the corresponding changes in the tryptophan emission. Both binding curves report the same unfolding transition, and the $\Delta G_{\text{mut}}$ obtained from the Trp unfolding curve is similar to that of L99A/T151BI (Table IV). Fig. 8b compares the binding isotherms of T4L-L99A to aB-D3 at pH 7.2, 37 °C reported by the bimane attached at sites 116 and 151. In this figure, the emission intensities of the bimane at both sites are normalized using the same absolute scale. Both the quenching and the recovery levels of the biphasic isotherm are altered for N116BI relative to T151BI, although the position of the dip along the x-axis is little changed. This is the expected pattern of changes in the binding curve if the

| T4L mutant | $n_1$ | $K_D$ | $F_1$ |
|------------|-------|-------|-------|
| D70N       | 0.25  | 0.05  | 0.8   |
| L99A/N116BI| 0.7   | 0.01  | 1.3   |
| L99A/T151BI| 0.24  | 0.02  | 0.9   |

$37^\circ\text{C and pH 7.2}$

**Table III**

Conformation of T4 Lysozyme Bound to $\alpha$-Crystallin

**DISCUSSION**

The purpose of this study was to provide a structural context for the thermodynamic analysis of the binding between the $\alpha$-crystallins and T4L. Complex formation is interpreted to reflect the balance between a number of free energy terms that include the free energy of refolding. This term refers to the free energy associated with the transition between the native state and that of the state recognized by the chaperone. From the native state, a protein can access a ladder of states with different extents of unfolding, the so-called excited states (29, 30). Two-mode binding models, proposed to describe the shape of the binding curves of the $\alpha$-crystallins to T4L, in conjuction with energetic considerations, were interpreted to imply that T4L is bound in at least two different excited states, one of which is more globally unfolded (13). In support of this interpretation, NMR studies of T4L-L99A reveal a significantly populated compact, excited state located 2 kcal/mol higher than the native state (31). To define the structural features of the states that trigger binding, we introduce a new fluorescence method for the detection of complex formation between sHSP and their substrates. Our results confirm that the $\alpha$-crystallins bind T4L in two different modes and suggest that this arises partly from the recognition of two distinct conformations of T4L.

Fluorescence Detection of Binding—The detection of binding using spin-labeled T4L relies on a change in the motional state of the spin label between the free and bound forms of T4L. Binding to the chaperone restricts the reorientation of the label relative to T4L on the nanosecond timescale. However, the spin label appears to have degenerate motional states in the two modes. Thus, their presence could only be inferred indirectly from the differential number of binding sites and dissociation constants that characterize their binding.

An advantage of fluorescent probes, in general, is their enhanced sensitivity to the solvent environment. Often, large shifts in wavelength and intensity changes are observed upon the transfer between two solvents of different dielectric constants. It is this property that prompted us to consider fluorescence as an alternative detection method to spin-labeling EPR. The sensitivity of the bimane to its protein context, demonstrated previously in T4L, provided the rationale for the choice of this particular probe (24).

Nevertheless, it is the alteration in the spectral properties of the bimane upon unfolding that is at the origin of the changes in the fluorescence characteristics reported in this paper. The dramatic change in the fluorescence emission intensity at a water-exposed site in the unfolded state is a novel finding and merits further investigation with regard to the underlying mechanism. Quenching of bimane fluorescence has been reported at sites in close proximity to tryptophan residues (25). This results from photo-induced electron transfer from the tryptophan to the bimane-excited state. In fact, our initial interpretation of the different emission characteristics of the bimane in the two modes is that it arises from two different binding environments on the $\alpha$-crystallin oligomer, one of which contains a tryptophan residue in close proximity to the bimane. However, the reduced quenching of the bimane attached at site 116 argues against this interpretation. The cor-
relation of the sign and magnitude of the change with unfolding-induced quenching supports the contention that the bimane fluorescence reflects the conformation of T4L.

The fluorescence binding assay has many advantages. The increased sensitivity allows a more optimal choice of the concentration of the substrate relative to the $K_D$ value. This is critical for accurate thermodynamic analysis as demonstrated in this report. The assay is easily portable to other proteins and requires only basic fluorescence instrumentation. However, changes in the bimane fluorescence upon binding may be protein-specific, and thus identifying the origin of these changes is essential for the interpretation of binding isotherms. Perhaps the most exciting advantage is the possibility of performing this assay in a high throughput fashion given the availability of fluorescence plate readers.

Mechanism of Chaperone Function and the Conformation of Bound T4L—The biphasic nature of the fluorescence binding isotherms strongly supports the two-mode binding model proposed by Mchaourab et al. (13) for the $\alpha$-crystallins. They also reproduce the phosphorylation, pH, and temperature-induced activation of binding (14). However, the binding parameters obtained from the analysis of the binding curves to $\alpha$B-D3 are different than those reported previously. The origin of the difference in $n_1$ is that none of the linear curves presented by Koteiche and Mchaourab consisted of single-mode binding due to the high concentration of T4L needed for EPR spectroscopy. The higher $n_1$ necessarily means that $K_D$ was underestimated. Another factor in the change of both $K_D$ is the small incremental destabilization due to the bimane relative to the spin label. We suspect that another contributor to the difference is the statistical superiority of fitting the direct binding curve versus the reciprocal representation.

In addition to confirming the two-mode nature of the binding by $\alpha$-crystallin, the results provide evidence that at least two different conformations of T4L are bound. At high molar ratio of $\alpha$B-D3 to T4L, corresponding to predominantly high affinity binding, a blue-shift in the $\lambda_{\text{max}}$ indicates the transfer to a buried environment. The lack of a substantial reduction in the emission intensity suggests that this bound state is not extensively unfolded. On the other hand, the fits of the various curves suggest an 80–90% quenching for the bimane bound in...
the low affinity mode. Assuming no further changes in the fluorescence are induced by the local environment, it appears that this T4L conformation is extensively unfolded. Another line of evidence supports unfolding as the determinant of the bimane fluorescence in the low affinity-bound state. A subset of residues in T4L has a higher quantum yield in the unfolded state relative to the folded state due to their proximity in the latter to a tryptophan side chain. These residues, such as Leu-121, Ala-129, and Leu-133 are mostly in a buried environment. Thus, cysteine substitution and the subsequent attachment of the bimane lead to a reduction of 4–6 kcal/mol in $\Delta G_{\text{unf}}$. Little is known about the detailed structure of these mutants; therefore, they were not used for quantitative analysis. Nevertheless, it is instructive that the emission intensity of the bimane increases when these mutants bind αB-D3 further confirming the correlation between the sign of the fluorescence change in unfolding and binding (data not shown).

An alternative interpretation of the data is that T4L has a similar extensively unfolded conformation in both modes. This implies that the change in the relative fluorescence upon transfer from the low to the high affinity mode is due to a distinct environment at the two binding sites. For instance, the high affinity site might present a more buried environment thereby increasing the quantum yield of the bimane. The increased affinity might reflect a cooperative effect arising from the binding of T4L by four $\alpha$-crystallin subunits ($n = 0.25$) versus a single subunit in the low affinity mode.

Although such a model is feasible based on the spectroscopic data, the thermodynamic analysis favors the two-conformation model. Particular residues in $\Delta G_{\text{unf}}$ seem to target binding through the low affinity mode suggesting the recognition of specific conformational states. This was previously observed for the binding of αA-crystallin (13), and the data presented in this report agree with this overall trend. For instance, even at 40 $\mu M$ a minor contribution from the low affinity binding is implied for D70N, whereas the 2 $\mu M$ isotherm of L99A is biphasic (data not shown). It is noted that this energetic threshold is expected to be protein-specific, because it presumably reflects the particular ladder of accessible states available to the protein. The two-conformation model is also consistent with previous studies that have shown that both crystallins can bind compact molten globule states (32–34). One of the complications in the above analysis is that the equilibrium data do not distinguish between the state recognized and the state bound. It is possible that a partially unfolded state is initially recognized and unfolds further as binding is completed.

Concluding Remarks—A model in which one of the $\alpha$-crystallin binding modes is specific for compact intermediate states is also favored by the type of unfolding events that may occur in the lens. Because little protein turnover occurs, non-native states are primarily populated due to fluctuations in the structure of lens proteins. Furthermore, protein damage that modifies particular amino acid side chains is unlikely to cause global unfolding but rather results in an increased propensity to occupy these predominantly compact non-native states, possibly leading to aggregation. These non-native states are the binding targets of the $\alpha$-crystallins if they were to maintain long-term lens transparency. Multiple lines of evidence have suggested a coupling between the properties of the oligomeric structure and the chaperone activity of sHSP (14, 18, 35–37). The remarkable divergence in the static and dynamic properties of the oligomeric structures of sHSP suggests that these proteins may differ in the details of their binding to non-native protein states (15, 37, 38). The extent to which two-mode binding is a general property of the superfamily is yet to be investigated. The binding assay we have introduced is ideal for examining this aspect.

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