Published in final edited form as:

J Mol Biol. 2008 January 25; 375(4): 1026–1039.

Structure and Orientation of T4 Lysozyme Bound to the Small Heat Shock Protein α -Crystallin

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Summary

We have determined the structural changes that accompany the formation of a stable complex between a destabilized mutant of T4 lysozyme (T4L) and the small heat-shock protein α -crystallin. Using pairs of fluorescence or spin label probes to fingerprint the T4L tertiary fold, we demonstrate that binding disrupts tertiary packing in the two domains as well as across the active site cleft. Furthermore, increased distances between i and i+4 residues of helices support a model in which the bound structure is not native-like but significantly unfolded. In the confines of the oligomer, T4L has a preferential orientation with residues in the more hydrophobic C-terminal domain sequestered in a buried environment while residues in the N-terminal domain are exposed to the aqueous solvent. Furthermore, EPR spectral lineshapes of sites in the N-terminal domain are narrower than in the folded, unbound T4L reflecting an unstructured backbone and an asymmetric pattern of contacts between T4L and α -crystallin. The net orientation is not affected by the location of the destabilizing mutation consistent with the notion that binding is not triggered by recognition of localized unfolding. Together, the structural and thermodynamic data indicate that the stably bound conformation of T4L is unfolded and support a model in which the two-modes of substrate binding originate from two discrete binding sites on the chaperone.

Keywords

α -crystallin; Small heat-shock proteins; Electron paramagnetic resonance spectroscopy; Fluorescence spectroscopy; site-directed spin labeling

Introduction

Although an overall thermodynamic bias directs the folding of a nascent polypeptide chain to the native state, the progress along the free energy hypersurface can be impeded by frequent population of aggregation-prone intermediates¹⁻⁴. Furthermore, these intermediates are continuously populated after folding is complete through equilibrium transitions from the native state⁵. In long-lived cellular systems, such as the ocular lens, accumulation of temperature-sensitive mutations or post-translational modifications enhances misfolding as well as the equilibrium population of non-native intermediates. In the crowded cellular environment, aggregation of proteins with exposed hydrophobic surfaces is favored by the high

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concentration and the excluded volume effect^{6,7}. The lens is a particularly extreme example with its minimal protein turnover and the consequent lack of repair of damaged, destabilized proteins^{8,9}. The most common form of lens opacity, age-related nuclear cataract, is associated with the formation of protein aggregates¹⁰.

A universal cellular mechanism for coping with misfolded or non-native proteins involves the expression of five classes of heat shock proteins that facilitate folding following the emergence of proteins from the ribosomes and subsequently monitor the level of aggregation-prone intermediates^{4,11}. All molecular chaperones recognize and bind proteins with exposed hydrophobic surfaces thereby suppressing their aggregation. The small heat-shock proteins (sHSP) superfamily consists of oligomeric proteins that have high binding capacity for non-native proteins reaching in some instances one to one binding of each subunit to a substrate of equal molecular weight¹². In the lens, two sHSP, α A- and α B-crystallin, play a critical role in maintaining lens transparency. They form polydisperse oligomers that undergo subunit exchange^{13,14}. By middle age α A- and α B-crystallin disappear from the water soluble fraction signifying the exhaustion of chaperone capacity¹⁵. In general, progressive shifts in the folding equilibria of proteins can overload the folding capacity of the cell and lead to protein aggregation¹⁶.

In the presence of folded but thermodynamically destabilized proteins, sHSP act as stability “sensors” binding the most destabilized proteins at higher levels^{17,18}. Recognition and binding of substrates by sHSP require transition to an activated state^{12,17,18} which for eukaryotic sHSP involves the dissociation into small multimers^{19,20}. The N-terminal domain has been implicated as the main substrate binding region^{21,22} although contacts occur with the conserved α -crystallin domain²³. sHSP bind destabilized mutants of T4 Lysozyme (T4L) in two modes differing in affinity and capacity¹⁸. The binding can be described by a thermodynamic model²⁰ where the activated state of the sHSP binds non-native T4L thereby coupling the substrate unfolding equilibrium to sHSP activation. The model proposes that the two modes of binding correspond to the recognition of distinct conformations of T4L¹⁸.

A number of studies have explored the structure of proteins bound to α -crystallin in complexes formed under conditions that induce substrate aggregation. The substrate conformations were found to be primarily slowly-aggregating molten globules and to maintain significant levels of residual secondary structure²⁴⁻²⁸. Because these assays are performed under non-equilibrium conditions, they may promote transient interactions with non-native states leading to kinetically trapped substrates. Fast-aggregating substrate intermediates on the other hand may escape binding by the chaperone. Furthermore, most mutations or post-translational modifications often lead to subtle increases in the free energy of unfolding (ΔG_{unf}) in contrast to the extreme destabilization of the native state (i.e. a negative ΔG_{unf}) characteristic of these assays. Thus, aggregation-assays do not capture the predominant equilibrium interactions of chaperones within the cellular environment that must precede nucleation of aggregation.

Here, we report the first direct investigation of the structure and orientation of a substrate, T4L, bound to a sHSP, α -crystallin. Complexes of defined stoichiometry between α -crystallin and T4L are formed under conditions where T4L does not aggregate and the equilibrium population of its native state is orders of magnitude higher than that of the ensemble of unfolded states. Distance constraints between spectroscopic probes are used to compare the structures of native and α -crystallin-bound T4L. In addition, analysis of the local environment across the T4L sequence in the chaperone complex reveals preferential regions of interactions and the overall orientation. Together, the results provide a novel perspective on the mechanism of recognition and binding by sHSP.

Results

Approach and general methodology

To determine the conformational state of T4L bound to α -crystallin, we analyzed proximities in pairs of spectroscopic probes introduced at selected sites that fingerprint the tertiary fold. The pairs consisted of either two nitroxide spin labels or a bimane and a tryptophan (trp). Trp quenches bimane fluorescence in the distance range below 15 Å²⁹. Spin labels undergo dipolar coupling in the 5-20 Å range which is manifested as broadening of the Electron Paramagnetic Resonance (EPR) lineshape³⁰. Changes in bimane emission intensity reflect proximity changes between the trp and the fluorophore. This approach allows detection of local structural movements upon complex formation. It also overcomes the non-specificity of circular dichroism or FT-IR²⁸ because the probes exclusively report on the structure of the substrate.

To promote binding of T4L to α -crystallin, the probes were introduced in the T4L-L99A background; a hydrophobic mutation in the C-terminal domain that significantly reduces the free energy of unfolding without perturbing the overall structure of T4L³¹. Several criteria were employed to select appropriate pairs. First, we needed to sample structural motifs and domains across the molecule. Second, replacement of endogenous residues for cysteine and/or trp should not result in significant changes in stability beyond that due to the destabilizing mutation. Therefore, only surface sites were considered for cysteine replacement and subsequent probe attachment. Third, the degree of bimane quenching is influenced by the distance and orientation of the trp. The quenching of bimane by tyrosine residues is a complicating factor and we avoided introducing cysteines in their vicinities where possible. Using these criteria, two bimane/trp pairs were introduced into the C-terminal (N116Bi/N132W, G113Bi/K83W) and N-terminal (V57Bi/K16W, L39Bi/Y25W) domains. In addition, another pair was designed to bridge the active site cleft between the C- and N-terminal domains (Q141Bi/E22W). Each pair has a C $_{\alpha}$ -C $_{\alpha}$ distance less than 10 Å according to the crystal structure (Supplementary Table 1) although the actual distance between the probes depends on their relative projection. Figure 1 shows the location of the bimane/trp pairs in the crystal structure of T4L-L99A.

α -crystallin binds T4L in two discrete modes with vastly different affinities leading to distinct bimane emission characteristics¹⁸. The manifestation of the two modes in the binding isotherm depends on the concentrations of the substrate and chaperone relative to the dissociation constants as well as the differences in the fluorescence emission in each mode. Therefore, the T4L concentrations were adjusted relative to the dissociation constants, K_{D1} and K_{D2} of the high and low affinity modes, to either include or eliminate contributions from low affinity binding. In addition, the majority of binding experiments were conducted with the triply phosphorylated analog of α B-crystallin, α B-D3 (S19D/S45D/S59D). α B-D3 has higher affinity for T4L than the WT which allows for quantitative analysis of two-mode binding isotherms¹⁸; however, experiments with α A-crystallin yielded qualitatively similar characteristics.

Pattern of bimane quenching in native T4L-L99A

Under conditions that favor the folded state, all five T4L bimane/trp mutants demonstrated substantial quenching of bimane fluorescence relative to the corresponding single mutant. This observation is consistent with the mutants being properly folded and suggests that the substitutions do not significantly alter the tertiary structure. The thermodynamic stabilities of single and double mutant T4L were analyzed by denaturant unfolding. In most cases both the bimane (485 nm) and intrinsic trp (350/320 nm) unfolding curves report similar thermodynamic parameters as detailed in Table 1. ΔG_{unf} of the bimane-labeled mutants clusters in a narrow range around that of T4L-L99A. Except for N116Bi/N132W, the deviations are well within the error of the non-linear least-squares fit of the curves.

Global unfolding alters the intensity of bimane emission at all sites reflecting changes in its environment between the folded and unfolded states. In the bimane/trp pairs, bimane fluorescence increases considerably in the unfolded state indicating that the main determinant of emission intensity, quenching by the neighboring trp, is completely removed during unfolding (Figure 2a). For the single mutants, the sign of intensity changes does not follow a simple pattern except at loop sites where subtle changes are observed (Supplementary Figure 1). At G113Bi and L39Bi, we observe an increase in bimane emission upon unfolding, suggesting a native quenching phenomenon. L39Bi and G113Bi are 61% and 84% quenched, respectively, relative to N116Bi, a surface site with no nearby trp or tyr residues. The quenching observed at L39Bi is likely due to a native tyrosine residue at site 25. However, the magnitude of quenching is six-fold less than trp-induced quenching as evidenced by L39Bi/Y25W double mutant. The source of quenching at G113Bi is more enigmatic. The distance between residue 113 and the closest trp 138 is within the quenching radius, but the projection of 113 away from the core is expected to result in minimal interaction. In any case, quenching is increased in the G113Bi/K83W double mutant allowing the use of this pair to monitor unfolding.

Bimane quenching is eliminated upon binding of trp/bimane pairs to α -crystallin

Upon addition of a saturating amount (50-fold excess) of α B-D3 (or α A-crystallin), corresponding to high affinity binding, bimane fluorescence increases for the double mutants suggesting a reduction in the extent of bimane quenching by the neighboring trp. This effect is illustrated in Figure 2b for N116Bi/N132W where the bimane emission intensity of the double mutant is almost identical to the corresponding single mutant in the bound state but severely quenched in native, free T4L. Table 2 (and Supplementary Table 2) quantitatively reports the intensity change for all five double mutants. The results indicate that under high affinity binding conditions, native tertiary contacts are disrupted in both the C- and N-terminal domains as well as at their interface near the active site cleft. The marginal deviations in bimane intensities between the single and double mutants in the bound state can be attributed to small differences in the efficiency of labeling between the mutants as the protein concentration was the same in all samples.

Bound T4L is unfolded in both binding modes

Because low affinity binding occurs following saturation of high affinity sites, it is not possible to directly analyze the structure of low-affinity bound T4L. However, if the structure of bound T4L is distinct in the two modes, a characteristic binding isotherm is expected for the bimane/trp double mutants. A more native-like structure (i.e. compact) in the low affinity mode will lead to a closer proximity between the trp and bimane. Therefore, F_2 , the low-affinity emission intensity, is expected to be significantly smaller than F_1 , the high-affinity emission intensity. In contrast, if T4L is unfolded in both modes F_1 and F_2 are significantly larger than F_N , the normalized emission intensity in the native state. The simulated isotherms corresponding to these two cases are compared in Figure 3a. An unfolded conformation in the low affinity mode versus a compact conformation in the high-affinity mode leads to a biphasic isotherm as shown in Figure 3b. This case is excluded by the results of Table 2.

Experimentally, the binding isotherms of α B-D3 (Figure 3c) and α A-crystallin (Figure 3d) to the double mutants demonstrated a monophasic increase in intensity. The fractional emission intensities of T4L in both modes extracted from non-linear least-squares analysis is significantly higher than that of free, folded T4L (Table 3). Thus, low-affinity binding also increases the separation between the trp and bimane. This pattern of fluorescence is consistent with a loss of tertiary structure in both binding modes. The differences between F_1 and F_2 reflect the more hydrophobic environment of T4L in the high affinity mode (discussed below) which leads to an enhanced quantum yield.

Tertiary and secondary structure unfolding of bound T4L

The binding-induced unfolding of T4L was further confirmed via analysis of proximity between pairs of spin labels. Spin labels attached to the double mutant N116C/N132C have a broadened EPR lineshape in the folded state consistent with the short distance (8.7 Å, Supplementary Figure 2) in the crystal structure (Figure 4a). Binding to α B-D3 (or α A-crystallin) eliminates broadening due to spin-spin proximity as indicated by the almost superimposable lineshapes of the double mutant to that of the sum of single mutants in the bound state in Figure 4b. Furthermore, the lineshapes are superimposable at molar ratios of α -crystallin to T4L corresponding to exclusive high-affinity binding and combined two-mode binding (Supplementary Figure 3) which supports the conclusion that binding induces distance increases regardless of binding mode. Loss of broadening due to spin-spin interactions is also observed for the pairs E22C/Q141C and Y25C/L39C (data not shown).

Pairs of spin labels were introduced at i, i+4 in helix B in the N-terminal domain (S44C/K48C) and helix H in the C-terminal domain (V131C/K135C) to assess secondary structure unfolding in the bound conformation. In solution, the EPR spectra reveal substantial dipolar broadening consistent with the expected distance in a helix (Figure 4a). Upon binding, the spectral features arising from dipolar coupling disappear as demonstrated by a significant increase in intensity (Figure 4b). The increased distance between i, i+4 suggests that loss of tertiary structure is accompanied by helical unfolding. However, the lineshape of bound S44C/K48C shows residual homogenous broadening relative to the sum of singles in the bound state. The origin of spectral broadening is spin-spin interactions between residues i and i+4 separated by less than 20 Å in an extended, unfolded peptide. Spin-spin interactions in this range can be detected by EPR as shown by the broadened lineshape of this double mutant in the presence of 4.5M guanidinium HCl (Supplementary Figure 4).

Bound T4L has a preferred orientation

In addition to reporting the increase in distance between spin labels in the bound state, the spectral lineshapes in Figure 4b describe the motional properties of spin labels³². At sites 116 and 132, rigid limit EPR spectra are indicative of a highly restrictive steric environment. In contrast, the dominant component in the EPR spectrum at sites 44 and 48 is highly mobile reflecting considerable motional freedom of the spin labels.

To probe whether these motional differences represent an asymmetric pattern of contacts between the chaperone and T4L, we took advantage of the sensitivity of bimane emission to the polarity of its environment. Specifically, the wavelength of maximum emission (λ_{max}) has been shown to shift to larger values upon transition from a hydrophobic to a hydrophilic environment³³. A set of single cysteine mutants sampling different regions of T4L-L99A was constructed to determine the wavelength of maximum emission under conditions corresponding to predominant high-affinity binding. Because the sites were solvent exposed in native, free T4L, the average emission maximum of bimane is 471 ± 3 nm. In the high-affinity complex with α B-D3 or α A-crystallin, induced shifts in λ_{max} are dependent upon the location of the probe. Overall, a bipolar distribution is observed with residues that map to the N-terminal domain (average bound λ_{max} 465 ± 2 nm) shifted less than those that map to the C-terminal domain (average bound λ_{max} 456 ± 3 nm). This suggests that bound T4L has a net orientation relative to the aqueous solvent with the unfolded N-terminal domain more exposed (Table 4 and Supplementary Table 3). Mapping λ_{max} of bound T4L onto a surface representation of T4L illustrates the polarized distribution in the bimane emission pattern between the C- and N-terminal domains (Figure 5). The shift in λ_{max} is almost identical at high and low T4L concentrations (Supplementary Figure 5) i.e. irrespective of contribution by the low affinity mode. Thus, low affinity binding does not significantly shift λ_{max} relative to native free T4L consistent with binding in a more solvent exposed region of the oligomer.

T4L orientation is not dependent on the location of the destabilizing mutation

Because L99 is located in the C-terminal domain, we investigated whether the net orientation reflects binding in the vicinity of the mutation due to local dynamic unfolding. To address this question, a mutation of similar nature, L46A, was introduced in the N-terminal domain. The crystal structure of L46A reveals that the mutation induces minor local repacking but the overall structure remains similar to the WT³⁴. The mutation reduces ΔG_{unf} relative to wild type T4L, albeit to a lesser extent than L99A (Figure 6a). Denaturant unfolding of T4L-L46A indicates a 2-3 kcal/mol greater stability than L99A (Table 1), consistent with previously published data³⁴. Reflecting its higher stability, the binding isotherm of L46A/T151Bi is characterized by an increase in K_D for both binding modes and an apparent reduction in the number of binding sites, n , relative to L99A/T151Bi (Figure 6b and Table 5). The reduction in n reflects the minor contribution of the low affinity mode which makes the binding isotherm under-determined for non-linear least squares analysis. Despite the differences in stability and binding, the shifts in bimane λ_{max} were insensitive to the location of the destabilizing mutation, resulting in identical λ_{max} patterns for both C- and N-terminal domain mutants (Supplementary Table 4).

Detailed analysis of the binding curves for the L46A (Supplementary Table 5) and L99A (Table 3) mutants conclusively shows that ΔG_{unf} is the main determinant of binding. Most L46A mutants bind only with high affinity at both 5 and 30 μM fixed concentration of T4L consistent with the greater stabilities of L46A relative to L99A. However, there are variations in the affinities of the mutants within a given background that do not correlate with ΔG_{unf} . These are particularly significant for N-terminal bimane sites in the L99A background (Table 3). It is likely that labeling T4L with bulky probes modulates binding in a site-specific manner.

EPR analysis confirms the unfolded structure of bound T4L and its asymmetric contacts in the complex

In agreement with the polarized distribution of bimane emission shifts, the EPR spectra of spin labeled V131C, N116C, V57C and K48C report distinct motional properties in the bound conformation (Figure 7). At site N116C, for instance, the spin label is highly immobilized and the λ_{max} of N116Bi is strongly blue shifted to 450 nm. Both results indicate that the attached probe is in a buried environment. V131Bi does not shift as strongly (final $\lambda_{\text{max}}=459\text{nm}$) and the corresponding EPR spectrum shows the titration of a mobile component (red arrow in Figure 7). Thus, there appears to be a direct correlation between the final bimane emission λ_{max} and the spin label mobility in the chaperone complex (Supplementary Figure 6).

A survey of the single T4L mutants labeled with iodoacetamide proxyl spin label revealed domain-specific motional properties in the bound conformation, regardless of binding mode. Spin labels introduced at sites in the C-terminal domain exhibit spectra characteristic of highly restricted motion (Figure 7a). In contrast, the spectra of sites in the N-terminal domain mutants are dominated by three sharp lines indicative of a highly mobile spin label, although a low field immobile component was also observed suggesting the presence of a second minor population of T4L where the spin label motion is restricted (Figure 7b).

The dominant mobile component indicates that this segment of the N-terminal domain does not directly contact α -crystallin. Furthermore, the lineshape is sharper than that of native, free T4L (inset, Figure 7b). The increased dynamics at the point of attachment of the spin label in the chaperone complex is consistent with the unfolding of this helix deduced from the observed proximity changes. Size exclusion chromatography before and after the EPR analysis did not detect significant dissociation of the complex (Supplementary Figure 7).

To confirm that the mobile lineshapes of residues in the N-terminal domain reflect a more solvent exposed location, we measured the accessibility of the spin labels to collision with

NiEDDA. The EPR spectra of bound T4L mutants V57C and N116C are shown in the absence and presence of 50mM NiEDDA in Figure 8b. For V57C, NiEDDA induces spectral broadening by T_2 relaxation. In contrast, the spectrum of the bound C-terminal mutant N116C does not demonstrate any changes in the presence of NiEDDA. Power saturation experiments under high affinity binding conditions (30:1, α B-D3:T4L) reveal minimal accessibility to NiEDDA ($\Pi=0.06$) although an increase in accessibility ($\Pi=0.12$) was observed under low affinity conditions. These results clearly indicate that the C-terminal domain of T4L is buried in the α B-D3 complex, while the N-terminal domain remains partially exposed to solvent.

Discussion

The spectroscopic analysis presented above captures the essential structural features of a model substrate bound to a sHSP. The fluorescence and EPR constraints reveal a loss of tertiary contacts defining the fold and relative orientation of both T4L domains. Specific segments of secondary structures are also disrupted making it unlikely that native-like conformations or even molten globule states are stably bound to the chaperone. Effectively, excess α -crystallin acts to unfold T4L-L99A, which is predominately folded when free in solution under the conditions of the binding assay. Previous studies from our laboratory demonstrated that binding of the native substrate β B1-crystallin to α -crystallin leads to dissociation of its dimeric structure³⁵. Because dissociation and unfolding of β B1-crystallin are coupled³⁶, this result implies that the bound conformation is likely to be extensively unfolded.

In the bound state, T4L is likely to adopt an ensemble of unfolded conformations. By comparing the spectroscopic parameters of the double mutants to the corresponding reference samples (sum of singles for the spin labels and the single bimane for the bimane/trp pair), we can conclude that the pairs are separated by distances outside their range of interactions in most of these conformations. If compact structures exist, their fractions are below the threshold of detection of the methods employed in this paper conservatively estimated at 15%.

Because sHSP, and chaperones in general, evolved to bind a wide variety of substrates, their promiscuity precludes interactions with specific sequences. Nevertheless, high-affinity bound T4L has a net orientation. Segments of the more polar N-terminal domain are exposed to the aqueous solvent without direct contacts with α -crystallin while the more hydrophobic C-terminal domain is occluded in solvent inaccessible regions. The lack of changes in orientation and asymmetry of contacts between the L46A and the L99A mutations imply that hydrophobicity of the sequence determines the orientation of the bound substrate.

Thermodynamic analysis of binding isotherms supports the conclusion that T4L is substantially unfolded in the bound state. Table 3 and Supplementary Table 5 confirm the substrate free energy of unfolding as the primary determinant of the level of binding. For two-state unfolding, this parameter reflects the equilibrium population of unfolded states. Thus, the correlation between ΔG_{unf} and affinities is consistent with preferential binding of unfolded T4L; a conclusion further supported by the lack of dependence of bound T4L orientation on the location of the destabilizing mutation.

We previously interpreted the biphasic binding isotherms as reflecting either the binding of two structurally distinct populations of T4L or binding in two different environments¹⁸. Within the resolution of the spectroscopic tools employed in this paper, the bound T4L appears to be unfolded in both modes. A more occluded environment in the high affinity mode is suggested by the larger shift in λ_{max} and the larger fractional intensity of the bimane relative to the low affinity mode. EPR solvent accessibility experiments of N116C report a small but noticeable increase in accessibility under conditions that promote low affinity binding. Together, these results provide evidence that the two modes are associated with topologically

distinct binding sites on the sHSP oligomer rather than binding of different conformations of T4L.

Although the structure of the α -crystallin oligomer has not been determined, insight into the assembly principles has been derived from crystal structures of Hsp16.5³⁷ and Hsp16.9³⁸. In both oligomers, the conserved C-terminal α -crystallin domain forms the solvent exposed outer shell while the variable N-terminal regions cluster in the core. In the context of this architecture, a feasible model of high-affinity binding places the hydrophobic C-terminal domain of T4L bound in the oligomer core while the N-terminal domain is closer to the outer shell.

Concluding remarks

A detailed perspective on the mechanism of α -crystallin binding to its substrates emerges from the structural and thermodynamic analysis. Figure 9 presents a schematic of the thermodynamic coupling model that describes the chaperone activity of sHSP. Our results support an unfolded structure for stably bound T4L. However, a transient recognition step that precedes binding may involve more compact or native-like conformations as suggested by kinetically-determined aggregation assays. This implies that subsequent unfolding occurs on the sHSP oligomer. A detailed investigation of the rate constants of T4L binding is required to test this hypothesis.

The T4L accessibility profile in the bound state strongly suggests that the high-affinity binding sites are inside the sHSP oligomers while low affinity binding seems to involve a surface location of T4L. CryoEM analysis of complexes between sHSP and T4L are currently underway to verify this conclusion.

Materials and Methods

Materials

Monobromobimane was purchased from Molecular Probes. 3-(2-Iodoacetamide)-proxyl spin label was purchased from Sigma-Aldrich.

Cloning and Site-directed mutagenesis

T4L mutants were created using complimentary oligonucleotide primers containing the desired mutation and amplified via polymerase chain reaction as previously described³². All mutants were cloned into pET20b+ expression vector. Plasmid DNA sequencing from transformed cells confirmed the presence of the desired mutations and the absence of unwanted changes. In this paper, mutants of T4L are named by specifying the original residue, the number of the residue, followed by the new residue. For bimane-labeled mutants, the suffix “Bi” is used.

Expression and Purification of T4L Mutants and α -Crystallin

T4L mutants were expressed in *E. coli* BL21(DE3) following standard protocols and using an induction temperature of 37°C for L46A mutants and 28-30°C for L99A mutants. T4L was purified using a two-step protocol and labeled as previously described, except that the 3-(2-Iodoacetamide)-proxyl was used as the spin label¹⁷. T4L concentrations were determined using an extinction coefficient of 1.23 cm²/mg for the single bimane (or spin-labeled) mutants and 1.55 cm²/mg for the bimane/trp pair mutants after subtracting the bimane contribution to the absorbance. Four L99A mutants expressed into inclusion bodies (K83W/G113C, K16W/V57C, L39C, Y25C/L39C). These mutants were expressed at 37°C and the proteins were refolded as previously described¹⁷.

Both α A- and the triply phosphorylated analog (S19D/S45D/S59D) of α B-crystallin, called α B-D3, were purified as previously described^{17,18}. Protein concentrations for α A- and α B-D3 were determined using an extinction coefficient of 0.834 and 0.947 cm²/mg, respectively.

Thermodynamic Analysis of T4L mutants

Denaturant-unfolding curves were constructed by monitoring tryptophan and bimane fluorescence as a function of guanidinium HCl at 37°C in pH 7.2 buffer on a Photon Technology International (PTI) L-format spectrofluorometer. ΔG_{unf} of L99A/V57Bi/K16W was determined at 30°C due to aggregation and precipitation at 37°C. The curves were fit with a two-state unfolding model using nonlinear least squares methods.

Bimane Fluorescence Binding Analysis

Binding curves for each bimane-labeled mutant were obtained by incubating defined ratios of α -crystallin:T4L for 2hrs at 37°C in pH 7.2 buffer and collecting the bimane emission spectrum on a PTI L-format spectrophotometer with excitation and emission slit widths of 1 nm and 0.5 nm, respectively. The emission spectrum was collected from 410 to 500 nm after excitation of bimane at 380 nm. Binding of L99A-V57Bi/K16W was performed at 30°C. Binding isotherms were generated by plotting bimane emission at 485 nm as a function of the [α -crystallin/T4L] ratio. The curves were fit using the Levenberg-Marquart nonlinear least squares method in the program Origin (OriginLab Inc.)¹⁸.

EPR Spectroscopy

Analysis of spin-labeled T4L was carried out on a Bruker EMX spectrometer at room temperature in pH 7.2 buffer. For binding, 30 μ M T4L was mixed with α A or α B-D3 at an α -crystallin:T4L ratio of 3:1 (low and high affinity binding) or 30:1 (high affinity binding) at 37°C for 2hrs. The complex was purified by SEC on a Superose 6 column to ensure complex formation and to remove any residual free T4L. Following chromatography, the complex was concentrated to the original volume for binding. For quantitative solvent accessibility determination, power saturation experiments were performed with a Bruker Elexsys 500 spectrometer in the presence of 50mM NiEDDA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Eye Institute, NIH grants R01-EY12683 to Hassane S. Mchaourab and T32-EY07135 to Derek P. Claxton. The authors thank Jared A. Godar for critical reading of the manuscript and assistance in Figures 1, 7 and 9; Dr. Hanane A. Koteiche for assistance in mutagenesis, discussions and critical reading of the manuscript.

Abbreviations used

sHSP, small heat shock protein; T4L, T4 lysozyme; trp, tryptophan; EPR, electron paramagnetic resonance; WT, wild type; CryoEM, cryoelectron microscopy; NiEDDA, nickel ethylenediaminediacetic acid.

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Figure 1. Crystal structure of T4L-L99A (PDB 1L90) showing the location of the bimane/trp double mutants. The C-terminal domain (blue) consists of residues 1-11 and 82-162. The N-terminal domain (green) consists of residues 12-58. The two domains are connected by a long helix (residues 60-80, gray).

Figure 2.

Unfolding- and binding-induced changes in bimane fluorescence. (a) Trp and bimane intensity changes upon chemical denaturation of N116Bi and N116Bi/N132W. The increase in the intensity for the double mutant reflects the increase in distance between the bimane and trp. (b) Increase in bimane emission intensity upon binding to α -crystallin. For N116Bi/N132W, it reflects the separation of the bimane/trp pair beyond 15 Å. In folded T4L the intensity of N116Bi/N132W is quenched relative to N116Bi while in the bound conformation the bimane intensity is the same.

Figure 3.

Similar structure of T4L in the high- and low-affinity binding modes. (a) Expected isotherms for two-mode binding assuming that bound T4L is more compact in the low affinity than high affinity mode. The parameters are $n_1=0.25$, $n_2=1$, $K_{D1}=0.01$ μ M. (b) Expected isotherm if T4L is more compact in the high affinity mode. For this panel $n_1=0.25$, $K_{D1}=0.3$ μ M, $n_2=1$, $K_{D2}=6$ μ M. Experimental binding isotherms of T4L-N116Bi/N132W to α B-D3 (c) and α A-crystallin (d) showing monotonic increase in fluorescence intensity.

Figure 4. Disruption of tertiary contacts and helix unfolding upon binding of T4L to α B-D3. (a) EPR spectra of double mutants that fingerprint both secondary (S44C/K48C) and tertiary (N116C/N132C) structure. Strong spin-spin coupling reflect close proximity in the free, native structure. (b) Upon binding, spectral broadening is significantly reduced, indicating extensive unfolding of T4L. The spectral scan width is 150 Gauss.

Figure 5. Mapping $\lambda_{\max}$ of bound bimane-labeled T4L on a surface representation of T4L reveals a bipolar distribution. This indicates that sequences corresponding to the N-terminal and C-terminal domains are in different environments.

Figure 6. ΔG_{unf} is a determinant of affinity to α -crystallin. (a) Denaturant unfolding curves of T4L-L46A and T4L-L99A highlighting the higher stability of the former. (b) Comparison of binding isotherms between L99A/T151Bi and L46A/T151Bi. The left shift of the former reflects higher affinity to $\alpha\text{B-D3}$.

Figure 7. EPR spectra of N- and C-terminal domain mutants in the chaperone complex. Spin labels in the C-terminal domain (a) are immobilized while those in the N-terminal domain (b) are highly mobile. The insets show the decreased linewidth in the complex relative to free, native T4L consistent with increased dynamics of the backbone.

Figure 8. Differential solvent accessibility of spin labels in the N- and C-terminal domains in the high-affinity bound conformation. (a) In native free T4L, spin labels at sites 57 and 116 have high collision frequency with NiEDDA. (b) In the complex, site 57 continues to be highly exposed while 116 has marginal accessibility as shown by the small accessibility parameter Π .

Figure 9. Model of substrate binding to sHSP. Schematic representation of the thermodynamic coupling model of three equilibria highlighting the loss of native structure of bound T4L and the two different sets of binding sites on the sHSP oligomer. Although not depicted in the low affinity bound state, T4L also occupies the high affinity binding sites within the oligomer.

Table 1

ΔG_{unf} and m , the denaturant dependence of ΔG_{unf} , of T4L mutants at 37°C, pH 7.2. ΔG_{Trp} and ΔG_{bim} are free energies of unfolding obtained from non-linear least-squares fits of intrinsic trp and bimane unfolding curves, respectively. m is the denaturant dependence of ΔG for the transition region. F_U and F_N are the fractional fluorescences in the unfolded and native states respectively. F_N is normalized to 1

Mutant	$\Delta G_{\text{Trp}} (+/-10\%)$	$\Delta \Delta G$	m	F_U/F_N	$\Delta G_{\text{bim}} (+/-10\%)$	m	$(F_U/F_N)_{\text{bim}}$
L99A	4.9		4.5	3.18			
G113Bi	5.5	0.6	4.9	3.62	5.2	4.9	4.85
G113Bi/K83W	4.9	0.0	4.5	2.86	4.9	4.4	5.59
Q141Bi	5.1	0.2	4.3	2.53	4.5	4.6	0.42
Q141Bi/E22W	5.4	0.5	4.6	3.22	5.7	5.1	3.41
N116Bi	5.1	0.2	5.0	3.08	4.7	5.2	0.54
N116Bi/N132W	6.3	1.4	4.8	3.29	6.3	5.2	9.94
T151Bi	4.7	-0.2	4.5	3.50	4.0	4.8	0.24
V57Bi	5.0	0.1	5.0	3.23	ND	ND	1
V57Bi/K16W [†]	4.8	-0.1	3.8	2.83	5.1	4.3	12.09
L39Bi	4.9	0.0	5.1	3.27	5.5	6.3	2.19
L39Bi/Y25W	4.9	0.0	4.8	2.67	4.1	4.1	13.77
Average ΔG_{Trp} for L99A mutants = 5.1 (+/- 0.4) kcal/mol							
L46A	7.1		4.5	3.36			
Q141Bi	8.2	1.1	5.0	3.45	6.7	4.6	0.42
N116Bi	7.3	0.2	4.6	3.57	5.7	3.8	0.31
T151Bi	6.6	-0.5	4.6	3.07	6.6	4.9	0.31
V57Bi	7	-0.1	4.4	3.31	ND	ND	1
L39Bi	6.4	-0.7	4.2	3.41	8	6.1	1.97
Average ΔG_{Trp} for L46A mutants = 7.1 (+/- 0.6) kcal/mol							

[†] at 30°C

Table 2

Bimane emission intensity at 485 nm in the absence (I_N) and presence (I_{Bound}) of α B-D3 at 37°C, pH 7.2. The molar ratio of α B-D3 to T4L is 50:1. The average and standard deviation are calculated from three independent measurements

Mutant	I_N	I_{Bound}
Q141Bi Q141Bi/E22W	1 +/- 0 0.2 +/- 0	2.3 +/- 0.1 2.4 +/- 0.1
G113Bi G113Bi/K83W	1 +/- 0 0.7 +/- 0.1	7.4 +/- 0.2 7.3 +/- 0.2
N116Bi N116Bi/N132W	1 +/- 0 0.04 +/- 0.01	1.3 +/- 0.1 1.2 +/- 0
V57Bi V57Bi/K16W	1 +/- 0 0.1 +/- 0	1.2 +/- 0.1 1.4 +/- 0.1
L39Bi L39Bi/Y25W	1 +/- 0.1 0.2 +/- 0.1	3.1 +/- 0.2 3.1 +/- 0.1

Table 3

Number of binding sites, n , and dissociation constants, K_D , of T4L-L99A mutants binding to α B-D3 at 37°C (unless indicated), pH 7.2. The parameters were obtained from the non-linear least squares fit of binding curves consisting of at least 25 data points. F_U and F_N are the relative fluorescence of T4L bound in the high and low affinity modes, respectively. F_U and F_N are the fractional fluorescences in the unfolded and native states respectively. F_N is normalized to 1. T4L concentration was fixed at 30 μ M (unless indicated)

L99A Mutant	n_U	K_{DU} (μ M)	n_N	K_{DN} (μ M)	F_U	F_N	ΔG_{TTP}	$(F_U/F_N)_{bin}$
Q141Bi	0.2	0.02	0.54	0.57	2.38	0.97	5.1	0.42
Q141Bi/E22W	0.2	0.02	0.59	1.81	20.46	12.84	5.4	3.41
G113Bi	0.32	0.02	1.2	0.81	9.96	1.82	5.5	4.85
G113Bi/K83W [†]	0.2	0.05	1.08	0.74	21.7	8.49	4.9	5.59
N116Bi	0.28	0.01	0.99	0.35	1.25	0.69	5.1	0.54
N116Bi/N132W	0.2	0.08	0.65	6.3	39.5	30.7	6.3	9.94
V57Bi	0.22	0.01	1.0	2.04	1.0	0.78	5.0	1
V57Bi/K16W [‡]	0.34	0.29	1.2	30.01	17.32	14.79	4.8	12.09
L39Bi	0.35	0.11	1.2	11.65	2.79	1.67	4.9	2.19
L39Bi/Y25W	0.2	0.05	1.2	14.3	19.4	17.4	4.9	13.77

[†] at 7.5 μ M

[‡] at 30°C

Table 4

Change in bimane wavelength of maximum emission (λ_{max}) upon binding to α B-D3. The average and standard deviation are calculated from three independent measurements

Domain	L99A mutant	Free λ_{max} (nm)	Bound λ_{max} (nm)	$\Delta\lambda_{\text{max}}$
C-Terminal	Q141Bi	474 +/- 1	458 +/- 1	16 +/- 1
	Q141Bi/E22W	470 +/- 1	458 +/- 1	12 +/- 1
	N116Bi	472 +/- 1	450 +/- 1	22 +/- 1
	N116Bi/N132W	468 +/- 1	451 +/- 1	17 +/- 1
	G113Bi	472 +/- 0	455 +/- 1	17 +/- 1
	G113Bi/K83W	470 +/- 2	456 +/- 1	14 +/- 2
	R76Bi	472 +/- 1	459 +/- 1	13 +/- 1
	P86Bi	471 +/- 1	456 +/- 1	15 +/- 1
	D92Bi	463 +/- 1	456 +/- 1	7 +/- 1
	V131Bi	470 +/- 1	459 +/- 1	11 +/- 1
	T151Bi	469 +/- 0	454 +/- 1	15 +/- 1
	K162Bi	472 +/- 1	456 +/- 1	16 +/- 1
N-Terminal	V57Bi	476 +/- 0	467 +/- 1	9 +/- 1
	V57Bi/K16W	471 +/- 1	466 +/- 1	5 +/- 1
	L39Bi	468 +/- 0	463 +/- 0	5 +/- 0
	L39Bi/Y25W	470 +/- 1	463 +/- 1	7 +/- 1
	K48Bi	471 +/- 1	463 +/- 1	8 +/- 1
	K65Bi	475 +/- 1	466 +/- 1	9 +/- 1

Table 5

Comparison of binding affinity between T4L-L99A and T4L-L46A

T4L mutant	ΔG_{unf}	n_i	$K_{Di} (\mu M)$	F_i
L99A/T151Bi	4.7	$n_1=0.26$	$K_{D1}=0.05$	$F_1=0.80$
		$n_2=1.2$	$K_{D2}=1.44$	$F_2=0.08$
L46A/T151Bi	6.6	$n_1=0.13$	$K_{D1}=0.34$	$F_1=0.90$
		$n_2=0.56$	$K_{D2}=5.84$	$F_2=0.05$