Photoincorporation of 4,4′-Bis(1-anilino-8-naphthalenesulfonic Acid) into the Apical Domain of GroEL: Specific Information from a Nonspecific Probe†

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ABSTRACT: The use of noncovalent hydrophobic probes such as bis-ANS has become increasingly popular in gaining structural information about protein structure and conformation. While these probes have provided rich information about protein conformation, specific structural information has been limited. In this report, we extend the usefulness of the probe bis-ANS by showing that it can be covalently photoincorporated into various proteins. Using the chaperonin GroEL, we have shown that it is possible to locate important hydrophobic surfaces through photoincorporation and peptide sequencing. It has been proposed that hydrophobic surfaces on the chaperonin may be responsible for the binding of unfolded polypeptides. We show here that photoincorporation of bis-ANS is able to locate a distinct hydrophobic surface on GroEL. Incorporation of the bis-ANS occurs within a 45 residue fragment of the monomer near the middle of the primary sequence. Interestingly, photoincorporation occurs within this fragment in both tetradecamers and assembly-competent monomers. From the three-dimensional structure of GroEL, this region maps to the apical domain (residues 191−376), which has been implicated in polypeptide binding [Fenton, W. A., Kashi, Y., Furtak, K., & Horwich, A. L. (1994) *Nature 371*, 614−619]. In addition, the fluorescent properties of the probe are retained including the excitation and emission maxima and the sensitivity to the polarity of its environment. These results suggest that photoincorporated bis-ANS may be a useful probe for protein structure and dynamics.

Proteins belonging to the class termed chaperonins have been implicated in such diverse functions as protein trafficking, protein assembly, and assisted protein folding (Gething & Sambrook, 1992; Hendrick & Hartl, 1993). Studies of chaperonin action have focused on the *Escherichia coli* chaperonins GroES and GroEL (Georgopoulos & Ang, 1990). GroEL is a homotetradecamer of 57 kDa subunits. The quaternary structure is such that two heptamers are stacked together to form a double doughnut-like structure. Due to this structural arrangement, there is a central cavity where unfolded polypeptides are believed to bind (Braig et al., 1994). Recently, a 2.8 Å X-ray crystal structure was determined for GroEL (Braig et al., 1994). From this structure, each monomer appears to be organized into three distinct domains: apical, intermediate, and equatorial. The equatorial domain accounts for the majority of the monomer−monomer interactions as well as the contact region for the two heptamers. The intermediate domain connects the equatorial domain with the apical domain. The flexible apical domain forms the end of the GroEL cylinder, and mutations in this region implicate it in polypeptide binding (Fenton et al., 1994).

Recent studies have shed light on the probable mechanism of GroEL and GroES in the assisted refolding of proteins (Azem et al., 1994; Schmidt et al., 1994; Todd et al., 1994; Weissman et al., 1994; Martin et al., 1993; Frydman et al., 1994). GroEL is quite promiscuous in polypeptide recognition and binding. Many different proteins are capable of binding to GroEL (Viitanen et al., 1992). The nature of partially folded polypeptides has led to the speculation that surfaces with hydrophobic character may be in part responsible for the binding of unfolded proteins to GroEL. It has been suggested that intermediates in the folding pathway of most proteins possess such hydrophobic surfaces (Ptitsyn et al., 1990). Recently, a thermodynamic model has been proposed and confirmed that invokes a hydrophobic partitioning of unfolded polypeptides onto GroEL (Zahn et al., 1994; Zahn & Pluckthün, 1994). These results highlight the importance of investigating the role of hydrophobic interactions in the mechanism of GroEL-assisted protein folding.

One method for monitoring the changes in hydrophobic surfaces in GroEL has been through the use of noncovalent probes. The use of these probes has not been restricted to GroEL, however. One such probe, bis-ANS,[1] was first described by Rosen and Weber 25 years ago (Rosen & Weber, 1969). Since then, bis-ANS has been used to study conformational changes in a number of proteins including hemagglutinin (Korte & Herrmann, 1994), glutaminyl-tRNA synthetase (Bhattacharyya et al., 1991), pregnancy-zone protein (Arbelaez et al., 1993), phytochrome (Choi et al., 1990), and rhodanese (Horowitz & Criscimagna, 1988). A similar probe, ANS, has been more widely used to gain similar information. ANS has been used in probing the molten globule state of apomyoglobin (Cocco & Lecomte, 1994), in determining the folding pathway of hen lysozyme (Itzhaki et al., 1994), for dynamic studies of insulin (Hua et al., 1993), in determining the ligand binding site in glutathione *S*-transferase P (Nishihira et al., 1993), and in probing the conformational states of β-lactamase (Goto &

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[1] Abbreviations: bis-ANS, 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); ANS, 1-anilino-8-naphthalenesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Fink, 1989). ANS fluorescence has also been used in the study of GroEL. Mizobata and Kawata (1994) used the binding of ANS to identify an unfolding intermediate in the guanidine unfolding of GroEL.

While these hydrophobic probes can yield much useful information about a system, there is a limited amount of specific information that can be gained from their use. This is due to the nonspecific and noncovalent nature of the hydrophobic interaction. These probes are usually found to be noncovalently bound in hydrophobic pockets of the protein. Therefore, acquiring specific information about the location of the noncovalently bound probe is difficult at best.

In this report, we show that the hydrophobic probe bis-ANS can be covalently incorporated into proteins, thereby potentially providing specific information from what was previously a nonspecific probe. Bis-ANS is shown to be incorporated into GroEL during illumination with UV light. The incorporation accompanies the formation of urea-induced, assembly-competent monomers. In addition, bis-ANS can be photoincorporated into tetradecameric GroEL under ionic strength conditions that were previously shown to increase hydrophobic exposure (Horowitz et al., 1995). In both cases, the probe has been localized within a 45 residue segment that is located in the apical domain of GroEL. This domain of the chaperonin has been implicated in binding unfolded polypeptide (Fenton et al., 1994).

EXPERIMENTAL PROCEDURES

Materials. Electrophoresis quality urea was purchased from Bio-Rad. All other reagents were of analytical grade. α-Chymotrypsin was purchased from Worthington Biochemical (Freehold, NJ). bis-ANS was purchased from Molecular Probes, Inc. (Eugene, OR). Bovine serum albumin, lactate dehydrogenase, and ribonuclease A were purchased from Sigma and used without further purification. Malate dehydrogenase was purchased from Boehringer Mannheim and used without further purification. Bovine liver rhodanese was purified as previously described (Miller et al., 1992).

Protein Purification. GroEL was purified as described previously with slight modification (Hendrix, 1979; Chandrasekhar et al., 1986). Purified GroEL was dialyzed against 50 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol, then made 10% (v/v) in glycerol, rapidly frozen in liquid nitrogen, and stored at −70 °C. Protein concentrations were determined by the method of Bradford (1976).

Photoincorporation of Bis-ANS into Proteins. Protein was dissolved in 50 mM Tris-HCl, pH 7.8. For the labeling reaction, protein (0.3 mg/mL) was added to a well of a microtiter plate containing 50 mM Tris-HCl, pH 7.8, and 50 μM bis-ANS. In the cases where monomers of GroEL were desired, urea was included at a final concentration of 2.5 M. For labeling of tetradecameric GroEL, spermidine (pH 8.0) was included at a final concentration of 5 mM. The total volume of the labeling reaction was 50 μL. The microtiter plate was placed on ice, and a hand-held UV lamp [Model UVS-11 Mineralight lamp, 115 V, 60 Hz, 0.16 A, 254 nm (Ultra-violet Products, Inc., San Gabriel, CA)] was placed approximately 1 cm above the samples. Samples were illuminated for 1 h. Aliquots of the samples were subjected to electrophoresis under either native or denaturing conditions as indicated in the figure legends. Photolabeled proteins were visualized on a UV light box (Chromato-vue Transilluminator Model TM-40, Ultra-violet Products, Inc.).

Limited Digestion of Bis-ANS-Labeled GroEL by Chymotrypsin. GroEL (1.1 mg/mL) was photolabeled with bis-ANS as described above. After the labeling reaction, chymotrypsin was added to a final concentration of 0.1% (w/w). The proteolysis was carried out at room temperature. Aliquots were removed at various times, and PMSF (in 95% ethanol) was added to a final concentration of 2.9 mM (final ethanol concentration, ~2%) to stop the proteolysis. The samples were then incubated on ice for 10 min. An equal volume of 2× SDS gel loading buffer was then added, and the samples were heated at 100 °C for 4 min. Samples were then stored at room temperature before separation on polyacrylamide gels.

Amino Acid Sequence Analysis of Peptide Fragments. Chymotryptic digests of bis-ANS-labeled GroEL were separated on 1.5 mm thick 12% SDS−polyacrylamide gels and electrophoretically transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA) using a semi-dry technique as described previously (Merrill et al., 1993). The membrane was then illuminated on a UV light box, and the positions of fluorescent peptides were marked with a pencil. The membrane was stained with 0.1% Amido Black-10B in 10% methanol and 2% acetic acid until the peptides were just visible. Peptides that corresponded to fluorescent bands were excised from the membrane, washed with 20% methano, and allowed to air-dry. The fragments were then analyzed on an Applied Biosystems 477A protein sequence analyzer (Applied Biosystems, Foster City, CA). Peptides were subjected to approximately 12 rounds of sequencing. Using this information, the sequences of the fragments were then compared to the published sequence of GroEL to determine their positions within the intact protein (Hemmingsen et al., 1988).

Effect of Photoincorporation on GroEL Reassembly. GroEL (0.84 mg/mL) was photolabeled in the presence of 2.5 M urea as described above. After photolabeling, the samples were applied to G50 Sephadex spin columns that had been preequilibrated with 50 mM Tris-HCl, pH 7.8, to remove the urea and unincorporated bis-ANS. Identical samples were treated in the dark as positive controls for the reassembly. After removal of the unincorporated bis-ANS and urea, the samples were incubated at room temperature for 4 h to allow for reassembly. Aliquots of the samples were then subjected to electrophoresis on native polyacrylamide gels by the procedure of Neahoff et al. (1986).

Photoincorporation of Bis-ANS into GroEL in the Presence of Unfolded Rhodanese. Unfolded rhodanese was prepared in 8.0 M urea as described previously (Mendoza et al., 1994). GroEL was labeled under the conditions described above with the addition of differing amounts of unfolded rhodanese (see Figure 7 legend). Unfolded rhodanese was added to GroEL in 2.0 M urea before the addition of bis-ANS in order that a complex could be formed before photoincorporation was initiated. It has been previously shown that a complex between GroEL and unfolded rhodanese was formed at this concentration of urea (Mendoza et al., 1994). After photolabeling, the products were separated on polyacrylamide gels using either native or denaturing conditions. Photolabeled proteins were visualized using a UV light box.

Fluorescence Measurements. GroEL (0.84 mg/ml) was photolabeled with bis-ANS, and the urea and unincorporated

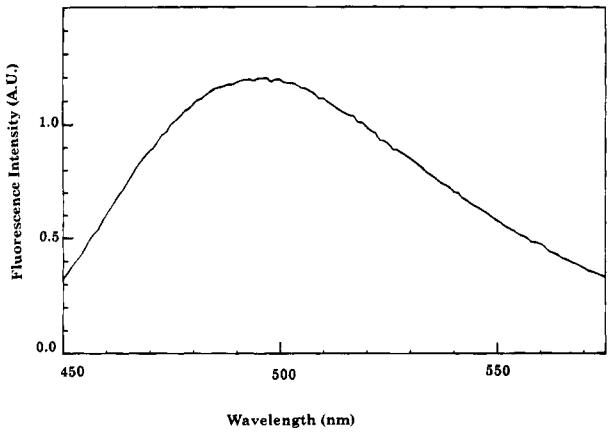

FIGURE 1: Photoincorporated bis-ANS retains its fluorescent properties. GroEL was photolabeled with bis-ANS as described in the text. The unincorporated bis-ANS was removed using a G-50 spin column. The emission spectrum was collected at a GroEL concentration of approximately 0.5 mg/mL. The sample was excited at 397 nm.

bis-ANS were removed as described above. Fluorescence spectra of labeled GroEL were then recorded at room temperature using an SLM 500C fluorometer (SLM Instruments, Urbana, IL). Excitation was at 397 nm, and the emission spectra were recorded over the range 400−600 nm. For urea denaturation experiments, aliquots of urea were added. After the samples were mixed thoroughly, the emission spectra were recorded. Sample spectra were corrected for dilution so that direct comparisons among various urea concentrations could be made.

RESULTS

Bis-ANS Can Be Photoincorporated into Proteins and the Adduct Remains Fluorescent. Brandt and co-workers showed that several fluorescent dyes could be coupled to biopolymers through photooxidation (Brandt et al., 1974). In order to see if bis-ANS could be incorporated into proteins, various proteins were irradiated with UV light in the presence of bis-ANS. Using SDS−PAGE to separate unincorporated bis-ANS, we were able to see that many proteins did become covalently labeled with bis-ANS (data not shown). Bis-ANS was able to be photoincorporated into bovine serum albumin, rhodanese, ribonuclease A, and lactate dehydrogenase in the absence of any denaturant. In the presence of urea, there was photoincorporation of bis-ANS into GroEL. Under the conditions used here, there was no incorporation of bis-ANS into GroES, malate dehydrogenase, or α-chymotrypsinogen. Since there was photoincorporation of bis-ANS into these dissimilar proteins, photolabeling with bis-ANS may provide information on hydrophobic pockets of many proteins. Figure 1 shows the fluorescence emission spectrum of bis-ANS that was photoincorporated into GroEL. GroEL was labeled with bis-ANS, and the unincorporated label was removed as described above. The labeled protein was excited at the excitation maximum, 397 nm (data not shown). There was a broad, asymmetric emission band with a maximum at 496 nm. This emission maximum is about 50 nm lower than the weak emission seen for bis-ANS free in solution. The values for the excitation and emission maxima are consistent with the values obtained from previous studies for bis-ANS that is nonspecifically bound to GroEL (Horowitz et al., 1995).

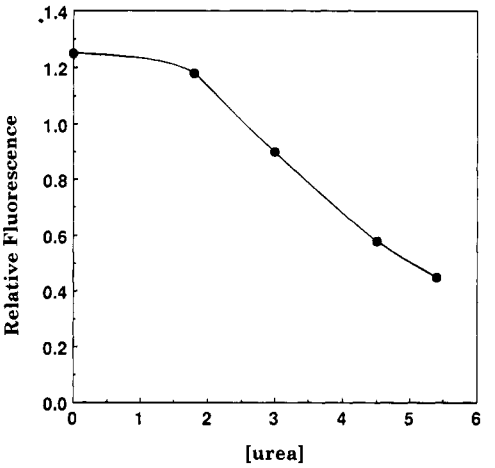

FIGURE 2: Urea denaturation of bis-ANS labeled-GroEL. Bis-ANS-labeled GroEL (~0.5 mg/mL) was treated with the indicated concentrations of urea as described in the text. The spectra were collected, and the intensities at the maxima are plotted.

The Bis-ANS−GroEL Covalent Adduct Remains Sensitive to the Polarity of Its General Environment. Figure 2 shows that the urea denaturation of bis-ANS-labeled GroEL was associated with changes in the bis-ANS spectrum. The denaturation follows a single, smooth transition with a midpoint between 3 and 3.5 M urea. As the urea concentration was increased from 1.8 M up to 5.4 M, there was a decrease in the fluorescence intensity (Figure 2) and a corresponding red shift in the fluorescence maximum (data not shown). The maximum shifts 10 nm (to about 505 nm). This is still substantially less than the maximum observed for free bis-ANS (550 nm). These effects are indicative of an increased solvent accessibility of the bis-ANS probe. This transition is consistent with the dissociation of the tetradecamer and subsequent unfolding of the monomers as shown previously (Mendoza et al., 1994). In this study, GroEL monomers are formed at 2.5 M urea. These monomers are stable and are able to undergo reassembly after the removal of the urea. An increase in the urea concentration above 2.5 M leads to the unfolding of the monomers. This unfolding appears to be irreversible.

Photoincorporation of Bis-ANS into GroEL Accompanies the Formation of Monomers. Figure 3 shows the [urea] dependence of the photoincorporation of bis-ANS into GroEL. GroEL was labeled in the presence of increasing urea concentrations as described under Experimental Procedures. After the fluorescent gel was photographed, the fluorescence intensities were quantified and plotted in Figure 3 as a function of the urea concentration. At urea concentrations below 1.5 M, there was very little incorporation of bis-ANS. Between 1.5 and 3.0 M urea, there was a significant increase in the photolabeling. At urea concentrations above 3 M, the fluorescence intensity decreases to approximately 40% of the maximum. This apparent "second maximum" is most likely insignificant and a result of the limitations of the photography. The decrease in labeling above 3 M urea is most likely due to the unfolding of the GroEL monomers with subsequent loss of organized hydrophobic surfaces (Mendoza et al., 1994).

Photolabeled GroEL Monomers Are Unable To Reassemble into Tetradecamers. GroEL monomers that have been labeled with bis-ANS in the presence of 2.5 M urea are unable to reassemble into tetradecamers. The urea

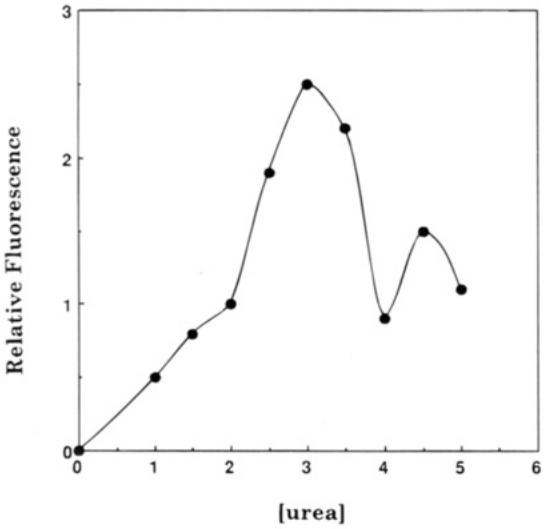

FIGURE 3: Urea dependence of bis-ANS photoincorporation into GroEL. GroEL was labeled with bis-ANS in the presence of various urea concentrations as indicated. Samples were separated on SDS gels and visualized on a UV light box. Plot of fluorescent intensities from the gel bands. The line is drawn as a guide to the eye and is not meant to imply any functional meaning. The bands on a negative from a photo of a fluorescent gel were captured using a CCD 505 video camera (CCTV Corp., New York, NY) under the control of NIH-Image software running on a MacIIxv computer. Intensities were measured using the densitometry options of the software and plotted as a function of urea concentration.

concentration used to make monomers for labeling with bis-ANS has been previously shown to induce the formation of monomers that are capable of reassembly after the removal of the urea (Mendoza et al., 1994). Figure 4a,b (top and bottom panels, respectively) shows the effect of the photoincorporation of bis-ANS on this reassembly process. Samples that have been labeled with bis-ANS remain in the monomeric form upon electrophoresis on native gels (Figure 4a, lane A). Figure 4b shows that there is a small amount of tetradecamer present in the labeled sample (lane A). However, there is no fluorescence detected in this tetradecamer in the fluorescent gel (Figure 4a, lane A). There is apparently some inhibition of tetradecamer formation due to the exposure of GroEL to UV light. A small amount of monomeric GroEL remains in the sample exposed to UV light in the absence of bis-ANS (Figure 4b, lane B). GroEL that has been treated with identical concentrations of urea and bis-ANS in the dark is fully reassembled (Figure 4b, lanes C and D).

Bis-ANS Can Be Photoincorporated into Tetradecameric GroEL in the Presence of Low Concentrations of Spermidine. Recent results have shown that low concentrations of spermidine (e.g., 5 mM) can increase the binding of bis-ANS to GroEL (Horowitz et al., 1995). At these concentrations of spermidine, the tetradecameric structure of GroEL remains intact. Figure 5a (top panel) shows the photolabeling of GroEL in the presence of 5 mM spermidine (denaturing gel). Lane A contains GroEL that was labeled in the presence of 2.5 M urea. As expected, there is significant labeling of this sample. Lane B contains GroEL that was incubated with bis-ANS in the absence of spermidine. There is no fluorescence detected in this sample. Lanes C−F show that there is photoincorporation of bis-ANS into GroEL in the presence of 1, 2, 5, and 10 mM spermidine, respectively. There is significant labeling, but there does not appear to be

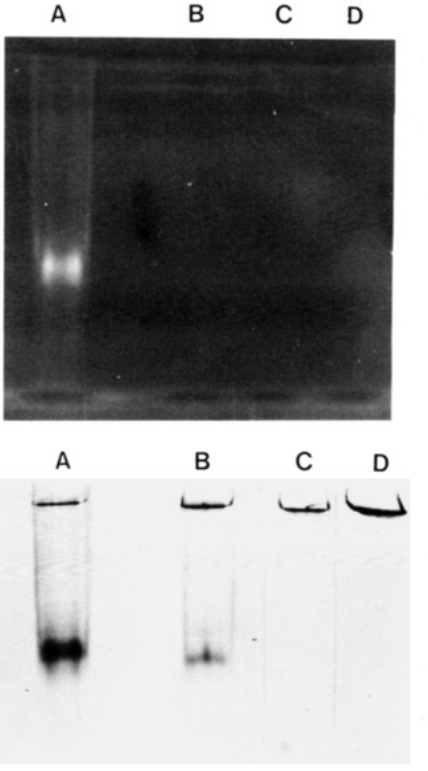

FIGURE 4: Reassembly of GroEL monomers is blocked by bis-ANS photoincorporation. Bis-ANS-labeled GroEL was allowed to undergo reassembly as described under Experimental Procedures. (a) Fluorescent native gel of reassembly products. The diffuse band in lane A corresponds to monomers. (b) Coomassie-stained gel from Figure 5a. The upper band corresponds to tetradecameric GroEL, while the lower band corresponds to monomers.

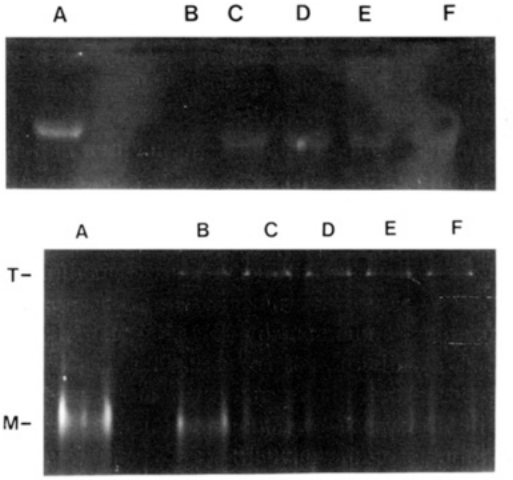

FIGURE 5: Photoincorporation of bis-ANS into tetradecameric GroEL in the presence of spermidine. Bis-ANS was photoincorporated into GroEL in the presence of 5 mM spermidine as described in the text. Aliquots of the labeling reaction were separated on (a) an SDS gel or (b) a native gel.

an increase in the degree of photolabeling above 1 mM spermidine.

Figure 5b (bottom panel) shows a nondenaturing gel of the samples from Figure 5a. The lanes correspond to the conditions used to prepare samples for the identical lanes in Figure 5a. Unlike GroEL labeled in the presence of urea, the samples labeled in the presence of spermidine remain as tetradecamers (lanes C−F). There is fluorescence in the sample that contained no spermidine. Because there was no fluorescence in the corresponding sample on the denatur-

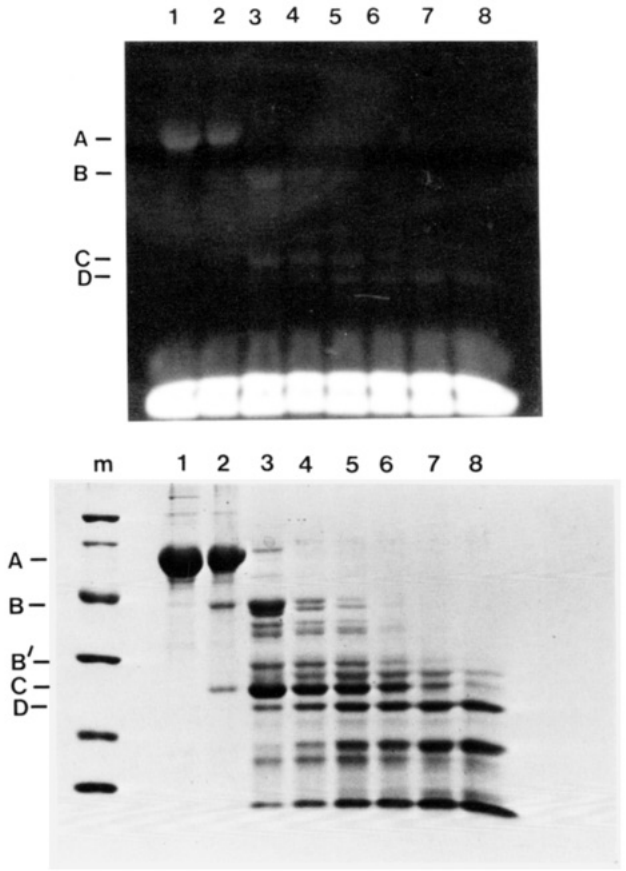

FIGURE 6: Localization of photoincorporated bis-ANS to the apical domain of GroEL. GroEL was photolabeled with bis-ANS in the presence of urea as described in the text. Labeled GroEL was treated with chymotrypsin. Aliquots were removed and separated on an SDS gel. Band A corresponds to the undigested GroEL. Fragments B−D locate the fluorescent peptides. Fragment B′ (panel b) corresponds to a previously sequenced fragment that does not contain bis-ANS. Lane 1 contains undigested GroEL. Lanes 2−8 correspond to samples incubated for 0, 15, 30, 45, 60, 90, and 120 min, respectively. (a) Fluorescent gel. The approximate molecular masses of the fluorescent bands are given in the text. (b) Coomassie-stained gel from panel a. The far left lane corresponds to the following molecular mass markers, from top to bottom: phosphorylase *b*, 97.4 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.5 kDa.

ing gel (Figure 5a, lane B), this must be bis-ANS that is noncovalently associated with GroEL. Photolabeling in the presence of low spermidine concentrations allowed for the incorporation of bis-ANS into native GroEL and, thus, will provide a useful avenue for the exploration of GroEL dynamics.

Photoincorporation of Bis-ANS Is Localized to a 45 Residue Segment of GroEL. The fluorescent peptides corresponding to bis-ANS-labeled fragments are shown in Figure 6a (top panel). There are four fluorescent bands, A, B, C, and D, corresponding to approximate molecular masses of 60, 46, 26, and 23 kDa, respectively. Peptide A corresponds to the parent GroEL. The intact protein is completely digested after 15 min (Figure 6a, lane 3). After 15 min, there appear three fragments, B, C, and D (Figure 6a, lane 3). Fragments B and C occur in approximately equal amounts, while there is very little peptide D. Fragment B was completely digested after 60 min (Figure 6a, lane 6). From 15 min to 120 min, there was a decreasing amount of peptide C and a corresponding increase in the amount of

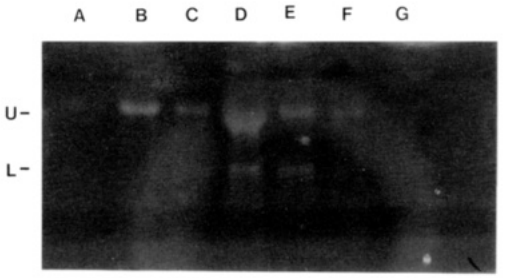

FIGURE 7: Photoincorporation of bis-ANS into both GroEL and rhodanese in a preformed binary complex. A binary complex between unfolded rhodanese and GroEL was formed prior to the photolabeling reaction. Photolabeling was performed as described under Experimental Procedures. The upper band in all lanes corresponds to GroEL while the lower band corresponds to rhodanese. Lane A contained no urea. Lane B contained no rhodanese. Lanes C and D contain a stoichiometric amount of rhodanese. Lane E contained a 5-fold excess of rhodanese. Lane F contained a stoichiometric amount of rhodanese and no urea. Lane G was a stoichiometric complex treated in the dark as a negative control.

peptide D. This suggests that peptide D is a fragment of peptide C.

Sequence analysis shows that peptide B begins at phenylalanine 204 and continues until the end of the protein, approximately 46 kDa. Peptide C corresponds to alanine 2 and continues to near isoleucine 249, approximately 26 kDa. Peptide D begins at arginine 18 and continues to near leucine 237, approximately 23 kDa.

Figure 6b (bottom panel) shows the Coomassie-stained polyacrylamide gel from Figure 6a. The fragment marked B′ has been previously sequenced (Horowitz et al., 1995) and has been shown to begin at arginine 268. Interestingly, this peptide does not appear to be labeled with bis-ANS since this fragment does not correspond to one of the fluorescent peptides in Figure 6a. It is possible, however, that some of the minor bands in Figure 6b are labeled with bis-ANS but are not detected by these methods. Using this information, it is possible to localize the bis-ANS incorporation to a fragment of GroEL corresponding approximately to phenylalanine 204 through at least isoleucine 249. This region is common to all fluorescent peptides and is absent in all other peptides sequenced to date (Horowitz et al., 1995).

The bis-ANS-labeled peptide (F204−I249) corresponds to a region of the apical domain in the three-dimensional structure (Braig et al., 1994). This hydrophobic region is comprised of a loop and strand of β-sheet. Mutational analysis of GroEL implicates this region in polypeptide binding (Fenton et al., 1994).

Bis-ANS Labeling of GroEL in the Presence of Unfolded Rhodanese Shows That Labeling Occurs in both GroEL and Rhodanese. It has previously been suggested that a similar hydrophobic probe, 1-anilino-8-naphthalenesulfonate, is bound to the molten globule state of rhodanese in a complex with GroEL (Martin et al., 1991). Figure 7 indicates that both unfolded rhodanese and GroEL contain sites in the complex that are capable of binding bis-ANS. Complexes between GroEL and unfolded rhodanese in the presence of 2 M urea are shown in lanes C−E. It has been previously shown that a complex between unfolded rhodanese and GroEL is formed at this urea concentration (Mendoza et al., 1994). These complexes were formed prior to the initiation of the labeling reaction to ensure that a complex was being formed. In a stoichiometric complex between GroEL and unfolded

rhodanese, there is significant labeling of both proteins (Figure 7, lanes C and D). Lanes C and D contain 1:1 complexes, with lane D having a 5-fold increase in the amount of protein over lane C. Interestingly, a 5-fold excess of unfolded rhodanese (Figure 7, lane F) was unable to block labeling of GroEL, suggesting that the site of labeling is not completely protected in the binary complex.

DISCUSSION

Through the use of photoincorporation, we have used the nonspecific probe bis-ANS to gain specific information about hydrophobic surfaces on the chaperonin GroEL. The use of hydrophobic probes to gain information about protein structural transitions has become increasingly popular. However, due to the noncovalent nature of the hydrophobic interaction, specific structural information from these probes has been limited at best. The present results show that bis-ANS can be incorporated into various proteins under conditions that may not require structural perturbation of the target protein and, therefore, specific information can be gained about hydrophobic surfaces that may be important in protein folding or protein conformational changes. Importantly, the incorporated probe retains its fluorescent properties and remains sensitive to the polarity of its environment.

Much speculation has led to the idea that hydrophobic surfaces are important in the recognition of unfolded polypeptides by the chaperonin GroEL. This model of protein recognition is attractive for several reasons. The low specificity of the interaction would account for the diverse range of unfolded proteins that can be recognized by GroEL (Viitanen et al., 1992). In addition, the so-called "molten globule" state, which is suggested to be an intermediate in the folding of many proteins, and a substrate for GroEL binding (Martin et al., 1991), displays an increased solvent accessibility of hydrophobic regions. One drawback to this model is that the hydrophobic surfaces on GroEL might be expected to cause self-aggregation in the absence of unfolded protein. However, recent reports suggest that the presentation of hydrophobic surfaces can be modulated by alterations in the quaternary structure (Mendoza et al., 1994), or by ionic triggering (Horowitz et al., 1995).

Recent studies of GroEL structure have made use of the binding of the hydrophobic probe bis-ANS (Mendoza et al., 1991a; Horowitz et al., 1995). However, in these reports, no information about the location of the regions of increased hydrophobic exposure could be gained. We have shown here that the region on GroEL that can be covalently labeled with bis-ANS under these conditions corresponds to the apical domain. The covalently attached probe is localized to a peptide fragment corresponding to residues 203−249. A Kyte−Doolittle (Kyte & Doolittle, 1982) analysis of GroEL using a 50 residue sliding window shows this peptide to lie within the most hydrophobic portion of the protein (Horowitz et al., 1995). Interestingly, bis-ANS is incorporated into the same peptide under conditions where GroEL was monomeric (2.5 M urea) or tetradecameric (5 mM spermidine). This result suggests that there is a distinct hydrophobic surface whose solvent exposure can be modulated by alterations in the quaternary structure or through an "ionic triggering". Mutations throughout this entire region have been reported to affect polypeptide binding by the chaperonin (Fenton et al., 1994). In light of these results, it is interesting that some bis-ANS could still be incorporated even with bound rhodanese which might expected to cover the hydrophobic surfaces. Previous results have shown that a GroEL tetradecamer might bind rhodanese only on one end of the cylindrical tetradecamer (Mendoza et al., 1991b). It is possible that labeling in the binary GroEL−rhodanese complex occurs within the apical domain(s) at the end of the GroEL cylinder opposite the site of bound rhodanese.

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REFERENCES

Arbelaez, L. F., Jensen, P. E., Shanbhag, V. T., & Stigbrand, T. (1993) *Eur. J. Biochem. 218*, 651−656.
Azem, A., Kessel, M., & Goloubinoff, P. (1994) *Science 265*, 653−656.
Bhattacharyya, T., Bhattacharyya, A., & Roy, S. (1991) *Eur. J. Biochem. 200*, 739−745.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Braig, K., Simon, M., Furuya, F., Hainfeld, J. F., & Horwich, A. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3978−3982.
Braig, K., Otwinowski, Z., Hedge, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., & Sigler, P. B. (1994) *Nature 371*, 578−586.
Brandt, J., Fredriksson, M., & Andersson, L.-O. (1974) *Biochemistry 13*, 4758−4764.
Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R. W., & Georgopoulos, C. (1986) *J. Biol. Chem. 261*, 12414−12419.
Choi, J. K., Kim, I. S., Kwon, T. I., Parker, W., & Song, P. S. (1990) *Biochemistry 29*, 6883−6891.
Cocco, M. J., & Lecomte, J. T. (1994) *Protein Sci. 3*, 267−281.
Fenton, W. A., Kashi, Y., Furtak, K., & Horwich, A. L. (1994) *Nature 371*, 614−619.
Frydman, J., Nimmesgern, E., Ohtsuka, K., & Hartl, F.-U. (1994) *Nature 370*, 111−117.
Georgopoulos, C. P., & Ang, D. (1990) *Semin. Cell Biol. 1*, 19−25.
Gething, M.-J., & Sambrook, J. (1992) *Nature 355*, 33−45.
Goto, Y., & Fink, A. L. (1989) *Biochemistry 28*, 945−952.
Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., Dennis, D. T., Georgopoulos, C. P., Hendrix, R. W., & Ellis, R. J. (1988) *Nature 333*, 330−334.
Hendrick, J. P., & Hartl, F.-U. (1993) *Annu. Rev. Biochem. 62*, 349−384.
Hendrix, R. W. (1979) *J. Mol. Biol. 129*, 375−392.
Horowitz, P. M., & Criscimagna, N. L. (1988) *J. Biol. Chem 263*, 10278−10283.
Horowitz, P. M., Hua, S., & Gibbons, D. L. (1995) *J. Biol. Chem. 270*, 1535−1542.
Hua, Q. X., Ladbury, J. E., & Weiss, M. A. (1993) *Biochemistry 32*, 1433−1442.
Itzhaki, L. S., Evans, P. A., Dobson, C. M., & Radford, S. E. (1994) *Biochemistry 33*, 5212−5220.
Korte, T., & Herrmann, A. (1994) *Eur. Biophys. J. 23*, 105−113.
Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105−132.
Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., & Hartl, F.-U. (1991) *Nature 352*, 36−42.
Martin, J., Mayhew, M., Langer, T., & Hartl, F.-U. (1993) *Nature 366*, 228−233.
Mendoza, J. A., Rogers, E., Lorimer, G. H., & Horowitz, P. M. (1991a) *J. Biol. Chem. 266*, 13044−13049.
Mendoza, J. A., Lorimer, G. H., & Horowitz, P. H. (1991b) *J. Biol. Chem. 266*, 16973−16976.

Mendoza, J. A., Demeler, B., & Horowitz, P. M. (1994) *J. Biol. Chem. 269*, 2447−2451.
Merrill, G. A., Butler, M. C., & Horowitz, P. M. (1993) *J. Biol. Chem. 268*, 15611−15620.
Miller, D. M., Kurzban, G. P., Mendoza, J. A., Chirgwin, J. M., Hardies, S. C., & Horowitz, P. M. (1992) *Biochim. Biophys. Acta 1121*, 286−292.
Mizobata, T., & Kawata, Y. (1994) *Biochim. Biophys. Acta 1209*, 83−88.
Neahoff, V., Cheong-Kim, K., & Altland, K. (1986) *Electrophoresis 7*, 56−57.
Nishihira, J., Ishibashi, T., Sakai, M., Tsuda, S., & Hikichi, K. (1993) *Arch. Biochem. Biophys. 302*, 128−133.
Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E., & Razgulyaev, O. I. (1990) *FEBS Lett. 262*, 20−24.
Rosen, C.-G., & Weber, G. (1969) *Biochemistry 8*, 3915−3920.
Schmidt, M., Rutkat, K., Rachel, R., Pfeifer, G., Jaenicke, R., Viitanen, P., Lorimer, G., & Buchner, J. (1994) *Science 265*, 656−659.
Todd, M. J., Viitanen, P. V., & Lorimer, G. H. (1994) *Science 265*, 659−666.
Viitanen, P. V., Gatenby, A. A., & Lorimer, G. H. (1992) *Protein Sci. 1*, 363−369.
Weissman, J. S., Kashi, Y., Fenton, W. A., & Horwich, A. L. (1994) *Cell 78*, 693−702.
Zahn, R., & Plückthun, A. (1994) *J. Mol. Biol. 242*, 165−174.
Zahn, R., Axmann, S. E., Rücknagel, K.-F., Jaeger, E., Laminet, A. A., & Plückthun, A. (1994) *J. Mol. Biol. 242*, 150−164.