# Protein Conformational Changes Induced by 1,1′-Bis(4-anilino-5-naphthalenesulfonic acid): Preferential Binding to the Molten Globule of DnaK†

Li Shi, Daniel R. Palleros, and Anthony L. Fink*

*Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064*

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ABSTRACT: 1,1′-Bis(4-anilino-5-naphthalenesulfonic acid) (bis-ANS), a hydrophobic fluorescent molecular probe which has been shown to bind to compact intermediate states of proteins (molten globules) and also to many nucleotide binding sites, induces a conformational change in DnaK by preferentially binding to its partially folded intermediate state (I) and thus shifting the equilibrium from favoring the native state (N) to favoring the I state. The conformational change was detected by CD, fluorescence emission, size exclusion chromatography, and small-angle X-ray scattering. The presence of bis-ANS significantly decreases the midpoint, $T_m$, of the initial transition (N → I) in the thermal unfolding of DnaK, resulting in the apparent destabilization of the native state of DnaK. There is a linear correlation between the apparent free energy (reflected by $T_m$) of this transition and the concentration of bis-ANS. Bis-ANS does not affect the midpoint of the transition for DnaK from the intermediate to the unfolded state (U). An additional small transition from I to I*, a more expanded intermediate state, was observed, suggesting that the thermal denaturation of DnaK proceeds via a four-state (N → I → I* → U) unfolding process. The addition of nucleotides, ADP or ATP, to the DnaK·bis-ANS complex causes a decrease in bis-ANS fluorescence emission due to the release of bound bis-ANS from the intermediate state of DnaK. This is due to preferential binding of the nucleotide to the native state of DnaK, resulting in a shift in the equilibrium from the intermediate toward the native state rather than the direct displacement of bis-ANS bound in the nucleotide binding site. Denaturation of DnaK induced by bis-ANS can be minimized by working at a temperature much lower than the $T_m$ of the protein, at low dye concentration, and in the presence of nucleotide. Under these conditions, bis-ANS binds to the native state of DnaK.

1,1′-Bis(4-anilino-5-naphthalenesulfonic acid) (bis-ANS),[1] a dimeric form of 8-anilino-1-naphthalenesulfonic acid (ANS) (Farris et al., 1978; Rosen & Weber, 1969), and ANS have been frequently used to probe hydrophobic sites in proteins (Mendoza et al., 1991; Musci et al., 1985; Musci & Berliner, 1985; Choi et al., 1990) and to study protein–substrate interactions (Horowitz & Criscimagna, 1985; Brand, 1970; Aoe et al., 1970; Takashi et al., 1977; Secnik et al., 1992) and protein conformational changes (Goto et al., 1979; Rodionova et al., 1989; Ptitsyn et al., 1990; Semisotnov et al., 1991; Bhattacharyya et al., 1991; Teschke et al., 1993). These dyes are minimally fluorescent in polar environments, such as aqueous solutions, but their fluorescence emission is dramatically increased in nonpolar environments. An increase in quantum yield and a blue shift in the emission maximum wavelength ($\lambda_{max}$) are generally assumed to reflect binding to hydrophobic sites in proteins.

It has frequently been suggested that ANS and bis-ANS bind to nucleotide binding sites on proteins (Takashi et al., 1977; Yoo et al., 1990; Griess et al., 1991) and are displaced on the addition of nucleotides (Rosen & Weber, 1969; Takashi et al., 1977; Secnik et al., 1990, 1992; Lee et al., 1991; Horowitz et al., 1984; Bohnert et al., 1982; Anderson, 1971). This assumption is based on the observation that on addition of the nucleotide the fluorescence enhancement of the dye is reduced. It has been reported that bis-ANS inhibits *Escherichia coli* RNA polymerase activity, and this inhibition can be *partially* prevented by preincubation of the enzyme with DNA and/or nucleoside triphosphates (Wu & Wu, 1978). However, the details of these processes are not clear.

We are interested in using a dye-displacement assay to measure substrate binding and dissociation in the 70-kDa heat shock (hsp70) family of molecular chaperones. Preliminary experiments suggested that the interaction of bis-ANS with DnaK (the hsp70 from *E. coli*) was complex and involved dye-induced conformational changes. We have previously shown that the thermal and guanidine hydrochloride-induced denaturations of DnaK proceed via a three-state (native, intermediate, and unfolded) unfolding process, in which the intermediate possesses some of the characteristics of a molten globule (Palleros et al., 1992, 1993). Such compact intermediates are known to preferentially bind ANS and bis-ANS, due to increased hydrophobic surface area compared to the native protein (Semisotnov et al., 1991; Goto & Fink, 1989). Therefore, under conditions in which both native (N) and intermediate (I) states of DnaK coexist, a preferential binding of bis-ANS to the I state would be expected.

In the present work, the conformation of DnaK in the presence of bis-ANS was investigated by fluorescence, far-UV circular dichroism, small-angle X-ray scattering (SAXS), and size-exclusion chromatography (SEC) HPLC. The results provide strong evidence that bis-ANS preferentially binds to

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* To whom correspondence should be addressed.
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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; bis-ANS, 1,1′-bis(4-anilino-5-naphthalenesulfonic acid), dipotassium salt; SAXS, small-angle X-ray scattering; SEC, size exclusion chromatography; CD, circular dichroism; N, native state; I, unfolding intermediate state; U, unfolded state; Gdn·HCl, guanidine hydrochloride; BSA, bovine serum albumin; DTT, dithiothreitol; ETE, energy-transfer efficiency; D, dye (bis-ANS).

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the I state and induces a conformational change in DnaK by shifting the equilibrium between N and I states and that nucleotides reduce the effect of bis-ANS by preferentially binding to the native protein. The possible generality of the effect of bis-ANS on protein conformation is discussed.

## EXPERIMENTAL PROCEDURES

*Materials.* The bis-ANS dipotassium salt was from Molecular Probes, Junction City, OR. The dye was used without further purification. The concentration of bis-ANS was determined by absorbance at 394 nm ($\epsilon_{394}$ = 16 000 $cm^{-1}$ $M^{-1}$ in water) or at 385 nm ($\epsilon_{385}$ = 16 790 $cm^{-1}$ $M^{-1}$ in water) (Farris et al., 1978). DnaK was isolated and purified by a modification of procedures previously described (Palleros et al., 1993). The concentration of DnaK was determined by absorbance using $\epsilon$ = 27 000 $cm^{-1}$ $M^{-1}$ at 280 nm (Palleros et al., 1993). Bovine brain hsp73 was isolated and purified as previously reported (Welch & Feramisco, 1985). The concentration of hsp73 was determined by absorbance using $\epsilon$ = 47 800 $cm^{-1}$ $M^{-1}$ at 280 nm (Palleros et al., 1991). ATP (disodium salt) was from Pharmacia LKB Biotechnology Inc. ADP (monosodium salt) was from Calbiochem. All nucleotide stock solutions were made in the range of 10 mM with 10 mM $MgCl_2$ and 20 mM potassium chloride in 20 mM Tris·HCl, pH = 7.5. Nucleotide concentrations were determined by absorbance using $\epsilon$ = 15 400 $cm^{-1}$ $M^{-1}$ at 259 nm (Fasman, 1983). Guanidine hydrochloride was from ICN Biochemicals. The concentration of guanidine hydrochloride stock solution was determined by the refraction index of the solution as described elsewhere (Pace et al., 1989). Sample stock solutions were made either in Tris buffer (20 mM Tris·HCl, pH = 7.2) or in phosphate buffer (0.02 M $Na_2HPO_4$, 0.20 M KCl, pH = 6.5).

*Fluorescence Measurements.* A Perkin-Elmer Model MPF-4 spectrophotometer equipped with a programmable temperature controller was used. The slit widths used for the excitation and emission were 4 and 10 nm, respectively. A 0.4- × 1.0-cm quartz cuvette was used, with the longer path parallel to the excitation light direction. Fluorescence intensity data were collected directly on an IBM computer connected to the spectrophotometer and analyzed by KaleidaGraph (Synergy Software).

Fluorescence emission intensities (at 485 nm) obtained from titrations with bis-ANS and DnaK were used for Scatchard and Hill plot fitting (Cantor & Schimmel, 1980). Based on the plot fitting, the number of molecules ($n$) of bis-ANS bound to DnaK and the dissociation constants ($K_d$) for both DnaK-(N)·bis-ANS and DnaK(I)·bis-ANS complexes were estimated as described below (Semisotnov et al., 1991; Bhattacharyya et al., 1991; Lee et al., 1991; Saha et al., 1992). For solutions of bis-ANS with concentration higher than 60 $\mu$M, the fluorescence intensities were corrected for the inner filter effect (Horowitz & Criscimagna, 1985; Calhun et al., 1983; Lakowicz, 1983).

The kinetic rate constants of DnaK conformational changes were obtained by fitting the data of time-dependent fluorescence intensity changes with the first-order kinetics equation. The experiments involved placing the protein with the majority of the total sample volume into the fluorescence cuvette, equilibrating the sample, and then adding a small aliquot of bis-ANS rapidly, followed by a quick stirring. The total dead time was in the range of 3–8 s.

The values of the midpoint ($T_m$) of the thermal unfolding transition were obtained by converting the fluorescence or CD data to free energy, $\Delta G$, versus temperature and determining $T_m$ from the temperature where $\Delta G$ = 0. The free energies ($\Delta G$) were obtained as described elsewhere (Pace et al., 1989).

Energy-transfer efficiency (ETE) values were obtained on the basis of the definition described by Stryer (1978): ETE = $1 - (Q_T/Q_0)$, where $Q_T$ and $Q_0$ are the quantum yields (or relative fluorescence intensities) of the energy donor in the presence and absence of the energy acceptor, respectively. The values of $Q_T$ and $Q_0$ were obtained by integrating the areas under the fluorescence spectra from 310 to 400 nm for the samples with and without bis-ANS, respectively.

*Dye Binding Studies.* The binding of bis-ANS to DnaK was monitored by the dye fluorescence intensity, shifts in $\lambda_{max}$ for bound dye, and changes in the efficiency of energy transfer. In all cases the protein concentration was 1.0 $\mu$M. Measurements were made at 20 °C and 37 °C. The fluorescence intensity data for the native state were measured at 20 °C within 3 s after mixing the dye and DnaK. For data at 37 °C or in the presence of Gdn·HCl, measurements were made after equilibration of the sample for 2 h. Identical values were observed in both cases at saturation. The values of fluorescence intensity for bis-ANS fully saturated with DnaK ($F_{sat}$) were determined as follows. Bis-ANS (1.0 $\mu$M) was titrated with increasing concentrations of DnaK, and the reciprocal of the intensity was plotted against the reciprocal of the DnaK concentration. The intercept at infinite protein concentration gives the fluorescence intensity at saturation (Bhattacharyya et al., 1991; Semisotnov et al., 1991; Lee et al., 1991). Scatchard plots were then constructed using $\nu = F/F_{sat}$ = bis-ANS bound per DnaK, where $F$ is the fluorescence intensity for a given concentration of bis-ANS and $F_{sat}$ is the value at saturation. The binding data (Figure 2, below) were fit to eq 1 for the native state, eq 2 for the intermediate state in 0.8 M Gdn·HCl, and eq 3 for the intermediate state at 37 °C:

$$\nu = n[ND]/([N] + [ND]) = n[D]/(K_{ND} + [D]) \quad (1)$$

$$\nu = (n_1[ID] + n_2[ID_2] + n_3[ID_3])/([I] + [ID] + [ID_2] + [ID_3])$$

$$= [D](n_1/K_1 + n_2[D]/K_2 + n_3[D]^2/K_3)/(1 + [D]/K_1 + [D]^2/K_2 + [D]^3/K_3) \quad (2)$$

$$\nu = ([ND] + [ID] + 2[ID_2] + 3[ID_3])/([N] + [I] + [ND] + [ID] + [ID_2] + [ID_3])$$

$$= [D](K_{NI}/K_{ND} + 1/K_1 + 2[D]/K_2 + 3[D]^2/K_3)/(K_{NI} + 1 + [D]K_{NI}/K_{ND} + [D]/K_1 + [D]^2/K_2 + [D]^3/K_3) \quad (3)$$

where

$$K_{NI} = [N]/[I],\ K_{ND} = [N][D]/[ND],\ K_{ID} = [I][D]/[ID],\ K_{ID2} = [ID][D]/[ID_2],\ K_{ID3} = [ID_2][D]/[ID_3],\ K_1 = [I][D]/[ID] = K_{1D},\ K_2 = [I][D]^2/[ID_2] = K_1K_{ID2},\ \text{and}\ K_3 = [I][D]^3/[ID_3] = K_2K_{ID3}$$

*Circular Dichroism and Small-Angle X-ray Scattering Measurements.* CD measurements were carried out on an Aviv Associates instrument (Lakewood, NJ), Model 60DS, equipped with a programmable temperature controller. A 250-$\mu$L cuvette with a 1-mm path was used. All samples were equilibrated at the desired temperature until no further time-dependent change in ellipticity was observed. Mean

Table 1: Parameters for Protein and Bis-ANS or ANS Complex Formation

| protein (state/conditions) | dye | max dye/protein ratio[a] ($\mu$M/$\mu$M) | $n^b$ | $K_d$ ($\mu$M) | $-\Delta FL^c$ | ref |
|---|---|---|---|---|---|---|
| DnaK (native) | bis-ANS | 189 | 1[d] | 7.0[d] | | this study |
| (compact intermediate) | | 200 | 3 | 2.0, 5.1, 39 | ~60% | this study |
| P22 coat protein (native) | bis-ANS | 50 | ~1.2 | ~7.2 | | Teschke & King, 1993 |
| (folding intermediate) | | | ~10.8 | ~2.1 | | |
| tRNA synthetase | bis-ANS | 120 | 0.9 | 10–11.5 | | Bhattacharyya et al., 1991 |
| λ-repressor | bis-ANS | 50 | 1.35–1.7 | 38–89 | | Saha et al., 1992 |
| RNA polymerase (weak binding) | bis-ANS | 5000 (100/0.02) | 34–36 | 20 | <40% | Wu & Wu, 1978 |
| (strong binding) | | | 16–18 | 2 | | |
| tubulin (strong binding) | bis-ANS | 50 (100/2) | 1 | 2 | | Prasad et al., 1986 |
| (weak binding) | | | 6 | 19 | | |
| (after 24 h) | | | 7 | 17 | | |
| nucleocapsid | bis-ANS | 3 | 3 | 11.6 | | Secnik et al., 1992 |
| | | 10 | 2–10 | <1 | ~90% | Secnik et al., 1990 |
| phytochrome | bis-ANS | 136.8 (520/3.8) | | | | Choi et al., 1990 |
| adrenal chromogranin A | bis-ANS | 15 (45/3) | | | ~50% | Yoo et al., 1990 |
| α-thrombin | bis-ANS | 15 000 (15/0.001) | | | | Musci & Berliner, 1985 |
| GroEL | bis-ANS | 5.5 (10/1.83) | 2.8 | 1.2 | ~45% | Mendoza et al., 1991 |
| bacteriophageT4 (burst) | bis-ANS | 100000(100.0.001) | 186 | 1.4 | | Griess et al., 1990 |
| (intact 0S41) | | 1900 (1.9/0.001) | 112 | 4.7 | ~94% | |
| protein kinase (RI) | bis-ANS | 10 | 5.4 | 1–10 | ~70% | Bohnert et al., 1982 |
| (RII) | | | 3.7 or 8.9 | 1–10 | | |
| yeast 40S ribosome | bis-ANS | ~148 | | | ~10% | Lee et al., 1991 |
| myosin S-1 | bis-ANS | ~50 | | | ~50% | Takashi et al., 1977 |
| α-lactalbumin (apo) | bis-ANS | ~1 | 1 | 20 | | Haezebrouck et al., 1992 |
| (apo) | bis-ANS | 9221 (450/0.049) | 1 | 4.7 | | Fitzgerald et al., 1989 |
| (apo) | bis-ANS | ~1.1 | 1 | 6.2 | | Musci et al., 1985 |
| (native) | bis-ANS | | 1 | 140 | | Haezebrouck et al., 1992 |
| (apo) | ANS | 9221 (450/0.049) | 1 | 64.6 | | Musci et al., 1985 |
| (apo) | ANS | | 1 | 78–86 | | Fitzgerald et al., 1989 |
| (native) | ANS | | 1 | 85 | | Rawitch, 1974 |
| (native) | ANS | | 1 | 200 | | Versee & Barel, 1976 |
| (native) | ANS | | | 920–980 | | Mulqueen et al., 1982 |
| PGK (H388Q strong binding) | ANS | 12.8 (90/7) | | ~2000 | ~30% | Dryden et al., 1992 |
| (wild-type weak binding) | | 12.5 (3000/240) | | | | Wiksell et al., 1982 |
| apo-cytochrome C | ANS | 250 | | | | Semisotonv et al., 1991 |
| β-lactamase | ANS | 250 | | | | Semisotonv et al., 1991 |
| (compact intermediate) | ANS | 100 | ≥2 | 25 | | Goto & Fink, 1989 |
| carbonic anhydrase | ANS | 20 (100/5) | 2 | 23.8–76.9 | | Ptitsyn et al., 1990 |
| transaldolase | ANS | 100 (2000/20) | 2.2 | 22 | | Brand, 1970 |
| fructose diphosphatase | ANS | 100 (200/2) | | 151–193 | ~20% | Aoe t al., 1970 |
| rhodanese (E) | ANS | 242 (182/0.75) | ~3 | ~158–200 | | Horowitz et al., 1985 |
| (ES) | | | ~1 | 1670 | | Horowitz et al., 1985 |

[a] The maximum ratios of dye concentration/protein concentration used in the references. [b] The number of molecules of dye bound per molecule of protein. [c] Dye fluorescence enhancement decrease induced by nucleotide. [d] At 20 °C. [e] From fluorescence $\lambda_{max}$ and intensity measurements at 37 °C.

residue weight ellipticities, $[\theta]_{MRW}$, were calculated as described elsewhere (Schmid, 1989). SAXS experiments were performed using the solution scattering station installed at BL10C at the Photon Factory, Tsukuba, Japan. The Kratky plots were obtained using the reported method of analysis (Kataoka et al., 1993; Glatter & Kratky, 1982).

*SEC-HPLC Measurements.* HPLC experiments were performed with a Beckman instrument using a BioSep-SEC 3000 silica column (600 × 7.8 mm, Phenomenex) at 22 °C and 20 mM phosphate buffer, pH = 6.5, with 200 mM NaCl as mobile phase with a flow rate of 1 mL/min.

## RESULTS

The existence of a compact (molten globule) intermediate in the denaturation of DnaK has been shown previously (Palleros et al., 1992, 1993). The various interactions of bis-ANS with DnaK described below are most simply interpreted in the framework of preferential binding of bis-ANS to the compact intermediate or molten globule of DnaK.

*Enhancement of bis-ANS Fluorescence Induced by DnaK.* The effects of different conformational states of DnaK on the fluorescence of the dye are shown in Figure 1. Almost no fluorescence emission of bis-ANS was observed in aqueous solution (trace 6), and the presence of unfolded (U) DnaK (2.2 M Gdn·HCl and 93 °C, traces 4 and 5, respectively) resulted in a very small increase in bis-ANS fluorescence. A modest increase in bis-ANS fluorescence emission and a blue shift are observed in the presence of native (N) DnaK (5 °C, trace 3), indicating some binding of bis-ANS to a hydrophobic site(s) on DnaK. In contrast, the presence of the compact intermediate (I) of DnaK (0.8 M Gdn·HCl and 49 °C, traces 1 and 2, respectively) induces a very substantial enhancement and blue shift in bis-ANS fluorescence emission, indicating strong binding of bis-ANS.

The number of molecules ($n$) of bis-ANS bound to DnaK and the dissociation constants ($K_d$) for both DnaK(N)·bis-ANS and DnaK(I)·bis-ANS complexes are listed in Table 1. The former were obtained from Scatchard plots (Figure 2A, inset). Dye binding to the native state was determined from experiments at 20 °C, which indicate the binding of a single dye molecule per protein. On the other hand, binding of bis-ANS to DnaK in the intermediate state (either at 37 °C or in the presence of 0.8 M Gdn·HCl) showed a stoichiometry of three dye molecules binding to the intermediate state at saturation. The dissociation constants for dye binding to the native and intermediate conformations were obtained from fitting the data to eqs 1–3, as appropriate (see Experimental Procedures). The dissociation constant was calculated to be

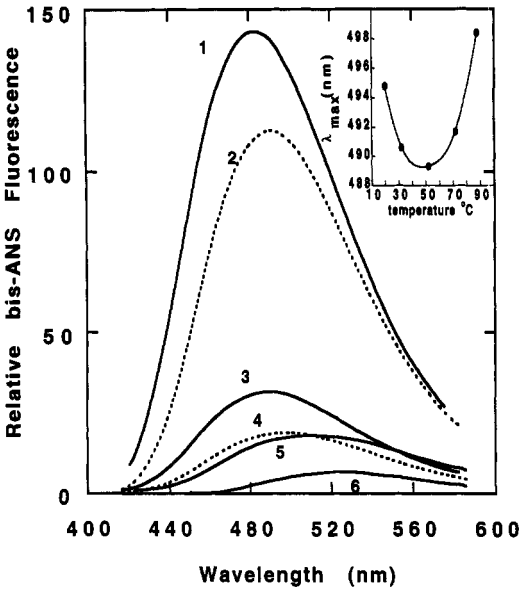



FIGURE 1: Enhancement of bis-ANS fluorescence by DnaK. (1) Compact intermediate of Dnak (49 °C). (2) Compact intermediate of DnaK (0.8 M Gdn·HCl, at 20 °C). (3) Native DnaK (5 °C). (4) Unfolded DnaK (2.2 M Gdn·HCl, at 20 °C). (5) Unfolded DnaK (93 °C). (6) Bis-ANS alone (no DnaK, 5 °C). For comparison, 1, 2, and 5 were extrapolated to the intensities at 5 °C. The concentrations of bis-ANS and DnaK were 18.3 and 1 $\mu$M, respectively, for all spectra. All the spectral measurements were carried out in Tris buffer (with 1 mM DTT). Spectrum 3 of the complex with native DnaK was scanned within 20 s after bis-ANS was added. All other samples were incubated for 2 h before the scan. Inset: changes in the fluorescence emission, $\lambda_{max}$, of the DnaK·bis-ANS complex with the thermal-induced unfolding of DnaK. DnaK (1 $\mu$M) was incubated with 22 $\mu$M bis-ANS at 20, 32, 52, 72, and 87 °C, respectively, for 20 min before the values of $\lambda_{max}$ were determined.

7.0 ± 1.2 $\mu$M for the native conformation at 20 °C. For the intermediate conformation, similar results were obtained for binding both in 0.8 M Gdn·HCl ($K_{ID}$ = 2.4 ± 1.2 $\mu$M; $K_{ID2}$ = 4.7 ● 0.9 $\mu$M; $K_{ID3}$ = 36 ± 9 $\mu$M) and at 37 °C ($K_{ID}$ = 2.0 ± 1.3 $\mu$M; $K_{ID2}$ = 5.1 ± 1.3 $\mu$M; $K_{ID3}$ = 39 ± 9 $\mu$M). The fits are shown in Figure 2A.

For comparison, the parameters of ANS and bis-ANS binding to some other proteins are also listed in Table 1. The analysis of the dye binding experiments also allowed comparison of the equilibrium constants for the conformational transition between N and I at 37 °C in the absence ($K_{NI}$ = 10.6 ± 0.05) and presence of bis-ANS. Because of the dye binding, the ratio of DnaK in the intermediate conformation (free and bound) to the native conformation ($K_{app}$) varies with the dye concentration, as shown in Figure 2B. The solid line in this plot is the theoretical fit using the above-determined dissociation constants. As noted, DnaK undergoes a transition to the I (molten globule-like) state in the 35–45 °C range (Palleros et al., 1992, 1993). In Figure 3, we show the effect of heating DnaK in the presence of bis-ANS to 53 °C, at which temperature the protein will be in its I state, and then cooling the solution to 20 °C. The decrease in temperature resulted in a linear increase in bis-ANS fluorescence emission, consistent with the expected effect of temperature on a stable complex of the DnaK intermediate with bis-ANS (i.e., very slow return to the native state).

The fluorescence emission, $\lambda_{max}$, for free bis-ANS in aqueous solution was 535 nm (Figure 1); this value does not change with bis-ANS concentration and temperature. The $\lambda_{max}$ of the complex between the DnaK and bis-ANS was observed

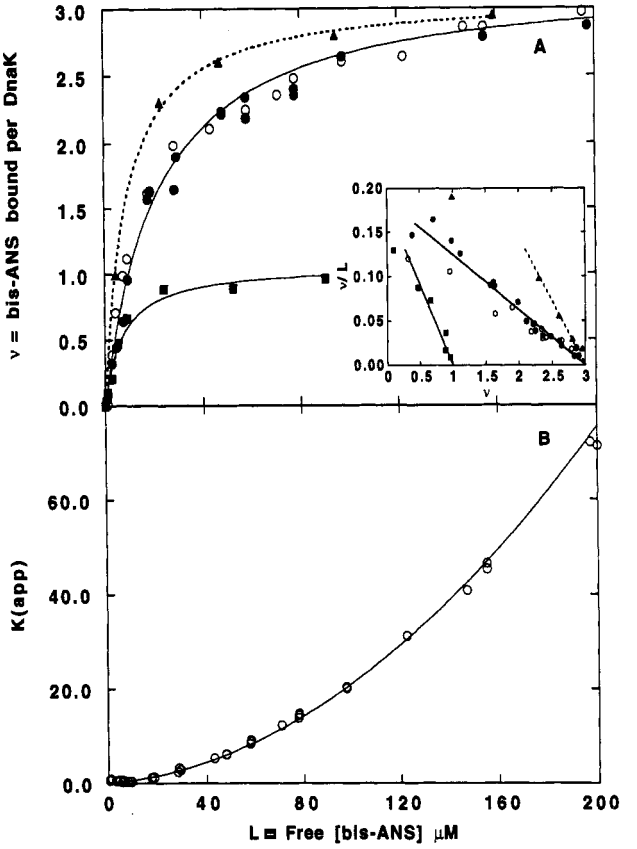



FIGURE 2: Binding of bis-ANS to the native and intermediate conformations of DnaK. (A) Bound bis-ANS as a function of free dye concentration. Inset: corresponding Scatchard plots. The filled squares are for the native state at 20 °C, the circles are for the intermediate at 37 °C (open circles are intensity data, filled circles are $\lambda_{max}$ data (corresponding to changes in $\lambda_{max}$ from 480 nm at low [bis-ANS] to 505 nm at saturation), and triangles are for the intermediate at 20 °C in 0.8 M Gdn·HCl. The solid lines are the theoretical curves drawn according to eqs 1, 2, and 3 using the values of the dissociation constants given in the Results section. (B) The ratio of the intermediate state (free and bound) to the native state (free and bound), $K_{app}$, as a function of bis-ANS concentration at 37 °C. $K_{app}$ is defined as $([I] + [ID] + [ID_2] + [ID_3])/([ND] + [N]) = (1 + [D]/K_1 + [D]^2/K_2 + [D^3]/K_3)/(K_{NI} + [D]K_{NI}/K_{ND})$.

to vary with the concentration of the dye (data not shown). A maximum blue shift to 480 nm was obtained with dye/protein ratio ≤ 0.01, and this increased to an upper limit of 505 nm at a dye/protein ratio ≳ 150. Similar fluorescence emission $\lambda_{max}$ increases with dye concentration have been reported when the dye was mixed with *E. coli* glutaminyl-tRNA synthetase (Bhattacharyya et al., 1991) and *lac* repressor (Lawson & York, 1987). At fixed bis-ANS concentration (22 $\mu$M), the $\lambda_{max}$ changes with the thermal unfolding of DnaK, passing through a minimum at the temperature corresponding to the I state of DnaK, indicating that the I state of DnaK is responsible for the maximum blue shift in bis-ANS fluorescence (Figure 1, inset).

*Kinetics of Bis-ANS Binding to DnaK.* Monitoring the bis-ANS fluorescence emission intensity as a function of time revealed that bis-ANS binds to DnaK and bovine cytosolic hsp73 (hsc70) in a biphasic process at temperatures below 50 °C (Figure 4A,B; data not shown for hsp73). The slow phase was monoexponential. When bis-ANS is mixed with the protein, there is a rapid jump in bis-ANS fluorescence, which is complete within the experimental dead time (1–3 s), followed by a slow, first-order increase in bis-ANS fluorescence with time. Simultaneously, a slow decrease in DnaK intrinsic

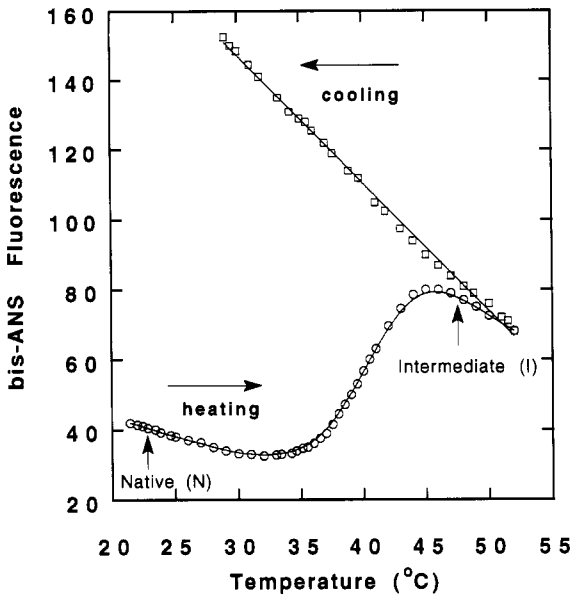



FIGURE 3: Stability of the DnaK folding intermediate–bis-ANS complex. Cooling of thermally denatured DnaK (1 $\mu$M) in the presence of bis-ANS (1.37 $\mu$M) followed by bis-ANS fluorescence emission at 485 nm in 100 mM Tris. The sample was heated (○) from 20 °C to 52 °C at a rate of 0.3 deg/min to generate the intermediate state and then allowed to cool down (□) to 20 °C, also at 0.3 deg/min. The cooling curve corresponds to the effect of temperature on the emission of the complex between dye and DnaK intermediate. The excitation wavelength was 394 nm.

(tryptophan and tyrosine) fluorescence was observed (Figure 4C). Similar time-dependent changes in the DnaK far-UV CD spectrum induced by bis-ANS were also observed by following the ellipticity at 222 nm (data not shown). The rates of the slow phase detected by bis-ANS fluorescence closely matched those observed by DnaK intrinsic fluorescence (Figure 4C), were dependent on bis-ANS concentration and temperature, and were much slower than expected for a simple dye binding process (in the range of milliseconds) (Brand, 1970) or dye quenching. A similar slow process was also observed when bis-ANS was mixed with staphylococcal nuclease (data not shown), and similar biphasic curves for bis-ANS binding to lactic dehydrogenase (Wu & Wu, 1978) and myosin fragment 1 (S-1) (Takashi et al., 1977) have been reported previously.

Figure 4A shows the effect of temperature on binding kinetics of bis-ANS to DnaK at fixed bis-ANS concentration. Only a single, rapid phase of bis-ANS binding was observed at 55 °C, conditions where only the compact intermediate exists (Palleros et al., 1992). The binding of bis-ANS to DnaK in 0.8 M Gdn·HCl at 20 °C (again, conditions where only the compact intermediate is populated; Palleros et al., 1993) showed similar rapid binding (data not shown). On the other hand, at 0.2 °C, only binding to the native state is observed (also very rapid). The small increase in amplitude of the initial phase at 37 °C compared to that at 0.2 °C and 20 °C is consistent with the amount of intermediate state calculated from the thermal unfolding transition to be present at 37 °C (see Discussion). Figures 4B and 4C show the effect of bis-ANS concentration on the binding kinetics of bis-ANS to DnaK at 37 °C, followed by bis-ANS fluorescence emission and DnaK intrinsic fluorescence, respectively. Excellent agreement in observed rates was noted between the two techniques. Assuming that dye binding is fast compared to the conformational changes, the dependence of rate on dye concentration can be explained by the following two concurrent

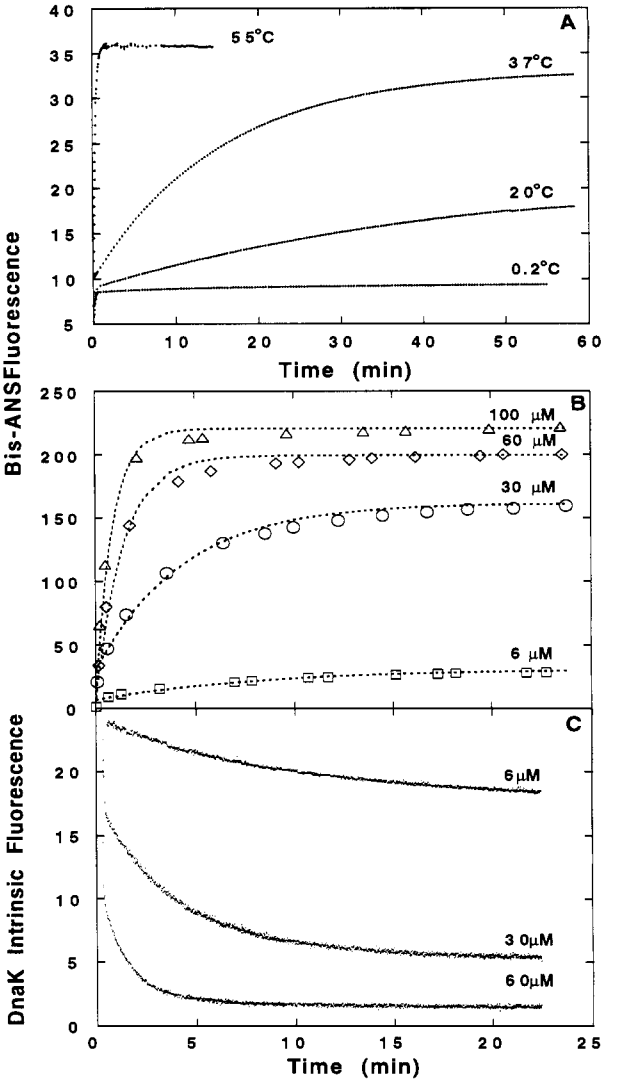



FIGURE 4: Binding kinetics of bis-ANS to DnaK. Effect of temperature and bis-ANS concentration. (A) Effect of temperature: bis-ANS (4 $\mu$M) fluorescence emission changes were followed at 485 nm at 55, 37, 20, and 0.2 °C. (B) Effect of bis-ANS concentration: bis-ANS (6, 30, 60, and 100 $\mu$M) fluorescence emission changes were followed at 485 nm at 37 °C. The open symbols are data (randomly selected from 1000 data points), and the dashed lines are theoretical curves drawn according to the model given in the text, using a value of 7.0 $\mu$M for the dissociation constant for dye binding to the native state and the rate constants given in the text. (C) Effect of bis-ANS concentration monitored by DnaK intrinsic (Trp and Tyr) fluorescence emission a 335 nm at 37 °C in the presence of 6, 30, and 60 $\mu$M bis-ANS. All experiments were performed with 1 $\mu$M DnaK in Tris buffer (no DTT). The excitation wavelengths were 394 nm for A and B, and 280 nm for C.

processes:[2]

$$N \xrightarrow{k_1} I \overset{K_{ID}}{\rightleftharpoons} ID \rightleftharpoons ID_3$$

$$N + D \overset{K_{ND}}{\rightleftharpoons} ND \xrightarrow{k_2} ID \rightleftharpoons ID_3$$

It can be shown that for the slow phase, $k_{obs} = k_1 + k_2[\text{bis-ANS}]/K_{ND}$, consistent with the observation that the rate is

[2] The binding of bis-ANS to the intermediate conformation is shown both directly to I and via isomerization of the native state with bound dye. This model is a slightly simplified version of the real system, in which multiple equilibria involving 1–3 molecules of dye binding to three independent sites may occur. However, assuming that these binding steps are fast, their omission does not affect the kinetic expression.

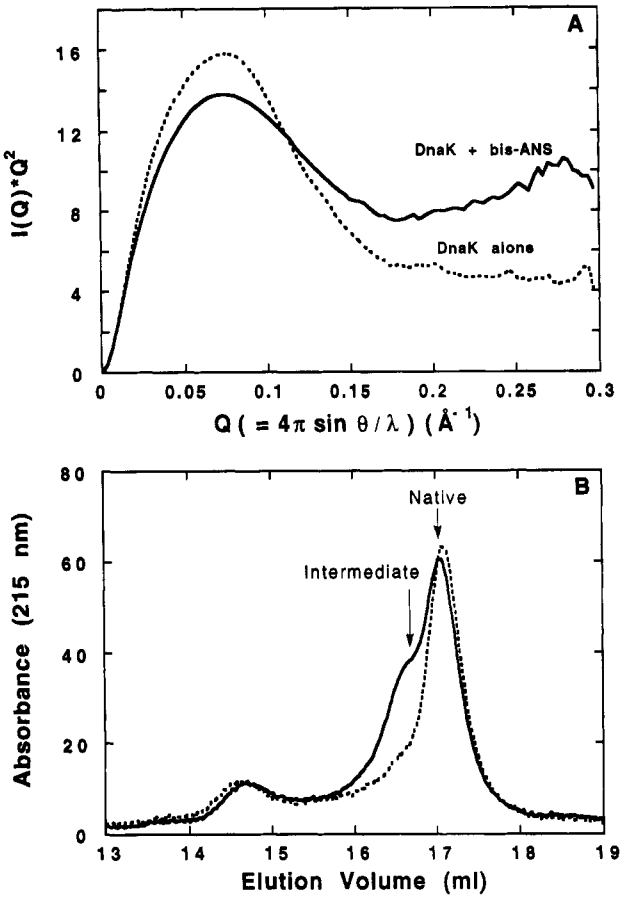



FIGURE 5: Bis-ANS-induced conformational change in DnaK. (A) Small-angle X-ray scattering Kratky plots of DnaK and DnaK-bis-ANS complex. DnaK (50 $\mu$M) solutions in the presence (solid line) and absence (dashed line) of bis-ANS (60 $\mu$M) were incubated at 36 °C for 30 min before SAXS measurements. $I(Q)$, scattering intensity, $\theta$, scattering angle, $\lambda$, X-ray, wavelength (1.488 Å). (B) SEC-HPLC traces of DnaK and DnaK·bis-ANS complex. DnaK (18 $\mu$M) solutions in the presence (solid line) and absence (dashed line) of bis-ANS (60 $\mu$M) were incubated at 37 °C for 40 min before injection. The arrows show where the intermediate and native states elute in the absence of bis-ANS. Phosphate buffer (20 mM) with 200 mM NaCl (pH = 6.5) was used as the mobile phase at 22 °C.

dependent on the dye concentration. Using the equilibrium and binding constants derived above, the values calculated for $k_1$ and $k_2$ were $(4 \pm 2) \times 10^{-4}$ and $(2 \pm 0.3) \times 10^{-3}$ s$^{-1}$, respectively, at 37 °C and $(2 \pm 1) \times 10^{-4}$ and $\leq 8 \times 10^{-5}$ s$^{-1}$, respectively at 20 °C. The theoretical curves in Figure 4B were drawn according to the above model. Excellent fits (not shown) were obtained for the data at 20 °C.

*Bis-ANS Induces a Conformational Change in DnaK.* The Kratky plot (Glatter & Kratky, 1982; Kataoka et al., 1993) of the SAXS data for DnaK at 36 °C showed a significant change in the shape and intensity of the peak corresponding to globular protein upon the addition of bis-ANS, indicating a conformational change in DnaK induced by bis-ANS (Figure 5A). The nature of the change in the Kratky plot indicates that in the presence of bis-ANS the protein becomes less compact with a less dense core, consistent with a molten globule-like conformation.

Further evidence for a major change in the conformation of DnaK in the presence of the dye at 37 °C was obtained from SEC-HPLC experiments. Incubation of DnaK with bis-ANS results in a new DnaK peak with a smaller elution volume of SEC-HPLC, suggesting an expansion in DnaK induced by bis-ANS binding (Figure 5B). The presence of free DnaK in this chromatogram reflects dissociation of the DnaK·bis-ANS complex during the HPLC run.

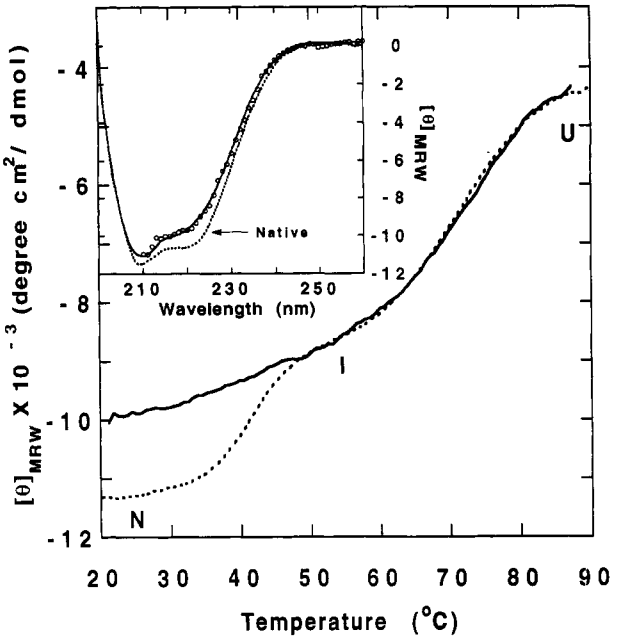



FIGURE 6: Effect of bis-ANS on DnaK thermal stability. DnaK thermal unfolding was followed by ellipticity at 222 nm in the presence and absence of bis-ANS. Temperature was increased at 0.3 deg/min. (1) 1.8 $\mu$M DnaK, 60 $\mu$M bis-ANS in Tris buffer (solid line). (2) 5.7 $\mu$M DnaK, no bis-ANS, in phosphate buffer (dashed line). Inset: DnaK (1.8 $\mu$M) far-UV CD spectral changes induced by bis-ANS. Top curve: 62 $\mu$M bis-NS. Bottom curve: no bis-ANS. Open circles: DnaK intermediate in 0.8 M Gdn·HCl, no bis-ANS. All the measurements in the presence of bis-ANS were made after samples had been equilibrated at 37 °C for 50 min and cooled to 20 °C for at least 20 min. N, native; I, intermediate; U, unfolded.

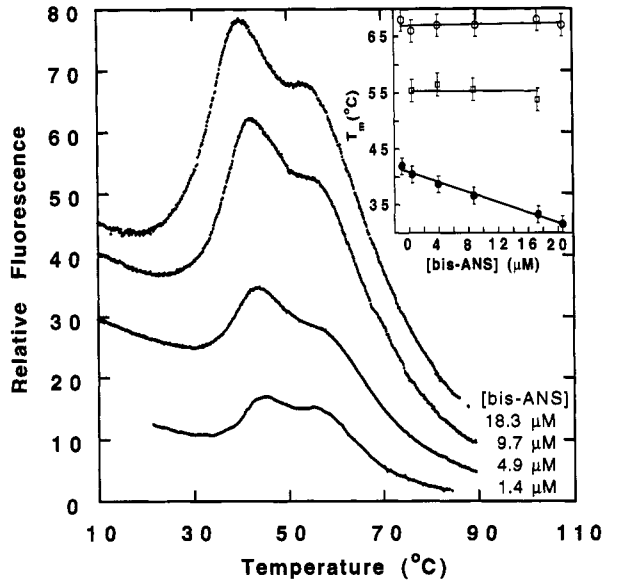



FIGURE 7: Effect of bis-ANS on DnaK thermal unfolding, $T_m$. DnaK (1 $\mu$M) thermal unfolding followed by bis-ANS fluorescence emission change at 485 nm in the presence of 1.4, 4.9, 9.7, and 18.3 $\mu$M bis-ANS in Tris buffer. Temperature change was 0.3 deg/min. The excitation wavelength was 394 nm. Inset: plots of $T_m$ vs bis-ANS concentration. Unfolding transitions from (●) native (N) to compact intermediate (I); (□) compact intermediate to the less compact intermediate (I*); and (○) less compact intermediate to unfolded state (U).

The thermally-induced unfolding of DnaK, with and without bis-ANS, was followed by CD (Figure 6) and by fluorescence (Figure 7). When monitored by the ellipticity at 222 nm in the absence of dye, DnaK showed an initial transition with a midpoint at 42 °C corresponding to the transition to a compact intermediate (Palleros et al., 1992); the subsequent unfolding

transition had a midpoint in the vicinity of 72 °C (Figure 6, dashed line). In contrast, in the presence of 60 μM bis-ANS, the first unfolding transition of DnaK from the native to the intermediate state was absent (Figure 6, solid line). The simplest explanation is that the unfolding started from the intermediate state rather than the native state. The CD trace in the presence of the dye (solid line) shown in Figure 6 was started after the bis-ANS and DnaK had been incubated for sufficient time (50 min at 37 °C and then 20 min at 20 °C) that equilibrium had been reached (i.e., no further time-dependent change in ellipticity was observed, and essentially all of the DnaK was in the intermediate conformation, complexed with bis-ANS). The inset of Figure 6 shows the far-UV CD spectra of DnaK in the presence and absence of bis-ANS after incubation for 50 min at 37 °C. The spectrum of DnaK with 60 μM bis-ANS is superimposable with that of the intermediate state of DnaK obtained in 0.8 M Gdn·HCl (Palleros et al., 1993) (Figure 6, inset, circles), indicating that bis-ANS induces a conformational change in DnaK from the native to the intermediate state. No CD signal was observed from the solution of bis-ANS alone. Since there is no CD signal in the wavelength region where bis-ANS absorbs (385 nm), we can assume that the CD contribution of bis-ANS, induced by binding to protein, is negligible in the far-UV CD spectra. A significant change in the DnaK fluorescence spectrum induced by bis-ANS was also observed due to energy transfer from DnaK to bis-ANS and conformational change in DnaK (see Figure 10).

Figure 7 shows the effect of bis-ANS concentration on the thermal unfolding transition of DnaK, as followed by bis-ANS fluorescence emission at 485 nm. Two major transitions were observed, that of N → I in the vicinity of 30–40 °C and that of I → U in the vicinity of 70 °C. An additional small transition between I and U in the vicinity of 55 °C was also observed; since this takes place at a higher temperature, it presumably reflects a transition between the compact intermediate I and a less compact state (I*) with additional bis-ANS affinity. The position of the $T_m$ for the N → I transition was observed to be linearly dependent on bis-ANS concentration, whereas the small transition (I → I*) and the major unfolding transition (I* → U) were independent of bis-ANS concentration (Figure 7, inset). In these experiments, the dye/protein molar ratio varied from 1.4 to 22, which are lower than those frequently used (Table 1).

*Nucleotide Effects on the Interaction of Bis-ANS with DnaK.* Nucleotides stabilize the native state of hsp70 to thermal and denaturant-induced unfolding (Palleros et al., 1991, 1992, 1993). No effect of ATP or ADP on the fluorescence spectrum of free bis-ANS in aqueous solution was observed. However, the addition of ATP or ADP to a solution of DnaK equilibrated with bis-ANS (i.e., the complex between the dye and the DnaK intermediate) significantly decreased the bis-ANS fluorescence emission intensity and induced an observable red shift in the bis-ANS fluorescence emission $\lambda_{max}$ (Figure 8, inset). Figure 8 shows the kinetic traces of the interaction of bis-ANS with DnaK before and after the addition of ATP at 37 °C, monitored by bis-ANS fluorescence emission at 485 nm. Exactly the same behavior of bis-ANS fluorescence was obtained when ADP was added to a DnaK and bis-ANS mixture (data not shown). The initial increase in fluorescene intensity (Figure 8 trace 1) reflects the dye-induced transition to the compact intermediate state. The subsequent decrease (trace 2) induced by the addition of ATP is most simply interpreted as the reverse transition due to the preferential binding of the nucleotide to the native state.

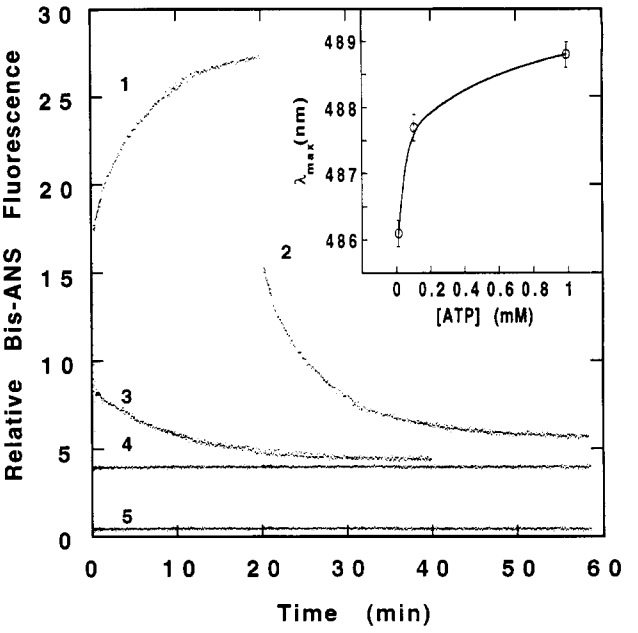



FIGURE 8: Effect of ATP on bis-ANS binding to the DnaK intermediate: Kinetics of bis-ANS fluorescence change before and after the addition of nucleotide at 37 °C. Bis-ANS fluorescence emission changes with time were followed at 485 nm with the excitation at 394 nm. (1) Bis-ANS was rapidly mixed with DnaK at zero time. (2) Mg/ATP was added at 20 min. (3) Bis-ANS and Mg/ATP were rapidly mixed with DnaK simultaneously at zero time. (4) Bis-ANS was rapidly mixed with DnaK at zero time after DnaK had been incubated with Mg/ATP at 37 °C for 1.5 h. (5) Bis-ANS was rapidly mixed with Mg/ATP (no DnaK) at zero time. The final concentrations were DnaK, 1 μM; bis-ANS, 1 μM; ATP, 1 mM; all in Tris buffer (pH = 7.2). Inset: ATP-induced change in the fluorescence emission $\lambda_{max}$ of the DnaK/bis-ANS mixture. Pre-equilibrated solutions of DnaK (0.64 μM) with 5 μM bis-ANS were incubated with 0.0, 0.1, and 1.0 mM ATP at 25 °C for 1 h before the value of $\lambda_{max}$ was determined.

The rate of this process ((1.13 ± 0.50) × $10^{-3}$ $s^{-1}$) was in excellent agreement with that of DnaK thermal refolding ((1.14 ± 0.05) × $10^{-3}$ $s^{-1}$) at 35 °C (Palleros et al., 1992). Conformation of the hypothesis that ATP induces a reverse transition from intermediate to native state is found in the observation that if ATP is first added to DnaK, it abolishes the fluorescence change seen on addition of bis-ANS (trace 4). When ATP and bis-ANS were added simultaneously, a small decrease in bis-ANS fluorescence was observed, with a rate ((1.64 ± 0.50) × $10^{-3}$ $s^{-1}$, trace 3) similar to that of trace 2 and corresponding to the transition of the small amount of intermediate state initially present to the N·ATP complex. It can be seen in Figure 8 that, although the addition of nucleotide induces a significant decrease in bis-ANS fluorescence enhancement, it does not completely reverse the fluorescence signal to the value expected for free bis-ANS (trace 5). It has been commonly observed that nucleotides do not completely reverse the increase in the fluorescence intensity of ANS and bis-ANS in protein solutions (Table 1).

ATP and ADP have previously been shown to have similar effects in stabilizing hsp70s to thermal and denaturant-induced denaturation (Palleros et al., 1991, 1992, 1993). The effects of ADP on the thermal and Gdn·HCl-induced unfolding of DnaK in the presence of bis-ANS have been followed by bis-ANS fluorescence emission, as shown in Figure 9. The presence of ADP shifted the bis-ANS fluorescence-detected transition midpoint to a higher temperature and a higher Gdn·HCl concentration. The presence of 1 mM ADP with 1.37 μM bis-ANS and 1 μM DnaK results in a shift in the transition midpoint from 40.3 to 60.4 °C. A small transition

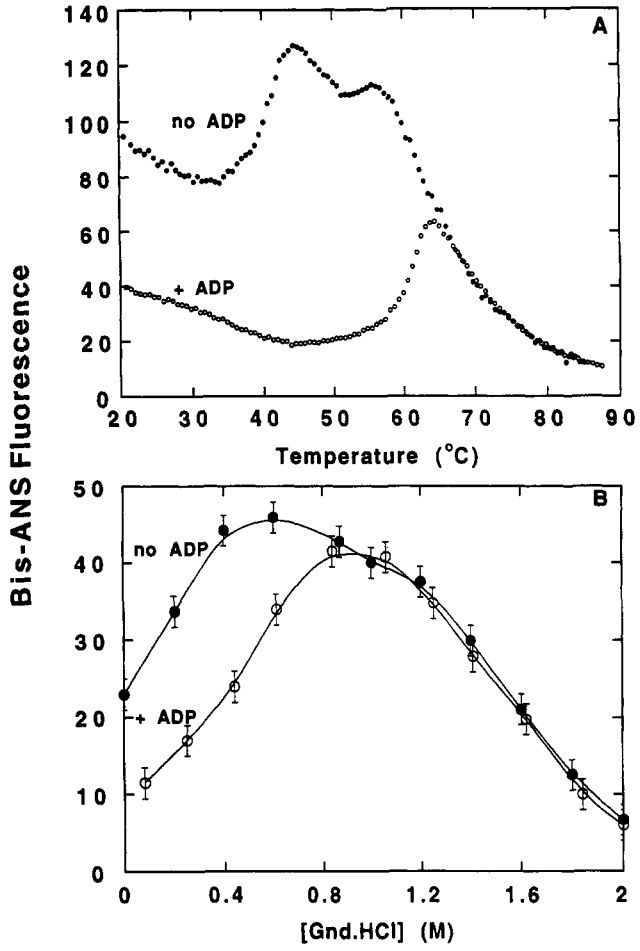



FIGURE 9: Effect of ADP on DnaK stability. DnaK unfolding followed by bis-ANS fluorescence emission at 485 nm. (A) Thermally induced DnaK unfolding. The rate of temperature increase was 0.3 deg/min. (B) Gdn·HCl-induced DnaK unfolding. Each of the samples containing various concentrations of Gdn·HCl was incubated at 20 °C for 2 h before the fluorescence measurement. The sample was 1 $\mu$M DnaK with 1.37 $\mu$M (panel A) or 0.65 $\mu$M bis-ANS (panel B) in phosphate buffer containing 1 mM DTT with (○) or without (●) 1 mM Mg/ADP. The excitation wavelength was 394 nm.

around 55 °C precedes this transition, I → I*, seen in the same temperature range in the absence of nucleotide. ADP (1 mM) shifts the midpoint for the first transition detected by bis-ANS in the Gdn·HCl-induced unfolding about 0.3 M higher.

*Fluorescence Energy-Transfer Experiments.* Tryptophan·bis-ANS makes a good fluorescence energy-transfer donor/acceptor combination due to the overlap of tryptophan emission and bis-ANS excitation. Energy-transfer experiments (Figure 10, excitation of Trp at 280 nm, emission by bis-ANS) at 37 °C show that the efficiency of energy transfer from DnaK to bis-ANS is a function of bis-ANS concentration and comes close to 100% when the bis-ANS concentration is higher than 90 $\mu$M. No significant change in the energy-transfer efficiency (ETE) (Trp at 298 nm → bis-ANS) was observed in the presence of ATP (from 0.62 to 1.1 mM at 20 °C) (data not shown). However, a significant difference in ETE (Trp at 298 nm → bis-ANS) between DnaK·bis-ANS (5%, Figure 11A) and hsp73·bis-ANS (35%, Figure 11B) was observed when 1 $\mu$M protein was mixed with 1.4 $\mu$M bis-ANS at 20 °C (i.e., native conformation).

## DISCUSSION

ANS and bis-ANS have been frequently used in protein studies (Brand, 1970; Aoe et al., 1970; Takashi et al., 1977; Wiksell & Larsson-Raznikiewicz, 1982; Horowitz & Criscimagna, 1985; Secnik et al., 1992; Dryden et al., 1992); however, possible effects of the dye on the conformation and stability of the protein have been largely overlooked, in that it is customarily assumed that if the dye is added to the native protein and binding is observed, it is to the native state. Table 1 shows that in many studies the dye concentration used may

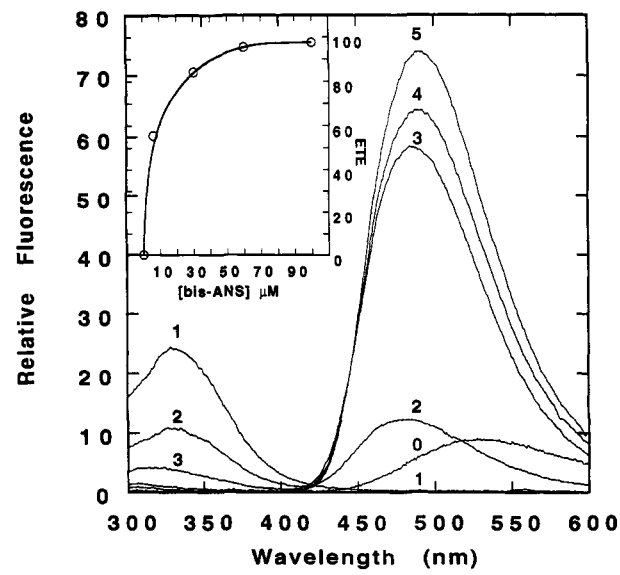



FIGURE 10: Fluorescence energy transfer from DnaK to bis-ANS. Fluorescence emission spectra of samples with 1 $\mu$M DnaK in the presence of (1) 0, (2) 6, (3) 30, (4) 60, and (5) 100 $\mu$M bis-ANS in Tris buffer were scanned at 37 °C. Spectrum 0 is that of bis-ANS (100 $\mu$M) along (no DnaK) scanned under the same conditions. The excitation wavelength was 280 nm. Inset; plot of energy-transfer efficiency (ETE) vs bis-ANS concentration for 1 $\mu$M DnaK at 37 °C. All measurements were carried out after the samples had been equilibrated for 3 h at 37 °C.

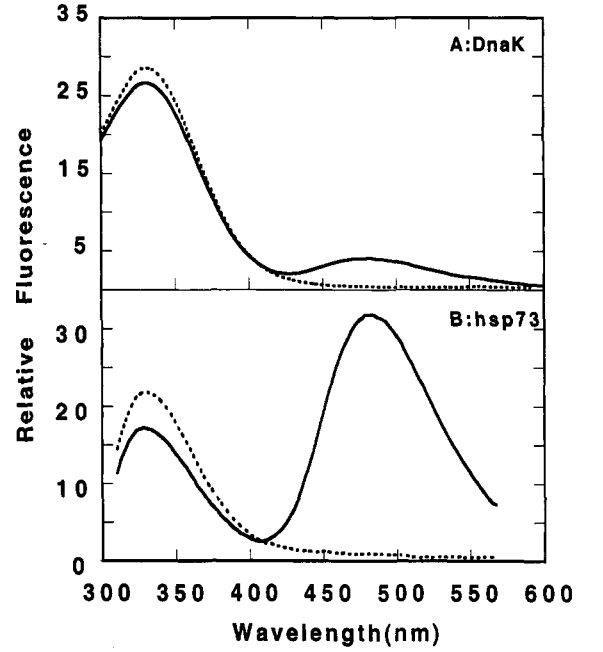
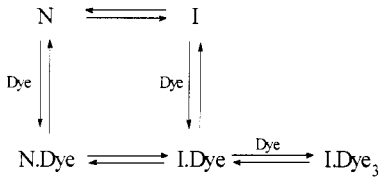



FIGURE 11: Comparison of the energy transfer from DnaK to bis-ANS and from hsp73 to bis-ANS. Spectra of samples with (A) 1 $\mu$M DnaK and (B) 1 $\mu$M hsp73 in presence of (solid line) 1.4 $\mu$M bis-ANS and (dashed line) absence of bis-ANS in 100 mM Tris·HCl buffer were scanned after the samples had been equilibrated for 2.5 h at 20 °C (under these experimental conditions, very little intermediate was formed; in separate experiments at 2 °C, similar results were observed for both hsp 73 and DnaK). The excitation wavelength was 298 nm.

Scheme 1




exceed the protein concentration by more than 2 orders of magnitude and that the $K_d$ values for the same dye are very different for different proteins and even vary for the same protein in different studies. The results reported here show that an external (hydrophobic) probe, especially at relatively high concentration, may significantly perturb the protein conformation by shifting underlying conformational equilibria.

*Bis-ANS Induces a Conformational Change in DnaK.* Previous studies have shown that ANS has maximum fluorescence emission when bound to compact intermediate or molten globule states of proteins (Goto et al., 1979; Rodionova et al., 1989; Goto & Fink, 1989; Ptitsyn et al., 1990; Semisotnov et al., 1991; Fink et al., 1993). Our results indicate that bis-ANS preferentially binds to the intermediate state, I, of DnaK and thus induces a conformational change in the protein. The enhancement of bis-ANS fluorescence by the I state of DnaK is much larger than that induced by the N state of DnaK, as shown in Figure 1. The facts that bis-ANS and the I state of DnaK form a very stable complex (Figure 3) and that DnaK unfolding, N → I, results in a maximum enhancement in bis-ANS fluorescence (Figure 7 and 9) indicate that bis-ANS binds preferentially to the intermediate state. The conformational change in DnaK induced by bis-ANS was also demonstrated by SAXS (Figure 5A), SEC-HPLC (Figure 5B), and far UV CD (Figure 6, inset) measurements.

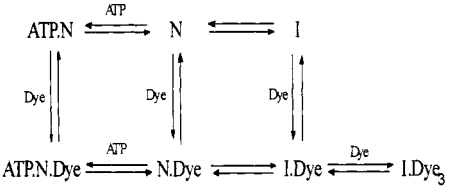
From the kinetics of bis-ANS binding to DnaK (Figure 4), the *slow* changes observed in bis-ANS fluorescence enhancement and DnaK intrinsic (Trp and Tyr) fluorescence intensity suggest that a conformational change occurred simultaneously with the binding of bis-ANS (the initial jump in fluorescence intensity at low temperature reflects the binding of bis-ANS to native DnaK and to any extant intermediate state of DnaK). In the absence of nucleotides, the initial transition in the thermal unfolding of DnaK begins around 35 °C. Calculations based on the unfolding monitored by CD (Figure 6A) and intrinsic fluorescence (Palleros et al., 1992) give an equilibrium constant of $9.44 \times 10^{-2}$ between the I and N states of DnaK at 37 °C. This means that at 37 °C, about 8.6% of DnaK is already in the I state, which was confirmed by monitoring the ATP-induced I → N transition followed by bis-ANS fluorescence after the simultaneous addition of ATP and bis-ANS to DnaK at 37 °C (Figure 8, trace 3). Thus, we conclude that the addition of bis-ANS shifts the balance from the N state toward the I state by preferentially binding to the I state and therefore induces the denaturation of DnaK, as shown in Scheme 1. In this process, the DnaK conformational changes are the rate-determining steps. This analysis (at 37 °C) also applies to the case at lower temperature, where the only difference is that at lower temperature the equilibrium between N and I is more in favor of the N state. At higher temperatures, such as 55 °C, as shown in Figure 4A, the binding of bis-ANS to DnaK is very fast, since almost all of the DnaK is present in the I state (Palleros et al., 1992). From the thermodynamic point of view, the binding of bis-ANS to the intermediate state of DnaK should affect the apparent free energy for the N → I transition. This is supported by the observation (Figure 7, inset) that the first thermal unfolding transition $T_m$, which corresponds to the N → I transition, decreases as a linear function of bis-ANS concentration.

*Bis-ANS-Monitored Thermal Unfolding Reveals an Additional Intermediate.* Our previous results indicated that the denaturation of DnaK proceeds via a three-state transition, N → I → U (Palleros et al., 1992, 1993). As can be seen from Figures 7 and 9A, when the thermal unfolding transition of DnaK in the presence of bis-ANS is monitored by the fluorescence emission of bis-ANS, three transitions, corresponding to four states, N → I → I* → U, are observed. The first major increase in bis-ANS fluorescence intensity corresponds to the previously reported transition from native to compact intermediate (Palleros et al., 1991, 1992), and its position is dependent on the dye concentration. The second transition, a smaller increase in bis-ANS fluorescence, which is independent of dye concentration, occurs in the vicinity of 55 °C and is not readily apparent when the transition is monitored in the absence of bis-ANS (Palleros et al., 1992), although the transition I → U, monitored by ellipticity at 222 nm (Figure 6), was quite broad and could be interpreted as two transitions. We attribute this second transition to one between the compact intermediate (I) and an additional, more expanded intermediate (I*). A recent calorimetric investigation of the thermal unfolding of DnaK also shows a four-state transition (Montgomery et al., 1993). Examination of Figure 9B, the Gdn·HCl-induced unfolding of DnaK in the presence of bis-ANS, suggests that a comparable intermediate may exist, with a midpoint around 1.1 M Gdn·HCl. The small initial transition observed by bis-ANS fluorescence in the presence of ADP (Figure 9A) is also centered around 55 °C. The third transition corresponds to a decrease in dye fluorescence and is the main unfolding transition, with $T_m$ around 70 °C.

*Different Modes of Bis-ANS Binding.* The difference between the $\lambda_{max}$ at low bis-ANS/DnaK ratios (~0.01) (480 nm) and at high bis-ANS/DnaK ratios (>150) (505 nm), is consistent with multiple bis-ANS binding sites. The data are most easily explained if the first molecule of dye to bind to the intermediate results in a $\lambda_{max}$ of 480 nm, and the binding of the second and third molecules of dye leads to a shift in $\lambda_{max}$ to 505 nm, due to the different environment in which the last two dyes are found. The hypothesis of two types of bis-ANS binding has also been proposed for binding to *E. coli* glutaminyl-tRNA synthetase (Bhattacharyya et al., 1991), *lac* repressor (Lawson & York, 1987), and lactate dehydrogenase (Wu & Wu, 1978), based on the change of bis-ANS $\lambda_{max}$ with dye concentration, and for bis-ANS binding to *E. coli* RNA polymerase (Martin et al., 1991) and tubulin (Prasad et al., 1986), based on the sigmoidal shape of protein titration curves.

The observation that $\lambda_{max}$ changes during thermal unfolding of DnaK, passing through a minimum at the temperature (50 °C, Figure 1, inset) corresponding to the I state of DnaK, reflects the formation of different protein–dye complexes at different temperatures. At low temperatures, the complex formed by binding of the dye to the native conformation of DnaK predominates and results in a small blue shift in bis-ANS fluorescence. In contrast, around 50 °C (conditions where only the compact intermediate exists), the complex formed by strong binding is dominant, corresponding to the maximum blue shift in bis-ANS fluorescence. The return of $\lambda_{max}$ to higher values at very high temperatures suggests that bis-ANS weakly binds to the unfolded protein, resulting a red shift, as shown by Figure 1, spectra 4 and 5.

Scheme 2




*Nucleotides Inhibit the Bis-ANS-Induced Conformational Change in DnaK.* Previous studies have suggested that ANS and bis-ANS dyes bind to nucleotide binding sites and could be displaced by nucleotides (Rosen & Weber, 1969; Takashi et al., 1977; Secnik et al., 1990, 1992; Yoo et al., 1990; Griess et al., 1991; Lee et al., 1991; Horowitz et al., 1984; Bohnert et al., 1982; Anderson, 1971). In contrast, our observations suggest that bis-ANS induces a conformational change in DnaK and that nucleotides can reverse the effect of bis-ANS by shifting the equilibrium between native and compact intermediate rather than displacing the bound bis-ANS. Baneck et al. (1992) have also reported that the addition of ATP to a DnaK/bis-ANS mixture results in a decrease in the intensity of bis-ANS fluorescence. However, we believe that their interpretation, involving a two-step conformational change induced by the binding of ATP and its subsequent hydrolysis, requires modification, since the data presented here suggest that the conformational change is due to the I → N transition of DnaK rather than a functional conformational change in a native protein.

Figure 9 demonstrates that nucleotide shifts the position of the N → I transition to higher temperature or higher denaturant concentration. The addition of bis-ANS to DnaK at 37 °C results in the slow formation of the complex between the dye and the compact intermediate state, manifested as an increase in fluorescence emission of the dye (Figure 8, trace 1). The subsequent addition of a large excess of nucleotide leads to a slow decrease in fluorescence, which we attribute to the preferential binding of the nucleotide to the native state and the concomitant release of bound bis-ANS from the intermediate form of DnaK (Figure 8, trace 2). The excellent match between the DnaK refolding rate (Palleros et al., 1992) and that of nucleotide-induced bis-ANS fluorescence decrease is consistent with this. Preincubation of DnaK with ATP results in complete disappearance of the slow increase in bis-ANS fluorescence emission, demonstrating the ability of ATP to stabilize the native state of DnaK and prevent is unfolding induced by bis-ANS (Figure 8, trace 4). The competition between ATP and bis-ANS for DnaK can be represented by Scheme 2. In this process, the rate-determining step is the transition N → I, whereas the equilibrium depends on the stability of both N·ATP and I·Dye and the concentrations of both ligands. As shown by Figures 8 and 9, the presence of nucleotide shifts the equilibrium toward the native state and thus induces the release of bis-ANS. In these experiments, the concentrations of ATP (1 mM) were all much higher than those of bis-ANS (1–1.4 $\mu$M). When bis-ANS concentration was increased to 16.4 $\mu$M in the presence of 53.5 $\mu$M ATP, a slow increase in bis-ANS fluorescence emission was still observed (data not shown).

From Figure 8 (traces 2, 3, and 4), it can be seen that nucleotide does not completely reverse the enhancement of bis-ANS fluorescence. This suggests that the ATP and bis-ANS binding sites of the native state of DnaK are independent and thus explains why ATP does not significantly affect the relative energy-transfer efficiency from DnaK Trp to bis-ANS, i.e., a ternary complex of ATP, dye, and native DnaK is formed (data not shown).

DnaK has only one tryptophan residue located in the N-terminal domain of DnaK. Hsp73 has two tryptophan residues, one located in the N-terminal domain and the other in the C-terminal domain. At low bis-ANS concentration (1.37 $\mu$M) and low temperature (20 °C), the difference between the energy-transfer efficiency of DnaK·bis-ANS (5%) and hsp73·bis-ANS (35%) (Figure 11) suggests that the binding of bis-ANS to the native protein may occur at a location in the C-terminal domain of DnaK, distant from the single tryptophan in the N-terminal domain, since the binding of bis-ANS to hsp73 results in more efficient energy transfer.

*Generality of Dye-Induced Conformational Changes.* That bis-ANS may induce conformational changes in other proteins can be inferred from the observation that both the far-UV CD and intrinsic fluorescence spectra of BSA showed analogous structural changes to DnaK in the presence of bis-ANS (L. Shi and A. Fink, unpublished results). In addition, it has recently been reported that bis-ANS inhibits the further refolding of phage P22 coat protein from its intermediate to the folded state (Teschke et al., 1993), also consistent with a mechanism analogous to that of DnaK (Scheme 1). Thus it is possible that bis-ANS-induced perturbation of native protein structure is a relatively common phenomenon.

In addition, our finding that nucleotides can inhibit such bis-ANS-induced perturbation by stabilizing the native state suggests that care should be given to interpreting observations that the addition of nucleotide results in a change in bis-ANS fluorescence of other proteins (Rosen & Weber, 1969; Takashi et al., 1977; Secnik et al., 1990; 1992; Lee et al., 1991; Bohnert et al., 1982; Anderson, 1971; Dryden et al., 1992; Wiksell & Larsson-Raznikiewicz, 1982). The possibility of a dye-induced conformational change thus offers an alternative interpretation to the hypothesis that bis-ANS is displaced by nucleotide in the same site (Takashi et al., 1977; Yoo et al., 1990; Secnik et al., 1990; Lawson & York, 1987), since for most of the cases the nucleotide did not completely reverse the ANS or bis-ANS fluorescence enhancement, as shown in Table 1. The possibility that ATP binds to a different site than the dye and that the release of bis-ANS is due to an alternate mechanism to direct competition was previously suggested (Yoo et al., 1990).

The effect of ANS dyes on protein conformation depends on the intrinsic stability of the protein under the experimental conditions, the presence of ligands which stabilize the native (or other) states, and the dye/protein ratio. The fluorescence emission of ANS was more intense when the dye was bound to H388Q mutant of phosphoglycerate kinase (PGK) than when it was bound to the wild-type PGK, and the curves of Gdn·HCl-induced unfolding show that H388Q is less stable than wild-type PGK (Dryden et al., 1992; Wiksell & Larsson-Raznikiewicz, 1982); similarly, bis-ANS has stronger affinity to apo-$\alpha$-lactalbumin than to the native $\alpha$-lactalbumin, as shown in Table 1.

In conclusion, under conditions where the native state is nominally predominant, the preferential binding of a dye such as bis-ANS to a compact intermediate state can effectively pull the equilibrium to favor the intermediate conformation. The presence of tight-binding ligands to the native state can shift the equilibrium back to the native conformation, regardless of whether the dye binds in the nucleotide binding site or not.

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