Analysis of protein folding using polarity-sensitive fluorescent probes

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Polarity-sensitive fluorescent probes like 8-anilino-1-naphthalenesulphonate (ANS) and 1,1′-*bis*(4-anilino-5-naphthalenesulphonic acid (*bis*-ANS) have been frequently used to detect equilibrium folding intermediates like the molten globule state, as the formation of the latter involves increase in hydrophobic exposure. The ability of these fluorescent probes to bind to the intermediate state thus provides a convenient method for detection of folding intermediates in a multiple-state folding transition. However, there is no convenient method available to quantitatively analyse the fluorescent changes detected as a function of denaturant concentration. Here we describe a new method for quantitative analysis that permits one to estimate free energy of folding and determine the relative population of the various states of protein in the solution.

PROTEIN folding pathways have been investigated using a wide variety of methods, including the use of polarity-sensitive fluorescent probes[1–5] like 8-anilino-1-naphthalenesulphonate (ANS) and 1,1′-*bis*(4-anilino-5-naphthalenesulphonic acid (*bis*-ANS). These probes are known to undergo an increase in fluorescence on binding to hydrophobic surfaces and have been used[1,3–5] to detect and study molten globule-like intermediate states in protein folding. While these studies have provided a convenient method for detection of folding intermediates involving hydrophobic exposure, a quantitative analysis of the data has not been reported as in the case of folding studies monitored by intrinsic fluorescence or CD spectroscopy. Here we present a model which explains the experimental data obtained in ANS binding studies at various denaturant concentrations and extracts useful information in relation to the energetics of protein folding. Application of the current method to bovine α-lactalbumin (α-LA) and diphtheria toxin (DT) permitted computation of $\Delta G(H_2O)$ values. In the case of α-LA where the $\Delta G(H_2O)$ values obtained by alternative methods were available, the values obtained here were found to be in good agreement with the literature values.

Commercially available bovine α-LA from Sigma was further purified according to the reported procedure[6]. α-LA at a final concentration of 1.5 μM was incubated with 30 μM *bis*-ANS for 5 min at 25° in different guanidine–HCl (GuHCl) concentrations (0, 0.5, 1, 1.4, 1.8, 2.2, 2.6, 3, 3.4, 3.8, 4.2, 4.6, 5, 5.5, 6, 7 M final concentration) prepared in 10 mM phosphate buffer pH 6.5, containing 0.1 M KCl. Degassed buffer was used for all fluorescence measurements. The fluorescence spectra were recorded with excitation wavelength set at 395 nm and emission at 500 nm noted. A slit width of 10 nm was used for both excitation and emission monochromator. All fluorescence measurements were carried out at 25°.

Purified DT[7] at a final concentration of 0.5 μM was incubated with 20 μM *bis*-ANS for 5 min at 25° in different GuHCl concentrations (0, 0.25, 0.5, 0.75, 1, 1.2, 1.4, 1.8, 2.2, 2.6, 3, 3.4, 3.8, 4.2, 4.6, 5, 5.5, 6, 7 M final concentration) prepared in 10 mM phosphate buffer pH 6.5, containing 0.1 M KCl. The fluorescence spectra were recorded as mentioned above.

Consider the transition $N \leftrightarrow I \leftrightarrow U$. We define three global parameters H_N, H_I and H_U for the states, N, I and U respectively, which would reflect the effective hydrophobic exposure of these two states such that:

$$F_N = H_N.C_N, \tag{1}$$

$$F_I = H_I.C_I, \tag{2}$$

$$F_U = H_U.C_U, \tag{3}$$

where F_N, F_I and F_U are the fluorescence signal intensities for the N, I and U states at concentrations C_N, C_I and C_U, respectively. Neglecting the fluorescence intensity due to the free probe in solution, the total intensity (F) can be given by eq. (4):

$$F = H_N.C_N + H_I.C_I + H_U.C_U, \tag{4}$$

$$C = C_N + C_I + C_U. \tag{5}$$

Let $K^L_{N\to I}$, $K^L_{N\to U}$ be the equilibrium constants and $\Delta G^L_{N\to I}$, $\Delta G^L_{N\to U}$ be the corresponding free energy changes for the transitions $N.L \leftrightarrow I.L$ and $N.L \leftrightarrow U.L$ where L represents the fluorescent probe which is bound to the protein. The free energy changes for these transitions can be given as

$$K^L_{N\to I} = \exp(-\Delta G^L_{N\to I}/RT) = C_I/C_N, \tag{6}$$

$$K^L_{N\to U} = \exp(-\Delta G^L_{N\to U}/RT) = C_U/C_N. \tag{7}$$

Solving the eqs (4)–(7) for F, we get

$$F = C\frac{(H_N + H_I.e^{-\Delta G^L_{N\to I}/RT} + H_U.e^{-\Delta G^L_{N\to U}/RT})}{(1+e^{-\Delta G^L_{N\to I}/RT} + e^{-\Delta G^L_{N\to U}/RT})}. \tag{8}$$

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Assuming a linear dependence[8] of ΔG^L on denaturant concentration ([D]), we get eqs (9) and (10). m represents the slope of the plot for free energy of unfolding vs denaturant concentration.

$$\Delta G^L_{N\to I} = \Delta G^L_{N\to I}(H_2O) - m_{N\to I}[D], \quad (9)$$

$$\Delta G^L_{N\to U} = \Delta G^L_{N\to U}(H_2O) - m_{N\to U}[D]. \quad (10)$$

Substituting eqs (9) and (10) into eq. (8), gives F in terms of seven unknowns, H_N, H_I, H_U. $\Delta G^L_{N\to I}$ (H_2O), $\Delta G^L_{N\to U}$ (H_2O), $m_{N\to I}$ and $m_{N\to U}$. The equation thus obtained can be used to determine the unknowns by fitting the experimental data points using nonlinear regression analysis. Once the unknowns are obtained, the relative population (f_N, f_I, f_U) of the three states can be determined as follows using eq. (6) and eq. (7),

$$f_N = \frac{C_N}{(C_N + C_N.e^{-\Delta G^L_{N\to I}/RT} + C_N.e^{-\Delta G^L_{N\to U}/RT})} = \frac{1}{(1+e^{-\Delta G^L_{N\to I}/RT} + e^{-\Delta G^L_{N\to U}/RT})}.$$

Similarly,

$$f_I = \frac{e^{-\Delta G^L_{N\to I}/RT}}{(1+e^{-\Delta G^L_{N\to I}/RT} + e^{-\Delta G^L_{N\to U}/RT})};$$

$$f_U = \frac{e^{-\Delta G^L_{N\to U}/RT}}{(1+e^{-\Delta G^L_{N\to I}/RT} + e^{-\Delta G^L_{N\to U}/RT})}. \quad (11)$$

The method presented here can provide accurate description of the relative population of the states. It can also provide very good estimates for the free energy of transition in cases where the free energy of binding for ANS is not large compared to the folding energy. It should also be noted that the free energy of transition from $N \to U$, calculated using this method would give good estimates for proteins which have low binding affinity towards the probe in the native and denatured state, which is true in many cases.

Changes in fluorescence of *bis*-ANS added to α-LA at different concentrations of GuHCl were recorded (Figure 1). A three-state transition has been observed during folding of α-LA and the equilibrium intermediate state (often referred to as the molten globule state) has been well characterized in case of α-LA[9,10]. The observation of a major peak at 1.9 M GuHCl supports the formation of a molten globule-like intermediate, referred to here as I state, since formation of molten globule state has been reported to involve hydrophobic exposure. We attempted to calculate the free energy of unfolding and the relative populations of the different states of α-LA by fitting the observed data to eq. (8) (Figure 1 *a*). We obtained values for $\Delta G^L(H_2O)$ as 4.0, 0.9 and 4.9 kcal mol^{-1} for the $N \to I$, $I \to U$ and $N \to U$ transitions, respectively, which are in good agreement with the reported[9] values ($\Delta G(H_2O)$) of 4.1, 2.5 and 6.5 kcal mol^{-1}. The relative populations of the three states are shown in Figure 1 *b*, calculated using eq. (11).

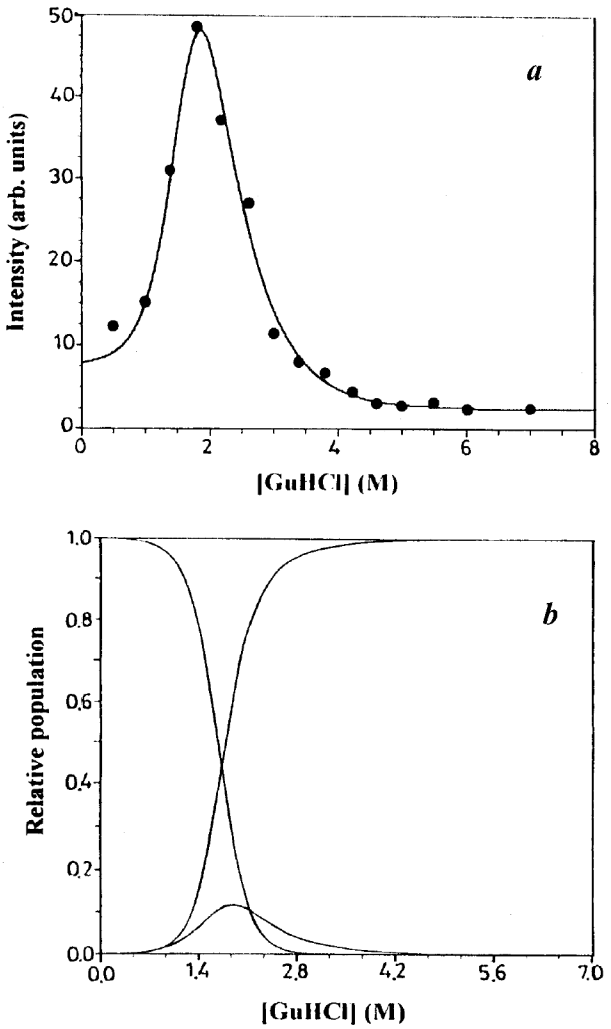

Figure 1. *a*, *bis*-ANS fluorescence data plotted against concentration of GuHCl fitted using eq. (8), for the three-state transition of α-LA at pH 6.5; *b*, Relative populations of the various states as determined using eq. (11), plotted against the denaturant concentration.

DT undergoes a pH-dependent conformational change so that the resulting folding intermediate can insert in membranes and subsequently translocate across the membrane. We recently reported that lowering of pH leads to hydrophobic exposure that can be monitored by hydrophobic photolabelling using diazofluorene as a photoactivable reagent[7]. The reagent binds and covalently labels the toxin, as the pH is lowered from 7.4 to 5.2. Analysis of this data using eq. (8) gave $\Delta G^L(H_2O)$ value of 0.7 kcal mol^{-1} for the $N \to I$ transition, indicating that this conformational transition that takes place in the low pH environment in the endosomes, is a facile process. We then studied the *bis*-ANS binding to DT to see if a low pH-like state can be detected during denatu-

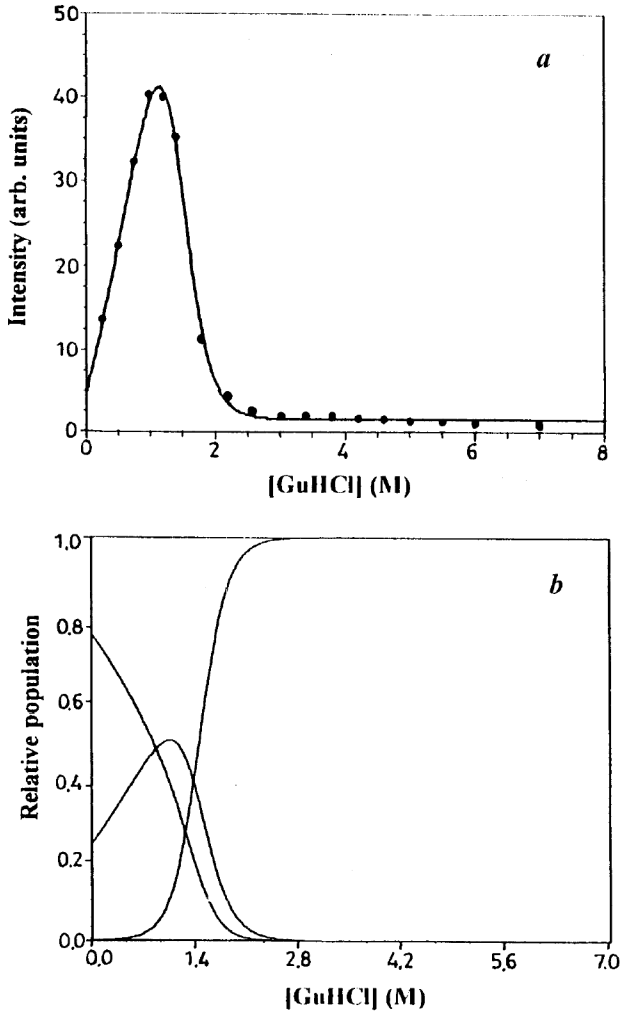

Figure 2. *a*, *bis*-ANS fluorescence data plotted against concentration of GuHCl fitted using eq. (8), for the three-state transition of DT at pH 6.5; *b*, Relative populations of the various states as determined using eq. (11), plotted against the denaturant concentration.

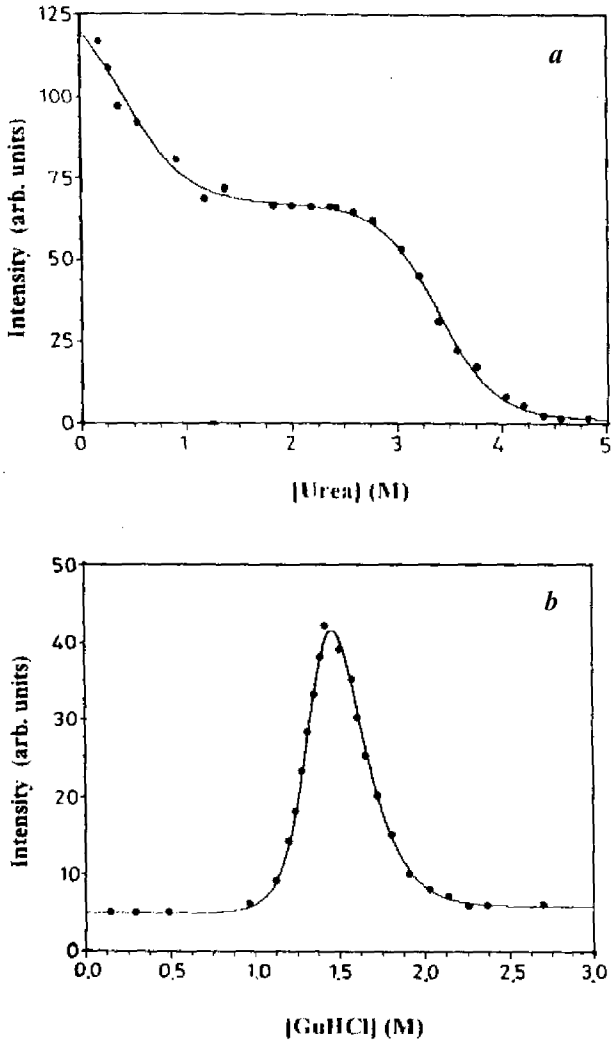

Figure 3. *a*, *bis*-ANS fluorescence data plotted against concentration of urea fitted using eq. (8), for GroEL (191–345); *b*, ANS fluorescence data plotted against concentration of GuHCl fitted using eq. (8) for bovine carbonic anhydrase.

ration by GuHCl. We then applied the same method for estimating $\Delta G^L(H_2O)$ for the three-state transition of DT using GuHCl as the denaturant (Figure 2 *a*). We obtained $\Delta G^L(H_2O)$ values of 0.6, 3.7 and 4.3 kcal mol^{-1} for the $N \rightarrow I$, $I \rightarrow U$ and $N \rightarrow U$ transitions, respectively. This implies almost 86% of the total free energy for folding is spent in the $I \rightarrow U$ transition and only 14% is spent in the $N \rightarrow I$ transition. The calculated value of $\Delta G^L(H_2O)$ for the $N \rightarrow U$ transition should be close to the actual value of $\Delta G(H_2O)$, since DT does not show substantial binding in its native or in the denatured state. The relative populations of the three states were also calculated (eq. (11)) and are shown in Figure 2 *b*.

To validate our method further, we compared the $\Delta G^L(H_2O)$ values determined from the current method with those of $\Delta G(H_2O)$ determined[5] from kinetic experiments for the transition states of GroEL (191–345) minichaperones. The plot of fluorescence vs concentration of urea is shown in Figure 3 *a*. Values of 0.8, 7.0 and 7.8 kcal mol^{-1} were obtained for $\Delta G^L(H_2O)$, compared to the reported $\Delta G(H_2O)$ values[5] of 1.6 ± 0.6, 3.8 ± 0.3 and 5.4 ± 0.6 kcal mol^{-1} for the $N \rightarrow I$, $I \rightarrow U$ and $N \rightarrow U$ transitions, respectively. $\Delta G^L(H_2O)$ has a lower value for $N \rightarrow I$ and a higher value for $I \rightarrow U$ transition compared to $\Delta G(H_2O)$, which implies that the intermediate is stabilized by the *bis*-ANS binding. The method was applied to the reported[11] fluorescence data for ANS binding of bovine carbonic anhydrase (Figure 3 *b*). The transition-free energies were calculated as 5.7, 8.7 and 14.4 kcal mol^{-1}, respectively for the $N \rightarrow I$, $I \rightarrow U$ and $N \rightarrow U$ transitions. The excellent fit obtained in all cases substantiates the validity of our method and indicates that this could be used as a simple method to

estimate free energy of folding ($\Delta G^{L}(H_2O)$) in solution and can be used to calculate the relative populations of the different states as illustrated earlier, in the case of α-LA and DT.

Previous studies have shown that ANS has maximum fluorescence emission when bound to molten globule states of proteins[1,3]. However, a quantitative model that could explain the ANS fluorescence data was not attempted before. Our present analysis suggests that simple binding studies with hydrophobic fluorescent probes can provide insight into the apparently complicated problem of protein folding. It should be noted that although the analysis presented here deals with only three state transitions, it can be extended to higher order transitions also, provided the probe is capable of detecting the intermediate states.

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