The Perturbations of the Native State of Goat α-Lactalbumin Induced by 1,1′-Bis(4-Anilino-5-Naphthalenesulfonate) Are Ca^{2+}-Dependent

Geertrui Vanderheeren, Ignace Hanssens, Katrien Noyelle, Herman Van Dael, and Marcel Joniau
Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, Kortrijk, Belgium

ABSTRACT In this work we have studied the interaction of the hydrophobic fluorescent probe 1,1′-bis(4-anilino-5-naphthalenesulfonate) (bis-ANS), with the native state of apo- and Ca^{2+}-bound goat α-lactalbumin (GLA). In 10 mM Tris-HCl, pH 7.5, at 4°C in 2 mM EGTA as well as at 37°C in 2 mM Ca^{2+}, the native protein is close to its thermal transition. Therefore, it can be expected that in both conditions the protein is equally susceptible to interaction with bis-ANS. Nevertheless, we have observed a number of interesting differences in the interaction of the dye with the apo and Ca^{2+} form. Native apo-GLA binds two bis-ANS molecules and in the complex with bis-ANS, the far-UV circular dichroism (CD) spectrum of apo-GLA becomes similar to that of the protein in the molten globule state. In contrast, native Ca^{2+}-GLA binds five bis-ANS molecules and the far-UV CD spectrum of native Ca^{2+}-GLA is conserved for the complex. In both cases, the high activation energies observed in kinetic experiments indicate that upon binding, large parts of the protein structure have to be reorganized. The reduced perturbation of the protein structure in the presence of Ca^{2+} can be attributed to local stabilization effects.

INTRODUCTION

For most globular proteins the folding mechanism is dominated by hydrophobic interactions. They are believed to play a main role in the formation of α-helices and β-sheet structures and also in the further folding to a structured active protein. Before reaching the definitive folds of the native state, an intermediate is formed with fluctuating tertiary structure. This partially folded protein is called a "molten globule." The residues in its hydrophobic core are accessible to external agents (Dolgikh et al., 1985; Ptitsyn, 1995; Kuwajima, 1996) and can effect binding interactions with hydrophobic probes, such as 1-anilinonaphthalene-8-sulfonate (ANS) or 1,1′-bis(4-anilino-5-naphthalenesulfonate) (bis-ANS). As the fluorescence quantum yield of ANS and bis-ANS greatly increases upon binding to hydrophobic sites, the interaction with molten globule-like intermediates lead to a large fluorescence increase. This property has been used to estimate the population of the molten globule state in protein folding studies (Ptitsyn et al., 1990; Teschke et al., 1993; Das and Surewicz, 1995).

In protein folding studies, α-lactalbumin has received considerable attention because the transitions among the compact native state, the molten globule state, and the more unfolded states can easily be realized and monitored by a large number of techniques (Kuwajima, 1989; Haynie and Freire, 1993). The protein has a native structure comparable to that of c-type lysozymes, but contains a Ca^{2+}-binding site. Ca^{2+} binding stabilizes the native conformation of α-lactalbumin. By heating the apo-protein, successive intermediately folded states are obtained (Griko et al., 1994). In a first stage the native conformation is converted into the molten globule state. At higher temperatures this intermediate, with its poorly defined tertiary but largely conserved α-helical structure, progressively unfolds through a series of conformations, each with less residual structure. Bis-ANS has been helpful in following the behavior of the hydrophobic domains along the folding pathway (Semisotnov, 1991; Vanderheeren and Hanssens, 1994; Tanaka et al., 1996). Two bis-ANS molecules strongly bind to bovine α-lactalbumin in its molten globule state. In stronger denaturing conditions the degeneration of the hydrophobic surface sites can be observed from the weakened dye-protein interaction.

Recently, the use of these probes for the characterization of the intermediate state has been questioned because they are suspected to induce changes in the native protein conformation. Coco and Lecomte (1994) revealed perturbations in the structure of native-like apomyoglobin upon ANS binding. Shi et al. (1994) observed that bis-ANS, which preferentially binds to the molten globule state of DnaK, shifts the equilibrium from native to molten globule. Engelhard and Evans (1995) observed that ANS, used in a kinetic study of the α-lactalbumin refolding process, stabilizes the dye-bound intermediates.

Despite these observations, few attempts have been made toward a systematic study of the structural perturbations caused by the binding of hydrophobic probes. In this work we examine the extent of the structural perturbations caused by the interaction of bis-ANS with native apo- and Ca^{2+}-bound goat α-lactalbumin (GLA) by static circular dichroism (CD), and by equilibrium and stopped-flow fluorescence spectroscopy. Goat α-lactalbumin is preferred to bovine α-lactalbumin because, at low temperatures, a clear native state of the apo-protein is obtained with spectroscopic properties similar to those of the Ca^{2+}-protein. Our results prove that the degree of interaction with bis-ANS, as well as the degree of the protein perturbation, depends on the presence of Ca^{2+}.

Received for publication 6 April 1998 and in final form 31 July 1998.

Address reprint requests to Dr. Ignace Hanssens, Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium. Tel.: 32-56-24.61.73; Fax: 32-56-24.69.97; E-mail: Ignace.Hanssens@kulak.ac.be.

0006-3495/98/11/2195/10 $2.00

MATERIALS AND METHODS

Materials

Goat α-lactalbumin is prepared from fresh milk whey. After addition of Tris and EDTA in a concentration of 50 and 1 mM, respectively, and adjustment of the pH to 7.5 with HCl, the whey is applied to a phenyl-Sepharose column from Pharmacia (Uppsala, Sweden). The apo-GLA binds hydrophobically to this column (Lindahl and Vogel, 1984), while the other whey proteins are eluted with the Tris buffer containing EDTA. To elute α-lactalbumin, the eluent is changed to 50 mM Tris, 1 mM Ca^{2+}, pH 7.5. The Ca^{2+}-GLA is subsequently demetallized as described earlier (Vanderheeren et al., 1996). Bis-ANS is obtained from Molecular Probes, Inc. (Eugene, OR). All experiments are performed in 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM Ca^{2+} or 2 mM EGTA. The mixtures of GLA and bis-ANS intended for equilibrium studies are incubated overnight at the desired temperature. The temperature in the different instruments is controlled by circulating water through the cell holder and is monitored by a thermocouple dipped into the solution.

Circular dichroism

The CD measurements are carried out on a Jasco J-600 spectropolarimeter (Tokyo, Japan). Cuvettes of 5 (or 10) mm and 1 mm are used for the near-UV and far-UV regions, respectively. The GLA concentration is ~25 μM. The protein-bis-ANS mixtures for thermal transition studies are preincubated overnight at the lowest temperature. At each temperature of the transition curve, the measurements are started 5 min after temperature equilibration of the sample. Evidence that equilibrium states are obtained is given by the fact that the ellipticity values are identical in heating and cooling runs, provided they have not been exposed to >70°C for several minutes.

Fluorescence measurements

The static fluorescence measurements are performed to obtain equilibrium binding data for the interaction of bis-ANS with GLA as well as to study energy transfer from GLA to bis-ANS. These measurements are carried out on an Aminco SPF-500 spectrofluorimeter (Rochester, NY). As described earlier (Vanderheeren and Hanssens, 1994), the equilibrium binding data for the interaction of bis-ANS with GLA are obtained from two fluorescence titration curves, both at equal pH, temperature, and Ca^{2+} content. First, the limit fluorescence increase of 1 μM bis-ANS is determined in the presence of increasing amounts of GLA. Next, that value is used to calculate the concentration of bound bis-ANS from the fluorescence increase on titrating 1 μM GLA with bis-ANS. For both titrations the excitation is at 465 nm, which is situated on the edge of the bis-ANS absorption peak. At this wavelength, even at 400 μM bis-ANS the absorbance does not exceed the value of 0.2. The fluorescence emission intensity between 515 and 550 nm is integrated. The background fluorescence of free bis-ANS is subtracted and the resulting fluorescence increase is corrected for inner filter effects.

If tryptophan and bis-ANS are close to each other they constitute a good fluorescence energy-transfer donor-acceptor pair. For these energy-transfer experiments the excitation is at 290 nm, the absorption maximum for tryptophan. The fluorescence emission intensity of tryptophan residues between 300 and 420 nm is measured and corrected for inner filter effects at the emission and excitation wavelengths.

Kinetic fluorescence studies

All kinetic studies are conducted using an SX.17 MV sequential mixing stopped-flow spectrophotometer from Applied Photophysics (Leatherhead, UK). The stopped-flow module and the observation cell with 2 mm pathlength are thermostated by circulating water. A double monochromator is used, one for excitation at 425 nm and one for emission at 485 nm. The dead time of the instrument is 1.8 ms. Typically, kinetics are measured 10 times in a 1:1 mixing ratio under identical conditions. The concentrations after mixing are 10 μM for GLA and 85–260 μM for bis-ANS. The fluorescence intensity, I, as a function of time, t, is averaged and is found to be fit by a monoexponential function:

$$I(t) = A + B \exp(-t/\tau). \quad (1)$$

The inverse of the relaxation time, τ, corresponds to the apparent rate constant, k_{app}. In each kinetic experiment the baseline indicating the fluorescence of bis-ANS in the absence of GLA is recorded in order to fix the global fluorescence increase due to the dye-protein interaction.

Photolabeling of bis-ANS to GLA

It has been shown that bis-ANS can be covalently photobound to various proteins (Gorovits et al., 1995; Seale et al., 1995). For some measurements we needed a large association of bis-ANS to native Ca^{2+}-GLA with small free dye concentration. To obtain this, a mixture of 35 μM GLA and 150 μM bis-ANS in Ca^{2+} buffer, thermostated at 37°C, is irradiated at a 366-nm wavelength for 2 h using a UV 131000 lamp from Desaga (Heidelberg, Germany). Next, the irradiated sample is applied to and eluted from a PD-10 column containing Sephadex G-25 (Pharmacia). The protein with the covalently bound bis-ANS passes through the column together with the void volume, while the free and noncovalently bound bis-ANS is retarded. In the eluate obtained in this way, on average 3.3 bis-ANS molecules are covalently bound at 1 Ca^{2+}-GLA molecule.

RESULTS

Thermal unfolding of GLA

Before studying the nature and the extent of the structural perturbations occurring when bis-ANS interacts with native apo- and Ca^{2+}-GLA, we first determined the thermal transitions of the protein in the presence and absence of Ca^{2+}. The unfolding of α-lactalbumin is readily monitored by the change of its near-UV ellipticity with temperature. Because of the immobilization of aromatic side chains by specific contacts, native α-lactalbumin shows a pronounced negative ellipticity near 270 nm. In the partially unfolded state of the protein, the interactions of the aromatic residues are randomized and the near-UV ellipticity approaches zero (Segawa and Sugai, 1983). The squares in Fig. 1 represent the mean residue ellipticity at 270 nm of GLA at different temperatures. From the figure it can be deduced that at pH 7.5 in a solution containing 10 mM Tris-HCl and 2 mM EGTA (Fig. 1, *filled squares*), the protein is in the fully native conformation only at temperatures below 10°C. By replacing EGTA for 2 mM Ca^{2+} (Fig. 1, *open squares*), the native protein conformation remains stable up to 55°C. The increased thermal stability resulting from Ca^{2+} binding is a general property of all α-lactalbumins (Acharya et al., 1989). Next, we concentrated on the comparison of the effects of bis-ANS interaction with native GLA at 4°C in 2 mM EGTA with those at 37°C in 2mM Ca^{2+}. In both conditions the protein is in the native state close to the thermal transition and shows the same spectroscopic properties. Therefore, although the temperatures are different, it can be expected to be equally susceptible to perturbing factors.

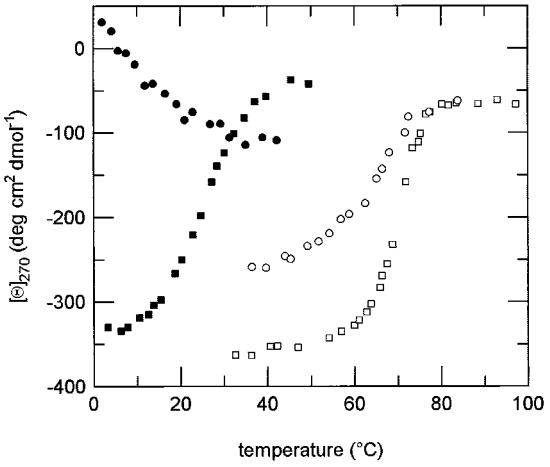

FIGURE 1 Thermal transition curves of GLA measured by the ellipticity changes at 270 nm. Conditions: 25 μM GLA in 10 mM Tris-HCl, pH 7.5 and 2 mM EGTA (■) or 2 mM Ca^{2+} (□), after equilibration with 60 μM bis-ANS in 2 mM EGTA (●), containing 3.3 photolabeled bis-ANS molecules per GLA in 2 mM Ca^{2+} (○).

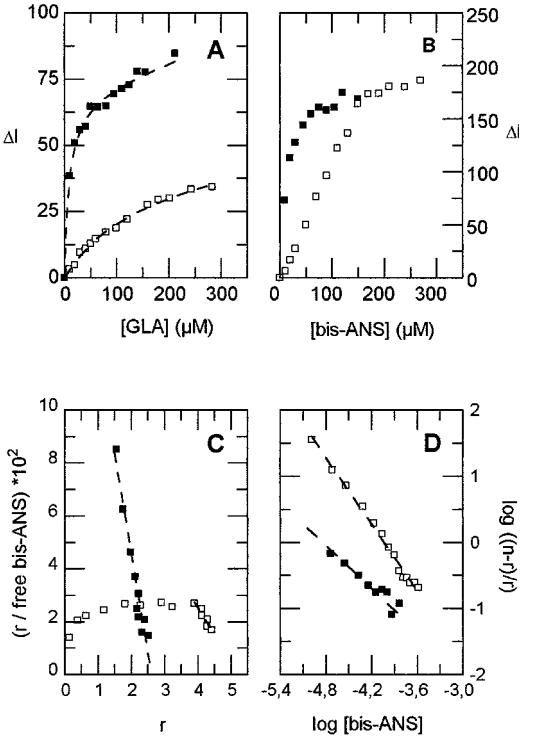

FIGURE 2 Fluorescence titrations of 1 μM bis-ANS with GLA (*A*) and 1 μM GLA with bis-ANS (*B*). Scatchard plots (*C*) and Hill plots (*D*) for the titration of 1 μM GLA with bis-ANS. Solvent conditions: 10 mM Tris-HCl, pH 7.5, 4°C and 2 mM EGTA (■), or 37°C and 2 mM Ca^{2+} (□). Fluorescence parameters: λ_{ex} = 465 nm, λ_{em} = 515–550 nm. Dashed lines represent optimal curve fittings.

Equilibrium binding of bis-ANS to GLA

First, we determined the binding properties of bis-ANS to GLA in the previously selected conditions. The data are obtained from two series of fluorescence titration curves, as described in Materials and Methods. The strong fluorescence increase at the start of the titrations of 1 μM bis-ANS with apo-GLA and of those of 1 μM apo-GLA with bis-ANS (Fig. 2, *A* and *B*, *filled squares*) are characteristic for the strong binding of (one or more) fluorophore molecules to the protein. As can be expected for such a strong binding, the fluorescence increase for the titration of apo-GLA with bis-ANS (Fig. 2 *B*, *filled squares*) readily tends to a limit. However, this limit is not obtained by the addition of apo-GLA to bis-ANS (Fig. 2 *A, filled squares*). It is judged that the most plausible explanation for this continued fluorescence increase at higher apo-GLA concentrations is self-association of the protein. By such self-association, bound bis-ANS is expected to be protected further from the water phase. This provokes a further increase of its fluorescence at protein concentrations for which the probe is already completely bound. Therefore, the maximum fluorescence increase of 1 μM bis-ANS at a low protein concentration (as is used in the experiments presented in Fig. 2 *B*) is estimated by the procedure elaborated previously (Vanderheeren and Hanssens, 1994). These maximum values, expressed in arbitrary units, are 66 ± 13 and 38 ± 7 for the binding of 1 μM bis-ANS to apo-GLA and Ca^{2+}-GLA, respectively. They are used to calculate the concentration of bound bis-ANS from the fluorescence increase observed on titrating 1 μM GLA with bis-ANS (Fig. 2 *B*).

The curve is slightly S-shaped for the titration of 1 μM native Ca^{2+}-GLA with bis-ANS at 37°C (Fig. 2 *B, open squares*). This is indicative of positive cooperativity for the dye binding, which is confirmed by the clear concave-downward shape of the Scatchard plot (Fig. 2 *C, open squares*). However, neither the hyperbolic titration curve nor the linear Scatchard plot gives any indication of cooperative behavior for the bis-ANS interaction with native apo-GLA at 4°C (Fig. 2, *B* and *C, filled squares*).

TABLE 1 Characteristics for the bis-ANS binding to GLA

		Scatchard Plot		Hill Plot	
Buffer	*T* (°C)	K_b (M^{-1})	*n*	K_b (M^{-1})	*m*
EGTA	4	(7.5 ± 1.5) 10^4	2.6 ± 0.6	(6.9 ± 1.4) 10^4	1.08 ± 0.1
	42	(4.5 ± 0.9) 10^4	1.8 ± 0.4	(3.9 ± 0.8) 10^4	1.04 ± 0.1
Ca^{2+}	37	n.d.*	5.3 ± 1.1	(1.1 ± 0.2) 10^4	1.67 ± 0.2

The data in 2 mM EGTA at 4°C and in 2 mM Ca^{2+} at 37°C are obtained from the Scatchard plot and the Hill plot of Fig. 2, *C* and *D*. The data in 2 mM EGTA at 42°C were derived from similar experiments.

*n.d., Not determinable from a nonlinear Scatchard plot.

From the abscis intercept of the Scatchard plot the total number of bis-ANS binding sites is found to be ~5.3 ± 1.1 for native Ca^{2+}-GLA and 2.6 ± 0.6 for native apo-GLA. In conditions when apo-GLA adopts the molten globule form (42°C) the number of binding sites measured equals 1.8 ± 0.4 (Table 1). To determine the corresponding degree of cooperativity, the binding data are plotted according to the Hill equation (Fig. 2 *D*).

$$\log[(n - r)/r] = -m \log K_b - m \log[\text{bis-ANS}] \quad (2)$$

where K_b is the average binding constant of bis-ANS to GLA, n is the number of binding sites, r is the number of sites occupied, and m is the Hill coefficient, which expresses the degree of cooperativity. The data of both titrations fit the linear relationship of the Hill equation. The Hill coefficients determined from the slopes are 1.08 ± 0.1 and 1.67 ± 0.2 for apo- and Ca^{2+}-GLA, respectively. The values confirm that for the bis-ANS interaction with native apo-GLA no cooperativity can be detected ($m \approx 1$), whereas for the dye interaction with native Ca^{2+}-GLA a positive cooperativity has been found ($m > 1$).

Kinetics of bis-ANS binding to GLA

The rate of the bis-ANS interaction with GLA has been determined from the dye fluorescence increase as a function of time. Fig. 3, *A* and *B* show examples of this fluorescence increase at different temperatures as a function of a logarithmic time scale, in the absence and presence of Ca^{2+}, respectively. All curves are characterized by an initial jump of the bis-ANS fluorescence within the dead time (1.8 ms) and by subsequent slow fluorescence increase. In 2 mM EGTA, the temperature range of the experiments partly coincides with the transition region of GLA and the early fluorescence jump gains importance with temperature (Fig. 3 *A*). This fluorescence jump is proportional to the fraction of apo-GLA in the molten globule state, which can be calculated from the transition curve of apo-GLA (Fig. 1, *filled squares*). As a conclusion we can state that the probe adsorption to partially unfolded GLA is very fast and results in an immediate fluorescence increase.

In 2 mM EGTA as well as in 2 mM Ca^{2+}, the fluorescence intensity of the slow phases fits Eq. 1 (Fig. 3, *A* and *B*). The corresponding apparent rate constants are presented in Fig. 4, *A* and *B*. At each temperature in 2 mM EGTA, these constants relate linearly to the square of the probe concentration. This concentration dependence proves that bis-ANS enhances the rate at which hydrophobic groups in native GLA become accessible, and therefore it indicates that bis-ANS directly interacts with native apo-GLA. In 2 mM Ca^{2+} the apparent rate constants do not depend on the bis-ANS concentration. This could be interpreted as though the probe only interacts with Ca^{2+}-GLA to the extent to which the conformational equilibrium of the protein shifts toward the denatured state. However, as bis-ANS directly interacts with compact apo-GLA, stimulating the structural conversion of the protein, it likely interacts in the same way with native Ca^{2+}-GLA. The small but significant initial fluorescence jump obtained on mixing bis-ANS with native Ca^{2+}-GLA (Fig. 3 *B*) is an experimental indication of this immediate interaction. Next, we will explain how this interaction mechanism results in a different bis-ANS concentration dependence of the apparent rate constants in the presence and absence of Ca^{2+}.

As the dye adsorption to the compact native protein is much faster than its penetration into the protein interior, the equilibrium of the former reaction step will continuously adapt to the progress of the overall reaction. If the perturbation of the native state is caused by the simultaneous penetration of two adsorbed bis-ANS molecules into GLA,

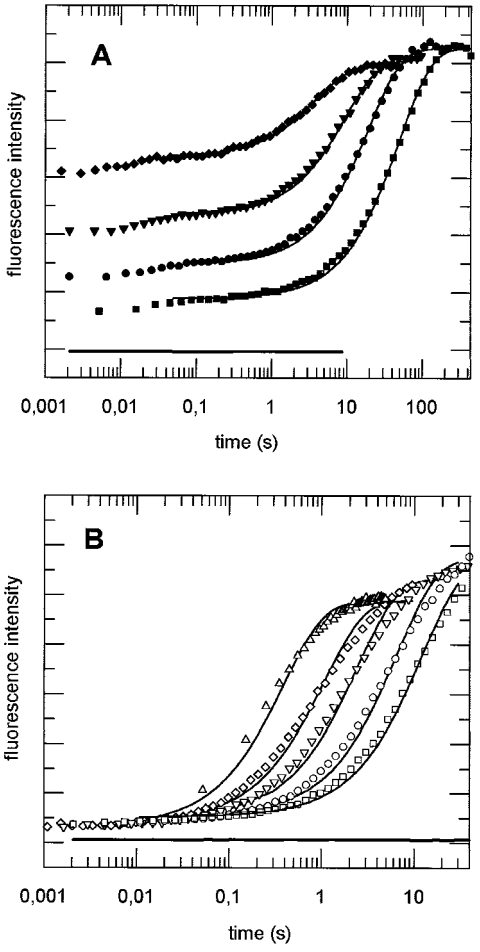

FIGURE 3 Fluorescence increase at different temperatures on mixing bis-ANS and GLA in a 1:1 mixing ratio as a function of a logarithmic time scale. After mixing, the concentrations are 10 μM for GLA and 187.5 μM for bis-ANS. Solvent conditions: 10 mM Tris-HCl, pH 7.5 and 2 mM EGTA (*A*) or 2 mM Ca^{2+} (*B*). In 2 mM EGTA the experimental temperatures are 12.4°C (■), 20.3°C (●]), 24.4°C (▼), and 30.0°C (♦). In 2 mM Ca^{2+} the experimental temperatures are 25.5°C (□), 30.1°C (○), 35.7°C (▽), 39.7°C (◇), and 45.7°C (△). The smooth line through the data is fitted according to a monoexponential function. The horizontal line below represents the fluorescence increase for 187.5 μM bis-ANS in the absence of GLA at all the considered temperatures. Fluorescence parameters: λ_{ex} = 425 nm, λ_{em} = 485 nm.

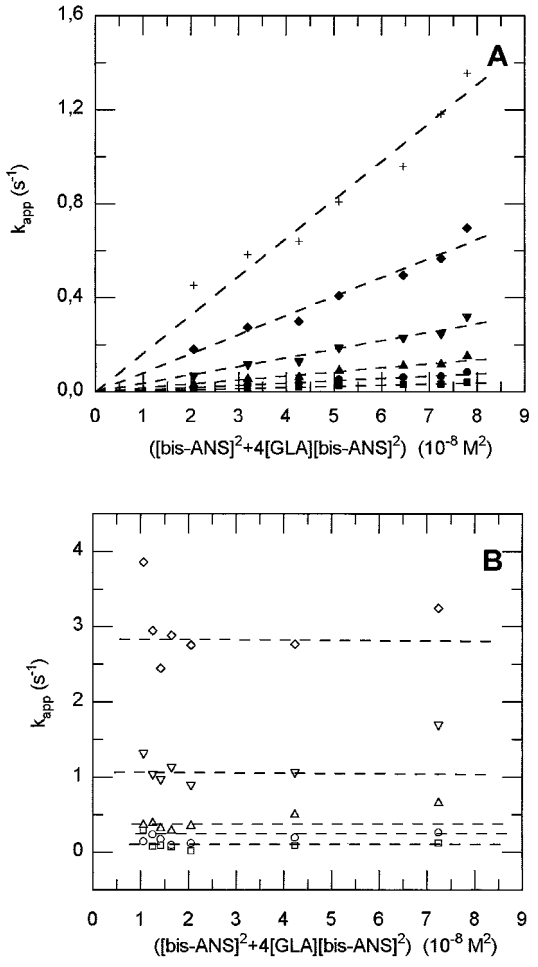

FIGURE 4 Apparent rate constants (k_{app}) as a function of the concentration term in the denominator of Eq. 3 in 2 mM EGTA (*A*) or 2 mM Ca^{2+} (*B*). In 2 mM EGTA the different temperatures are 12.1°C (■), 17.1°C (●), 21.0°C (▲), 25.8°C (▼), 30.8°C (♦), and 35.0°C (+). In 2 mM Ca^{2+} the different temperatures are 25.0°C (□), 30°C (○), 35.1°C (△), 39.9°C (▽), and 45.0°C (◇).

one can write

$$GLA_N + 2\ \text{bis-ANS} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} GLA_N \cdot 2\text{bis-ANS}_{nfl}$$

$$GLA_N \cdot 2\text{bis-ANS}_{nfl} \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} GLA_I \cdot 2\text{bis-ANS}_{fl}$$

In this reaction outline, GLA_N refers to native GLA, $GLA_N \cdot 2\text{bis-ANS}_{nfl}$ represents an intermediate nonfluorescent complex of native GLA and the probe, and $GLA_I \cdot 2\text{bis-ANS}_{fl}$ refers to the transformed complex in which the probe molecules interact with the hydrophobic interior of the protein, and therefore show fluorescence increase. In an analogy with the calculations made for comparable two-step systems (Bernasconi, 1976) the apparent rate constant (k_{app}) for the fluorescence increase can be written as

$$k_{app} = \frac{k_2 K_1([\text{bis-ANS}]^2 + 4[\text{GLA}][\text{bis-ANS}]^2)}{1 + K_1([\text{bis-ANS}]^2 + 4[\text{GLA}][\text{bis-ANS}]^2)} + k_{-2} \quad (3)$$

where K_1, the equilibrium constant for the first reaction step, equals k_1/k_{-1}; k_2 and k_{-2} are the rate constants for the direct and reversed second step.

If $1 \gg K_1$ ([bis-ANS]^2 + 4[GLA][bis-ANS]^2), Eq. 3 transforms to

$$k_{app} = k_2 K_1([\text{bis-ANS}]^2 + 4[\text{GLA}][\text{bis-ANS}]^2) + k_{-2} \quad (4)$$

The linear relation between the rate constant of native apo-GLA and the concentration term shown in Fig. 4 *A* is in agreement with this expression. At each temperature the combined constant $k_2 K_1$ is determined from the slopes of these straight lines and listed in Table 2. The ordinate intercepts at the different temperatures are near zero, indicating that the rate constants for the inverse reaction of step 2 (k_{-2} values) are very small.

If $1 \ll K_1$ ([bis-ANS]^2 + 4[GLA][bis-ANS]^2), Eq. 3 reduces to

$$k_{app} = k_2 + k_{-2}, \quad (5)$$

and the apparent rate constant does not depend on the concentration of the reagents.

For the Ca^{2+}-bound protein more than two bis-ANS molecules bind to one GLA. Therefore, in Eq. 3 additional terms will appear. Nevertheless, analogous to our previous reasoning, the expression reduces to Eq. 5 if the combined value of the concentration terms is importantly >1. At each temperature the mean value for k_{app} ($\approx k_2$) is calculated and shown in Table 2.

Conformational change induced by bis-ANS

The slow kinetic phase in the fluorescence increase of bis-ANS on its interaction with compact native GLA can be related to hydrophobic contacts that are created between the protein and the probe. The question that arises is to what degree this interaction induces perturbations or more pronounced conformational changes in GLA.

From the previous kinetic analysis we deduced the combined constant k_2K_1 for the interaction with native apo-GLA and k_2 for the interaction with native Ca^{2+}-GLA (Table 2). The Arrhenius plots representing the logarithm of these constants as a function of $1/T$ (Fig. 5) are linear in the considered temperature regions. From the slopes it can be

TABLE 2 Rate constants for apo-GLA and Ca^{2+}-GLA

apo-GLA		Ca^{2+}-GLA	
T (°C)	k_2K_1 (s^{-1} l^2 mol^{-2})	T (°C)	k_2 (s^{-1})
12.1	$5.31\ 10^5$	25.0	$8.13\ 10^{-2}$
17.1	$1.16\ 10^6$	30.0	$1.66\ 10^{-1}$
21.0	$2.10\ 10^6$	35.1	$3.92\ 10^{-1}$
25.8	$4.39\ 10^6$	39.9	1.13
30.8	$9.20\ 10^6$	45.0	2.96
35.0	$2.00\ 10^7$	50.0	8.18

The constant for apo-GLA is a combined constant k_2K_1 obtained from the slopes of the curves in Fig. 4 *A*. The rate constant k_2 for Ca^{2+}-GLA is determined from the curves in Fig. 4 *B*.

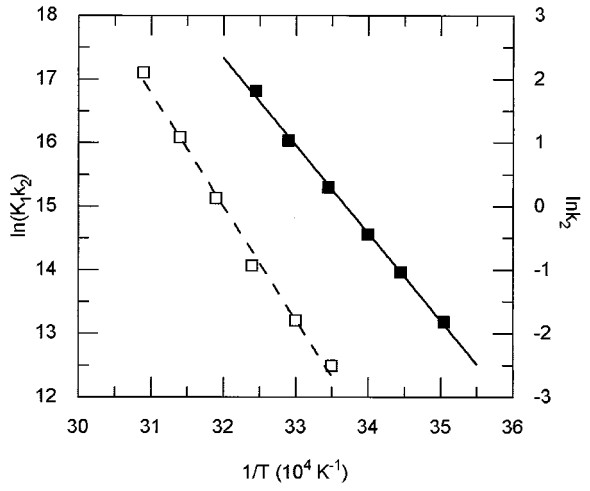

FIGURE 5 Arrhenius plots of the combined constants k_2K_1 in 2 mM EGTA (■) and the mean apparent rate constant k_2 in 2 mM Ca^{2+} (□). The values of the combined constants k_2K_1 at different temperatures in 2 mM EGTA are obtained from the slopes of the lines in Fig. 4 *A*. The values of the mean apparent constants k_2 in 2 mM Ca^{2+} are obtained from Fig. 4 *B*.

deduced that the activation energy in 2 mM EGTA is 110 kJ/mol and amounts to 148 kJ/mol in 2 mM Ca^{2+}. The mean temperatures of the Arrhenius plots are 25°C and 37°C, respectively; therefore, the values of the activation energy actually relate to these temperatures. To compare the activation energy with the enthalpy needed for the transition from the native state to the molten globule state, the latter quantity is calculated from the transition curve in Fig. 1 (*filled squares*). The calculated enthalpy at 25°C is 154 kJ/mol, considerably larger than the activation energy for the bis-ANS penetration into native apo-GLA. The different enthalpy gain for obtaining the molten globule state and the activated complex interacting with bis-ANS may be due partially to a different degree of fluidization at both states, and partially to the realization of hydrophobic contacts with bis-ANS in the activated complex. As the contribution of hydrophobic contacts in the activated complex is endothermic, the enthalpy gain, which has to be ascribed to the fluidization of the protein, is larger for the molten globule state than for the activated complex and will be even larger than the calculated values. It can be concluded that the native state of apo-GLA does not fully fluidize, as it would in the molten globule state, upon penetration of bis-ANS into the hydrophobic interior.

For Ca^{2+}-GLA we know the activation energy for the bis-ANS penetration at 37°C. To compare this value with the transition enthalpy we need to extrapolate the latter to the same temperature. The value for δC_p related to the transition enthalpy of bovine α-lactalbumin is 4 kJ/K · mol (Vanderheeren et al., 1996). If this quantity is also valid for GLA, the transition enthalpy at 37°C will amount to 202 kJ/mol. Again, the transition enthalpy is significantly larger than the activation energy (148 kJ/mol). As a consequence, native Ca^{2+}-GLA, at the kinetic transition state that allows bis-ANS to penetrate in between hydrophobic residues, is not fluidized to the degree of a molten globule.

Information on the conformational changes that occur in the GLA-bis-ANS complexes is gathered from protein ellipticity in the far- and near-UV wavelength regions (Fig. 6, *A* and *B*). In the absence of bis-ANS the far- and near-UV CD spectra of native apo-GLA at 4°C and Ca^{2+}-GLA at 37°C are quite similar, in agreement with their similar states. In 60 μM bis-ANS, the absolute values for the far-UV ellipticity of apo-GLA at 4°C (Fig. 6 *A*) are decreased at wavelengths near 222 nm, and increased near 208 nm, as compared to the native state. A comparable behavior is observed in the molten globule state of GLA as shown by the far-UV spectrum of apo-GLA at 42°C. The similar shape of both far-UV CD spectra indicates that the penetration of the dye in native apo-GLA causes changes in the secondary structure comparable to the formation of a molten

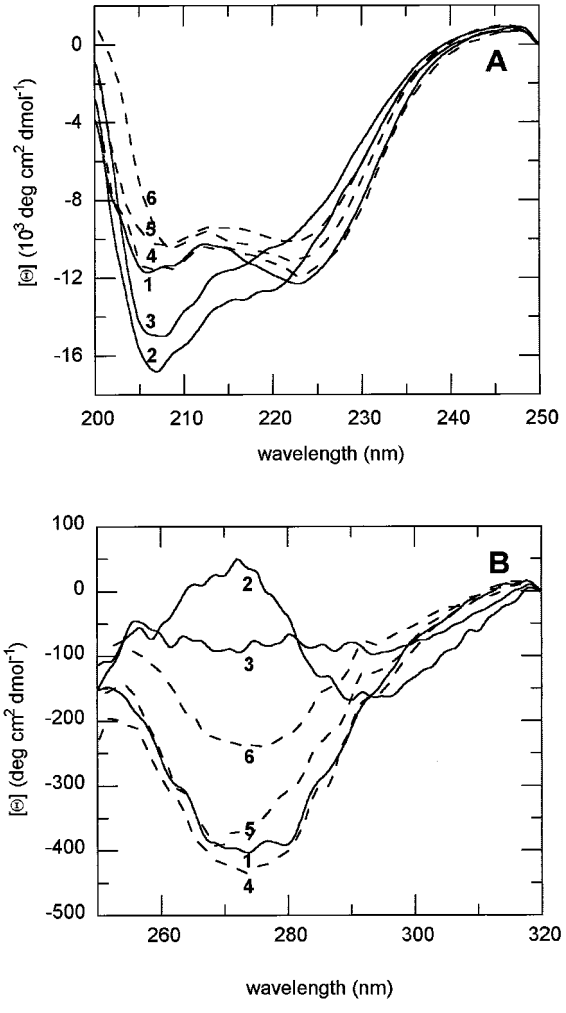

FIGURE 6 Circular dichroism spectra of GLA at different conditions in the far (*A*) and near (*B*) ultraviolet regions. In 2 mM EGTA at 4°C (1), in 2 mM EGTA and 60 μM bis-ANS at 4°C (2), in 2 mM EGTA at 42°C (3), in 2 mM Ca^{2+} at 37°C (4), in 2 mM Ca^{2+} and 60 μM bis-ANS at 37°C (5), containing 3.3 photolabeled bis-ANS molecules per GLA in 2 mM Ca^{2+} at 37°C (6).

globule. It is likely that the changes are local unfoldings that must allow some expansion of the protein. Also, the near-UV properties of apo-GLA change substantially by the addition of 60 μM bis-ANS. However, the resulting near-UV spectrum is clearly more structured than the spectrum of the molten globule state obtained at 42°C (Fig. 6 *B*). This indicates that the aromatic side chains of apo-GLA at 4°C preferentially conserve more anisotropic interactions in 60 μM bis-ANS than in the thermally induced molten globule state, and thus remain relatively immobile. This idea agrees with the fact that the activation energy for the bis-ANS penetration does not exceed the transition enthalpy. The immobility of the aromatic residues within the complex is also proven from the temperature dependence of their ellipticity at 270 nm (Fig. 1, *filled circles*). The ellipticity value progressively decreases as a function of the temperature and approaches the value of the thermally induced molten globule. Visibly, this curve represents a part of the thermal unfolding of the GLA-bis-ANS complex, for which the transition is shifted to a lower temperature than for the original apo-GLA.

At 37°C and 2 mM Ca^{2+}, the far- and near-UV CD spectra hardly change by the presence of 60 μM bis-ANS, indicating that the conformation of the compact native Ca^{2+}-GLA remains intact. From Fig. 2 *B* it is obvious that this limited influence may be related to the weak dye interaction in these conditions. To obtain saturation, high dye concentrations (>100 μM) would be required. Because of the strong light absorption of bis-ANS at 270 nm, CD measurements are compromised in such conditions. To circumvent the problem we have photolabeled the protein with the interacting dye, which allows observations at relatively low free dye concentrations. As explained in Materials and Methods, by photolabeling and chromatography we obtained a sample with 3.3 bis-ANS molecules irreversibly bound per Ca^{2+}-GLA molecule. Although its number of bound probe molecules is larger than that for apo-GLA at saturation, the far-UV CD spectrum of the labeled Ca^{2+}-GLA · bis-ANS complex (Fig. 6 *A*) shows the typical shape of native GLA with conserved secondary structure elements. Similarly, the residual ellipticity in the near-UV (Fig. 6 *B*) indicates that the bound dye molecules only partially disrupt the tertiary structure. In addition, the stability of the tertiary structure is lowered, as shown by the decrease in transition temperature from 70°C to 62°C (Fig. 1, *open circles*).

Fluorescence energy transfer experiments

Bis-ANS and tryptophan close to each other constitute a good fluorescence energy-transfer donor/acceptor pair because of the overlap of tryptophan emission and bis-ANS excitation. Fig. 7 shows the influence of bis-ANS on the tryptophan fluorescence spectra of originally compact native GLA. The pronounced decrease of the tryptophan fluorescence intensity (Fig. 7 *A*), obtained for apo-GLA in the presence of bis-ANS at 4°C, shows that an important part of

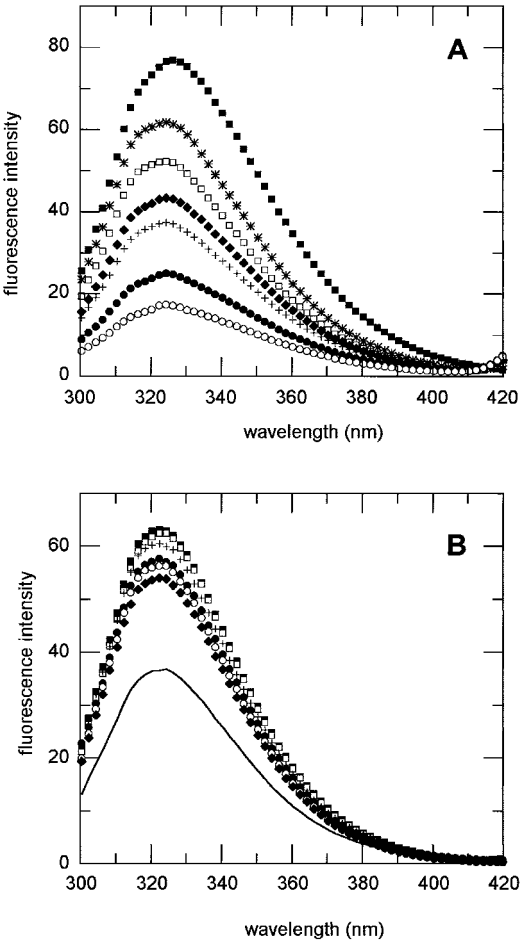

FIGURE 7 Reduction of the tryptophan emission fluorescence as a consequence of energy transfer to bis-ANS. (*A*) Fluorescence spectra of 10 μM GLA in 2 mM EGTA, at 4°C and increasing bis-ANS concentration: 0 μM (■), 5 μM (*), 10 μM (□), 15 μM (♦), 20 μM (+), 30 μM (●), and 40 μM (○). (*B*) Fluorescence spectra of 10 μM GLA in 2 mM Ca^{2+}, 37°C and increasing bis-ANS concentration: 0 μM (■), 10 μM (□), 20 μM (+), 30 μM (●), 40 μM (○), and 50 μM (♦) or containing 3.3 photolabeled bis-ANS molecules per GLA (*solid line*). Fluorescence parameters: λ_{ex} = 290 nm, λ_{em} = 300–420 nm.

the tryptophan fluorescence energy is transferred to the dye. The interaction of bis-ANS with Ca^{2+}-GLA at 37°C does not result in a comparable energy transfer (Fig. 7 *B*). Even the tryptophan fluorescence of the bis-ANS-labeled Ca^{2+}-GLA is not reduced to the same degree as in the case of apo-GLA. These data agree with the observations from the CD measurements, making it clear that bis-ANS induces more conformational change in native apo-GLA than in the native Ca^{2+}-protein. Indeed, dye molecules binding to buried tryptophan residues induce more conformational changes than the dye molecules, which are more distant from the tryptophan groups and therefore are more peripherally located.

DISCUSSION

The present study reveals a number of interesting differences between the interaction of bis-ANS with native apo-GLA and native Ca^{2+}-GLA in conditions in which both are

equally far from their thermal transition temperature. Native apo-GLA at 4°C binds to two bis-ANS molecules, the same number as is observed for apo-GLA in its molten globule state at 42°C (Table 1). The interaction with the dye at 4°C induces a far-UV CD spectrum of the native apo-protein that corresponds to that of the molten globule conformation (Fig. 6 *A*). However, the near-UV CD spectrum (Fig. 6 *B*) indicates that the aromatic side chains are more preferentially oriented in this dye-induced conformation than in the temperature-induced molten globule. The efficient fluorescence quenching (Fig. 7 *A*) indicates that the binding of bis-ANS to the apo-protein occurs at locations in the vicinity of the tryptophan groups. Although the hydrophobic contacts, leading to an increased fluorescence intensity of bis-ANS, are realized at a rate second-order in dye concentration (Fig. 4 *A*), no cooperativity effects are detected from the binding equilibria (Fig. 2).

In contrast, up to five dye molecules are involved in the complex formation of bis-ANS with native Ca^{2+}-bound GLA, whereas in this case the secondary and tertiary structures are less disturbed (Fig. 6). Correspondingly, the relatively small reduction of the tryptophan fluorescence suggests that in the complex with Ca^{2+}-GLA, the dye molecules are located far from those aromatic groups (Fig. 7 *B*). Finally, the equilibrium study clearly shows cooperativity for the dye binding (Fig. 2), although the rate of the fluorescence increase is characterized by an apparent zero order in the dye concentrations (Fig. 4 *B*).

Further discussions will focus on the dye-induced conformational changes, kinetics, and cooperativity of the bis-ANS binding, respectively.

Bis-ANS-induced conformational changes

By its interaction at 4°C, bis-ANS induces a change in conformation of the native apo-GLA comparable to that of the native-to-molten globule state transition (Fig. 6 *A*). However, the conformation adopted by the molecule is not that of a molten globule, as shown by the structured CD spectrum in the near-UV (Fig. 6 *B*), which is indicative of immobilized tyrosine and tryptophan nuclei. At 37°C in the presence of Ca^{2+} the effect of bis-ANS on the protein conformation is not so pronounced. Even at high dye concentrations the secondary and tertiary structures of the native state are better conserved than in the absence of Ca^{2+}. The hydrophobic interaction, which is presumably the major contributor to bis-ANS binding, is endothermic and favored by higher temperatures. Therefore, the smaller effect that bis-ANS has on the conformational change of the Ca^{2+}-protein at 37°C than on the apo-protein at 4°C is not the consequence of a weakened interaction tendency of the probe. The smaller perturbations of Ca^{2+}-GLA are rather the consequence of a reduction of the probe access to hydrophobic regions of the protein. Nevertheless, native apo-GLA and Ca^{2+}-GLA are at the considered temperatures close to their thermal destabilization (Fig. 1, *squares*). The observation that the native apo-protein as well as the native Ca^{2+}-protein go through a high energetic transition state in the realization of hydrophobic contacts with bis-ANS (Fig. 5) confirms that in both conditions large parts of the global protein structure are being reorganized. Therefore, the reduction of the perturbation in the final Ca^{2+}-GLA · bis-ANS complex is not due to the stabilization of GLA as a whole. It is rather due to stiffening of local structure by Ca^{2+}. The inspection of the protein structure proves the ability for such a local effect. One of the hydrophobic clusters of α-lactalbumin consists of Phe-53, Trp-60, Tyr-103, and Trp-104 (Fig. 8). The cluster is situated in the crevice that divides the protein in two structural halves and contains aromatic groups that belong to both halves. The Ca^{2+}-binding loop of

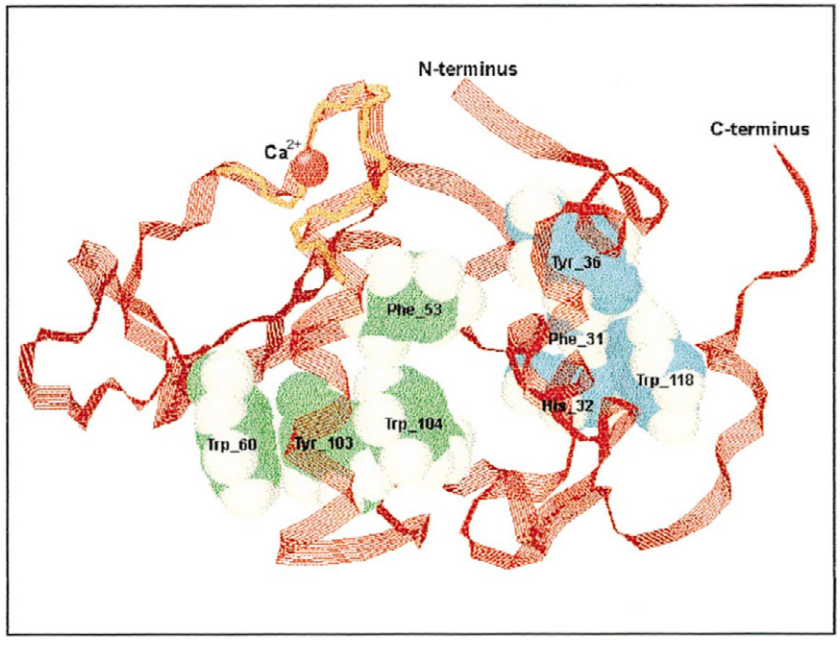

FIGURE 8 The α-lactalbumin structure. The strand shows the peptide backbone with the Ca^{2+}-binding site that stabilizes the native structure. The clusters of aromatic residues are space-filled. The picture was created using the structure of baboon α-lactalbumin (PDB code 1ALC).

α-lactalbumins (with ligand residues Lys-79, Asp-82, Asp-84, Asp-87, and Asp-88) contributes to the amino-terminal side of a 3_{10}-helix (residues 76–82) and to the carboxyl-terminus of the α-helix C (residues 86–99) (Acharya et al., 1989). As either of the two helical structures belong to a different half of the protein, Ca^{2+} binding closes the crevice, preventing the access of bis-ANS to the hydrophobic residues. Other indications of this local stabilization are observed in our earlier studies on bovine α-lactalbumin in the absence of a hydrophobic probe (Vanderheeren and Hanssens, 1994; Vanderheeren et al., 1996); we found that local fragments of the secondary structure are dissipated in the molten globule state of the apo-protein, but are conserved at temperatures above the thermal transition of the Ca^{2+}-bound protein.

Kinetics of the bis-ANS binding

The kinetics of the interaction of bis-ANS with GLA are characterized by an immediate fast jump of the fluorescence followed by a slow fluorescence increase (Fig. 3, *A* and *B*). A comparable two or even multiphasic time dependence of the fluorescence increase has also been observed for the interaction of bis-ANS with other proteins that undergo conformational changes leading to the exposition of hydrophobic regions (Shi et al., 1994; Bethell et al., 1995). In our study the immediate fluorescence increase is proportional to the fraction of apo-GLA in the molten globule state. This proves that the hydrophobic clusters of the molten globule are accessible to external reagents within the dead time (1.8 ms). In contrast, the slow phase of the fluorescence increase is related to the process by which adsorbed bis-ANS induces a conformational change in native GLA, apo- or Ca^{2+}-bound, and comes in contact with its hydrophobic groups. The apparent rate constants of the slow phase are proportional to the square of the bis-ANS concentration for apo-GLA, but are independent of the bis-ANS concentration for Ca^{2+}-GLA. These results indicate that in the case of apo-GLA Eq. 4 should be applied, and in the case of Ca^{2+}-GLA, Eq. 5. From this we can conclude that the absorption constant K_1 is much smaller for the interaction of bis-ANS with apo-GLA than with Ca^{2+}-GLA. The small value of K_1 in the absence of Ca^{2+} could be explained by the electrostatic repulsion between the protein and the dye, which are both negatively charged under the conditions of the experiment. The pronounced stabilization of the transformed complex "apo-GLA_I · 2bis-ANS_{fl}" is the result of large favorable hydrophobic interactions. The effects of the addition of 2 mM Ca^{2+} are not restricted to the saturation of the Ca^{2+} binding site, but also include the formation of a layer of counterions shielding the multiple negative charges on the protein surface. Therefore, the primary adsorption equilibrium between the native Ca^{2+}-protein and bis-ANS becomes important, as suggested by Eq. 5.

Cooperativity of the bis-ANS binding

In most studies of binding equilibria of hydrophobic dyes with proteins, the plots of bound/free dye versus the bound dye concentration (Scatchard plots) are linear (Semisotnov et al., 1991; Vanderheeren and Hanssens, 1994; Shi et al., 1994) in accordance with our observations for the interaction between bis-ANS and apo-GLA (Fig. 2 *C, filled squares*). These linear Scatchard plots agree with an interaction model in which dye binding at one site is independent of its binding at another. Fig. 2 *C* (*filled squares*) therefore indicates that the bis-ANS molecules independently bind to native apo-GLA with nearly equal strength. The slope of the Hill plot (Fig. 2 *D, filled squares*) confirms the presence of independent binding sites. A positive cooperativity for the dye's fluorescence increase, as observed for the interaction of bis-ANS with Ca^{2+}-GLA (Fig. 2, *C* and *D, open squares*), is also found for the binding of the probe with tubulin (Ward and Timasheff, 1994). Positive cooperativity implies that the binding of the first bis-ANS molecule increases the affinity of the next.

Some data from the kinetic experiments seem to be contradictory to data obtained from the equilibrium studies. From the slow phase fluorescence increase we have deduced that the penetration of bis-ANS in native GLA requires the simultaneous interaction of two or more adsorbed dye molecules in accordance with Eqs. 4 and 5. Therefore, positive cooperativities could be expected. In the equilibrium study, positive cooperativity is observed for the interaction of bis-ANS with native Ca^{2+}-GLA, but the dye interaction with apo-GLA lacks cooperativity. The lack of cooperativity indicates the reaction steps that dominate in the kinetics of the complex formation with apo-GLA cannot be used to describe the equilibrium. This includes that dissociation of "apo-GLA_I · 2bis-ANS_{fl}" does not follow the reverse path of its formation and that the two dye molecules do not release simultaneously. The independent behavior of the two dye molecules on dissociation agrees with the presence of two clusters of aromatic groups in α-lactalbumins. As described earlier, the cluster containing Phe-53, Trp-60, Tyr-103, and Trp-104 is situated in the crevice of the molecule; the cluster containing Phe-31, His-32, Tyr-36, and Trp-118 is situated in the domain containing the NH_2- and COOH-terminal fragments (Acharya et al., 1989) (Fig. 8).

In contrast, the fact that Ca^{2+}-GLA binds more bis-ANS molecules than the number of available hydrophobic sites should imply that the bis-ANS molecules adsorbed to the protein interact with each other. The tendency of bis-ANS to associate is favored by the presence of Ca^{2+} and can also be observed in solution in the absence of protein (Vanderheeren and Hanssens, 1994). By the mutual interaction of the adsorbed dye molecules, the Hill coefficient obtained from the relation between the free dye concentration and the binding constant (Eq. 2) is larger than 1, which is also expressed by the positive cooperativity.

We thank Mark Devenijn for providing us with whey from fresh goat milk. The expert assistance of Wim Noppe with the purification and decalcification of goat α-lactalbumin is gratefully acknowledged. We thank Dr. Johan Desmet for performing the visualization of the aromatic clusters in α-lactalbumin.

This work was supported by grants from the Research Council of the Katholieke Universiteit Leuven and from the Flemish Research Fund.

REFERENCES

Acharya, K. R., D. I. Stuart, N. P. C. Walker, M. Lewis, and D. C. Philips. 1989. Refined structure of baboon α-lactalbumin at 1.7 Å resolution. *J. Mol. Biol.* 208:99–127.

Bernasconi, C. F. 1976. Relaxation Kinetics. Academic Press, New York.

Bethell, R. C., N. M. Gray, and C. R. Penn. 1995. The kinetics of the acid-induced conformational change of influenza virus haemagglutinin can be followed using 1,1′-bis(4-anilino-5-naphtalenesulphonic acid). *Biochem. Biophys. Res. Commun.* 206:355–361.

Coco, M. J., and J. T. Lecomte. 1994. The native state of apomyoglobin described by proton NMR spectroscopy: interaction with the paramagnetic probe HyTEMPO and the fluorescent probe ANS. *Protein Sci.* 3:267–281.

Das, K. P., and W. K. Surewicz. 1995. Temperature induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystallin. *FEBS Lett.* 369:321–325.

Dolgikh, D. A., L. V. Abaturov, I. A. Bolotina, E. V. Brazhnikov, V. E. Bychkova, R. I. Gilmanshin, Yu. O. Lebedev, G. V. Semisotnov, E. I. Tiktopulo, and O. B. Ptitsyn. 1985. Compact state of a protein with pronounced small scale mobility: α-lactalbumin. *Eur. Biophys. J.* 13: 109–122.

Engelhard, M., and P. A. Evans. 1995. Kinetics of interaction of partially folded proteins with a hydrophobic dye: evidence that the molten globule character is maximal in early folding intermediates. *Protein Sci.* 4:1553–1562.

Gorovits, B. M., J. W. Seale, and P. M. Horowitz. 1995. Residual structure in urea-denatured chaperonin GroEL. *Biochemistry*. 34:13928–13933.

Griko, Y. V., E. Freire, and P. L. Privalov. 1994. Energetics of the α-lactalbumin states: a calorimetric and statistical thermodynamic study. *Biochemistry*. 33:1889–1899.

Haynie, D. T., and E. Freire. 1993. Structural energetics of the molten globule state. *Proteins: Struct., Funct., Genet.* 16:115–140.

Kuwajima, K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular protein structure. *Proteins: Struct., Funct., Genet.* 6:87–103.

Kuwajima, K. 1996. The molten globule state of α-lactalbumin. *FASEB J.* 10:102–109.

Lindahl, L., and H. J. Vogel. 1984. Metal-ion-dependent hydrophobic interaction chromatography of α-lactalbumin. *Anal. Biochem.* 140: 394–402.

Ptitsyn, O. B. 1995. Molten globule and protein folding. *Adv. Protein Chem.* 47:83–229.

Ptitsyn, O. B., R. H. Pain, G. V. Semisotnov, E. Zerovnik, and O. I. Razgulyaev. 1990. Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* 262:20–24.

Seale, W. S., J. L. Martinez, and P. M. Horowitz. 1995. Photoincorporation of 4,4′-bis(1-anilino-8-napthalenesulfonic acid) into the apical domain of GroEL. *Biochemistry*. 35:7443–7449.

Segawa, T., and S. Sugai. 1983. Interactions of divalent metal ions with bovine, human, and goat α-lactalbumin. *J. Biochem.* 93:1321–1328.

Semisotnov, G. V., N. A. Rodionova, O. I. Razgulyaev, V. N. Uversky, A. F. Gripas, and R. I. Gilmanshin. 1991. Study of the molten globule intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers*. 31:119–128.

Shi, L., D. R. Palleros, and A. L. Fink. 1994. Protein conformational change induced by 1,1′-bis(4-anilino-5-naphthalenesulfonic acid): preferential binding to the molten globule of DnaK. *Biochemistry*. 33: 7536–7546.

Tanaka, N., K. Nakajima, and S. Kungi. 1996. The pressure-induced structural change of bovine α-lactalbumin as studied by fluorescence hydrophobic probe. *Int. J. Pept. Protein Res.* 48:259–264.

Teschke, C. M., J. King, and P. E. Prevelige. 1993. Folding of the phage P22 coat protein in vitro. *Biochemistry*. 32:10839–10847.

Vanderheeren, G., and I. Hanssens. 1994. Thermal unfolding of bovine α-lactalbumin. *J. Biol. Chem.* 269:7090–7094.

Vanderheeren, G., I. Hanssens, W. Meijberg, and A. Van Aerschot. 1996. Thermodynamic characterization of the partially unfolded state of Ca^{2+}-loaded α-lactalbumin: evidence that partial unfolding can precede Ca^{2+} release. *Biochemistry*. 35:16753–16759.

Ward, L. D., and S. N. Timasheff. 1994. Cooperative multiple binding of bis-ANS and daunomycin to tubulin. *Biochemistry*. 33:11891–11899.