New insight on β -lactoglobulin binding sites by 1-anilinonaphthalene-8-sulfonate fluorescence decay

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Abstract

The fluorescence time decay parameters of the β -lactoglobulin-1-anilinonaphthalene-8-sulfonate complex have been investigated under physical and chemical perturbations ($2 < \text{pH} < 8$ and added electrolyte $0 < \text{NaCl} < 0.5 \text{ M}$) to obtain new insight on the nature of the protein binding interactions. A double exponential decay of the bound probe lifetime has been confirmed by the presence of a longer component, 11 to 14.5 ns, and a shorter component, 2.5 to 3.5 ns. The two lifetimes are ascribed to different binding modes associated also with different exposure to the solvent; in particular, the longer component is attributed to binding inside the hydrophobic beta barrel, while a “surface” site is suggested for the shorter component. A detailed analysis of the lifetime fractional intensities correlates the binding constants with ionic strength and supports the presence of electrostatic effects at both sites. A Debye–Hückel approach, applied to extrapolate the electrostatic free energy contribution vs. pH at vanishing ionic strength, gives interesting clues on the effective charge felt by the ANS ligands in the proximity of each site. In particular, binding is found to parallel the aspartate and glutamate titrations between pH 3 and pH 4.5; the “surface” site mainly responds to the presence of these local titrating charges while the “internal” site more closely follows the overall protein net charge.

Keywords: ANS; β -lactoglobulin; binding sites; electrostatic interactions

β -Lactoglobulin, a 162 residues globular protein whose proper functionality has not been clarified yet, in spite of the great number of studies performed on this protein, is present in very large quantities in the milk of several mammals, thus suggesting its nutritional role. Its interaction with a great variety of hydrophobic ligands, such as retinol (Futerman & Heller, 1972; Dufour et al., 1990; Dufour & Haertlé, 1991; Cho et al., 1994; Narayan & Berliner, 1997; Lange et al., 1998), fatty acids and triglycerides (Dufour et al., 1990; Frapin et al., 1993; Narayan & Berliner, 1997; Qin et al., 1998b; Wu et al., 1999), has led to its inclusion in the lipocalin superfamily (Brownlow et al., 1997). This family includes transport proteins, such as the retinol binding protein, the odorant binding protein, and the major urinary protein, which all share the common structural feature of a β -barrel calyx, built from eight antiparallel β -sheets, arranged as an ideal site for hydrophobic ligands (Brownlow et al., 1997).

Since β -lactoglobulin appears to lack specificity for particular ligands and since several studies suggest that more than one binding site exists, it seems interesting to further investigate the nature and the general features of the protein binding sites. A thorough

study is carried out here with a “test” ligand, ANS, a molecule that shares both hydrophilic and hydrophobic characters and whose fluorescence response appears as a convenient and sensitive tool apt to investigate the nature of β -lactoglobulin binding regions. While ANS is only weakly fluorescent in aqueous solutions, its fluorescence is greatly enhanced when it is buried in the protein matrix and screened from water quenching. Small, albeit significant, changes in ANS fluorescence, when changing the protein environment, appear to reflect a modified ligand–protein interaction, which can be due to changes in the probe response (fluorescence quantum yield) and to different free energy values of the association process (ligand strength). Different solvent conditions have been explored here both by changing electrolyte concentration, with the addition of NaCl salt, and by changing the pH from 2 to 8 and, thereby, largely modifying protein charges and ligand interactions. In this paper, the dynamic fluorescence technique is the main tool adopted to measure the fluorescence decay of ANS bound to β -lactoglobulin under the different solution conditions stated above. One of the aims of the paper is that of exploiting ANS fluorescence data in terms of lifetimes and fractional intensities of the dye bound to the BLG binding sites in the light of known bare protein structural data. Finally, a new approach is developed here leading to estimates of the ANS-BLG association constants vs. ionic strength and suggesting a significant role of the local charges in the proximity of the two sites.

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Results

Lifetimes

Under all the pH and ionic strength conditions here examined a three exponential decay has been found to give a very good fit. The shortest decay τ_F is due to free ANS, while the other two lifetimes are attributed to BLG bound ANS molecules as previously reported (D'Alfonso et al., 1999). The free ANS lifetime is found to be independent of pH and ionic strength values; therefore, it has been fixed at 0.26 ns. Concerning the longer bound ANS, lifetime component τ_1 is shown vs. the square root of the ionic strength in Figure 1. It is apparent that a decrease occurs at increasing salt concentration, and its extent is related to the pH of the solution. In particular, at the lowest examined pH value, 2.2, the decrease of τ_1 when adding salt from $I = 0.01$ M to $I = 0.5$ M is about 30% (13 to 10 ns), while it is reduced to about 3% only at pH values close to that of the isoelectric point (from 14.1 to 13.6 ns). At still higher pH values, where the so-called Tanford transition is known to take place (Qin et al., 1998a), the ionic strength induced lifetime shortening reaches 8%, with τ_1 decreasing from 14 ns, at low ionic strength, to 13 ns at 0.5 M NaCl. The pH dependence of the lifetime (shown in Fig. 2A) is common to all the examined ionic strengths. The spread of the data represents the extent of the change induced by salt concentration. It can be seen that τ_1 decreases monotonically away from the native state (pH 6.3), where it has its maximum value, $\tau_1 = 14.5$ ns, while its minimum value is attained in the acid condition (pH 2.2) with $\tau_1 = 11.3$ ns. Also τ_2 decreases with increasing ionic strength at all the examined pH values, though a higher noise level present in this lifetime component makes it more difficult to assess the extent of the variation. When viewed vs. pH (Fig. 2B), the minimum value of the short lifetime is observed near the isoelectric point while the maximum values are found at the acid and alkaline extremes. The ratio of the long to the short ANS-bound lifetimes τ_1/τ_2 is found to be almost constant when plotted vs. ionic strength, and bell shaped if plotted vs. pH, with a maximum value of 5.5 ± 0.5 around the isoelectric point.

Fig. 1. Bound ANS fluorescence lifetime τ_1 vs. the square root of the ionic strength. The symbols refer to the different pH values: pH 2.2, square; pH 3.3, circle; pH 4.3, pointing up triangle; pH 5.2, pointing down triangle; pH 6.3, diamond; pH 7.3, star; pH 8.1, cross.

Fig. 2. **A:** Bound ANS fluorescence lifetime τ_1 vs. pH. **B:** Bound ANS fluorescence lifetime τ_2 vs. pH. The symbols refer to the ionic strength values: $I = 0.01$ M, square; $I = 0.02$ M, circle; $I = 0.03$ M, pointing up triangle; $I = 0.06$ M, pointing down triangle; $I = 0.1$ M, diamond; $I = 0.25$ M, star; $I = 0.5$ M, cross.

The largest change is found between pH 6.3 and pH 2.2, where τ_1/τ_2 decreases from 5.5 ± 0.5 down to 4 ± 0.25 , respectively.

Fractional intensities

As reported in Materials and methods, the fractional intensities are related to the fractions of population that decay with the corresponding lifetimes. Their behavior is influenced both by the value of the lifetime and by the concentration of excited ANS molecules, which decay with the given time constant. In Figure 3, the bound

Fig. 3. Fractional intensities of ANS bound to BLG. The top fractional intensity f_1 refers to τ_1 and the bottom one f_F to the free dye lifetime. The symbols refer to the different pH values: pH 2.2, square; pH 3.3, circle; pH 4.3, pointing up triangle; pH 5.2, pointing down triangle; pH 6.3, diamond; pH 7.3, star; pH 8.1, cross.

ANS fractional intensities f_1 and f_2 , together with the free dye fractional intensity f_F , are shown vs. the square root of the ionic strength at each pH examined. The good data repeatability is shown in the figure since each salt concentration is the result of two different preparations. The longer bound lifetime fractional intensity decreases at increasing ionic strength varying from 80 to 65% on the average, f_2 increases at increasing ionic strength, while the free dye f_F contributes at most by 6% to the total intensity. The behavior of a single fractional intensity cannot directly be related to a change in the binding properties of the site associated to that component since the sum is normalized to unity, and further relations are needed.

Discussion

Lifetimes

Fluorescence of ANS bound to BLG shows two distinct lifetimes confirming that two binding modes are involved in the binding as already suggested in a previous work (D'Alfonso et al., 1999). In fact, we have shown that the affinities of ANS for the two sites, referred to as "site 1," longer decay time, and "site 2," shorter lifetime decay, appear to be of comparable strength since a substantial weight for both lifetimes is always detected when performing ANS titrations on a wide range of protein concentrations (2 mM–10 μ M) at pH 2, pH 6 and pH 8, corresponding to different degrees of dimerization (Townend et al., 1960; Verheul et al., 1999). The lifetime values were found to be constant during the titration and the fractional intensities followed a simple mass-action law. This result suggests that none of the BLG binding sites is significantly influenced by protein dimerization, as confirmed also by fluorescence measurements performed vs. temperature (see Figs. 3 and 5 in D'Alfonso et al., 1999).

The presence of two binding sites has been inferred also from several data in the literature. This information was obtained by competitive and simultaneous binding of retinol and fatty acids (Narayan & Berliner, 1997) or protoporphyrins (Dufour et al., 1990), SDS molecules (Lovrien & Anderson, 1969), etc. From NMR data, two hydrophobic patches have been found to be conserved down to pH 2.2 (Ragona et al., 1997; Fogolari et al., 1998; Uhrinova et al., 2000) when compared with the X-ray structure (Brownlow et al., 1997).

One of these sites, "site 1," is an "internal" site localized in the beta barrel of the protein and it is the putative site for ligands such as retinol (Cho et al., 1994), dodecanoic acid (Qin et al., 1998b), palmitate (Wu et al., 1999). The other site, "site 2," is a "surface" site (Dufour et al., 1994; Lange et al., 1998) located near the interface between the beta barrel and the alpha helix away from the dimer interface. In a recent review by Sawyer et al. (1998), all the studied BLG ligands are reported together with the values of their dissociation constants. The comparison between the strength of the association constants and the structure of the ligands suggests that elongated ligands are favored in the barrel site. ANS seems to reasonably fit the beta-barrel, in agreement also with the observed binding which shows intermediate strength (Sawyer et al., 1998; D'Alfonso et al., 1999).

A further suggestion on the presence of two distinct sites stems from the different dependence of each component of the bound ANS fluorescence decay upon electrostatic effects and protein charge. The observed fluorescence lifetime dependence could be ascribed to different conformations of the BLG binding sites and/or

to a different degree of protection against solvent water quenching under the examined conditions. The low ANS concentration used throughout this work rules out the influence of ANS self-association on the change of ANS lifetime (Uversky et al., 1998). Moreover, recent works on ANS fluorescence suggest that the enhancement of the ANS fluorescence upon binding to proteins results from the protection of the naphthalene moiety from water molecules (Matulis et al., 1999), or from a greater rigidity imposed by the geometry of the "internal" site (Kirk et al., 1996). Both pH and ionic strength have been found to induce conformational changes in BLG, such as the Tanford transition (Qin et al., 1998a) which can be detected either by the tryptophan intrinsic fluorescence (Renard et al., 1998) or by circular dichroism (D'Alfonso et al., 1999), and to influence protein aggregation (Aymard et al., 1996). The BLG sites appear to be protected from the solvent and prone to the ANS binding to a different degree. In particular, at pH 6.3 the τ_1 value suggests that "site 1" reaches the maximum screening with respect to water quenching (Fig. 2A), and the conformation observed at the lowest ionic strength seems to be the most impenetrable to solvent at all the examined pH values. At pH 2, especially for "site 1," there seems to be the larger conformational changes (Fig. 1). CD, NMR, and other techniques also confirm the particular character of this acid but still native BLG state (Uhrinova et al., 2000). A previously envisioned, transition to a molten globule state at low pH has been recently ruled out since a high degree of ordered structure has been observed (Ragona et al., 1997).

The τ_1/τ_2 ratio is found to depend upon pH, ranging from a value of 4, at pH 2.2, up to a maximum value of about 6 at pH 6.3, while the dependence of τ_1/τ_2 upon ionic strength is much weaker (the change being at most 0.5 at each pH examined). This suggests that the conformational changes induced by altering the protein charge by varying the pH are more effective on ligand properties than the effects of electrostatic screening induced by the ionic strength.

Fractional intensities

More detailed information on the binding characteristics vs. the solution parameters (pH, ionic strength) can be pursued by employing the approximation of Equation 11. The ratio of the two fractional intensities associated to the BLG bound ANS is related to the fluorescence quantum yield of ANS bound to the i^{th} site, ϕ_i , by

$$\frac{f_1}{f_2} = \frac{\epsilon_1 c_{B1} \phi_1}{\epsilon_2 c_{B2} \phi_2} \approx \frac{K_1 \phi_1}{K_2 \phi_2} \quad (1)$$

where it has been assumed that $n_1 = n_2 = 1$ (Hamdan et al., 1996) and that the extinction coefficients for the two bound ANS species do not differ appreciably, $\epsilon_1 = \epsilon_2$. The latter assumption is based on absorption measurements performed under conditions such that the relative occupancies of sites 1 and 2 ranged from a ratio of 1 to 1 to a ratio of 3 to 1, respectively. It is therefore possible to obtain information about the mutual strength of the two binding sites from the f_1/f_2 ratio, which is closely connected with the association constant ratio according to

$$\frac{K_1}{K_2} \approx \frac{f_1/f_2}{(\tau_1/\tau_2)(\tau_{R2}/\tau_{R1})} \quad (2)$$

where $\tau_{Ri} = \tau_i/\phi_i$ is the radiative lifetime of ANS bound at the i^{th} site.

One can obtain another useful relation on the binding constants by considering the ratio between the ANS bound fractional intensities sum, $f_1 + f_2$, and the free ANS fractional intensity f_F as follows:

$$\frac{f_1 + f_2}{f_F} = \frac{c_{B1} \epsilon_1 \phi_1 + c_{B2} \epsilon_2 \phi_2}{c_F \epsilon_F \phi_F} = P \frac{\epsilon_B}{\epsilon_F} \frac{\phi_1}{\phi_F} \left[K_1 + \frac{K_2}{\phi_1/\phi_2} \right] \quad (3)$$

where ϵ_B is the bound ANS extinction coefficient and Equation 11 has been used. Then,

$$\left[K_1 + K_2 \left(\frac{\tau_2}{\tau_1} \frac{\tau_{R1}}{\tau_{R2}} \right) \right] \approx \left[\frac{f_1 + f_2}{f_F} \right] \left(\frac{\tau_F}{\tau_1} \frac{\tau_{R1}}{\tau_{RF}} \right) \left(\frac{\epsilon_F}{\tau_1} \frac{1}{P} \right). \quad (4)$$

By combining Equations 2 and 4, it is possible to single out the separate values of K_1 and K_2 , since ϵ_F , ϵ_B , P , f_i , and τ_i are known quantities, once the radiative lifetimes are available. To this aim, a direct measure of the bound ANS quantum yield by steady state fluorescence does not seem to be easily accessible, since the measured value, as a matter of fact, is the average of the yields of the individual sites each weighted by the corresponding association constant (D'Alfonso et al., 1999). Some help can be obtained from data on the fluorescence quantum yields and lifetimes of ANS in water ethanol mixtures (Robinson et al., 1978), which allow estimates of the radiative and nonradiative decay rates, k_R and k_{nR} , respectively. Using the data on ethanol-water mixtures as an indication of ANS-protein fluorescence properties, one can draw some semi-quantitative estimates of the radiative decay rates in the system. By inspecting the data of Robinson et al., it can be noticed that lifetime changes from 0.25 ns, in water, to 11 ns, in ethanol, correspond to a threefold magnification of k_R (from $1.2 \times 10^7 \text{ s}^{-1}$ to $3.7 \times 10^7 \text{ s}^{-1}$), while k_{nR} , which does not enter in our expressions, decreases by two orders of magnitude (from $\sim 400 \times 10^7 \text{ s}^{-1}$ to $5.3 \times 10^7 \text{ s}^{-1}$). Since the largest contribution to the different decay properties can be ascribed to the nonradiative term, then k_R can be interpolated vs. the fluorescence lifetime values leading then to estimates of the ANS decay rates under the conditions reported in this paper. Concerning the site with the longer lifetime, its change is found to range from 11 to 14.5 ns. The interpolated curve of k_R (Robinson et al., 1978) vs. fluorescence lifetime is almost flat in this range thus leading to a nearly constant value of $k_R \sim 3.8 \times 10^7 \text{ s}^{-1}$. The shorter lifetime changes from 2.5 to 3 ns, whereas k_R changes only slightly from 2.25 to $2.5 \times 10^7 \text{ s}^{-1}$. Thus, the ratio of the longer to shorter radiative lifetime, appearing in Equation 2, can be taken to be essentially constant vs. pH and equal to $\tau_{R2}/\tau_{R1} = 1.6 \pm 0.1$. In Equation 4, the ratio of the “site 1” radiative lifetime to the free ANS radiative lifetime is also needed. Again, this can be estimated from the work by Robinson et al. to be close to $\tau_{RF}/\tau_{R1} \approx 3.8 \times 10^7 \text{ s}^{-1}/1.2 \times 10^7 \text{ s}^{-1} = 3.2 \pm 0.15$.

In Figure 4 the natural logarithms of K_1 and K_2 are shown vs. ionic strength. At each pH value examined, the binding affinities for the two sites are of comparable strength. For each site, the binding strength strongly depends upon pH, showing the highest affinity at pH 2, a minimum value around pH 6 and a new increase toward pH 8. In particular, $\ln K_1$, corresponding to the “internal”

Fig. 4. Natural logarithm of (A) K_1 and (B) K_2 vs. the square root of the ionic strength. The symbols refer to the different pH values: pH 2.2, square; pH 3.3, circle; pH 4.3, pointing up triangle; pH 5.2, pointing down triangle; pH 6.3, diamond; pH 7.3, star; pH 8.1, cross. The dotted lines are best fits obtained by using Equation 5 as described in the text.

site and shown in Figure 4A, displays decreasing values vs. ionic strength from pH 2.2 to pH 5.2, while an opposite trend is found at pH values larger than the isoelectric value. Since ANS carries a negative charge, its electrostatic interaction with BLG is expected to be more favorable between pH 2.2 and pH 5.2, whereas, above 5.2, the opposite trend should be related to the changed sign of the electrostatic interaction. Larger ionic strengths were found to decrease the ANS-BLG affinities of the surface site at pH 2.2 and at pH 3.3 only, while the opposite is found above pH 4.3.

To obtain a more quantitative insight in the description of ANS binding behavior at each site, the interaction between ANS and BLG is taken to be given by two contributions: electrostatic (ΔG_{el}) and nonelectrostatic (ΔG_{nel}). The former should contain either a dependence upon the charge on the protein (since K_1 and K_2 change vs. pH) and a dependence on ionic strength via a Debye–Hückel screening term (since K_1 and K_2 change vs. ionic strength), such as

$$\ln(K) = -\Delta G_{nel} - \Delta G_{el} = -\Delta G_{nel} - \Delta G_{el}(I=0) \exp(-\rho \sqrt{I}) \quad (5)$$

where ΔG_{nel} and ΔG_{el} are in RT units and ρ and $\Delta G_{el}(0)$ are related to the characteristic Debye screening length l_D at the ionic strength I through

$$\rho = \sqrt{\frac{8\pi e^2 N_{AV}}{1,000 k_B T}} \frac{l_D}{\sqrt{\delta}} \quad \Delta G_{el}(0) = \frac{Z_{ANS} Z_{BLG}}{RT \delta l_D} \quad (6)$$

where δ is the medium dielectric constant, T is the solution temperature, Z_{ANS} is the charge of the ligand (here equal to $-e$ at all the examined pH), and Z_{BLG} is the protein charge.

Analysis of the binding sites

From the obtained electrostatic dependence of K_1 and K_2 , one is led to suggest that different values of the local charges are involved at the two sites. In particular, Equation 5 can be employed to fit $\ln K_1$ and $\ln K_2$ vs. ionic strength at different pH values thus giving the parameters of the interaction, i.e., nonelectrostatic and electrostatic free energies, and the Debye parameter. Assuming that the Debye parameter ρ remains almost constant vs. pH, then, by fitting the sum of the logarithms of the association constants vs. ionic strength at different pH values, one should obtain the exponent for each site: $\rho_1 = 3.16 \text{ M}^{-1/2}$ and $\rho_2 = 1.13 \text{ M}^{-1/2}$. The Debye length for each site can be obtained if the dielectric constant is known: while for the “surface” site it might be reasonable to assume a value close to that of the bulk water $\delta = 78.5$, thus leading to $l_D = 3.73 \text{ \AA}$, for the “internal” site lower values of the dielectric constant should be more appropriate. For the “internal” sites in proteins, it is believed that δ can assume values as low as 30 (Warshel, 1987), leading to distances of closest approach located between 6.5 to 10.4 $\text{\AA}$, with the latter value being the highest value when $\delta = 78.5$. The best fit parameters for the two sites as obtained with mean ρ values are listed in Table 1. The nonelectrostatic free energy contributions for the two sites are almost constant vs. pH. The electrostatic contribution vs. pH shows a similar trend for both sites: the free energy increases from negative to positive values from pH 2.2 up to pH 6.3, then it shows a small decrease in the region of the Tanford transition (Tanford et al., 1959; Qin et al., 1998a). The different behavior of the two sites, as already pointed out, is revealed by pH value corresponding to the free energy sign change, from negative to positive, occurring at pH 5.2 for “site 1” and between pH 3.3 and 4.3 for “site 2.”

To discuss the pH dependence of the binding affinities, a comparison between the Protein Data Bank deposited BLG structures at physiological pH (Brownlow et al., 1997) and acid pH (Kuwata et al., 1999; Uhrinova et al., 2000) can be made by assuming that the residues involved in the two sites are those reported in the literature (Ragona et al., 1997; Fogolari et al., 1998). The entrance to the protein calyx (internal site) appears to be wider at pH 2, and therefore a higher ANS affinity is expected due to the better fit to the beta barrel, and a weaker screening from the solvent molecules justify a lower ANS lifetime value. The region around the external site at pH 2 seems to be less compact due to a different orientation of the α -helix with respect to the calyx (Kuwata et al., 1999;

Uhrinova et al., 2000). ANS binding to the external site at acid pH might be enhanced by this more favorable conformation. Concerning the effects due to local charge differences between the two sites, also the location of the charge residues on the protein has been explored. While the lysines and the arginine residues carry always positive charges in the pH range explored, the glutamate and the aspartate residues are deprotonated between pH 4 and pH 4.5 (Cantor & Schimmel, 1980; Arakawa & Timasheff, 1987) with the exception of Glutamate 89 (Qin et al., 1998a) whose pK, is around pH 7.2. At pH lower than the pK, the 15 glutamate residues and the 10 aspartate residues are neutral, and only positive charges are present, while at pH values higher than their pK they become negative and the net charge on the protein changes sign approximately at pH 5.3 (Basch & Timasheff, 1967) depending on the ionic strength. It is tempting to correlate the behavior of the $\Delta G_{el}(0)$ with the titration of Glu and Asp residues. Both in the proximity of the “surface” hydrophobic site and of the β -barrel entrance several positive charges and several Glu and Asp residues are present with the consequent local net charge change as the pH changes. In Figure 5 the best fit values of $\Delta G_{el}^{(1)}(0)$ and $\Delta G_{el}^{(2)}(0)$ are shown vs. pH. The dotted lines represent the interpolating curves (excluding the points above pH 7.5) obtained when assuming the well-known sigmoidal behavior describing the titration of a protein residue in the explored pH range:

$$\Delta G_{el}^{(i)}(0) = \Delta G_f^{(i)} - \frac{\Delta G_f^{(i)} - \Delta G_i^{(i)}}{(1 + 10^{\text{pH} - \text{pK}})} \quad (7)$$

where $\Delta G_i^{(i)}$ and $\Delta G_f^{(i)}$ represent the values of $\Delta G_{el}^{(i)}(0)$ at the two extremes of the transition. The resulting good fit suggests that residues with similar pK values are involved in the process. In particular, since the pK value of the titration is found to lie at $\text{pH } 4.6 \pm 0.1$ for the “internal” site, and at $\text{pH } 4.0 \pm 0.1$ for the “surface” site, it appears that aspartate and glutamate residues are those involved. On the other hand, the electrostatic free energy

Table 1. Electrostatic and nonelectrostatic free energies for the two BLG binding sites as obtained from best fit of $\ln K_1$ and $\ln K_2$ vs. ionic strength with the equation: $\ln K = -\Delta G_{nel} - \Delta G_{el}(0)\exp(-\rho\sqrt{I})$ and by assuming mean ρ values, $\rho_1 = 3.16 \text{ M}^{-1/2}$ and $\rho_2 = 1.13 \text{ M}^{-1/2}$

pH	$\Delta G_{nel}^{(1)}$	$\Delta G_{el}^{(1)}(0)$	$\Delta G_{nel}^{(2)}$	$\Delta G_{el}^{(2)}(0)$
2.2	-7.3	-1.1	-8.5	-0.35
3.3	-6.3	-1.5	-8.2	-0.17
4.3	-6.6	-0.71	-8.6	0.71
5.2	-6.4	-0.0022	-8.3	1.3
6.3	-6.2	0.25	-7.9	1.4
7.3	-6.5	0.30	-7.9	1.2
8.1	-6.5	-0.25	-7.8	0.86

Fig. 5. Electrostatic free energies for the two different binding sites vs. pH. Internal site (site 1), square; external site (site 2), circle. The dotted lines are best fits obtained as described in the text. The pK of the titration is found to correspond to pH 4.6 for the “internal” site, and to pH 4.0 for the “surface” site.

behavior vs. pH indicates that the net charge in the proximity of each site is different.

In particular, while the “surface” site is mainly influenced by the titrating residues, for the “internal” site the local charge follows the behavior of the net protein charge (isoelectric point at pH 5.2), and this can also be influenced by the nearby located Glu89, which displays an anomalous pK value.

Conclusions

The binding of ANS to BLG investigated by means of dynamic fluorescence shows lifetime changes vs. the solution conditions (ionic strength and pH) that suggest different degrees of shielding of the bound ANS molecules and different BLG binding site characteristics. The analysis performed on fluorescence data, combined with existing structural hints on the binding site locations, has permitted to obtain semi-quantitative information on the nature of the BLG binding sites. The interaction of ANS with BLG is largely influenced by the nature of the two sites on the protein as found from spectroscopic properties (quantum yield, fluorescence lifetime), in spite of the observed little change in the strength of the interaction, corresponding to association constants differing by a factor of three, at most.

Nonetheless, a remarkable behavior of the two sites is found when inspecting the association constants vs. ionic strength: the local net charges are given by two different balances between positive and negative (or neutral) residues vs. the pH. In particular, for the “internal” site, the local charge follows the same behavior vs. pH as that of the net protein charge, while for the “surface” site it follows the titration of the aspartate and glutamate residues leading to a protein-probe interaction that becomes attractive below pH 4.

Materials and methods

Materials

β -Lactoglobulin B (BLG) has been purchased by Sigma Chemical Co. (St. Louis, Missouri), lot n°13H7150 and it has been used without further purification. The lyophilized protein has been dissolved in a suitable buffer at the desired concentration and subsequently dialyzed with an Amicon diafiltration unit (YM3 membrane with 3 kD of cutoff) to restore the buffer pH value, which becomes altered when employing high protein concentrations. 1-Anilino-8-naphthalene sulfonate (ANS) ammonium salt has been purchased by Fluka Chemical Co. (Milwaukee, Wisconsin) (Neu-Ulm, CH). Protein and dye concentrations have been checked photometrically by the molar extinction coefficient at the absorption peak, $\epsilon(280) = 17,600 \text{ cm}^{-1} \text{ M}^{-1}$ for BLG and $\epsilon(350) = 5,000 \text{ cm}^{-1} \text{ M}^{-1}$ for free ANS in solution.

All the reagents used in buffer preparation were of analytical grade. The buffers used in dynamic fluorescence titrations are: $\text{H}_3\text{PO}_4/\text{NaOH}$ pH 2.2, ionic strength $I = 0.007 \text{ M}$; $\text{H}_3\text{PO}_4/\text{NaOH}$ pH 3.3, $I = 0.010 \text{ M}$; $\text{CH}_3\text{COOH}/\text{NaOH}$ pH 4.3, $I = 0.010 \text{ M}$; $\text{CH}_3\text{COOH}/\text{NaOH}$ pH 5.2, $I = 0.010 \text{ M}$; $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ pH 6.3, $I = 0.010 \text{ M}$; $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ pH 7.3, $I = 0.010 \text{ M}$; and $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4/\text{NaOH}$ pH 8.1, $I = 0.010 \text{ M}$. Stock solutions of 2 M NaCl, each dissolved in the proper buffer, were used to raise ionic strength from 0.01 M to about 0.5 M. Each salt concentration has been prepared twice to check data reproducibility. BLG and ANS

concentrations have been held constant during each measurement at 150 and 5 μM , respectively.

Equipment

Dynamic fluorescence measurements have been performed with a frequency modulated phase fluorimeter (Digital K2, I.S.S., Urbana Illinois). The excitation was accomplished by the 80 mW output of the 363.7 nm line of an Argon ion laser (2025, Spectra Physics, Mountain View, California). For further details, see Collini et al. (1995). At least 15 data at logarithmically spaced frequencies in the range 2–180 MHz with a cross-correlation frequency of 80 Hz have been acquired for lifetime measurements. The convenient accuracy for phase angles and modulation ratios has been of 0.2 and 0.004°, respectively. The sample cell has been thermostated at $25^\circ\text{C} \pm 0.5$ during each measurements by means of a Haake thermostating bath and the sample temperature has been monitored through a thermocouple directly inserted in the cuvette. Lifetime measurements have been performed under the magic angle conditions and a 435 nm long pass filter has been employed to cut light scattering. A solution of glycogen in bidistilled water has been used as reference sample. Lifetime data fitting has been accomplished by an I.S.S. routine based on the Marquardt least-squares minimization.

Procedures

Fluorescence lifetimes have been obtained by recording the ANS fluorescence decay in the frequency domain. When a radio-frequency modulated light beam impinges on a sample, the resulting fluorescence emission is modulated at the same frequency although phase shifted and reduced in modulation amplitude due to the finiteness of the excited state. The phase shift angle φ and the modulation ratio M collected at each frequency are related to the time domain fluorescence intensity through a Fourier transforms:

$$\text{tg}(\varphi(\omega)) = \frac{\text{Im}(F(I_\omega))}{\text{Re}(F(I_\omega))} \quad M(\omega) = |F(I_\omega)| \quad (8)$$

with

$$F(I_\omega) = \int_0^\infty I(t) \exp(-i\omega t) dt. \quad (9)$$

Fluorescence lifetimes have been analyzed in terms of sums of three discrete exponential components, with the lifetimes values τ_i and their corresponding fractional intensities f_i as unknown parameters, according to the expression:

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \quad \text{and} \quad f_i = \frac{\alpha_i \tau_i}{\sum \alpha_i \tau_i} \quad (10)$$

where α_i represent the pre-exponential factors. The fractional intensity is proportional to the concentration of the emitting species, to its quantum yield, and to the molar extinction coefficient (D'Alfonso et al., 1999). The bound ANS concentration at each

site c_{Bi} is directly related to the association constant K_i and to the number of sites n_i :

$$c_{Bi} = \frac{n_i K_i P c_F}{1 + K_i c_F} \approx n_i K_i P c_F \quad (11)$$

where P is the protein concentration and c_F is the uncomplexed dye concentration. Since the product $K_i c_F$ weights at most 0.1 at the concentrations used here and employing the value of K_i typical of ANS-BLG interaction (D'Alfonso et al., 1999), its influence on the denominator of Equation 11 has been neglected.

References

- Arakawa T, Timasheff SN. 1987. Abnormal solubility behavior of β -lactoglobulin: Salting-in by glycine and NaCl. *Biochemistry* 26:5147–5153.
- Aymard P, Durand D, Nicolai T. 1996. The effect of temperature and ionic strength on the dimerization of β -lactoglobulin. *Int J Biol Macromol* 19:213–221.
- Basch JJ, Timasheff SN. 1967. Hydrogen ion equilibria of the genetic variants of bovine β -lactoglobulin. *Arch Biochem Biophys* 118:37–47.
- Brownlow S, Cabral JHM, Cooper R, Flower DR, Yewdall SJ, Polikarpov I, North ACT, Sawyer L. 1997. Bovine β -lactoglobulin at 1.8 Å resolution—Still an enigmatic lipocalin. *Structure* 5:481–495.
- Cantor CR, Schimmel PR. 1980. Structure of proteins. In: *Biophysical chemistry. The conformation of biological macromolecules. Part I*. San Francisco: WH Freeman and Company. pp 47–51.
- Cho Y, Batt CA, Sawyer L. 1994. Probing the retinol-binding site of bovine β -lactoglobulin. *J Biol Chem* 269:11102–11107.
- Collini M, Chirico G, Baldini G, Bianchi ME. 1995. Conformation of short DNA fragments by modulated fluorescence polarization anisotropy. *Biopolymers* 36:211–225.
- D'Alfonso L, Collini M, Baldini G. 1999. Evidence of heterogeneous 1-anilino-8-sulfonate binding to β -lactoglobulin from fluorescence spectroscopy. *Biochim Biophys Acta* 1432:194–202.
- Dufour E, Genot C, Haertlé T. 1994. Beta-lactoglobulin binding properties during its folding changes studied by fluorescence spectroscopy. *Biochim Biophys Acta* 1205:105–112.
- Dufour E, Haertlé T. 1991. Binding of retinoids and beta-carotene to beta-lactoglobulin. Influence of protein modifications. *Biochim Biophys Acta* 1079:316–320.
- Dufour E, Marden MC, Haertlé T. 1990. β -lactoglobulin binds retinol and protoporphyrin IX at two different binding sites. *FEBS Lett* 277:223–226.
- Fogolari F, Ragona L, Zetta L, Romagnoli S, De Kruif KG, Molinari H. 1998. Monomeric bovine β -lactoglobulin adopts a β -barrel fold at pH 2. *FEBS Lett* 436:149–154.
- Frapin D, Dufour E, Haertlé T. 1993. Probing the fatty acid binding site of beta-lactoglobulins. *J Protein Chem* 12:443–449.
- Futterman S, Heller J. 1972. The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β -lactoglobulin, and the retinol-binding protein of human plasma. *J Biol Chem* 247:5168–5172.
- Hamdan M, Curcuruto O, Molinari H, Zetta L, Ragona L. 1996. Electrospray mass spectrometry: Complexation between 1-anilino-8-sulphonate and proteins. *J Mass Spectrom* 31:1261–1264.
- Kirk WR, Kurian E, Prendergast FG. 1996. Characterization of the sources of protein-ligand affinity: 1-Sulfonato-8-(1')anilino-8-naphthalene binding to intestinal fatty acid binding protein. *Biophys J* 70:69–83.
- Kuwata K, Hoshino M, Forge V, Era S, Batt CA, Goto Y. 1999. Solution structure and dynamics of bovine β -lactoglobulin A. *Protein Sci* 8:2541–2545.
- Lange DC, Kothari R, Patel RC, Patel SC. 1998. Retinol and retinoic acid bind to a surface cleft in bovine β -lactoglobulin: A method of binding site determination using fluorescence resonance energy transfer. *Biophys Chem* 74:45–51.
- Lovrien R, Anderson W. 1969. Resolution of binding sites in β -lactoglobulin. *Arch Biochem Biophys* 131:139–144.
- Matulis D, Baumann CG, Bloomfield VA, Lovrien RE. 1999. 1-Anilino-8-naphthalene sulfonate as a protein conformational tightening agent. *Biopolymers* 49:451–458.
- Narayan M, Berliner LJ. 1997. Fatty acids and retinoids bind independently and simultaneously to β -lactoglobulin. *Biochemistry* 36:1906–1911.
- Qin BY, Bewley MC, Creamer LK, Baker HM, Baker EN, Bewley GB. 1998a. Structural basis of the Tanford transition of bovine β -lactoglobulin. *Biochemistry* 37:14014–14023.
- Qin BY, Creamer LK, Baker EN, Jameson GB. 1998b. 12-Bromododecanoic acid binds inside the calyx of bovine β -lactoglobulin. *FEBS Lett* 438:272–278.
- Ragona L, Pusterla F, Zetta L, Monaco HL, Molinari H. 1997. Identification of a conserved hydrophobic cluster in partially folded bovine β -lactoglobulin at pH 2. *Fold Des* 2:281–290.
- Renard D, Lefebvre J, Griffin MCA, Griffin WG. 1998. Effects of pH and salt environment on the association of β -lactoglobulin revealed by intrinsic fluorescence studies. *Int J Biol Macromol* 22:41–49.
- Robinson GW, Robbins RJ, Fleming GR, Morris JM, Knight AEW, Morrison RJS. 1978. Picosecond studies of the fluorescence probe molecule 8-anilino-1-naphthalenesulfonic acid. *J Am Chem Soc* 100:7145–7150.
- Sawyer L, Brownlow S, Polikarpov I, Wu S. 1998. β -Lactoglobulin: Structural studies, biological clues. *Int Dairy J* 8:65–72.
- Tanford C, Bunville LG, Nozaki Y. 1959. The reversible transformation of β -lactoglobulin at pH 7.5. *J Am Chem Soc* 81:4032–4035.
- Townend R, Weinberger L, Timasheff SN. 1960. Molecular interactions in β -lactoglobulin. IV. The dissociation of β -lactoglobulin below pH 3.5. *J Am Chem Soc* 82:3175–3179.
- Uhrinova S, Smith MH, Jameson GB, Uhrin D, Sawyer L, Barlow PN. 2000. Structural changes accompanying pH-induced dissociation of the β -lactoglobulin dimer. *Biochemistry* 39:3565–3574.
- Uversky VN, Winter S, Löber G. 1998. Self-association of 8-anilino-1-naphthalene-sulfonate molecules: Spectroscopic characterization and application to the investigation of protein folding. *Biochim Biophys Acta* 1388:133–142.
- Verheul M, Pederson JS, Roefs SPFM, de Kruif KG. 1999. Association behavior of native β -lactoglobulin. *Biopolymers* 49:11–20.
- Warshel A. 1987. What about protein polarity? *Nature* 330:15–16.
- Wu S, Perez MD, Puyol P, Sawyer L. 1999. β -lactoglobulin binds palmitate within its central cavity. *J Biol Chem* 274:170–174.