Mapping fatty acid binding to β -lactoglobulin: Ligand binding is restricted by modification of Cys 121

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Abstract

Native β -lactoglobulin (Blg) binds 1 mole of palmitic acid per mole of protein with a dissociation constant of $0.6 \mu\text{M}$ for the primary fatty acid binding site. Chemical modification of Cys 121, which lies at the external putative hydrophobic binding site of Blg, does not affect retinol or 4,4'-bis 1-(phenylamino)-8-naphthalenesulfonate (bis-ANS) binding to the protein, indicating that the incorporated appendages do not perturb the internal hydrophobic site within the β -barrel of Blg (i.e., the retinoid site is unaffected). On the other hand, methylation of Cys 121, reduces the affinity of Blg for palmitic acid by 10-fold as monitored by intrinsic fluorescence. Modification of the Cys 121 with methylmethanethiosulfonate or a thiol-specific spin label appears to either further weaken or totally eliminate fatty acid binding, respectively, due to steric hindrance. Furthermore, this binding pattern has been independently verified using a spin labeled fatty acid analog and monitoring ESR as well as by bis-ANS fluorescence when bound to the protein. These results suggest that fatty acids bind at the "external site" of β -lactoglobulin, between the sole α -helix and the β -barrel. In addition, structural stability studies of native and chemically modified Blg appear to confirm this observation as well.

Keywords: β -lactoglobulin; electron spin resonance; fatty acids; fluorescence; retinol; spin labels

β -lactoglobulin (Blg)¹ is a small, globular whey protein found in the milks of many mammals including cow, camel, pig, dog, etc. (Pervaiz & Brew, 1985). Although bovine Blg can occur as a dimer at neutral pH (subunit molecular weight $\sim 18,400$ daltons), it undergoes monomerization at $\text{pH} \leq 2$ and can also octamerize between pH 4 and 5 under appropriate conditions (McKenzie, 1971). The protein has been shown to bind a number of hydrophobic ligands in vitro such as retinol, retinoic acid (Futterman & Heller, 1972; Fugate & Song, 1980; Puyol et al., 1991), SDS and long chain fatty acids (Seibles, 1969; Jones & Wilkinson, 1976; Spector & Fletcher, 1970; Perez et al., 1989), protoporphyrin (Dufour et al., 1990), heme-CO complexes (Marden et al., 1994), aromatic compounds (Robillard & Wishnia, 1972a, 1972b; Dufour et al., 1992) and alkanone flavors

(O'Neill & Kinsella, 1987). As a result of these observations as well as its remarkable acid stability, it has been hypothesized to serve as a carrier molecule in vivo for the transport of retinoids and fatty acids in developing neonates (Papiz et al., 1986). Furthermore, it has been proposed as an attractive candidate for protein engineering, whereby it could be suitably modified for purposes of transport and delivery of nutrients to specific targets within the alimentary tract (Sawyer, 1987).

Retinoids and fatty acids have been hypothesized (Frapin et al., 1993; Dufour et al., 1994) and later proven to bind independently and simultaneously to β -lactoglobulin (Narayan & Berliner, 1997), although there had been earlier reports suggesting overlapping or identical binding sites for these two lipophiles (Creamer, 1995). The location of the retinoid binding site is well established (Cho et al., 1994) and lies inside the β -barrel of the protein. On the other hand, there were two locations hypothesized for fatty acid binding: 1) an internal site within the β -barrel and perhaps overlapping, or adjacent to, the retinoid binding site (Creamer, 1995); 2) external hydrophobic site between the sole α -helix and the β -barrel (Frapin et al., 1993; Dufour et al., 1994). In order to map the fatty acid site(s), we have probed both the internal (within the β -barrel) and external (putative) hydrophobic site(s) of Blg by chemical modi-

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¹Abbreviations: Blg, (β -lactoglobulin); bis-ANS, 4,4'-bis 1-(phenylamino)-8-naphthalenesulfonate; 5-DSA, 5-doxylosteic acid; ESR, electron spin resonance; MTS, 1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methylmethanethiosulfonate; MMTS, methylmethanethiosulfonate; DTNB, dithio-bis-nitrobenzoic acid; SDS, sodium dodecyl sulfate; Cys, cysteine.

Fig. 1. Structures of thiol modification reagents used in this study. (A) methyl iodide; (B) methylmethanethiosulfonate; (C) [(1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfonate] (MTSL).

fication studies of Cys 121 using intrinsic and extrinsic fluorescence as well as electron spin resonance spectroscopy.

Results

Influence of chemical modification upon the binding of retinol and bis-ANS to Blg

Cys 121 of Blg² was covalently modified by three reagents which varied in length and bulk as shown in Figure 1. The degree of thiol labeling was always $\geq 92\%$ as verified by both DTNB assay and ESR spin quantitations. Figure 2 shows a moderate to strongly immobilized spectrum of Blg spin labeled with MTSL [(1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethiosulfonate] at Cys 121 with a hyperfine extrema separation parameter, $2T_{||}$, of 57 ± 0.5 G. Upon addition of palmitic acid, the spectral features remained virtually *unchanged* within experimental error (data not shown). The fluorescence emission maximum ($\lambda_{\text{em}}^{\text{max}}$) was 338 ± 1 nm; λ_{ex} was 280 nm. In Blg, the contributions from tyrosine are negligible (compared with tryptophan fluorescence) allowing excitation at 280 nm for all three Cys 121 modified derivatives indicating that the global tertiary structure of Blg was relatively unaffected by these modifications. Far-UV CD spectra of the modified protein were identical to that of native Blg, indicating the secondary structure was unperturbed (data not shown). Furthermore, specific ligand binding results discussed below also appear to indicate no major change within the β -barrel of the protein.

The influence of Cys 121 modifications on the binding properties of the internal (β -barrel) hydrophobic site were examined

Fig. 2. X-band ESR spectrum of spin-labeled Blg. All solutions were at pH 7, 0.05 M Tris-HCl and at 20 °C. Spectrometer conditions are noted in Materials and methods.

using the specific ligand retinol (Papiz et al., 1986; Cho et al., 1994). Figure 3A, B, and C depict observed intrinsic fluorescence profiles ($\lambda_{\text{ex}} = 280$ nm) of Blg, Blg-S-CH₃, and Blg-S-SCH₃, respectively, corrected for retinol absorbance at the excitation wavelength, with increasing retinol which manifests itself in an energy transfer interaction between protein tryptophan(s) and the ligand, resulting in an apparent quenching of intrinsic protein fluorescence. Table 1 summarizes the binding parameters derived from nonlinear regression analysis fits to these titrations. Note that both the stoichiometry and nanomolar binding affinity of retinol to protein was *unaffected* by modification of Cys 121.

We also monitored the binding of NBD-stearic acid which was displaced when adding either retinol or bis-ANS (which also appears to bind within the β -barrel of the protein), but *not* palmitic acid (M. Narayan & L.J. Berliner, unpubl. obs.). Addition of protein to a fixed concentration of bis-ANS results in an enhancement of fluorescence emission (Fig. 4A–C) which was fit to a simple one-site binding scheme, yielding apparent binding constants in the 9–15 μM range for these derivatives (Table 2).

Binding of fatty acids to native and chemically modified Blg

Typical intrinsic fluorescence emission intensity titration profiles ($\lambda_{\text{ex}} = 280$ nm) for the binding of palmitic acid to Blg at pH 8.5 and 7.0 are shown in Figure 5A and B, respectively. There is approximately a 10% enhancement in fluorescence emission upon binding palmitic acid, in agreement with previous results of Frapin et al. (1993). In order to obtain accurate equilibrium parameters we measured this binding at significantly lower protein concentrations ($\sim 1 \mu\text{M}$) which are summarized in Table 3. It is clear that the Blg:lipophile interaction is *unaffected* over the 6.5–8.5 pH range (at higher pH the protein polymerizes) and presumably the degree of protonation (85%) of Cys 121, which has a pK_{app} of 9.35 (Kella & Kinsella, 1988).

We then monitored the binding of palmitic acid or 5-doxydstearic acid (5-DSA) to both spin-labeled protein (Blg-S-MTSL) and to protein that had been modified with either methylmethanethiosulfonate or methyl iodide. Table 4 lists the dissociation constants for this binding as monitored by both fluorescence and ESR spectroscopy. The spin labeled protein did not appear to bind to palmitic acid up to 20 μM concentration whereas thiomethyl-Blg (Blg-S-SCH₃) showed a decreased affinity ($K_d \sim 25 \mu\text{M}$) vs. native

²Native β -lactoglobulin is referred to as either Blg or Blg-SH where Blg-SH refers to the free thiol group of Cys 121 in Blg. S-X refers to a covalently modified Cys 121 where X is either -CH₃, or -SCH₃, or -MTSL.

Fig. 3. Intrinsic fluorescence emission profiles for the binding of retinol to native and modified Bg. (A) Bg-SH (1.47 μ M); (B) Bg-S-CH₃ (1.42 μ M); (C) Bg-S-SCH₃ (2.47 μ M). (λ_{ex} = 280 nm) All other conditions were identical to those in Figure 2.

Table 1. Dissociation constants and summary of binding stoichiometries for the interaction of retinol with native and modified β -lactoglobulin^a

Interaction of retinol with	K_d (nM)	Stoichiometry
Bg-SH	65 $\pm$ 12	1.0 $\pm$ 0.1
Bg-S-CH ₃	79 $\pm$ 8	1.0 $\pm$ 0.1
Bg-S-SCH ₃	58 $\pm$ 4	1.0 $\pm$ 0.1
Bg-S-MTSL ^b	70 $\pm$ 7	1.0 $\pm$ 0.1

^aConditions were 0.05 M Tris-HCl, pH 7, 20°C.

^bMTSL = (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-.

Bg which binds palmitic acid with a $K_d \approx 0.6 \mu$ M. Furthermore, simple methylation of Cys 121 (Bg-S-CH₃) results in a 10-fold reduction in the affinity for palmitic acid as compared to native Bg. Thus the interaction between the ligand and the protein becomes weaker with increasing bulkiness of the modifier group.

To independently verify these results, we also monitored palmitic acid binding to Bg-SH and Bg-S-X complexed with the extrinsic fluorophore, bis-ANS, as the reporter group. Figure 6A and B depict observed fluorescence emission intensity profiles for the titration of palmitic acid into fixed concentrations of either bis-ANS:Bg or bis-ANS:Bg-S-SCH₃. Control experiments, which were performed to determine whether palmitic acid:bis-ANS binary complex formation contributed to the observed fluorescence, were negative. Upon addition of palmitic acid to native Bg:bis-ANS complex, the bound fluorophore was quenched in a biphasic manner. Although this is indicative of palmitic acid to protein stoichiometries >1, possibly reflecting weaker binding of the lipophile to secondary fatty acid binding sites as shown earlier by Spector & Fletcher (1970), it also may contain contributions from the onset of micelle formation. On the other hand, there was no observable quenching when palmitic acid was incubated with bis-ANS:Bg-S-SCH₃, indicating that binding was very weak or obliterated. Although these data were somewhat less reliable compared with the other experiments above, they are nonetheless consistent with both the results from intrinsic fluorescence and ESR (Table 4). Consequently, Table 5 summarizes the binding parameters for palmitic acid binding to bis-ANS:protein complexes. A dissociation constant of $5 \pm 3 \mu$ M was fit to the binding of palmitic acid to the bis-ANS:Bg-S-CH₃ complex (data not shown) which agrees well with the value obtained for the first phase binding of palmitic acid to bis-ANS:Bg complex.

Discussion

We have probed the hydrophobic ligand binding properties of β -lactoglobulin by chemical modification of Cys 121 and as a function of the pH of the surrounding medium. Cys 121 lies between the sole α -helix of the protein and its β -sheets (Papiz et al., 1986; Monaco et al., 1987; Brownlow et al., 1997). The residue plays an important role in the polymerization of Bg as well as disulfide exchange that is known to take place in the protein during heating and at alkaline pH (Cairli et al., 1994). Chemical modification of Cys 121 was performed through a choice of three probes which varied both in length as well as bulkiness. Modification of

Fig. 4. Fluorescence emission profiles for the binding of bis-ANS (1–1.5 μ M) to native and modified Blg. (A) Blg-SH; (B) Blg-S-CH₃; (C) Blg-S-MTSL. Solid line represents a theoretical one-site binding profile. Fluorescence parameters were: λ_{ex} = 385 nm, λ_{em} = 485 nm from a scan over 400–600 nm. All other conditions were identical to those in Figure 2.

Table 2. Dissociation constants and summary of binding stoichiometries for the interaction of bis-ANS with native and modified β -lactoglobulin^a

Interaction of Bis-ANS with	K_d (μ M)	Stoichiometry
Blg-SH	12 ± 1.5	0.9 ± 0.1
Blg-S-CH ₃	14 ± 2.0	0.9 ± 0.2
Blg-S-MTSL ^b	11.5 ± 2.0	0.9 ± 0.1

^aConditions were 0.05 M Tris-HCl, pH 7, 20 °C.

^bMTSL = (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-.

Cys 121 did not appear to alter the tertiary structure of the protein as evidenced by fluorescence spectroscopy. In order to evaluate the effect of chemical modification of the thiol on the internal hydrophobic site of Blg (i.e., within the β -barrel), we probed the retinol binding properties of both native and chemically modified protein. Retinol binds to Blg, Blg-S-CH₃, Blg-S-SCH₃, and Blg-S-MTSL with essentially the same affinity (Table 1) indicating that these covalent linkers do not perturb the retinoid binding site. The binding of bis-ANS, a large fluorescent probe which is frequently used to probe hydrophobic sites on proteins (Musci & Berliner, 1985), and also binds within the barrel of the protein at the internal site (M. Narayan & L.J. Berliner, unpubl. obs.), is also unaffected by chemical modification of Cys 121 (Table 2).

It has been long established that β -lactoglobulin strongly binds 1 mole of long-chain fatty acids (myristic, palmitic, stearic acid, etc.) per mole of monomeric protein (Spector & Fletcher, 1970; Frapin et al., 1993; Dufour et al., 1994). This binding was sensitive to pH in that the protein was unable to bind fatty acids at the primary fatty acid binding site below pH 3.5. A lysine residue was hypothesized to be involved in the binding process (Creamer, 1995) whereby at neutral pH the carboxylate group of the fatty acid salt bridged to the positively charged ϵ -amino group. Although there has been no conclusive X-ray structural proof for the location of the fatty acid binding site on Blg, it was proposed to lie within the β -barrel of the protein at the retinoid site (perhaps overlapping it), based on the enhanced structural stability of the protein toward urea denaturation in the presence of SDS or palmitic acid (Creamer, 1995). Alternatively, results from intrinsic fluorescence studies of fatty acid binding to the Blg:retinol complex suggested that the fatty acid site might lie in the groove between the α -helix and β -sheets of the protein (Frapin et al., 1993; Dufour et al., 1994).

We investigated the fatty acid binding properties of both native Blg as well as the chemically modified protein. A study of the binding of palmitic acid to the native protein as function of pH indicated that increasing the pH from 6.5 to 8.5 had no significant effect on the binding of the fatty acid to the protein, although Cys 121 of Blg becomes increasingly electronegative in this pH range due to deprotonation of its thiol (Kella & Kinsella, 1988). This suggests that any "hydrogen bonding" between the carboxyl of the fatty acid and Cys 121 does not affect the affinity of the protein for the lipophile. Next, we looked at "steric effects" by covalently modifying Cys 121 of Blg by attaching groups of various lengths and bulk which might be expected to protrude into the external putative hydrophobic site on the protein. As the size of the modifier progressively increased from a methyl group to a pyrroline nitroxide spin label moiety,

Fig. 5. Intrinsic fluorescence emission profiles for the binding of palmitic acid to native Blg (7.4 μM). (A) pH 8.5; (B) pH 7.0. Fluorescence parameters were: $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 350 \text{ nm}$ from a scan over 300–450 nm. All other conditions were identical to those in Figure 3. The pH was checked at the beginning and end of each titration and found to remain constant within <0.02 pH units.

the fatty acid binding affinity progressively decreased and was finally obliterated, presumably due to complete steric hindrance. We verified this independently by bis-ANS fluorescence. Although the binding of palmitic acid to bis-ANS:Blg was weaker ($K_d = 5 \pm 2 \mu\text{M}$) than to Blg alone ($K_d \sim 0.6 \mu\text{M}$), the bis-ANS was *not displaced* suggesting that the two ligands bind at *separate sites*. A weakened fatty acid affinity might also be explained by a conformational change of the protein upon bis-ANS binding which was manifested at the fatty acid binding

Table 3. Dissociation constants for the interaction of palmitic acid with β -lactoglobulin with pH^a

pH	K_d (μM)
8.5	0.4 ± 0.2
7.0	0.6 ± 0.3
6.5	0.5 ± 0.1

^aProtein concentrations were $\sim 1 \mu\text{M}$, 0.05 M Tris-HCl, 20 °C.

site. However, remarkably, the binding affinity of palmitic acid to bis-ANS:Blg and bis-ANS:Blg-S-CH₃ was identical, suggesting that a methyl group was not sufficient to perturb the external hydrophobic site to any extent. Upon titration of palmitic acid to bis-ANS:Blg-S-SCH₃ complex, bis-ANS fluorescence was essentially unchanged (within error) indicating either the obliteration of lipophile binding to this type of complex or severely weakened binding. The explanation might lie in the fact that, given the weaker affinity of Blg-S-SCH₃ for palmitic acid ($K_d = 29 \mu\text{M}$) coupled with the fact that the presence of bis-ANS weakens the interaction between palmitic acid and native Blg, the fatty acid binding was extremely weak and consequently not detected.

Finally, we also monitored the structural stability of Blg in urea in the presence and absence of various ligands after Creamer (1995). The results (data not shown) showed a slight enhancement in stability when Blg was complexed with SDS or palmitic acid, however were not as pronounced as the near-UV CD and intrinsic fluorescence quenching results of Creamer (1995), who monitored the structural stability of Blg complexes at a 3:1 ratio of ligand to protein, where the slight increase in stability here may be explained by a secondary location of the fatty acid on the protein which is also consistent with our chemical modification results (see Table 6).

Taking into account the three considerations above, it appears very likely that the fatty acid binding site lies in the groove be-

Table 4. Dissociation constants for the interaction of palmitic acid and 5-doxylstearic acid with β -lactoglobulin measured from intrinsic protein fluorescence and ESR titration (where noted)^a

Fatty acid:protein	K_d (μM)
Palmitic:Blg-S-CH ₃	5.5 ± 1
Palmitic:Blg-S-SCH ₃	29 ± 4
5-DSA:Blg-SSCH ₃ ^b (ESR titration)	23 ± 3
Palmitic:Blg-S-MTSL ^c	n.d. ^d

^aConditions were 0.05 M Tris-HCl, pH 7, 20 °C.

^b K_d for 5-DSA binding Blg is $0.6 \mu\text{M}$ (Narayan & Berliner, 1997).

^cMTSL, (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-.

^dn.d., could not be determined.

Fig. 6. Fluorescence emission profiles for the titration of palmitic acid to native and modified bis-ANS:Blg complexes. (A) bis-ANS (0.85 μ M):Blg; (B) bis-ANS(0.95 μ M):Blg-S-SCH₃. Fluorescence parameters were: λ_{ex} = 385 nm, λ_{em} = 485 nm from a scan over 400–600 nm. All other conditions were identical to those in Figure 2.

tween the α -helix and the β -barrel of the protein at the external pocket. Figure 7 depicts a ribbon structure of Blg from the X-ray work of Brownlow et al. (1997) showing the putative fatty acid and retinoid site(s) relative to Cys 121.

Given the fact that Blg is able to bind different retinoids and fatty acids independently, and that the location of the fatty acid site appears to be at the external hydrophobic binding site on the protein, it seems reasonable that Blg may be a good candidate for genetic

Table 5. Dissociation constants for the interaction of palmitic acid with native and modified bis-ANS:Blg complexes^a

Palmitic acid binding to	K_d (μ M)
Bis-ANS:Blg-SH	5 ± 2^b
Bis-ANS:Blg-S-CH ₃	5 ± 3
Bis-ANS:Blg-S-SCH ₃	n.d. ^c

^aConditions were 0.05 M Tris-HCl, pH 7, 20°.

^bFit to the first binding phase in Figure 6 because the latter phases extend into micelle formation.

^cn.d., could not be determined.

engineering whereby, with rational modifications, it can serve as a carrier for purposes of nutrition or drug-delivery.

Materials and methods

Materials

Bovine β -lactoglobulin (Lot 51H7210, L0130 3 $\times$ crystallized), all-*trans*-retinol, 5-DSA and bis-ANS were obtained from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were of analytical reagent grade.

Preparation of ligand solutions

Retinol, bis-ANS and fatty acid stock solutions were freshly prepared using absolute ethanol (which had been purged with nitrogen gas) and were stored at -70°C in the dark. The concentrations of retinol and bis-ANS were determined spectrophotometrically using extinction coefficients $\epsilon_{325\text{nm}} = 46,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Dufour & Haertlé, 1991) and $\epsilon_{385\text{nm}} = 16,790 \text{ M}^{-1} \text{ cm}^{-1}$, respectively (Musci & Berliner, 1986).

Chemical modification and spin labeling of Cys 121 in Blg

A solution of Blg (10–150 μ M) was incubated at 4°C in the dark with a 2- to 10-fold excess of methylating agent (MMTS or CH₃I)

Table 6. Structural stability results for native and modified Blg in the presence and absence of fatty acid ligands^a

Blg sample	Spectroscopic method	Mid-point of urea denaturation (M)
Native	Fluorescence/CD ^b	4.4
+ SDS/Palmitic acid	Fluorescence/CD ^b	5.8
Native	Fluorescence	3.6
+ Palmitic acid ^c	Fluorescence	4.2
+ SDS ^c	Fluorescence	4.5

^aConditions were 0.05 M Tris-HCl, pH 7, 20°.

^bNear-UV CD and fluorescence quenching results using acrylamide taken from Creamer (1995).

^cGiven the binding affinity of palmitic acid and SDS for Blg, the 1:1 molar ratios used in this study correspond to >95% bound lipophile.

Fig. 7. Ribbon structure of bovine milk Blg (Brookhaven Protein Data Bank file no. BEB1) adapted from the X-ray work of Brownlow et al. (1997) showing the putative fatty acid and retinoid site(s) relative to Cys 121 (which has its sidechains denoted).

for 30 to 60 min followed by exhaustive dialysis against buffer (50-mM Tris-HCl adjusted to pH 7). Spin labeled Cys 121 was prepared by mixing 50–200 μ M Blg in Tris-HCl buffer (pH 7.5–8) with a 2- to 10-fold excess of the thiol specific spin label, MTSL [(1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)-methanethio-sulfonate], in acetonitrile for 10 to 60 min at 4°C in the dark (Berliner et al., 1982). The final concentration of acetonitrile was always <3% (v/v). The solution was centrifuged to remove a small amount of precipitate and then dialyzed exhaustively against 50 mM Tris-HCl (pH 7). ESR spectra of the dialysates were continuously monitored to check for free label which can also occasionally dimerize to form a biradical (which can be eliminated by using smaller initial label concentrations and further dialysis).

Stoichiometry of chemical modification

The stoichiometry of Cys 121 labeling was determined by the DTNB assay at pH 8.3 using an extinction coefficient $\epsilon_{412\text{nm}} = 11,400 \text{ M}^{-1} \text{ cm}^{-1}$ (Robyt & White, 1990) and by the ESR “spin count” technique for spin labeled thiols (Berliner, 1978). The number of spins was calculated from a double integrated spectrum of spin labeled protein which was compared against a standard solution of spin label. Protein concentration was determined spectrophotometrically using $\epsilon_{280\text{nm}} = 17,600 \text{ M}^{-1} \text{ cm}^{-1}$ (Dufour et al., 1992). All modified species, ligand bound species and the methylated protein were characterized by fluorescence, CD and retinol binding and compared with the native species. No significant differences were found in the secondary and tertiary structure.

Fatty acid, bis-ANS, and retinol binding to Blg

Small aliquots of stock fatty acid or retinol solution were titrated into a cuvette containing a given volume of the protein solution.

The protein was always a monomer under the protein concentrations and other conditions used in this work (Zimmermann et al., 1970). The final ethanol concentration was always <1% (v/v). Fluorescence spectra were obtained on a Perkin-Elmer LS 50B spectrofluorimeter at 20°C. For the bis-ANS binding studies, protein was titrated into a fixed bis-ANS solution at pH 7, in 50 mM Tris-HCl. Ligand plus protein absorbance were always <0.1 at the excitation wavelength used; emission spectra were corrected for secondary inner filter effects.

ESR measurements

X-band ESR spectra were measured in quartz capillaries (1 mm internal diameter) at ambient temperature (ca. $22 \pm 1^\circ\text{C}$) on a Varian E-9 spectrometer. Typical instrument settings were: microwave power, 20 mW; modulation frequency, 100 kHz; field set, 3380 G; scan range, 100 G; modulation amplitude, 1 G; time constant, 0.3 s; and scan time, 8 min. Measurements of the hyperfine extrema, $2T_{\parallel}$ ($\pm 0.5 \text{ G}$), were measured from “high gain” spectra which were typically recorded at 2- to 4-fold higher modulation amplitude and 4- to 10-fold higher receiver gain. A given volume of 5-DSA stock solution (in ethanol), diluted into a buffer (50 mM Tris-HCl at pH 7), was placed in the capillary tube and its ESR spectrum recorded. Then, small aliquots of native or chemically modified Blg were added sequentially. The final concentration of ethanol cosolvent was less than 1% (v/v) under these conditions.

Data analysis

For the ESR measurements, the high field line peak height was monitored as a quantitative indicator of free ligand. For the intrinsic fluorescence titrations, the observed emission intensities were converted to a response factor, which is defined as the fractional quenching(enhancement) between the free protein and the saturated ligand:protein complex. The data were fit to a simple hyperbolic binding model by nonlinear regression analysis using DeltaGraph (DeltaPoint, Inc, Monterey, California).

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