Fatty Acids and Retinoids Bind Independently and Simultaneously to β-Lactoglobulin

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ABSTRACT: β-Lactoglobulin (Blg) binds 1 mol of a fatty acid spin-label analog, 5-doxylstearic acid (5-DSA), per mole of protein with a dissociation constant $K_d = 0.8\ \mu$M for the strongest binding site. There are also several weaker sites for this ligand. Blg saturated with either retinol or retinoic acid binds 5-DSA with essentially equal affinity ($K_d = 0.6$ and 1 μM, respectively). Palmitic acid and SDS displace bound 5-DSA from Blg. However, unlike palmitic acid, 5-DSA binding does not enhance the structural stability of Blg to urea denaturation. The spin-labeled fatty acid also binds to the protein at low pH, presumably at secondary fatty acid binding sites. These results suggest that Blg binds at least two different types of hydrophobic ligands simultaneously.

β-Lactoglobulin (Blg)[1] is a small, globular whey protein found in the milks of many mammals including cow, horse, pig, dog, etc. (Pervaiz & Brew, 1985). Bovine Blg occurs as a dimer at neutral pH with a subunit molecular weight of ~18 000 daltons (Papiz *et al*., 1986). The protein undergoes octamerization at pH 4−5 (McKenzie, 1971). The multimerization process is complex although monomers predominate at low pH or at low concentrations (<20 μM) at neutral pH (Zimmerman *et al*., 1970). Blg has been shown to bind a variety of hydrophobic substances in vitro which include retinol (Futterman & Heller, 1972), retinoic acid, long-chain fatty acids (Spector & Fletcher, 1970), protoporphyrin (Dufour *et al*., 1990), heme−CO complexes (Marden *et al*., 1994), and aromatic compounds (Robillard & Wishnia, 1972; Dufour *et al*., 1992). Blg has been postulated to serve as a carrier for retinol in neonates (Sawyer *et al*., 1985). This carrier property, along with its remarkable acid stability, makes it an attractive candidate for protein engineering whereby Blg (or other proteins) could be modified or designed to serve as transporters for delivering important nutrients to specific targets in the alimentary tract (Sawyer, 1987).

Much effort has been devoted in the past in delineating the various ligand binding sites on Blg (Frapin *et al*., 1993; Creamer, 1995). It has been shown that retinal is bound within the β-barrel of the molecule, also implying the binding of retinol at the same site (Cho *et al*., 1994). Frapin *et al*. (1993) and Dufour *et al*. (1994) suggested that fatty acids may bind at an external binding pocket based on fluorescence results which suggested that fatty acids could bind to Blg complexed with retinol. On the other hand Creamer (1995), demonstrated enhanced stability of the protein to urea denaturation when complexed with either palmitic acid or low concentrations of SDS and concluded that the protein must bind fatty acids within the β-barrel at or overlapping the retinol binding site. In order to resolve this discrepancy we have probed the binding of fatty acid analogs to Blg and to Blg:retinoid complexes by fluorescence and electron spin resonance spectroscopy.

MATERIALS AND METHODS

Materials. Bovine β-lactoglobulin (Lot 51H7210, L0130, 3× crystallized), *all-trans*-retinol, retinoic acid, 5-DSA, 12-DSA, and 16-DSA were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade.

Preparation of Delipidated Blg. Blg was delipidated by employing the method of Chen (1967). Protein concentration was determined spectrophotometrically using an extinction coefficient of $\epsilon_{278nm} = 17\,600\ M^{-1}\ cm^{-1}$ (Dufour *et al*., 1992). Protein solutions were centrifuged before use. At the protein concentration range used in these experiments, Blg exists predominantly as the monomer (Zimmermann *et al*., 1970), although it is important to note that both the dimer and monomer are able to bind retinoids with equal affinity (Fugate & Song, 1980).

Preparation of Ligand Solutions. Retinol, retinoic acid, 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 retinoic acid were determined spectrophotometrically using extinction coefficients $\epsilon_{325nm} = 46\,000\ M^{-1}\ cm^{-1}$ and $\epsilon_{350nm} = 45\,000\ M^{-1}\ cm^{-1}$ respectively (Dufour & Haertle, 1991).

ESR Measurements. ESR spectra were measured in quartz capillaries (1 mm i.d.) at ambient temperature (*ca*. 22 ± 1 °C) on a Varian E-9 X-band spectrometer. Typical instrument settings were as follows: 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, 4 min. Measurements of the hyperfine extrema, $2T_{||}$ (±0.5 G), were taken from "high gain" spectra

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[1] Abbreviations: Blg, β-lactoglobulin; 5-DSA, 5-doxylstearic acid; 12-DSA, 12-doxylstearic acid; 16-DSA, 16-doxylstearic acid; ESR, electron spin resonance; cmc, critical micelle concentration.

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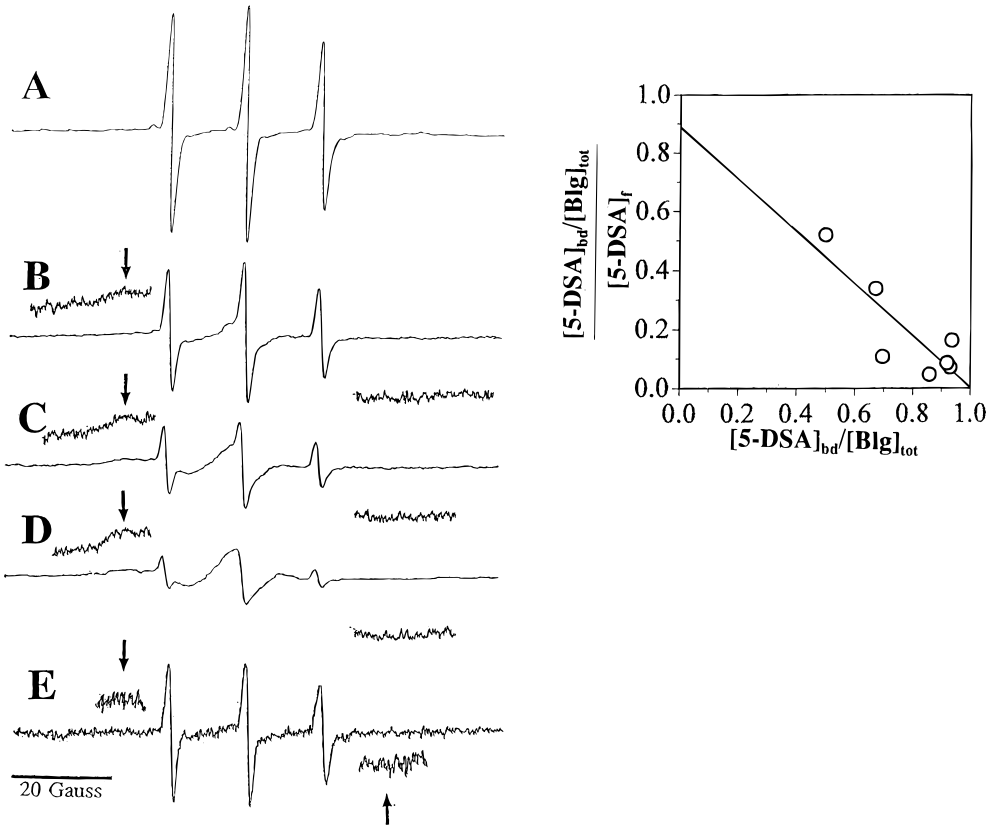

FIGURE 1: X-band ESR spectra of 5-DSA:Blg complexes. (A) 23 μM 5-DSA alone; (B) 11.5 μM Blg plus 23 μM 5-DSA; (C) 23 μM Blg plus 23 μM 5-DSA; (D) 46 μM Blg plus 23 μM 5-DSA; (E) 80 μM Blg plus 23 μM 16-DSA. (Inset) Scatchard plot of 5-DSA binding to Blg where $[5\text{-DSA}]_{bd}$ = bound ligand, $[5\text{-DSA}]_f$ = free ligand, and $[Blg]_{tot}$ = total protein. All solutions were pH 7, 0.1 M phosphate buffer.

measured immediately after the normal 100 G scan. High gain spectra were typically recorded at 2−4-fold higher modulation amplitude and 4−10-fold higher receiver gain. A given volume of DSA stock solution was placed in the capillary tube, and its ESR spectrum was recorded. Then, small aliquots of Blg or Blg complexed with either retinol or retinoic acid were added. The final concentration of ethanol was less than 0.4% (v/v) under these conditions.

Ascorbate Reduction Kinetics. A solution of DSA or DSA:protein complex was incubated with an ~50-fold excess of ascorbic acid (pH 7, 0.1 M phosphate) and the ESR spectra recorded every 120 s in a flat cell.

Fluorescence Measurements. Fluorescence spectra were obtained on a Perkin-Elmer LS 50B spectrofluorimeter at 20 °C. Small aliquots of concentrated spin-labeled fatty acid stock solution (in ethanol) were titrated into a cuvette containing Blg. The final ethanol concentration was always <1% (v/v).

RESULTS

Binding of Spin-Labeled Fatty Acids to Blg. A typical ESR spectrum of free 23 μM 5-DSA at pH 7 (0.1 M phosphate) is shown in Figure 1A. Upon the addition of increasing concentrations of delipidated Blg, there was a decrease in the free 5-DSA spectral component as monitored by a diminution of the narrow high field peak (Figures 1B−D) with a concomitant increase in a broad, strongly immobilized spectral component of bound 5-DSA.[2] The hyperfine extrema separation parameter, $2T_{||}$, which is a measure of the mobility of the label, was 53.5 ± 0.5 G in this case (arrows, Figures 1B−D). A Scatchard plot of this binding is shown in the inset to Figure 1. When nondelipidated protein was used the reduction in the free ligand signal was somewhat less, which reflects the ~0.5 mol:mole of palmitic acid to protein present in commercial preparations (Perez *et al*., 1989). When Blg binds to 12- and 16-DSA, the interaction is considerably weaker as shown in Figure 1E where, for example, at a 3-fold excess of protein to fatty acid concentration, a considerable amount of free ligand remained *vs* that in Figure 1C and D.

We also confirmed this binding behavior by intrinsic fluorescence spectroscopy. Figure 2A and B depict binding profiles for 5-DSA and 16-DSA binding to delipidated Blg, respectively, as monitored by tryptophan emission at 344 nm. The biphasic data for 5-DSA binding to Blg (Figure 2A) was fit to two classes of independent sites: the first site enhances intrinsic fluorescence, while the second class quenches fluorescence. On the other hand 12- and 16-DSA binding was characterized by hyperbolic quenching behavior (Figure 2B). Table 1 summarizes the binding results for spin-labeled fatty binding to Blg by ESR and fluorescence. It is clear that Blg possesses a single, strong binding site for 5-DSA with a dissociation constant around 0.8 μM. There may also be some weaker, perhaps nonspecific sites for this ligand; however, this apparent binding falls in that concentration range where DSA micelles begin to form, making the analysis more complicated. The tight (K_d = 0.8 μM) binding site agrees well with the results of Spector and

[2] One can assume here that the peak height of the free line is a direct measure of 5-DSA concentration since it did not overlap the bound spectral component and since the line width did not change over the course of the titration.

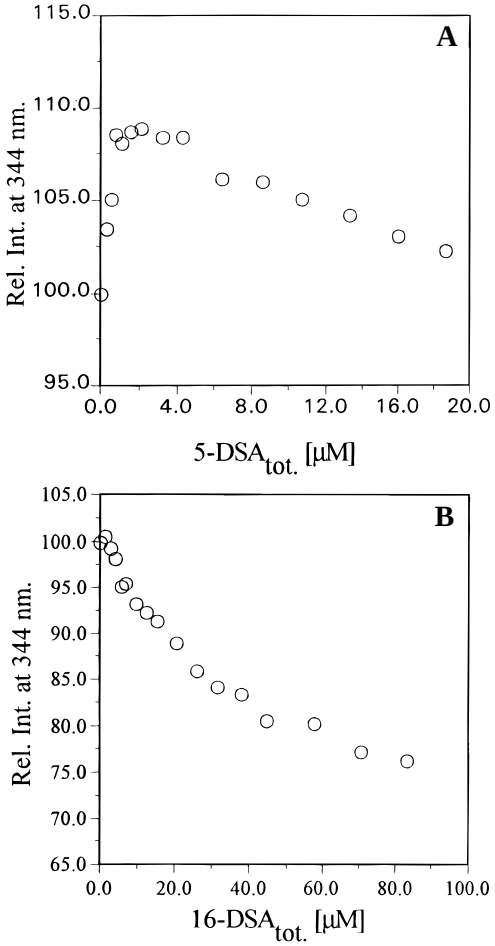

FIGURE 2: Intrinsic fluorescence emission profiles for the binding of 5- and 16-doxyl spin-labeled fatty acids to 3 μM Blg. (A) 5-DSA; (B) 16-DSA. Emission intensities were normalized to protein alone. All other conditions were identical to those in Figure 1. Fluorescence parameters were $\lambda_{ex} = 280$ nm, $\lambda_{em} = 344$ nm from a scan over 300−450 nm.

Table 1: Dissociation Constants and Summary of Binding Stoichiometries for the Interaction of Fatty Acid Analogs with β-Lactoglobulin[a]

	ESR		fluorescence			
ligand	K_d (μM)	n	K_{d1} (μM)	n	K_{d2} (μM)	n
5-DSA	0.8 ± 0.3	1 ± 0.1	0.4 ± 0.2	1 ± 0.02	43 ± 5	1.7 ± 0.5
12-DSA	397 ± 4	7 ± 1	no strong site		412 ± 12	7.2 ± 0.7
16-DSA	nd	nd	no strong site		278 ± 8	6.6 ± 1.2

[a] nd, could not be determined. Conditions were 0.1 M potassium phosphate, pH 7.

Fletcher (1970) and Frapin *et al*. (1993). On the other hand, the results with 12- and 16-DSA were quite different from those for 5-DSA. While there was no evidence for a strong binding site, there was evidence for the presence of multiple sites with dissociation constants >100 μM, which also correlates well with the secondary binding sites found by Spector and Fletcher (1970).

In order to examine the solvent accessibility of the strongly and weakly immobilized spectral components, representing the various bound states of the spin-labeled fatty acids, we measured the ascorbate reduction kinetics. Table 2 lists the half-lives of nitroxide reduction in the presence of 1 mM ascorbate as monitored by the psuedo-first-order steady decrease in ESR signal peak height with time. Note that the half-lives for 12-DSA in the absence and presence of excess Blg were almost identical while 5-DSA was partially protected from reduction in the binary complex with Blg as evidenced by a 50% longer half-life (Table 2).

Table 2: Kinetics of the Reduction of the Nitroxide Moiety of Spin-Labeled Fatty Acid Analog by Ascorbate[a]

fatty acid:Blg	half-life (s)
5-DSA:Blg, 24 μM:80 μM	2900 ± 150
5-DSA (alone), 24 μM	2050 ± 80
12-DSA:Blg, 24 μM:80 μM	2000 ± 200
12-DSA (alone), 24 μM	1940 ± 60

[a] Conditions were 0.1 M potassium phosphate, pH 7.

Competitive Binding of 5-DSA to Blg:Retinol Complex. Figure 3A again depicts an ESR spectrum of 23 μM 5-DSA. Figure 3B−D depicts ESR spectra of 5-DSA:Blg:retinol complexes at varying ratios, which should be compared with the set in Figure 1B−D which contain no retinol. Note that the hyperfine extrema separation parameter, $2T_{||}$, of 54 ± 0.5 G (arrows) was essentially identical to the spectrum of 5-DSA:Blg complex shown in Figures 1C,D. The spin-labeled fatty acid even binds to Blg at saturating retinol concentrations as shown in the Scatchard plot for a 5-DSA titration of Blg:retinol (1:1) complex (Figure 3, upper inset). Similar results were found for retinoic acid (data not shown). Table 3 summarizes the dissociation constants calculated for 5-DSA binding to Blg, Blg:retinol, and Blg:retinoic acid complex, respectively. In summary, there was very little change, if any, in these binding constants under a variety of conditions. Large excesses of retinol (≥100 × Blg:5-DSA) were ineffective in displacing 5-DSA from the complex. However, upon addition of palmitic acid or SDS to 5-DSA:Blg:retinol (1:1:1), only free, unbound 5-DSA appeared in the ESR spectrum, consistent with competitive displacement *only* at the fatty acid binding site (Figure 3E). Finally, we monitored the fluorescence of retinol complexed to Blg as a function of added 5-DSA. Partial, but not complete, quenching of retinol fluorescence was observed (Figure 3, lower inset) with a $K_d \approx 1$ μM, consistent with simultaneous binding of retinol and 5-DSA, which was essentially identical to the value reported in Table 3.

Binding of Fatty Acids to Blg at Low pH and in Retinol Micelles. It was previously suggested that Blg was unable to bind fatty acids at low pH since the carboxyl group of the fatty acid was protonated at pH's <4.5 (Frapin *et al*., 1993). Figure 4A shows a spectrum of 23 μM 5-DSA at pH 2. Under these conditions there is spin−spin dipolar and Heisenberg exchange broadening due to the presence of micelles of DSA formed at this pH (Rehfeld *et al*., 1978). Upon addition of Blg, a composite spectrum of 5-DSA:protein and micellar 5-DSA appears (see Figure 4B). The "apparent increase" in intensity is due in part to solubilization of some free 5-DSA molecules from the micelle. Finally, Figure 4C shows a spectrum of 5-DSA in excess retinol (pH 7) where the strongly immobilized component ($2T_{||}$ = 64 ± 0.5 G) reflects an immobilized nitroxide on a slowly tumbling macromolecular aggregate. Upon addition of Blg (Figure 4D), the spectrum converts to that of 5-DSA:Blg ($2T_{||}$ = 54 ± 0.5 G), similar to that found earlier in Figure 1C (although some contribution to the overall motion from aggregated micellar retinol is still present).

Urea Denaturation Studies of Blg:5-DSA Complex. We examined the urea denaturation of 5-DSA:Blg complex by

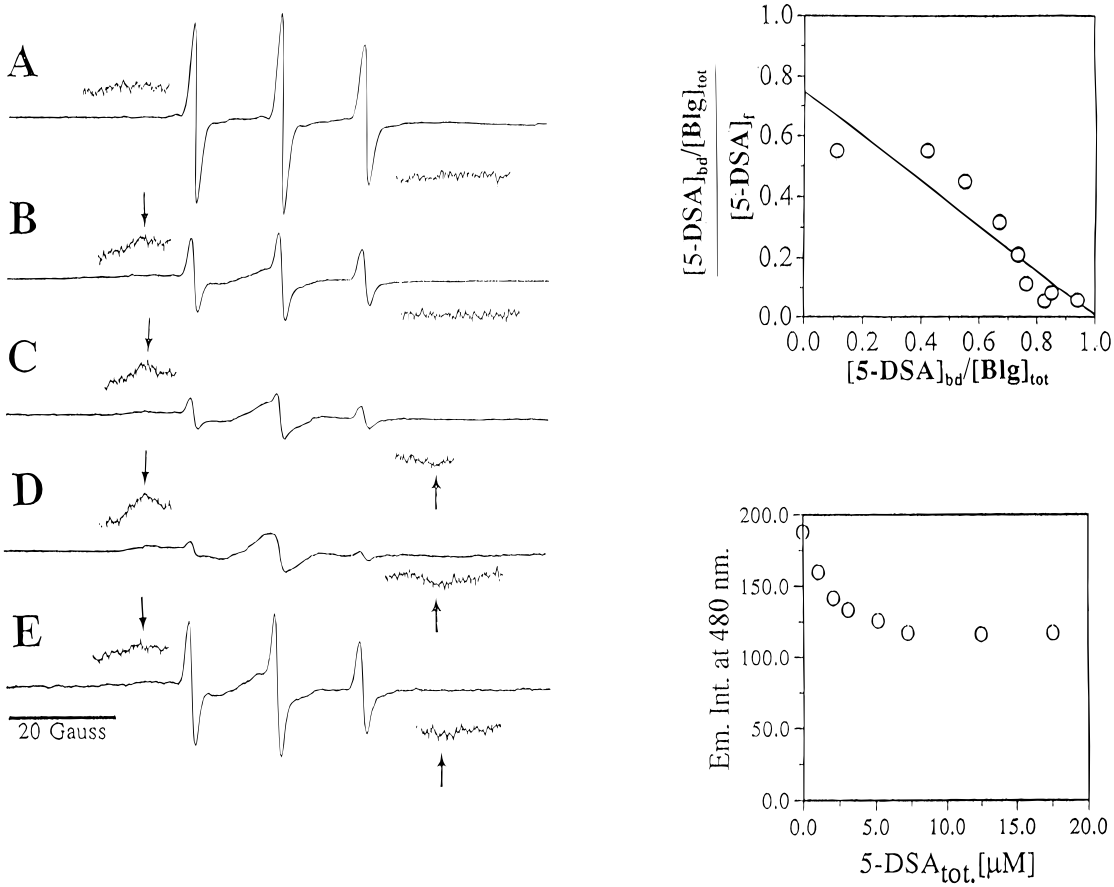

FIGURE 3: ESR spectra of Blg:retinol:5-DSA complexes. (A) 23 μM 5-DSA alone; (B) 13 μM Blg:13 μM retinol:23 μM 5-DSA; (C) 24.6 μM Blg:24.6 μM retinol:23 μM 5-DSA; (D) 48 μM Blg:48 μM retinol:23 μM 5-DSA; (E) 21 μM Blg:21 μM retinol:21 μM 5-DSA plus 60 μM SDS. (Upper Inset) Scatchard plot of 5-DSA binding to (1:1) Blg:retinol complex. (Lower Inset) Fluorescence emission profile of retinol (2.9 μM):Blg (2.5 μM) complex with increasing concentrations of 5-DSA. Fluorescence parameters were $\lambda_{ex} = 325$ nm, $\lambda_{em} = 480$ nm from a scan over 400−550 nm. All other conditions were identical to those in Figure 1.

Table 3: Dissociation Constants and Stoichiometry for 5-DSA Binding to Retinoid:Blg Complexes[a]

complex	K_d (μM)	n
Blg:retinol, 23 μM:23 μM	0.6 ± 0.1	1.0 ± 0.1
Blg:retinoic acid, 23 μM:23 μM	1.0 ± 0.02	1.2 ± 0.3

[a] Conditions were 0.1 M potassium phosphate buffer, pH 7.

ESR, since it was previously shown by intrinsic fluorescence spectroscopy that the binding of palmitic acid or SDS (<100 μM) to Blg enhances protein structural stability (Creamer, 1995). Figure 5 is a plot of the high-field line peak height of (unbound) freely tumbling 5-DSA as a function of increasing urea concentration which presumably represents increasing unfolded Blg that no longer binds fatty acid. The peak height increased with added denaturant until ca. 7 M urea at which concentration the 5-DSA was totally displaced from the protein. A control experiment (in order to correct for any viscosity effects on the free 5-DSA spectrum), indicated that over this urea concentration range, the viscosity effects were negligible. The midpoint of unfolding of 5-DSA:Blg complex was estimated at 4.4 M urea.

DISCUSSION

The binding of spin-labeled fatty acid analogs to Blg provides useful insights into the hydrophobic binding topography of this protein. Both the fluorescence and ESR results suggest that 5-DSA binds to Blg with an affinity similar to that found with stearic and palmitic acids (Spector & Fletcher, 1970). Remarkably, the protein did not appear to have a strong affinity for either 12- or 16-DSA. One model suggests that there is a specific locus for the 5-doxyl moiety in the hydrophobic binding region of the protein and consequently the position of the doxyl moiety on the fatty acid chain is critical. Otherwise a large part of the fatty acid analog is sterically prohibited from binding.[3] Thus in the case of 12- or 16-DSA, the doxyl moiety lies outside the binding region (i.e., into the solvent) and is freely tumbling since it is restricted from binding at the lipid site. The fluorescence results do not support this latter argument since strong binding was not detected for 12- and 16-DSA (Table 1). Recall that for 5-DSA binding to Blg a biphasic binding isotherm was observed by fluorescence which appeared first as an emission enhancement, followed by a quenching step which was also common to 12- and 16-DSA (Figure 2A). This fluorescence enhancement was observed for natural fatty acids by Frapin *et al*. (1993). Lastly, kinetic studies of 5-DSA nitroxide reduction with ascorbate showed that Blg bound 12-DSA was as exposed to solvent as free 12-DSA in comparison to Blg bound 5-DSA which was less exposed when incubated with excess Blg.

The cmc of 5-DSA under the conditions of our experiments is about 35 μM above which the monomeric concentration of 5-DSA remains constant (Rehfeld *et al*., 1978). Blg appears to bind fatty acids in both their monomeric and

[3] It also is relevant to note that dicarboxylic fatty acids do not bind to Blg (Frapin *et al*., 1993).

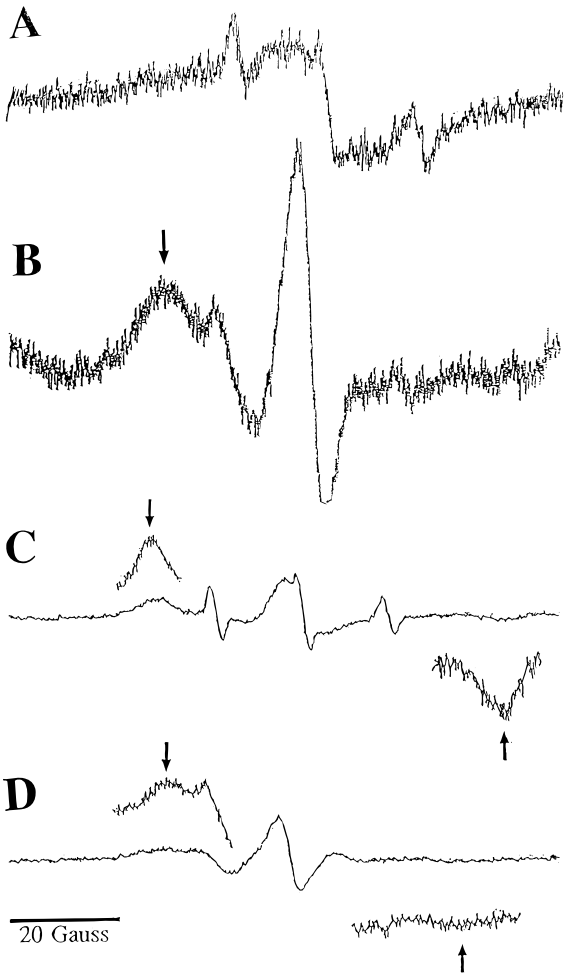

FIGURE 4: ESR spectra at low and neutral pH. (A) 23 μM 5-DSA alone (pH 2, 50 mM NaCl); (B) 23 μM (1:1) 5-DSA:Blg (pH 2, 50 mM NaCl). Spectra A and B were measured at the same spectrometer gain. (C) 23 μM 5-DSA in 1.2 mM retinol (pH 7, 0.1 M phosphate); (D) sample C plus 23 μM Blg. Spectra C and D were measured at the same spectrometer gain.

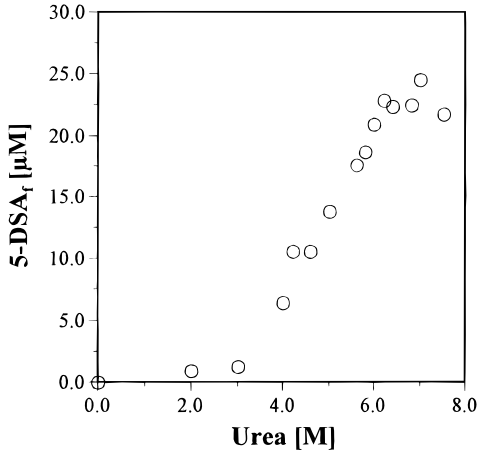

FIGURE 5: Urea denaturation of 23 μM 5-DSA:Blg complex. The unfolding was monitored by the ESR high-field line peak height of free 5-DSA as a function of added urea. All other conditions were identical to those in Figure 1.

micellar states (Spector & Fletcher, 1970). There is a single strong binding step in the monomeric region followed by multiple sites in the micellar phase which are difficult to quantitate accurately. To our knowledge, there has been no hypothesis forwarded so far as to the location or nature of micellar fatty acid binding site(s) on Blg.

The retinol binding site was suggested to lie inside the β-barrel from chemical modification studies (Cho *et al*., 1994), however a controversy remained as to whether Blg could bind fatty acids simultaneously at the strong hydrophobic site(s). Frapin *et al*. (1993) and Dufour *et al*. (1994) suggested that an external, independent fatty acid binding site lies in a hydrophobic groove along the sole α-helix of the protein. On the other hand, Creamer (1995) suggested that fatty acids (or detergents such as SDS) bind at the hydrophobic barrel of the protein at a site that presumably overlaps the retinol binding site. However, the ESR results presented in this study support *simultaneous binding* of retinoids and fatty acids to Blg. Yet it is understandable that a steady-state fluorescence study might miss this binding since the data are more difficult to interpret when several ligands bind simultaneously as well as the problems of aggregation, light scattering and overlapping conformational changes that need to be sorted out. On the other hand, ESR monitors the binding directly under conditions where both fatty acid and protein are in the monomeric state. Furthermore, these results showed that 5-DSA binds to Blg with equal affinity in the absence *or presence* of retinoids since large excesses of retinol (1500K_d) were unable to displace 5-DSA from the protein while palmitic acid or SDS displaced the spin-label competitively (Figure 3E). Consequently our results suggest the presence of at least two strong hydrophobic sites on Blg. Neither retinol nor fatty acid can occupy both sites in the absence of the other ligand as evidenced from the binding stoichiometry of 5-DSA to Blg in the absence and presence of retinol.

We also observed the binding of fatty acid analogs to Blg at pH 2, in contrast to the contention (from intrinsic fluorescence studies) that fatty acids are unable to bind due to the lack of a crucial salt bridge between Lys 70 and the ionized carboxyl group of the fatty acid (Frapin *et al*., 1993). However it is possible again that the fluorescence was simply too insensitive to fatty acids binding at low pH. Although it was more difficult to determine a dissociation constant or binding stoichiometry for 5-DSA:Blg by ESR at low pH (due to problems in correcting for the exchange broadened component), a qualitative inspection of the data suggested that the binding was much weaker and may, in fact, reflect binding to Blg secondary sites instead (Figure 4B).

Previous urea denaturation studies supported an enhanced protein stability upon binding of SDS or palmitic acids (Cremer, 1995). It was hypothesized that this resulted from either increased hydrophobic interactions between the β-barrel and the lipophile wherein displacement of water molecules from the barrel contributed to stabilization. In contrast, however, our ESR studies showed that 5-DSA was *unable* to impart additional stabilization to Blg. Unless interaction of the 5-doxyl group with the protein somehow blocks the promotion of enhanced stabilization, it is ironic since 5-DSA binds to Blg with an affinity quite similar to that of palmitic acid.

Since both retinol/retinoic acid and fatty acids bind simultaneously to Blg and also that SDS or palmitic acid binding enhances protein stability, it may be possible that the hydrophobic β-barrel of Blg can accommodate two distinct ligands. Another domain for the fatty acid binding site could comprise the second putative hydrophobic site between the α-helix and the β-barrel of the protein (Monaco *et al*., 1987; Frapin *et al*., 1993). The retinol binding site

has been identified from chemical modification studies to be within the barrel (Cho *et al*., 1994). Lys 70, which lies close to the carboxyl group of retinoic acid, may especially interact with one or both of these ligands (retinoic acid or fatty acid). It is pertinent to note that for pig Blg, where Lys 70 is absent, fatty acid binding is also absent (Frapin *et al*., 1993). In addition, retinoic acid binds with weaker affinity to the K70M bovine Blg mutants (Cho *et al*., 1994). Lastly, it is interesting to note that there is also Lys 69 in Blg which may play a role in binding these ligands. Future site-directed mutagenesis and X-ray studies of other Blg: ligand complexes may help to refine the details of these binding interactions. Most significantly, the results presented herein have shown that Blg is able to bind several physiologically relevant ligands simultaneously and can thus serve as a potential carrier protein.

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