**Interactions of α-Lactalbumin with Fatty Acids and Spin Label Analogs**

(Received for publication, May 29, 1997, and in revised form, September 2, 1997)

Kevin M. Cawthern, Mahesh Narayan, Dipankar Chaudhuri, Eugene A. Permyakov, and Lawrence J. Berliner

*From the Departments of Chemistry and Medical Biochemistry and the Biophysics Program, The Ohio State University, Columbus, Ohio 43210*

Bovine α-lactalbumin (α-LA) has been shown by intrinsic protein fluorescence and electron spin resonance methods to interact with the spin-labeled fatty acid analog, 5-doxylstearic acid, as well as stearic acid. An intrinsic fluorescence titration of various α-LA forms with 5-doxylstearic acid causes first an increase and then a decrease in emission intensity with concomitant shifts in tryptophan emission wavelength. In some cases, up to three steps in the fluorescence titration curves were visible, which were fit to apparent binding steps from $10^{-6}$ to $10^{-4}$ M. The binding parameters of 5-doxylstearic acid for apo- and Ca$^{2+}$-α-LA were an order of magnitude different from one another; the stronger one, apo-α-lactalbumin, exhibited a $K_d$ of 35 μM. Electron spin resonance titrations of 5-doxylstearic acid-loaded apo-α-LA with stearate (micelles) seem to suggest separate binding loci if α-LA indeed binds stearate at these concentrations. The titration of α-LA by stearic acid results in a fluorescence emission red shift and an apparent stepwise increase in fluorescence intensity. Lipid-protein association occurred at concentrations at which stearic acid micelles and aggregates begin to form in the absence of protein. Nonetheless, the relatively strong association between stearic acid and apo-α-LA was also confirmed by means of the fluorescent indicator acrylodan fatty acid binding protein, in which addition of stearic acid micelles and aggregates begin to form in the absence of protein. Nonetheless, the relatively strong association between stearic acid and apo-α-LA was also confirmed by means of the fluorescent indicator acrylodan fatty acid binding protein, in which addition of α-LA to the stearate-loaded indicator protein reverses the decrease in fluorescence of the acrylodan chromophore conjugated to the protein.

Many milk protein components, particularly the whey proteins and caseins, are known to be lipophilic under certain conditions. Interactions between lipids and β-lactoglobulin (Blg)$^1$ have been reported (1–3), as well as a structural homology between milk β-lactoglobulin and plasma retinol binding protein (4). A role for Blg as a retinol transport protein in the neonatal small intestine has been suggested (4, 5), as has a role in assisting product uptake of milk fat digestion (3).

Another milk protein with potential roles in lipid transport is α-lactalbumin (α-LA),$^1$ an acidic whey protein of $M_r \approx 14,200$, which is expressed during lactogenesis in the mammary gland. The primary function of this protein is to modulate acceptor specificity in the lactose synthase complex of mammary cells (6). α-LA has lipophilic properties, interacting with hydrophobic peptides, such as melittin (7), model lipid membranes (8–12), and hydrophobic chromatographic supports (13). However, significant association between α-LA and fatty acid had not been observed with these lipids to date (3). This is perplexing because significant quantities of α-LA were found to be associated with milk fat globule membranes, suggesting a possible role for this protein in fat digestion or transport.

α-LA and β-lactoglobulin are structurally distinct, as α-LAs possess homologies to c-type lysozymes (14, 15). Another significant difference is that α-LA has a strong Ca$^{2+}$-binding site, which competitively binds Na$^{+}$, K$^+$, Mg$^{2+}$ (16–19), and several distinct Zn$^{2+}$-binding sites (20, 21). Changes in the metal-bound state (or pH) of the protein have been shown to modulate both the conformation of α-LA and its interactions with hydrophobic ligands. For example, apo-α-LA binds more effectively to model membranes (9) and possesses higher affinity for hydrophobic probes than does Ca$^{2+}$-α-LA (7, 13, 22). Furthermore, under acidic conditions (molten globule), α-LA associates readily with these lipophiles (23, 24).

We have speculated recently that α-LA may also function as a transport protein for lipophilic substances (25) because it is also possible that earlier studies of the low affinity calcium form of the protein (3) may have missed strong α-LA-fatty acid association. The present work examines the association of α-lactalbumin with fatty acid spin-labeled analogs by several physical methods.

**EXPERIMENTAL PROCEDURES**

*Materials—* Bovine α-LA (lot 128F-8140), 5-doxylstearic acid (5-DSA), and 16-doxylstearic acid (16-DSA) were from Sigma. Apo α-LA was devoid of calcium as verified by intrinsic fluorescence prior to use (19). The protein was found to be in excess of 97% pure by silver-stained SDS-polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography. Acrylodan intestinal fatty acid binding (ADIFAB) protein (A-3880) was purchased from Molecular Probes, Inc. (Eugene, OR). Bis-tris propane (BTP) was purchased from Research Organics, Inc. (lot E7907). Stearic acid was from Mallinckrodt Inc. (Paris, KY) and Nu Chek Prep, Inc. (Elysian, MN); palmitic acid was from Fisher (Pittsburgh, PA) and Nu Chek Prep, Inc.

*Methods—* All glassware, including ESR cells and quartz tubes, was rinsed with EGTA and buffer solutions before use. Stock solutions of the doxylstearic acid; cmc, critical micelle concentration.
protein were prepared in 10 mM BTP, 1.5 mM EGTA, pH 7.4. Fluorescence spectra were recorded on a Perkin-Elmer LS-50B spectrophotometer. Intrinsic fluorescence excitation for α-LA was fixed at 280 nm, where, despite the fact that α-LA contains four tryptophans, the Tyr contribution to total protein fluorescence is negligible (18), and excitation for ADIFAB protein was fixed at 390 nm. α-LA concentration was measured spectrophotometrically on a Kontron 930 spectrophotometer using an extinction coefficient of $E_{280nm}^\text{protein} = 20.1$ (26). The fatty acids were dissolved in 50% (v/v) ethanol at elevated temperatures near the melting point and then diluted with aqueous buffer as stock solutions for intrinsic fluorescence titration experiments. The titration data were fit to various binding schemes by nonlinear regression (27). The theoretical curves corresponding to the chosen binding schemes were calculated numerically and fit to the experimental data by variation of the binding parameters. The precision in binding parameters is estimated conservatively to be within one-half order of magnitude for the best model applied to the fit.

ESR Measurements—ESR spectra were measured on a Varian Associates E-9 spectrometer at 22 °C. Typical instrument settings were as follows: microwave power, 20 mW; 100 kHz modulation amplitude, 0.63 G; field set, 3380 G; sweep width, 100 G; scan time, 4 min; time constant, 1 s. An aliquot of 5-DSA solution in the above buffer (containing 20% ethanol) was pipetted into a quartz ESR tube and measured, followed by an aliquot of apo-protein that was allowed to equilibrate before the spectrum was obtained. The high field line peak height was monitored as a quantitative indicator of free ligand. Binding isotherms before the spectrum was obtained. The high field line peak height was followed by an aliquot of apo-protein that was allowed to equilibrate.

RESULTS

ESR Titrations with 5-DSA—Fig. 1A shows an X-band ESR spectrum of 485 μM apo-α-LA and 30 μM 5-DSA (A) and 1.9 mM Ca$^{2+}$-loaded α-LA plus 30 μM 5-DSA (B). Conditions were pH 7.4 (1.5 mM EDTA, 10 mM BTP). C, molten globule 485 μM apo-α-LA and 30 μM 5-DSA at pH 2 in 10 mM BTP. High gain spectral bound spectral components are designated by arrows.

ESR peak height with increasing [5-DSA] and the lack of Heisenberg exchange, which would arise from DSA aggregates. ADIFAB protein were determined using molar extinction coefficients $\epsilon = 16,900$ M$^{-1}$ cm$^{-1}$ (280 nm) and $\epsilon = 10,600$ M$^{-1}$ cm$^{-1}$ (360 nm), respectively (29). Since ADIFAB fluorescence is sensitive to the presence of ethanol, fatty acid stock solutions were prepared (as recommended by the manufacturer) as the soap with 4 mM KOH.

Estimation of cmc for 5-DSA—The cmc of 5-DSA is about 35 μM in buffer solutions similar to those used here (28). Above this level, the concentration of 5-DSA monomers remains constant. Therefore, to probe interactions between α-LA and 5-DSA monomers, one must work below the cmc of the ligand. This is complicated when the affinity of the ligand for the protein is less than its tendency to form micelles, requiring the use of specific cosolvents that favor dispersal of the ligand into monomers. Since relatively low concentrations of ethanol can solubilize fatty acids, we have used this solvent at concentrations up to 20% (v/v), which corresponds to a cmc of 50 μM for 5-DSA and has negligible effects on the protein tertiary structure as monitored by intrinsic fluorescence. The free 5-DSA was always below the cmc as confirmed by linearity in ESR peak height with increasing [5-DSA] and the lack of Heisenberg exchange, which would arise from DSA aggregates.

ADIFAB Binding—Protein and acrylodan concentrations for ADIFAB protein were determined using molar extinction coefficients $\epsilon = 16,900$ M$^{-1}$ cm$^{-1}$ (280 nm) and $\epsilon = 10,600$ M$^{-1}$ cm$^{-1}$ (360 nm), respectively (29). Since ADIFAB fluorescence is sensitive to the presence of ethanol, fatty acid stock solutions were prepared (as recommended by the manufacturer) as the soap with 4 mM KOH.

receiver gain were typically set at 3–5 times the normal value to enhance the bound, immobilized component. The maximum hyperfine extrema, or $2T_2$, serves as a semiquantitative measure of the rotational correlation time of the nitroxyl spin label complexed to the macromolecule.

**Fig. 2.** ESR binding isotherm from titration of 5-DSA with apo-α-LA. The ESR spectra were analyzed as noted under “Experimental Procedures.” The concentrations of bound and free spin label (5-DSA<sub>bound</sub> and 5-DSA<sub>free</sub>, respectively) were calculated from the changes in the high field peak of the three line spectral component of the free 5-DSA signal. The upper panel represents the goodness of fit. All other conditions were as in Fig. 1.
best fit two identical independent binding sites with an dissociation constant of 35 μM for apo-α-lactalbumin. We were unable to measure an accurate dissociation constant for 5-DSA binding to Ca\(^{2+}\)-α-LA since the concentrations of either spin probe or protein required to observe any significant binding by ESR were essentially experimentally impossible. We estimated a very rough \(K_d\) of 300–500 μM, which actually compares well with the values measured by intrinsic fluorescence (see below).

When we saturated acid (pH 2) with the values measured by intrinsic fluorescence (see below). 

**FIG. 1**

**FIG. 2**

**FIG. 3**

**FIG. 4**

**FIG. 5**

The experimental data were fit either a simple one-site binding scheme,

\[
\alpha\text{-LA} + 5\text{-DSA} \rightleftharpoons \alpha\text{-LA} \cdot 5\text{-DSA}
\]  

(Eq. 1)

or to a more general cooperative scheme (where sigmoid-like binding curves were observed),

\[
\alpha\text{-LA} + n\text{-5-DSA} \rightleftharpoons \alpha\text{-LA} \cdot n\text{-5-DASa}
\]  

(Eq. 2)

where we obtained values of apparent dissociation constants, \(K_1\), \(K_2\), and \(K_3\) and some corresponding \(n\) values (which should not be viewed as stoichiometries, but rather as an indicator of cooperativity). The apparent dissociation constants for the last two phases of the titration curves in Fig. 4 were 9–25 \(×\) \(10^{-6}\) M for apo-α-LA, 5–10 \(×\) \(10^{-5}\) M, for Ca\(^{2+}\)-α-LA and 2.8 \(×\) \(10^{-4}\) for
Ca²⁺Zn²⁺-α-LA, respectively, all with n values in the 3–4 range. The apparent dissociation constant related to the initial small increase in emission intensity (+) were all within 0.2–1.6 × 10⁻⁶ M for Ca²⁺-α-LA, reflecting the high end of the range.

Fig. 5 shows a spectrofluorometric titration of pH 2 (acid-form) α-LA by 5-DSA, where the protein is in the molten globule state (30). Note that increasing 5-DSA induces a quenching of tryptophan emission intensity although no appreciable spectral shifts were observed (data not shown). The titration curve best fit a simple single site binding scheme adequately, with an apparent dissociation constant of 0.3 × 10⁻⁶ M, in good agreement with the ESR results presented earlier (Fig. 1C).

Stearic Acid Binding—Compared with the 5-DSA results, which exhibited complex titration behavior (Fig. 4A), binding of stearic acid to Ca²⁺-α-LA (Fig. 6) caused a progressive, monotonic increase in tryptophan emission (+). The red shift in spectral position (after the initial jump) resembled that found for 5-DSA, although a much larger red shift was observed with stearate ( ), Fig. 6). Both an emission intensity increase (ΔI) and a red shift were observed when titrating the apo and Zn²⁺-α-LA, Ca²⁺ forms as well (data not shown), in contrast to the decreased emission (ΔF) and blue shift observed with 5-DSA over the same ligand:α-LA range (up to 20:1) (Fig. 4B). The apparent binding parameters obtained from the experiment in Fig. 5 were 0.4 × 10⁻⁶ M for the initial phase (low stearate: Ca²⁺-α-LA) followed by value(s) in the 1.1–2 × 10⁻⁶ M range; the latter generally agree with the weaker constant K values estimated for (spin labeled) 5-DSA binding by ESR. For apo-α-LA, a K of 2.3 × 10⁻⁸ M (n = 1) was found, whereas acid α-LA (pH 2) showed no measurable emission intensity changes, making an accurate fit difficult.

Micelle Formation (cmc)—The concentrations in the fluorescence work were similar to those at which detectable macro-molecular aggregates form (approximately 2–4 μM stearic acid). Since this concentration range is close to some of the apparent Kₐ values from the apo-α-LA titrations, the cmc could be higher in the presence of α-lactalbumin. Although we cannot completely rule out the possibility that the stearate results were partly consistent with micelle formation contributing to the fluorescence data, this was most likely not the case with 5-DSA binding, since the cmcs reported for DSA acid (35 μM in 0.13 M NaCl, 0.02 M phosphate, pH 7.4, 37 °C) are at least 10–20-fold higher than for stearic acid; hence, aggregates form at much higher concentrations as well (28). In addition, no evidence of exchange broadening was observed that was easily distinguished from the asymmetric line broadening reflecting ligand (nitroxy) immobilization (28).

ADIFAB Binding Experiments—In an effort to independently verify this high affinity binding between fatty acids and α-LA, the fluorescence-sensitive ADIFAB protein was employed. Fig. 7A shows a titration of ADIFAB protein by stearic acid (in the presence of 1 mM EGTA), where the pronounced decrease in fluorescence intensity at 420 nm was fit to a single site binding constant of 8 × 10⁻⁸ M (n = 1), which was close to the value reported earlier (Kₐ = 8 × 10⁻⁸ M) (31). Subsequent titration of the stearic acid-loaded protein by apo-α-LA resulted in an increase of ADIFAB fluorescence (Fig. 7B). The titration data were fit to a competitive binding scheme,

\[ K₁ \, \text{ADIFAB-protein} \, + \, \text{SA} \rightleftharpoons K₁ \, \text{ADIFAB-protein} \cdot \text{SA} \quad (\text{Eq. 3}) \]

\[ K₂ \, \alpha-\text{LA} \, + \, \text{SA} \rightleftharpoons K₂ \, \alpha-\text{LA} \cdot \text{SA} \quad (\text{Eq. 4}) \]

where SA is stearic acid. The constant K comes from the titration in Fig. 7A above; K was evaluated as 0.2 × 10⁻⁶ M, which is roughly within an order of magnitude of the binding.

---

**Fig. 5.** Spectrofluorometric titration of α-lactalbumin by 5-doxylstearic acid at pH 2 (50 mM glycine). Excitation was fixed at 280 nm. There was no spectral shift in this titration. Protein concentration, 1.3 μM.

**Fig. 6.** Spectrofluorometric titration of Ca²⁺-loaded α-lactalbumin (Ca²⁺-protein = 1000:1) by stearic acid (SA). Conditions were 50 mM bis-tris propane, pH 7.5, at 22 °C. Protein concentration, 1.6 μM. Excitation was fixed at 280 nm.

**Fig. 7.** A, spectrofluorometric titration of 0.26 μM ADIFAB protein with stearic acid. The curve is the best fit of the data by a one-site binding scheme. B, 0.26 μM ADIFAB protein saturated with 0.84 μM stearic acid with apo-α-lactalbumin. The curve is the best fit of the data by a one-site competition binding scheme. Conditions were 50 mM Tris-HCl, pH 7.4; 1 mM EGTA; 20 °C. The acrylodan probe was excited at 390 nm.
constant measured by intrinsic fluorescence (2.3 × 10⁻⁶ m). Recall that the ADIFAB data above is for a simple one-site competition scheme, whereas α-LA possesses multiple (possibly cooperative) binding sites. Nonetheless, the return of fluorescence intensity in this experiment was approximately 50%.

**DISCUSSION**

The results clearly show that α-LA binds both stearic acid and its spin-labeled (doxyl) analog. The binding data are summarized in Table I. We have introduced a novel use of the ADIFAB reagent to measure fatty acid binding to proteins by competitive binding. This should prove to be a sensitive method for other systems as well.

There are at least two binding sites in apo-α-LA, as evidenced by both the ESR and fluorescence titrations shown above. The intrinsic fluorescence emission red shift and general increase resembles studies in which α-LA was transferred from aqueous media and embedded in the surface of liposomes (11, 12). Although there are several mechanisms to explain the fluorescence changes, one model could even be consistent with identical binding sites for both stearic acid and 5-DSA. Spin labels can cause contact quenching of tryptophan residues when the nitroxide group is bound at or near the indole ring. In the case of stearic acid, the observed emission enhancements by might be due to reduced solvent accessibility. Nonetheless, the ESR experiments with stearate (micelles) seem to suggest separate binding loci if α-LA indeed binds stearate at these concentrations. Certainly, the intrinsic and ADIFAB fluorescence data confirm strong α-LA-stearate complex formation.

Certainly, the binding constant of the second phase of the 5-DSA fluorescence titrations for apo-loaded α-LA was in generally good agreement with that of Ca²⁺-loaded α-LA (i.e., within an order of magnitude). The first phase (i.e., a small fluorescence emission enhancement in every case) was reproducible, but it was difficult to reconcile with the ESR equilibria studies. This type of behavior has been noted earlier in studies with phospholipase A₂ by Jain and co-workers (32). In any case, the fluorescence titrations clearly indicate fatty acid binding effects on the protein.

Apo-α-LA had a substantially stronger $K_d$, in good agreement with the ESR studies (Fig. 2). The experiments were complicated by the tendency of stearic acid to form dimers and higher aggregates (particularly micelles) at concentrations above 2–6 μM. The ESR results suggest that only two lipid molecules may bind per protein molecule, although α-LA binding to micellar lipid aggregates may be more similar to liposome binding.

BLG is frequently a minor contaminant (2–5%) in apo-α-LA preparations, and it is extremely difficult to remove. We recently showed by ESR that 5-DSA binds to BLG in the 0.8 μM range and that both 5-DSA binding and palmitate binding to BLG cause intrinsic fluorescence emission enhancements (33). Such minor contaminants would be transparent in the apo-α-LA binding experiments (and, in this study, with the Ca²⁺-loaded α-LA binding experiments) but would be quite prominent in intrinsic fluorescence titrations in the sub-μM to 1 μM range. Nonetheless, the ADIFAB studies showed conclusively that stearate binds to apo-α-LA with high affinity.

Last, the biological relevance of this binding should be considered in nutrition and other transport processes, such as neonatal gastroenterology, where the gut or small intestine may have a lower pH, favoring the molten globule or apo-like forms.

**Acknowledgment**—We are grateful to Dmitry Veprinsev for many helpful discussions.

**REFERENCES**

1. Spector, A. A., and Fletcher, J. E. J. (1970) Lipids 5, 403–411
2. Fugate, R. D., and Song, P. (1988) Biochim. Biophys. Acta. 1025, 28–42
3. Puyol, P., Perez, M. D., Ena, J. M., and Valvo, M. (1991) Agric. Biol. Chem. 55, 2515–2520
4. Papiz, M. Z., Sawyer, L., Eliopoulis, E. E., North, A. C. T., Newcomer, M. E., and Kraulis, P. J. (1986) Nature 324, 383–385
5. Godovac-Zimmermann, J. (1988) Trends Biochem. Sci. 13, 64–66
6. Hill, R. L., and Brew, K. (1975) Adv. Enzymol. Relat. Areas Mol. Biol. 43, 411–490
7. Permyakov, E. A., Grishchenko, V. M., Kalinichenko, L. P., Oslov, N. Y., Kuwajima, K., and Sugai, S. (1991) Biochem. Chem. 39, 111–117
8. Hanssens, I., van Ceunebroeck, J. C., Pottel, H., Preaux, G., and van Cauweleart, F. (1985) Biochim. Biophys. Acta. 1817, 154–166
9. Berliner, L. J., and Koga, K. (1987) Biochemistry 26, 3096–3099
10. Permyakov, E. A., Kreinem, D. I., Kalinichenko, L. P., and Shnyrov, Y. V. (1988) Gen. Physiol. Biophys. 7, 95–107
11. Grishchenko, V. M., Kalinichenko, L. P., Seikus, G. Y., Veprinsev, D. B., Cathrern, K. M., Berliner, L. J., and Permyakov, E. A. (1996) Biochim. Biophys. Acta. 130, 453–466
12. Cathrern, K. M., Permyakov, E. A., and Berliner, L. J. (1996) Protein Sci. 5, 1394–1405
13. Lindahl, L., and Vogel, H. J. (1984) Anal. Biochem. 140, 394–402
14. Acharya, K. R., Statta, D. I., Walker, N. P. C., Lewis, M., and Phillips, D. C. (1989) J. Mol. Biol. 208, 99–127
15. Acharya, K. R., Ben, J. D., Statta, D. I., Phillips, D. C., and Fenna, R. E. (1991) J. Mol. Biol. 217, 571–581
16. Hirooka, Y., Segawa, T., Kuwajima, K., Sugai, S., and Murai, N. (1980) Biochem. Biophys. Res. Commun. 95, 1089–1104
17. Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., and Burstein, E. A. (1981) Biochem. Biophys. Res. Commun. 100, 191–197
18. Permyakov, E. A., Morozova, L. A., and Burstein, E. A. (1985) Biochim. Biophys. Acta. 21, 21–31
19. Murakami, K., Andree, P. J., and Berliner, L. J. (1982) Biochemistry 21, 4585–4590
20. Murakami, K., and Berliner, L. J. (1983) Biochemistry 22, 3370–3374
21. Permyakov, E. A., Shnyrov, Y. V., Kalinichenko, L. P., Kuchar, A., Rezyer, I. L., and Berliner, L. J. (1991) J. Protein Chem. 10, 577–584
22. Musci, G., and Berliner, L. J. (1985) Biochemistry 24, 3852–3856
23. Hanssens, I., Kuwajima, K., and van Cauweleart, F. H. (1980) Biochim. Biophys. Acta. 602, 539–557
24. Mulqueen, P. M., and Kronman, M. J. (1982) Arch. Biochem. Biophys. 215, 38–50
25. Hirau, Y., Permyakov, E. A., and Berliner, L. J. (1995) J. Protein Chem. 11, 51–57
26. Kronman, M. J., Andrette, R. E., and Vitols, R. (1964) Biochemistry 3, 1152–1160
27. Reich, J. G., Wangermann, G., Falck, M., and Rohde, K. (1972) Eur. J. Biochem. 26, 368–379
28. Rehfeld, S. J., Eatough, D. J., and Plachy, W. Z. (1978) J. Lipid Res. 19, 841–849
29. Richer, G. V., Ogata, R. T., and Kleinfeld, A. M. (1992) J. Biol. Chem. 267, 23445–23501
30. Kuwajima, K. (1989) Proteins Struct. Funct. Genet. 6, 87–103
31. Anel, A., Richier, G. V., and Kleinfeld, A. M. (1993) Biochemistry 32, 530–536
32. Volwerk, J. J., Filthuth, E., Griffith, O. H., and Jain, M. K. (1994) Biochemistry 33, 3464–3474
33. Narayan, M., and Berliner, L. J. (1997) Biochemistry 36, 190–1911