α-Lactalbumin Binding to Membranes: Evidence for a Partially Buried Protein†

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ABSTRACT: The self-incorporation of apo-α-lactalbumin (α-LA) into single unilamellar vesicles (SUV) of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine was demonstrated by column chromatographic analyses on Sephadex G-200 (10 mM Tris-HCl, pH 7.4, 26–28 °C) and by intrinsic fluorescence emission of SUV-bound α-LA. It was shown that apo-α-LA slowly incorporated into the DMPC vesicle bilayer after equilibrating different mixtures of protein and SUV for several hours. The intrinsic fluorescence properties of the bound apo-α-LA were altered only slightly (λ_{max}^{em} = 333 nm vs. 337 nm in aqueous solution). The large blue shift in apo-α-LA fluorescence in solution induced by monovalent cations, such as Na(I), was almost completely prevented when apo-α-LA was membrane bound. Furthermore, the addition of calcium caused a slow conversion from apo-α-LA to Ca(II)–α-LA by a mechanism consistent with passive diffusion of Ca(II) into the bilayer interior to the (buried) calcium binding site. The release of Ca(II)–α-LA from the membrane is discussed with reference to α-LA release from the smooth endoplasmic reticulum in vivo.

α-Lactalbumin (α-LA)[1] is the modifier protein of the lactose synthase complex in the mammary cell. Recently, α-LA was shown to be a calcium binding protein (Hiraoka et al., 1980; Permyakov et al., 1981; Murakami et al., 1982), which was related to its stabilization against thermal unfolding at 37 °C (Musci & Berliner, 1985b). Furthermore, it was shown that cation binding to the strong calcium site resulted in the decreased exposure of the hydrophobic binding region on the α-LA surface, which was believed to be important in the α-LA–galactosyltransferase interaction (Lindahl & Vogel, 1984; Musci & Berliner, 1985a). In vivo, α-LA is biosynthesized and stored on the smooth endoplasmic reticulum (ER) and then transported to the Golgi lumen after stimulation by prolactin for lactose biosynthesis and milk secretion (Hill & Brew, 1975). Since both the smooth ER surface and the environment of galactosyltransferase in the Golgi lumen are composed of membranes, it was of interest to examine the interaction of α-LA with model membrane vesicles. Considering also the possibility that α-LA is synthesized on the smooth ER as the apo conformer, it is intriguing to test whether calcium binding to α-LA might be the initial release step from the smooth ER. Furthermore, since galactosyltransferase has been shown to be a membrane-bound enzyme, the interaction of α-LA with the membrane at this site is also significant to understanding both the lactose synthase reaction and the subsequent release of α-LA into the secreted media (Mitranic et al., 1983).

Hanssens et al. (1980, 1983, 1985) and Herreman et al. (1981) have examined both the apo conformer and calcium conformer of α-LA with DMPC SUV and the changes induced by pH and ionic strength. The unseparated vesicle–protein mixture was studied at neutral pH by a variety of optical methods. At acid pH 2–4 they observed only micelle formation. While conclusions were drawn about the vesicle-bound protein, attempts to separate and isolate the α-LA–SUV by Sepharose 6B chromatography either were unsuccessful or were not exploited spectroscopically. In addition, the majority of the experiments were made at 23 °C, where DMPC was just slightly above the broad gel–liquid-crystalline phase transition (14–27 °C), a potentially nonphysiologically relevant state (Hanssens et al., 1985; Lentz et al., 1976). We have taken an entirely different approach to examining α-LA–SUV interactions by isolating and purifying α-LA–SUV and examining in great detail the nature of the α-LA membrane interaction and the effects of Ca(II) and NaCl on the α-LA–SUV complex. Quantitative data are presented for partitioning ratios between free and SUV-bound α-LA as well as rate constants for Ca(II)–α-LA diffusion out of the bilayer. Some additional conclusions about the nature of the α-LA–SUV interaction may shed light on the previously reported studies.

Materials and Methods

Chemicals. Bovine α-LA (lot 52F-80751), obtained from Sigma Chemical Co., typically contained 0.3 mol of Ca(II)/mol of protein. Protein demetalization was carried out by the method of Koga and Berliner (1985) to yield an essentially calcium-free protein (to within 2%). DMPC (lot C140-58) and DPPC (lot C160-72) were obtained from Avanti Chemicals. Sephadex G-200 (40–120 μm) was obtained from Pharmacia Chemicals. All other chemicals were of reagent grade. All buffers were demetalized by pretreatment with Chelex 100; all glassware was prewashed with acid and rinsed with Chelex 100 treated double-distilled water. Small unilamellar vesicles (SUV) of phosphatidylcholine were made by sonication of a multilamellar dispersion (under N_2) with a Branson sonicator after the method of Bangham et al. (1965). The sonication time required was ca. 1 h for the solution to

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[1] Abbreviations: α-LA, α-lactalbumin; SUV, small unilamellar vesicle(s); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; I_{360}/I_{max}, ratio of the fluorescence emission intensity at 360 nm to the fluorescence intensity at the emission maximum; UDP-Gal, uridine 5′-diphosphate galactose; Glc, glucose; SDS, sodium dodecyl sulfate; CIDNP, chemically induced dynamic nuclear polarization; bis-ANS, 4,4′-bis[1-(phenylamino)naphthalene-8-sulfonate]; ER, endoplasmic reticulum.

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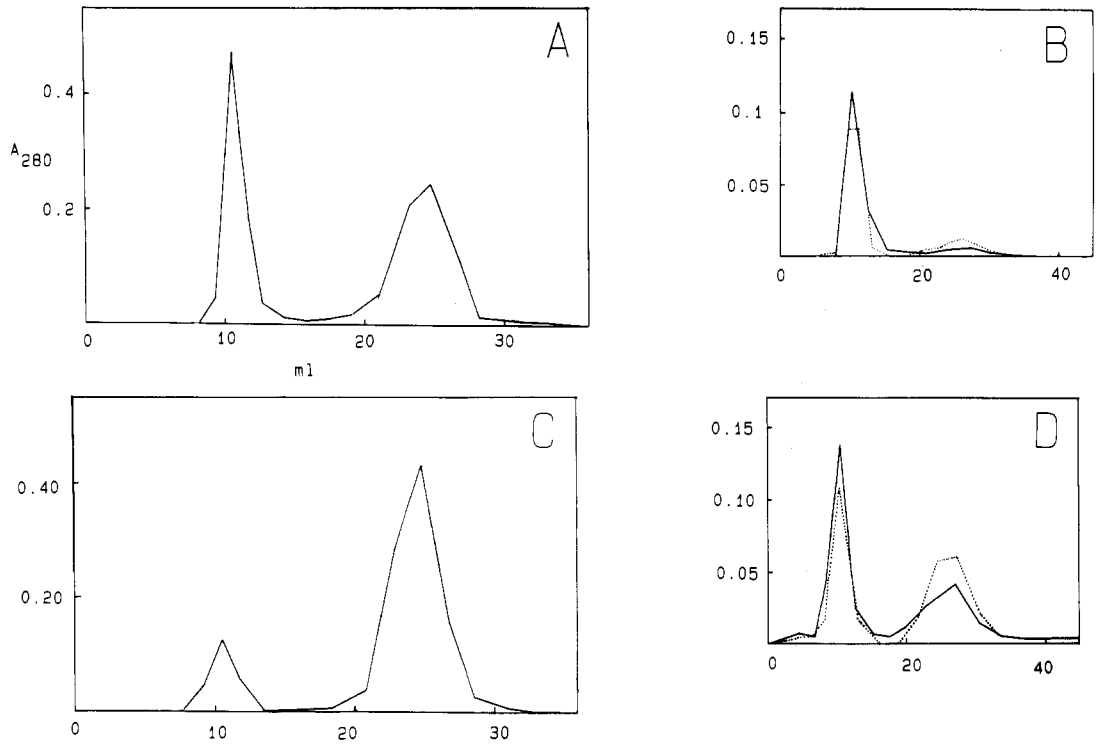

FIGURE 1: Elution profiles of α-LA–DMPC SUV on Sephadex G-200 (0.2 × 30 cm). Conditions: 10 mM Tris-HCl, pH 7.4, 26–28 °C. (A) After incubation of 123 μM apo-α-LA and 9.1 mM DMPC SUV for 14 h at 26–28 °C. (B) Rechromatography of the apo-α-LA–DMPC peak (i.e., void volume) in (A) at 0.8 (—) and 6 h (···) after elution from the column in (A). The applied sample was 21 μM in apo-α-LA. (C) After incubation of 123 μM Ca(II)–α-LA and 9.1 μM DMPC SUV for 15 h at 26–28 °C. (D) Rechromatography of the apo-α-LA–DMPC peak in (A) first incubated in 0.2 mM Ca(II) for 0.8 (—) and 4 h (···).

clarify; these were done at ca. 25 °C for DMPC and 50 °C for DPPC.

Methods. Intrinsic fluorescence was measured on a Perkin-Elmer Model MPF-44A spectrofluorometer at 26 °C, reported as uncorrected spectra. Protein concentration was estimated with E_{280} = 2.01 mg mL^{-1} cm^{-1}. The chromatographically isolated α-LA–SUV were quantitated from the absorbance at 280 nm of the void volume fractions (Sephadex G-200). When a fraction eluted as a cloudy suspension, it was first clarified with SDS, and the absorbance was then corrected for residual scattering by calibrating at 330 nm.

RESULTS

Incorporation of α-LA into DMPC Vesicles. In a typical experiment, DMPC SUV were mixed with apo-α-LA, incubated at 26–28 °C (10 mM Tris), pH 7.4, for 10–15 h, and subsequently chromatographed on a Sephadex G-200 column (0.2 × 30 cm). The experiments were repeated several times over various incubation times to ensure that complete equilibration was approached.[2] Figure 1A depicts a typical elution pattern of an apo-α-LA–DMPC SUV mixture as monitored at 280 nm. Two well-separated peaks were observed; an α-LA–SUV peak at the void volume and another which coeluted with free α-LA monomer. The ratio of bound α-LA to free α-LA in this experiment was 0.43:0.57. Figure 1B depicts the rechromatography of the void volume fraction (i.e., membrane-bound α-LA fraction) from Figure 1A. Essentially, all of the α-LA-containing material eluted with the void volume when the sample was applied within 0.8 h after elution from the first column (solid line). There was a slight increase in free α-LA if a delay of 6 h elapsed between the two columns (dotted line). Each fraction was characterized by intrinsic fluorescence spectroscopy. The λ_{max}^{em} values of free α-LA (pH 7.4, 10 mM Tris, 26 °C) were 337 and 323 nm for apo-α-LA and Ca(II)–α-LA, respectively. The free apo-α-LA fraction had precisely the same λ_{max} value (337 nm) as did the free apo-α-LA, which shifted instantly to 324 nm upon addition of Ca(II) (Murakami et al., 1982). The results also confirmed that there was no Ca(II) contamination in the apo-α-LA–DMPC systems and that the conformational integrity of apo-α-LA was maintained. On the other hand, the bound α-LA–SUV fraction displayed a somewhat different λ_{max}^{em} value (333 nm) from the other forms. It was also unaffected by the addition of EGTA; that is, the differences in fluorescence from free apo-α-LA were not due to a small contamination of Ca(II) but were specifically from membrane-bound apo-α-LA. The column chromatography and fluorescence results showed that apo-α-LA could penetrate into the lipid region of the DMPC SUV without any special treatment (i.e., sonication) in ca. 10–15 h, forming rather stable complexes that could be isolated by column chromatography. After removal of free α-LA in the first chromatography, only a very slow equilibrium shift was observed, as reflected by the minor free apo-α-LA peak (Figure 1B). Figure 1C depicts the elution patterns of the Ca(II)–α-LA–DMPC SUV system. There were two peaks at the same position as in Figure 1A, but the ratio of bound α-LA to free α-LA was much smaller, i.e., 0.14:0.86. The λ_{max} values were 330 and 323 nm for the bound α-LA and free α-LA fractions, respectively. The λ_{max} value of the free fraction did not change with the addition of Ca(II), confirming that there was no apo-α-LA in the sample. Therefore, this λ_{max} value (330 nm) was specific for membrane-bound Ca(II)–α-LA. Furthermore, comparison between parts A and C of Figure 1 clearly shows that apo-α-LA has

[2] When these mixtures were sonicated for 5–30 min, incorporation of α-LA into the SUV was more rapid; however, we could not rule out the possibilities of alterations to the SUV structure and some irreversible denaturation of the protein caused by the sonication process (K. Koga and L. J. Berliner, unpublished results).

Table I: Chromatography of α-LA–SUV

membrane system	concn[a] of Ca(II) (mM)	chromatography [time after Ca(II) addn] (min)	fraction of bound α-LA (%)
DMPC	0.20	50	53
	0.20	250	38
	0.74	520	23
	1.9	10	49
	0	50	90
	0	360	79
DPPC	0.29	460	87

[a] Concentrations of α-LA were 15–37 μM.

a higher affinity for DMPC SUV than does Ca(II)–α-LA. This column chromatography method proved to be an excellent technique for preparing α-LA–membrane samples that contain no free α-LA.

Effects of Ca(II) on Membrane-Bound α-LA. Figure 1D depicts a typical elution pattern after the addition of 0.2 M Ca(II) to a pure membrane-bound apo-α-LA sample. The elutions were done at 0.8 and 4 h, respectively, after calcium addition (α-LA concentration was 2–6 μM). Again, we observed only two peaks, which eluted at the same positions as earlier (Figure 1A). A free α-LA fraction appeared upon addition of Ca(II) to apo-α-LA–SUV, which increased with time (compare with the results in Figure 1B). Table I summarizes the results of the additions of several concentrations of Ca(II). We also monitored these slow processes by following the intrinsic fluorescence of α-LA. Figure 2A depicts a typical result which shows that the λ_{max} gradually decreased with time, the rate being dependent upon the added Ca(II) concentration. Note that a control (membrane-bound apo-α-LA alone) did not change over a period of 9 h. These results were in good agreement with the column chromatography studies above of Ca(II)–α-LA release from DMPC SUV (Table I). Logarithmic replots of the data in Figure 2A were made by plotting $\ln [F_t - F_d)/(F_0 - F_d)]$ vs. t, where F_t is the λ_{max} at time t, F_0 is the λ_{max} immediately after the addition of Ca(II),[3] and F_d is the λ_{max} of Ca(II)–α-LA. It was assumed that the final λ_{max} value is that of free Ca(II)–α-LA. The log plots were linear, from which a first-order rate constant k_{obsd} was calculated for convenience of presentation. The rate constants, which increased with increasing Ca(II) concentration, are plotted in Figure 2B. The reverse step, i.e., calcium removal by EDTA, also followed behavior that was consistent with the slow, passive diffusion of the chelating agent and/or the Ca(II) cation through the membrane (data not shown).

Similar experiments were carried out with apo-α-LA–DPPC SUV systems (initial DPPC:α-LA molar ratio was 96). After chromatography at 26–28 °C, the ratio of bound to free α-LA was 0.27:0.73. Rechromatography at 7.6 h after the addition of 1.29 mM Ca(II) yielded a bound to free α-LA ratio of 0.87:0.13 (figure not shown). In this system, in spite of the addition of Ca(II), almost all of the α-LA remained in the membrane, as long as the DPPC was in the gel state. Furthermore, the intrinsic fluorescence (e.g., λ_{max}^{em} value) did not change until the temperature was raised above the gel–liquid-crystalline transition temperature for DPPC SUV, ca. 40 °C (where it shifted from 331 to 329 nm).

Experiments were also carried out with DMPC SUV in buffers containing 0.1 M NaCl. Column chromatography showed a ratio of bound to free α-LA of 0.20:0.80. Rechromatography was also done after Ca(II) addition. The results showed that NaCl decreased the affinity of apo-α-LA to DMPC SUV.

[3] Since a small fraction of free apo-α-LA was always present (see Figure 1B), a small (immediate) drop in λ_{max}^{em} occurs due to the rapidly formed free Ca(II)–α-LA.

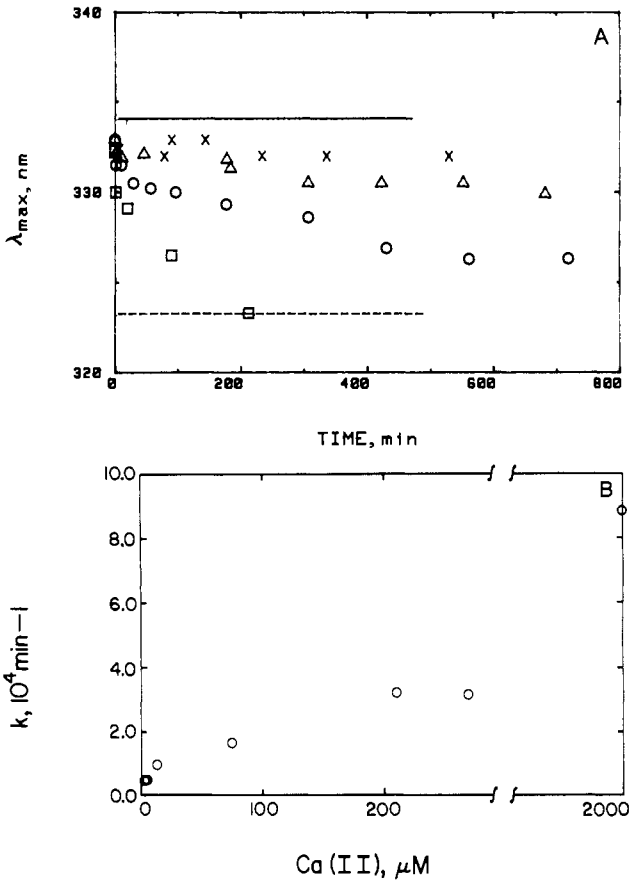

FIGURE 2: (A) Time dependence of the shift in α-LA intrinsic fluorescence emission wavelength for apo-α-LA–DMPC complex after addition of varying Ca(II) concentrations: (×) 0 μM; (△) 4.2 μM; (○) 74 μM; (□) 2000 μM. For reference the λ_{max}^{em} of membrane-free apo-α-LA (—) and λ_{max}^{em} of Ca(II)-α-LA (- - -) are shown. (B) First-order rate constants for the λ_{max}^{em} vs. time curves as a function of added Ca(II) concentration. Bound α-LA concentration was 2–3 μM. Buffer: 10 mM Tris-HCl, pH 7.4, 26 °C. λ_{em} = 280 nm.

Table II: Summary of Intrinsic Fluorescence Characteristics for Free and Membrane-Bound α-LA[a]

	λ_{max}^{em}	I_{360}/I_{max}
free (aqueous)		
apo-α-LA	337	0.74
apo-α-LA, 0.1 M NaCl	323	0.34
Ca(II)–α-LA	323	0.41
Ca(II)–α-LA, 0.1 M NaCl	321	0.33
DMPC SUV bound		
apo-α-LA	333	0.59
Ca(II)–α-LA	330[b]	0.60
apo-α-LA, 0.1 M NaCl	330–331[b]	0.57

[a] 0.01 M Tris buffer, pH 7.4, 26 °C, excitation wavelength 280 nm. [b] These samples became cloudy with time, which probably accounts for their slightly lower λ_{max}^{em} value as a result of slight light-scattering effects that could not be eliminated.

A summary of the intrinsic fluorescence parameters for free and bound α-LA are shown in Table II. The λ_{max} value in 0.1 M NaCl and 10 mM Tris-HCl, pH 7.4 at 26 °C, were 323 and 321 nm for apo-α-LA and Ca(II)–α-LA, respectively. The λ_{max} for bound α-LA was 330–331 nm. Note that, in contrast to the large difference in λ_{max} for free α-LA with and without 0.1 M NaCl, these values for bound α-LA were very close. The results in Table II do not agree totally with those of Hanssens et al. (1985), who found an almost complete identity between λ_{max}^{em} for α-LA in the presence and absence of

DMPC vesicles at pH 7. However, since they were unable to separate free α-LA from membrane-bound protein, the observed fluoroscence parameters contained significant contributions from free α-LA.

Discussion

The results presented here show direct physical evidence for the binding of apo-α-LA to DMPC and DPPC SUV. These SUV serve as good model systems for smooth ER membranes (and many other biological membranes as well), which are composed highly in DMPC or choline-containing phosphatides (Vance & Vance, 1985). The results further demonstrate that the protein must be buried (at least partially the calcium binding locus) in the lipid interior of the bilayer. That is, the rate of calcium binding to membrane-bound apo-α-LA was dependent on Ca(II) concentration consistent with the slow passive diffusion of calcium cations through the bilayer, similar to the results of Vanderkooi and Martonosi (1971) on the permeability of DMPC vesicles for calcium. If the results were dependent on protein movement to the cytosol first, the rate constants measured in Figure 2B would have been totally independent of Ca(II) concentration since calcium binding to cytosolic α-LA is essentially instantaneous. Furthermore, the intrinsic fluorescence parameters reflected a protein in a more hydrophobic apolar environment, i.e., more comparable to the fluorescence emission of apo-α-LA in solution. This is also supported from bis-ANS binding studies by Musci and Berliner (1985a) that demonstrated the presence of a strongly hydrophobic region in the apo conformer of the protein. If any protein were trapped in the interior (cytosol) of the vesicle, its fluorescence characteristic would have reflected that of the free protein in solution rather than in an apolar environment. This was quite noticeable with the vesicles formed in 0.1 M NaCl. While Na(I) induces significant blue shifts in apo-α-LA intrinsic fluorescence in solution, it was *not* observed in the SUV-bound form (Table II). Thus the calcium binding site must be buried deep in the bilayer structure; whether a portion of the protein extends into the solvent is not determinable from the experiments presented here. If the calcium site were exposed, we would have expected an immediate shift in λ_{max}^{em} to 323 nm (Table II).

The relative affinities for apo-α-LA and Ca(II)-α-LA for bilayer vesicles were obvious from the chromatography experiments. Upon conversion to the calcium form, α-LA was slowly released from the SUV. Whether this phenomenon is paralleled in α-LA release from the smooth ER membrane, of course, remains to be proven more thoroughly. Several other mechanisms exist in the mammary cell system, such as facilitated protein transport, which might be kinetically more significant. The details of the interaction of α-LA and galactosyltransferase at the membrane interface pose some interesting questions. Previous work by Berliner et al. (1984), Lindahl and Vogel (1984), and Musci and Berliner (1985a,b) has implicated one or more important hydrophobic binding regions in α-LA and galactosyltransferase. On the other hand, it would seem counterproductive if α-LA were buried deep in the membrane, inaccessible to the galactosyltransferase active site and its water-soluble substrates [UDP-Gal, Glc, Zn(II)]. Rather, one might expect the α-LA–galactosyltransferase interface to be exposed to the aqueous environment. Since the physiologically active form of α-LA in the Golgi lumen is undoubtedly the calcium conformer (where its membrane affinity is significantly reduced), the latter model would appear most plausible. Another model to consider for lactose biosynthesis is α-LA binding to the membrane in order to orient it more effectively for catalysis. On the other hand, the rapid turnover and secretion of α-LA in lactation suggests only weak binding to the Golgi lumen if at all. Further experiments are in progress, such as surface-sensitive laser photo-CIDNP NMR techniques to explore structural aspects of the lipid binding behavior of α-LA.

Acknowledgments

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