Protein Science (1996), 5:1394–1405. Cambridge University Press. Printed in the USA.

Membrane-bound states of α-lactalbumin: Implications for the protein stability and conformation

KEVIN M. CAWTHERN,[1] EUGENE PERMYAKOV,[2] AND LAWRENCE J. BERLINER
Department of Chemistry, The Ohio State University, Columbus, Ohio, 43210

(RECEIVED March 6, 1996; ACCEPTED May 1, 1996)

Abstract

α-Lactalbumin (α-LA) associates with dimyristoylphosphatidylcholine (DMPC) or egg lecithin (EPC) liposomes. Thermal denaturation of isolated DMPC or EPC α-LA complexes was dependent on the metal bound state of the protein. The intrinsic fluorescence of thermally denatured DMPC-α-LA was sensitive to two thermal transitions: the T_c of the lipid vesicles, and the denaturation of the protein. Quenching experiments suggested that tryptophan accessibility increased upon protein-DMPC association, in contrast with earlier suggestions that the limited emission red shift upon association with the liposome was due to partial insertion of tryptophan into the apolar phase of the bilayer (Hanssens I et al., 1985, *Biochim Biophys Acta 817*:154–166). On the other hand, above the protein transition (70 °C), the spectral blue shifts and reduced accessibility to quencher suggested that tryptophan interacts significantly with the apolar phase of either DMPC and EPC. At pH 2, where the protein inserts into the bilayer rapidly, the isolated DMPC–α-LA complex showed a distinct fluorescence thermal transition between 40 and 60 °C, consistent with a partially inserted form that possesses some degree of tertiary structure and unfolds cooperatively. This result is significant in light of earlier findings of increased helicity for the acid form, i.e., molten globule state of the protein (Hanssens I et al., 1985, *Biochim Biophys Acta 817*:154–166). These results suggest a model where a limited expansion of conformation occurs upon association with the membrane at neutral pH and physiological temperatures, with a concomitant increase in the exposure of tryptophan to external quenchers; i.e., the current data do not support a model where an apolar, tryptophan-containing surface is covered by the lipid phase of the bilayer.

Keywords: α-lactalbumin; fluorescence; lactose synthase; membranes; molten globule

α-Lactalbumin is the modifier protein of the lactose synthetase complex (E.C.2.4.1.22) (Brodbeck & Ebner, 1966; for reviews, see Ebner, 1970, 1973; Hill & Brew, 1975). The complex formed between galactosyltransferase, α-LA, nucleotide substrate, and metal ion cofactor increases the affinity of GT for glucose as the galactosyl acceptor, shifting the specificity of GT from protein glycosylation to the production of lactose. α-LA has been shown to be highly homologous to the *c*-type lysozymes, the two sharing similar primary, secondary, and tertiary structures (Brew et al., 1967; Browne et al., 1969; Stuart et al., 1986; Acharya et al., 1989, 1991). Although it is generally accepted that α-LA evolved from *c*-type lysozyme, its function, i.e., in lactose synthesis, is clearly distinct from the lytic activity of its ancestral protein (McKenzie & White, 1991).

Native α-LA is typically isolated with stoichiometric levels of calcium bound at a unique, strong Ca^{2+}-binding loop that has been observed in all crystal structures of α-LA reported to date, but this structural feature is absent in most *c*-type lysozymes (Hiraoka et al., 1980; Stuart et al., 1986; Acharya et al., 1989, 1991). The binding of calcium stabilizes the protein against thermal as well as guanidine HCl and urea denaturation (Hiraoka et al., 1980; Permyakov et al., 1985). The so-called Ca^{2+}-binding "elbow" has been also shown to bind Mg^{2+}, Na^+, and K^+ weakly (Permyakov et al., 1981a, 1981b, 1985). Fluorescence measurements show that the removal of calcium at physiological temperatures induces a conformational change, which is accompanied both by increased emission intensity and a red shift (Permyakov

Reprint requests to: Lawrence J. Berliner, Department of Chemistry, The Ohio State University, 120 West 18th Avenue, Columbus, Ohio, 43210; e-mail: berliner.2@osu.edu.

[1] Present address: Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405.

[2] On leave from the Institute of Biological Instrumentation, Russian Academy of Sciences, Pushchino, Moscow region, 142292, Russia.

Abbreviations: α-LA, α-lactalbumin; bis-ANS, bis-4,4′-[1-(phenylamino)-8-naphthalenesulfonate]; EPC, egg lecithin; DMPC, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; GT, galactosyl transferase; MG, molten globule; SUVs, small unilamellar vesicles.

et al., 1981a, 1985; Murakami et al., 1982; Ostrovsky et al., 1988), increased affinity for the fluorescent hydrophobic probe, bis-ANS (Musci & Berliner, 1985a), and retardation on phenyl-Sepharose (Lindahl & Vogel, 1984). The apo-form also shows a higher maximal activity than Ca^{2+}-LA in the lactose synthase complex (Musci & Berliner, 1985b). Two conserved tryptophan residues on α-LA have been implicated in the interaction with GT (Barman, 1972; Barman & Bagshaw, 1972; Bell et al., 1975; Grobler et al., 1994), although a putative role of tryptophan in the control of protein function in vitro is unproven at present.

α-LA conformation is also modulated by other metal ions and pH (Murakami & Berliner, 1983; Permyakov, 1985, 1988b; Aramini et al., 1996). In particular, native α-LA possesses a relatively strong secondary Zn^{2+} site ($K_{assoc} = 5 \times 10^5\ M^{-1}$), which causes subtle conformational changes upon binding to the calcium-loaded protein as monitored by intrinsic fluorescence (Permyakov et al., 1991; Ren et al., 1993) and increased affinity for bis-ANS (Musci & Berliner, 1985a). Further conformational changes occur at higher zinc levels (characterized by a sigmoidal isotherm between 1:1 and 40:1 Zn^{2+}:α-LA) where protein stability to denaturants and heat is sharply reduced (Permyakov et al., 1985). Typically, protein aggregation occurs at zinc to protein ratios in excess of 40:1 (Permyakov et al., 1985, 1991). The potential importance of the apo-like state to the regulation of lactose synthase has been suggested by Musci and Berliner (1985b), noting that manganese and other secondary cationic activators have been implicated in the regulation of lactose generation in Golgi vesicles (Kuhn et al., 1980; Navratnam et al., 1986, 1988; Witsell et al., 1990). Recently, the strong Zn^{2+} site in human α-LA has been identified in the crystal, where zinc ion bridges the "lysozyme-like cleft" region at ca. 17 Å from the Ca^{2+} binding loop (Ren et al., 1993). This location is proximal to the region in the cleft suggested to bind glucose in the lactose synthase complex (Grobler et al., 1994). It is also known that Zn^{2+}, Ca^{2+}-α-LA supports lactose biosynthesis, even in the absence of manganese (Musci & Berliner, 1985b).

At acidic pH values (<3), or in the presence of moderate amounts of denaturants at neutral pH, α-LA adopts the so-called "molten globule" state, a compact, intermediate protein state between native and unfolded, which is characterized by a conserved secondary structure but fluctuating tertiary structure (Dolgikh et al., 1981; Ohgushi & Wada, 1983). There has been considerable interest in the MG state as a general compact intermediate along the folding pathway of globular proteins (Ptitsyn, 1987, 1992; Kuwajima, 1989; Creighton, 1990). Thermal unfolding between the MG and random coil (U) states is thermally diffuse or immeasurable, occurring noncooperatively among various isolated secondary (helical) structural elements. It undergoes an expansion of ca. 10–20% compared with a 40% increase in radius of gyration for the random coil in guanidinium hydrochloride (Kronman et al., 1964; Gast et al., 1982; Damaschun et al., 1986; Pfeil, 1987). There is also a greater exposure of hydrophobic residues, as suggested by an increased affinity of the MG state for hydrophobic fluorescent probes (Mulqueen & Kronman, 1982). Internal hydrophobic contacts maintain the compact size of the MG protein, giving rise to weak association between the compact core of residual native structure and other more mobile secondary structural elements (Baum et al., 1989; Alexandrescu et al., 1993; Chyan et al., 1993).

The interaction of α-LA with phospholipid membranes and model vesicles is of interest because membranes have been implicated in the protein folding pathway (Ptitsyn, 1992). It is also of interest because the interaction of α-LA and GT with lipid has been suggested to be important for regulating levels of lactogenesis in model systems (Mitranic et al., 1983, 1988; Mitranic & Moscarello, 1985). In particular, neutral and positively charged lipids activate GT in both the presence and absence of α-LA, whereas negatively charged lipids are inhibitory. This interest has lead to substantial progress in defining the effects of lipid on the physical and functional state of α-LA. Previous physical studies of α-LA/vesicle mixtures have provided some valuable information on the nature and extent of interactions of various α-LA conformations with the lipid (Hanssens et al., 1980, 1983, 1985; Herreman et al., 1981a, 1981b; Ameloot et al., 1984; Berliner & Koga, 1987; Permyakov et al., 1988a). Fluorescent results with probes placed in the bilayer suggested that the lipid was not strongly perturbed upon interaction with α-LA at neutral pH, and that the association was largely electrostatic with the vesicle surface (Hanssens et al., 1980, 1983, 1985; Herreman et al., 1981a, 1981b; Ameloot et al., 1984), as evidenced by the absence of an energy transfer band with a protein tryptophan (Brown, 1984). On the other hand, at acidic pH (<4), it was found that protein insertion into the bilayer is pronounced, exhibiting strong protein-probe energy transfer (Herreman et al., 1981a, 1981b) and a marked tendency to solubilize the lipid into micellar complexes (Hanssens et al., 1980, 1983; Dangreau et al., 1982), suggesting that, at low pH, α-LA is an intrinsic membrane protein (Brown, 1984).

However, the physical state of the different metal-bound forms of α-LA isolated on a liposome have not been carefully examined. For example, apo-α-LA is known to interact more strongly with PC liposomes at neutral pH than does the calcium protein (Berliner & Koga, 1987). It was suggested that partial insertion of tryptophan residues into the bilayer can account for the limited red shifts observed in the intrinsic protein fluorescence (Hanssens et al., 1980, 1985). Such questions have been addressed here by probing intrinsic fluorescence in the presence of soluble quenchers (e.g., Lakey et al., 1991; Meers & Mealy, 1993; Volwerk et al., 1994).

Results

Temperature dependence of fluorescence for isolated α-LA-vesicle complexes at neutral pH

Figure 1 depicts a typical elution profile from Sephadex G-200 chromatography at 4 °C of a preincubated mixture of Ca^{2+}-α-LA with DMPC vesicles. The first fraction from the column contains the lipid and protein complex, which in this case was ca. 220:1 molar ratio of lipid to protein. For these ratios, no change in morphology of the vesicles is induced by the protein at neutral pH (Hanssens et al., 1980). Only freshly isolated α-LA–lipid complexes were used, with fluorescence studies being completed within 2 h of isolation (Berliner & Koga, 1987). Complexes were chromatographed at temperatures (4 °C) below the lipid gel–liquid crystal transition, (T_c = 19.9 °C for DMPC SUVs, Lentz et al., 1987); therefore, slow release of the protein from the vesicle was negligible during the isolation process (Berliner & Koga, 1987). The free unbound protein fraction from the second peak was used for controls.

Figure 2 depicts the temperature dependence of tryptophan fluorescence for Ca^{2+}(1 mM)-loaded α-LA bound to DMPC or

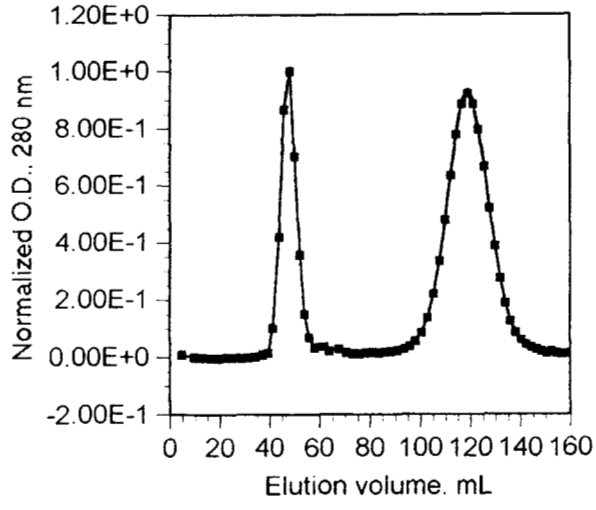

Fig. 1. Chromatography of a mixture of Ca^{2+}-α-LA with DMPC vesicles on Sephadex G-200. The column (2.5 cm × 22.5 cm) was pre-equilibrated with 1 mM $CaCl_2$ in KCl/HEPES (150 mM/50 mM/pH 7.4) at 4 °C. Prior to loading, a 5-mL mixture of 176 μM α-LA, 1 mM $CaCl_2$, and 23.5 mM DMPC was pre-incubated at 4 °C for 15 h. Upon isolation, the vesicle-associated fraction contained an estimated 26 μM protein, 1.0 mM calcium, and 5.8 mM DMPC (estimated lipid-protein was 220:1). These values were typical for isolated complexes of α-LA and lipid.

lecithin liposomes, and free Ca^{2+}-α-LA in solution. Calcium concentrations in excess of the protein concentration were employed to compensate for possible weak, nonspecific adsorption to the excess lipid, as well as to provide significant inhibition against formation of the apo-state (Ikeguchi et al., 1986). The plots of emission maximum (Fig. 2A) and relative emission intensity versus temperature for DMPC-bound Ca^{2+}-α-LA (Fig. 2B) exhibit two apparent transitions. A somewhat broad, low temperature transition between 16 and 20 °C was attributed to the lipid SUVs, which is lower than the known gel-liquid crystal transition for hydrated DMPC (Lentz et al., 1987). The appearance of this low-temperature transition reflects the sensitivity of the bound protein to changes in the lipid state, which is absent in the plots for α-LA-lecithin SUVs (gel-transition temperature < −10 °C) and for free protein.

At higher temperatures, between 50 and 70 °C, a more pronounced transition is detected where the free Ca^{2+}-protein exhibits a red shift (Fig. 2A) and an abrupt increase in emission intensity at 350 nm (Fig. 2B) with midpoint near 65 °C. We have attributed this red shift to the thermal unfolding of the protein between native-like (N′) and higher temperature (H′) state,[3] where certain solvent-shielded tryptophan residues become more accessible to water (Lakowicz, 1983; Permyakov, 1993). Comparing the emission maxima in Figure 2A, we note that the shift in the maximum exhibited during the unfolding transition was much smaller for SUV-bound α-LA (DMPC, <7 nm to red; EPC, <4 nm to blue) than for free protein (16 nm to red).

[3] Because we know something about the conformation, but we do not know the detailed three-dimensional structure of the lipid-bound form of the protein, the terms N′ and H′ state refer to some state of native (N) and thermally unfolded (H) state of α-LA when bound to the membrane.

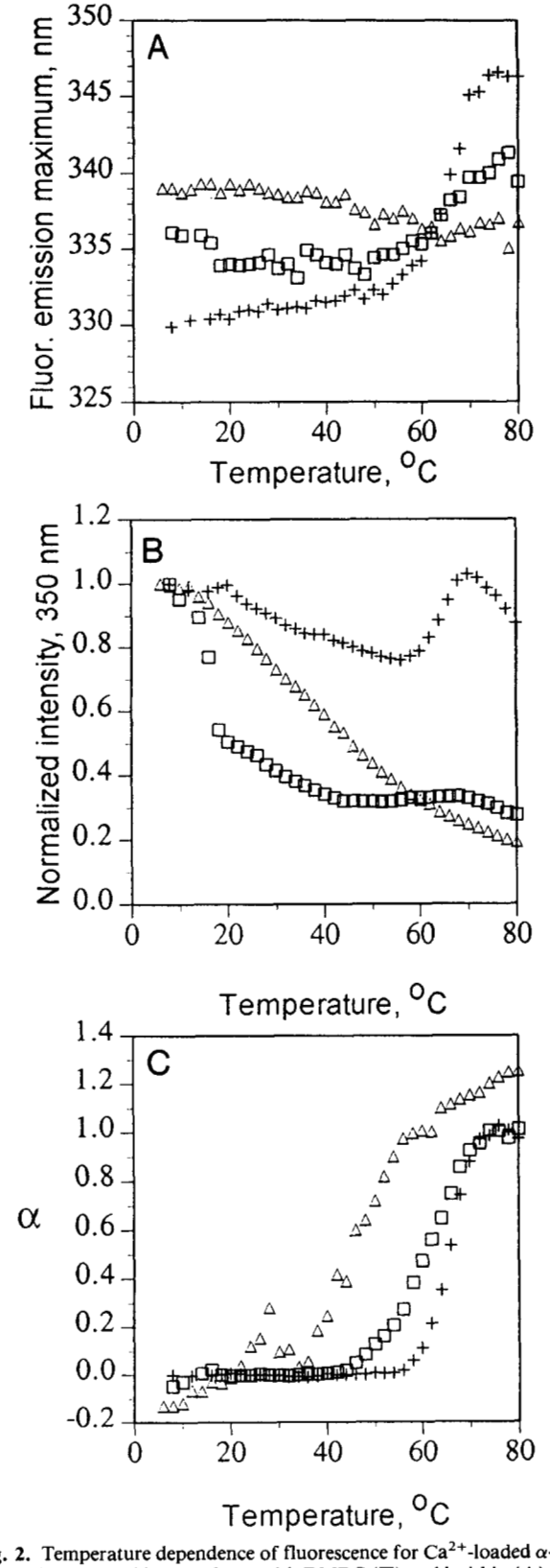

Fig. 2. Temperature dependence of fluorescence for Ca^{2+}-loaded α-LA in solution (+) and in complexes with DMPC (□) and lecithin (△) vesicles. **A:** Tryptophan fluorescence maximum position. **B:** Normalized fluorescence intensity at 350 nm. **C:** Fractional conversion (α) from the native to thermally denatured state; 50 mM HEPES, pH 7.4; 150 mM KCl. Protein concentration was between 2 and 4 μM; excitation was fixed at 280 nm.

Because the unfolding process between N′- and H′-state α-LA bound to liposomes is accompanied by smaller changes in tryptophan fluorescence, the tryptophan environments in N′- and H′-states in the SUV-bound state appear to be very different than the free protein. For example, below the protein thermal transition (e.g., 25 °C, Fig. 2A), the spectra of DMPC/Ca^{2+}-α-LA complexes are red shifted by 4–5 nm with respect to the free protein spectra, whereas for EPC complexes, the red shift is ca. 9 nm. In contrast, at temperatures above the protein thermal transition (80 °C, Fig. 2A), the maximum for membrane-bound H′-state α-LA is blue-shifted compared with the free protein. Here, the simplest interpretation is that a significant fraction of the tryptophans are less solvent-accessible on SUV-bound versus free protein in solution. The two effects, i.e., increased tryptophan exposure in the N′-state and decreased exposure in the H′-state, for Ca^{2+}-α-LA/DMPC yield a net red shift of ~7 nm versus 16 nm upon unfolding. The effects were also pronounced enough for Ca^{2+}-α-LA on EPC that a ≤4-nm blue shift was observed upon unfolding.

Also note that Figure 2B shows that Ca^{2+}-α-LA/DMPC unfolds at slightly lower temperatures than free protein, suggesting that the protein is somewhat destabilized by association with DMPC. For EPC SUVs (Fig. 2B,C), the protein was destabilized to an even greater extent. Figure 2C shows the fraction of conversion from the native to thermally denatured states (as described in Data analysis) for Ca^{2+}-α-LA, derived from the data in Figure 2B, where again vesicle-bound protein unfolding shifts to lower temperatures. This summary figure (Fig. 2C) shows again the destabilization of the protein upon lipid binding.

Figure 3 summarizes the temperature dependence data for apo-α-LA. Because these measurements required 150 mM KCl to stabilize the SUVs (Huang, 1969), the protein was actually in a partially stabilized apo-form (Permyakov & Kreimer, 1986; Permyakov et al., 1985). The DMPC- or EPC-associated proteins in the N′-state were also red-shifted compared with the free protein (Fig. 3A). In the H′-state (≥45 °C), membrane-bound apo-protein was blue-shifted relative to the vesicle-free species (Fig. 3A). As observed earlier for Ca^{2+}-α-LA, the shift in emission maximum during unfolding of SUV-bound apo-α-LA was small (3 nm on DMPC, <1 nm on EPC) compared with that for apo-α-LA in solution (~16 nm). For apo-α-LA:DMPC, the proximity of the protein thermal transition (~20–40 °C) to the lipid transition (8–18 °C) made precise assignment of the exact unfolding temperature limits difficult (Fig. 4A,B). With EPC, the protein transition was difficult to discern from either emission maxima (<1 nm, Fig. 3A) or intensity (Fig. 3B).

Probing tryptophan exposure with external quenchers

Figure 4A depicts Stern–Volmer plots for iodide quenching of intrinsic fluorescence of Ca^{2+}-loaded α-LA in the N-state (20 °C), bound to DMPC liposomes or free in solution. The data were described adequately by a single-component equation. Quenching parameters are listed in Table 1. Note that iodide quenching is smaller for free Ca^{2+}-α-LA, due presumably to inaccessibility of tryptophan, in agreement with previous fluorescence investigations (Sommers & Kronman, 1980). The quenching constant for Ca^{2+}-α-LA:DMPC is twofold larger than for Ca^{2+}-α-LA in solution (Table 1), consistent with a more expanded conformation of N′-state Ca^{2+}-α-LA when bound to liposomes.

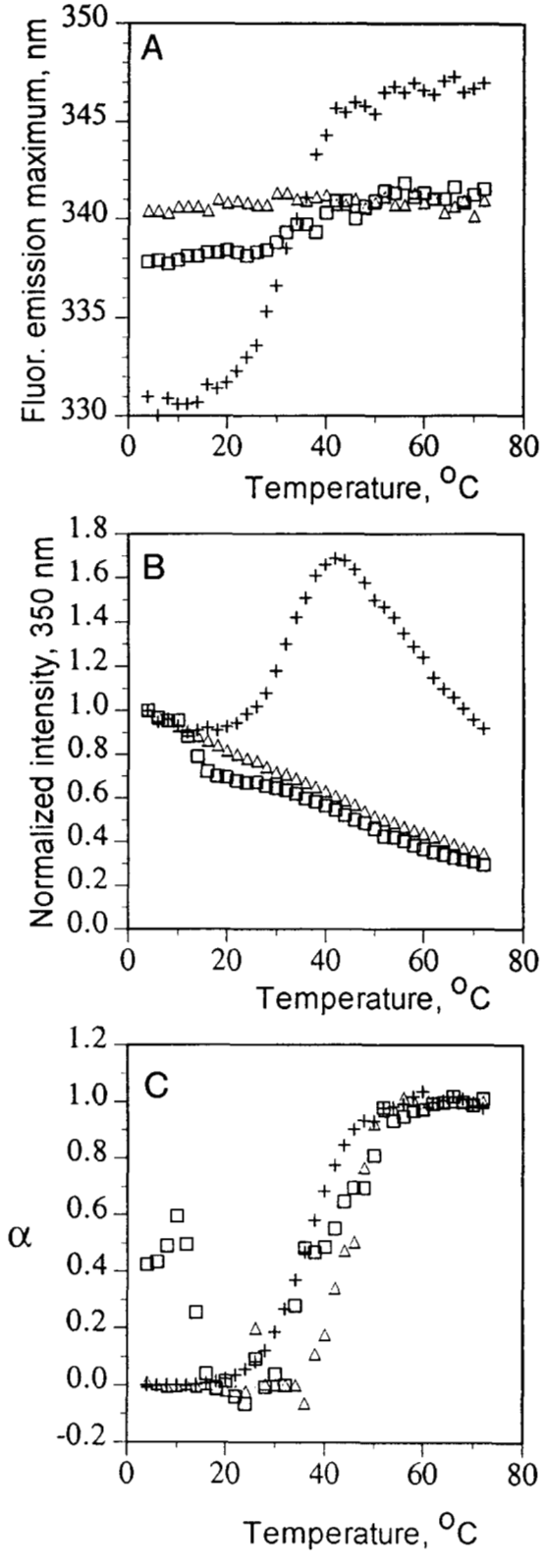

Fig. 3. Temperature dependence of apo-α-LA fluorescence in solution (+) and in complexes with DMPC (□) and lecithin (△) vesicles. **A:** Tryptophan fluorescence maximum position. **B:** Normalized fluorescence intensity at 350 nm. **C:** Fractional conversion (α) from the native to thermally denatured state; 50 mM HEPES, pH 7.4; 1 mM EGTA, 150 mM KCl. Protein concentration, 2–4 μM. Fluorescence excitation was at 280 nm.

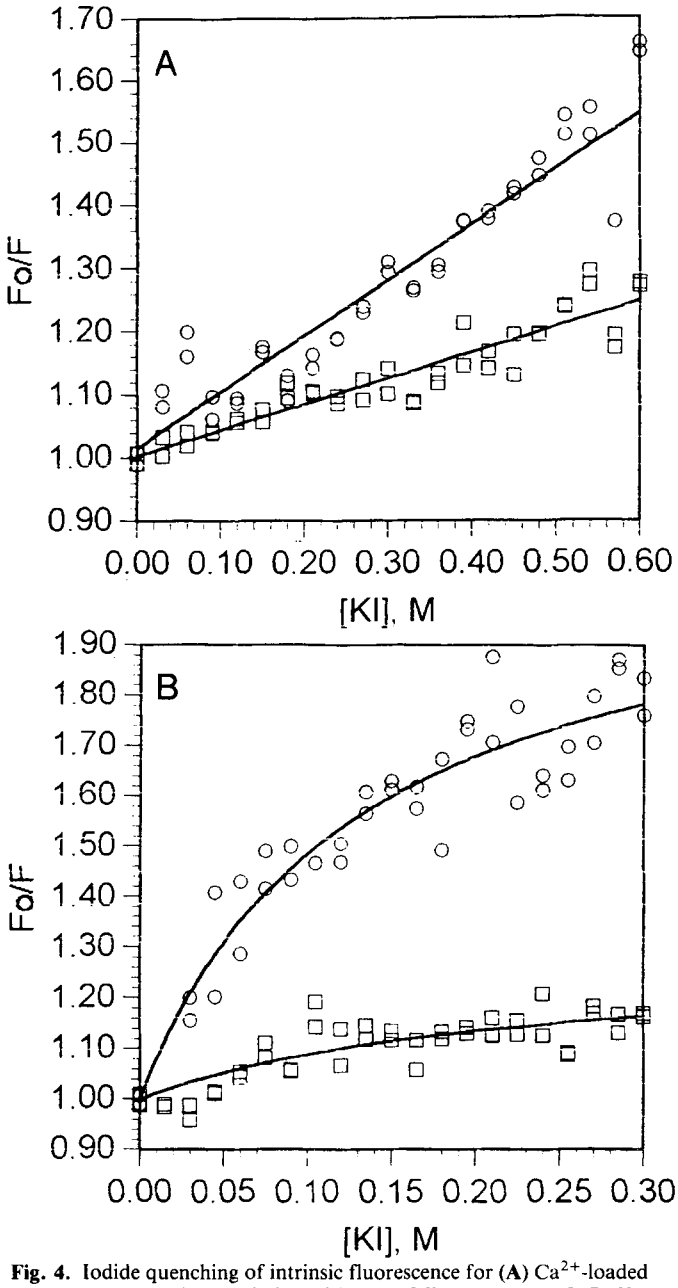

Fig. 4. Iodide quenching of intrinsic fluorescence for **(A)** Ca^{2+}-loaded and **(B)** apo-α-LA in association with DMPC liposomes, 20 °C. Buffer was 50 mM HEPES, 150 mM KCl, pH 7.4, with the following additions. **A:** 1 mM $CaCl_2$. **B:** 1 mM EGTA. For vesicle-associated (○) and vesicle-free (□) protein, metal-bound state was maintained by isolation in the presence of (A) 1 mM $CaCl_2$, or (B) 1 mM EGTA. Emission was monitored at 350 nm, excitation was 280 nm. Results of the fit to a single- and dual-component Stern-Volmer plots (Equations 1 and 2) are given in Table 1.

This trend is even more dramatic for N′-state apo-α-LA bound to DMPC at 20 °C. The KI quenching data were, however, nonlinear, as depicted in Figure 4B for the free and DMPC-associated protein. The downward curvature suggests a two-component fit (Lehrer, 1971; Eftink, 1991). That is, the fluorescence of the free protein arises from two tryptophan populations (Table 1): one that is highly accessible ($K_1 = 8.6\ M^{-1}$, approximately 23% of the emission at 350 nm), and the other that is buried ($K_2 = 0\ M^{-1}$, 77% of emission at 350 nm). For apo-α-LA:DMPC, the exposed population had a K_1 of 18.8 M^{-1} (48% of the 350 nm emission). Note that both the fraction of accessible tryptophans and the collisional constant were twofold greater than that found for the free apo-protein. Overall, the liposome-bound protein exhibited more tryptophan accessibility than free α-LA.

Table 1. *Quenching of tryptophan fluorescence of α-lactalbumin by KI at 20 °C*[a]

Protein state	Conditions	K_1 (M^{-1})	K_2 (M^{-1})	f_1
Ca-loaded	DMPC liposomes	0.82 ± 0.11		
	Control	0.40 ± 0.05		
Apo	DMPC liposomes	18.8 ± 3.5	0.22 ± 0.10	0.48
	Control	8.6 ± 1.8	0	0.23
Acidic	DMPC liposomes	13.7 ± 2.5	0	0.93
	Control	6.8 ± 0.4		

[a] Buffer was either 50 mM HEPES, pH 7.4, or 50 mM glycine, pH 2.0; all buffers contained 150 mM KCl. K_1 and K_2 are Stern-Volmer constants (see the Materials and methods); f_1 is the contribution of the first emission component.

The results with acrylamide quenching at 20 °C are shown in Figure 5A. Both free and DMPC-bound Ca^{2+}-α-LA fit well to a single-component model, but there was significant upward curvature from a static component. DMPC-bound Ca^{2+}-α-LA shows significantly higher tryptophan exposure compared to free protein (K = 11.4 versus 8.7 M^{-1}, Table 2). Similar trends in the collisional and static parameters were observed for the apoprotein (Fig. 5A). In summary, the data for membrane-bound Ca^{2+}- and apo-α-LA at 20 °C are consistent with increased tryptophan exposure.

The iodide quenching data are summarized in Table 3 for H′-state SUV-bound Ca^{2+}-loaded and apo-α-LA at 70 °C. The data are consistent with the emission blue shifts reported above for SUV-bound protein, suggesting that tryptophan residues are less accessible to quencher. The collisional parameters were smaller for SUV-bound protein, consistent with protection of protein tryptophans from quencher by the lipid at these higher temperatures.

Table 2. *Quenching of tryptophan fluorescence of α-lactalbumin by acrylamide, 20 °C*[a]

Protein state	Conditions	K (M^{-1})	V (M^{-1})
Ca-loaded	DMPC liposomes	11.4	1.7
	Control	8.7	1.7
Apo	DMPC liposomes	9.5	2.4
	Control	9.1	1.7
Acidic	DMPC liposomes	14.8	0
	Control	11.0	4.0

[a] All buffers were as in Table 1. Data were fit to Equation 3 (see text) due to the presence of upward curvature in the Stern-Volmer plots. Two-component fits did not produce parameters that differed significantly; therefore, only a single-component fit was employed. K is the collisional parameter and V is the static constant.

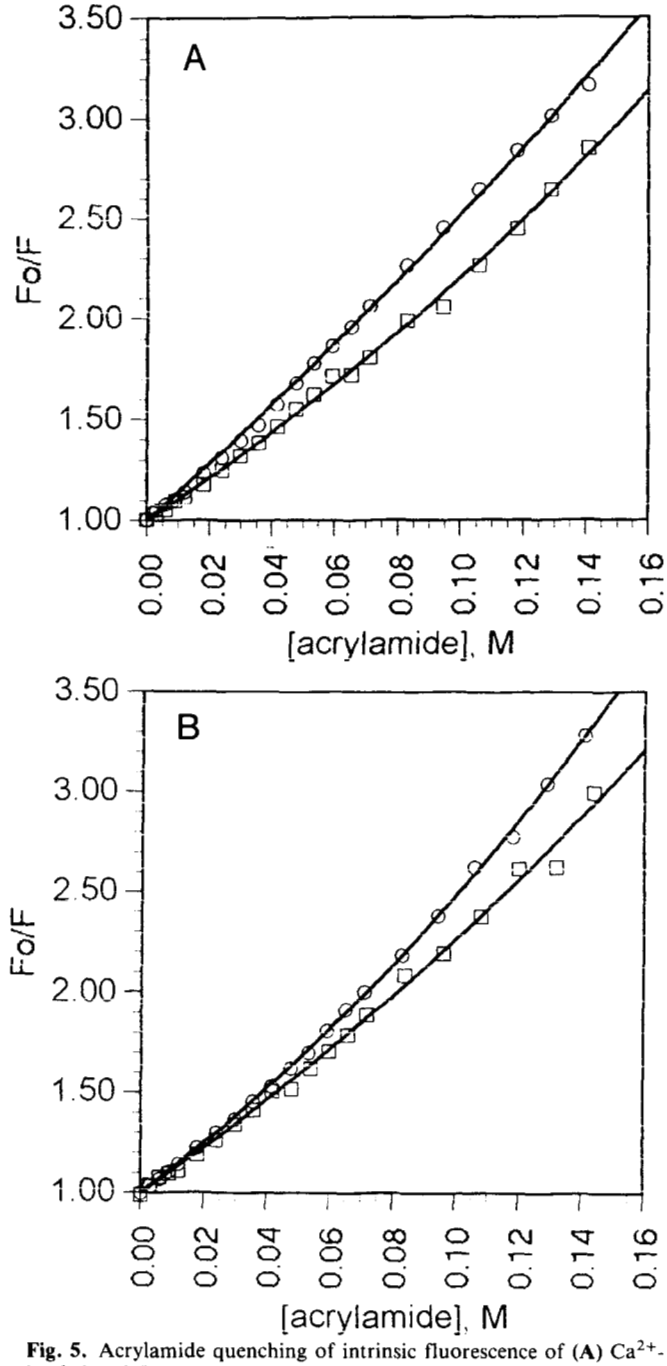

Fig. 5. Acrylamide quenching of intrinsic fluorescence of (**A**) Ca^{2+}-loaded and (**B**) apo-α-LA in association with DMPC vesicles, 20 °C. Buffer was 50 mM HEPES, 150 mM KCl, pH 7.4. For vesicle-associated (○) and vesicle-free (□) protein, metal-bound state was maintained by isolation in the presence of (A) 1 mM $CaCl_2$, or (B) 1 mM EGTA. Emission was monitored at 350 nm, excitation at 280 nm. Parameters for the single-component Stern-Volmer plot with static quenching (Equation 3) are given in Table 2.

Temperature dependence of Zn^{2+}/Ca^{2+} loaded α-LA on DMPC liposomes

Results similar to those for the Ca^{2+}-α-LA case on SUVs were obtained with the Zn^{2+}(54 μM)/Ca^{2+}(1 mM)-loaded α-LA on liposomes (Fig. 6). Membrane-bound complexes of Zn^{2+}/Ca^{2+}-α-LA were impossible to isolate at room temperature due to a tendency of the zinc-loaded protein to precipitate, which was especially pronounced at ≥40:1 zinc:protein. As a precaution, all Zn^{2+}/Ca^{2+}-loaded α-LA samples complexed with liposomes were isolated at 4 °C, where they are thermally stable (Permyakov et al., 1991). Figure 6A shows a plot of emission maximum versus temperature for SUV-bound and free protein. Membrane interaction shifts the protein thermal transition toward lower temperatures; again, the lipid transition is also detected by the protein. The shift in emission maxima is smaller than that for the free protein. Figure 6B shows the corresponding emission intensity plot with temperature, which is replotted as fractional conversion in Figure 6C; a ca. 5 °C lowering of the protein transition temperatures was observed for the Zn^{2+}/Ca^{2+}-α-LA bound to SUVs.

Table 3. *Quenching of tryptophan fluorescence of α-lactalbumin by KI*[a]

Protein state	Conditions	K (M^{-1})
Ca-loaded	DMPC liposomes	3.4 ± 0.3
	Control	3.8 ± 0.1
Apo	DMPC liposomes	3.5 ± 0.2
	Control	4.3 ± 0.3
Acidic	DMPC liposomes	10.3 ± 0.6
	Control	8.6 ± 0.9

[a] Buffers were 50 mM HEPES, pH 7.4, or 50 mM glycine, pH 2.0; all buffers contained 150 mM KCl; 70 °C. K is the Stern-Volmer constant.

Temperature dependence and quenching for the acid α-LA on liposomes

Figure 7 depicts results of temperature-dependence fluorescence studies for free and DMPC-bound acid-state α-LA, incubated at pH 2 (150 mM KCl/50 mM glycine) for 1 min in order to avoid morphology changes in the liposomes (Hanssens et al., 1980). The free (unbound) protein fluorescence parameters (Fig. 7A,B) were constant with temperature, aside from trivial thermal quenching, which is in agreement with the notion that the acidic protein is devoid of tertiary structure (Kuwajima, 1989). However, the α-LA-vesicle complexes gave a distinct thermal transition near 40 °C, both in plots of spectral emission maximum and intensity (Fig. 7A,B), suggesting that the vesicle-associated protein possesses some degree of tertiary structure on the liposome.

Lactose synthase activity for α-LA in association with liposomes

We measured the rate of lactose synthesis by the coupled assay of Fitzgerald et al. (1970) for four different α-LA:liposome complexes of varying protein:lipid ratio. In every case, 3/4 to several hours was required to regain the maximal expected activity. The results are consistent with a slow metal-dependent release of α-LA from lipid-protein complexes found earlier by Berliner and Koga (1987). The complexes were assayed in the presence of 3.5 mM Mn^{2+}, which is similar to the Ca^{2+} levels that induce

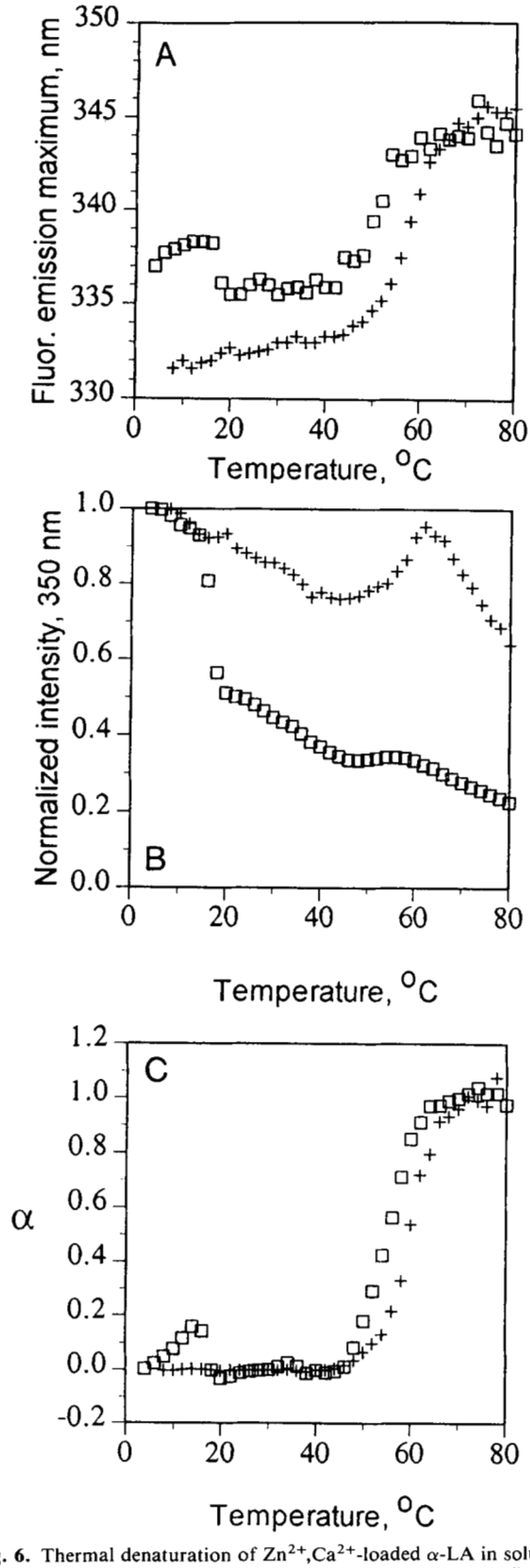

Fig. 6. Thermal denaturation of Zn^{2+},Ca^{2+}-loaded α-LA in solution (+) and in complexes with DMPC (□) vesicles. **A:** Tryptophan fluorescence maximum position. **B:** Normalized fluorescence intensity at 350 nm. **C:** Fractional conversion (α) from the native to thermally denatured state; 50 mM HEPES, pH 7.4; 1 mM $CaCl_2$, 54 μM $ZnCl_2$, 150 mM KCl. Protein concentration, 2–4 μM. Fluorescence was excited at 280 nm.

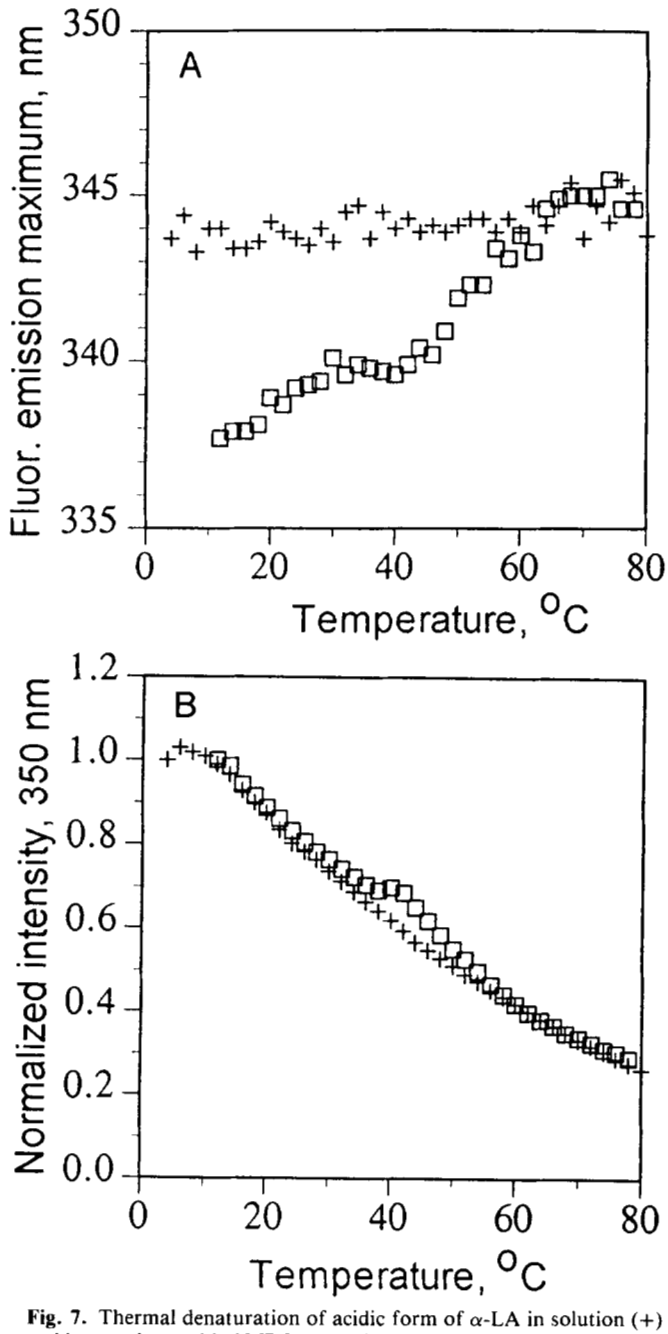

Fig. 7. Thermal denaturation of acidic form of α-LA in solution (+) and in complexes with DMPC (□) vesicles. **A:** Tryptophan fluorescence maximum position. **B:** Normalized fluorescence intensity at 350 nm, 50 mM glycine, pH 2.0; 150 mM KCl. Protein concentration was again maintained between 20 and 40 μM. Excitation was at 280 nm.

slow release of α-LA from DMPC liposomes (Berliner & Koga, 1987).

Discussion

Results at neutral pH

Overall, the thermal unfolding data for various α-LA–DMPC complexes at neutral pH supports the hypothesis that the pro-

tein binds to the vesicles without global unfolding or major changes in stability (Hanssens et al., 1985), because the protein transition occurs at temperatures near that for the free protein. For Ca^{2+}-α-LA bound to DMPC liposomes, the transition was within 5–10 °C of that for the free protein, suggesting that the protein is still calcium bound (Berliner & Koga, 1987). With EPC liposomes (which are composed of a mixture of acyl chain lengths), the protein transition was broader and hence more difficult to assign, perhaps due to the irregular lipid surface presented to the protein. Nonetheless, the relatively invariant positions of the thermal transitions on DMPC versus solution correlates well with earlier observations at neutral pH, which showed that the association between DMPC and α-LA was mainly electrostatic and did not disrupt either the lipid surface or native state of the protein severely (Hanssens et al., 1985).

The fluorescence spectra of α-LA-bound SUVs prior to the protein transition (N′-state) were red-shifted versus the free protein, suggesting a partially expanded protein structure on the liposome with increased tryptophan solvent exposure. The effect was similar to a "partial unfolding" at these low temperatures (Sommers & Kronman, 1980). Above the main transition, the lipid-associated protein in its high-temperature (H′) state exhibited emission maxima that were blue-shifted versus free protein, suggesting an interaction between some fraction of the (four) tryptophan(s) and the lipid bilayer. The blue shifts at 80 °C (in either the DMPC or EPC complex) are consistent with a possible penetration of tryptophan into the apolar bilayer region, and further indicate that the thermally unfolded protein remains firmly associated with the liposome.

The net spectral shift upon thermal unfolding of SUV-bound protein was less compared with the shift for free α-LA in solution. The emission intensity changes during protein unfolding (Figs. 2B, 3B) were also consistent with an expanded protein conformation in the N′-state when bound to liposomes. As with the net spectral shifts, the increase in normalized emission intensity of the SUV-bound protein over the unfolding transition was less than for free α-LA in solution. The positions of the transitions on the liposome were shifted only slightly and were broader than the corresponding free α-LA in solution. For Ca^{2+}-α-LA bound to DMPC, the unfolding occurred at lower temperatures, suggesting that the vesicle-bound N′-state protein was already partially expanded (Fig. 3A). In the case of apo-α-LA bound to EPC vesicles (Fig. 3A), the N′ and H′-states had very similar fluorescence characteristics, because the thermal transition was not obvious in the fluorescence spectra. In general, the N′-state of lipid-bound α-LA appears to be expanded and partially destabilized compared with the free protein. In addition, the protein thermal transitions for both the DMPC and EPC complexes were somewhat broader than the sharp unfolding transition of the free protein, which might arise from a less cooperative nature for unfolding events on the liposome, or may simply result from several characteristically distinct transitions over a range of temperatures. In either case, we must consider some degree of heterogeneity of the N′-state of the SUV-bound protein in our interpretations.

Similar observations, reported previously, were ambiguous because the experiments were performed on unseparated mixtures of complex, lipid, and α-LA (Hanssens et al., 1980, 1985). Both the increased quantum yield and red shift were proposed to arise from a conformational change resembling protein unfolding. The thermal denaturation of α-LA as monitored by fluorescence has been shown to be accompanied by large quantum yield increases arising from the reduction of internal quenching mechanisms, e.g., the N-state of bovine α-LA involves energy transfer from Trp 26 and Trp 104 to Trp 60, which is quenched by two vicinal disulfides (61–77, 73–91) (Sommers & Kronman, 1980). During unfolding, a loss in internal energy transfer quenching results, along with abolition of the near-UV CD spectrum (Sommers & Kronman, 1980). Studies with guinea pig α-LA support this hypothesis, due to the lack of Trp 60. Consequently, there is no increase in emission intensity at 350 nm upon unfolding, despite it containing three other conserved tryptophans (Sommers & Kronman, 1980). Internal quenching mechanisms are well known; for example, energy transfer from tryptophan to the heme groups occurs in cytochrome *c* and hemoglobin, which results in increased emission quantum yield upon unfolding (Eftink, 1994).

Trp 60 and Trp 104 also form the lining of the well-characterized hydrophobic box region of α-LA (Berliner & Koga, 1987; Alexandrescu et al., 1992). Although it would seem reasonable to suggest that these residues insert into the apolar phase of the bilayer (Hanssens et al., 1985), the increased tryptophan accessibility observed in both iodide and acrylamide quenching studies are inconsistent with this model. In fact, the increase in tryptophan exposure for liposome-bound versus free protein is difficult to rationalize in any model where some fraction of the tryptophans are occluded from solvent (Hanssens et al., 1985). Note also that single-component quenching behavior was found with Ca^{2+}-α-LA, suggesting that accessibility changes were uniform for all tryptophans. On the other hand, apo-α-LA exhibited two-component quenching behavior, although substantially greater tryptophan exposure was observed with the liposome-associated form. Lastly, quenching parameters for both the Ca^{2+}- and apo-forms are almost identical, indicating that the unfolded state is similar for either form. Overall, these observations imply that the tryptophan environment is averaged by thermal unfolding and that some tryptophans probably insert into the bilayer. Of course, quenching studies cannot detect buried tryptophans accurately (either in the free protein or upon interaction with the lipid region) because both cases would be characterized by very small collisional quenching parameters. In these cases, one expects that the fluorescence spectrum of the N-state should remain mostly blue-shifted; however, this was not the case with our data.

From careful inspection of the baboon Ca^{2+}-α-LA crystal structure (Acharya et al., 1989), we noted that the calcium-binding loop lies on a surface distant from the site of the hydrophobic box region. Unless a large-scale disruption and realignment of protein tertiary structure occurred, it is nearly impossible to construct a model where both regions contact the lipid simultaneously. At both low (Kim & Kim, 1986) and neutral pH (Berliner & Koga, 1987), the data suggest that the calcium-binding loop region is associated with or buried in the lipid. If α-LA binding was peripheral, as suggested by Dangreau et al. (1982) and Hanssens et al. (1985), the hydrophobic box region would not be associated with lipid and consequently might be accessible to quenchers in the bulk medium. Conversely, the increased tryptophan exposure in the pre-transition temperature region does not fit a model in which the hydrophobic box ("tryptophan face") tightly associates with the bilayer, because this would place the calcium-binding loop region in the bulk medium where it would bind calcium rapidly, not extremely slowly, as

shown by Berliner and Koga (1987). Overall, the model that best fits the current data has the hydrophobic box (tryptophan face) exposed to the solvent. Only upon full thermal unfolding of the protein (H′-state) could a significant fraction of the tryptophans associate with or insert into the lipid phase of the bilayer.

We also note that a second hydrophobic cluster on α-LA, consisting of Phe 31, His 32, and Trp 118 (Acharya et al., 1989, 1991; Alexandrescu et al., 1992), has been proposed to be significant in GT interactions with during lactose synthesis (Barman, 1972; Barman & Bagshaw, 1972; Bell et al., 1975; Grobler et al., 1994). We observed no lactose synthase activity for vesicle-bound α-LA in the presence of GT, suggesting either a disruption of this second hydrophobic cluster upon vesicle binding, or steric limitations to productive GT α-LA association on the bilayer. The most exposed tryptophan was shown to be residue 118 from NMR (Alexandrescu et al., 1992), lifetime (Ostrovsky et al., 1988), and steady-state fluorescence measurements (Sommers & Kronman, 1980). The fluorescence results suggested that this residue accounts for almost 50% of the total fluorescence emission in both N- and H-state α-LA, and that it is substantially red-shifted during thermal melting (e.g., the emission maximum shifts from 326 to 343 nm). Therefore, it is conceivable that Trp 118 is relatively close to the lipid bilayer in the N′-state. Because the binding of N′-state apo-α-LA to DMPC SUVs is neither accompanied by a complete denaturation of the protein nor a measurable loss of tryptophan accessibility, the simplest explanation for the loss of lactose synthase activity is that the interacting residues are too close to the lipid surface to allow access to GT.

Our attempts to examine the effects of Zn^{2+} on the affinity of Ca^{2+}-α-LA for membranes was thwarted by both protein aggregation and instability at high zinc:protein ratios. The results reflect the influence of zinc on protein denaturation (Permyakov et al., 1991). Consequently, it appears unlikely that high levels of Zn^{2+} would induce significant protein:liposome interactions, because the precipitation we observed in lipid mixtures above 25 °C was accompanied by no significant accumulation on the vesicles. However, we were able to isolate Zn^{2+}-loaded Ca^{2+}-α-LA:SUV complexes at 4 °C, as described above.

The MG state on the liposome?

From the results above, we conclude that α-LA-DMPC complexes (at both neutral and acid pH) exhibit protein fluorescence thermal transitions. At low temperature, the fluorescence of DMPC-bound acid α-LA was blue-shifted versus that for free protein, implying that tryptophan residues were less accessible in the membrane-bound case. Also, the static quenching parameter for acrylamide is in agreement with lower tryptophan accessibility when bound to SUVs. The collisional quenching constants for iodide and acrylamide were larger for membrane-bound protein, which does not support decreased exposure. However, it is possible that an increase in fluorescence lifetime occurs upon association between the protein and lipid, which could also account for the increased collisional constants (Eftink, 1991). The static parameter V (acrylamide results) is insensitive to changes in the lifetime and represents the molar volume of chromophore available for preassociation with quencher (Eftink & Ghiron, 1976; Eftink, 1991). This parameter reported significant reduction in exposure for the SUV-bound protein. This apparent contradiction here in some of the quenching results for acid α-LA will require extensive lifetime studies, which are more complicated for multi-tryptophan proteins (Ostrovsky et al., 1988). If this transition can be verified in the future by other techniques as well, such as mutagenesis to single or double tryptophan mutants, it seriously calls into question the hypothesis that MG intermediates are present in proteins bound to the membrane (Kuwajima, 1989). The acid form clearly inserts spontaneously into lipid bilayers as reflected by a very favorable free energy of association between lipid and protein. The protein is stabilized by the lipid as reflected in a lower free energy for unfolding. Note that a transition consistently appears in the temperature-dependent fluorescence data, in agreement with this simple interpretation.

It is also pertinent to consider whether increasing temperature simply induces protein dissociation from the vesicle. In several cases, we rechromatographed the thermally denatured complexes (albeit at lower temperature), where little or no additional dissociation was observed. However, had protein dissociation occurred, the fluorescence parameters of the free and vesicle bound protein would be the same at the completion of the thermal transition. This was not the case for either the Ca^{2+}-, apo-, or Zn^{2+}-loaded Ca^{2+}-forms of α-LA (Figs. 2, 3, 6). Although the acid form, both free and vesicle-bound, displayed similar fluorescence parameters at high temperature (Fig. 7), it was, in fact, the lipid complexation that *stabilized* some element of tertiary structure in the protein as discussed above. Consequently, temperature-induced protein dissociation appears to be unlikely.

This work is significant in light of the recent hypothesis that, when proteins are translocated across the membrane, they must be an non-native or MG state (Ptitsyn, 1992). At both neutral and acid pH, we see little evidence suggesting the existence of the MG state bound to DMPC vesicles. The presence of a thermal transition suggests cooperative unfolding events, where no such cooperativity exists between the MG and U-states (Kuwajima, 1989; Creighton, 1990). Thus, in any case where a thermal transition is observed, the classically described MG state is unlikely. For example, the work of Lala et al. (1995) suggested that the CD of lipid-bound apo-α-LA "resembled" the MG state; however, the results presented here indicate that CD is not necessarily a totally reliable criterion for the existence of the MG state. Consequently, it is imperative to check the thermal unfolding by other criteria.[4] A similar problem may have occurred in the work of Yutani et al. (1992) with apo-α-LA in low ionic strength buffers. Furthermore, this work shows that, at neutral pH, both the Ca^{2+}- and apo-forms of α-LA bind to membranes. Perhaps the fact that Ca^{2+}-α-LA does not bind as strongly relates to one of its physiological roles in protein folding by serving to release the apo- or some intermediate form of the protein after translocation.

Materials and methods

Materials

Bovine α-LA (lot 128F-8140) was purchased from Sigma Chemical Co. (St. Louis, Missouri) and used without further purification. The purity of the protein preparation was estimated to

[4] We attempted DSC and CD on these complexes, but were not able to attain sufficient sensitivity due to the low levels of total protein in the isolate α-LA:SUV complexes. Furthermore, the DSC showed only the lipid transition.

be in excess of 97% by SDS-PAGE and reverse-phase HPLC. DMPC powder (lots 140PC-156, -158) and EPC (chloroform solution, dried under N_2 prior to use, lot EPC-152) were from Avanti Polar Lipids, Inc. GT was isolated as described previously (Grunwald & Berliner, 1978). The following materials were obtained from Sigma Chemical Company: pyruvate kinase/lactate dehydrogenase mixture (PK/LDH, ammonium sulfate suspension, lot 31H-6108, ≥700 IU/mL rabbit muscle PK, ≥1,000 IU/mL rabbit muscle LDH); β-nicotinamide dinucleotide, reduced form, disodium salt (NADH, 97% pure, grade III from yeast, lot 49F-71001); phosphoenolpyruvate, tri(monocyclohexylammonium) salt (PEP, 99% pure, lot 68F-3792); uridine diphospho-galactose, disodium salt (UDP-galactose, 98% pure, lot 17F-7015). All other chemicals were of reagent grade or purer, and were used without further purification.

Preparation of liposomes

Phospholipid SUVs were prepared according to the method of Huang (1969). Sonication of a 3% phospholipid suspension in 150 mM KCl and the desired buffer was performed to clarity (30–45 min) under nitrogen with a Branson Sonifier 250 at temperatures above the lipid-transition temperature (Huang & Thompson, 1970). The clarified mixture was centrifuged at room temperature to remove titanium fragments (16,600 × *g*, 10 min), then the supernatant was applied to a lipid-saturated Sepharose CL-4B column in the sonication buffer. SUVs were pooled from the second eluted peak, and were used immediately. Lipid concentration was estimated by optical density at 300 nm using an approximate "extinction" coefficient of 38 M^{-1} cm^{-1} (Huang, 1969), in good agreement with independent results from a two-phase ferrithiocyanate assay of lyophilized aliquots resuspended in chloroform (New, 1990).

Isolation of lipid–protein complexes

The vesicle-bound α-LA was prepared by a procedure described previously (Berliner & Koga, 1987). Lipid/protein mixtures (typically 100–150 lipid:protein at 2–25 mM lipid) were preincubated for a minimum of 2 h at 4 °C. The protein–vesicle complex was separated from free protein by chromatography on Sephadex G-200, also at 4 °C (see Fig. 1). The lipid:protein ratio of the isolated, pooled void fractions was roughly estimated by determining the ratio of optical densities at 300 nm (see above) and 280 nm from a solvent-corrected absorbance spectrum using an extinction coefficient for the protein of $\epsilon_{1\%,280} = 20.1$ (Kronman & Andreotti, 1964). Values were typically between 200 and 2,000 molar ratio of lipid:protein.

Fluorescence spectra

Fluorescence measurements were performed on a Perkin-Elmer Model LS-50B spectrofluorimeter. Excitation at 280 nm was used because Kronman and Holmes (1971) had shown previously that the tyrosine contribution to the intrinsic fluorescence spectra of α-LA was negligible. Also, excitation at 295 nm was not very feasible due both to low protein quantum yield and the presence of significant scattering by the SUVs. Excitation and emission slit widths were typically between 8 and 10 nm. Spectra were smoothed (binomial filtering, 32–37 points) and were analyzed directly without further correction, as a matter of convenience. Temperature was monitored inside the sample cell with a model 201 thermometer and sheathed DT-471-SD silicon diode probe (Lake Shore Cryotronics, Westerville, Ohio). The heating rate was between 0.5 and 1 C°/min, and spectra were collected every 2 °C over the entire temperature range.

Data analysis

Temperature dependence of fluorescence data was analyzed as described by Permyakov and Burstein (1984) (see also Eftink, 1994 for an alternate analysis). Plots of intrinsic protein fluorescence intensity at a selected wavelength versus temperature typically exhibit a transition superimposed upon on thermal background quenching. Assuming that the transition is of a two-state type, such as a thermal unfolding process between the N-state and H-state (which can be either the random-coil unfolded state, a compact intermediate, such as the MG state, or a mixture of the two), for a single tryptophan protein, the trivial background quenching obeys the following relationship (Bushueva, et al., 1978):

$$1/F_\lambda = a + b \cdot (T/\eta), \tag{1}$$

where F_λ is the intensity at a fixed wavelength, T is temperature in K, and η is viscosity of the solvent in cP. Equation 1 applies to each observable state bracketing a transition region, and this reciprocal intensity relationship holds for multi-tryptophan proteins such as bovine α-LA as well (Permyakov & Burstein, 1984). The regions of the plot above and below the main transition are adequately approximated by two distinct linear (pre- and post-transition) regions.

The transition region between these linear regions is described by:

$$F_\lambda = (1 - \alpha)(F_\lambda)_{N,T} + \alpha(F_\lambda)_{H,T}, \tag{2}$$

where F_λ is the observed fluorescence intensity at a given temperature; $(F_\lambda)_{N,T}$ and $(F_\lambda)_{H,T}$ are the fixed wavelength intensities for the N-state and H-state region. The last two quantities can be estimated by extrapolation of the data from the linear regions of the reciprocal plot into the transition region. Solution of Equations 1 and 2 provide an estimate of α, the fractional conversion or fraction denatured, which is plotted versus temperature.

For data exhibiting only small changes in reciprocal intensity upon unfolding, fluorescence phase plots were employed that make use of changes in the spectral shape during a transition from one state to another (Kreimer et al., 1985; Permyakov, 1993). At each temperature measured, the emission intensities at two different wavelengths, $F_{\lambda 1}$ and $F_{\lambda 2}$ (in this case, 330 and 350 nm) were plotted with $F_{\lambda 1}$ as ordinate and $F_{\lambda 2}$ as abscissa. The paired $(F_{\lambda 1}, F_{\lambda 2})$ data for the N-state and H-state lie along separate straight lines due to the distinct shape of the spectra in each of these states (Permyakov, 1993). Both lines intersect the origin and the points between the two lines represent data in the transition region. The limits of the thermal transition region can be estimated from these plots, due to their sensitivity to spectral shape changes in the transition region.

Quenching experiments and analysis

For the potassium iodide (KI) experiments, solutions were prepared so that the series was balanced to constant total ionic strength. No sodium thiosulfate was used in these experiments to inhibit iodide oxidation, because monovalent cations bind weakly to apo α-LA, affecting quantum yield (K_d about 10 mM, see Permyakov et al., 1985). The presence of triiodide was undetectable in freshly made solutions. No correction was necessary for the excluded volume of the vesicles, because the uncertainty in volume was typically less than 3–5% (based upon SUV volume estimates by Huang, 1969).

Emission intensity data at 350 nm for quenching by KI or acrylamide was corrected for dilution and inner filter effects (for acrylamide), then fit to theoretical curves computed according to a single-, or dual-component model, or single-component model with static quenching (Lehrer, 1971; Eftink & Ghiron, 1976; Eftink, 1991; Permyakov, 1993):

$$F = F_0/(1 + K[Q]), \text{ or } F_0/F = (1 + K[Q]), \quad (3)$$

$$F = F_0[f_1/(1 + K_1[Q]) + (1 - f_1)/(1 + K_2[Q])], \quad (4)$$

$$F = F_0\,[1/(1 + K_1[Q])]\exp(-V[Q]), \quad (5)$$

where F is the fluorescence intensity at a fixed wavelength; F_0 is the fluorescence intensity in the absence of quencher; $[Q]$ is quencher concentration; K is Stern–Volmer parameter for the single emitting center model; K_1 and K_2 are Stern–Volmer parameters for the dual emitting centers model; and f_1 is the fractional contribution of the first center to the total fluorescence intensity. Curve-fitting software for Equations 3, 4, and 5 was developed and employed to estimate both the parameters and their error ranges according to a published algorithm (Reich et al., 1972). Alternatively, fits of Equations 3, 4, and 5 were performed using the Levenberg–Marquardt procedure (Press et al., 1986).

Acknowledgments

This work was supported by grants from the U.S.P.H.S., the International Science Foundation (MJ3000), and NATO (LG 930245 and 940412). We are grateful to Patricia J. Anderson for helpful comments.

References

Acharya KR, Ren J, Stuart DI, Phillips DC, Fenna RE. 1991. Crystal structure of human α-lactalbumin at 1.7 Å resolution. *J Mol Biol 221*:571–581.

Acharya KR, Stuart DI, Walker NPC, Lewis M, Phillips DC. 1989. Refined structure of baboon α-lactalbumin at 1.7 Å resolution. *J Mol Biol 208*: 99–127.

Alexandrescu AT, Broadhurst RW, Wormald C, Chyan C, Baum J, Dobson CM. 1992. ^{1}H-NMR assignments and local environments of aromatic residues in bovine, human and guinea pig variants of α-lactalbumin. *Eur J Biochem 210*:699–709.

Alexandrescu AT, Evans PA, Pitkeathly M, Baum J, Dobson CM. 1993. Structure and dynamics of the acid-denatured molten globule state of α-lactalbumin: A two-dimensional NMR study. *Biochemistry 32*:1707–1718.

Ameloot M, Hendrickx H, Herreman W, Pottel H, Van Cauwelaert F, Van der Meer WF. 1984. Effect of orientational order on the decay of the fluorescence anisotropy in membrane suspensions. Experimental verification on unilamellar vesicles and lipid/α-lactalbumin complexes. *Biophys J 46*:525–539.

Aramini JM, Hiraoki T, Vogel HJ. 1996. NMR and stopped-flow studies of metal ion binding to α-lactabumins. *Biochem Biophys Acta 1293*:72–82.

Barman TE. 1972. The modification of the tryptophan residues of bovine α-lactalbumin with 2-hydroxy-5-nitrobenzylbromide and with dimethyl(2-hydroxy-5-nitrobenzyl) sulphonium bromide. I. Characterization of the modified protein. *Biochim Biophys Acta 258*:297–313.

Barman TE, Bagshaw W. 1972. The modification of the tryptophan residues of bovine α-lactalbumin with 2-hydroxy-5-nitrobenzylbromide and with dimethyl(2-hydroxy-5-nitrobenzyl)sulphonium bromide. II. Effect on the specifier protein activity. *Biochim Biophys Acta 278*:491–500.

Baum J, Dobson CM, Evans PA, Hanley C. 1989. Characterization of a partly folded protein by NMR methods: Studies on the molten globule state of guinea pig α-lactalbumin. *Biochemistry 28*:7–13.

Bell JE, Castellino FJ, Trayer IP, Hill RL. 1975. Modification of α-lactalbumin with *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide. *J Biol Chem 250*:7579–7585.

Berliner LJ, Koga K. 1987. α-Lactalbumin binding to membranes: Evidence for a partially buried protein. *Biochemistry 26*:3006–3009.

Brew K, Vanaman TC, Hill RL. 1967. Comparison of the amino acid sequence of bovine α-lactalbumin and hen's egg white lysozyme. *J Biol Chem 242*:3747–3749.

Brodbeck U, Ebner KE. 1966. Resolution of a soluble lactose synthetase into two protein components and solubilization of microsomal lactose synthetase. *J Biol Chem 241*:762–764.

Brown EM. 1984. Interactions of β-lactoglobulin and α-lactalbumin with lipids: A review. *J Dairy Sci 67*:713–722.

Browne WJ, North ACT, Phillips DC, Brew K, Vanaman TC, Hill RL. 1969. A possible three-dimensional structure of bovine α-lactalbumin based on that of hen's egg-white lysozyme. *J Mol Biol 42*:65–86.

Bushueva TL, Busel EP, Burstein EA. 1978. Relationship of thermal quenching of protein fluorescence to intramolecular structural mobility. *Biochem Biophys Acta 534*:141–152.

Chyan CL, Wormald C, Dobson CM, Evans P, Baum J. 1993. Structure and stability of the molten globule state of guinea pig α-lactalbumin: A hydrogen exchange study. *Biochemistry 32*:5681–5691.

Creighton TE. 1990. Protein folding. *Biochem J 270*:1–16.

Damaschun G, Gernat C, Damaschun H, Bychkova VE, Ptitsyn OB. 1986. Comparison of intramolecular packing in native and "molten globule" states. *Int J Biol Macromol 8*:226–230.

Dangreau H, Joniau M, DeCuyper M, Hanssens I. 1982. An intramolecular excimer-forming probe used to study the interaction of α-lactalbumin with model membranes. *Biochemistry 21*:3594–3598.

Dolgikh DA, Gilmanshin RI, Brazhnikov EV, Bychkova VO, Semisotnov GV, Venjaminov SY, Ptitsyn OB. 1981. α-Lactalbumin: Compact state with fluctuating tertiary structure. *FEBS Lett 136*:311–315.

Ebner KE. 1970. A biological role for α-lactalbumin as a component of an enzyme requiring two proteins. *Acc Chem Res 3*:41–47.

Ebner KE. 1973. Lactose synthetase. *The Enzymes 9*:363–377.

Eftink MR. 1991. Fluorescence quenching reactions: Probing biological macromolecular structures. In: Dewey TG, ed. *Biophysical and biochemical aspects of fluorescence spectroscopy*. New York: Plenum. pp 1–41.

Eftink MR. 1994. The use of fluorescence methods to monitor unfolding transitions in proteins. *Biophys J 66*:482–501.

Eftink MR, Ghiron CA. 1976. Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. *Biochemistry 15*:672–680.

Fitzgerald DK, Colvin B, Mawal R, Ebner KE. 1970. Enzymic assay for galactosyl transferase activity of lactose synthetase and α-lactalbumin in purified and crude systems. *Anal Biochem 36*:43–60.

Gast K, Zirwer D, Welfe H, Bychkova VE, Ptitsyn OB. 1986. Quasielastic light scattering from human lactalbumin: Comparison of molecular dimensions in native and "molten globule" states. *Int J Biol Macromol 8*:231–236.

Grobler JA, Wang M, Pike ACW, Brew K. 1994. Study by mutagenesis of the roles of two aromatic clusters of α-lactalbumin in aspects of its action in the lactose synthase system. *J Biol Chem 269*:5106–5114.

Grunwald J, Berliner LJ. 1978. Immobilized bovine lactose synthetase—A method of topographical analysis of the active site. *Biochim Biophys Acta 523*:53–58.

Hanssens I, Herreman W, Van Ceunebroeck JC, Dangreau H, Gielens C, Preaux G, Van Cauwelaert FH. 1983. Interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. III. Influence of the temperature and of the lipid-to-protein molar ratio on the complex formation. *Biochim Biophys Acta 728*:293–304.

Hanssens I, Houthuys C, Herreman W, Van Cauwelaert FH. 1980. Interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. I. A microcalorimetric and fluorescence study. *Biochim Biophys Acta 602*:539–557.

Hanssens I, van Ceunebroeck JC, Pottel H, Preaux G, van Cauwelaert F.

1985. Influence of the protein conformation on the interaction between α-lactalbumin and dimyristoylphosphatidylcholine vesicles. *Biochim Biophys Acta 817*:154–166.

Herreman W, Van Cauwelaert F, Hanssens I. 1981a. Use of energy transfer to study the interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. *Biochem International 2*:237–242.

Herreman W, Van Tornout P, Van Cauwelaert FH, Hanssens I. 1981b. Interaction of α-lactalbumin with dimyristoyl phosphatidylcholine vesicles. II. A fluorescence polarization study. *Biochim Biophys Acta 640*:419–429.

Hill RL, Brew K. 1975. Lactose synthetase. *Adv Enzymol Relat Areas Mol Biol 43*:411–490.

Hiraoka Y, Segawa T, Kuwajima K, Sugai S, Murai N. 1980. α-Lactalbumin: A calcium metalloprotein. *Biochem Biophys Res Commun 95*:1098–1104.

Huang C. 1969. Studies on phosphatidylcholine vesicles. Formation and characteristics. *Biochemistry 8*:344–352.

Huang C, Thompson TE. 1970. Preparation of homogeneous, single-walled phosphatidylcholine vesicles. *Methods Enzymol 32*:485–489.

Ikeguchi M, Kuwajima K, Sugai S. 1986. Ca^{2+}-induced alteration in the unfolding behavior of α-lactalbumin. *J Biochem 99*:1191–1201.

Kim J, Kim H. 1986. Fusion of phospholipid vesicles induced by α-lactalbumin at acidic pH. *Biochemistry 25*:7867–7874.

Kronman MJ, Andreotti RE. 1964. Inter- and intramolecular interactions of α-lactalbumin. I. The apparent heterogeneity at acid pH. *Biochemistry 3*:1145–1151.

Kronman MJ, Holmes L. 1971. The fluorescence of native, denatured and reduced-denatured proteins. *Photochemistry and Photobiology 14*:113–134.

Kuhn NJ, Carrick DT, Wilde CJ. 1980. Lactose synthesis: The possibilities of regulation. *J Dairy Sci 63*:328–336.

Kuwajima K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular protein structure. *Proteins Struct Funct Genet 6*:87–103.

Lakowicz J. 1983. *Principles of fluorescence spectroscopy*. New York: Plenum.

Lakey JH, Massotte D, Heitz F, Dasseux JL, Faucon JF, Parker MW, Pattus F. 1991. Membrane insertion of the pore-forming domain of colicin A. A spectroscopic study. *Eur J Biochem 196*:599–607.

Lala AK, Kaul P, Ratnam PB. 1995. Membrane–protein and the molten globule state—Interaction of α-lactalbumin with membranes. *J Protein Chem 14*:601–609.

Lehrer SS. 1971. Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry 10*:3254–3263.

Lentz BR, Carpenter TJ, Alford DR. 1987. Spontaneous fusion of phosphatidylcholine small unilamellar vesicles in the fluid phase. *Biochemistry 26*:5389–5397.

Lindahl L, Vogel HJ. 1984. Metal-ion-dependent hydrophobic-interaction chromatography of α-lactalbumins. *Anal Biochem 140*:394–402.

McKenzie HA, White FH. 1991. Lysozyme and α-lactalbumin: Structure, function and interrelationships. *Adv Protein Chem 41*:173–315.

Meers P, Mealy T. 1993. Relationship between annexin V tryptophan exposure, calcium and phospholipid binding. *Biochemistry 32*:5411–5418.

Mitranic MM, Boggs JM, Moscarello MA. 1983. Modulation of bovine milk galactosyltransferase activity by lipids. *J Biol Chem 258*:8630–8636.

Mitranic MM, Moscarello MA. 1985. The effect of acidic lipids on the activity of bovine milk galactosyltransferase in vesicles of different phosphatidylethanolamines. *Biochim Biophys Acta 816*:182–186.

Mitranic MM, Paquet MR, Moscarello MA. 1988. The interaction of bovine milk galactosyltransferase with lipid and α-lactalbumin. *Biochim Biophys Acta 958*:277–284.

Mulqueen PM, Kronman MJ. 1982. The binding of naphthalene dyes to the N and A conformers of bovine α-lactalbumin. *Arch Biochem Biophys 215*:28–39.

Murakami K, Andree PJ, Berliner LJ. 1982. Metal ion binding to α-lactalbumin. *Biochemistry 21*:5488–5494.

Murakami K, Berliner LJ. 1983. A distinct zinc binding site in the α-lactalbumins regulates calcium binding. Is there a physiological role for this control? *Biochemistry 22*:3370–3374.

Musci G, Berliner LJ. 1985a. Probing different conformational states of bovine α-lactalbumin: Fluorescence studies with 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate]. *Biochemistry 24*:3852–3856.

Musci G, Berliner LJ. 1985b. Physiological roles of zinc and calcium binding to α-lactalbumin in lactose biosynthesis. *Biochemistry 24*:6945–6948.

Navratnam N, Virk SS, Ward S, Kuhn NJ. 1986. Cationic activation of galactosyltransferase from rat mammary golgi membranes by polyamines and by basic peptides and proteins. *Biochem J 239*:423–433.

Navratnam N, Ward S, Fisher C, Kuhn NJ, Keen JN, Findlay JBC. 1988. Purification, properties and cation activation of galactosyltransferase from lactating-rat mammary Golgi membranes. *J Biochem 171*:623–629.

New RRC. 1990. *Liposomes—A practical approach*. New York: IRL Press.

Ohgushi M, Wada A. 1983. "Molten-globule state:" A compact form of globular proteins with mobile side-chains. *FEBS Lett 164*:21–24.

Ostrovsky AV, Kalinichenko LP, Emelyanenko VI, Klimanov AV, Permyakov EA. 1988. Environment of tryptophan residues in various conformational states of α-lactalbumin studied by time-resolved and steady-state fluorescence spectroscopy. *Biophys Chem 30*:105–115.

Permyakov EA. 1993. *Luminescence spectroscopy of proteins*. Boca Raton, Florida: CRC Press.

Permyakov EA, Burstein EA. 1984. Some aspects of studies of thermal transitions in proteins by means of their intrinsic fluorescence. *Biophys Chem 19*:265–271.

Permyakov EA, Kalinichenko LP, Morozova LA, Yarmolenko VV, Burstein EA. 1981a. α-Lactalbumin binds magnesium ions: Study by means of intrinsic fluorescence technique. *Biochem Biophys Res Commun 102*:1–7.

Permyakov EA, Kreimer DI. 1986. Effects of pH, temperature and Ca^{2+} content on the conformation of α-lactalbumin in a medium modelling physiological conditions. *Gen Physiol Biophys 5*:377–390.

Permyakov EA, Kreimer DI, Kalinichenko LP, Shnyrov VL. 1988a. Interaction of calcium binding proteins, parvalbumin and α-lactalbumin, with dipalmitoyl-phosphatidylcholine vesicles. *Gen Physiol Biophys 7*:95–107.

Permyakov EA, Morozova LA, Burstein EA. 1985. Cation binding effects on the pH, thermal and urea denaturations in α-lactalbumin. *Biophys Chem 21*:21–31.

Permyakov EA, Morozova LA, Kalinichenko LP, Derezhkov VY. 1988b. Interaction of α-lactalbumin with Cu^{2+}. *Biophys Chem 32*:37–42.

Permyakov EA, Shnyrov VL, Kalinichenko LP, Kuchar A, Reyzer IL, Berliner LJ. 1991. Binding of Zn(II) ions to α-lactalbumin. *J Protein Chem 10*:577–584.

Permyakov EA, Yarmolenko VV, Kalinichenko LP, Morozova LA, Burstein EA. 1981b. Calcium binding to α-lactalbumin: Structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes. *Biochem Biophys Res Commun 100*:191–197.

Pfeil W. 1987. Is the thermally denatured protein unfolded? The example of α-lactalbumin. *Biochim Biophys Acta 911*:114–116.

Press WH, Flannery BP, Teukolsky SA, Vetterling WT. 1986. *Numerical recipes in C*. Cambridge, UK: Cambridge University Press.

Ptitsyn O. 1987. Protein folding: Hypotheses and experiments. *J Protein Chem 6*:273–293.

Ptitsyn O. 1992. The molten globule state. In: Creighton TE, ed. *Protein folding*. New York: Freeman. pp 243–300.

Reich JG, Wangermann G, Falck M, Rohde K. 1972. A general strategy for parameter estimation from isosteric and allosteric-kinetic data and binding measurements. *Eur J Biochem 26*:368–379.

Ren J, Stuart DI, Acharya KR. 1993. α-Lactalbumin possesses a distinct zinc binding site. *J Biol Chem 268*:19292–19298.

Sommers PB, Kronman MJ. 1980. Comparative fluorescence properties of bovine, goat, human and guinea pig α-lactalbumin. Characterization of the environments of individual tryptophan residues in partially folded conformers. *Biophys Chem 11*:217–232.

Stuart DI, Acharya KR, Walker NPC, Smith SG, Lewis M, Phillips DC. 1986. α-Lactalbumin possesses a novel calcium binding loop. *Nature 324*:84–87.

Volwerk JJ, Filthruth E, Griffith OH, Jain MK. 1994. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* at the lipid–water interface: Interfacial binding, catalysis and activation. *Biochemistry 33*:3464–3474.

Witsell DL, Casey CE, Neville MC. 1990. Divalent cation activation of galactosyltransferase in native mammary Golgi vesicles. *J Biol Chem 265*: 15731–15737.

Yutani K, Ogasahara K, Kuwajima K. 1992. Absence of the thermal transition in apo-α-lactalbumin in the molten globule state. A study by differential scanning microcalorimetry. *J Mol Biol 228*:347–350.