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Interaction of Human α-Lactalbumin with Fatty Acids: Determination of Binding Parameters

C. Barbana*, M. D. Perez, C. Pocovi, L. Sanchez, and Z. Wehbi

Tecnologia y Bioquimica de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, 50013-Zaragoza, Spain; fax: (34-976) 761-590; E-mail: barbana@unizar.es

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Abstract—The interaction of holo- and apo-forms of human α-lactalbumin with fatty acids was studied by a partition equilibrium method. Apo-α-lactalbumin, obtained by treatment with EDTA, displays one binding site for fatty acids, the association constants for oleic and palmitic acids being $1.9 \cdot 10^6$ and $4.2 \cdot 10^5$ M^{-1}, respectively. However, holo-α-lactalbumin was unable to bind fatty acids as measured by this technique. Likewise, no fatty acids bound to holo-α-lactalbumin, isolated using nondenaturing conditions, were detected by gas chromatography. These results demonstrate that the conformational change induced in α-lactalbumin by the removal of calcium enables the protein to interact with fatty acids.

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α-Lactalbumin is an acidic protein of about 14.2 kD molecular mass, which is expressed in the mammary gland during lactogenesis. It is the most abundant protein in human whey, where its concentration is about 1.7 mg/ml [1]. α-Lactalbumin has the ability to bind different metal cations [2, 3]. The human protein possesses a strong Ca^{2+}-binding site with an apparent binding constant of $3.0 \cdot 10^8$ M^{-1} [4] and a second, weaker-binding site, 7.9 Å away from the first site [5]. Several other metal cations such as Mn^{2+}, Cd^{2+}, Na^+, and K^+ have been found to bind to the Ca^{2+}-binding site [6].

The binding of calcium to α-lactalbumin has pronounced effects on its stability to heat and chemical denaturants as well as on other physicochemical properties [2, 3, 7, 8]. Removal of the tightly bound Ca^{2+} from α-lactalbumin using chelators causes a pronounced conformational change as seen by fluorescence and circular dichroism measurements [6, 8, 9]. The protein adopts a conformation, called molten globule state, which is an intermediate between native and unfolded protein [4, 10]. These changes closely resemble those occurring during acid denaturation of the native protein [3, 10].

The primary function of α-lactalbumin is to participate in lactose biosynthesis as the regulatory component of the lactose synthetase complex. α-Lactalbumin increases the affinity of galactosyltransferase for glucose as the galactosyl acceptor, so that lactose is synthesized at the physiological concentrations of glucose present in the mammary gland [11].

A novel approach to the biological function of human α-lactalbumin was suggested in the last decade. It was proposed that a particular form of the protein induces apoptosis-like death in tumor and immature cells but spares most healthy differentiated cells [12, 13]. The conversion of α-lactalbumin to the active apoptotic form required the removal of calcium by treatment with EDTA and the passage of the apo-protein throughout an ion-exchange column that had been conditioned previously with oleic acid [14, 15]. The active form of the protein, called "human α-lactalbumin made lethal to tumor cells" (HAMLET), is a molecular complex of partially unfolded human α-lactalbumin and oleic acid. Since then, the interaction of α-lactalbumin with fatty acid conditioned matrices as well as the complexes obtained after protein elution from oleic acid conditioned columns has been the object of several studies [16-19].

It has also been reported that bovine α-lactalbumin is able to interact with fatty acids in solution. It binds 5-doxylstearic, stearic, palmitic, and oleic acids [20-22], the binding parameters depending upon the protein state.

* To whom correspondence should be addressed.

Studies performed with bovine α-lactalbumin indicated that the apo-form displays one binding site for fatty acids, the association constant being of the order of 10^6 M^{-1} [20, 21]. The binding of fatty acids to bovine apo-α-lactalbumin produced a decrease in thermal stability of the protein [7, 22], whereas no effect was observed on its resistance to tryptic degradation [8, 9].

The aim of this work was to extend the study of the interaction of α-lactalbumin with fatty acids to the homologous protein obtained from human milk. This study was performed by a partition equilibrium method using labeled fatty acids dissolved in heptane.

MATERIALS AND METHODS

Materials. Oleic and palmitic acids, Sephadexes G-100, G-50, and G-25, and EDTA were provided by Sigma (USA). [^{14}C]Oleic and [^{14}C]palmitic acids were obtained from Amersham International (U.K.). The HP-FFAP column was purchased from Agilent Technologies (USA). Human milk samples were kindly supplied by Miguel Servet Hospital (Zaragoza, Spain).

Isolation of human α-lactalbumin. Human milk samples were skimmed by centrifugation at 2000*g* for 30 min at 4°C. Whey was obtained by ultracentrifugation at 105,000*g* for 2 h at 4°C after adding $CaCl_2$ to a final concentration of 1% (w/v). α-Lactalbumin was isolated by gel filtration chromatography on a Sephadex G-100 column (90 × 5 cm) equilibrated with 25 mM sodium acetate, 50 mM NaCl buffer, pH 6.5, at 4°C. Fractions enriched in α-lactalbumin were pooled, concentrated in ultrafiltration cells with YM-3 Diaflo membranes (Amicon, USA), and chromatographed under the same conditions on a Sephadex G-50 column (75 × 3 cm). Fractions obtained from this chromatography were dialyzed against distilled water and lyophilized. The α-lactalbumin obtained was 99% pure as determined by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed using 8-25% polyacrylamide gels on a Phast system (Pharmacia, Sweden). Dialyzed protein samples dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% SDS, and 0.01% bromophenol blue were boiled for 5 min. Proteins were stained by immersing the gels in 0.1% Coomassie Blue R dissolved in water–methanol–acetic acid (6 : 3 : 1) for 2 h. Destaining was performed in a mixture of methanol, acetic acid, distilled water, and glycerol (3 : 1 : 5 : 1).

Preparation of apo-α-lactalbumin. Calcium bound to holo-α-lactalbumin was removed to obtain the apo-form as described by Murakami et al. [10]. Briefly, 12 mg of α-lactalbumin was dissolved in 1.2 ml of 10 mM Tris-HCl buffer, pH 8.5, containing 100 mM EDTA. The mixture was incubated at room temperature for 1 h, and then it was applied to a Sephadex G-25 column (25 × 1 cm) equilibrated with the same buffer. Fractions eluted in the void volume of the column were pooled and concentrated to 10 mg/ml in ultrafiltration cells with YM-3 Diaflo membranes.

Characterization of apo-α-lactalbumin by size-exclusion chromatography. A volume of 0.6 ml of human holo- or apo-α-lactalbumin (10 mg/ml) in 10 mM Tris-HCl buffer, pH 8.5, was applied to a Sephadex G-50 column (75 × 1.5 cm) equilibrated with the same buffer. The flow rate was of 30 ml/h and 3 ml fractions were collected. Fractions obtained were analyzed by SDS-PAGE. The column was previously calibrated under the same conditions using a mixture of commercial bovine serum albumin, β-lactoglobulin, and α-lactalbumin.

Characterization of apo-α-lactalbumin by fluorescence spectroscopy. Fluorescence spectra of holo- and apo-α-lactalbumin solutions were recorded in the ratio mode at 25°C with a Varian Cary-Eclipse spectrofluorimeter using a quartz cuvette with 1 cm excitation path length. Intrinsic tryptophan fluorescence was recorded between 300 and 400 nm (excitation at 290 nm). The excitation and emission bandwidths were set to 5 nm. Protein concentration was 10 μM in 10 mM Tris-HCl buffer, pH 8.5.

Study of fatty acids bound to α-lactalbumin by gas–liquid chromatography. Lipids bound to human α-lactalbumin (15 mg of protein) were extracted with chloroform–methanol (2 : 1 v/v) and *trans*-esterified with H_2SO_4 (1%) in anhydrous methanol for 2 h at 80°C as described by Perez et al. [23]. Fatty acid methyl esters were extracted with heptane and analyzed by gas–liquid chromatography in an HP-FFAP column (30 m × 0.53 mm) using *n*-heptadecanoic acid as an internal standard. The column temperature was programmed at 180°C for 20 min, then from 180 to 200°C at 10°C/min, and finally at 200°C for 9 min. Chromatographic peaks were identified using appropriate standards.

Interaction of α-lactalbumin with fatty acids by partition equilibrium. Binding constants and the number of binding sites for the interaction between α-lactalbumin and fatty acids were determined by a partition equilibrium method [24]. A volume of 600 μl of protein solution (175 μM) in 10 mM Tris-HCl buffer, pH 8.5, was incubated overnight with 600 μl of solution of labeled fatty acids (2-300 μM) in heptane with end-over-end agitation at 37°C. After centrifugation, radioactivity was determined in 200 μl samples of organic and aqueous phases by liquid scintillation counting. The partition ratio of free fatty acids between heptane and protein-free buffered salt solution was determined under the same conditions. Then, the concentration of bound fatty acid in each aqueous phase of protein samples was calculated as the difference between the total concentration and the unbound concentration of fatty acid in each aqueous phase. The binding parameters were calculated using the method of Scatchard [25].

RESULTS

The holo- and apo-forms of human α-lactalbumin were characterized by size-exclusion chromatography, SDS-PAGE, and fluorescence spectroscopy. The Sephadex G-50 chromatography profile of holo-α-lactalbumin showed a symmetric peak with an elution volume as expected from its molecular mass. However, the profile of the apo-form resulted in two peaks. The major peak, which accounted for about 87% of total protein, corresponded to the elution volume of holo-α-lactalbumin. The minor peak was eluted ahead of the major peak indicating the formation of protein aggregates (Fig. 1). The analysis of chromatographic fractions of apo-α-lactalbumin by SDS-PAGE showed that the minor peak contained electrophoretic bands mainly between 20 and 35 kD and the major peak contained α-lactalbumin of the molecular mass of 14 kD (Fig. 2).

The holo- and apo-forms of human α-lactalbumin were also characterized by fluorescence spectroscopy (Fig. 3). The fluorescence intensity of the spectrum of holo-α-lactalbumin reaches its maximum at 325 nm. The removal of calcium bound to the protein caused an increase in fluorescence intensity and shifted the wavelength maximum to a significantly higher value (335 nm).

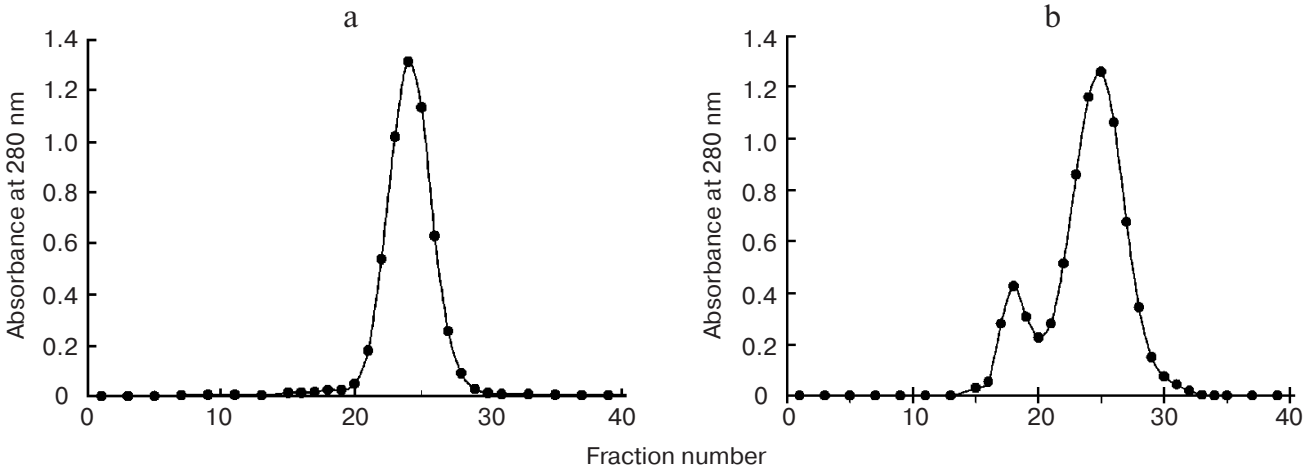

Fig. 1. Chromatographic profile on Sephadex G-50 of human holo- (a) and apo-α-lactalbumin (b).

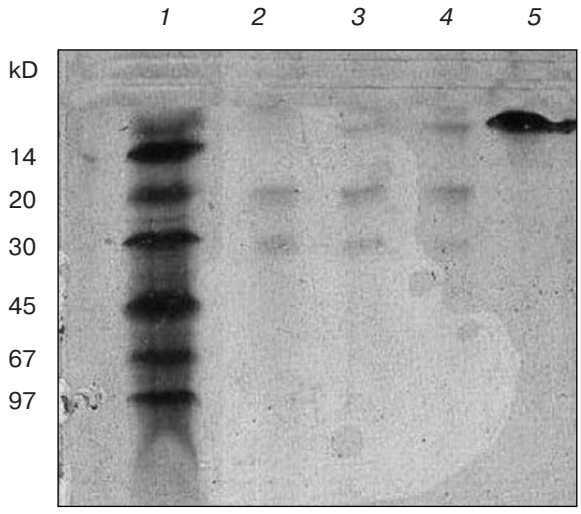

Fig. 2. SDS-PAGE of fractions obtained after Sephadex G-50 chromatography of human apo-α-lactalbumin: *1*) molecular weight markers; *2-4*) fractions 17-19; *5*) fraction 25.

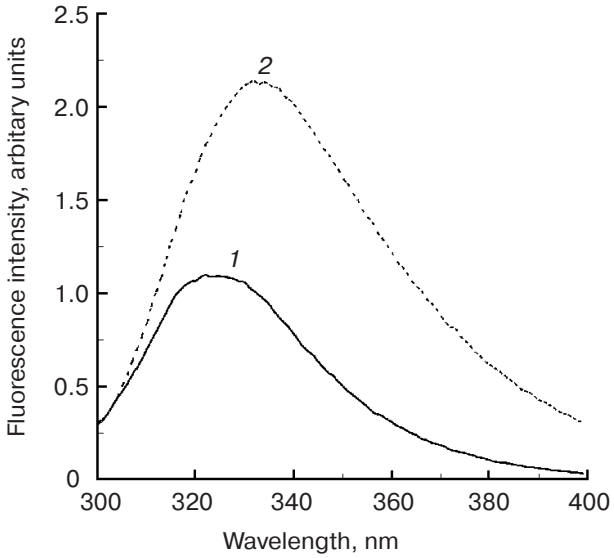

Fig. 3. Fluorescence spectra of human holo- (*1*) and apo-α-lactalbumin (*2*). Spectra were recorded for 10 μM protein solution in 10 mM Tris-HCl buffer, pH 8.5, at 25°C. Fluorescence excitation was at 290 nm.

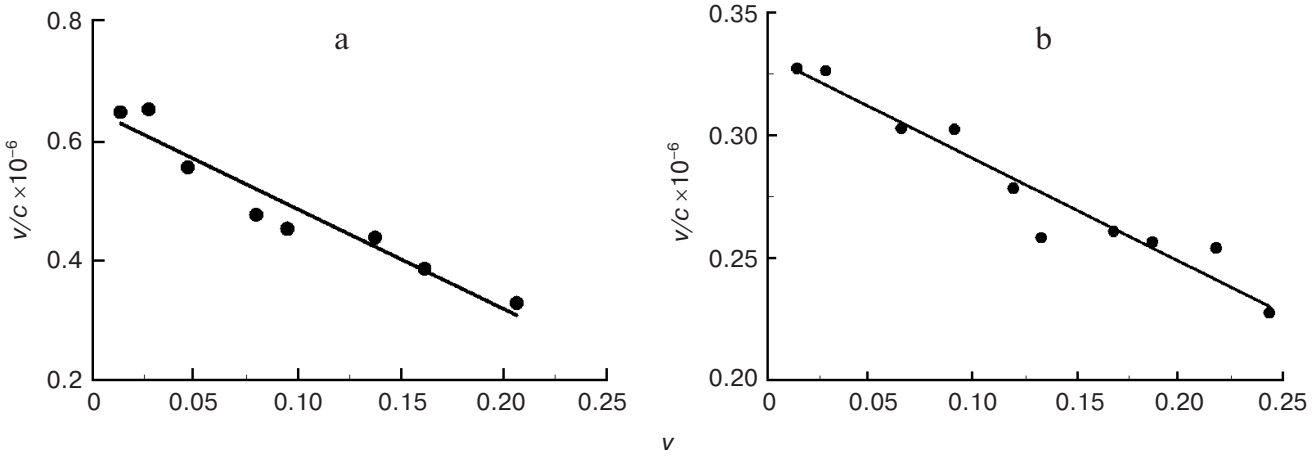

Fig. 4. Scatchard plots for the binding of oleic (a) and palmitic (b) acids to human apo-α-lactalbumin by partition equilibrium: *v* is the concentration of fatty acid bound to the protein per mole of protein, and *c* is the concentration of fatty acid not bound to the protein in the aqueous phase. Each value represents the average of three determinations.

Results obtained using the partition equilibrium method indicate that human holo-α-lactalbumin binds neither palmitic nor oleic acid. Likewise, no fatty acids bound to human α-lactalbumin, isolated using nondenaturing conditions, were detected by gas–liquid chromatography (results not shown). In contrast, the calcium-free form of the protein showed the ability to interact with both fatty acids studied. The Scatchard plots obtained for the interaction of apo-α-lactalbumin with oleic and palmitic acids are shown in Fig. 4. Human apo-α-lactalbumin displays about 0.4 and 0.8 binding sites with apparent affinity binding constants of $1.9 \cdot 10^6$ and $4.2 \cdot 10^5$ M^{-1} for oleic and palmitic acids, respectively. The apparent stoichiometry lower than one indicates that no more than one molecule of ligand is bound per α-lactalbumin molecule.

DISCUSSION

In this work, we found that the emission maximum of the holo-form is blue-shifted by about 10 nm with respect to that of the apo-form. This result indicates that tryptophan residues are more solvent exposed in the apo-form and therefore, reflect a conformational change in the protein caused by the removal of the calcium bound to the protein [21]. These results agree well with earlier studies in which a wavelength shift of 11 nm was reported for human apo-α-lactalbumin [11].

As can be observed by size-exclusion chromatography and SDS-PAGE, the removal of calcium bound to α-lactalbumin also induces the formation of protein aggregates of 20 and 35 kD, which represent about 13% of the total protein. This fact has been attributed to the conformational change produced in the protein that exposes hydrophobic side chains of amino acids increasing its surface hydrophobicity and leading to oligomerization due to hydrophobic interactions [2]. Using mass spectrometry, Yang et al. [22] indicated that a small amount of dimer coexists with monomer in bovine apo-α-lactalbumin solutions at neutral pH.

The interaction of α-lactalbumin with fatty acids has been studied using the partition equilibrium method. This method has been widely used in the study of fatty acid binding with other proteins [24, 26]. The heptane used does not denature the protein and avoids the interference produced by the formation of fatty acid micelles in the aqueous phase [26]. Therefore, differences in the concentration of fatty acids in the aqueous phase in the presence and in the absence of protein correspond to the concentration of fatty acid bound to the protein.

We found that holo-α-lactalbumin, isolated from milk, neither had fatty acids bound nor had the ability to bind them *in vitro*. These results are in a good agreement with those we previously obtained for the holo-form of bovine α-lactalbumin [21]. However, our results are different from those reported by Cawthern et al. [20] because they observed that bovine holo-α-lactalbumin could interact with stearic acid, although with very low affinity. The lack of interaction between human and bovine α-lactalbumin and fatty acids has also been indicated using size-exclusion chromatography and autoradiography of whey incubated with radio-labeled fatty acids [23]. In that work the only protein with fatty acid binding capacity in human whey was serum albumin and in bovine whey, β-lactoglobulin and serum albumin.

In contrast, the apo-form of human α-lactalbumin showed the ability to interact with fatty acids, the apparent affinity binding constants being $1.9 \cdot 10^6$ and $4.2 \cdot 10^5$ M^{-1} for oleic and palmitic acid, respectively. These results are similar to those we obtained with bovine α-lactalbumin, which has also one binding site for fatty

acids with association constants of $4.6 \cdot 10^6$ and $5.4 \cdot 10^5$ M^{-1} for oleic and palmitic acid, respectively [21]. Similarly, using intrinsic protein fluorescence, a high affinity binding site was reported for the binding of stearic acid to bovine apo-α-lactalbumin with a dissociation constant of $2.3 \cdot 10^{-6}$ M [20].

Svensson et al. [16] studied the interaction of human apo-α-lactalbumin obtained by treatment with EDTA with fatty acids differing in the degree of saturation, the carbon chain length, and the *cis/trans*-conformation. The protein was applied to ion-exchange matrixes conditioned with the individual fatty acids and complexes eluted with 1 M NaCl. They found that unsaturated C18 fatty acids in the *cis*-conformation formed stable complexes with apo-α-lactalbumin with a high yield, oleic and vaccenic acids being the most effective. Unsaturated *cis*-fatty acids with shorter or longer carbon chain formed stable complexes, but with yields lower than that of oleic acid. However, when using columns conditioned with saturated fatty acids such as stearic or palmitic acids, the protein was not retained in the column, indicating lack of interaction. These findings demonstrated that apo-α-lactalbumin interacts in a very stereo-specific manner with fatty acids in conditioned matrices [17, 18]. This high stereo-specificity was not observed in our work and by others when the protein was incubated with fatty acids as high affinity-binding constants were obtained for both saturated and unsaturated *cis*-fatty acids [20, 21].

The binding stoichiometry of complexes obtained after elution of human α-lactalbumin from a column conditioned with oleic acid was determined by gas chromatography/mass spectrometry and found to be between 0.6 and 1.3. These results are similar to those observed in our work and those reported for the apo-form of bovine α-lactalbumin [20, 21].

The tentative location of fatty acid-binding sites in α-lactalbumin has been identified in the three-dimensional structure of the protein [16-18]. The fatty acid could be located in the interface between the α-helical and the β-sheet domains. This region, which is conserved in human and bovine α-lactalbumins, undergoes a significant structural change upon calcium release from the protein [27, 28]. In addition, this hydrophobic pocket is capped by basic residues, which might coordinate the polar head groups of the fatty acid, thus orientating the lipid [17]. Thus, it is likely that interaction of apo-α-lactalbumin with fatty acids involved both hydrophobic interactions with the lipid tail and electrostatic interactions with the negatively charged head group of fatty acid as has been also indicated for other fatty-acid binding proteins [29].

It has been speculated that the interaction of apo-α-lactalbumin with fatty acids could have some biological implications. The conditions existing in the gastrointestinal tract could provide an environment fulfilling the requirements necessary for the conversion of α-lactalbumin into the active apoptotic form. The low pH of the stomach is known to cause partial unfolding of α-lactalbumin by calcium removal, and lipids are hydrolyzed there by acid lipases releasing oleic acid. Therefore, it has been suggested that the active form of α-lactalbumin may reduce the pool of potentially malignant cells in the gut, inducing their apoptosis [15, 16].

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