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Molecular Characterization of α–Lactalbumin Folding Variants That Induce Apoptosis in Tumor Cells*

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This study characterized a protein complex in human milk that induces apoptosis in tumor cells but spares healthy cells. The active fraction was purified from casein by anion exchange chromatography. Unlike other casein components the active fraction was retained by the ion exchanger and eluted after a high salt gradient. The active fraction showed N-terminal amino acid sequence identity with human milk α-lactalbumin and mass spectrometry ruled out post-translational modifications. Size exclusion chromatography resolved monomers and oligomers of α-lactalbumin that were characterized using UV absorbance, fluorescence, and circular dichroism spectroscopy. The high molecular weight oligomers were kinetically stable against dissociation into monomers and were found to have an essentially retained secondary structure but a less well organized tertiary structure. Comparison with native monomeric and molten globule α-lactalbumin showed that the active fraction contains oligomers of α-lactalbumin that have undergone a conformational switch toward a molten globule-like state. Oligomerization appears to conserve α-lactalbumin in a state with molten globule-like properties at physiological conditions. The results suggest differences in biological properties between folding variants of α-lactalbumin.

Human milk provides the newborn child with exquisite nutrition, and a mucosal immune system. Breastfeeding protects against respiratory and gastrointestinal infections, due to the presence in milk of molecules with anti-microbial activity: antibodies, potentially bactericidal molecules like lysozyme and lactoferrin (1), fatty acids that lyse bacteria and viral particles (2, 3), and glycoconjugates that inhibit bacterial adherence to epithelial cells (4, 5). Epidemiological studies have shown that breastfeeding protects also against cancer (6), suggesting that milk contains molecules with anti-tumor activity. We recently observed that a protein fraction of human milk induces apoptosis in tumor cells but not in mature healthy cells (7). Surprisingly, the main protein constituent of this fraction was α-lactalbumin.

Monomeric α-lactalbumin is secreted by the mammary epithelium and is the major whey protein of human milk (8). Its main known function is to change the acceptor specificity of β-galactosyltransferase from GlcNAc to Glc, thus enabling the synthesis of lactose in milk (9, 10). The crystal structure of α-lactalbumin has been solved (11) (Fig. 1). It is a metalloprotein with high affinity for Ca^{2+} and other divalent cations (12, 13), and Ca^{2+} is essential for the folding and structural stability of α-lactalbumin (14, 15). At low pH, α-lactalbumin forms a relatively stable protein folding variant (16). This form, the molten globule, has native-like secondary structure but less well defined tertiary structure, and larger stokes radius (17). Similar states are formed at elevated temperatures, by reduction of disulfide bonds or by removal of calcium at neutral pH (18–20).

Unlike monomeric α-lactalbumin from whey, the apoptosis-inducing component was purified from the casein fraction (precipitated at pH 4.3), and behaved as a multimeric protein rather than a monomer. Furthermore, native monomeric α-lactalbumin from human milk whey was inactive in the apoptosis assay. These observations suggested that the active fraction contains an alternative molecular form of α-lactalbumin. Here we show, using mass spectrometry, gel filtration, UV absorption, fluorescence, and CD spectroscopy, that the apoptosis-inducing form of α-lactalbumin is a mixture of monomeric and multimeric forms with molten globule-like properties and suggest that folding variants of α-lactalbumin differ in biologic activity.

MATERIALS AND METHODS

Chemicals—ANS[1] ammonium salt was from Fluka, Buchs, Switzerland. Ammonium sulfate, Tris, calcium chloride, HCl, sodium chloride, methanol, acetic acid, glycine, sodium barbitone, acetonitrile, trifluoroacetic acid, sinapinic acid, and potassium phosphate were from Merck, Darmstadt, Germany. EDTA, SDS, bromphenol blue, and glycerol were from Sigma. Potassium oxalate was from Riedel-de Haen, Seelze, Germany. Agarose (Sea Kem GTG) was from Bioproducts, Rockland, MI. PAGE ready gels were from Bio-Rad. RPMI 1640 cell culture media, fetal calf serum, nonessential amino acids, sodium pyruvate. and gentamicin were from Life Technologies, Paisley, United Kingdom. The Biotin Labeling kit was from Boehringer Mannheim, GmbH, Germany. All chemicals were of the highest grade commercially available.

α-Lactalbumin—Native, monomeric α-lactalbumin was purified from human milk by ammonium sulfate precipitation. The ammonium sul-

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[1] The abbreviations used are: ANS, 8-anilinonaphtalene-1-sulfonic acid; PAGE, polyacrylamide gel electrophoresis; FPLC; fast protein liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF, matrix assisted laser desorbtion ionization-time of flight mass spectrometry; PBS, phosphate-buffered saline.

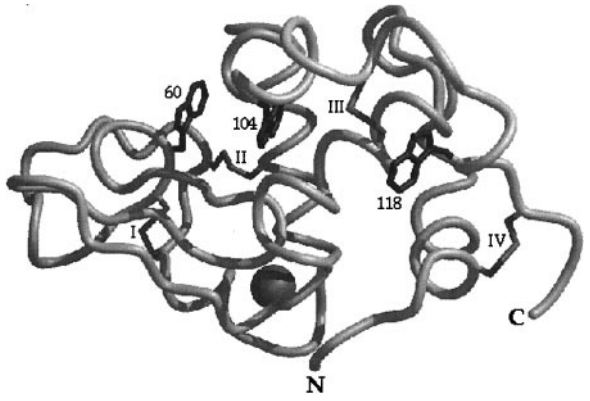

FIG. 1. **Three-dimensional structure of native human α-lactalbumin.** The backbone, the three tryptophan side chains, and the tightly bound Ca^{2+} ion are shown. The disulfide bonds are indicated with *roman numerals* as follows: *I,* 61–77; *II,* 73–91; *III,* 28–111; *IV,* 6–120. This figure was generated using UCSF software MidasPlus (37). α-lactalbumin is a 14-kDa protein with four α-helices formed by residues 1–37 and 85–123. The triple-stranded β-sheet is found at one side of the protein (residue 38–84) and unfolds more easily than the α-helical region (38). A high affinity calcium-binding site is formed by the carboxylates of Asp-82, Asp-87, and Asp-88, the carbonyl oxygens of Lys-79 and Asp-84, and two water molecules (11).

fate was added as a salt, 264 g/liter milk, and the mixture was incubated overnight at 4 °C. The mixture was centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont Instruments, Wilmington, DE) at 5000 × *g* for 15 min. The whey fraction was collected, lyophilized, and dissolved in 50 mM Tris/HCl with 35 mM EDTA, pH 7.5. A 400-ml phenyl-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) was packed in 50 mM Tris/HCl with 1 mM EDTA, pH 7.5, 25 °C and a 500-ml sample was loaded onto the column. The column was first washed with 50 mM Tris/HCl with 1 mM EDTA, pH 7.5, and α-lactalbumin was then eluted from the column with 50 mM Tris/HCl with 1 mM $CaCl_2$, pH 7.5, thus yielding the native, Ca^{2+} bound form of α-lactalbumin.

The classical molten globule state was obtained by lowering the pH of a solution of native monomeric α-lactalbumin to 2.0 by adding 0.1 M HCl. This material was used as a control sample in subsequent spectroscopic studies. In the cellular experiments, EDTA (0.14 mM/mg) was added to α-lactalbumin to remove Ca^{2+} and thus form the apo state, which is molten globule-like.

Casein Precipitation—Frozen human milk was thawed and centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont Instruments) at 2500 × *g* for 15 min; the upper fat layer was removed. Casein was isolated by an overnight incubation at 4 °C with 10% potassium oxalate followed by a second overnight incubation at 4 °C after lowering the pH to 4.3 using 1 M hydrochloric acid and heating the solution to 32 °C for 2 h. The casein precipitate was harvested by centrifugation at 5000 × *g* for 15 min, washed by 3–5 cycles of centrifugation, and resuspension in distilled water and lyophilized.

Anion Exchange Chromatography—Casein was fractionated on DEAE-Trisacryl M (Biosepra, Villeneuve la Garenne, France) using an FPLC instrument (Pharmacia Biotech Inc.) with increasing NaCl gradient. The sample was loaded in buffer A (0.01 M Tris-HCl, pH 8.5) at 25 °C and eluted by increasing proportions of buffer B (buffer A containing 1 M NaCl). Gradient program: start 15% B; from 0 to 60 ml: linear gradient from 60 to 90 ml: 30% B at 90 ml: 100% B; for 10 min; thereafter 100% A. Flow rate: 1 ml/min, recorder: 0.1 cm/min. The buffers were degassed and filtered through 0.22-μm filters before use. The peaks were monitored at 280 nm, and the fraction size was 3 ml. The eluate was desalted by dialysis (Spectra/Por, Spectrum Medical Industries, Laguna Hills, CA, membrane cut off 3.5 kDa) against distilled water for at least 48 h and lyophilized.

Size Exclusion Chromatography—The active fraction from anion exchange chromatography was subjected to size exclusion separation on Sephadex G-50 column (Pharmacia Biotech, Uppsala, Sweden, 93 × 2.5 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0, 25 °C. The flow rate was 0.5 ml/min, peaks were monitored at 280 nm and 3-ml fractions were collected and pooled. The pools were desalted by dialysis (membrane cut off 3.5 kDa) against distilled water for at least 48 h and lyophilized before further analysis in subsequent experiments.

Further gel filtration of the active fraction from the anion exchange chromatography was performed on a Superose 12 column (Pharmacia Biotech, 30 × 1.0 cm) in 10 mM Tris/HCl, pH 7.5, 25 °C with 0.15 M NaCl. The flow rate was 0.3 ml/min, the fraction size was 0.5 ml and peaks were monitored at 280 nm. Observed peaks were collected, desalted by dialysis against distilled water, and lyophilized.

PAGE—Analytical PAGE was performed using 4–20% polyacrylamide precast gels on a Bio-Rad Mini Protean II cell. To 10 μl of the lyophilized fractions from anion exchange chromatography or gel filtrations dissolved in distilled water (5–10 mg/ml), an equal volume of sample buffer (13.1% 0.5 M Tris-HCl, pH 6.8, 10.5% glycerol, and 0.05% bromphenol blue) was added. Samples (20 μl) were then loaded onto the gel, which was run in Tris glycine buffer, pH 8.3, with 0.1% SDS at 200 V constant voltage for 40 min. Proteins were stained by immersing the gel in 0.1% Coomassie Blue solution in water/methanol/acetic acid (5:4:1) for 0.5 h. Destaining was by several changes in 40% methanol, 10% acetic acid until a clear background was obtained.

N-terminal Amino Acid Analysis—After PAGE, the protein bands of peak K from the G-50 column were transferred by Western blotting onto polyvinylidene difluoride membranes. The protein bands were visualized by Coomassie Blue staining and the stained bands were cut out for protein sequencing. Protein sequencing was also performed on an aliquot of each of the peaks 1–4 from the Superose-12 column directly. All samples were subjected to Edman degradation performed in an automated pulse-liquid sequencer (Applied Biosystems model 477A).

ESI-MS—Peak K was analyzed on a VG Bio-Q ESI-MS (Fisons/VG, Manchester, UK) equipped with an atmospheric pressure electrospray ion source and a quadruple mass analyzer with a maximum mass range of 4000. The mass spectrometer was scanned from m/z 600 to 2000 in 10 s. The mass resolution was set to 500. The data system was operated as a multichannel analyzer and 5 scans were averaged to obtain the final spectrum. The electrospray carrier solvent was 1% acetic acid in acetonitrile/water, 1:1, and the flow rate was 2–4 μl/min. The sample was dissolved at a concentration of 10–20 pmol/μl in the carrier solvent and 5 μl was injected. The molecular weight of sample components was estimated from the m/z values of series of ions as described earlier.

MALDI-TOF—Peak K was analyzed by MALDI mass spectrometry on an LDI 1700 time of flight mass spectrometer equipped with a pulsed nitrogen laser (337 nm) (Biomolecular Separations Inc., Reno, NE). The laser power was set to 8.6 microjoule and the spectrum was the sum of 140 laser shots. Sinapinic acid was used as a matrix and bovine serum albumin was used as the external standard. About 100 μg of the protein was dissolved in 50 μl of water and 0.1% trifluoroacetic acid. 10 μl of this solution was mixed with 10 μl of 50 mM sinapinic acid. The probe was loaded with 0.8 μl of the sample mixture, vacuum dried, loaded with another 0.8 μl of sample, and vacuum dried again before being inserted into the mass spectrometer.

Spectroscopic Analysis—Prior to spectroscopic analysis, the proteins or protein fractions were dialyzed against doubly distilled water and lyophilized. Stock solutions of each sample were prepared by dissolving the lyophilized material in 10 mM potassium phosphate buffer at pH 7.5. The concentrations of the stock solutions were determined using amino acid analysis after acid hydrolysis. The spectra were recorded on solutions prepared by diluting aliquots of stock solution into 10 mM potassium phosphate buffer at pH 7.5. All spectra were recorded at 25 °C.

UV Absorbance Spectroscopy—UV absorbance spectra were recorded at room temperature on a GBC UV/VIS 920 spectrophotometer, in a quartz cuvette with 1-cm path length.

Fluorescence Spectroscopy—Fluorescence spectra were recorded at 25 °C on a Perkin-Elmer LS-50B spectrometer using a quartz cuvette with 1-cm excitation path length. Intrinsic (tryptophan) fluorescence emission spectra were recorded between 305 and 530 nm (step 1 nm) with excitation at 295 nm. The excitation band width was 3 nm and the emission band width was 5 nm. ANS fluorescence emission spectra were recorded between 400 and 600 nm (step 1 nm) with excitation at 385 nm. Both the excitation and emission bandpass were set to 5 nm.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were obtained using a JASCO J-720 spectropolarimeter with a JASCO PTC-343 Peltier-type thermostated cell holder. Quartz cuvettes were used with 1-cm path length in the near UV range and 1 and 0.1-mm path length in the far UV range. Near UV spectra were recorded between 320 and 240 nm, and far UV spectra between 250 and 182 nm. The wavelength step was 1 nm, the response time was 4 s, and the scan rate was 10 nm/min. Six scans were recorded and averaged for each spectrum. Baseline spectra were recorded with pure buffer in each cuvette and subtracted from the protein spectra. The mean residue ellipticity θ_m (mdeg × cm^2 × $dmol^{-1}$) was calculated from the recorded ellipticity, θ, as $\theta_m = \theta/(c{\cdot}n{\cdot}l)$, where c is the protein concentration in M,

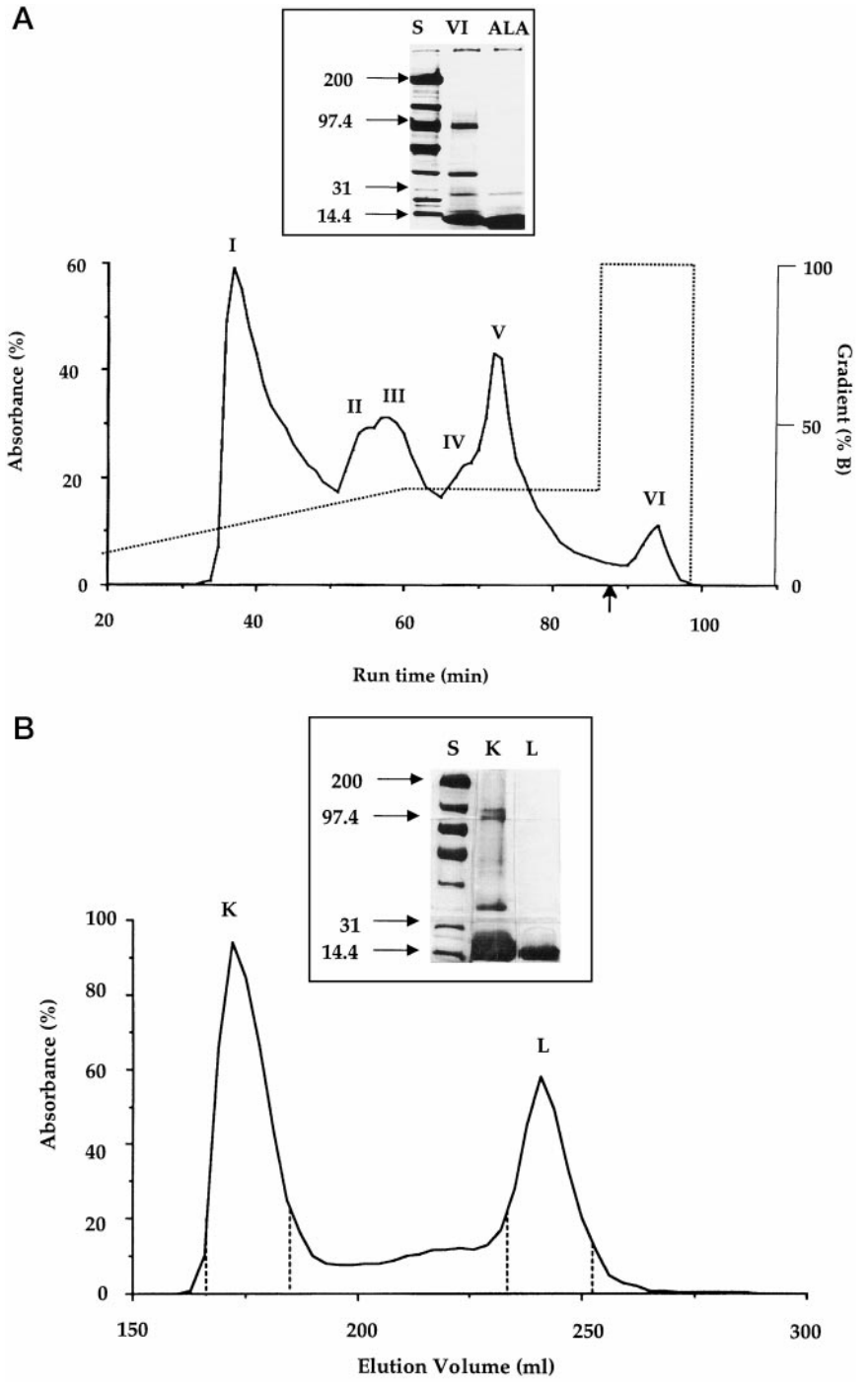

FIG. 2. **Anion exchange chromatography and gel filtration.** *Panel A,* ion-exchange chromatogram of human casein. 30 mg of casein was fractionated using a DEAE-Trisacryl column attached to a FPLC system using a stepwise NaCl gradient, at 25 °C. The apoptosis-inducing fraction eluted after 1 M NaCl indicated by the *arrow*. *Inset,* PAGE of the apoptosis inducing fraction VI and monomeric α-lactalbumin (*ALA*). The molecular weight standards (*lane S*) are: myosin (M_r = 200,000), β-galactosidase (M_r = 116, 250), phosphorylase *b* (M_r = 97, 400), bovine serum albumin (M_r = 66, 200), ovalbumin (M_r = 45,000), carbonic anhydrase (M_r = 31,000), soybean trypsin inhibitor (M_r = 21,500), and lysozyme (M_r = 14,400), respectively. *Panel B,* size exclusion chromatogram of fraction VI from the ion-exchange column. 100 mg was applied to a Sephadex G-50 column (93 × 2.5 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0, 25 °C. The flow rate was 30 ml/h and the fraction size was 3 ml. Two well separated peaks were obtained. *Inset,* PAGE of peaks K and L. The molecular weight standards are as in *panel A*. *Panel C,* size exclusion chromatogram of fraction VI on a Superose 12 column. Fraction VI (10 mg) was suspended in 500 μl of 10 mM potassium phosphate buffer, pH 7.5, 25 °C (flow rate 0.3 ml/min, fraction size 0.5 ml). Peak 1 eluted with the void volume (8 ml), peak 2 eluted at 9.4–11.6 ml corresponding to a molecular mass of 150–400 kDa, peak 3 eluted at 13.8–14.2 ml corresponding to a mass of 30–42 kDa, and peak 4 eluted at 15.0–15.2 ml, corresponding to monomeric α-lactalbumin, 14 kDa. *Inset,* PAGE of peaks 1–4, monomeric α-lactalbumin (*ALA*) and fraction VI. The molecular weight standards (*S*) are as in *panel A*.

n the number of residues in the protein (123 in this case), *l* the path length in m, and θ the ellipticy in degrees.

Tumor Cell Line—The L1210 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA), cultured in 25-cm^2 flasks (Falcon, Becton Dickinson, NJ) in RPMI 1640 supplemented with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, 50 μg of gentamicin/ml, kept at 37 °C in a humidified atmosphere containing 5% CO_2, with change of medium every 3 days. The cells were harvested from the culture flasks by centrifugation (200 × *g* for 10 min). The cell pellet was resuspended in medium and seeded into 24-well plates, 2 × 10^6/well (Falcon, Becton Dickinson, NJ).

Cells were exposed to the different forms of α-lactalbumin, with medium as a control. At time 0, 100 μl of medium was aspirated from each well, replaced by 100 μl of the different experimental solutions and incubated at 37 °C in an atmosphere of 5% CO_2 for 6 h. Cells were harvested from the 24-well plates by aspiration, resuspended in PBS (5 ml), washed, and resuspended in 1 ml of PBS.

Cell Viability—For analysis, 30 μl of the washed cell suspension was mixed with 30 μl of a 0.2% trypan blue solution and the number of stained cells (dead cells) per 100 cells was determined by interference contrast microscopy (Ortolux II, Leitz Wetzlar, Germany).

DNA Fragmentation—Oligonucleosome length DNA fragments were detected by agarose gel electrophoresis. The remainder of the washed cell suspension (970 μl, 2 × 10^6/ml) was lysed in 5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0, at 4 °C for 1 h and centrifuged at 13,000 × *g* for 15 min. DNA was ethanol precipitated overnight in −20 °C, treated with RNase proteinase K, and loaded on 1.8% agarose gels, and electrophoresed with constant voltage set at 50 V overnight. DNA fragments were visualized with ethidium bromide using a 305-nm UV-light source and photographed using Polaroid type 55 positive-negative film.

Intracellular Distribution of Biotinylated Fraction VI—L1210 cell suspension (4 × 10^6 cells/ml, 95 μl) were incubated at room temperature with 5 μl of biotinylated fraction VI or native monomeric α-lactalbumin (5 mg/ml, biotinylated according to the manufacturer's instructions), and then washed in PBS with centrifugation at 320 × *g* for 10

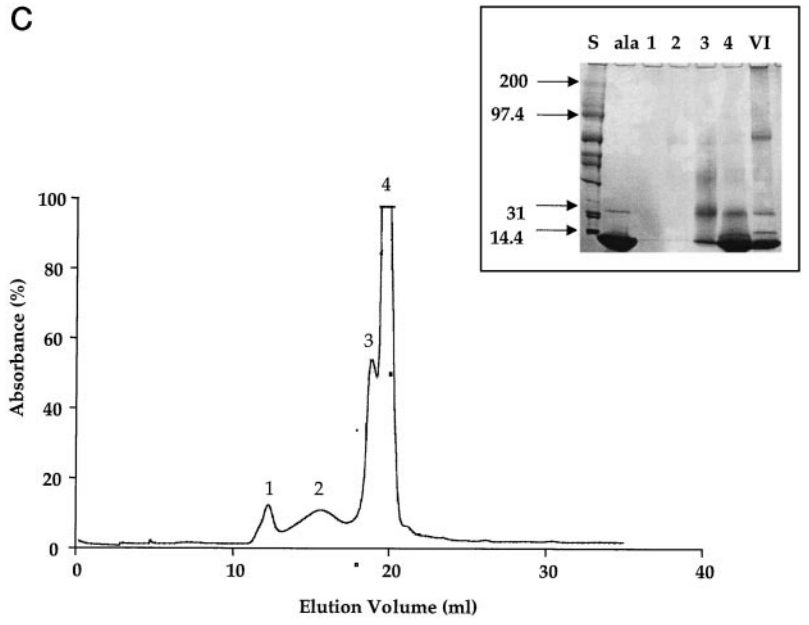

FIG. 2—*continued*

min to remove unbound protein. To detect intracellular protein, cells exposed to biotinylated protein were permeabilized with saponin to allow entry of fluorescein isothiocyanate-conjugated streptavidin. Cells harvested by centrifugation at 320 × *g* were fixed by suspension in phosphate-buffered paraformaldehyde (4%) (21) for 5 min at room temperature, washed in PBS, and permeabilized with 0.1% saponin in PBS. After washing in 0.1% saponin, fluorescein isothiocyanate-conjugated streptavidin (1:100 in 0.1% saponin, 100 μl) was added and the cells were incubated for 30 min at room temperature. The cells were washed twice in PBS/saponin and once in PBS, mounted on a glass slide and analyzed in a Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad, Hemel-Hempstead, UK) attached to a Nikon Diaphot inverted microscope (Nikon, Japan).

RESULTS

Isolation of an Apoptosis-inducing Complex from Human Milk—Human milk samples from four donors were separated into casein and whey and the fractions were tested for apoptosis induction in L1210 leukemia cells. The activity precipitated with casein and further purification was from the casein fraction. Following anion-exchange chromatography, six fractions were collected and tested for apoptosis induction. Fractions I-V completely lacked activity. Fraction VI that eluted with 1 M NaCl contained all of the apoptosis inducing activity (Fig. 3*A*). PAGE of fraction VI revealed one major band in the 14-kDa region, and additional bands in the molecular mass range of 30, 60, and 100 kDa (Fig. 2*A, inset*). After heat treatment (100 °C, 5 min), fraction VI lost its activity (data not shown).

Size Exclusion Chromatography—Fraction VI was applied on a Sephadex G-50 column. Two well separated peaks (*K* and *L*, Fig. 2*B*) were obtained. Peak K eluted near the void volume and contained all of the apoptotic activity, peak L eluted at the position of a 14-kDa protein and had no effect on cell viability. PAGE of peak K showed one major band at about 14 kDa, and additional bands of about 30, 60, and 100 kDa. Peak L gave only one band at 14 kDa (Fig. 2*B, inset*).

N-terminal Amino Acid Sequence Analysis—The different bands of peak K were subjected to N-terminal amino acid sequence analysis (Table I). The first 30 residues of the band at 14 kDa and the first 9 residues of the 30-kDa band were identical to the N-terminal sequence of human α-lactalbumin, except for residue 6, which was not detected. The main N-terminal sequence of the 60-kDa band and the two 100-kDa bands was also identical to human α-lactalbumin, but some sequencing cycles showed heterogeneity. These results suggested that peak K contained α-lactalbumin complexes of increasing molecular size

TABLE I

N-terminal amino acid sequence of the protein bands of peaks K and 1–4 and human α-lactalbumin (HLA)

Unk, indicates unknown; according to published results, residue 6 in α-lactalbumin is cysteine. Residues shown below the 60- and 100-kDa sequence of peak K are other possible candidates.

	Sequences
HLA	
14 kDa	Lys-Gln-Phe-Thr-Lys-Cys-Glu-Leu-Ser-Gln-
	-Leu-Leu-Lys-Asp-Ile-Asp-Gly-Tyr-Gly-Gly-
	-Ile-Alα-Leu-Pro-Pro-Leu-Ile-Asp-Thr-Met-
Peak K	
14 kDa	Lys-Gln-Phe-Thr-Lys-Unk-Glu-Leu-Ser-Gln-
	-Leu-Leu-Lys-Asp-Ile-Asp-Gly-Tyr-Gly-Gly-
	-Ile-Alα-Leu-Pro-Pro-Leu-Ile-Asp-Thr-Met-
30 kDa	Lys-Gln-Phe-Thr-Lys-Unk-Glu-Leu-Ser-Gln-
60 kDa	Lys-Gln-Phe-Leu-Lys-
	Arg Pro Lys Thr Pro
100 kDa	Lys-Gln-Phe-Thr-Unk-Unk-Glu-Leu-Unk-Gln-
	Asn Ile Ser Val
	Tyr Asn
Peak 1	Lys-Gln-Phe-Thr-Lys-Unk
Peak 2	Lys-Gln-Phe-Thr-Lys-Unk
Peak 3	Lys-Gln-Phe-Thr-Lys-Unk
Peak 4	Lys-Gln-Phe-Thr-Lys-Unk

Induction of Apoptosis—The effect on cell viability and induction of DNA fragmentation was compared between the different milk fractions (Fig. 3*A*). A rapid reduction of cell viability from >95% to <10% occurred after exposure of the L1210 cells to casein, fraction VI, and peak K (Fig. 3*A*), and oligonucleosome length DNA fragments were formed in those cells. Whey, casein fractions I-V, and native monomeric α-lactalbumin did not reduce cell viability or induce DNA fragmentation (Fig. 3*A*).

Nuclear Uptake of Fraction VI—The localization of fraction VI in the L1210 cells was examined using biotinylated material in confocal microscopy (Fig. 3*B*). Fraction VI was shown to bind to the cell surface, to enter the cytoplasm and finally to accumulate in the nuclei of the L1210 cells (Fig. 3*B*). Native monomeric α-lactalbumin did not accumulate in the cell nuclei, and only showed surface binding (Fig. 3*B*). The apoprotein did not induce DNA fragmentation in L1210 cells.

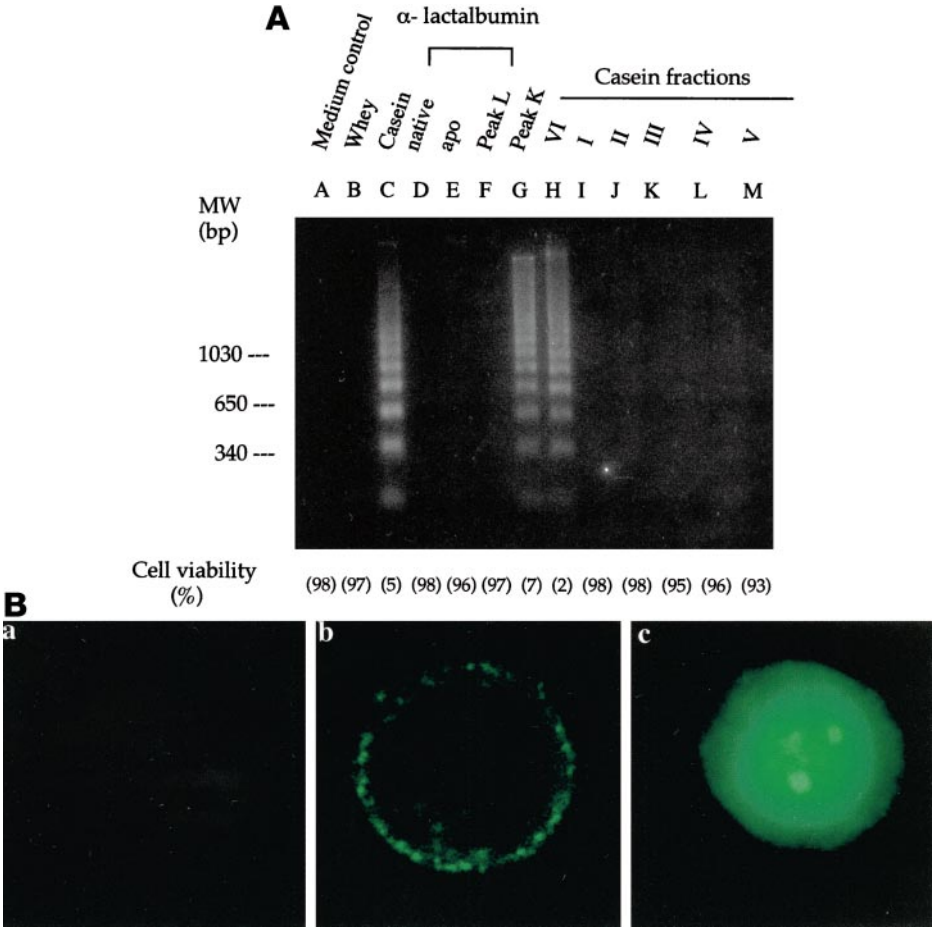

FIG. 3. **Induction of apoptosis and cellular localization.** *Panel A,* the different milk fractions were dissolved in cell culture medium and added to L1210 cells, the viability and induction of DNA fragmentation was measured after 6 h of incubation at 37 °C, 5% CO_2. Cell viability was measured using trypan blue exclusion and oligonucleosome length DNA fragments were detected using gel electrophoresis. The inactive fractions were tested at 5 mg/ml (whey, casein fraction I-V, G-50 peak L, and native and molten globule-like monomeric α-lactalbumin). The active fractions were tested at 0.5 mg/ml (casein, casein fraction VI, and G-50 peak K). The cell viability in % of the control is shown *under* each lane (mean of three separate experiments). *Panel B,* nuclear localization of fraction VI. The localization of fraction VI and native α-lactalbumin in the L1210 cells was examined using biotinylated material in confocal microscopy. Cells were exposed to the biotinylated material for 6 h and stained with fluorescein isothiocyanate-conjugated streptavidin. Fraction VI readily accumulated in the nuclei of L1210 cells (*c*), whereas native α-lactalbumin bound to the surface of the cells but was not located to the nuclei (*b*). No staining was seen with streptavidin alone (*a*).

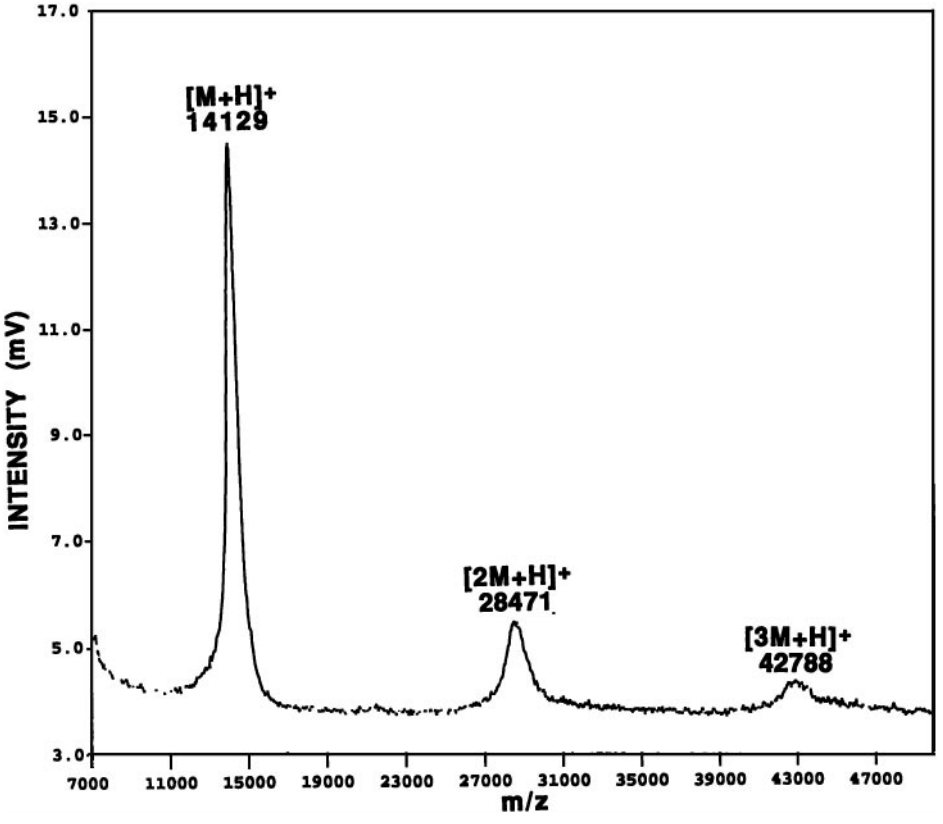

FIG. 4. **Mass spectrometry.** MALDI-MS of peak K. 100 μg of peak K was dissolved in 50 μl of water and 0.1% trifluoroacetic acid. 10 μl of this solution was mixed with 10 μl of 50 mM sinapinic acid and the probe was loaded twice with 0.8 μl before insertion into the mass spectrometer.

Mass Spectrometry—The results above suggested that the active molecular form of α-lactalbumin may have different post-translational modifications and/or different conformation as compared with native α-lactalbumin. Peak K was therefore analyzed by ESI-MS and MALDI-TOF, native α-lactalbumin was used as a control. The estimated molecular mass of the major component (14.088 kDa) was close to the molecular mass of α-lactalbumin calculated from the amino acid sequence (14.078 kDa). The small mass differences ruled out post-translational modifications such as phosphorylation and glycosylation. MALDI-TOF of peak K showed a major peak close to 14 kDa consistent with monomeric α-lactalbumin, but peaks consistent with dimeric and trimeric forms (28 and 42 kDa) were also seen (Fig. 4). These results support the conclusion from ESI-MS of lack of post-translational modifications, and in addition showed the possibility of formation of multimeric forms of α-lactalbumin.

These results suggested that the apoptosis inducing activity was dependent on a high molecular weight complex, containing α-lactalbumin. Mass spectrometry ruled out post-translational modifications which indicated that the difference between native, monomeric α-lactalbumin and the apoptosis inducing complex was conformational.

Size Exclusion Chromatography—To facilitate spectroscopic analysis of the different oligomeric states of α-lactalbumin, fraction VI was applied on a Superose-12 column. Four peaks were obtained (Fig. 2*C*). Peak 1 eluted with the void volume, peak 2 eluted as 150–400 kDa, peak 3 as 30–42 kDa, and peak 4 eluted as a 14-kDa protein, at the same position as the native, native α-lactalbumin control. SDS-PAGE of peak 1 showed a faint band larger than 200 kDa, peak 2 had a faint band at 90 kDa, peak 3 had bands at 14, 30, and 66 kDa, and peak 4 gave one band at 14 kDa and one at 31 kDa (Fig. 2*C, inset*).

The stability of the peaks was investigated by reinjection on the Superose-12 column, after lyophilization and resuspension in 10 mM Tris/HCl, pH 7.5. No dissociation into monomers was detected for the multimers in peaks 1–3. Peak 4 remained a stable monomer (data not shown). N-terminal amino acid sequence analysis of peaks 1–4 from the Superose-12 column showed the presence of α-lactalbumin in all peaks. Peak 4 was >95% pure. Peaks 1, 2, and 3 showed a higher background (steps 1–10), but no dominating second sequence suggestive of another protein (Table I).

Spectroscopic Characterization—Fraction VI and peaks 1–4 from the Superose-12 column were analyzed by spectroscopic techniques. Monomeric α-lactalbumin in the native or the acid-induced molten globule state were included as controls.

By UV absorption spectroscopy α-lactalbumin and fraction VI showed virtually identical spectra (Fig. 5*A*), except for the elevated background absorbance for fraction VI indicating that the complex scattered light more than native α-lactalbumin. Peaks 1, 2, 3, and 4 strongly resembled native, monomeric α-lactalbumin, but with elevated backgrounds. Peak 1 had the highest background absorbance as expected for very large complexes (Fig. 5*B*).

Tryptophan fluorescence spectra were recorded to compare the degree of folding between the molecular forms of α-lactalbumin. Native human α-lactalbumin had its intensity maximum at 335 nm and a shoulder at 320 nm, indicative of tryptophan residues in a folded hydrophobic core. The pH 2 molten globule and fraction VI had an intensity maximum at 340 nm and a shoulder at 355 nm, indicating that the tryptophans in fraction VI and the pH 2 molten globule are more accessible to the solvent compared with native α-lactalbumin (Fig. 6, *A* and *B*). Peaks 1, 2, and 3 showed the same intensity maxima as fraction VI and the pH 2 molten globule α-lactalbumin, but the intensity at 355 nm was almost as high as at 340 nm. Peak 4 strongly resembled native α-lactalbumin but with an additional weak shoulder at 355 nm. (Fig. 6*B*). The results indicate that tryptophan residues are shielded from solvent in the monomer and in peak 4, but are more solvent exposed in the pH 2 molten globule, in fraction VI and in peaks 1–3.

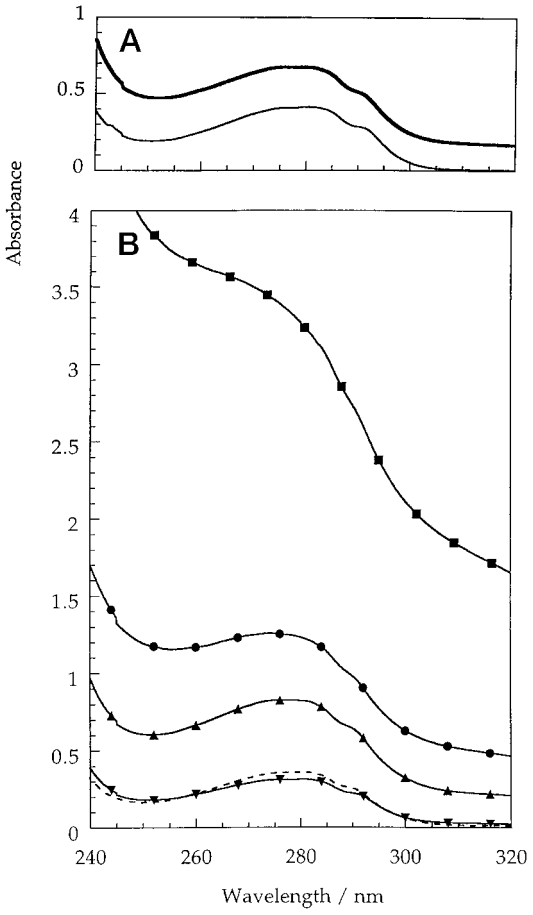

FIG. 5. **UV spectra.** UV absorbance spectra were recorded at 25 °C on a GBC UV/VIS 920 spectrophotometer, in a quartz cuvette with 1-cm path length. *Panel A* shows fraction VI (*solid black line*) and native, monomeric α-lactalbumin (*thin black line*). *Panel B* shows peaks 1–4 (peak 1, ■; peak 2, ●; peak 3, ▲; peak 4, ▼) and pH 2 molten globule α-lactalbumin (*dashed line*). All spectra were recorded for 1 mg/ml protein solutions, corresponding to 70 μM monomer.

ANS was used as a probe of solvent accessible hydrophobic surfaces. ANS was titrated into each sample in steps of 0.25 equivalents, relative to the monomer concentration. The spectra at 1.5 equivalents of ANS are shown (Fig. 7, *A* and *B*). Native monomeric α-lactalbumin did not bind ANS as shown by the low intensity of the spectrum with a maximum at 515 nm strongly resembling the spectrum of ANS added to pure buffer (Fig. 7*A*). The pH 2 molten globule α-lactalbumin showed the most significant ANS binding with the maximum at 475 nm and significantly enhanced intensity (Fig. 7*B*). The fraction VI spectrum was blue-shifted compared with native α-lactalbumin with the intensity maximum at 475 nm and increased quantum yield, indicating that ANS binds to fraction VI (Fig. 7*A*). The ANS fluorescence spectra of peaks 1–3 showed intensity maxima at 475 nm, and for peaks 2 and 3 a strongly enhanced quantum yield, indicating significant ANS binding. Peak 4 was virtually identical to native α-lactalbumin with low intensity and a non-shifted maximum at 515 nm (Fig. 7*B*). The results indicate exposed hydrophobic surfaces in the pH 2 molten globule, fraction VI and peaks 1–3, but not in peak 4 or the native monomer.

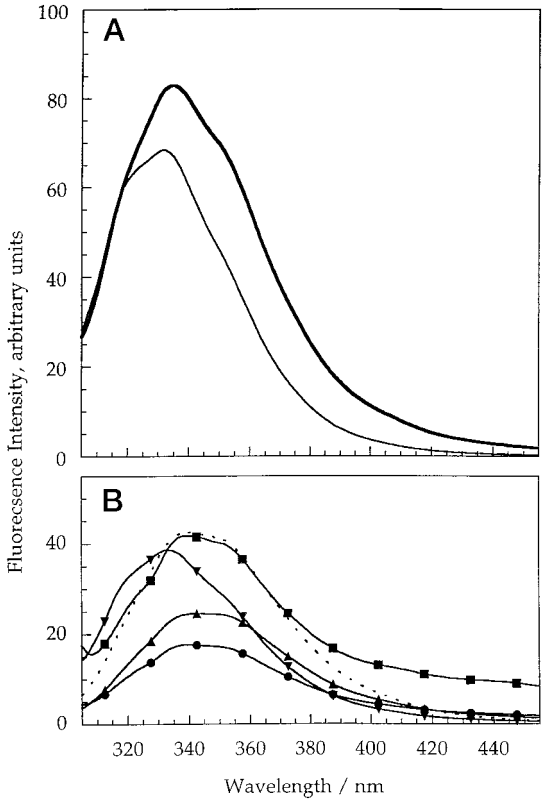

FIG. 6. **Intrinsic fluorescence spectra.** *Panel A,* fluorescence spectra for fraction VI (*solid line*) and native, monomeric α-lactalbumin (*thin line*). *Panel B,* fluorescence spectra for peaks 1–4 (peak 1, ■; peak 2, ●; peak 3, ▲; peak 4, ▼) and pH 2 molten globule α-lactalbumin (*dashed line*). All spectra were recorded for 0.3 mg/ml protein solutions, corresponding to 20 μM monomer, in 10 mM potassium phosphate buffer at pH 7.5, 25 °C.

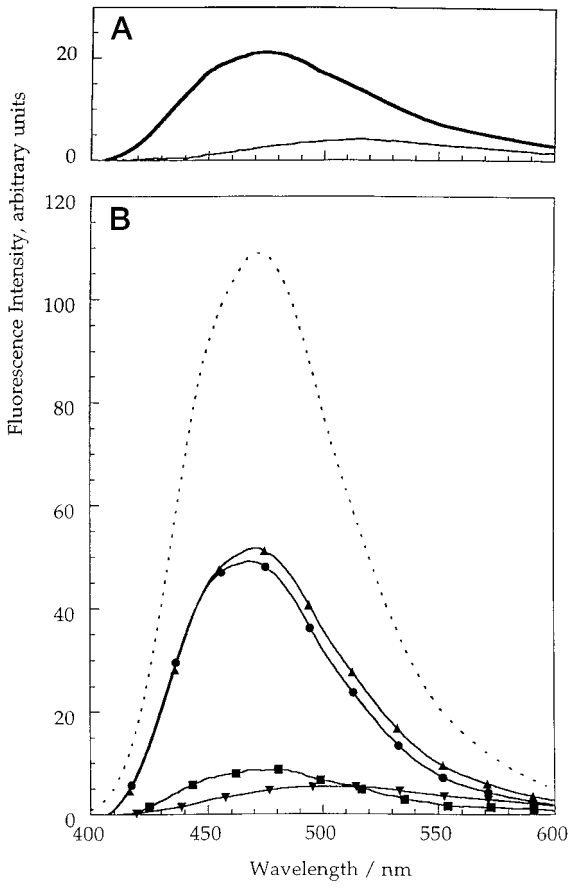

FIG. 7. **ANS fluorescence spectra.** *Panel A,* ANS titration of fraction VI (*solid line*) and native, monomeric α-lactalbumin (*thin line*). *Panel B,* ANS titration of peaks 1–4 (peak 1, ■; peak 2, ●; peak 3, ▲, peak 4, ▼) and pH 2 molten globule α-lactalbumin (*dashed line*). The figure shows spectra obtained with 1.5 μM ANS. The protein concentration was 0.3 mg/ml, corresponding to 20 μM monomer, in 10 mM potassium phosphate buffer at pH 7.5, 25 °C.

The secondary structure content in the different samples was compared by far UV CD spectroscopy. Native α-lactalbumin showed a double dip at 208 and 228 nm. The spectrum is broader than for many other proteins composed of mixed α-helices (double dip at 208 and 222 nm) and β-sheets (single dip at 215 nm). This may be due to influences from aromatic groups at the higher wavelengths. The far UV CD spectra did not indicate any major differences in secondary structure between native α-lactalbumin, the pH 2 molten globule, fraction VI, and peaks 1–4, although the analysis becomes uncertain for peaks 1 and 2 due to the higher background observed in the amino acid analysis (Fig. 8, *A* and *B*). An analysis of the spectra using the Selcon program (22) gave no differences in secondary structure beyond the error limits when comparing any of the samples studied.

Near UV CD spectroscopy was used to study rigidity *versus* flexibility of aromatic side chains. Native α-lactalbumin had a minimum at 270 nm arising from tyrosine residues and a maximum at 294 nm arising from tryptophan residues. The near UV CD spectrum of the pH 2 molten globule showed the characteristic loss of signal, indicating less restrained tyrosines and tryptophans. Fraction VI had a spectrum similar to native α-lactalbumin but with less signal, indicating that the motion of tyrosines and tryptophans is less restrained (Fig. 9*A*). Peaks 1, 2, and 3 showed similar spectra as the pH 2 molten globule, but peak 4 was almost identical to native α-lactalbumin (Fig. 9*B*). The results point to a higher degree of mobility of aromatic residues in fraction VI and peaks 1–3 as compared with peak 4 and native α-lactalbumin. The results also confirm the presence of α-lactalbumin in all samples.

DISCUSSION

α-Lactalbumin is present in the milk of all mammals. Pig, sheep, and goat milk contain multiple forms of α-lactalbumin, that vary in amino acid sequence (23), but only one molecular form of human α-lactalbumin has been described. This 14-kDa protein is a major constituent of human milk whey, and occurs at concentrations around 2 mg/ml (8). The present study showed that α-lactalbumin complexes of larger molecular size could be purified from human milk casein. The complexes were characterized as α-lactalbumin multimers that induces apoptosis in tumor cells. The apoptosis-inducing form was isolated from casein by ion exchange chromatography. N-terminal amino acid sequencing showed identity with α-lactalbumin and gel filtration demonstrated the presence of multimers that were kinetically stable toward dissociation. Mass spectrometry excluded post-translational modifications such as glycosylation and phosphorylation indicating that activity was associated with changes in secondary or tertiary structure. Spectroscopic analyses showed that the multimers in the apoptosis-inducing fraction had properties strongly resembling those of the molten globule state. These results show that the apoptosis-inducing fraction consists of α-lactalbumin in monomeric and oligomeric states. The oligomers constitute novel folding variants with molten globule-like properties.

Fraction VI had interesting and unusual effects on the L1210 leukemia cells. Like casein, it induced apoptosis, as shown by a drastic reduction in tumor cell viability and by the fragmentation of DNA. The whey fraction and the other casein fractions

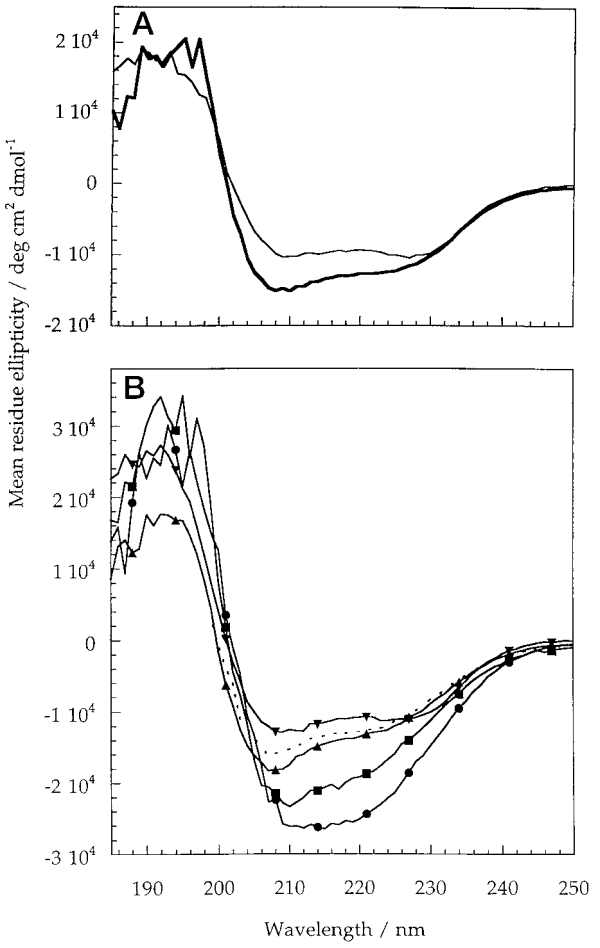

FIG. 8. **Far UV CD spectra.** *Panel A,* circular dichroism spectra in the far UV region of fraction VI (*solid line*) and native, monomeric α-lactalbumin (*thin line*). *Panel B,* CD spectra of peaks 1–4 (peak 1, ■; peak 2, ●; peak 3, ▲; peak 4, ▼) and pH 2 molten globule α-lactalbumin. The protein concentration was 0.14 mg/ml, corresponding to 10 μM monomer, in 10 mM potassium phosphate buffer at pH 7.5, 25 °C.

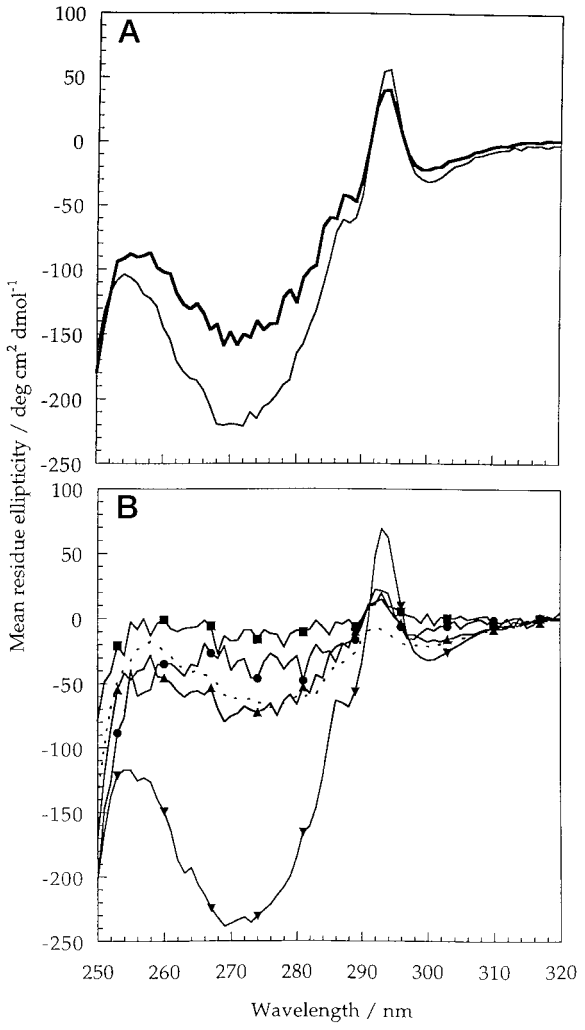

FIG. 9. **Near UV CD spectra.** *Panel A,* circular dichroism spectra in the near UV region for fraction VI (*solid line*) and native, monomeric α-lactalbumin (*thin line*). *Panel B,* CD spectra in the near UV region for peaks 1–4 (peak 1, ■; peak 2, ●; peak 3, ▲; peak 4, ▼) and pH 2 molten globule α-lactalbumin (*dashed line*). The protein concentration was 1.0 mg/ml, corresponding to 70 μM monomer, in 10 mM potassium phosphate buffer at pH 7.5, 25 °C.

lacked this activity, as did the α-lactalbumin both in the native and in the molten globule-like state. This might be explained by the presence of Ca^{2+} in the cell culture medium reverting apo-α-lactalbumin back to its native fold. Consequently, the apoptosis inducing activity appeared to be specific for the molecular complex in fraction VI. This activity was partly explained by cellular localization studies. The distribution of the active fraction was examined by confocal microscopy after biotinylation of the protein, with native α-lactalbumin as a negative control. Fraction VI was shown to bind the cell surface, to enter the cytoplasm and accumulate in the cell nuclei. Nuclear uptake was rapid and was detected at the time of DNA fragmentation. Furthermore, it was specific for the tumor cells; primary cultures of non-transformed cells showed no nuclear uptake (24). We propose that the interaction with the nucleus is critical for the induction of DNA fragmentation, since inhibition of nuclear uptake with wheat germ agglutinin rescued cells from DNA fragmentation.

While there was no difference in cell surface binding between native α-lactalbumin and fraction VI, native α-lactalbumin did not accumulate in the cell nuclei. This is consistent with previous studies suggesting that the molten globule state is important for translocation of proteins across phospholipid bilayers (25). While native α-lactalbumin interacted with negatively charged membranes only at pH values below the isoelectric point, partially unfolded, molten globule-like conformers of α-lactalbumin bind to phospholipid membranes also at higher pH values (26). This may be a consequence of the surface accessible hydrophobic residues in the partially unfolded state. Based on the present study we propose that nuclear uptake and DNA fragmentation require that α-lactalbumin is oligomerized and preserved in the molten globule-like state.

The term "molten globule" was introduced to describe a stable folding variant of α-lactalbumin, which has native-like secondary structure but less well defined tertiary structure (17, 27). Molten globules are formed under acidic conditions, and similar states are formed at neutral pH upon removal of the tightly bound Ca^{2+} ion, reduction of the disulfide bonds, or at elevated temperatures (18–20). Molten globules have also been proposed as kinetic intermediates observed during protein folding (28–30).

In this study, spectroscopic analysis of native monomeric α-lactalbumin and the pH 2 molten globule control, agreed with earlier reports. The fluorescence spectrum for native α-lactalbumin showed tryptophan residues in a folded hydrophobic core, and the small effects on the ANS spectrum suggested that no larger hydrophobic surfaces were exposed in the native monomer. The far UV CD spectrum was typical for native

α-lactalbumin, and the near UV CD spectrum had the characteristic tyrosine dip and tryptophan peak (17). In the pH 2 molten globule the tryptophan residues were more accessible to the solvent and ANS binding was strong indicating interaction with hydrophobic surfaces. The far UV CD spectrum showed no difference in secondary structure between the pH 2 molten globule and native α-lactalbumin, but the near UV CD spectrum showed the characteristic loss of signal, indicating less restrained tyrosines and tryptophans (17).

The spectroscopic characterization of fraction VI showed a large contribution of the native, but there were, however, distinct spectral differences compared with the native α-lactalbumin. The intrinsic fluorescence spectrum indicated solvent accessible tryptophan residues in fraction VI, and the large intensity increase and wavelength decrease in the ANS spectrum of fraction VI suggested that ANS bound to hydrophobic surfaces which had become accessible on oligomerization. Peak 1 from the Superose-12 column contained the largest multimers, and had intrinsic fluorescence, far UV CD, and near UV CD spectra similar to those of α-lactalbumin in the pH 2 molten globule state. The ANS fluorescence spectrum showed very low intensity compared with the pH 2 molten globule, possibly due to inaccessibility of hydrophobic surfaces to ANS in the large aggregates or due to fluorescence quenching arising from molecular collisions. Peaks 2 and 3 bound ANS strongly and the fluorescence and UV CD spectra resembled the pH 2 molten globule α-lactalbumin. Peak 4, that eluted at the same volume as native, monomeric α-lactalbumin behaved as native monomeric α-lactalbumin. The fraction VI spectra could be generated by a weighted summation of the spectra for peaks 1–4.

Protein folding variants have recently been proposed to differ in biologic function. The conformational switch of the prion protein leads to the formation of the disease causing isoform (31–33). The two isoforms have the same amino acid sequence and no post-translational modifications distinguish the two (34, 35). The prion protein first changes to the molten globule state and then proceeds to a non-reversible β-sheet rich form (33, 36). In this study, we have identified a new example of a protein which acquires novel functions after conformational switching. Like in the prion system, the two molecular forms of α-lactalbumin had identical amino acid sequence, with no post-translational modifications as detected by mass spectrometry. We propose that the relative folding instability of α-lactalbumin determines its ability to attain new essential functions.

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