

A folding variant of α -lactalbumin with bactericidal activity against *Streptococcus pneumoniae*

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Summary

This study describes an α -lactalbumin folding variant from human milk with bactericidal activity against antibiotic-resistant and -susceptible strains of *Streptococcus pneumoniae*. The active complex precipitated with the casein fraction at pH 4.6 and was purified from casein by a combination of anion exchange and gel chromatography. Unlike other casein components, the active complex was retained on the ion-exchange matrix and eluted only with high salt. The eluted fraction showed N-terminal and mass spectrometric identity with human milk α -lactalbumin, but native α -lactalbumin had no bactericidal effect. Spectroscopic analysis demonstrated that the active form of the molecule was in a different folding state, with secondary structure identical to α -lactalbumin from human milk whey, but fluctuating tertiary structure. Native α -lactalbumin could be converted to the active bactericidal form by ion-exchange chromatography in the presence of a cofactor from human milk casein, characterized as a C18:1 fatty acid. Analysis of the antibacterial spectrum showed selectivity for streptococci; Gram-negative and other Gram-positive bacteria were resistant. The folding variant of α -lactalbumin is a new example of naturally occurring molecules with antimicrobial activity.

Introduction

Human milk provides the breast-fed child not just with nutrients, but with a mucosal immune system. Milk contains a wide array of molecules with antimicrobial activity: antibodies to bacterial, viral and protozoal antigens (Gillin *et al.*, 1983; Hanson *et al.*, 1985; Redhead *et al.*, 1990; Ogra and Rassin, 1995); bactericidal molecules such as lysozyme and lactoferrin (Reiter, 1984; Lönnnerdal, 1985); fatty acids that lyse bacteria and viral particles (Kabara *et al.*, 1972; Sarkar *et al.*, 1973; Kabara, 1980); and glycoconjugates that inhibit bacterial adherence to epithelial cells (Andersson *et al.*, 1983; Svanborg *et al.*, 1991; Kunz and Rudloff, 1993; Newburg, 1999). These components reach mucosal surfaces in the respiratory and gastrointestinal tracts of the breast-fed child and are thought to interfere with various steps in disease pathogenesis at these sites (Ofek *et al.*, 1977; Andersson *et al.*, 1986; Kunz and Rudloff, 1993). As a result, breast-feeding protects the infant from infection caused by a broad range of mucosal pathogens (Cunningham, 1977; Goldman and Goldblum, 1980; Saarinen, 1982; Cunningham *et al.*, 1991; Aniansson *et al.*, 1994; Golding *et al.*, 1997; Hylander *et al.*, 1998).

Streptococcus pneumoniae and *Haemophilus influenzae* are common causes of upper respiratory tract infections (URI). They start their interaction with the host at the respiratory tract mucosa by attaching to mucins (Davies *et al.*, 1995) and epithelial cells (Andersson *et al.*, 1983; Kunz and Rudloff, 1993; Cundell *et al.*, 1995). Some colonized individuals become asymptomatic carriers, whereas others develop symptomatic infections such as otitis, sinusitis or pneumonia. Breast-feeding reduces the rate of such infections, but the protective mechanisms are poorly understood. There are several classes of molecules in human milk that interact with *S. pneumoniae* and *H. influenzae* and may contribute to the reduced frequency of URI (Saarinen, 1982; Aniansson *et al.*, 1994). These include secretory IgA antibodies to capsular polysaccharides, to cell wall polysaccharides of *S. pneumoniae* (Andersson von Rosen *et al.*, 1996) and to *H. influenzae* LPS or capsule (Kassim *et al.*, 1989; Kauppi *et al.*, 1993). The lactoseries of oligosaccharides, including lacto-*N*-tetraose, provides receptor sites for *S. pneumoniae* and inhibit bacterial adherence to the respiratory tract epithelium (Andersson *et al.*, 1983; Svanborg *et al.*, 1991; Kunz and Rudloff, 1993). However, breast-fed children do not appear to

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have reduced nasopharyngeal carriage of these bacteria, suggesting that the protective effect of human milk influences the progression from colonization to disease rather than colonization *per se*.

When exposed to low pH, milk separates into casein and whey. During our early studies on the antibacterial effects of human milk, we observed that the casein fraction had strong antibacterial activity against *S. pneumoniae* (Aniansson *et al.*, 1990; Håkansson *et al.*, 1996). This study describes the purification of the active molecular complex from human milk casein and identifies it as a novel form of α -lactalbumin. The active complex contains a folding variant of human α -lactalbumin in a molten globule-like state complexed with a C18:1 fatty acid. In this molecular form, α -lactalbumin kills both antibiotic-sensitive and -resistant strains of *S. pneumoniae*, but has little or no activity against other bacterial species.

Results

Bactericidal activity of human casein fractions

Human milk casein fractions from three donors were tested for bactericidal activity against *S. pneumoniae* 10175. Bacteria in suspension were mixed with the fractions, and the viability was determined after 0, 30 and 180 min (Table 1). At a concentration of about 1 mg ml⁻¹, the casein fractions reduced the viability of *S. pneumoniae* in suspension from 10⁷ cfu ml⁻¹ to about 10 cfu ml⁻¹ in 30 min.

All of the bactericidal activity precipitated with the casein; none remained in the whey fractions (Table 1).

Fractionation of human milk casein

The casein fractions were subjected to ion-exchange chromatography and tested for antibacterial activity. The ion-exchange fractionation profile for the casein sample AD

Table 1. Killing of *S. pneumoniae* by human milk fractions.

Samples ^b	Viable bacteria (cfu ml ⁻¹) ^a			
	AD ^c	HE ^c	NO ^c	Buffer ^d
Whey	4 × 10 ⁶	2 × 10 ⁶	3 × 10 ⁶	2 × 10 ⁶
Casein	< 10	< 10	2 × 10 ¹	1 × 10 ⁷
Casein fractions				
I	4 × 10 ⁸	5 × 10 ⁸	4 × 10 ⁸	3 × 10 ⁸
II	4 × 10 ⁸	4 × 10 ⁸	4 × 10 ⁸	3 × 10 ⁸
III	< 10	< 10	< 10	3 × 10 ⁸

a. Bacteria were incubated with the samples for 30 min, and viable counts were scored after overnight culture. Numbers represent mean values of three experiments.

b. Milk fractions were tested at a concentration of 1 mg ml⁻¹.

c. Milk samples from three different donors.

d. Untreated bacteria in buffer were used as a control.

is shown in Fig. 1A. Three main peaks were collected. Peaks I and II, which eluted at 0.12 M and 0.29 M NaCl respectively, were inactive in the bioassay (Table 1). The bactericidal activity was concentrated in peak III, containing material that bound so strongly to the column that it eluted only after 1 M NaCl. Peak III reduced the viability of *S. pneumoniae* from 3 × 10⁸ cfu ml⁻¹ to < 10 cfu ml⁻¹ in 30 min and was more active than the casein on a weight basis.

The other casein samples (HE and NO) were separated under similar conditions. The bactericidal activity was again found in peak III (Table 1), whereas the other peaks lacked a bactericidal effect against *S. pneumoniae* even at 5 mg ml⁻¹ and 4 h of incubation.

Purification of the active component

Isolation of the bactericidal component was continued from peak III of the casein sample AD. Analytical polyacrylamide gradient gel electrophoresis (PAGE), run under non-reducing and non-denaturing conditions, revealed one major band in the 14 kDa region and additional bands in the molecular weight range of 30, 60 and 100 kDa (Fig. 1B). Further purification was attempted using size exclusion chromatography on the Sephadex G-50 column, but the active fraction retained an oligomeric pattern and could not be purified to homogeneity.

N-terminal amino acid sequence analysis

The oligomeric pattern and molecular weights that were multiples of 14 kDa suggested that fraction III contained one major protein. N-terminal amino acid sequence analysis of the different bands showed that the sequence of the first 30 residues of the 14 kDa band and the first nine 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 in fraction III or in the α -lactalbumin control. The main N-terminal sequences of the 60 kDa band and the two 100 kDa bands were also identical to human α -lactalbumin, but some sequencing cycles showed heterogeneity.

α -Lactalbumin from milk whey lacks bactericidal activity

As fraction III and human α -lactalbumin showed sequence identity, their bactericidal activities were compared. Fraction III (0.25 mg ml⁻¹) completely killed an inoculum of 10⁶ *S. pneumoniae* in 30 min. In contrast, α -lactalbumin from human milk whey had no bactericidal effect even at a concentration of 10 mg ml⁻¹ (Table 2). Subsequent studies aimed at characterizing fraction III and the structural basis for the difference in antimicrobial activity compared with human α -lactalbumin.

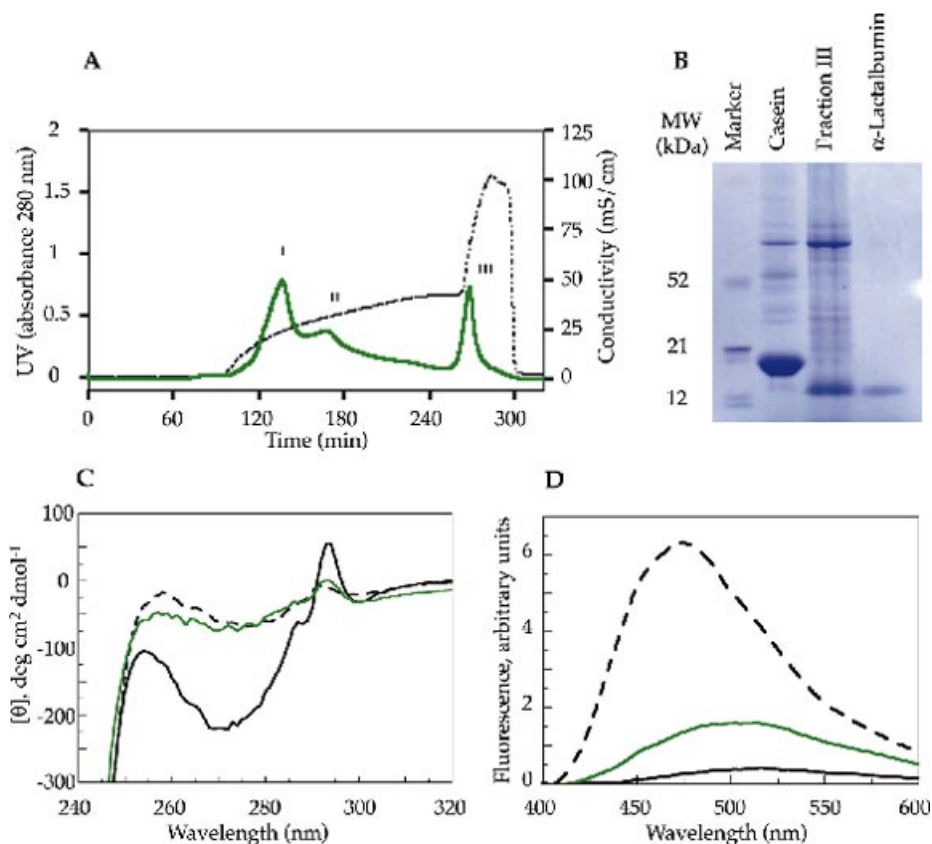


Fig. 1. Purification of the bactericidal component from human casein.

A. Ion-exchange chromatography of human milk casein. Fractionation of casein on the DEAE-Trisacryl M ion-exchange column using a NaCl gradient. Eluted fractions were monitored at 280 nm (green line) and pooled as indicated (I, II and III).

B. SDS-PAGE of casein, fraction III and α -lactalbumin. The molecular weight standards (lane 1) are myosin ($M_r = 188\,000$), phosphorylase b ($M_r = 97\,000$), glutamic dehydrogenase ($M_r = 52\,000$), carbonic anhydrase ($M_r = 33\,000$), myoglobin ($M_r = 21\,000$) and lysozyme ($M_r = 12\,000$). Staining of fraction III (lane 2) revealed bands with a molecular mass of 14, 30, 60 and 100 kDa, and α -lactalbumin ran as a single band at 14 kDa.

C. Near-UV CD spectrum of fraction III. Native α -lactalbumin (solid black line) showed the characteristic 270 nm tyrosine minimum and the 294 nm tryptophan maximum. EDTA-treated apo- α -lactalbumin (dashed black line) showed the characteristic loss of signal in the tyrosine and tryptophan regions. Fraction III (green line) strongly resembled the apo control, indicating a partially unfolded conformation of the protein.

D. ANS fluorescence spectrum of fraction III, at 1.5 equivalents of ANS. The apo- α -lactalbumin control (dashed black line) and fraction III (green line) bound ANS as seen by increased intensity and an intensity maximum shifted to 480 nm. The native α -lactalbumin (solid black line) 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 in buffer.

Mass spectrometry of fraction III and human α -lactalbumin

Post-translational modifications might explain the difference between the active and the inactive forms of human α -lactalbumin. No such differences were detected using electrospray ion-mass spectrometry (ESI-MS). The estimated molecular mass of the major component of the active fraction (14 088 kDa) was close to the molecular mass of α -lactalbumin (14 061 kDa), as calculated from the amino acid sequence. The small mass differences ruled out post-translational modifications, such as phosphorylation or methylation, and proposed that no difference in covalent structure was present between the major component of fraction III and human α -lactalbumin.

Matrix-assisted laser desorption/ionization (MALDI) mass

spectrometry of fraction III showed a major peak close to 14 kDa, consistent with monomeric α -lactalbumin, but also peaks at 28 kDa and 42 kDa, consistent with the di- and trimeric forms. These results support the conclusion from ESI-MS that the 14 kDa component was monomeric α -lactalbumin and also showed the presence of oligomeric forms of α -lactalbumin.

Spectroscopic analysis of the active fraction

α -Lactalbumin is known to form a variety of folding states under different solvent conditions. A partially unfolded state, the apo state, is formed at neutral pH if the Ca^{2+} ion is removed by EDTA (Ku wajima *et al.*, 1985; Pfeil, 1987; Ku wajima, 1996). This state has essentially retained

Table 2. Protein folding variants of α -lactalbumin and bactericidal activity for *S. pneumoniae*.

Sample	Viable bacteria (cfu ml ⁻¹) ^a			
	Concentration (mg ml ⁻¹)			
	0	0.25	0.5	0.75
Fraction III	1 × 10 ⁸	2 × 10 ⁴	< 100	< 100
Native α -lactalbumin	5 × 10 ⁷	5 × 10 ⁷	3 × 10 ⁷	7 × 10 ⁷
Converted apo- α -lactalbumin ^b	5 × 10 ⁷	2 × 10 ³	< 100	< 100
Lipid from converted apo- α -lact ^c	5 × 10 ⁷	3 × 10 ⁵	4 × 10 ³	< 100
Mixture of converted apo- α -lact and native α -lactalbumin ^d	5 × 10 ⁷	4 × 10 ³	< 100	< 100
Mixture of lipid from converted apo- α -lact and native α -lactalbumin ^d	5 × 10 ⁷	2 × 10 ⁶	8 × 10 ⁴	6 × 10 ³
Converted native α -lactalbumin ^b	1 × 10 ⁸	1 × 10 ⁷	7 × 10 ⁴	1 × 10 ⁴
Lipid from converted native α -lact ^c	1 × 10 ⁸	9 × 10 ⁵	4 × 10 ⁴	4 × 10 ³
	0	0.05	0.1	0.25
Palmitic acid (C16:0) ^e	1 × 10 ⁸	8 × 10 ⁷	8 × 10 ⁷	5 × 10 ⁷
Stearic acid (C18:0) ^e	1 × 10 ⁸	1 × 10 ⁸	5 × 10 ⁷	2 × 10 ⁶
Oleic acid (C18:1) ^e	1 × 10 ⁸	8 × 10 ⁷	9 × 10 ⁷	1 × 10 ⁴

a. Bacteria were incubated with the samples for 2 h, and viable counts were scored after overnight culture. Numbers represent mean values of three experiments.

b. Native or apo- α -lactalbumin was subjected to ion-exchange chromatography on C18:1 conditioned matrices. The fractions eluting after 1 M NaCl were dialysed, lyophilized, resuspended to the appropriate concentration and tested for bactericidal activity.

c. Lipids were extracted from the converted apo or native α -lactalbumins by organic extraction. Lipids found in 0.25, 0.5 or 0.75 mg ml⁻¹ of the converted material (0.018, 0.036 and 0.054 mg ml⁻¹ respectively) were added to the bacteria, and viable counts were scored.

d. Converted apo- α -lactalbumin or lipid from converted apo- α -lactalbumin was mixed with 0.25, 0.5 or 0.75 mg ml⁻¹ native α -lactalbumin for 20 min at room temperature before addition to the bacteria. Native α -lactalbumin decreased the activity of lipids extracted from converted apo- α -lactalbumin but did not affect the activity of the converted material *per se*.

e. Pure free fatty acids were tested at 0.05, 0.1 and 0.25 mg ml⁻¹, and viable counts were scored.

secondary structure, but fluctuating tertiary structure (Dolgikh *et al.*, 1981). The change in fold results in less restrained tryptophans and tyrosines as well as exposure of hydrophobic surfaces, and can be detected by spectroscopic methods, such as near UV circular dichroism (CD) and fluorescence spectra, and by the interaction with hydrophobic dyes such as ANS (Dolgikh *et al.*, 1981).

The conformation of fraction III was examined using UV CD and ANS spectroscopy (Fig. 1C and D). α -Lactalbumin from human milk whey was used as a control of the native state, and EDTA-treated α -lactalbumin was used as a control of the partially unfolded apo state. Far-UV CD spectra showed that the secondary structure of fraction III was similar to α -lactalbumin (not shown). Using near-UV CD, the tertiary structure was shown to be more fluctuating, with a high degree of concordance with α -lactalbumin in the partially unfolded state (Fig. 1C). Fraction III was also shown to expose hydrophobic surfaces not available in the native protein (Fig. 1D)

Conversion of native α -lactalbumin to the active form by anion-exchange chromatography

These results suggested that fraction III differed from

human α -lactalbumin in non-covalent structure and/or conformation. If so, it should be possible to activate native α -lactalbumin by converting it under the same conditions as used for purification of the active component from casein. A full description of the conversion and characterization of the converted material is given by M. Svensson *et al.* (submitted).

Native or apo- α -lactalbumin was loaded onto a casein-conditioned ion-exchange matrix. Apo α -lactalbumin was obtained by removal of Ca²⁺ with EDTA, and the change in fold was confirmed by spectroscopy (Fig. 2B and C). Apo- α -lactalbumin bound strongly to the column, and a sharp peak containing close to 90% of added material eluted only after 1 M NaCl (Fig. 2A). Using CD and ANS spectroscopy, the eluate was similar to the apo- α -lactalbumin control (Fig. 2B and C). It was active against *S. pneumoniae* and reduced the viability of 1 × 10⁷ cfu ml⁻¹ to < 100 cfu ml⁻¹ in 2 h at 0.5 mg ml⁻¹. Ion-exchange chromatography of native α -lactalbumin on a casein-conditioned column gave a different result (Fig. 2A). Most of the native α -lactalbumin was not retained on the casein-conditioned matrix. A broad peak containing only 30–40% of the starting material eluted with high salt, but the eluted material retained the native conformation (Fig. 2B and C). This

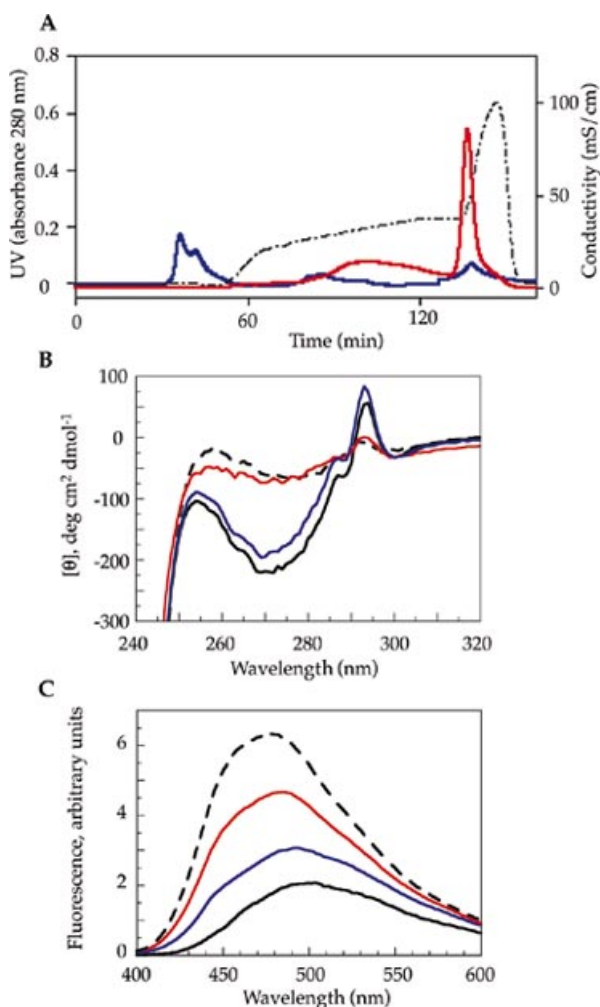


Fig. 2. Conversion of α -lactalbumin to the active form requires a cofactor.

A. Ion-exchange chromatography using a casein-conditioned column. Native or apo- α -lactalbumin was subjected to ion-exchange chromatography on a column previously exposed to human casein. More than 95% of the apo protein (red line) was retained on the column and eluted as a sharp peak at 1 M NaCl. In contrast, most of the native α -lactalbumin eluted in the void volume (blue line) with only a small fraction eluting at 1 M NaCl.

B. Near-UV CD spectrum of material eluted from the casein-conditioned column. Apo (dashed black line) and native (solid black line) α -lactalbumin controls are as in Fig. 1. The apo- α -lactalbumin eluate from the casein-conditioned column (red line) had a spectrum similar to that of the apo control. The native α -lactalbumin eluate from the casein-conditioned column (blue line) had a spectrum similar to that of the native control.

C. ANS fluorescence spectrum of material eluted from the casein-conditioned column. Apo (dashed black line) and native (solid black line) α -lactalbumin controls are as in Fig. 1. The apo- α -lactalbumin eluate (red line) bound ANS, but the native α -lactalbumin eluate (blue line) did not.

material had no bactericidal activity against *S. pneumoniae*, even at a concentration of 1 mg ml^{-1} . This demonstrated that α -lactalbumin must be in the apo state in order to convert to the active form.

This result was surprising, as the apo form of α -lactalbumin is known to revert to the native fold at physiologic solvent conditions. The converted material contained apo- α -lactalbumin that was stable at neutral pH and in the presence of Ca^{2+} , suggesting that a cofactor was present in the complex and that this cofactor stabilized the molecule in the apo state. Furthermore, these observations suggested that the cofactor was derived from casein and bound to the column matrix.

The cofactor was identified by chemical extraction of the casein-conditioned matrix. Solvents known to elute proteins released only residual α -lactalbumin; no other proteins were detected. Organic solvents, on the other hand, released lipids from the column matrix (Fig. 3A). The lipid extract was shown by thin-layer chromatography (TLC) to contain phospholipids, monoglycerides, diglycerides, cholesterol, free fatty acids and triglycerides. The free fatty acid content was shown to decrease gradually on casein-conditioned matrices used for purification of the active complex, suggesting that free fatty acids were the cofactor that became incorporated into the α -lactalbumin complex (Fig. 3A). Individual free fatty acid species were identified using gas chromatography combined with mass spectrometry (not shown), and C18:1, C16:0 and C14:0 fatty acids were shown to predominate.

New ion-exchange matrices were subsequently pre-conditioned with C18:1, C16:0 or C14:0 and exposed to α -lactalbumin in the native or apo state. α -Lactalbumin was shown to convert to the active form only on the C18:1 pre-conditioned column and when applied in the apo form. The protein was retained on the column and eluted as a sharp peak after 1 M NaCl, with a yield of about 90% (Fig. 3B). The near-UV CD spectrum and ANS binding of the active fraction strongly resembled the apo- α -lactalbumin control (Fig. 3C and D). This material had similar or stronger activity against *S. pneumoniae* as the casein-derived material (Table 2). In contrast, the C18:1 conditioned column could not activate native α -lactalbumin. Most of the material eluted in the void volume with a small peak after 1 M NaCl (Fig. 3B), which showed native tertiary structure (Fig. 3C and D) and had little bactericidal activity.

We have thus identified two key factors that regulate the novel functional state of α -lactalbumin. Removal of the Ca^{2+} ion was required to open up the protein, and oleic acid served to stabilize and preserve the bactericidal conformation.

Bactericidal activity of free fatty acids

Long-chained free fatty acids have been reported to kill *S. pneumoniae* and other bacteria (Kabara *et al.*, 1972). The bactericidal activity of C18:1, C16:0 and C14:0 fatty acids against *S. pneumoniae* 10175 was compared in concentrations that occur in the converted α -lactalbumin fraction

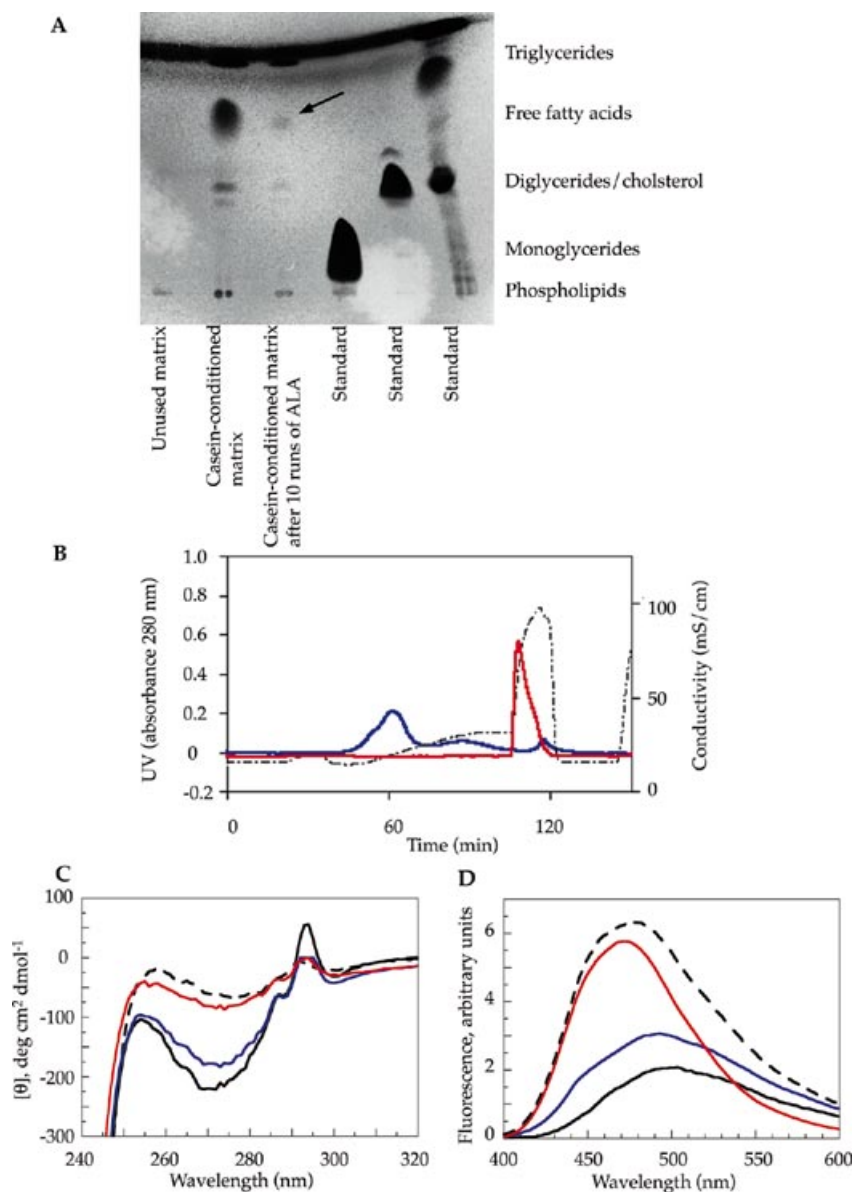


Fig. 3. The cofactor was identified as a C 18:1 fatty acid.

A. TLC of lipid extracts from ion-exchange matrices. New matrix contained essentially no lipids. The casein-conditioned matrix contained phospholipids, diglycerides, cholesterol, free fatty acids and triglycerides. After 10 runs of α -lactalbumin over the casein-conditioned matrix, the total lipid content had decreased, with the most drastic change in the free fatty acid band (see arrow).

B. Ion-exchange chromatography on a C18:1 conditioned column. Native α -lactalbumin or EDTA-treated apo- α -lactalbumin was subjected to ion-exchange chromatography on a C18:1 fatty acid conditioned column. Apo- α -lactalbumin (red line) bound to the column and eluted at 1 M NaCl. Most of the native protein (blue line) eluted in the void volume, with a small fraction eluting at 1 M NaCl.

C. Near-UV CD spectrum of material eluted off the C18:1 column. Apo (dashed black line) and native (solid black line) α -lactalbumin controls are as in Fig. 1. The apo- α -lactalbumin eluate (red line) had a spectrum similar to that of the apo control. The native α -lactalbumin eluate (blue line) retained the native conformation.

D. ANS fluorescence spectrum of material eluted from the C18:1 column. Apo (dashed black line) and native (solid black line) α -lactalbumin controls are as in Fig. 1. Like the apo control, the apo- α -lactalbumin eluate from the C18:1 conditioned column (red line) bound ANS. The native α -lactalbumin eluate (blue line) retained the native folded conformation.

(50–100 μ g lipid mg⁻¹ converted α -lactalbumin). No bactericidal activity was detected in this concentration range (Table 2).

Subsequently, lipid extracts from the active fraction were examined. Lipids extracted from 0.5 mg ml⁻¹ of the active fraction were not active, whereas the corresponding concentration of the active fraction (0.5 mg ml⁻¹) killed 5×10^7 bacteria in 2 h (Table 2). Mixtures of α -lactalbumin and lipid extracts from the active fraction had no bactericidal activity in this concentration range. Thus the lipid did not appear to account for the activity of the complex. The inactivity of the mixtures suggested that the activated lipid-protein complex has unique molecular features not present in the lipid or in the protein.

Antibacterial spectrum of fraction III and the active fraction

S. pneumoniae strains were more sensitive than other bacterial species to the bactericidal effect of fraction III and the converted α -lactalbumin. This included penicillin-resistant and -sensitive isolates differing in capsular type and resistance to other antibiotics (Table 3). All strains tested were killed at concentrations < 0.5 mg ml⁻¹ after 2 h of incubation. The fractions had no bactericidal activity against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus sanguis*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus parainfluenzae*, *Enterobacter cloacae*,

Table 3. Bactericidal activity of Fraction III and converted apo- α -lactalbumin against penicillin-sensitive and -resistant strains of *S. pneumoniae*.

Bacterial strain	Capsular type	Viable bacteria (cfu ml ⁻¹) ^a		
		Control	Fraction III	Converted apo- α -lactalbumin
A. Penicillin sensitive^b				
L14060-92 (3)	3	1 × 10 ⁵	< 10	< 100
L15256-92 (3)	3	2 × 10 ⁵	< 10	< 100
L14326-92 (3)	3	2 × 10 ⁵	< 10	1 × 10 ²
L15006-92 (6)	6	2 × 10 ⁴	< 10	< 100
CCUG10175 (19)	19	1 × 10 ⁶	< 10	< 100
L14117-92 (19)	19	2 × 10 ⁵	< 10	< 100
L14612-92 (19)	19	1 × 10 ⁵	3 × 10 ¹	< 100
L14423-92 (23)	23	6 × 10 ⁵	< 10	< 100
L14007-92 (23)	23	5 × 10 ⁴	< 10	< 100
B. Penicillin resistant^c				
SA 44165 (6)	6	5 × 10 ³	< 10	< 100
L14091-92 (14)	14	5 × 10 ⁵	< 10	1 × 10 ³
L1828 (14)	14	2 × 10 ⁶	< 10	< 100
L317-93 (15)	15	1 × 10 ⁴	< 10	< 100
L1017-93 (15)	15	5 × 10 ⁵	< 10	< 100
L859 (19)	19	3 × 10 ⁵	< 10	100
L963 (19)	19	4 × 10 ⁶	< 10	1 × 10 ²
L14030-92 (23)	23	2 × 10 ⁶	2 × 10 ¹	< 100
BN241-94 (23)	23	5 × 10 ⁴	< 10	< 100
DK 84/87 (23)	23	5 × 10 ⁶	< 10	< 100

a. Bacteria were incubated with the fraction III (1 mg ml⁻¹) for 2 h, and viable counts were scored after overnight culture. Numbers represent mean values of three experiments.

b. MIC value < 0.06 mg ml⁻¹.

c. MIC value > 2 mg ml⁻¹. All strains were multiply resistant against penicillin and one or more of tetracycline, erythromycin, cotrimoxazole and chloramphenicol.

Enterococcus faecalis or group B streptococci (2 mg ml⁻¹). A slight decrease in viability (from 10⁷ to about 10⁴ cfu ml⁻¹) was observed for *H. influenzae*, *Moraxella catharralis*, *Streptococcus mitis* and *Streptococcus pyogenes* at 2 mg ml⁻¹ converted α -lactalbumin, but there was no effect at lower concentrations.

Discussion

This study identified a folding variant of α -lactalbumin with strong bactericidal activity against *S. pneumoniae*. The active form of the molecule had retained secondary structure but fluctuating tertiary structure, compared with the native form of α -lactalbumin from human milk whey. Hydrophobic surfaces were exposed, as shown by the interaction with the hydrophobic dye ANS, and tryptophan residues were accessible to solvent. α -Lactalbumin from human milk whey lacked bactericidal activity, but could be converted to the active form. Conversion required that the protein was in the apo state and the presence of a stabilizing component from casein, identified as a C18:1 fatty acid. The activated complex killed *S. pneumoniae* regardless of capsular type and with similar efficiency against penicillin-resistant and -sensitive strains.

The active component was isolated from human milk casein by anion exchange chromatography followed by size exclusion chromatography. The purification was

monitored as the bactericidal activity of each fraction after removing salts and free carbohydrates by dialysis. The active component bound strongly to the ion-exchange column and eluted only with 1 M NaCl. The SDS-PAGE profile showed a major band at 14 kDa and minor bands in the 30, 60 and 100 kDa regions. N-terminal sequence analysis of each band showed identity to α -lactalbumin, but α -lactalbumin from human milk whey lacked bactericidal activity. We then sought to identify the structural basis for this difference. To exclude post-translational modifications, the molecular weight of MAL was analysed by mass spectrometry. By ESI-MS, the molecular weights of MAL (14.088 kDa) and α -lactalbumin (14.061 kDa) were found to be similar, and the small differences ruled out most known post-translational modifications, such as phosphorylation. Further analysis by MALDI-MS showed peaks consistent with mono- (14 kDa), di- (28 kDa) and trimers (42 kDa) of α -lactalbumin. This supported the conclusion from ESI-MS and also proved that the active fraction contained oligomeric forms of human α -lactalbumin.

The well-known native form of α -lactalbumin is a monomeric, 14 kDa protein with a single high-affinity Ca²⁺ binding site. High-resolution crystal structures from several species, including man, show the presence of four α -helices and one triple-stranded β -sheet in the C-terminal end of the molecule (Acharya *et al.*, 1991). The high-affinity Ca²⁺ binding site is co-ordinated by the side-chain carboxylates of

Asp-82, Asp-87 and Asp-88, and the carbonyl oxygens of Lys-79 and Asp-84, with two water molecules. When exposed to low pH or to other conditions that remove the tightly bound Ca^{2+} ion, the molecule undergoes a conformational change to the so-called apo form (Dolgikh *et al.*, 1981; Kuwajima *et al.*, 1985; Pfeil, 1987; Schulman *et al.*, 1995; Kuwajima, 1996; Wu *et al.*, 1996).

The folding state of α -lactalbumin in the active fraction was examined using spectroscopic techniques. Far-UV CD spectra showed a secondary structure similar to native α -lactalbumin. A decreased signal in near-UV CD spectroscopy indicated a more fluctuating tertiary structure, and ANS fluorescence spectroscopy showed that hydrophobic surfaces were exposed. These results suggested that the active fraction contained α -lactalbumin in a partially unfolded molten globule-like state, but unlike the apo form of α -lactalbumin, which reverts to the native conformation when solvent conditions are normalized; fraction III was stable at neutral pH and in the presence of Ca^{2+} . We concluded that the purification procedure with precipitation of casein at low pH combined with the ion-exchange chromatography somehow conserved the protein in the partially unfolded conformation and stabilized its activity.

To prove that the bactericidal activity was conformation dependent, we sought to convert α -lactalbumin into the active form by altering its fold. α -Lactalbumin purified from human milk whey by ammonium sulphate precipitation followed by phenyl-Sepharose chromatography and gel filtration was >95% pure by Western blot and was used as the starting material. The apo state was induced by EDTA treatment, and native or apo was subjected to ion-exchange chromatography on a column previously exposed to casein. This procedure was found to convert >90% of apo- α -lactalbumin to the active form, and spectroscopic analysis showed that the activated material retained a partially unfolded state. Native α -lactalbumin could not be converted to the active state.

Two features of the conversion process suggested that the active complex contained a cofactor present in casein. First, conversion of apo- α -lactalbumin could not be achieved on a clean column matrix. Second, the yield of converted material decreased with repeated runs, suggesting that the stabilizing factor was consumed and integrated in the active complex. C18:1 fatty acid was identified as the cofactor by extraction of the column matrix, identification of the fatty acids and conditioning of clean column matrices with each of the fatty acids. Conversion to the active state was achieved with apo- α -lactalbumin on a C18:1 conditioned column. The other fatty acids were inactive, and the native, folded protein could not be activated. We conclude that the bactericidal component is a folding variant of human α -lactalbumin, stabilized by the C18:1 fatty acid.

The active fraction had broad bactericidal activity against all tested strains of *S. pneumoniae*. Mutants lacking

capsule, autolysin, pneumolysin and competence factors were killed with similar efficiency. Staphylococci or Enterococci, and most Gram-negative bacteria were completely resistant to this effect, while other streptococci formed an intermediate group. The bactericidal activity against *S. pneumoniae* was independent of the resistance to penicillin and other antibiotics. These observations predict that the cellular targets of fraction III are present in *S. pneumoniae* and certain streptococci, but not in other bacteria. Furthermore, these targets differ from molecules that determine the sensitivity of *S. pneumoniae* to antibacterial agents.

The activity of fraction III and converted α -lactalbumin was not, however, restricted to bacteria. Tumour cells and immature cells were also sensitive to this fraction and were shown to undergo apoptosis (Håkansson *et al.*, 1995; Svensson *et al.*, 1999). There was a rapid loss of viability accompanied by DNA fragmentation. Healthy, differentiated cells, in contrast, were resistant to the effects of fraction III. The structural basis for activity was similar for bacteria and cells, in that monomeric α -lactalbumin was inactive, but could be converted to the active form under the conditions described (M. Svensson *et al.*, submitted). These observations suggest that there are structural similarities between the pneumococcal surface and human cells, which allow the α -lactalbumin folding variant to find a target in both cell types.

The treatment of pneumococcal infections has become a global problem because of the rapid dissemination of resistant strains. The reported frequencies of penicillin-resistant pneumococci in different countries and patient groups vary between 5% and 60% (Klugman, 1990; Echariz-Aviles *et al.*, 1998; Huebner *et al.*, 1998; Jacobs *et al.*, 1998). In addition, up to 50% of penicillin-resistant pneumococci may have reduced sensitivity to other antimicrobial agents in the β -lactam group. There is an immediate need for novel approaches to the prevention and treatment of pneumococcal infections. The α -lactalbumin folding variant can kill both antibiotic-sensitive and -resistant strains of *S. pneumoniae*. This is but one example of the many naturally occurring antibacterial compounds that should be explored as novel antimicrobials.

Experimental procedures

Reagents

Distilled sterilized water was used for the preparation of buffers, culture media, eluents, chromatographic and electrophoresis solutions. Sodium phosphate, sodium chloride, Tris-HCl, glycine, hydrochloric acid and acetic acid were from Merck; choline was from Sigma. The Amberlite, monobed desalting resin was from BDH Limited, and the DEAE-Trisacryl M ion-exchanger was from BioSeptra. The dialysis membranes (Spectra/Por) were from Spectrum Medical Industries. The

Sephadex gel (G-50) for gel filtration chromatography was from Pharmacia.

Reagents for electrophoresis were either from Sigma (glycerol, SDS) or from Bio-Rad (gels, bromophenol blue, Coomassie blue, molecular weight standards). Polyvinylidene difluoride (PVDF) membranes (Pro Blott) were from Applied Biosystems, and the nitrocellulose sheets were from Bio-Rad. Lactoferrin and lysozyme were from Sigma.

Bacteria

S. pneumoniae 10175 (CCUG 10175, Culture Collection, University of Göteborg, Sweden) was used during the isolation of the antibacterial fraction from human milk casein. The strain was kept lyophilized, transferred to blood agar plates, cultured for 9 h at 37°C in liquid medium (Lacks and Hotchkiss, 1960), harvested by centrifugation and suspended in 1 ml of PBS containing 1% choline chloride.

The antibacterial spectrum of the active fraction was tested using different penicillin-sensitive and penicillin-resistant clinical isolates. *S. pneumoniae* SA 44165, BN241-94 and DK 84/87 were kindly provided by Dr J. Henrichsen, Statens Serum Institut, Copenhagen, Denmark. The remaining penicillin-resistant and -sensitive strains were from the Clinical Bacteriology Laboratory, Lund University Hospital, Sweden. The minimal inhibitory concentrations (MICs) for penicillin were calculated by the disk diffusion method (*E*-test, AB Biodisk).

The antibacterial spectrum of the milk fraction was tested further using strains of *E. coli*, *P. aeruginosa*, *E. faecalis*, *E. cloache*, *S. aureus*, *S. epidermidis*, *S. mitis*, *S. sanguis*, *S. mutans*, *S. pyogenes*, group B streptococci, *K. pneumoniae*, *H. influenzae*, *H. parainfluenzae* and *M. catharralis*. These strains were provided by the Clinical Bacteriology Laboratory, Lund University Hospital, Lund, Sweden.

Bacteria were stored in fetal calf serum (FCS) at -80°C, transferred to blood agar plates and grown overnight at 37°C in an atmosphere of 5% CO₂. *H. influenzae* were cultured on chocolate agar plates. Staphylococci, Enterococci, *E. coli*, *K. pneumoniae*, *P. monocytogenes* and *P. aeruginosa* were scraped off the plates into PBS. The remaining bacteria were cultured in liquid growth medium [Todd-Hewitt for group B streptococci *S. mitis*, *S. pyogenes*, *S. mutans* and *S. sanguis*, pneumococcal-4 medium for *S. pneumoniae* (Lacks and Hotchkiss, 1960) and Haemophilus-1 medium for *H. influenzae* and *M. catharralis* (Branefors-Helander, 1972)], harvested in early log phase at 37°C using centrifugation and suspended in PBS (or NaCl containing 1% choline chloride for the pneumococcal strains).

Bactericidal activity of human milk and milk components

The bactericidal activity of human milk components was tested in suspension. Bacteria (0.1 ml, 10⁷-10⁹ cfu ml⁻¹) were mixed with buffer (0.9 ml of PBS) or with the component to be tested. The mixtures were incubated at 37°C, and 0.1 ml samples were withdrawn after 0 min, 30 min, 1 h, 2 h or 4 h. Samples were serially diluted, and the relevant dilutions were plated on blood agar or haematin agar (*H. influenzae* and *M. catharralis*). The number of colonies was scored after

overnight incubation at 37°C in 5% CO₂, and the concentration of viable bacteria was determined.

Casein precipitation

Frozen milk from three donors (AD, HE and NO) was thawed and centrifuged. Casein was prepared from about 2 l of milk, as has been described previously (Mellander, 1947). Briefly, about 27 ml of 10% potassium oxalate was added to each litre of fat-free milk. After overnight incubation at 4°C, the precipitate was discarded. To the supernatant, 1 mM HCl was slowly added to a final pH of 4.6. The solution was then heated to 30°C for 1 h and left overnight at 4°C. The casein precipitate was separated by centrifugation and washed by three to five cycles of resuspension in distilled water and centrifugation. Finally, the precipitate was lyophilized and resuspended to the concentration required for each experiment.

Whey fractions

The pH of the whey fractions was readjusted with PBS to 6.8. The fractions were desalted over the monobead ion-exchange resin (equilibrated and eluted with water) and lyophilized. For each experiment, the lyophilized fractions were diluted with PBS to the appropriate concentration.

Anion-exchange chromatography

The casein fractions were subjected to anion-exchange chromatography on a column (14 cm × 1.6 cm) packed with DEAE-Trisacryl M (BioSeptra) attached to a Biologic chromatography system (Bio-Rad). The matrix was eluted using a NaCl gradient. Running conditions were: buffer A, 0.1 M NaCl, 10 mM Tris-HCl, pH 8.5; buffer B, buffer A containing 1 M NaCl. Gradient programme: from start to 15 ml, 0% B; from 15 ml to 55 ml, 0-15% B; from 55 ml to 75 ml, 15% B; at 75 ml, 100% B for 10 min; from 85 ml to 115 ml, 0% B; at 115 ml, 100% B for 20 min; from 135 ml to the end, 0% B. The flow rate was 1 ml min⁻¹, and absorbance was monitored at 280 nm. The eluted protein fractions were desalted by dialysis (Spectra/Por; membrane cut-off 3.5 kDa, boiled four times in doubly distilled water before use) against distilled water for at least 48 h and lyophilized.

Polyacrylamide gradient gel electrophoresis (PAGGE)

Analytical PAGGE was performed using 4-20% polyacrylamide precast gels on a Bio-Rad Mini Protean II cell, as has been described previously (Svensson *et al.*, 1999). Proteins were stained with Coomassie blue.

Amino acid sequencing

Proteins were transferred by Western blotting to PVDF membranes and visualized using Coomassie blue staining. The PVDF-immobilized protein bands were subjected to Edman degradation (Matsudaira, 1987) in an automated pulse-liquid sequencer (Applied Biosystems model 477A).

Mass spectrometry

Purified proteins were analysed by ESI-MS on a VG Bio-Q ESI-MS (Fisons/VG) equipped with an atmospheric pressure electrospray ion source and a quadrupole mass analyser with a maximum mass range of 4000, as has been described previously (Svensson *et al.*, 1999). The sample was dissolved at a concentration of 10–20 pmol μl^{-1} 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 (Beavis and Chait, 1990).

The purified antibacterial proteins were analysed further using MALDI mass spectrometry (Keough *et al.*, 1992) on a LDI 1700 time of flight mass spectrometer equipped with a pulsed nitrogen laser (337 nm; Biomolecular Separations). About 100 μg of the protein fraction to be analysed was dissolved in 50 μl of water and 0.1% trifluoroacetic acid. This solution (10 μl) 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.

Purification of α -lactalbumin

Native α -lactalbumin was purified from human milk by ammonium sulphate precipitation (Lindahl and Vogel, 1984). The ammonium sulphate was added as a salt (264 g l^{-1} milk), and the mixture was incubated over night at +4°C. The mixture was centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont Instruments) at 5000 g for 15 min. The supernatant was collected, and Tris-HCl and EDTA, pH 7.5, was added to a final concentration of 50 mM and 35 mM respectively. A 400 ml phenyl-Sepharose column (Pharmacia Biotech) was packed in 50 mM Tris-HCl with 1 mM EDTA, pH 7.5, 25°C, and 250 ml of 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 and 1 mM CaCl_2 , pH 7.5, thus yielding the native, Ca^{2+} -bound form of α -lactalbumin.

Native α -lactalbumin was purified further by passing it over a Sephadex G-50 column. The column (93 $\text{cm} \times 2.5 \text{ cm}$) was equilibrated with 10 mM Tris-HCl, 50 mM NaCl buffer, pH 8.5. The flow rate was 0.8 ml min^{-1} , and peaks were monitored at 280 nm. The fractions were desalted by dialysis (Spectra/Por, Spectrum Medical Industries; membrane cut-off 3.5 kDa, boiled four times in doubly distilled water before use) against distilled water for at least 48 h and lyophilized.

Native α -lactalbumin from human milk whey was converted to the apo form by treating native α -lactalbumin with EDTA (0.14 mmol mg^{-1}), which removes Ca^{2+} and causes partial unfolding to the molten globule-like apo form. This preparation, together with native α -lactalbumin, was used as controls in the spectroscopic studies.

Conversion of α -lactalbumin to the active form

Native and apo- α -lactalbumin were subjected to anion-exchange chromatography on a DEAE-Trisacryl M column previously exposed to casein or conditioned with 10 mg of fatty acid. Residual protein attached to the column was

removed by extensive washing with the NaCl gradient as described.

α -Lactalbumin in the native or in the apo state was applied to the casein-conditioned column (25 mg dissolved in 10 ml of 10 mM Tris-HCl, pH 8.5, with or without EDTA) and eluted using the above running conditions.

Ten milligrams of palmitic acid (16:0), stearic acid (18:0), myristic acid (14:0) or oleic acid (18:1) were dissolved in 500 μl of 99.5% ethanol, using sonication (3 min using a bath sonicator; Branson 2200). After the addition of 10 ml of 10 mM Tris-HCl, pH 8.5, the different lipid solutions were applied to three separate newly packed DEAE-Trisacryl M matrices. Native or apo- α -lactalbumin (25 mg dissolved in 10 ml of 10 mM Tris-HCl, pH 8.5, with or without EDTA) was applied to each matrix and eluted as described above.

The peaks were monitored at 280 nm, and the fractions were desalted by dialysis (Spectra/Por, Spectrum Medical Industries; membrane cut-off 3.5 kDa boiled four times in doubly distilled water before use) against distilled water for at least 48 h and lyophilized.

Spectroscopic analysis

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 aliquots of stock solution in 10 mM potassium phosphate buffer at pH 7.5 at 25°C.

ANS fluorescence spectra were recorded at 25°C on a Perkin-Elmer LS-50B spectrometer using a quartz cuvette with 1 cm excitation path length between 400 and 700 nm (step 1 nm) with excitation at 385 nm. Both the excitation and emission bandpass were set to 5 nm.

Circular dichroism (CD) spectra were studied 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. Spectra were recorded between 300 and 240 nm (wavelength step 1 nm, response time 4 s and scan rate 10 nm min^{-1}). Six scans were recorded and averaged for each spectrum. The mean residue ellipticity $[\Theta]$ ($\text{mdeg} \times \text{cm}^2 \times \text{dmol}^{-1}$) was calculated from the recorded ellipticity, θ , as: $[\Theta] = \theta / (c \cdot n \cdot l)$, where c is the protein concentration in M, n is the number of residues in the protein (123 in this case), l is the path length in m and θ is the ellipticity in degrees.

Identification of lipids on the casein-conditioned matrix

To elute lipids from the ion-exchange matrix, organic solvents and a method modified from Bligh and Dyer (1959) were used. Casein-conditioned matrix (2 ml) was dissolved in chloroform:methanol:water [1:2:0.8 (v/v)] and incubated at 37°C for 1.5 h. Chloroform:water [1:1 (v/v)] was added, the solution was thoroughly mixed, and the two phases were separated overnight in a separation funnel. The organic phase was collected and dried under nitrogen. Preparative scale lipid separation was achieved using silica gel glass plates. Lipids were dissolved in chloroform (3 mg per 30 μl), and 10 μl was applied in triplicate to the plates (1 mg on each lane). The

plates were developed using petroleum ether/diethyl ether/ acetic acid/methanol [80:20:1:2 (v/v/v/v)], and the lipids were visualized by iodine vapours. Bands containing defined lipid species were scraped off the plates, and the lipids were eluted from the silica gel with chloroform/methanol [2:1, 1:1, 1:2 (v/v)]. The band containing fatty acids was analysed further on a Varian gas chromatograph (model 3500) equipped with a split/splitless injector and a flame ionization detector. The fatty acids were separated on a (0.25 mm inner diameter) fused silica capillary column coated with FFAP (Chrompack). The column temperature was programmed from 140°C to 240°C at 8°C min⁻¹. Chromatograms were evaluated using a Varian integrator model 4290.

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