β-Lactoglobulin and α-lactalbumin as potential modulators of mammary cellular activity

A Ca^{2+}-responsive model system using acid phosphoprotein phosphatases

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Summary. It has been observed that β-lactoglobulin of cow's milk inhibits the hydrolysis of p-nitrophenyl phosphate by phosphoprotein phosphatases from bovine spleen and lactating mammary gland. Kinetic studies indicate that the inhibition of p-nitrophenyl phosphate hydrolysis involves binding to the enzyme by β-lactoglobulin. Alkaline denaturation of the β-lactoglobulin molecule decreases the inhibition. This inhibition is reversed by Ca^{2+}-binding to sites on β-lactoglobulin with K_D equal to 3 mM, or by salt concentration > 100 mM.

α-Lactalbumin functions in a similar fashion with both the mammary and spleen enzymes. Since both these whey proteins and the milk phosphatase are secreted by Golgi, this in vitro reaction may serve as a model system for enzyme regulation in mammary secretory vesicles where the $[Ca^{2+}]_{free}$ concentration approaches 5 to 8 mM. Formation of enzyme-protein complexes in Golgi could in turn be effected by the binding of a ligand such as Ca^{2+} to "weak" sites. Thus protein—protein interactions which limit the activity of an enzyme may in turn be modulated by Ca^{2+} binding to the protein.

Keywords: Acid/phosphoprotein phosphatase; β-Lactoglobulin; Enzyme inhibition; Ca^{2+}-binding; Mammary gland; α-Lactalbumin.

Abbreviations: K_D dissociation constant for a protein—ligand interaction; p-NPP p-nitrophenyl phosphate; β-Lg β-lactoglobulin (β-Lg A, β-Lg B, β-Lg C genetic variants).

Introduction

The synthesis and secretion of milk involve the acquisition by the mammary gland of a wide variety of precursors, their assemblage into lactose, protein and lipid, and a unique ultrastructural mechanism for the dual secretion of skim milk and milk fat globules. The relative constancy of the composition of the milks of each species of mammal argues for a hierarchial system of control mechanisms for these processes. Computer modeling of the mammary secretory process has brought some insight into factors which may limit milk secretion (Waghorn and Baldwin et al. 1984), however our knowledge of biochemical regulatory mechanisms in mammary gland is still rudimentary. The complexity of milk biosynthesis and secretion, coupled with the total dependency of the neonate on the product, argue that the production of a major milk component is not frivolous. Furthermore, as our knowledge of control mechanisms increases, the interdependencies which occur in the milk secretory system have become apparent. Starting from these premises we and many others have considered what possible biochemical role the milk protein β-lactoglobulin may play in the synthesis and secretion of milks containing this protein. α-Lactalbumin, the major biochemically functional whey protein, has long been known to participate in the lactose synthetase system (Brodbeck et al. 1967). These proteins (β-lactoglobulin and α-lactalbumin) contain appropriate signal sequences which cause their insertion into the lumen of the correct segment of endoplasmic reticulum, leading to their secretion into skim milk through Golgi vesicles (Mepham et al. 1984, Shappell et al. 1986). It is within the Golgi vesicles that α-lactalbumin specifies lactose synthesis and wherein β-lac-

toglobulin may function as well, should it have any biochemical role within the mammary gland. Following the assumption that like α-lactalbumin, β-lactoglobulin may regulate an enzyme activity in Golgi, we have investigated one system in some detail. We observed that β-lactoglobulin, the major whey protein of cow's milk, inhibits the hydrolysis of p-nitrophenyl phosphate by phosphoprotein phosphatase (phospho-protein phosphohydrolase, EC 3.1.3.16). β-Lactoglobulin has been well characterized with regard to its chemical and physical properties and is homologous to retinol binding proteins (for a review, see Farrell 1988), yet no biological activity has been ascribed to this molecule. It is hoped that the investigation of the mechanism of this inhibitory effect might yield some insight into the reason for the occurrence of β-lactoglobulin and other whey proteins in milk and mammary gland.

Materials and methods

Material

Bovine β-lactoglobulin genetic variants A, B, and C, as well as α-lactalbumin, were prepared by the method of Aschaffenburg and Drewry (1957); β-lactoglobulins were recrystallized 4×. α-Lactalbumin was also prepared as described by Quarfoth and Jenness (1975) and as described by Robbins and Holmes (1972); purified lactoferrin was from M. L. Groves of this laboratory. α_{s1}-Casein was prepared as previously described (Thompson 1966). Bovine serum albumin and horse heart cytochrome *c* were purchased from Sigma Chemical Company[1]; bovine γ-globulin and ovalbumin were purchased from Nutritional Biochemicals. Calf intestine alkaline phosphatase, potato acid phosphatase, p-nitrophenyl sulfate, p-nitrophenyl β-glucuronate, snail aryl sulfatase/β-glucuronidase, p-nitrophenyl phosphate (Grade A) and p-nitrophenol were all purchased from Calbiochem. The o-carboxyphenyl phosphate was purchased from Worthington Biochemical Corporation.

Bovine spleen phosphoprotein phosphatase was prepared by the method of Revel and Racker (1960) and the mammary gland enzyme by the method of Farrell et al. (1988 a). Stock solutions of these enzymes were stored at − 20 °C in 2 mM sodium acetate and 5 mM sodium chloride, pH 5.9, for several months without a detectable loss of activity.

Methods

Lactose synthetase was assayed as previously described (Palmiter 1969) using a microsomal preparation from lactating rat mammary gland as the source of the galactosyl transferase (Leung et al. 1990). The enzymatic assays for the hydrolysis of p-nitrophenyl phosphate by phosphoprotein phosphatase were conducted at pH 6.0 and 25 °C. A typical 3 ml reaction mixture contained: 30 μmoles of ammonium acetate, 10 μmoles of p-nitrophenyl phosphate, 0.5 ml of enzyme solution (0.3 ml of the stock enzyme solution diluted to 25 ml with buffer), and β-lactoglobulin, from 2 to 6 mg, when required. The time course of the reaction was followed by measuring the release of p-nitrophenol at 410 nm on a Gilford Model 2000 Recording Spectrophotometer. The rate of hydrolysis with respect to time was linear, and the initial velocity is expressed as nmoles of p-nitrophenol liberated per ml per min, as calculated from a standard curve for p-nitrophenol at the given pH and ionic strength (Farrell et al. 1988 a). When o-carboxyphenyl phosphate was used as the substrate, the release of o-carboxyphenol was followed at 300 nm (Zittle et al. 1960). Casein dephosphorylation was measured by following the release of inorganic phosphate as previously described (Farrell et al. 1988 a, Summer 1944).

Sucrose density gradient centrifugation was carried out by the method of Martin and Ames (1961). Continuous gradients of 5 to 20% and 5 to 11% sucrose were prepared at 5 °C in 10 mM sodium acetate, pH 6.0. Samples of 200 μl containing 7.5 μl of stock phosphoprotein phosphatase solution ± 100 μg of β-lactoglobulin were carefully layered on top of the gradients in 4.0 ml centrifuge tubes. The tubes were then centrifuged for 17 h at 200,000 **g** in a Beckman SW56 rotor at 5 °C. Fractions of 5 drops each were collected by puncturing and draining the tubes with a 22 gauge needle. Enzymatic activity was monitored by measuring the increase in A_{410} after a 10 min incubation of each fraction with 0.5 ml of 10 mM p-nitrophenyl phosphate, pH 6.0, in ammonium acetate followed by addition of 2 ml of 0.1 N NaOH. Samples of cytochrome *c* and ovalbumin, 200 μg each, were used as markers and their positions detected by determining the A_{225} of the fractions after dilution with water.

The binding of Ca^{2+} to β-Lg was studied using the murexide dye spectrophotometric assay developed by Scarpa (1972). Stock $CaCl_2$ solutions (1 M) were determined by atomic absorption analysis and all cuvettes and glass were washed carefully with HNO_3.

Nonlinear regression analyses of standard Michaelis curves, and variance of parameters with concentration were carried out using the program Abcus which is based on the Gauss-Newton iterative method and which was developed by Dr. William Damert of this Center. Choices between fits of models and statistical methods of analysis of the nonlinear fits are as described by Meites (1974). For plots of velocity, v, against concentration of substrate [S] which deviated from standard patterns, data were fitted with equations originally derived from Wyman's Theory of Thermodynamic Linkage (1964) and derived for Ca^{2+}-induced interactions of caseins (Farrell et al. 1988 b). These models were subsequently adapted for use in enzyme kinetics experiments (Farrell et al. 1990 b). The simplest expression used in these experiments is an expansion of the general Michaelis expression:

$$V_{obs} = \frac{V_{max}[S]^n}{K_m + [S]^n}. \quad (1)$$

For enzyme kinetics in general n = 1; for binding studies $V = \bar{v}$ (moles of ligand bound/mole of protein), S is replaced by L, and $K_m = K_D$. In the latter case n can be varied by integer values and the best value with the lowest error chosen statistically (Meites 1974). Isoelectric focusing was conducted using the Pharmacia Phast-Gel System. The broad range ampholines ranging in isoelectric point from 3.0 to 9.0 were used. Standard proteins ranged from pI 3.5 to pI 8.65. Staining for phosphatase activity followed the procedure of Chen and Chen (1988).

Results

Conditions of the inhibition assay

The activity of both spleen and mammary phosphoprotein phosphatase was enhanced by reducing agents

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

such as 1 mM 2-mercaptoethanol, and the hydrolysis of p-nitrophenyl phosphate (p-NPP) was increased two-fold as previously reported (Farrell et al. 1988 a). Optimal enzymatic activity toward p-NPP was obtained at pH 6.0 with either 10 or 100 mM acetate. Hence the concentration of acetate employed had no effect on the activity of phosphoprotein phosphatase. Because β-lactoglobulin (β-Lg) contains one free sulfhydryl (per monomer) as well as two disulfide bonds, reducing agents were excluded in order to avoid any complications arising from the interaction of the mercaptoethanol and the β-Lg. A pH profile of the inhibition is given in (Fig. 1), and was obtained using o-carboxyphenyl phosphate as the substrate since the detection of o-carboxyphenol is not pH dependent in this range as is that of p-nitrophenol. The greatest relative inhibition apparently occurs near pH 5.5 with this substrate.

Kinetic study of the inhibition

Variation of the initial velocity of the hydrolysis of p-NPP by spleen phosphoprotein phosphatase was studied as a function of substrate concentration in the presence and absence of β-Lg A. Initially the β-Lg A concentrations were 39.2 and 19.6 μM. The results of a typical experiment are plotted in (Fig. 2). K_m (Table 1) calculated from three such experiments was 1.38 ± 0.08 mM and K_i was 19.6 ± 5.0 μM. The inhibition is diminished by increasing substrate concentration, and V_{max} appears to be unchanged by the β-Lg. These initial results are suggestive of classical competitive inhibition involving the formation of an enzyme-inhibitor (E-I) complex, at these concentrations of β-Lg.

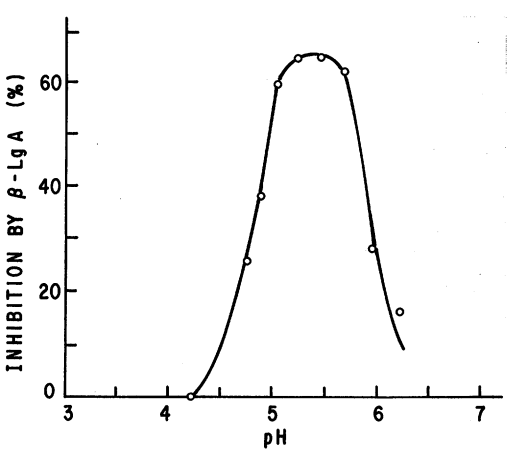


Fig. 1. The effect of pH on the inhibition by β-Lg A of phosphoprotein phosphatase hydrolysis of o-carboxyphenyl phosphate. The 3 ml reaction mixtures contained 30 μmoles ammonium acetate 10 μmoles o-carboxyphenyl phosphate, 118 n moles of β-Lg A adjusted to the desired pH, and 0.5 ml of the stock enzyme solution diluted 0.3 to 25 ml. The reactions were carried out as described in Materials and methods by recording the change in A_{300}

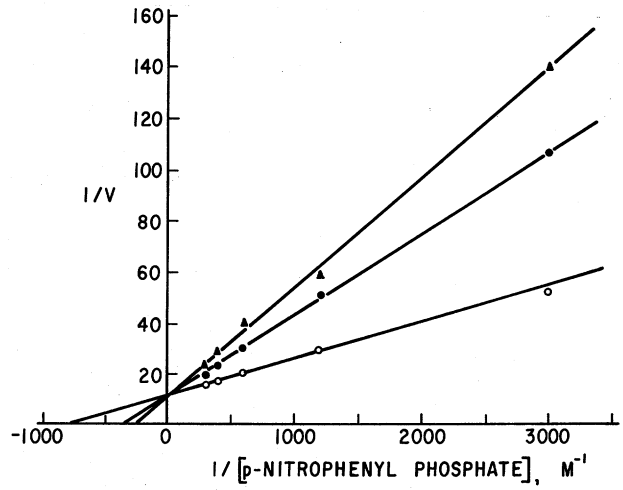


Fig. 2. Lineweaver-Burk analysis of the β-Lg inhibition of the hydrolysis of p-NPP by phosphoprotein phosphatase. The slopes and intercepts of the line were obtained from nonlinear regression analysis of the data points. Each point represents the average of three determinations. All reactions were carried out as described in Table 1, except that β-Lg A was added: ○—○ no β-Lg added; ●—● 0.333 mg/ml β-Lg A added (19.6 μM); ▲—▲ 0.667 mg/ml β-Lg A added (39.2 μM). The i/v ordinate represents the reciprocal of the initial velocity expressed as n moles of p-nitrophenol liberated per ml per min times 10^{-3}

Table 1. Effect of β-Lg on the kinetic parameters of spleen phosphoprotein phosphatase

Protein added	V_{max} [a]	K_m (mM)	K_i (μM)
10 mM acetate			
Buffer only	81.3 ± 0.8 [b]	1.38 [b] ± 0.08	–
β-Lg A	80.6	2.89 [c]	18 [d]
β-Lg B	82.0	2.50 [c]	24 [d]
β-Lg C	81.3	1.44 [c]	450 [d]
100 mM acetate			
Buffer only	80.2 ± 0.8 [b]	1.30 [b]	–
β-Lg A	83.6	1.40	–

[a] Velocity expressed as n moles of p-nitrophenol liberated/ml/min. The reaction mixtures were prepared as described in Material and methods with β-Lg at 19.6 μM

[b] Average of three determinations; other values two determinations, averaged

[c] Values represent "apparent" K_m (K_{map}) reflecting the change in calculated value due to the presence of β-Lg

[d] Apparent K_i calculated from $K_{map} = K_m + \frac{K_m}{K_i}[I]$. These are for comparison of genetic variants at this specific concentration (Segel 1975)

Electrostatic effects on the inhibition

The inhibition by β-Lg of the hydrolysis of p-NPP by spleen or mammary phosphoprotein phosphatase was drastically decreased in 100 mM acetate (or 10 mM acetate + 90 mM chloride). At 10 mM, acetate does not decrease the inhibitory action of β-Lg A; either sodium or ammonium cations can be employed. In the absence of β-Lg, the calculated value of K_m is not effected by ionic strength (Table 1). Data are for the spleen enzyme.

Effect of genetic variants of β-Lg on enzymatic activity

Three genetic variants of bovine β-Lg (β-Lg A, B, and C) were tested for their ability to inhibit the action of phosphoprotein phosphatase on p-NPP. The results shown in Table 1 indicate that β-Lg A > B > C in inhibitory effect for both spleen and mammary enzymes. The differences observed may be related to the structural changes induced by the amino acid substitutions.

Denaturation of β-Lg

β-Lg is susceptible to base denaturation (Pessen et al. 1985) and when treated overnight at 5 °C at pH 9.5 (NH_4OH), a partially reversible denaturation of the β-Lg occurs. The inhibitory response of base treated β-Lg A was compared (after neutralization with acetic acid) with native β-Lg A, which had been treated overnight with the same molarity of ammonium acetate (10 mM) at pH 6.0. This partial denaturation caused the inhibition to drop from 35% in the case of the "native molecule" to 8% in the case of the base treated β-Lg. Hence, effective inhibition of the hydrolysis of p-NPP by phosphoprotein phosphatase may require the native conformation of the β-Lg A molecule.

Enzyme specificity of the inhibition

The enzymes potato acid phosphatase and calf intestine alkaline phosphatase were tested to determine whether or not they could be inhibited by β-Lg A. At pH 6.0 in 10 mM ammonium acetate, the hydrolysis of p-NPP by potato acid phosphatase was not inhibited by β-Lg. At pH 8.0 in 10 mM *tris* hydroxymethyl aminomethane, β-Lg A did not inhibit the hydrolysis of p-NPP by calf intestine alkaline phosphatase. In addition, the hydrolysis of p-nitrophenyl sulfate and p-nitrophenyl β-glucuronide by snail aryl sulfatase and β-glucuronidase were not inhibited by β-Lg A. In contrast, the same degree of inhibition by β-Lg was observed with an acid phosphoprotein phosphatase purified from bovine mammary gland (Farrell et al. 1988 a).

Demonstration of an enzyme-inhibitor complex

Sucrose density gradient centrifugation was used to detect a spleen phosphoprotein phosphatase-β-Lg A complex. The best resolution was obtained on gradients of 5 to 11% and the results of two separate experiments have been averaged in Fig. 3. In each experiment, two tubes contained phosphoprotein phosphatase alone, two contained enzyme + β-Lg and two contained a mixture of cytochrome *c* and ovalbumin. The enzyme alone moves slightly ahead of cytochrome *c*, while in the presence of β-Lg at pH 6.0 in 10 mM acetate buffer a complex is formed which now moves somewhat ahead of ovalbumin. Note also that for an identical amount of enzyme the area under the curve of the complex is decreased showing β-Lg A inhibition after centrifugation.

Kinetic evidence for enzyme – inhibitor interactions

In order to further test the hypothesis that an enzyme-inhibitor complex was involved in the inhibition, expansion of the experiments shown in Fig. 2 to a 7 × 5 plot (7 concentrations of β-Lg at the 5 concentrations of p-NPP used in Fig. 2) was carried out at higher β-Lg concentrations. The slopes obtained from these experiments were computed and deviated from classical behavior; they are plotted as a function of β-Lg (inhibitor) concentration in Fig. 4. The resulting curve resembles a saturation binding isotherm with the degree of inhibition reaching a limit; when analyzed as such

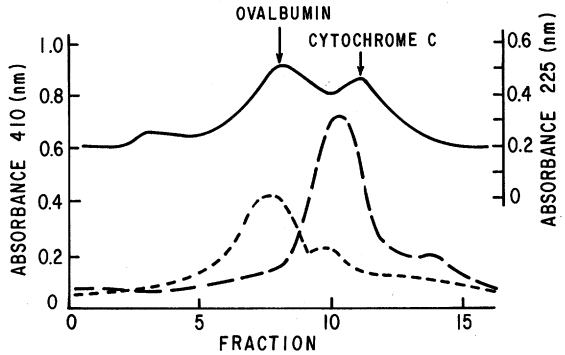


Fig. 3. The interaction of β-Lg with spleen phosphoprotein phosphatase as demonstrated by sucrose density centrifugation. The sucrose gradients were from 5 to 11% and the samples were treated as described in Material and methods. After centrifugation, fractions of 5 drops each were collected and assayed for enzymatic activity by measuring the A_{410} of the p-nitrophenol produced. The curves represent enzyme alone (— —), ovalbumin and cytochrome *c* (————), and enzyme plus β-Lg (– – – – –). The position of the marker proteins was determined by measuring the absorbance at 225 nm

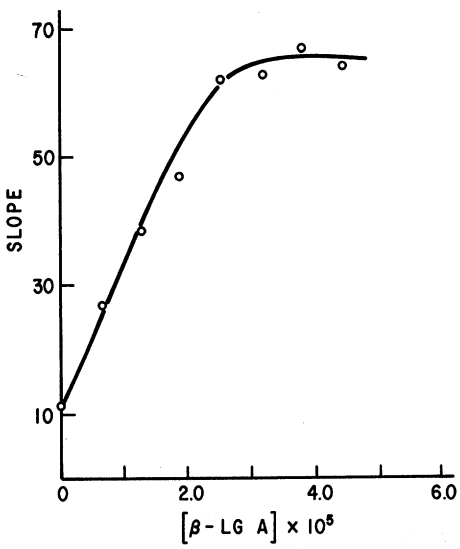


Fig. 4. Replot of slope against β-Lg concentration obtained by extending the experiments shown in Fig. 2 to higher β-Lg concentrations. The slopes (K_m/V_m) were calculated as described in Material and methods, and the data were then fit with Eq. (1) to determine K_I

by Eq. (1) a $K_{0.5}$ of 15 ± 4 μM was calculated from three experiments. Sedimentation velocity studies of the binding of β-Lg to cytochrome *c* showed similar saturation binding behavior (Brown and Farrell 1978). The degree of aggregation of β-Lg per se, as judged by sedimentation velocity, is not influenced by p-NPP under the conditions tested; it behaves as a dimer as predicted by the literature for β-Lg (Pessen et al. 1985). Thus, formation of a reversible β-lactoglobulin-enzyme complex capable of limiting the enzymatic hydrolysis of the substrate seems plausible.

Effect of β-lactoglobulin on casein and ATP hydrolysis

The dephosphorylation of α_{s1}-casein by both spleen and mammary phosphoprotein phosphatases was studied in the presence and absence of the activator 2-mercaptoethanol. In 150 mM acetate, pH 5.9, at an α_{s1}-casein concentration of 128 μM (monomer concentration) no inhibition of the reaction by β-Lg A at 20 μM was observed. When the reaction was carried out in 10 mM ammonium acetate at the same concentration of α_{s1}-casein and extended for up to 3 h, no inhibition was observed either in the presence or absence of the activator. β-Lg A thus inhibits the hydrolysis of p-NPP, but does not inhibit the dephosphorylation of α_{s1}-casein. Experiments carried out in 10 mM ammonium acetate showed only 3 ± 1% inhibition of the initial velocity of either spleen or mammary phosphoprotein phosphatase. In contrast, ATP hydrolysis by both enzymes was inhibited (20%) under conditions in which p-NPP hydrolysis is inhibited to 40% by β-Lg.

Effect of other proteins on enzymatic activity

Other milk proteins were tested for their ability to inhibit this system on an equal weight basis, bovine lactoferrin, bovine serum albumin and bovine γ-globulin were without effect. In fact, these proteins appeared to enhance the activity slightly. This observation can be explained by the "sparing effect" of added protein on the enzyme activity in general (Farrell et al. 1990). α-Lactalbumin, which has been shown to participate in the synthesis of lactose (Brodbeck et al. 1967), exhibited inconsistent behavior with respect to phosphoprotein phosphatase inhibition. Although similar in lactose synthetase activity, four different preparations of α-lactalbumin yielded inhibitions ranging from zero to 70%. Older studies by Robbins and Homes (1972) have reported that some physical chemical properties of α-lactalbumin depended greatly upon the method of preparation of the protein. More recent evidence has shown that the susceptibility of α-lactalbumin to denaturation is related to its degree of saturation with Ca^{2+} (Berliner and Johnson 1988). The best preparation of α-lactalbumin, with respect to phosphatase inhibition, yielded competitive inhibition of the spleen enzyme with a K_i of 24.1 μM.

Effect of Ca^{2+} on inhibition

The inhibition of spleen (and mammary) phosphoprotein phosphatases by β-Lg was studied as a function of Ca^{2+} concentration (Fig. 5). At low Ca^{2+} concentration little or no effect on the inhibition is seen, but as the concentration of Ca^{2+} approaches 3 to 5 mM a significant lessening of the inhibition occurs. The data of Fig. 5 were analyzed with Eq. (1) and the best fits were found for n = 2 and K_D equal to 3.68 mM. Similar results were found for Ca^{2+}-saturated α-lactalbumin. The results are shown in Table 2.

Binding of Ca^{2+} to β-lactoglobulin

The results of Fig. 5 can be taken to indicate that Ca^{2+} binds to β-Lg, thus alleviating inhibition. Studies of the binding of Ca^{2+} to β-Lg were conducted using the murexide dye binding assay. The data are shown in Fig. 6 and were analyzed using Eq. (1) except $\bar{v}$ (moles of ligand bound/moles of β-Lg monomer) replaces V_{max}. The results of the analysis are given in Table 2.

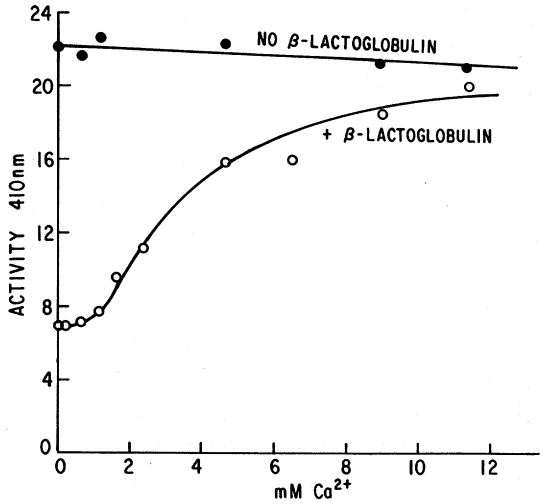


Fig. 5. Ca^{2+} alleviation of the inhibition of mammary phosphoprotein phosphatase by β-lactoglobulin. Upper line: effect of Ca^{2+} alone on enzyme activity; lower curve: showing inhibition by β-Lg A (39.2 μM) and restoration of activity with increased Ca^{2+} concentration. Data were analyzed with Eq. (1); n = 2. In these experiments the buffer was 10 mM Pipes (piperazine-N-N′bis(2-ethanesulfonic acid) which has no affinity for Ca^{2+}

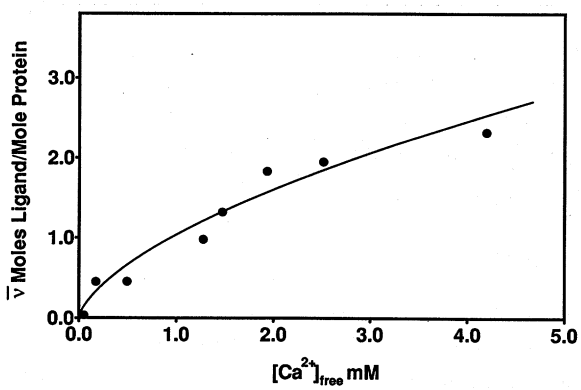


Fig. 6. Binding of Ca^{2+} to β-Lg using the murexide dye binding method of Scarpa (1972). Concentrations of β-Lg, 348 μM; buffer 10 mM Pipes as in Fig. 5

Table 2. Analysis of effects of Ca^{2+} on alleviation of β-lactoglobulin and α-lactalbumin inhibition of phosphoprotein phosphatase, and comparison with Ca^{2+} binding data

Protein	Kinetic data[a]		Binding data	
	K_D (mM)	n	K_D (mM)	$\bar{v}$
β-lactoglobulin	3.67	2	3.14	4[b]
α-lactalbumin	5.91	2	3 to 6	~ 4[c]

[a] Analysis of data shown in Fig. 5 using Eq. (1)
[b] This study
[c] Berliner and Johnson (1988)

Table 3. Comparison of isoelectric points of selected phosphatases and proteins[a]

Protein	pI
α-lactalbumin	4.5
β-lactoglobulin	5.1
Spleen phosphoprotein phosphatase	8.7
Mammary phosphoprotein phosphatase	8.7
Cytochrome *c*	10.6
Potato acid phosphatase	6.6

[a] Estimated on the same system following the method of Chen and Chen (1988)

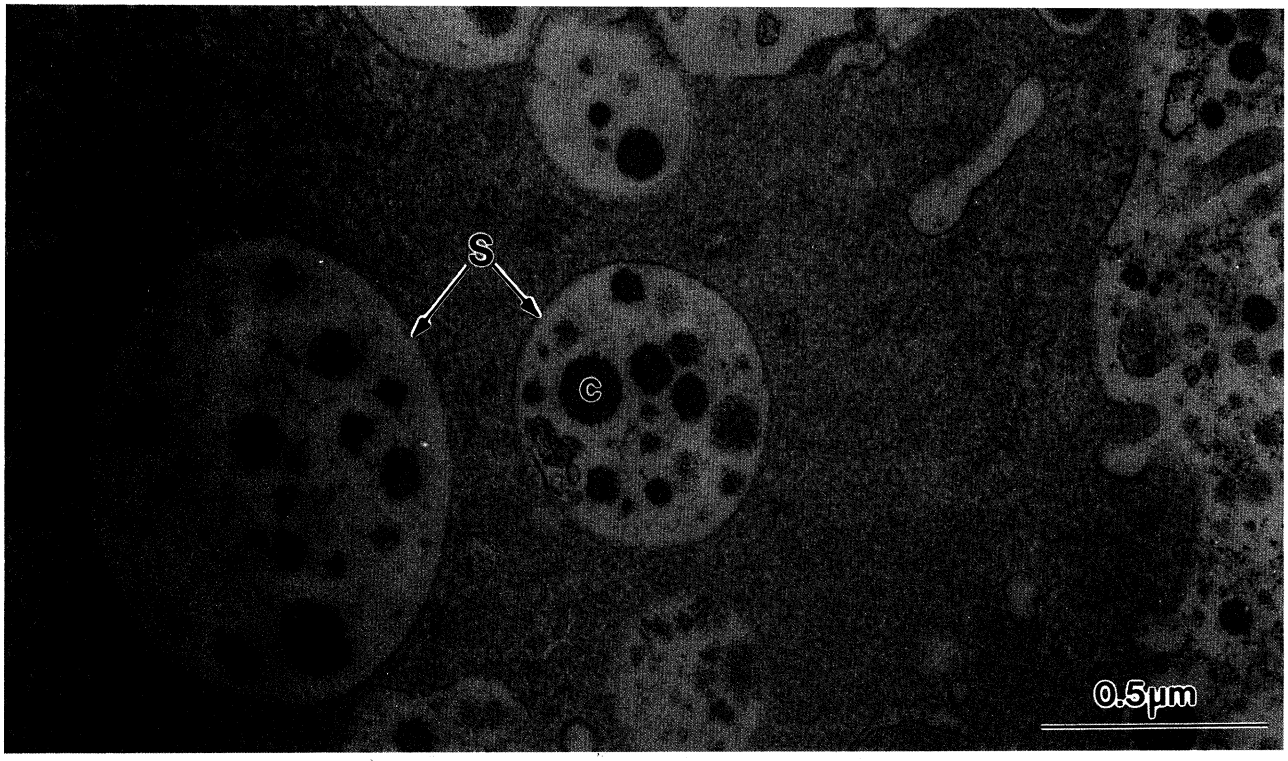
Isoelectric points of the proteins

The isoelectric points of the spleen and mammary phosphoprotein phosphatases and potato acid phosphatase were estimated by isoelectric focusing following the procedures of Chen and Chen (1988). Results showed the two phosphoprotein phosphatases to be basic. Their calculated pIs. are compared with those of β-Lg and cytochrome *c* and potato acid phosphatase in Table 3.

Discussion

β-Lactoglobulin and α-lactalbumin inhibit the hydrolysis of p-NPP by the phosphoprotein phosphatases of bovine spleen and mammary gland. The mammary enzyme is nearly identical in its properties to the well characterized acid phosphatase of bovine milk (Farrell et al. 1988 a, Andrews and Pallavicini 1973, Shahani et al. 1973). In lactating rat mammary gland, acid phosphatase is secreted via the lumen of the rough endoplasmic reticulum and through Golgi derived secretory vesicles as shown in Figs. 7 and 8 (Leung et al. 1990). Similar results were found for bovine mammary gland (unpubl. observation). Thus the route of secretion of phosphatase parallels that of the whey proteins (Shappell et al. 1986). Examination of the mechanism of inhibition of the phosphoprotein phosphatases may shed some light on the possible physiological relevance of these observations.

The greatest inhibition occurs near the pH of optimum enzyme activity (pH 5.6). The two major genetic polymorphs of β-Lg (A and B) inhibit the enzyme to differing degrees (A > B), while a third polymorph (β-Lg C) yields little inhibition. These differences are significant since β-Lg A has been shown to undergo a specific octamerization below pH 5.0; β-Lg B undergoes this reaction to a lesser extent, while the amino acid differences in C do not permit this reaction to



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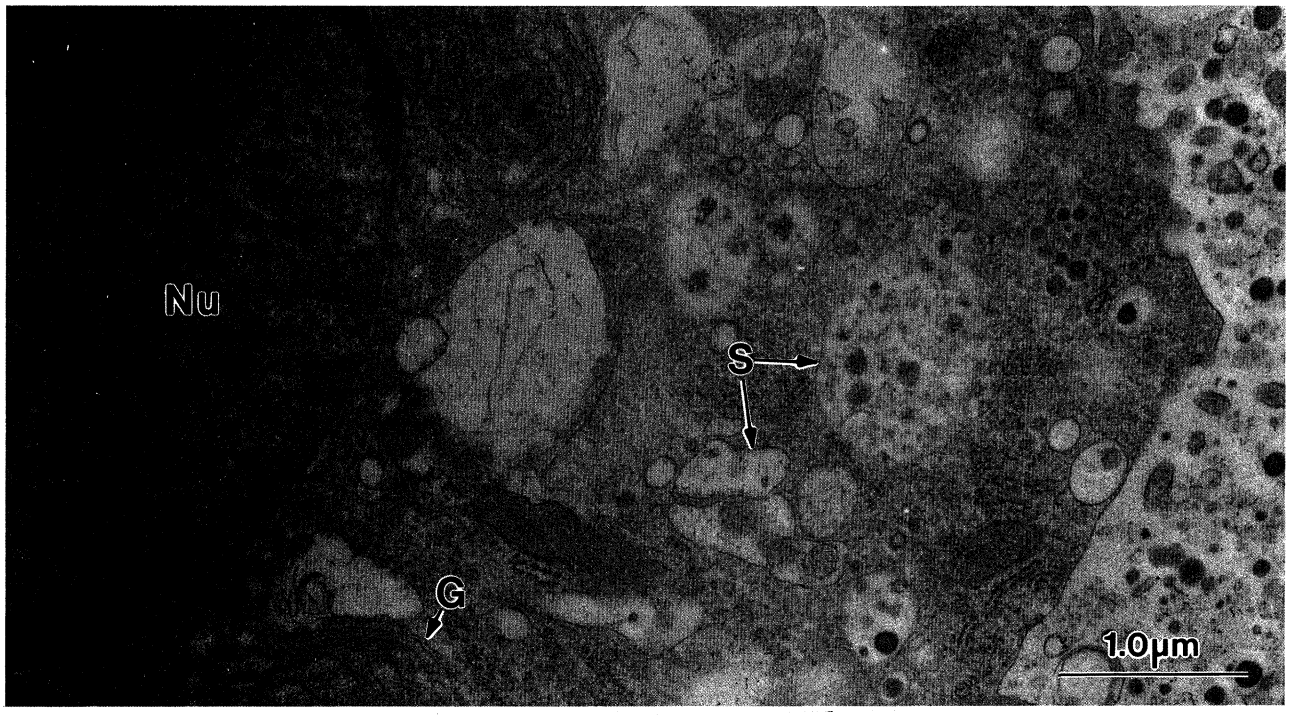
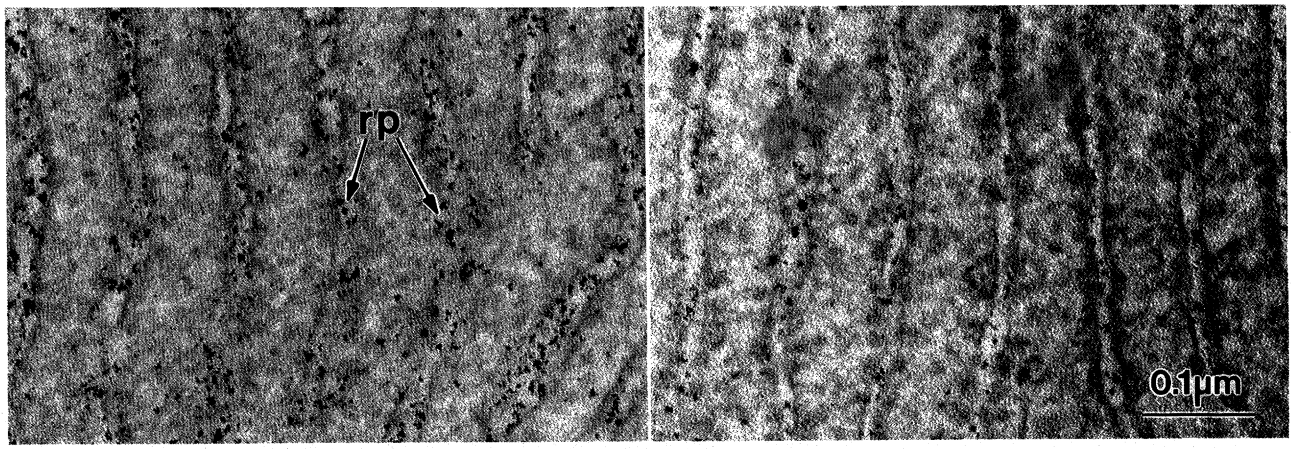


b

Fig. 7. Histochemical evidence for the route of secretion of milk acid phosphatase. **a** In the presence of p-NPP, reaction product of acid phosphatase was found near casein micelles, both in intracellular secretory vesicles and in milk. **b** In the absence of p-NPP, less stain was observed in secretory vesicles and in milk. *c* Casein micelle, *S* secretory vesicle, *G* Golgi vesicle (Leung et al. 1990)



a b

Fig. 8. Englarged area of mammary secretory cells showing reaction products on RER. **a** Complete mixture. **b** Control tissue incubated without p-NPP. *rp* Reaction product (Leung et al. 1990)

occur (Pessen et al. 1985). This physical aggregation does not occur to any great extent under the conditions of the enzymatic assay of phosphoprotein phosphatase but the conformational changes related to it do (Pessen et al. 1985). Hence, the "carboxyl-rich area" responsible for the octamerization reaction in β-Lg A could participate in the inhibition of p-NPP hydrolysis, particularly if protein—protein interactions play a role in the inhibition. The pH profile of the inhibition, which follows well defined conformational changes in β-Lg, and the effect of increased ionic strength would argue in favor of a β-Lg—enzyme interaction. In addition, the pH denaturation experiment demonstrates the requirement for some native conformation of the β-Lg. The initial series of kinetic experiments showed strong evidence for classical competitive inhibition by β-Lg; a β-Lg—phosphoprotein phosphatase interaction was demonstrated on sucrose density gradients. The expansion of the kinetic experiments yielded (Fig. 4) a curve for the replot of the slope against inhibitor which could be analyzed as a binding isotherm; $K_{0.5}$ for inhibition (I_{50}) was calculated to be 15 μM. Brown and Farrell (1978) showed a strong interaction between cytochrome *c* and β-Lg. A K_D of 20 μM can be calculated from their ultracentrifuge data; this value is within the range calculated from the β-Lg-phosphatase kinetic data (15 ± 4 μM from Fig. 4 and 19 μM from Fig. 2). The estimated value of pI for the spleen and mammary enzymes is 8.7, while that of the cytochrome *c* is 10.6. Thus all three proteins which show a strong interaction with whey protein have a net positive charge at the pH of the assay, while β-Lg exhibits a net negative charge. Since both the inhibition of phosphoprotein phosphatase by β-Lg and its binding to cytochrome *c* (Brown and Farrell 1978) are diminished by buffer or salt at 100 mM, electrostatic interactions appear to be the driving force for these reactions. From chromatographic and spectral data (Farrell et al. 1987) it would appear that β-Lg has a strong affinity for the aromatic compounds tested as substrates. Since the K_D for the p-NPP complex with β-Lg is 31 μM and K_m for p-NPP reaction with phosphoprotein phosphatase is only 1.4 mM, it would be expected that at very low substrate concentrations some inhibition could occur based on binding considerations alone. Recalculation of K_m, taking binding into account, would change K_m from 1.38 mM to 1.36 mM, a reduction of only 2%. In fact, acting as competitive inhibitors both β-Lg and α-lactalbumin increase the apparent or calculated K_{map}. The most likely explanation for the inhibition observed appears to be that the β-Lg-p-NPP complex serves as the inhibitor, however E-I complexes are observed in the absence of p-NPP (Fig. 3) so that only β-Lg and enzyme are required. In addition, potato acid phosphatase and alkaline phosphatase which have acidic pIs are not inhibited, even though β-Lg binds p-NPP under these assay conditions.

An interesting aspect of these experiments is the inability of β-Lg to inhibit dephosphorylation of α_{s1}-casein. In 10 mM acetate α_{s1}-casein is essentially monomeric at the concentrations tested (1 to 3 mg/ml). The percent inhibition of a reaction can be calculated if the concentration of substrate and inhibitor as well as K_m and K_i are known (Segel 1975):

$$\%i = \frac{[I]}{[I] + K_i + \frac{K_i}{K_m}[S]} \times 100. \qquad (2)$$

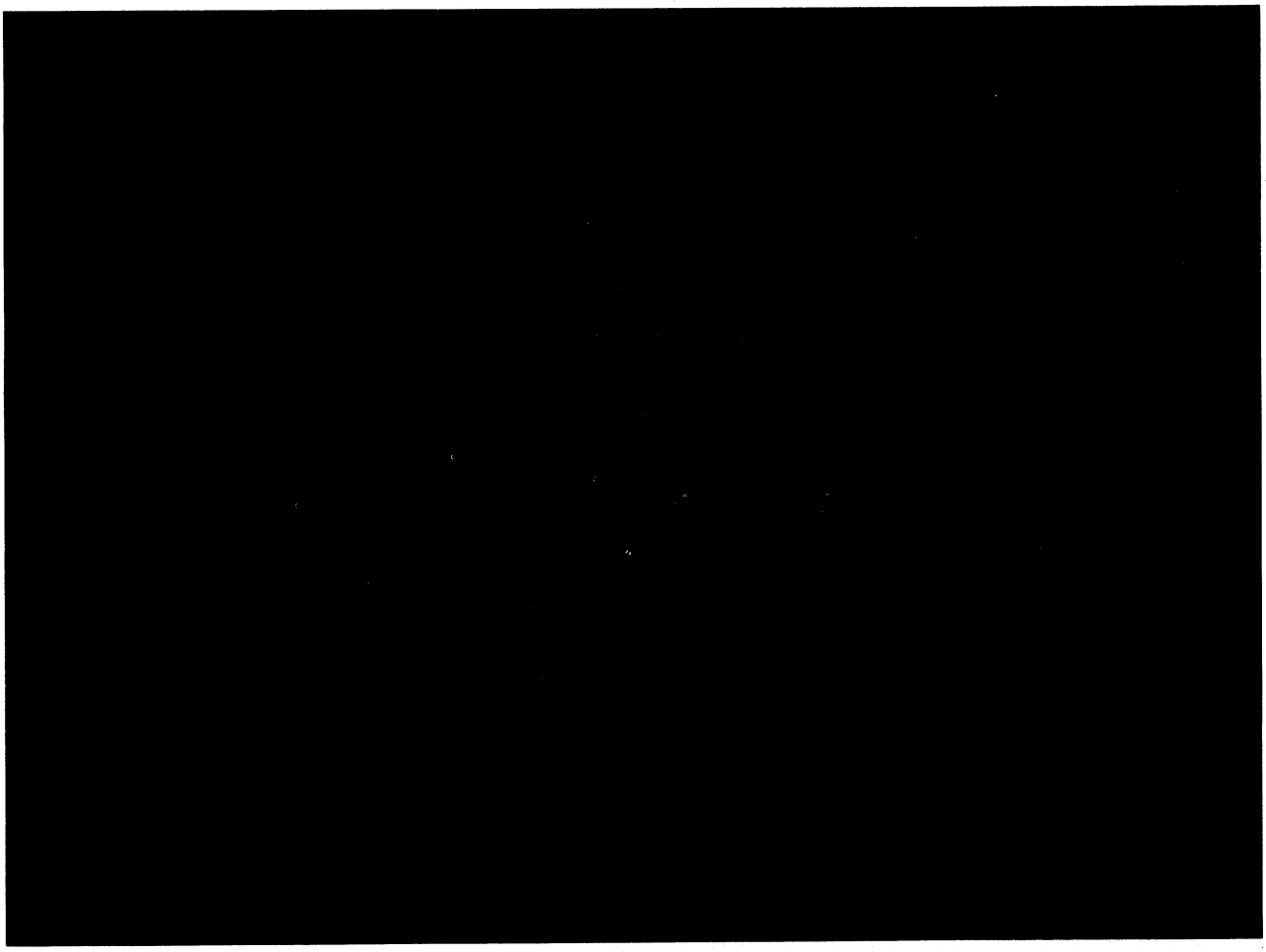


Fig. 9. Three dimensional molecular model of α_{s1}-casein, backbone and side chains in cyan, PSer residues in red (Farrell et al. 1990 a)

Thus for β-Lg A at 19 μM, with a K_i of 20 μM a [p-NPP] of 1,000 μM and a K_m of 1,380 μM, a 35% inhibition is predicted, and 40% observed. A similar case may be made for the observed inhibition of ATP hydrolysis. In contrast for α_{s1}-casein at 128 μM (monomeric) and with a K_m of 42 or 65 μM for mammary and spleen enzymes respectively (Farrell et al. 1988 a) an inhibition of 20 to 25% is predicted. Experimentally no inhibition was observed. A likely explanation for this lies in the nature of the substrate. Three dimensional molecular modeling predictions (Farrell et al. 1990 a) show that 6 of 8 serine phosphate residues of α_{s1}-casein lie in close proximity to each other on the right shoulder of the molecule. This region is bounded by Pro-27 and Pro-75 (Fig. 9). Thus when one phosphate is hydrolyzed, the enzyme is in such close proximity to 5 other possible substrates that the E-S complex does not directly disassociate and so the phosphoprotein phosphatase can go on to rapidly dephosphorylate other adjacent residues. This being the case, the 6 phosphate residues may represent a cooperative class of sites and so $K_m \approx K^n = K^6$ (Wyman 1964); the true value for K_m is thus closer to 2 μM. Inserting this value into Eq. (2), the predicted percent inhibition drops to 1 to 2% in line with the experimental observations. Here it is assumed that K_i for casein is similar to that for p-NPP (20 μM) and to K_D for the β-Lg-cytochrome *c* reaction which is also 20 μM. Although bovine serum albumin, bovine lactoferrin and bovine γ-globulin did not affect the reaction, the results obtained with α-lactalbumin cast doubt on the biological significance of this inhibition reaction with respect to β-Lg. Ingebritson and Cohen (1983) have pointed out that the true substrates for phosphoprotein phosphatases may be phosphorylated enzymes and that the dephosphorylation of these enzymes by phosphoprotein phosphatases may be of importance in regulating their activity. However, there is no known biological

role for the phosphoprotein phosphatases examined in this study, although the enzyme has long been recognized as a component of milk.

If the reactions between β-Lg (or α-lactalbumin) and the milk/mammary phosphoprotein phosphatases are primarily electrostatic in nature, then the abolishment of inhibition by 100 mM salt is expected. What is unexpected is the ability of Ca^{2+} at only 12 mM to block binding of the proteins to the enzyme (Fig. 5). The demonstration that β-Lg has Ca^{2+} binding sites ($K_D = 3.14\,\mu M$) and that $K_{D0.5} = 3.67$ mM for reversal of inhibition confirms that Ca^{2+} binding is involved. These binding sites are equivalent in strength to those of casein (Farrell et al. 1988 b), but much weaker than those of calmodulin or the high affinity Ca^{2+} site of α-lactalbumin (Berliner and Johnson 1988). The key point here is that the K_Ds fall in the range of free Ca^{2+} concentrations (5 to 8 mM) in milk and presumably in Golgi vesicles (Holt 1985). Interestingly the total $Na^+ + K^+$ concentration is 62.4 mM which is the mid-range for Na^+ or K^+ alleviation of inhibition of phosphatase by β-Lg. The above information argues that there could be a physiological role for this reaction. The milk acid phosphatase and the mammary enzyme (Farrell et al. 1988 a) are similar in nature and hydrolyze a variety of phosphates ranging from ATP to β-glycerol phosphate. Leung et al. (1990) demonstrated the occurrence of acid phosphatase in Golgi vesicles and in milk. It could be speculated that the phosphatases of milk serve a purpose in regulating P_i content by dephosphorylating casein or other organic phosphates. Their activity in turn would be modulated directly by whey proteins, indirectly by Ca^{2+}, K^+, and Na^+ and additionally through product inhibition by P_i released.

The above speculation is based on in vitro experiments but these observations provide an interesting model for demonstrating how ligand binding to "weak" sites could regulate enzyme activity in vivo, and perhaps help to control the ionic environment of milk itself.

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Biochemical and immunological comparison of bovine butyrophilin with a butyrophilin-like glycoprotein in guinea pig milk-fat-globule membrane

Evidence that the guinea pig protein is developmentally regulated and specifically expressed in lactating mammary tissue

Summary. A glycoprotein of apparent M_r 63,000 in the fat globule membrane (FGM) of guinea pig milk has been purified, characterized and compared with bovine butyrophilin. The two proteins resist extraction from FGM with non-ionic detergents, they share common epitopes and have similar but not identical amino acid compositions and N-terminal sequences. Digestion of the proteins with specific proteinases leads to the release of some similar peptides. The guinea pig protein therefore appears to be closely related to bovine butyrophilin. The distribution of the guinea pig protein was determined in tissues and milk by a combination of immunoblotting techniques and immunofluorescence microscopy. The guinea pig protein was detected in lactating mammary tissue and accounted for approximately 0.3% of tissue proteins. No evidence for the expression of this protein was obtained in mammary tissue from virgin or mid-pregnant animals, or in kidney, uterus, spleen, heart, pancreas, intestine, liver, lung, brain, ovary, sublingual salivary gland, skin, anal apocrine sweat glands or blood cells. The 63,000 Da guinea pig protein is therefore a member of a family of butyrophilin-like proteins which are specifically expressed in mammary epithelial cells during lactation. The results are discussed with reference to the possible role of butyrophilin in milk secretion, and the use of butyrophilin as a tissue-specific marker of differentiation in the mammary gland.

Keywords: Butyrophilin; Fat secretion; Mammary gland; Lactation.

Abbreviations: FGM fat-globule membrane; PMSF phenylmethylsulphonyl fluoride; SDS sodium dodecyl sulphate; TBS Tris-buffered saline.

Introduction

Butyrophilin is an acidic glycoprotein associated with the membrane surrounding fat droplets in bovine milk (Franke et al. 1981, Heid et al. 1983, Jack and Mather 1990). The protein has an apparent M_r of 67,000 [the M_r of the bovine butyrophilin polypeptide is 56,460 (determined indirectly from the amino acid sequence derived from a full-length cDNA) and the M_r of fully processed butyrophilin is approximately 60,000 (Jack and Mather 1990); for practical convenience the M_rs referred to in this paper are the *apparent* values determined by SDS/polyacrylamide gel electrophoresis]. Butyrophilin exists in several isoelectric variants, contains 'N-linked' oligosaccharide and tightly bound fatty acids (Mather et al. 1980, Franke et al. 1981, Keenan et al. 1982, Heid et al. 1983, Johnson et al. 1985). Butyrophilin is an integral membrane protein with a single membrane-spanning domain (Jack and Mather 1990). Surveys of bovine tissues with specific antibodies (Franke et al. 1981) and cDNA (Jack and Mather 1990) suggest that expression of the butyrophilin gene is restricted to the mammary gland. Furthermore, butyrophilin appears to be developmentally regulated, since it was not detected with antibodies in mammary tissue from virgin animals (Franke et al. 1981) and specific mRNA increases dramatically in late pregnancy (Jack