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COOPERATIVITY OF THE CONCANAVALIN A INHIBITION OF BOVINE MILK FAT GLOBULE MEMBRANE 5'-NUCLEOTIDASE

RESPONSE TO EXTRACTION OF NUCLEOTIDASE AND OF PUTATIVE CYTOPLASMIC SURFACE COAT COMPONENTS *

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Summary

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) of bovine milk fat globules can be solubilized by deoxycholate from either isolated globule membranes or washed cream. The solubilized and membrane-bound enzymes exhibit similar K_m values and are inhibited by concanavalin A by an apparent noncompetitive process. The soluble enzyme shows positive cooperativity for the inhibition (Hill coefficient of 2) at 37°C, but the membrane enzyme exhibits essentially no cooperative effect. At lower temperatures (5 or 20°C) the cooperative effect in the inhibition of the soluble enzyme is lost. Colchicine and cytochalasin D failed to induce cooperativity of the concanavalin A inhibition of the membrane enzyme, but induction of cooperativity occurred when membranes were extracted with glycine/EDTA/mercaptoethanol, releasing a major protein component with a polypeptide molecular weight of 155 000. We suggest that the interaction of this component with the membrane imposes restraints on the behavior of the nucleotidase which are reflected in the cooperativity of the inhibition of the enzyme by concanavalin A.

Introduction

Based on perturbations with microfilament and microtubule-specific drugs [1—6] and morphological observations [7], studies on the mobility of cell

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surface antigens and lectin receptors have implicated cytoplasmic cytoskeletal elements in the control of cell surface organization and properties. We have recently suggested that the ectoenzyme 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) can be used as a cell surface 'reporter' by observing the inhibition of the enzyme by the plant lectin concanavalin A [8]. The inhibition can be seen with intact cells [9] and is sensitive to the 'state' of the cell [8]. For example, the inhibition process is not cooperative in 13762 ascites rat mammary adenocarcinoma cells, but cooperativity is induced by treatment of the cells with cytochalasins, colchicine, dibucaine and ionophore A23187 plus Ca^{2+} [8], agents which alter the agglutinability, morphology and receptor mobilities in many cell types [2]. Cell surface envelopes isolated from 13762 ascites cells 'stabilized' by Zn^{2+} treatment show no cooperativity [9]. If the envelopes are extracted with glycine/EDTA/mercaptoethanol at pH 9.5, cooperativity is induced. This extraction solubilizes cytoskeletal proteins, including actin, α-actinin and actin binding protein associated in a complex with the interior surface of the envelopes [10]. The implication of all of these studies is that association of cytoskeletal elements with the plasma membrane alters certain membrane properties which are reflected in the behavior of the nucleotidase.

We have now performed similar studies on the bovine milk fat globule membrane, which is a derivative of the apical surface of the secretory epithelial cells of mammary gland [11]. Although this membrane contains little, if any, actin [12], it does have a coat of material at the inner membrane surface [12]. Extraction and drug perturbation studies on milk fat globule membrane show that the cooperativity of the nucleotidase inhibition is sensitive to extractions that affect the putative milk fat globule membrane coat polypeptides, but is insensitive to microfilament and microtubule-perturbing drugs.

Experimental procedures

Materials. Raw cream was obtained from the Oklahoma State University Dairy. All enzymes and chemicals for enzyme assays were products of Sigma Chemical Company. Highly purified lectins were obtained from Miles-Yeda, Ltd. Chemicals for electrophoresis were obtained from Eastman or Bio-Rad. Other chemicals were reagent grade or of the highest purity available.

Preparation of milk fat globule membranes. Raw cream was washed by three suspensions in 0.25 M sucrose, 10 mM imidazole, 2 mM $MgCl_2$ (pH 7.0) at room temperature [13]. The washed cream was suspended to a concentration of 33% in the same buffer and subjected to one cycle of freezing at −20°C for 20 h and thawing at 37°C. Membranes were isolated from the thawed 33% cream suspension by centrifugation at 40 000 × *g* for 1 h at 5°C and washed twice by centrifugation. Membranes were dialyzed overnight against water at 4°C before use for protein and enzyme assays.

Enzyme assays. 5′-Nucleotidase activity for isolated membranes and deoxycholate-solubilized enzyme was determined spectrophotometrically by coupling the reaction of 5′-nucleotidase to the deamination of adenosine as described by Ipata [14]. Except where noted, the assay medium included 0.1 mM 5′-AMP, 1 mM $MgCl_2$, 0.1 mM glycerol 2-phosphate, 2 units adenosine deaminase,

50 mM Tris-HCl (pH 7.9), and the enzyme sample in a final volume of 3.0 ml.

In some experiments, as noted, 5′-nucleotidase activity was assayed by a modification of the method of Morré [15] in which the reaction mixture (final volume of 0.4 ml) consisted of 50 mM Tris-HCl (pH 7.9), 10 mM AMP, 1 mM $MgCl_2$, and appropriate enzyme sample. The reaction mixture was incubated at 37°C for 15 min and the reaction terminated by addition of 0.2 ml 0.32% $HClO_4$. Liberated phosphate was determined by the method of Lazarus and Chou [16].

Preliminary studies indicated that preincubation in the assay medium was not necessary and that optimal enzyme activity was obtained with 0—1 mM Mg^{2+} and pH 7.5—8 for both membrane and soluble enzyme.

For enzyme assays at different temperatures (5—40°C), the temperature of the cuvettes was controlled by a circulating constant temperature bath. Reaction temperatures were measured by insertion to a thermocouple directly into the cuvettes. In all measurements reported the initial rates of reaction were linear for several minutes and proportional to the amount of protein used.

Solubilization of 5′-nucleotidase. For a typical enzyme preparation 17.5 mg membrane protein was suspended at 37°C for 2 h in 42 ml 0.25% (w/v) deoxycholate, 50 mM Tris-HCl (pH 7.9). The membrane suspension was centrifuged at 100 000 × *g* for 1 h and the supernatant collected. About 95% of the 5′-nucleotidase activity was present in the supernatant. The 5′-nucleotidase activity could also be extracted directly from washed cream by suspending 14 g washed cream (28—35 mg protein) in 42 ml 1.0% deoxycholate/50 mM Tris, pH 7.9. After centrifugation at 100 000 × *g* for 1 h, about 95% of the enzyme activity remained in the supernatant.

Preparation of divalent concanavalin A. Succinyl-concanavalin A was prepared by a modification of the method of Gunther et al. [17]. At room temperature 20 mg concanavalin A were dissolved in 4 ml saturated sodium acetate. The protein solution was added to 1 ml saturated sodium acetate containing 3 mg succinate anhydride. The solution was stirred at 4°C for 1 h, dialyzed overnight against water and lyophilized. This procedure was repeated twice to obtain succinyl-concanavalin A.

Membrane perturbations. Colchicine or cytochalasin D was incubated with milk fat globule membrane for 30 min at 37°C. Subsequently, concanavalin A was added and the mixture incubated an additional 10 min at 37°C prior to assaying at the same temperature.

$MgCl_2$, KCl/10 mM Tris-HCl (pH 7.4) and glycine/EDTA/mercaptoethanol (pH 9.5) extractions were performed at 4°C for 16 h. Aliquots from the extraction tubes were incubated for 15 min at 37°C. Concanavalin A was then added and incubated another 10 min at 37°C before assaying at the same temperature.

Electrophoresis of extracted samples. Milk fat globule membrane samples from the various extractions were centrifuged at 100 000 × *g* 1 h and the pellets resuspended in 0.25 M sucrose, 10 mM imidazole, 2 mM $MgCl_2$ (pH 7.0). Membrane and supernate samples were solubilized by incubating at 100°C after addition of an equal volume of solution containing 0.125 M Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate and 5% β-mercaptoethanol. Electrophoresis was performed on slab gels (13.5 cm × 16.0 cm × 0.75 mm) prepared with a linear 5—15% acrylamide gradient using the gel buffer system of King and Laemmli [18].

Results

Concanavalin A perturbation of membrane 5'-nucleotidase

The addition of concanavalin A to the membranes caused inhibition of the 5'-nucleotidase activity of isolated milk fat globule membrane. The concentration required for half-maximal inhibition of the enzyme by concanavalin A was 250 μg/mg membrane protein. If membrane samples were incubated in the presence of concanavalin A and 50 mM α-methylmannoside, at the highest concanavalin A concentrations used 90% of the inhibition was reversed by this competitive inhibitor for the saccharide binding site. Both the inhibition reaction and the reversal process proceeded rapidly and were virtually complete within 5 min. A Hill plot for the inactivation by concanavalin A of the membrane-bound 5'-nucleotidase gave a Hill coefficient of 1.0 indicating no apparent cooperativity in the inhibition process for the membrane-bound enzyme.

Treatment with a fairly high concentration of concanavalin A on membrane 5'-nucleotidase activity at substrate concentrations from 0.02 to 0.2 mM AMP gave a double-reciprocal plot showing apparent non-competitive inhibition. In the absence of concanavalin A the apparent K_m for the membrane-bound enzyme was 25 μM with a V of 15 μmol/h per mg protein. In the presence of 250 μg concanavalin A/mg protein the V decreased to 4.2 μmol/h per mg protein with no change in K_m.

Effects of detergents on 5'-nucleotidase

Addition of 0.05% deoxycholate to milk fat globule membranes increased 5'-nucleotidase activity by about 10%. Activity decreased to 80% of its original value in 1% deoxycholate and even further at higher concentrations. In contrast, dodecyl sarcosinate and dodecyl sulfate produced substantial inactivation of the enzyme at concentrations as low as 0.1 and 0.2%, respectively.

Fig. 1 shows the degree of solubilization of membrane protein and 5'-nucleotidase activity after treatment with increasing concentrations of deoxycholate at 37°C for 2 h. The deoxycholate concentration which gave maximal enzyme solubilization with minimal solubilization of membrane protein was 0.25% (6 mg detergent/mg protein). At lower temperatures the yield of solubilized enzyme was reduced. After treatment with 0.25% deoxycholate at 37°C, at least 95% of the enzyme activity and only 20% of the total membrane protein was solubilized. These conditions were subsequently used routinely to solubilize the enzyme. The procedure permitted an almost complete separation of the enzyme from the membrane. Essentially all of the 5'-nucleotidase activity of washed cream could be solubilized by a similar treatment. However, the 1% deoxycholate concentration necessary for this step was four times that required for comparable solubilization from membranes.

Effects of lectins on detergent-solubilized membranes

The deoxycholate-solubilized enzyme is also inhibited by high concentrations of concanavalin A, with 400 μg concanavalin A/mg solubilized protein required to give 50% inhibition. The increased concentration necessary for half-maximal inhibition is probably due to the interaction of the enzyme with

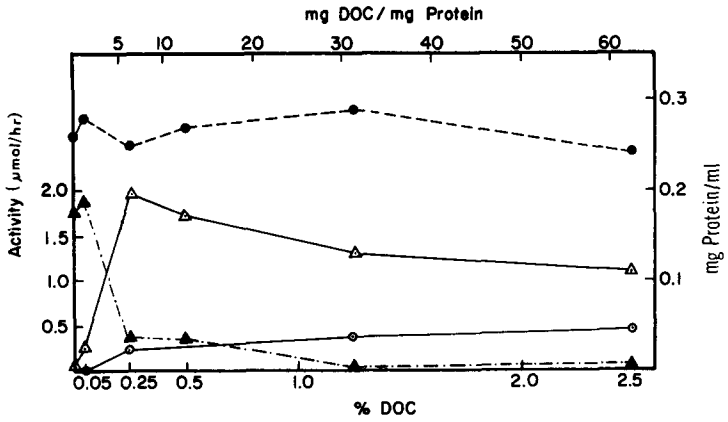

Fig. 1. Solubilization of membrane protein and 5′-nucleotidase activity with deoxycholate. Membrane samples were suspended in 50 mM Tris-HCl (pH 7.9) containing various concentrations of deoxycholate. After incubation for 2 h at 37°C, the suspensions were centrifuged at 100 000 × *g* for 1 h. The supernatant protein (○———○) was determined by the Lowry method and solubilized enzyme activity (△———△) was measured by the spectrophotometric method. The precipitate protein (●- - - - -●) and enzyme activity (▲- - - - -▲) were determined similarly.

deoxycholate. The Hill plot for these data reveals a significant difference between membrane-bound and deoxycholate-solubilized enzyme. The slope of the Hill plot for the soluble enzyme is 1.9, indicating a process involving a positive cooperative interaction between the enzyme and the lectin.

Results of kinetic studies on deoxycholate-solubilized enzyme in the presence of concanavalin A were similar to those for membrane-bound enzyme. Deoxycholate-solubilized enzyme shows apparently non-competitive inhibition of 5′-nucleotidase activity in the presence of concanavalin A. The apparent K_m was 25 μM with a V of 13 μmol/h per mg protein. Upon addition of 500 μg concanavalin A/mg solubilized protein, the V decreases to 4.9 μmol/h per mg protein.

To distinguish whether the modification of the cooperativity of the 5′-nucleotidase inhibition was due to general perturbations of carbohydrate moieties or due specifically to the binding of the tetravalent concanavalin A to specific carbohydrate residues, the effect of lectins specific for other monosaccharides commonly found in glycoproteins were studied. Soybean agglutinin showed no significant inhibition of 5′-nucleotidase activity. The inhibition of the enzyme by wheat germ agglutinin was only 10—20% of that observed with equivalent concanavalin A concentrations on a molar basis. No positive cooperativity was detected in the wheat germ lectin enzyme inhibition.

To assess the role of the multivalency of concanavalin A in mediating the cooperativity of enzyme inhibition, succinylated lectin (divalent) was prepared [17] and used in inhibition studies. The divalent concanavalin A from two separate preparations did not inhibit deoxycholate-solubilized 5′-nucleotidase activity at concentrations as high as 1 mg divalent lectin/mg protein. Typical inhibition was observed with native concanavalin A controls. In contrast membrane-bound 5′-nucleotidase activity was inhibited 30% by the same succinylated concanavalin A preparations under conditions (190 mg lectin/mg

membrane protein) in which the native concanavalin A gave 70% inhibition. The amount of modified lectin preparation required for minimum agglutination of SA-180 cells was increased 4—5-fold over native concanavalin A.

Effect of temperature on enzyme activity and lectin perturbations

Changes of nucleotidase activity with temperature were measured between 5 and 40°C. The hydrolysis rate of AMP at each temperature was linear over the range of enzyme concentrations used. Temperature effects on enzyme-lectin interactions were studied by comparing concanavalin A inhibition curves of 5′-nucleotidase activity obtained by incubating and assaying at 5, 20 and 37°C. Assay of deoxycholate-solubilized enzyme by the spectrophotometric method showed no positive cooperativity at 5 or 20°C, with the Hill coefficients being 1.0 and 1.05, respectively. At 37°C the same enzyme preparation had a Hill coefficient of 1.96 for concanavalin A inhibition. When assayed by the phosphate release method, membrane-bound enzyme had a Hill coefficient of about 1.2 for all three temperatures, whereas deoxycholate-solubilized enzyme had Hill coefficients of 1.8 at 37°C and 1.12 at 20°C.

Effects of putative cytoskeletal perturbants on the cooperativity of the concanavalin A inhibition of 5′-nucleotidase

Either colchicine, which disrupts microtubules, or cytochalasins, which alter microfilament organization, will induce cooperativity for the concanavalin A inhibition of 5′-nucleotidase in 13762 ascites cells [8], but neither is effective with milk fat globule membranes (Table II). However, extractions of milk fat globule membranes with glycine/EDTA/mercaptoethanol does induce cooperativity (Table II), as it does with 13762 ascites cell surface envelopes [9]. Since the milk fat globule membrane does not contain significant quantities of the cytoskeletal proteins removed by extraction from the ascites membranes, it was of interest to determine what components were being extracted. Electrophoresis on polyacrylamide gels in dodecyl sulfate showed that the predominant component eluted from milk fat globule membrane was one with a molecular weight of 155 000 (Fig. 2).

TABLE I

EFFECT OF TEMPERATURE ON HILL COEFFICIENTS OF 5′-NUCLEOTIDASE

S, spectrophotometric assay; P, phosphate assay.

Enzyme preparation	Assay method	Temperature (°C)	Hill coefficient
Membrane-bound enzyme			
(in homogenized membrane particles)	S	37	1.0
	P	37	1.26
	P	20	1.14
	P	5	1.26
Deoxycholate-solubilized enzyme	S	37	1.96
(in 100 000 × *g* supernatant	S	20	1.05
	S	5	1.0
	P	37	1.8
	P	20	1.12

TABLE II

PROPERTIES OF 5'-NUCLEOTIDASE OF VARIOUS MEMBRANE SOURCES

Colch, colchicine; DOC, deoxycholate; GEM, glycine/EDTA/mercaptoethanol; CD, cytochalasin D; TX-100, Trixon X-100; NC, non-competitive.

Membrane source perturbation	K_m (μM)	Inhibition characteristics	
		Kinetics	Hill coefficient
13762 cells	25	NC	1.0
Colch, CD	—	—	2.0
13762 envelopes	25	NC	1.1
DOC	25	NC	1.9
GEM extraction	25	NC	1.8
Milk fat globule membrane	25	NC	1.0
Colch, CD	—	—	1.1
DOC	—	—	1.9
GEM extraction	25	NC	1.9

The induction of cooperativity is not due to non-specific membrane perturbation by the extraction. Neither 1.5 M $MgCl_2$ nor 1 M KCl, which have also been used to extract milk fat globule membrane components [13,19], were effective in inducing cooperativity. These treatments also did not extract significant quantities of the 155 000 dalton polypeptide (data not shown).

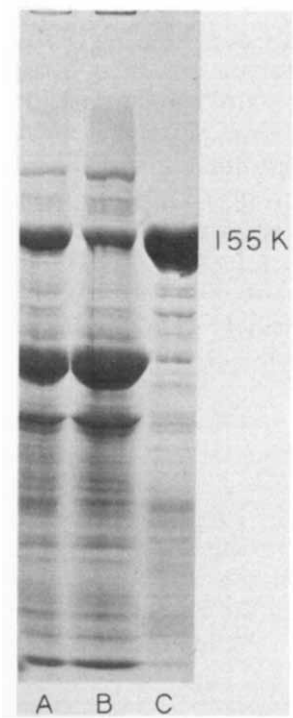

Fig. 2. Electrophoresis of milk fat globule membranes extracted with glycine/EDTA/mercaptoethanol. (A) Milk fat globule membranes; (B) glycine/EDTA/mercaptoethanol pellet; (C) glycine/EDTA/mercaptoethanol supernatant.

Discussion

Table II compares results from this study with those obtained for 13762 ascites cells and membranes. In spite of the differences in the systems the parallels are quite striking. Extraction with deoxycholate or glycine/EDTA/mercaptoethanol induced cooperativity in either 13762 or milk fat globule membranes. These observations are consistent with our previous suggestion that the induction of cooperativity results from a release of restraints on the membrane enzyme [8,9,20,21]. The nature of these restrains is still unclear. Actin and tubulin, which have been postulated to play restraining roles in other systems [1,2], are not present in significant amounts in bovine milk fat globule membranes. However, these membranes do have associated coat material resembling, at least superficially in the electron microscope [22], that found in association with erythrocyte membranes [23] and ghosts or envelopes from more complex mammalian cells [10]. This material is resistant to extraction with high ionic strength buffers and detergents [22], behavior similar to that of associated cytoskeletal material of Sarcoma envelopes [10]. However, it has a very different protein composition; the primary constituents are polypeptides of molecular weights 155 000 and 67 000 [22]. Our extraction studies with glycine/EDTA/β-mercaptoethanol indicate that the 155 000 molecular weight component can be substantially removed from the milk fat globule membrane under conditions which cause induction of cooperativity. It is tempting to speculate that this 155 000 dalton polypeptide plays a part in controlling membrane functions in the milk fat globule membrane, possibly contributing to the secretion process, since actin-containing elements which are active in other secretion processes and are virtually ubiquitous components of isolated plasma membranes [24], are absent from milk fat globule membrane. It has been suggested that xanthine oxidase is the 155 000 dalton component of milk fat globule membrane [25], but this does not eliminate the possibility of overlapping electrophoresis bands containing more than one polypeptide. Alternatively, it is possible that a more minor polypeptide(s) released by glycine/EDTA/mercaptoethanol is responsible for binding the 155 000 dalton polypeptide to the membrane and altering the behavior of 5′-nucleotidase. Further investigations of milk fat globule membrane structure and the cooperativity phenomenon are needed to settle these questions.

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