J. Membrane Biol. 142, 137–144 (1994)

The Journal of
Membrane
Biology

Reconstitution of 5′-Nucleotidase of Bull Seminal Plasma in Spin-labeled Liposomes

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Received: 11 May 1994

Abstract. Seminal plasma separated from freshly ejaculated bull semen contains vesicles with a 5′-nucleotidase activity incorporated as an ectoenzyme anchored by glycosyl phosphatidylinositol (GPI). After its extraction from bull seminal plasma vesicles, the protein was purified and reconstituted into hen egg yolk lecithin liposomes obtained through prolonged dialysis of buffered n-octylglucoside detergent solutions of lipid, protein and various effectors against detergent-free solutions. Gel filtration experiments showed that the enzyme incorporated into liposomes in a dimeric form with its two subunits linked by disulfide bridges. In the presence of reduced glutathione, the protein dissociated into monomers and failed to incorporate into liposomes. Electron spin resonance (ESR) experiments, performed with liposomes containing electron spin labels localized at the hydrophilic lipid headgroups (5-doxyl stearic acid) or in the hydrophobic lipid hydrocarbon chains (16-doxyl stearic acid), demonstrated that the incorporation of 5′-nucleotidase resulted in the immobilization of the spin probes. Furthermore, the spectral parameters obtained before and after treatment of 5′-nucleotidase-containing liposomes with phosphatidylinositol-specific phospholipase C (PI-PLC) indicated that the liposome membrane bilayer did not contain protein segments. This supports the well-known ecto-localization of 5′-nucleotidase and rules out a previously reported possibility of a proteic transmembrane anchoring of the enzyme.

Key words: 5′-Nucleotidase — Liposomes — GPI-anchored proteins — Ectoenzymes — Electron spin probes — Disulfide bridges

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Introduction

5′-Nucleotidase is a ubiquitous glycoprotein whose cytosolic forms are involved in the metabolic control of the intracellular levels of nucleoside 5′-monophosphates (Worku, Luzio & Newby, 1984; Itoh, Oka & Ozasa, 1986; Madrid-Marina & Fox, 1986; Truong, Collinson & Lowenstein, 1988; Skladanowski & Newby, 1990; Le Hir, 1991; Yamazaki, Truong & Lowenstein, 1991). A membrane-bound enzyme containing a glycosylphosphatidylinositol anchor has also been described in various biological sources. This type of enzyme, now referred to as ecto-5′-nucleotidase, seems to be part of a cascade of hydrolytic enzymes that metabolize the extracellular trinucleotides in nucleosides and inorganic phosphate (Bailyes et al., 1984; Thompson, Reudi & Low, 1987; Meghji, Middleton & Newby, 1988; Misumi et al., 1990; Flocke & Mannherz, 1991; Volknandt et al., 1991).

The observation that GPI-specific phospholipase C or D can release 5′-nucleotidase from membranes less effectively than other ectoenzymes, taken together with other experimental data suggesting a possible transmembrane localization of the protein (Zimmermann, 1992 and references therein), indicate that detailed information on the type and extent of the interactions between GPI-anchored 5′-nucleotidase and membrane lipid bilayer is still lacking.

To give insight into such interactions and to study the molecular and structural properties of a mammalian GPI-anchored 5′-nucleotidase, we have reconstituted the ecto-5′-nucleotidase of bull seminal plasma into hen egg yolk liposomes containing fatty acids bearing an electron spin label localized at two different positions: the first to report on the interior (16-SASL) and the second on the polar heads region of the membrane bilayer (5-SASL). The ESR spectral data demonstrate that the GPI-anchor of 5′-nucleotidase of bull seminal plasma was inserted into the lipid bilayer of the liposome membrane and that

no protein segment was embedded within the membrane, thus ruling out the possibility for a proteic transmembrane anchoring of the enzyme.

Finally, when the reconstitution of 5′-nucleotidase in liposomes was carried out in the presence of reduced glutathione, the protein molecules dissociated into monomers of low specific activity, which failed to incorporate into liposomes. This indicates that the dimeric form of the enzyme linked by disulfide bridges is the proper molecular organization of 5′-nucleotidase, both for the expression of the AMPase activity as well as for its incorporation into a lipid bilayer.

Materials and Methods

Materials

Bull semen was kindly provided by Dr. Augusto Chiacchierini from the artificial insemination center, Centro Tori di Perugia, Italy and by Drs. Paolo Ghinassi and Franca Farabegoli from Società Semen Italy, Centro di Diegaro, Cesena, Italy. 1-*O*-*n*-octyl-β-D-glucopyranoside (*n*-octyl glucoside), α-chymotrypsin from bovine pancreas (EC 3.4.21.1), adenosine deaminase from calf intestine (EC 3.5.4.4), oxidized and reduced glutathione, 5′-AMP and the protease inhibitor set were purchased from Boehringer (Milan, Italy). L-α-phosphatidylcholine from frozen egg yolk and dithiothreitol were obtained from Sigma (Milan). Phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* was supplied from Calbiochem Inalco (Milan). 5-Doxyl stearic acid and 16-doxyl stearic acid were provided by Syva (Palo Alto, CA). All chemicals used were of the highest analytical grade available.

Localization of 5′-AMPase Activity in Bull Seminal Plasma (BSP) Vesicles

Freshly ejaculated bull semen (usually 2 ml) was incubated at 32°C for 30 min to allow complete liquefaction. The semen was then centrifuged at 600 × *g* to sediment spermatozoa and the supernatant constituting the seminal plasma removed. Vesicles normally present in BSP were recovered by centrifuging BSP at 105,000 × *g* for 2 hr at 4°C in a Beckman 15/50B ultracentrifuge equipped with a SW 50.1 rotor. The supernatant was removed, the membrane pellet resuspended in 50 mM Tris/maleate buffer (pH 7.4) and used for the cytochemical detection of 5′-nucleotidase with 5′-AMP as substrate and $CeCl_3$ as a capturing agent, according to Robinson and Karnovsky (1983). Aspecific phosphatase activity was monitored using β-glycerophosphate as substrate.

A Philips 400 TEM operated at 60 kV was used.

Purification of 5′-Nucleotidase from Bull Seminal Plasma Vesicles

Membrane pellets obtained from centrifugation of 100 ml BSP at 105,000 × *g* for 2.5 hr were incubated for 2 hr at 37°C in 20 mM HEPES/NaOH buffer (pH 7.5) containing 50 mM sodium cholate and 0.1 mM protease inhibitor cocktail. The mixture was then centrifuged at 50,000 × *g* for 30 min and the supernatant was used for the purification of 5′-nucleotidase according to Fini et al. (1983).

Incorporation of 5′-Nucleotidase in Egg Yolk Lecithin Liposomes

Typically, 12 mg of dried egg yolk lecithin, 60 μg of 5- or 16-SASL and 150 μg of 5′-nucleotidase were dissolved in 12 ml of 20 mM HEPES/NaOH buffer (pH 7.4) containing 2 mM *n*-octyl glucoside. The approximate molar ratio of spin probe to lipid was 1:100. The lipidic dispersion was sonicated (50 W for 2 min) and dialyzed overnight in a Spectrapore 1 bag against 20 mM HEPES/NaOH buffer (pH 7.4) containing Bio-Beads SM-2 to facilitate detergent removal. Liposomes were separated from the reconstitution mixture by gel filtration chromatography on a Bio-Gel A-0.5 m column (1.4 cm id × 25 cm) equilibrated and eluted with 150 mM NaCl in 20 mM HEPES/NaOH buffer (pH 7.4) at a flow rate of 20 ml/hr. Liposome-containing fractions were detected by absorbance at 280 nm. Liposomes of uniform size were obtained by forcing the vesicle suspension through a 200 nm pore size polycarbonate filter under a nitrogen pressure in an EXTRUDER apparatus (Lipex, Vancouver, Canada). Spin-labeled liposomes not containing 5′-nucleotidase were prepared in the same manner, except that 5′-nucleotidase was not included in the lecithin/detergent solution. The reconstitution of 5′-nucleotidase into liposomes not containing spin labels was performed in the same way, with the omission of 5- or 16-SASL.

Electron Spin Resonance Experiments

To perform ESR experiments, spin-labeled samples were placed into 0.8 mm glass capillaries which were in turn placed into a standard ESR quartz tube. The latter was then placed into the temperature-controlled Dewar flask filling the central part of the resonant cavity of the spectrometer. ESR spectra were recorded at 25°C by an X-band Varian E-109 spectrometer using a 100 kHz modulation frequency for conventional first harmonic in-phase absorption spectra. Second harmonic, Saturation Transfer ESR (ST-ESR) spectra were run according to procedures and calibrations previously reported (Bruno et al., 1985; Bruno, Gliozzi & Cannistraro, 1986).

Treatment of Liposomes Containing 5′-Nucleotidase with PI-PLC

Liposomes containing 5′-nucleotidase, usually suspended in 0.5 ml 150 mM NaCl in 20 mM HEPES/NaOH buffer (pH 7.5), were incubated for 2 h at 37°C in the presence of 0.15 units of PI-PLC. After incubation, the suspension was applied to a Bio-Gel A-0.5 m column (1.5 cm ID × 31 cm) previously equilibrated with the same buffer, and 1 ml fractions were collected and assayed for protein concentration and enzyme activity. The fractions containing the liposome peak were combined and transferred to a dialysis bag and the suspension was then concentrated to 0.5 ml by embedding the bag in PEG powder for several hours.

Native 5′-nucleotidase containing the GPI anchor could be distinguished from the soluble protein released after treatment with PI-PLC by differential partition into Triton X-114 aqueous solutions, under the experimental conditions reported by Hooper and Bashir (1991).

Determination of 5′-Nucleotidase Activity

The enzymatic reaction was carried out at 25°C with 5′-AMP as substrate by measuring spectrophotometrically at 265 nm the amount of inosine produced in the presence of an adenosine deaminase excess, according to Ipata (1967). The standard reaction mixture contained in a final volume of 1 ml: 150 mM NaCl, 1 μg of adenosine deaminase, 50 mM Tris/Cl buffer (pH 7.5) and the proper amount of 5′-nucleotidase. A decrease in optical density of 1 corresponds to 0.123 μmol of 5′-AMP hydrolyzed under the experimental conditions adopted. One unit

of activity is the amount of enzyme that hydrolyzes 1 mmol of AMP per minute.

Protein Determination

Protein was determined by the bicinchoninic acid reagent according to Smith et al. (1985) using ribonuclease A and BSA as standards.

Abbreviations

GPI, glycosyl phosphatidylinositol; GSH, reduced glutathione; GSSG, oxidized glutathione; PI-PLC, phosphatidylinositol-specific phospholipase C; 5-SASL, 5-doxyl stearic acid; 16-SASL, 16-doxyl stearic acid.

Results

Localization of 5′-Nucleotidase in Bull Seminal Plasma Vesicles

As shown in Fig. 1*A*, bull seminal plasma contains bilayered vesicles of different sizes. The appearance of dense granules after the incubation of the vesicles in the presence of 5′-AMP (*B*) and their absence when the assay is carried out with β-glycerophosphate (the pattern is similar to that shown in *A*) indicate that the 5′-nucleotidase activity is localized on the vesicle membrane and is clearly distinguishable from aspecific phosphatase activities.

Preparation of Liposomes Containing 5′-Nucleotidase

The reconstitution of 5′-nucleotidase into egg yolk lecithin in most preparations resulted in the incorporation of over 50% of the initial activity under a molar ratio lipid:protein of 1:10,000 (*see* Fig. 4*A*). The incubation of the 5′-nucleotidase-containing liposomes in 50 mM sodium cholate in 20 mM HEPES/NaOH buffer (pH 7.5) produced disassembly of the vesicles without any subsequent increase in 5′-nucleotidase activity, thus indicating that during the reconstitution process the enzyme had not been trapped inside the lumen of liposomes.

Localization of 5′-Nucleotidase in Liposomes

The elution pattern of the gel filtration of 5′-nucleotidase-containing liposomes previously treated with PI-PLC showed that over 90% of the liposome-incorporated activity was released within a peak corresponding to about 158 kD, (Fig. 2).

As our prior studies had indicated the presence of disulfide bridges between the subunits of the purified dimeric protein (Fini et al., 1985), we investigated whether the molecular form obtained by the gel filtration of the liposome preparation containing 5′-nucleotidase and treated with PI-PLC corresponded to the dimeric protein with disulfide bridges without the phospholipid anchor. To this end, the peak in Fig. 2 corresponding to an M_r of approximately 158 kD was collected, concentrated on an Amicon YM10 membrane, and an aliquot was incubated overnight in the presence of 20 mM GSH followed by gel filtration on Bio-Gel A-0.5 m (Fig. 3). The elution profile consists of a single peak corresponding to an M_r of approximately 65 kD which contains practically the total protein applied on the column and about 10% of the initial activity. The inhibition of the AMPase activity is strictly linked to the dissociating effect of GSH. In fact, upon dialysis against buffer without 20 mM GSH and traces of M-mercaptoethanol, the protein subunits were able to reassociate with the formation of a 158 kD dimeric form which contained over 40% of the initial AMPase activity.

Incorporation of 5′-Nucleotidase into Liposomes in the Presence of GSH or GSSG

Figure 4*A* refers to the gel filtration of liposomes obtained after dialysis of a sonicated solution containing 20 mM HEPES buffer (pH 7.5), 2 mM *n*-octylglucoside, 1 mg/ml egg yolk lecithin and enzyme. As can be seen, over 50% of 5′-nucleotidase activity is incorporated into the vesicles. On the contrary, when the reconstitution is carried out in the presence of 5 mM GSH (Fig. 4*B*), the AMPase activity is not incorporated into the liposomes and most of the protein is localized in the peak corresponding to 65 kD with a specific activity of 44.6 U/mg, whereas a minor fraction of protein is present in the 158 kD peak with a specific activity of 160 U/mg.

Finally, in the presence of GSSG, the enzyme was incorporated into liposomes in the same way as the native enzyme, without any stimulatory or dissociating effect (*not shown*).

ESR of Spin-labeled Liposomes

Figure 5 shows the conventional ESR spectrum obtained from liposomes which had been labeled with 16-SASL. The spectrum indicates that the spin label undergoes an almost isotropic reorientation with a moderate immobilization. The pattern obtained when 5′-nucleotidase is added to the liposomes is very similar (*not shown*). To estimate the degree of immobilization of the spin label, we have evaluated the correlation time (τ_c) according to the expression:

$$\tau_c = 6.5 \times 10^{-10}\, \Delta H_0 \left(\sqrt{\frac{h(0)}{h(-1)}} - 1 \right)$$

where $\Delta H(0)$, $h(0)$ and $h(-1)$ are indicated in Fig. 5.

The inset of Fig. 5 shows that such a correlation time is slightly increased in the presence of 5′-nucleotidase. Furthermore, no significant change in τ_c is registered when 5′-nucleotidase-containing liposomes are treated with PI-PLC.

5-SASL spin-labeled liposomes with or without 5′-nucleotidase presented a conventional ESR spectrum

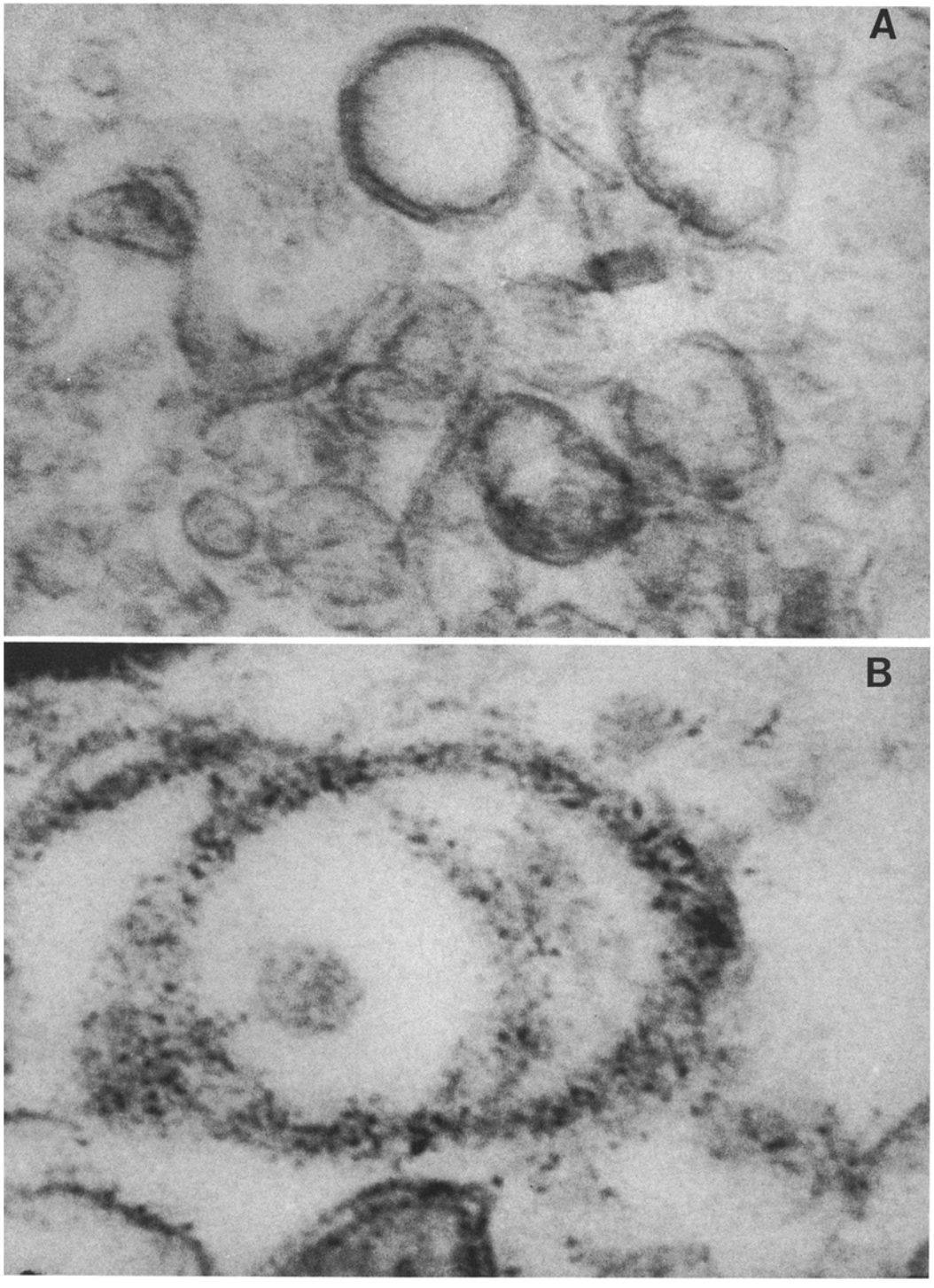


Fig. 1. Electron microscopy of bull seminal plasma. (*A*) Authentic bull seminal plasma, original magnification 50,000×. (*B*) Electron micrography of 5′-nucleotidase cytochemical reaction carried out on bull seminal vesicles incubated in a medium containing cerium chloride and 5′-AMP. The dense reaction at the vesicle surface is clearly observable. Original magnification 70,000×.

typical of an anisotropic motion in the slow motion regime. In such cases, ST-ESR should be applied to study the dynamics of the spin-labeled system. ST-ESR spectra can be analyzed by simulation methods to obtain the rotational correlation time. However, in most ST-ESR investigations reported and even in the presence of anisotropic motion, the rotational correlation times are estimated from published calibration curves for the spectral parameters C′/C and L″/L (*see* Fig. 6) which were obtained for isotropic reorientation. The correlation times

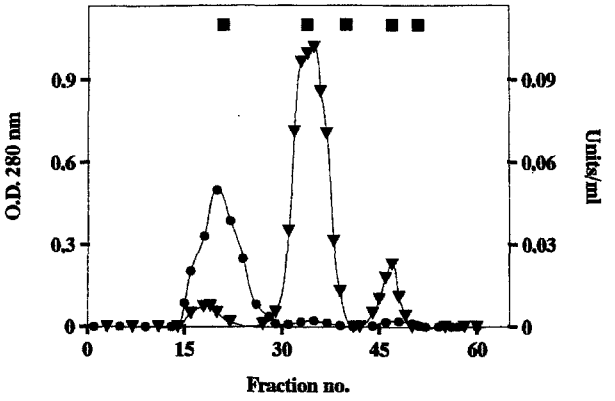


Fig. 2. Gel filtration chromatography of liposomes containing 5′-nucleotidase treated with PI-PLC. Liposomes containing 5′-nucleotidase were treated with PI-PLC as described in Materials and Methods. The suspension was then loaded on a Bio-Gel A-0.5 m column (1.5 cm ID × 31 cm) previously equilibrated with 150 mM NaCl in 20 mM HEPES/NaOH buffer (pH 7.4). The column was eluted with the same buffer at a flow rate of 20 ml/hr and 1 ml fractions were collected. The molecular weights of standards are in descending order: blue dextran (about 2,000,000); bovine gamma globulin (158,000); rabbit phosphorylase b (97,000); bovine serum albumin (68,000) and chicken ovalbumin (44,000).

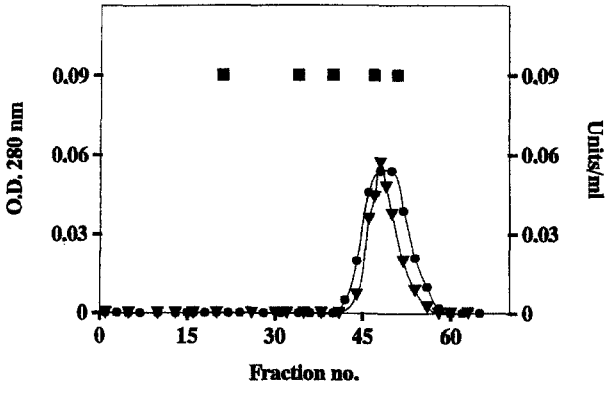
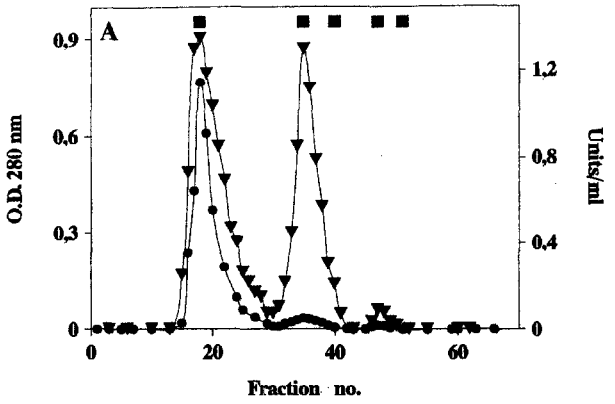


Fig. 3. Gel filtration chromatography of the 158 kD peak of Fig. 2 after treatment with reduced glutathione. The 158 kD peak of Fig. 2 was concentrated to 0.5 ml through an Amicon YM 10 membrane and dialyzed overnight against 20 mM GSH in 20 mM HEPES/NaOH buffer (pH 7.4) containing 150 mM NaCl. The mixture was then applied to a Bio-Gel A-0.5 m column previously equilibrated with 150 mM NaCl and 5 mM GSH in 20 mM HEPES/NaOH buffer (pH 7.4). Column sizes and chromatographic conditions were the same as those described in Fig. 2.



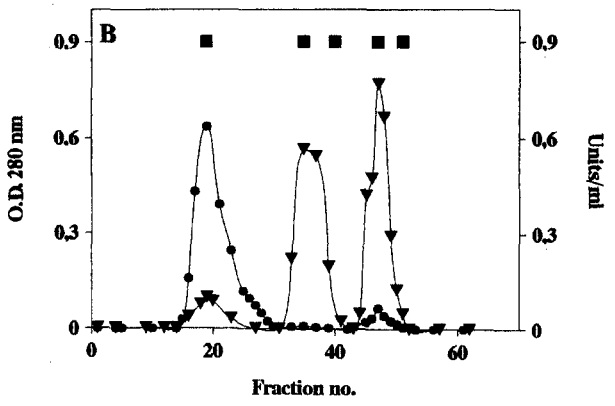


Fig. 4. Bio-Gel A-0.5 m gel filtration chromatography of the dialyzed 5′-nucleotidase-egg yolk lecithin-detergent mixture after liposome formation. The liposomes were prepared as described in Materials and Methods. (*A*) Typical gel filtration chromatography pattern of the liposome suspension recovered from the dialysis bag. The chromatographic conditions were the same as those reported in Fig. 2. The peak eluted at V_o contains 5′-nucleotidase incorporated into liposomes. The peak eluted in correspondence of bovine gamma globulin (158 kD) contains 5′-nucleotidase not incorporated in the vesicles. (*B*) Gel filtration profile of the incorporation of 5′-nucleotidase in liposomes prepared in a reconstitution mixture containing 5 mM GSH, giving 70% inhibition of AMPase activity. In this case, the enzyme was poorly incorporated into liposomes. Triangles represent enzyme activity and the continuous line is the OD profile at 278 nm. Peaks are from left to right: liposomes, dimeric 5′-nucleotidase and glutathione-reduced monomeric 5′-nucleotidase. Activity under the third peak represents less than 10% of the initial amount.

estimated according to such a procedure are reported in the inset of Fig. 5. In general, for all the samples analyzed, the τ_c values calculated from L″/L and C′/C differ by about one order of magnitude. This behavior suggests the presence of anisotropic motion and in such cases the calibration curves do not provide a correct τ_c value (Bruno et al., 1986); however, the trend of τ_c with the addition of 5′-nucleotidase and PI-PLC is meaningful. Both τ_c values again indicate that 5′-nucleotidase incorporation in liposomes decreases the overall mobility of the spin labels. Furthermore, the addition of PI-PLC to this system does not alter τ_c values.

Discussion

The cDNAs of 5′-nucleotidase from four different vertebrates have been cloned and sequenced in the past three years. In this manner it was possible to gain information

on: the primary structure of 5′-nucleotidase from rat liver (Misumi et al., 1990*a*), human placenta (Misumi et al., 1990*b*), electric ray (Volknandt et al., 1991) and bovine liver (Suzuki et al., 1993); on the molecular mass; on the number and position of the potential N-glycosylation sites and on the position of the serine residue of the mature protein to which the GPI-anchor is attached. However, the tertiary structure as well as the details of the molecular organization of the mature protein at the membrane level are still unknown.

Our Fourier transform infrared study (Fini et al., 1992) has shown that the secondary structure of 5′-nucleotidase of bull seminal plasma contains 54% β-sheet, 18% α-helix, 22% β-turns and 6% unordered structure and that changes in the tertiary structure were responsible for the thermal behavior observed in the presence of reducing and metal-chelating agents.

The ecto-enzyme nature of GPI-anchored 5′-nucleotidase has been known for some time (Low, 1987; Stochaj et al., 1989); however, the possibility of the presence of hydrophobic protein transmembrane segments has been reported (Zachoscki et al., 1981; Dieckhoff et al., 1987).

The results of the gel filtration experiments described here showed that in the presence of 10 mM GSH, which dissociates the dimeric protein into monomers with inhibition of the AMPase activity, the enzyme was not incorporated into liposomes, while the authentic dimeric protein with intact disulfide bridges incorporated into the liposomes without any modification of the AMPase activity. The monomeric protein's failure to incorporate into the liposomes in the presence of 10 mM GSH poses questions about the modality by which the enzyme is transferred to the membrane in vivo.

It has been shown that after the GPI-anchor attachment, membrane proteins are usually transferred to the plasma membrane by conventional vesicular transport (Ferguson & Williams, 1988); however, the procedure for the insertion of a GPI-anchored protein into the membrane in vivo is relatively unknown.

Our observation of the effect of physiological intracellular concentrations of GSH (2–20 mM) on the incorporation of bull seminal plasma 5′-nucleotidase into liposomes induces us to suppose that the protein vesicular transport system in vivo should not contain GSH, since this reducing agent proved it could impair 5′-nucleotidase insertion into the liposomal membrane. Furthermore, inside the transport vesicles in vivo, two 5′-nucleotidase monomers could possibly interact with formation of a dimeric protein molecule which is subsequently inserted into the membrane. Alternatively, after their insertion into the membrane, due to their lateral mobility, the GPI-anchored 5′-nucleotidase monomers could migrate until they reach the proper distance for the formation of a disulfide bridge, thus resulting in the mature dimeric ecto-enzyme.

The ESR and electron microscopy experiments described in the present paper showed that in artificial lipid vesicles the GPI anchor of 5′-nucleotidase was inserted into the membrane bilayer with the protein moiety containing both the AMPase and the PI-PLC activity sites remaining outside of the liposome. ESR data concerning spin-labeled liposomes ± 5′-nucleotidase indicated that the spin reporter bound to 5-SASL was strongly immobilized, whereas, due to its localization in a more fluid environment towards the interior of the phospholipid bilayer, the label bound to 16-SASL proved to be weakly immobilized. Furthermore, the coincidence between the ESR spectra of liposomes containing 5′-nucleotidase and those of liposomes containing 5′-nucleotidase subsequently treated with PI-PLC, strongly indicated that the protein moiety of the enzyme did not penetrate the lipid bilayer. This result was supported by two additional observations: (i) after the treatment with PI-PLC, the 5′-nucleotidase activity associated with liposomes was recovered by gel filtration within a peak corresponding to an M_r of about 158 kD and in Triton X-114 partition experiments the same activity was almost all localized in the aqueous phase; (ii) no protein material or AMPase activity was detectable in the clear solutions obtained after 50 mM sodium cholate treatment of liposomes containing 5′-nucleotidase which had been previously treated with PI-PLC.

Taken together, the results of the gel filtration experiments carried out after the treatment of liposomes containing 5′-nucleotidase with PI-PLC, the recovery of the dimeric soluble protein (158 kD) and the formation of subunits of 65 kD after treatment with GSH, indicated that the dimeric structure with subunits linked by disulfide bridges constituted the biologically active form of the membrane-bound 5′-nucleotidase which also corresponded to the main molecular form extracted by detergents with high critical micellar concentration from the vesicles contained in bull seminal plasma.

In a recent study, we have demonstrated that the dimeric enzyme contained two zinc ions both essential to the 5′-nucleotidase activity, suggesting that each subunit might contain one active center (Fini, Coli & Floridi, 1991). The almost complete loss of the AMPase activity following the dissociation of the protein into subunits, together with the monomers' failure to incorporate into liposomes in the presence of the dissociating effector GSH, observed in the present study, indicate that the protein is functionally active only when it is structurally organized as a dimer with its subunits linked by disulfide bridges. On the other hand, reduction of the disulfide bridges proved to induce dramatic modifications in the tertiary structure of the protein (Fini et al., 1992). Therefore, the enzymatic activity of 5′-nucleotidase of bull seminal plasma depends not only on the endogenous zinc ions but also on the presence of intact intersubunit disulfide bridges. However, the possibility of intrachain disulfide bridges should not be disregarded.

Results of immunofluorescence studies to determine

	LIPOSOME	LIP.+5'N	LIP.+5'N+PIPLC
τ_c (ns)	1.13	1.21	1.23

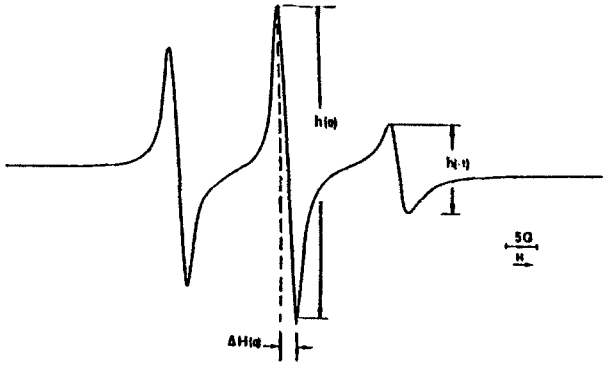


Fig. 5. Conventional ESR spectrum of egg yolk lecithin liposomes spin-labeled with 16-SASL. The rotational correlation times (τ_c, ns) of authentic spin-labeled liposomes (*liposome*), spin-labeled liposomes containing 5′-nucleotidase (*lip + 5′-N*) and of spin-labeled liposomes recovered after treatment of spin-labeled liposomes containing 5′-nucleotidase with PI-PLC (*lip + 5′-N + PI-PLC*) are shown in the inset.

the biological function of 5′-nucleotidase in the spermatozoon have indicated a localization of the protein at the acrosomal cap domain where it appeared to be bound to the membrane by a GPI anchor (*in preparation*).

All information obtained leads to the characterization of 5′-nucleotidase as a multifunctional protein whose role in the sperm should also be defined in view of its possible intervention in the egg-sperm recognition process.

The functional role of 5′-nucleotidase in bull seminal plasma, which is very rich in this enzyme, is still largely obscure; certainly it is released in the seminal

	LIPOSOME	LIP.+5'N	LIP.+5'N+PIPLC
τ (L'/L)	0.10	0.30	0.34
τ (C'/C)	1.22	4.55	4.50

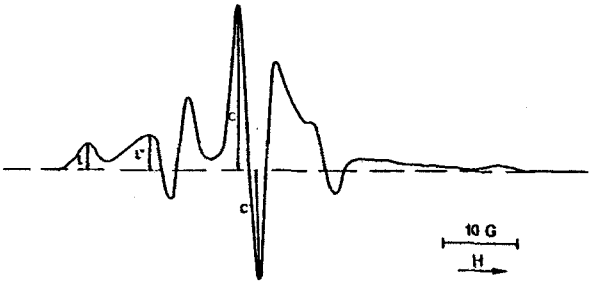


Fig. 6. ST-ESR spectrum of liposomes spin-labeled with 5-SASL. The inset shows the $\tau_{c(L'/L)}$ and $\tau_{c(C'/C)}$ values for authentic 5-SASL spin-labeled liposomes (*liposome*), 5-SASL spin-labeled liposomes containing 5′-nucleotidase (*lip + 5′-N*) and 5-SASL spin-labeled liposomes recovered after treatment of 5-SASL spin-labeled liposomes containing 5′-nucleotidase with PI-PLC (*lip + 5′-N + PI-PLC*). τ_c values are given in msec.

plasma during semen ejaculation and still too little is known of the spermatozoon-plasma relationship to assign definite roles. However, the data shown here indicated that the incorporation of 5′-nucleotidase into membranes was impaired by GSH, thus suggesting that the redox state of the cell as well as the cellular energy charge could have a role in the modulation of the 5′-nucleotidase activity both at intracellular and membrane levels.

We thank Mr. Marcello Coli and Dr. Maria Grazia Cantelmi, University of Perugia, for their skillful technical assistance; Dr. Paolo Ghinassi and Dr. Franca Farabegoli, Semen Italy, Diegaro, Cesena, Italy and Dr. Augusto Chiacchierini, Centro Tori Perugia, Italy, for kindly supplying the bull semen; Dr. Maria Grazia Rambotti, Department of Experimental Medicine, University of Perugia, Italy, for her skillful assistance in the electron microscopy experiments; Ms. Darlene I. Morosi for her helpful suggestions.

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