# Release of the glycosylphosphatidylinositol-anchored enzyme ecto-5′-nucleotidase by phospholipase C: catalytic activation and modulation by the lipid bilayer

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Many hydrolytic enzymes are attached to the extracellular face of the plasma membrane of eukaryotic cells by a glycosylphosphatidylinositol (GPI) anchor. Little is currently known about the consequences for enzyme function of anchor cleavage by phosphatidylinositol-specific phospholipase C. We have examined this question for the GPI-anchored protein 5′-nucleotidase (5′-ribonucleotide phosphohydrolase; EC 3.1.3.5), both in the native lymphocyte plasma membrane, and following purification and reconstitution into defined lipid bilayer vesicles, using *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC). Membrane-bound, detergent-solubilized and cleaved 5′-nucleotidase all obeyed Michaelis–Menten kinetics, with a $K_m$ for 5′-AMP in the range 11–16 $\mu$M. The GPI anchor was removed from essentially all 5′-nucleotidase molecules, indicating that there is no phospholipase-resistant pool of enzyme. However, the phospholipase was much less efficient at cleaving the GPI anchor when 5′-nucleotidase was present in detergent solution, dimyristoyl phosphatidylcholine, egg phosphatidylethanolamine and sphingomyelin, compared with the native plasma membrane, egg phosphatidylcholine and a sphingolipid/cholesterol-rich mixture. Lipid molecular properties and bilayer packing may affect the ability of PI-PLC to gain access to the GPI anchor. Catalytic activation, characterized by an increase in $V_{max}$, was observed following PI-PLC cleavage of reconstituted 5′-nucleotidase from vesicles of several different lipids. The highest degree of activation was noted for 5′-nucleotidase in egg phosphatidylethanolamine. An increase in $V_{max}$ was also noted for a sphingolipid/cholesterol-rich mixture, the native plasma membrane and egg phosphatidylcholine, whereas vesicles of sphingomyelin and dimyristoyl phosphatidylcholine showed little activation. $K_m$ generally remained unchanged following cleavage, except in the case of the sphingolipid/cholesterol-rich mixture. Insertion of the GPI anchor into a lipid bilayer appears to reduce the catalytic efficiency of 5′-nucleotidase, possibly via a conformational change in the enzyme, and activity is restored on release from the membrane.

## INTRODUCTION

Covalent modification of proteins with a glycosylphosphatidylinositol (GPI) anchor is commonly employed as a mode of membrane attachment in a wide range of eukaryotes, including parasitic protozoa, the yeast *Saccharomyces cerevisiae*, higher plants and mammals [1,2]. GPI-anchored proteins are characterized by the presence of a hydrophobic peptide sequence at the C-terminus, which is removed by a putative transamidase enzyme in the endoplasmic reticulum that also attaches the preformed anchor [3,4]. Well over 100 proteins are known to be GPI-anchored; they are a functionally diverse group, encompassing extracellular-coat proteins, hydrolytic enzymes, adhesion proteins, surface antigens and receptors.

The exact function of the GPI anchor has been the subject of speculation [5–7]. The anchor confers rapid lateral mobility to some plasma-membrane proteins, and it has been proposed that the anchor enables the proteins themselves to pack tightly, which may be especially important for protozoan surface-coat glycoproteins. The GPI anchor appears to act as an intracellular targetting signal in polarized epithelial cells. GPI-anchored proteins are sorted into glycolipid-enriched membrane subdomains prior to transport to the apical-membrane surface [8]. Because of their insolubility in Triton X-100 at 4 °C, these domains can be isolated as detergent-insoluble glycolipid-enriched complexes (DIGs). DIGs also exist in reconstituted liposomal systems [9], and it was suggested that acyl-chain interactions are important in their formation. It has also been suggested that certain GPI-anchored proteins, e.g. the folate receptor, are involved in the high-affinity cellular uptake of small molecules by the process of potocytosis, which involves specialized membrane invaginations known as caveolae [6]. GPI anchors are also involved in transmembrane signalling. The products of phospholipase cleavage of the anchor, inositol phosphoglycans, are proposed to be mediators in the action of insulin and several other agents, whereas crosslinking of GPI-anchored proteins by antibody can stimulate T-lymphocyte activation [7,10–12]. Simons and Ikonen [13] have proposed that GPI-anchored proteins exist at the membrane surface within sphingolipid-cholesterol 'rafts', which serve as relay stations in transmembrane signalling. Finally, hydrolysis of the anchor by specific phospholipases C and D results in release of the protein in soluble form, and this process may play a role in modulating the display and function of GPI-anchored proteins at the cell surface.

The GPI-anchored ectoenzyme 5′-nucleotidase (5′-NTase; systemic name, 5′-ribonucleoside phosphohydrolase; EC 3.1.3.5) is found on the plasma membrane of many cell types, and is

Abbreviations used: DIG, detergent-insoluble glycolipid-enriched complex; DMPC, dimyristoyl phosphatidylcholine; GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; 5′-NTase, ecto-5′-nucleotidase; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; SCRL, sphingolipid/cholesterol-rich liposomes; SM, sphingomyelin; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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widely distributed from plants to mammals (reviewed in [14]). The enzyme converts extracellular 5′-AMP to adenosine, which acts via adenosine receptors in a variety of physiological signalling processes. 5′-NTase plays an important role in purine salvage in lymphocytes, where it is known as CD73, and has also been implicated in transmembrane signalling *in vitro* in T-lymphocytes [12]. The enzyme is N-glycosylated, and binding of a variety of lectins to N-linked carbohydrates on 5′-NTase is known to inhibit activity [15,16], suggesting that some communication exists between the glycan chain(s) and the catalytic site.

Previous work in our laboratory led to the isolation and reconstitution of porcine-lymphocyte 5′-NTase into lipid bilayers, where it retains enzymic activity that is inhibited by lectin binding [15,17]. Reconstitution of purified GPI-anchored proteins into bilayers of defined phospholipids provides a powerful tool to delineate the effects of membrane properties on the behaviour and interactions of this class of proteins. More recently, we explored the cleavage of 5′-NTase and two other GPI-anchored enzymes, acetylcholinesterase and alkaline phosphatase, by bacterial phosphatidylinositol (PI)-specific phospholipases [18]. 5′-NTase showed an increase in activity of 20–25 % following release from the lymphocyte membrane surface by phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis*. In the present study, we have characterized the catalytic activation of 5′-NTase kinetically, in the native lymphocyte plasma membrane and also following reconstitution of purified protein into bilayers of several different lipids. Results indicate that enzymic activation results from lowered catalytic turnover when the GPI anchor is inserted into membrane lipids, suggesting that the GPI anchor may affect the protein conformation of 5′-NTase. In addition, we show that the physicochemical properties of the membrane can modulate both the susceptibility of the GPI anchor to PI-PLC cleavage, and the extent of catalytic activation observed on anchor removal.

## MATERIALS AND METHODS

### Materials

Egg phosphatidylethanolamine (PE) and egg phosphatidylcholine (PC) were supplied by Avanti Polar Lipids (Alabaster, AL, U.S.A.). CHAPS, dimyristoyl phosphatidylcholine (DMPC), sphingomyelin (SM; from bovine erythrocytes and bovine brain), cholesterol, galactocerebrosides (type II from bovine brain) and Triton X-114 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant *B. thuringiensis* PI-PLC (250–300 units/ml; expressed in *B. subtilis*) was obtained from Oxford GlycoSciences Inc. (Bedford, MA, U.S.A.). One unit of enzyme activity released 1 $\mu$mol of $P_i$ from phosphatidylinositol per min at 37 °C, pH 7.5. $Na^{125}I$ was purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.).

### General methods

The protein content of plasma membrane and partially purified 5′-NTase was determined by the method of Peterson [19] using BSA (Sigma; crystallized and lyophilized) as a standard. SDS/PAGE was carried out in polyacrylamide gels according to Laemmli [20], and was followed by silver staining or autoradiography. Purified 5′-NTase was radiolabelled on tyrosine residues with $^{125}I$ using Iodobeads (Pierce Chemical Co., Rockford, IL, U.S.A.), according to the manufacturer's instructions.

### Purification of porcine-lymphocyte 5′-NTase

Porcine mesenteric lymph nodes were supplied by the Meat Laboratory at the University of Guelph within a few minutes of slaughter, and plasma-membrane vesicles were prepared according to the method of Maeda et al. [21]. Briefly, homogenized porcine mesenteric lymph nodes (30 g) were layered over a sucrose cushion (41 %, w/v), which was followed by ultracentrifugation at 95000 ***g***. Lymphocyte 5′-NTase was purified as described previously [15,17], by solubilizing plasma membrane in 50 mM CHAPS and performing two sequential affinity-chromatography steps, the first using lentil lectin–Sepharose 4B (Pharmacia Canada, Baie D'Urfé, QC, Canada), the second using 5′-AMP–Sepharose (Sigma). Purified 5′-NTase solution in CHAPS (1 ml; the 5′-AMP column eluate) was concentrated to approx. 5–10 $\mu$l using a Microcon 30 microconcentrator (30 kDa cutoff; Amicon Inc., Beverly, MA, U.S.A.), which was pretreated with 1 % (w/v) powdered milk to prevent non-specific binding of protein to the membrane. The concentrated 5′-NTase sample was diluted with 0.5 ml of distilled water and concentrated once more to reduce the CHAPS concentration. The final volume was 70 $\mu$l, representing a 14-fold concentration. The protein content of the concentrated 5′-NTase sample was determined by the method of Bradford [22], using BSA as a standard.

### Reconstitution of porcine-lymphocyte 5′-NTase

Purified 5′-NTase was reconstituted into lipid bilayer vesicles using a modification of the detergent-dialysis technique described previously [15,17,18]. A mixture of the desired lipids (1–3 mg) in methanol/$CHCl_3$ (1:4, v/v) was evaporated to dryness in a small glass tube using $N_2$ gas, and pumped under vacuum for 1 h to remove all traces of organic solvent. The dried lipid was dissolved in 12.5 mM CHAPS in 50 mM Tris/HCl/0.1 M NaCl/0.2 mM dithiothreitol/0.02 % sodium azide/0.7 mM $CaCl_2$/0.7 mM $MgCl_2$/0.7 mM $MnCl_2$ (pH 7.4), and mixed with purified 5′-NTase in the same buffer. The detergent was removed by dialysis in Spectrapor 4 tubing (12–14 kDa cutoff) against three changes (a total of 3 l) of 20 mM Tris/HCl buffer (pH 7.4). The resulting lipid bilayer vesicles had a final lipid/protein ratio of 150–200:1 (w/w). Purified 5′-NTase was reconstituted into the following lipid systems: DMPC, egg PC, SM, egg PE and sphingolipid/cholesterol-rich liposomes (SCRL; consisting of egg PC:egg PE:SM:cerebrosides:cholesterol in 1:1:1:1:2 molar proportions) [9].

### Cleavage of detergent-solubilized 5′-NTase by PI-PLC

Recombinant *B. thuringiensis* PI-PLC expressed in *B. subtilis* was used to cleave the GPI anchor of 5′-NTase. Experiments were carried out to determine the concentration of PI-PLC that effected maximal cleavage of purified detergent-solubilized 5′-NTase, and the concentration of PI-PLC required to release 50 % of the 5′-NTase in soluble form (defined as the $EC_{50}$). Increasing concentrations of PI-PLC in 50 mM Tris/HCl (pH 7.4) were incubated for 90 min at 37 °C with purified 5′-NTase in 12.5 mM CHAPS. The GPI-anchored form of 5′-NTase in CHAPS was separated from the soluble form by two-phase separation in Triton X-114, based on a modification of the method of Bordier [23]. Briefly, a 20 $\mu$l aliquot of 5′-NTase in CHAPS was made up to 50 $\mu$l with 50 mM Tris/HCl (pH 7.4) containing increasing concentrations of PI-PLC. Following incubation for 90 min at 37 °C, 150 $\mu$l of 50 mM Tris/HCl (pH 7.4) and 20 $\mu$l of pre-condensed Triton X-114 were added. The mixture was cooled on ice for 3 min, warmed to 37 °C for 5 min, and then centrifuged at 14700 ***g*** for 1 min at 37 °C in a microcentrifuge to separate the phases. The upper aqueous phase and the lower detergent phase were removed into separate

microcentrifuge tubes. Triton X-114 (20 $\mu$l) was added to the upper aqueous phase, 200 $\mu$l of 50 mM Tris/HCl (pH 7.4) was added to the lower detergent phase, and a second phase separation was performed on each of the two original phases. Following this, the two upper phases were combined (400 $\mu$l in total), as were the two lower phases (40 $\mu$l, mixed with 360 $\mu$l of 50 mM Tris/HCl to a total of 400 $\mu$l), and the 5′-NTase activity was determined using 20 $\mu$l aliquots of each.

### Cleavage of membrane-bound 5′-NTase by PI-PLC

Previous work in our laboratory showed that *B. thuringiensis* PI-PLC was inactivated in a time-dependent fashion following adsorption to DMPC bilayers; however, loss of activity could be completely prevented by addition of 1 % (w/v) BSA to cleavage reaction mixtures containing lipid vesicles [18]. To determine the phospholipase concentration at which maximal cleavage of 5′-NTase occurred, and the $EC_{50}$, increasing concentrations of PI-PLC in 50 mM Tris/HCl/1 % (w/v) BSA (pH 7.4) were incubated for 90 min at 37 °C with either lymphocyte plasma-membrane vesicles or purified 5′-NTase reconstituted into various lipids. Following incubation, the lymphocyte plasma membrane or lipid vesicles were collected by centrifugation (41000 ***g*** for 10 min), and the pellet and supernatant were assayed for enzymic activity of the GPI-anchored form and the cleaved soluble form of 5′-NTase, respectively (see below).

### Kinetic analysis of 5′-NTase enzymic activity

Lymphocyte plasma-membrane vesicles, purified 5′-NTase in CHAPS or purified 5′-NTase reconstituted into various lipids were incubated for 90 min at 37 °C, with the appropriate concentration of PI-PLC that yielded maximal cleavage. Aliquots were removed for kinetic analysis of 5′-NTase activity. The enzymic activity of 5′-NTase was determined by measuring the release of [2-$^3$H]adenosine from 5′-[2-$^3$H]AMP, as described by Sharom et al. [15] and optimized by Loe et al. [17]. The initial rate of substrate hydrolysis was determined for increasing concentrations of the substrate, 5′-AMP, and the kinetic data were fitted to the Michaelis–Menten equation using the CurveFit function of the SigmaPlot programme (SPSS Inc., Chicago, IL, U.S.A.).

## RESULTS

### Purification and reconstitution of porcine-lymphocyte 5′-NTase

Porcine-lymphocyte 5′-NTase was successfully purified using a two-step affinity-chromatography procedure, first on lentil lectin (which isolates $\alpha$-D-mannose-containing glycoproteins), then on Sepharose containing the covalently bound substrate, 5′-AMP (Table 1). The zwitterionic detergent CHAPS proved highly suitable for the purification procedure, resulting in a high degree of solubilization of the lymphocyte plasma membrane (approx. 80 % of the total protein). Retention of 5′-NTase activity during the procedure was also high; 26 % of the catalytic activity of the CHAPS extract was recovered in the final purified, concentrated enzyme preparation. SDS/PAGE analysis of highly purified 5′-NTase following $^{125}$I-radiolabelling resulted in a major band, with an apparent $M_r$ of 81000 (Figure 1, lanes 4 and 5). It is important to note that 5′-NTase is a low-abundance protein in the lymphocyte membrane (see Figure 1), and a typical purification protocol yielded less than 50 $\mu$g of enzyme. However, 5′-NTase is highly catalytically active, and nanogram amounts could be readily measured using the assay system described in the Materials and Methods section. The apparent level of purification of 5′-NTase was > 400-fold from the plasma membrane and approx. 280-fold from the CHAPS extract (Table 1).

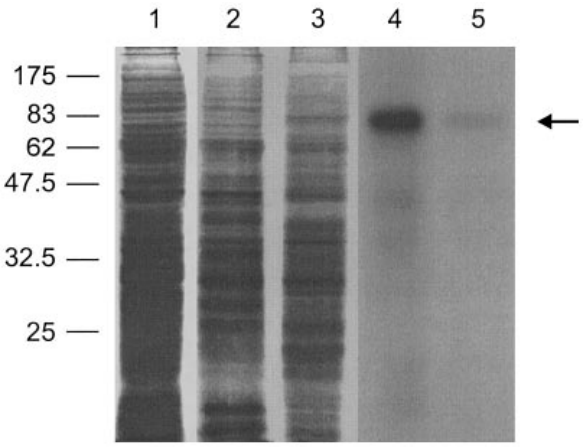


**Figure 1 SDS/PAGE analysis of purified porcine-lymphocyte 5′-NTase**

Lymphocyte plasma membrane (lane 1, 15 $\mu$g of protein), CHAPS-solubilized plasma membrane (lane 2, 10 $\mu$g of protein) and the glycoprotein fraction (lane 3, 10 $\mu$g of protein) were subjected to SDS/PAGE analysis in a 12% gel, followed by staining with silver. $^{125}$I-Labelled, purified 5′-NTase in CHAPS solution (lane 4), and DMPC vesicles containing reconstituted $^{125}$I-5′-NTase (lane 5) were separated in a 12% gel, and detected by autoradiography. The position of molecular-mass markers is indicated on the left, and the 5′-NTase band is indicated by an arrow.

**Table 1 Purification of 5′-NTase from porcine lymphocytes**

Samples from various stages of the purification procedure were assayed for 5′-NTase activity as described in the Materials and Methods section. Activity is expressed as 5′-AMP-hydrolysed and is presented as the mean ± S.E.M. ($n = 3$).

| Stage of purification | Protein (mg) | Total 5′-NTase activity ($\mu$mol/min) | 5′-NTase specific activity ($\mu$mol/min per mg of protein) | Fold purification |
|---|---|---|---|---|
| Plasma membrane | 63.8 ± 0.8 | 1.89 ± 0.01 | 0.0297 ± 0.0006 | 1 |
| CHAPS extract | 49.8 ± 1.6 | 2.20 ± 0.02 | 0.0442 ± 0.0017 | 1.5 |
| Lentil lectin column eluate | 7.0 ± 0.15 | 2.33 ± 0.02 | 0.334 ± 0.011 | 11.2 |
| 5′-AMP column eluate | 0.0458 ± 0.0015 | 0.566 ± 0.015 | 12.36 ± 0.73 | 416 |

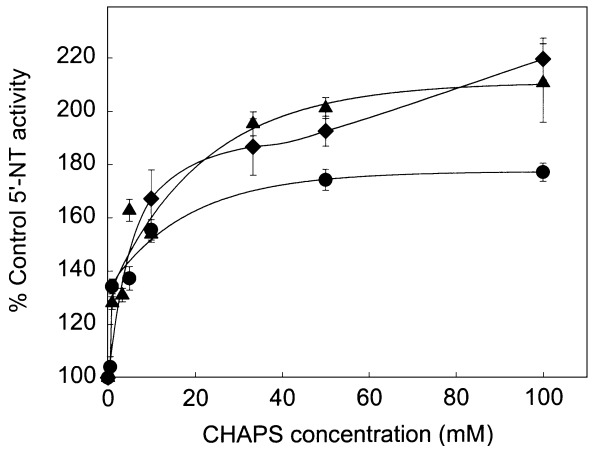


**Figure 2 Symmetry of reconstitution of 5′-NTase into various lipids**

5′-NTase (5′-NT) activity following reconstitution into various lipids (DMPC, ●; SCRL, ▲; egg PE, ◆) was determined in the presence of increasing concentrations of CHAPS, which permeabilizes the vesicles and reveals cryptic inward-facing enzyme. Data points are presented as the mean ± S.D. for duplicate determinations of 5′-NTase activity, as a percentage of control samples with no added detergent.

### Reconstitution of purified 5′-NTase

Purified 5′-NTase was reconstituted into lipid bilayers of various natural and synthetic phospholipids, including DMPC, egg PC, SM, egg PE and a sphingolipid/cholesterol-rich mixture, using a detergent-dialysis technique developed previously in our laboratory. Over 95 % of the 5′-NTase enzymic activity was removed from the reconstitution mixtures following harvesting of the vesicles by centrifugation. Reconstitution resulted in large unilamellar vesicles, as assessed by fluorescence microscopy in the presence of the lipid-soluble fluorescent marker 1-anilinonaphthalene-8-sulphonic acid. PE species have a tendency to shift from the bilayer phase to the hexagonal ($H_{II}$) phase as the temperature is increased. These two phases can readily be distinguished by their characteristic $^{31}$P-NMR spectra [24]. For egg PE, the temperature at which the lipid starts to convert from the bilayer phase to the hexagonal phase is just below 40 °C, and depends on the acyl-chain composition of the particular PE. $^{31}$P-NMR experiments showed that the egg PE used in this study was largely in the bilayer phase at 37 °C. The transition from the bilayer to the hexagonal phase started to occur at 38–39 °C, and was largely complete by 45 °C.

The symmetry of 5′-NTase reconstitution was assessed by addition of increasing concentrations of CHAPS to the lipid structures. The assay for 5′-NTase measures the activity of outward-facing enzyme, and the 5′-NTase facing the vesicle lumen remains cryptic unless the bilayer is permeabilized to 5′-AMP by the addition of detergent. 5′-NTase was inserted into the vesicles approximately symmetrically for DMPC, SCRL and egg PE, since 56 %, 48 % and 46 %, respectively, of the total enzyme activity was measurable in the absence of detergent, indicating that it is present at the outer surface of the vesicle. The balance of the enzyme activity (44–54 % of the total) remained cryptic until after the addition of permeabilizing amounts of CHAPS (Figure 2). Reconstituted 5′-NTase retained full catalytic activity for over 5 weeks when the reconstituted systems were stored at 4 °C in 20 mM Tris/HCl buffer (pH 7.4), emphasizing the stability of the enzyme under these conditions.

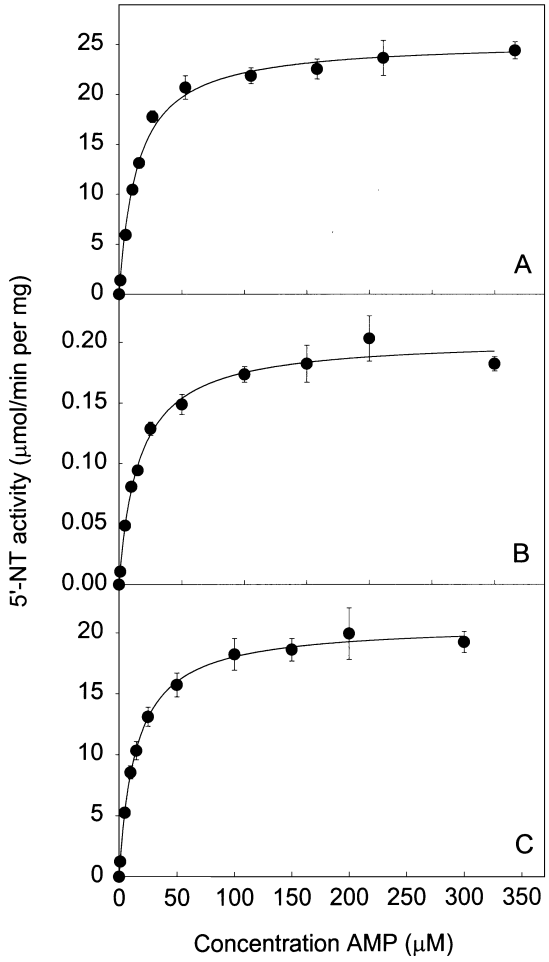


**Figure 3 Kinetics of catalysis by detergent-solubilized and membrane-bound 5′-NTase**

The initial rate of AMP hydrolysis were determined for purified 5′-NTase (5′-NT) in CHAPS solution (**A**), porcine-lymphocyte plasma-membrane vesicles (**B**) and purified 5′-NTase reconstituted into DMPC vesicles (**C**). Data points are presented as the means ± S.E.M. of triplicate determinations of enzymic activity. The solid lines represent the best fit of the data points to the Michaelis–Menten equation, as determined by non-linear regression analysis.

### Kinetics of AMP hydrolysis by detergent-solubilized and membrane-bound 5′-NTase

A major objective of this study was to determine whether 5′-NTase underwent a change in kinetic parameters following cleavage from a membrane surface by PI-PLC. The first step was, therefore, to characterize the kinetics of purified 5′-NTase in both detergent solution and a membrane environment. 5′-NTase is a homodimer with interchain disulphide bridges, which are essential for enzymic activity (reviewed in [14]). We have shown previously that 5′-NTase remains in the dimeric form (as indicated by gel-filtration FPLC) following solubilization of lymphocyte plasma membrane by CHAPS [17]. Initial rate measurements were carried out using purified 5′-NTase in CHAPS solution, in the absence of added lipids, using a 5′-AMP concentration range from 1 $\mu$M to over 300 $\mu$M. The data were fitted to the Michaelis–Menten equation by non-linear regression analysis (Figure 3A), and the kinetic parameters $K_m$ and $V_{max}$ were extracted (Table 2). The same experiment was carried out for 5′-NTase in native lymphocyte plasma membrane (Figure

**Table 2 Summary of kinetic parameters for 5′-NTase before and after cleavage by PI-PLC**

| | $K_m$ ($\mu$M) | | | $V_{max}$ ($\mu$mol/min per mg of protein) | | |
|---|---|---|---|---|---|---|
| | Bound | Cleaved | $\Delta K_m$ (%) | Bound | Cleaved | $\Delta V_{max}$ (%) |
| Plasma membrane | 13.4 ± 1.0 | 13.4 ± 1.0 | 0* | 0.124 ± 0.003 | 0.150 ± 0.003 | 21 |
| CHAPS | 14.9 ± 1.0 | 16.5 ± 1.0 | +11* | 28.2 ± 0.5 | 34.7 ± 0.5 | 23 |
| DMPC | 14.6 ± 0.6 | 13.6 ± 0.6 | −9* | 20.7 ± 0.2 | 22.4 ± 0.2 | 8 |
| Egg PC | 11.1 ± 0.5 | 12.5 ± 0.7 | +13* | 22.6 ± 0.2 | 26.2 ± 0.4 | 16 |
| Egg PE | 13.7 ± 1.8 | 14.0 ± 1.0 | +2* | 13.4 ± 0.3 | 23.3 ± 0.4 | 74 |
| SCRL | 15.7 ± 1.2 | 11.2 ± 0.5 | −28 | 15.3 ± 0.3 | 18.6 ± 0.2 | 21 |
| SM | 11.0 ± 0.9 | 11.2 ± 0.6 | +2* | 20.2 ± 0.4 | 20.9 ± 0.2 | 4 |

* Not significantly different.

3B) and reconstituted DMPC vesicles (Figure 3C). In each case, the kinetic data fitted well to the Michaelis–Menten equation, so it appears that 5′-NTase follows classical Michaelis–Menten kinetics despite existing as a homodimer.

### Cleavage of detergent-solubilized and membrane-bound 5′-NTase by PI-PLC

Purified 5′-NTase, both in CHAPS solution and reconstituted into various lipids, was incubated with increasing concentrations of recombinant PI-PLC from *B. thuringiensis*. We previously showed that the form of 5′-NTase that exists following PI-PLC treatment is hydrophilic, as assessed by Triton X-114 partitioning, indicating that the GPI anchor had been removed from the enzyme [18]. The hydrophilic soluble form of 5′-NTase was separated from the remaining enzyme with an intact anchor, by either Triton X-114 partitioning (in the case of CHAPS-solubilized 5′-NTase), or by centrifugation (in the case of membrane-bound 5′-NTase). For 5′-NTase solubilized in CHAPS, cleavage by PI-PLC resulted in loss of enzymic activity from the hydrophobic detergent layer and its appearance in the aqueous phase (Figure 4A). In the case of plasma-membrane or reconstituted 5′-NTase, the disappearance of activity from the pellet corresponded with its appearance in the supernatant (Figures 4B and 4C). The GPI anchor of essentially all 5′-NTase molecules was cleaved by PI-PLC, whether the enzyme was solubilized in detergent solution (Figure 4A), reconstituted into lipid bilayer vesicles of DMPC (Figure 4C) or present in native lymphocyte plasma-membrane vesicles (Figure 4B). This indicates that there is no pool of lymphocyte 5′-NTase that is intrinsically resistant to PI-PLC cleavage.

Phospholipase cleavage of 5′-NTase was further characterized by determination of the quantitative parameter $EC_{50}$, defined as the concentration of PI-PLC required for removal of the GPI anchor from 50 % of the 5′-NTase pool. For 5′-NTase solubilized in CHAPS, the $EC_{50}$ was estimated from the curve representing enzyme activity in the Triton X-114 layer (Figure 4A). For 5′-NTase in native plasma membrane, or reconstituted into various lipid systems, the $EC_{50}$ was determined from the curves for enzyme activity of the pellet, as shown in Figures 3(B) and 3(C). As indicated in Table 3, the $EC_{50}$ values for cleavage of reconstituted 5′-NTase spanned a very broad range, from 0.008 units/ml for egg PC to 1.5 units/ml for SM. Lipids fell into two distinct groups; those with low $EC_{50}$ values, and those with high $EC_{50}$ values. Native plasma membrane, egg PC and SCRL all showed high efficiency of 5′-NTase cleavage, with $EC_{50}$ values in the range 0.008–0.03 units/ml. On the other hand, PI-PLC worked much less efficiently on 5′-NTase when it was present in CHAPS micelles, DMPC, egg PE and SM, where $EC_{50}$ values fell in the range 0.53–1.5 units/ml. This wide difference in susceptibility to cleavage suggests that the properties of the host lipid, such as bilayer packing, fluidity and head-group molecular properties, may affect the ability of PI-PLC to gain access to the GPI anchor of 5′-NTase.

### Activation of 5′-NTase following cleavage from various membrane systems

Substantial catalytic activation (approx. 21 %) was observed for lymphocyte plasma-membrane 5′-NTase following cleavage of the GPI anchor by PI-PLC and release in soluble form (Figure 5A). Activation following PI-PLC treatment was also noted for 5′-NTase in CHAPS micelles (Figure 5B), and after reconstitution into certain lipids and lipid mixtures (Figures 5C–5F). In all cases, the $V_{max}$ (Table 2) and catalytic-centre activity (Table 4) of the ecto-enzyme were increased following release from the membrane. The $V_{max}$ of soluble 5′-NTase after release was more or less independent of the lipid system initially used to anchor the protein. The $K_m$ of 5′-NTase for AMP hydrolysis remained essentially unchanged following release from the membrane for all lipids except SCRL, where a 28 % decrease in $K_m$ was noted (Table 2). The largest degrees of activation were observed for 5′-NTase reconstituted into egg PE (74 %) and SCRL (21 %). Only a small activation of the enzyme was noted following cleavage of 5′-NTase from vesicles of DMPC and SM. The observation of activation in detergent solution indicates that insertion of the GPI anchor into a lipid bilayer is not in itself strictly necessary for catalytic activation following anchor cleavage.

The catalytic-centre activity of soluble 5′-NTase lacking the GPI anchor fell in a narrow range (27–38 molecules·$s^{-1}$, mean 31.9 molecules·$s^{-1}$; Table 4), no matter which lipid system the enzyme had originally been reconstituted into. The catalytic-centre activity for cleaved 5′-NTase in the presence of CHAPS micelles was significantly higher (Table 4), which may reflect the effect of detergent on the enzyme. The catalytic-centre activity of 5′-NTase was decreased on reconstitution into certain lipids, especially egg PE and SCRL, and release from the membrane surface following PI-PLC cleavage restored the enzymic activity (Table 4). These results suggest that the kinetic characteristics of 5′-NTase are affected by the presence of the GPI anchor. The intact anchor, when inserted into a detergent micelle, lipid bilayer or membrane, may change the conformation of the protein to reduce its catalytic efficiency. This reduction in efficiency appears to depend on the nature of the bilayer or membrane into which the anchor is inserted.

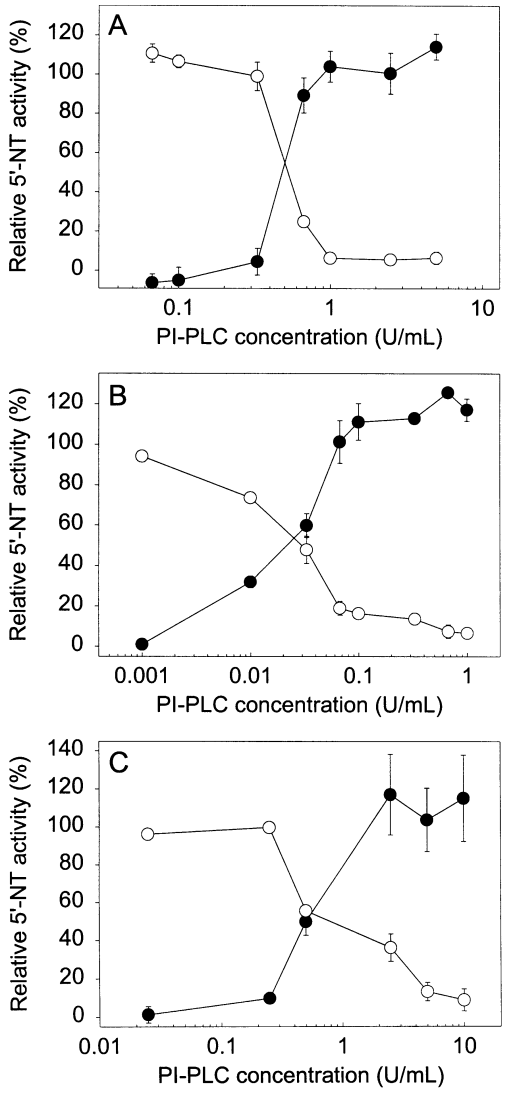


**Figure 4 Cleavage of detergent-solubilized and membrane-bound 5′-NTase by PI-PLC**

(**A**) Purified 5′-NTase (5′-NT) in CHAPS solution was incubated with increasing concentrations of *B. thuringiensis* PI-PLC for 90 min at 37 °C, and enzyme retaining an intact GPI anchor was separated from the cleaved enzyme using Triton X-114 extraction. Enzyme activities in the lower detergent phase (○) and the upper aqueous phase (●) were then determined as described in the Materials and Methods section. Plasma-membrane vesicles (**B**) and DMPC vesicles containing reconstituted 5′-NTase (**C**) were incubated with increasing concentrations of PI-PLC for 90 min at 37 °C, and the membrane-bound enzyme was separated from the cleaved soluble enzyme by centrifugation. Enzyme activities in the membrane pellet (○) and the soluble supernatant (●) were then determined as described in the Materials and Methods section. Data points represent the mean ± S.D. for duplicate determinations. U/mL, units/ml.

## DISCUSSION

Release of GPI-anchored proteins by endogenous phospholipases has been proposed to play an important role in regulation of their surface activity, and may also generate second messengers that initiate transmembrane signalling processes [7,10,11,25]. It is, therefore, important to have a detailed understanding of the potential consequences of such release. Our approach in the present work was to use the purified GPI-anchored ecto-enzyme 5′-NTase, reconstituted into defined phospholipids. The advantage of using such lipid systems is that many biochemical and biophysical parameters, such as lipid:protein ratio, acyl-chain length and fluidity, and head-group charge, may be strictly controlled.

**Table 3 Release of detergent-solubilized and membrane-bound 5′-NTase by PI-PLC**

The $EC_{50}$ was defined as the concentration of PI-PLC required to release 50% of the 5′-NTase in soluble form. For CHAPS-solubilized 5′-NTase, 5′-NTase with an intact GPI anchor was separated from soluble 5′-NTase from which the anchor had been cleaved using Triton X-114. For membrane-bound 5′-NTase, the value of $EC_{50}$ was determined from a plot of enzyme activity in the pellet versus PI-PLC concentration, and was similar to the value determined from a plot of enzyme activity remaining in the supernatant versus PI-PLC concentration.

| 5′-NTase sample | $EC_{50}$ (units/ml) |
|---|---|
| Plasma membrane | 0.03 |
| CHAPS solution | 0.53 |
| DMPC | 0.7 |
| Egg PC | 0.008 |
| Egg PE | 0.85 |
| SCRL | 0.01 |
| SM | 1.5 |

Porcine lymphocyte 5′-NTase is a disulphide-linked homodimer in its native state [14], and remains in this form during purification and reconstitution [17]. Results from the present study indicated that 5′-NTase obeyed classical Michaelis–Menten kinetics whether it was present in native plasma membrane, solubilized in detergent or reconstituted with phospholipids, indicating that there is no allosteric communication between the two monomers. Experiments also showed that 5′-NTase, whether it was membrane-bound, in detergent solution or reconstituted, was completely cleaved by the action of PI-PLC. Therefore, no phospholipase-resistant pool of enzyme exists for 5′-NTase from this source. The resistance of some GPI-anchored proteins (e.g. human erythrocyte acetylcholinesterase, alkaline phosphatase) to release by PI-PLC has been demonstrated to arise from a change in the anchor structure via covalent modification of the inositol ring by esterification of an additional fatty-acid moiety [26]. Such modified anchors may remain susceptible to cleavage by glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) [27]. Highly PI-PLC-resistant populations have been reported for 5′-NTase in hepatocytes [28], and in the plasma membrane of liver from different species [29]. Since in the case of porcine-lymphocyte 5′-NTase the entire population was cleaved by PI-PLC, covalent modification does not appear to be involved.

Koelsch et al. [30] reported that aminopeptidase P of rat small-intestine brush-border membrane vesicles, which had a normal unmodified anchor structure, was resistant to cleavage by PI-PLC, but could be released by GPI-PLD. The anchor could be made accessible to PI-PLC by treatment of the membrane vesicles with papain, sonication or solubilization with detergent. It was proposed that the GPI anchor of aminopeptidase P is sterically hindered, and only accessible to PI-PLC after disturbance of the membrane structure. Similar arguments were used to explain the PI-PLC resistance of alkaline phosphatase in mouse brush-border membrane vesicles [31].

We used $EC_{50}$ (the concentration of phospholipase required to release 50% of the 5′-NTase in soluble form) as a quantitative measure of the ease of cleavage of the 5′-NTase anchor in several different lipid environments by PI-PLC. Results showed that $EC_{50}$ values varied over a range of almost 200-fold (see Table 3), depending on the lipid chosen for reconstitution. The lipid:protein ratio of the reconstituted preparations was con-

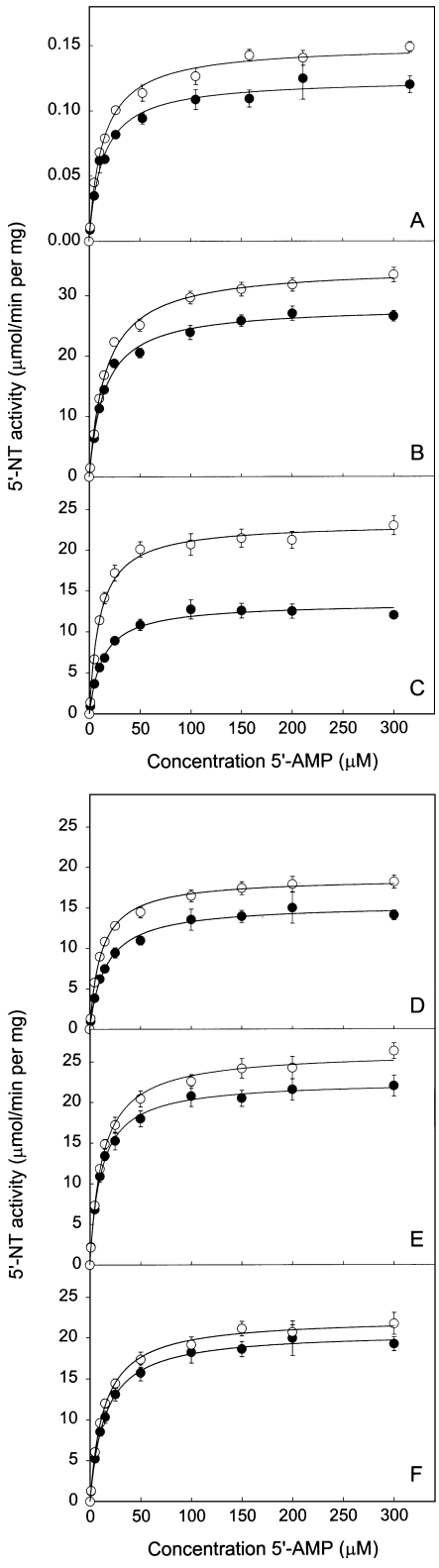


**Figure 5 Kinetics of 5′-NTase before and after cleavage of the GPI anchor by PI-PLC**

Plasma-membrane vesicles, 5′-NTase (5′-NT) solubilized in CHAPS and 5′-NTase reconstituted into various lipids were treated for 90 min at 37 °C with the appropriate concentration of *B. thuringiensis* PI-PLC necessary for complete cleavage of the GPI anchor (see Figure 4). The initial rate of AMP hydrolysis was determined on identical samples before (●) and after (○) anchor cleavage, for native lymphocyte plasma-membrane vesicles (**A**), CHAPS-solubilized 5′-NTase (**B**), and 5′-NTase reconstituted into egg PE (**C**), SCRL (**D**), egg PC (**E**) and DMPC (**F**). Data points are presented as the means ± S.E.M. of triplicate determinations of enzymic activity. The solid lines represent the best fit of the data points to the Michaelis–Menten equation, as determined by non-linear regression analysis.

**Table 4 Catalytic-centre activities for 5′-NTase before and after cleavage by PI-PLC**

| | $k_{cat}$ (molecules · $s^{-1}$) | |
|---|---|---|
| | Bound | Cleaved |
| CHAPS | 40.4 ± 0.7 | 49.7 ± 0.7 |
| DMPC | 29.6 ± 0.3 | 32.1 ± 0.3 |
| Egg PC | 32.3 ± 0.3 | 37.5 ± 0.5 |
| Egg PE | 19.2 ± 0.4 | 33.3 ± 0.6 |
| SCRL | 22.0 ± 0.4 | 26.6 ± 0.3 |
| SM | 28.9 ± 0.5 | 30.0 ± 0.3 |

trolled to be in the range 150–200:1 in all cases, and, given the low protein content, steric crowding effects on the membrane surface should not have been a factor. It appears, therefore, that the properties of the lipid alone can modulate cleavage by PI-PLC. Three systems (native membrane, egg PC and SCRL) allowed PI-PLC to cleave 5′-NTase very easily, yielding low $EC_{50}$ values, whereas three lipids (DMPC, egg PE and SM) and CHAPS micelles had high $EC_{50}$ values, indicating that phospholipase cleavage was considerably more difficult in these environments. We have shown that the susceptibility of GPI-anchored proteins to PI-PLC cleavage may depend on bilayer surface charge [18]; however, in the present case, the lipids and detergent used were all zwitterionic, so differences in head-group charge cannot account for the observed variations. Head-group structure also does not seem to play a role, since large differences in $EC_{50}$ values were observed among the choline-containing lipids (PC and SM). This suggests that properties of the lipid acyl chains (packing, fluidity) may be important in determining susceptibility to phospholipase cleavage. In this regard, we have previously shown that the ability of PI-PLC to cleave 5′-NTase is greatly lowered when the host lipid is in the rigid, tightly packed gel phase, compared with the fluid, loosely packed liquid crystalline phase [18]. It is possible that a tight packing density and low surface deformability may restrict the ability of the phospholipase to gain access to the GPI anchor, which is located within the polar interfacial region of the bilayer.

There have been other reports that the susceptibility of GPI-anchored proteins to attack by phospholipase C is dependent on membrane lipid composition. In Chinese hamster ovary cells deficient in sphingolipids, the GPI-anchored antigen CD14 became hypersensitive to PI-PLC cleavage, relative to the same cells when supplemented with sphingolipids in the medium [32]. These results suggested that interaction of GPI-anchored proteins with sphingolipids, probably by formation of DIGs, can reduce the accessibility of the anchor to phospholipase action.

The results of the present study indicate that 5′-NTase is catalytically activated following release from a number of different membrane systems. Catalytic activation of GPI-anchored proteins following release from the membrane surface by specific phospholipases has been reported for several enzymes. The hyaluronidase activity of the PH-20 protein present in the plasma membrane of guinea pig sperm is released into the medium by PI-PLC with a large increase in enzymic activity [33].

Early work by Low and Finean [34] reported recovery of substantially increased 5′-NTase activity in the soluble supernatant following treatment of intact pig lymphocytes with *Staphylococcus aureus* PI-PLC. Dipeptidase from porcine-kidney microvillar membrane is also activated following removal of the GPI anchor [35]. In this case, release of the enzyme from the membrane surface resulted in a 10-fold decrease in the $K_m$ of the enzyme, whereas $V_{max}$ remained essentially unchanged. The authors suggested that insertion of the GPI anchor into a lipid bilayer may result in conformational restraints on the active site, which are relaxed when the protein is released by PI-PLC cleavage, resulting in an increase in the affinity of the enzyme for its substrate. Interestingly, 5′-NTase present in the same membrane did not show any apparent activation, which suggests that different constraints on the activity of porcine 5′-NTase exist in the plasma membrane of lymphocytes relative to kidney cells in the pig.

In the case of porcine-lymphocyte 5′-NTase, kinetic analysis indicated that catalytic activation is primarily the result of an increase in $V_{max}$ (or $k_{cat}$) of the enzyme, rather than $K_m$ (Tables 3 and 4). $V_{max}$ appears to be lowered when the GPI anchor of the enzyme is inserted into various lipids, especially egg PE. As might be expected, the $V_{max}$ of soluble 5′-NTase, after release from the membrane, is more or less independent of the lipid system initially used to anchor the protein. Overall, the data indicate that the catalytic efficiency of 5′-NTase is reduced when its GPI anchor is inserted into a membrane environment or a detergent micelle. This restriction is relieved following release of 5′-NTase in soluble form by the action of PI-PLC. The kinetic properties of chicken-gizzard 5′-NTase were also reported to be different depending on whether the enzyme was detergent-solubilized or cleaved by *B. thuringiensis* PI-PLC [36]. In this case, 5′-NTase with the anchor removed showed both an increase in $V_{max}$ and a 2-fold decrease in $K_m$ compared with intact 5′-NTase in deoxycholate solution. Clearly, 5′-NTase behaves differently depending on the species and tissue from which it is isolated.

The affinity of membrane-bound 5′-NTase for the substrate 5′-AMP was quite high, with $K_m$ in the range 11–16 $\mu$M, depending on the lipid system employed (see Table 2). This affinity remains essentially unchanged following PI-PLC release for the native plasma membrane and all the lipid bilayer systems used in this study, with the exception of SCRL. The $K_m$ of 5′-NTase in SCRL bilayers was higher than for other lipids, and a significant decrease was observed following cleavage (Table 2), which suggests that a conformational constraint on substrate binding might exist in this case. The lipid composition of SCRL has been shown to encourage the formation of DIGs into which GPI-anchored proteins preferentially locate [9], and this might lead to some conformational restriction that would affect $K_m$. 5′-NTase has been reported to be present in DIGs in intestinal epithelial cells [37].

It is possible that insertion of both GPI anchors of the 5′-NTase homodimer into the bilayer imposes some conformational constraints on the protein structure, which are relieved following cleavage of one anchor. This explanation was suggested for dipeptidase [35], where maximal catalytic activation was observed at a PI-PLC concentration 10-fold less than that required for complete release from the membrane. In contrast, in the present study, increased activity of 5′-NTase coincided with maximal release of the enzyme into the supernatant, making it unlikely that this mechanism is responsible for activation.

Alterations in protein structure and function following anchor cleavage have also been observed for GPI-anchored proteins with no enzymic activity. Two yeast cAMP receptor proteins displayed an approx. 10-fold decrease in the $K_m$ for cAMP following treatment with PI-PLC or GPI-PLD [38]. Wang et al. [39] reported that covalent fatty-acyl modification of the inositol ring of a folate receptor variant was associated with high binding affinity for reduced folates. PI-PLC cleavage of the modified anchor after mild base treatment led to a large decrease in substrate binding affinity, whereas anchor removal from an unmodified folate receptor had no effect, suggesting that the modified anchor can influence protein conformation or topology with respect to the membrane.

Removal of the GPI anchor from Thy-1 (a GPI-anchored lymphocyte antigen) by phospholipase C or D triggered a major change in structure of the protein, which greatly reduced binding of both polyclonal and monoclonal antibodies to the protein [40]. Experimental evidence and molecular-dynamics simulations indicated that removal of the phospholipid portion of the GPI anchor causes a conformational change in the remaining glycan, which in turn leads to a change in conformation on the opposite face of the Thy-1 protein. Changes in antibody binding following cleavage of the GPI anchor have also been noted for carcinoembryonic antigen [41]. Barboni et al. [40] suggested that the GPI anchor might generally alter the conformation of proteins to which it is attached. It is possible that, as in the case of Thy-1, cleavage of the GPI anchor of 5′-NTase alters the protein conformation, which in turn increases the catalytic activity of the enzyme.

We reported previously that reconstituted 5′-NTase demonstrated a decrease in activation energy when the bilayer was converted from the solid gel phase to the fluid liquid-crystalline phase [18]. 5′-NTase from rat enterocytes also displayed a break point on Arrhenius plots, which coincided with a lipid thermotropic transition [42]. These results suggest that the protein portion of 5′-NTase may be in direct contact with the lipid bilayer. Evidence from studies of the GPI-anchored lymphocyte antigen Thy-1 suggest that this may indeed be the case. The glycan portion of the GPI anchor of Thy-1 is predicted to lie either between the lipid surface and the protein in a tightly folded conformation [40], or in a carbohydrate-binding pocket within the protein itself [43], and it may thus impose a particular conformation on the protein. In both models, the protein domain of Thy-1 is visualized as being very close to, or in contact with, the bilayer. If this is true of GPI-anchored proteins in general, it would provide a mechanism for transmission of structural changes from the membrane surface to the protein.

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