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Ectoenzymes of the kidney microvillar membrane

Differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor

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The pattern of solubilization of nine kidney microvillar ectoenzymes by a range of detergents distinguished two classes of membrane proteins: those released from the membrane by bacterial phosphatidylinositol-specific phospholipase C and those not so released. The latter group of transmembrane proteins were solubilized efficiently (> 80%) by all the detergents examined. In contrast, proteins released by phosphatidylinositol-specific phospholipase C were solubilized effectively only by octyl glucoside, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate and sodium deoxycholate. Octyl glucoside solubilized the amphipathic forms of the ectoenzymes examined, suggesting that this may be a useful detergent in the purification of glycosyl-phosphatidylinositol-anchored ectoenzymes.

INTRODUCTION

The kidney microvillar membrane contains a battery of ectoenzymes that includes peptidases, phosphatases and glycosidases (reviewed in Kenny & Maroux, 1982; Semenza, 1986; Kenny & Turner, 1987). Several of these ectoenzymes, such as endopeptidase-24.11, are anchored by a transmembrane stretch of hydrophobic amino acid residues (Semenza, 1986; Devault *et al.*, 1987; Malfroy *et al.*, 1987), whereas others, such as alkaline phosphatase, are anchored in the outer leaflet of the bilayer by a covalently attached glycosyl-PI moiety (Cross, 1987; Low, 1987). The solubilization of ectoenzymes in a functional form has commonly been achieved by using proteinases, such as trypsin and papain (see, e.g., George & Kenny, 1973; Danielsen *et al.*, 1980); in addition, those ectoenzymes anchored via glycosyl-PI can be released from the membrane by bacterial PI-PLC (Thompson *et al.*, 1987). However, release of ectoenzymes by proteinases or phospholipases produces the hydrophilic form of the protein.

Often, for structural studies, the amphipathic form of a protein, which retains the membrane anchor, is required. In this case the ectoenzyme is usually solubilized from the membrane with a non-ionic neutral detergent such as Triton X-100 (see, e.g., Macnair & Kenny, 1979; Fulcher & Kenny, 1983). However, those ectoenzymes that are anchored via a glycosyl-PI moiety are relatively resistant to solubilization by such detergents (Kim & Campbell, 1983; Hooper *et al.*, 1987), and have generally been solubilized in their amphipathic form by the use of organic solvents such as butan-1-ol (Saseyima *et al.*, 1975; Campbell *et al.*, 1984; Malik & Low, 1986). These problems prompted us to compare the ability of a range of detergents to solubilize the amphipathic forms of glycosyl-PI-anchored kidney microvillar ectoenzymes.

Here we show that detergents with a high CMC (e.g. octyl glucoside, CHAPS and sodium deoxycholate) can solubilize substantial amounts of those ectoenzymes in pig kidney microvillar membranes that are also susceptible to release by bacterial PI-PLC. The detergent-solubilized enzymes displayed amphipathic properties on phase separation in Triton X-114. Also, glycosyl-PI-anchored ectoenzymes gave a pattern of detergent solubilization quite distinct from those ectoenzymes that are not released from the membrane by bacterial PI-PLC. This latter group included endopeptidase-24.11, aminopeptidases A and N, dipeptidyl peptidase IV and, in contrast with a previous report (Nakabayashi & Ikezawa, 1986), alkaline phosphodiesterase I.

MATERIALS AND METHODS

Materials

PI-PLC from *Bacillus thuringiensis* and from *Staphylococcus aureus* were purified as described previously (Malik & Low, 1986; Hooper *et al.*, 1987) and were gifts from Dr. M. G. Low. Units of PI-PLC activity are μmol/min. Sodium deoxycholate was purchased from BDH Chemicals, Poole, Dorset, U.K. All other detergents were purchased from Sigma Chemical Co. Triton X-114 was pre-condensed before use (Bordier, 1981). Pig kidneys and all other materials were obtained from sources previously noted.

Preparation of pig kidney microvillar membranes and solubilization of membrane proteins by detergents and phospholipases

All operations were carried out at 4 °C unless stated otherwise. Microvilli were prepared from pig kidney cortex by the method of Booth & Kenny (1974), except that the 15000 *g* centrifugation steps were each extended from 12 min to 15 min. Before incubation with detergent or phospholipase, the microvillar membranes were centrifuged at 31000 *g* for 1.5 h and then resuspended in 10 mM-Hepes/NaOH buffer, pH 7.4, to give a protein concentration of approx. 2.5 mg/ml. Portions of deter-

Abbreviations used: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; CMC, critical micellar concentration; octyl glucoside, n-octyl β-D-glucopyranoside; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

gent or phospholipase were added where appropriate, and the membranes were incubated with shaking for 1 h at 4 °C or 37 °C respectively. After solubilization of the protein, the incubation mixtures were centrifuged at 31000 *g* for 1.5 h. Enzyme activities were determined in the total incubation mixture and in the supernatant after centrifugation at 31000 *g*; the solubilized activity was expressed as a percentage of the total (100 %) activity in the original incubation mixture.

Enzyme assays

Trehalase was assayed by the method of Dahlqvist (1964). Alkaline phosphodiesterase I was assayed by the spectrophotometric method of Brightwell & Tappel (1968) with 4-nitrophenyl thymidine 5′-monophosphate as substrate. 5′-Nucleotidase was assayed with 5′-AMP as substrate (Heinonen & Lahti, 1981). Endopeptidase-24.11, aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, renal dipeptidase, alkaline phosphatase and protein were assayed as described previously (Fulcher & Kenny, 1983; Matsas *et al.*, 1985; Hooper *et al.*, 1987).

RESULTS AND DISCUSSION

Release of ectoenzymes from pig kidney microvillar membranes by bacterial PI-PLC

The nine ectoenzymes studied were all enriched in the pig kidney microvillar membrane preparation (Table 1). When this membrane preparation was incubated with PI-PLC from *B. thuringiensis*, substantial release of alkaline phosphatase, renal dipeptidase, trehalase and 5′-nucleotidase was observed, but negligible release of endopeptidase-24.11, aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV or alkaline phosphodiesterase I (Table 1). Similar results were obtained with *S. aureus* PI-PLC (results not shown).

Although more than 70 % of the alkaline phosphatase, renal dipeptidase and trehalase activities were released by *B. thuringiensis* PI-PLC, only 37 % of the 5′-nucleotidase activity was so released (Table 1), perhaps reflecting two distinct populations of the enzyme. A similar partial release of 5′-nucleotidase by bacterial PI-PLC has also been observed with rat liver cells (Low & Finean, 1978; Shukla *et al.*, 1980).

Solubilization of ectoenzymes from pig kidney microvillar membranes by detergents

The solubilization of the microvillar enzymes by a number of detergents was compared. Fig. 1 reveals that only the detergents of high CMC (octyl glucoside, CHAPS and sodium deoxycholate) were effective at releasing substantial amounts of the glycosyl-PI-anchored proteins into the high-speed supernatant. In contrast, all the detergents were equally effective at solubilizing those enzymes not released by PI-PLC. From Fig. 1, the mean ratio of activity solubilized by octyl glucoside to that solubilized by Triton X-100 was 4.0 (range 1.7–6.3) for glycosyl-PI-anchored ectoenzymes, but 1.2 (range 1.0–1.3) for the remainder. The ratio of total protein solubilized by the two detergents was 1.3.

The solubilization of pig kidney microvillar ectoenzymes by octyl glucoside was examined in more detail (Fig. 2). For all the ectoenzymes studied, a concentration of 60 mM-octyl glucoside appeared to provide maximum solubilization; no further release was observed at a detergent concentration of 100 mM. At concentrations of octyl glucoside lower than 60 mM the amount of each enzyme solubilized substantially decreased, with minimal (< 10 %) enzyme activity being solubilized at concentrations below the CMC value.

Phase separation of soluble and membrane forms of the ectoenzymes in Triton X-114

Phase separation of untreated microvillar membranes (Table 2*a*) and octyl glucoside-solubilized membranes (Table 2*b*) in Triton X-114 (Bordier, 1981) resulted in most of the ectoenzyme activities partitioning into the detergent-rich phase, indicative of an amphipathic character. Recently, octyl glucoside has been used to solubilize rat intestinal trehalase with a recovery of 77 %.

Table 1. Specific activity, enrichment and release by PI-PLC of ectoenzymes in pig kidney microvillar membranes

Enzymes were assayed as described in the Materials and methods section. The specific activity and enrichment results are the means ± S.E.M. for three separate microvillar membrane preparations. The maximal activity released by PI-PLC was that released from pig kidney microvillar membranes upon incubation with *B. thuringiensis* PI-PLC (1 unit/ml) under the conditions described in the Materials and methods section, and the results are the means for duplicate incubations with PI-PLC.

Enzyme	EC number	Specific activity (nmol/min per mg)	Enrichment over homogenate (fold)	Maximal activity released by PI-PLC (%)
Alkaline phosphatase	3.1.3.1	146 ± 1	5.5 ± 0.1	82.6
5′-Nucleotidase	3.1.3.5	39 ± 5	2.5 ± 0.2	36.5
Trehalase	3.2.1.28	82 ± 12	4.6 ± 1.0	70.5
Renal dipeptidase	3.4.13.11	127 ± 30	5.3 + 0.2	82.3
Aminopeptidase N	3.4.11.2	524 ± 36	6.0 ± 0.5	0.0
Aminopeptidase A	3.4.11.7	126 ± 9	2.6 ± 0.3	0.0
Dipeptidyl peptidase IV	3.4.14.5	606 ± 65	4.3 ± 0.1	0.0
Endopeptidase-24.11	3.4.24.11	390 ± 25	5.3 ± 0.5	0.0
Alkaline phosphodiesterase I	3.1.4.1	70 ± 14	4.8 ± 0.2	0.3

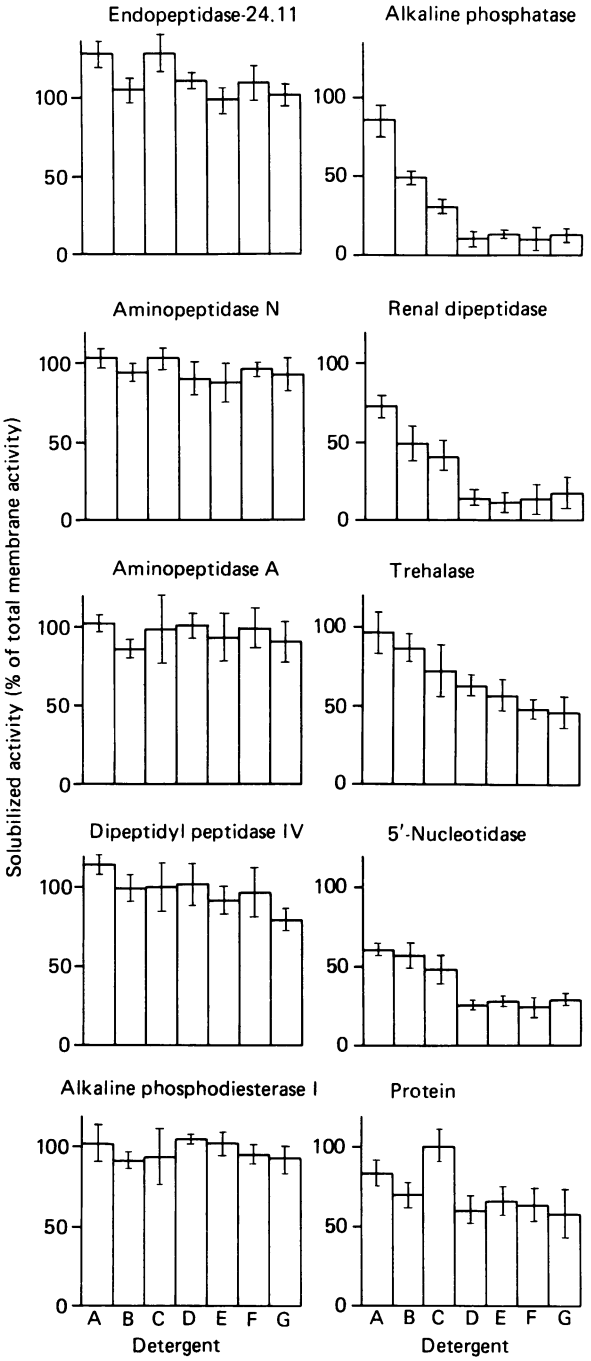

Fig. 1. Solubilization of pig kidney microvillar ectoenzymes by detergents

Pig kidney microvillar membrane fraction (approx. 2.5 mg of protein/ml) was incubated with detergent at the concentrations listed as described in the Materials and methods section. The results are the means ± S.E.M. for four separate experiments with each detergent, and have been corrected for any activation or inhibition caused by individual detergents in the enzyme assays. The various detergents together with the CMC and the concentration used are as follows: A, octyl glucoside (CMC, 25.0 mM; concentration used, 60 mM); B, CHAPS (8 mM; 20 mM); C, sodium deoxycholate (4–6 mM; 8.9 mM); D, Nonidet P-40 (0.29 mM; 6.1 mM); E, Triton X-100 (0.24 mM; 5.9 mM); F, Triton X-114 (0.20 mM; 6.9 mM); G, Emulphogene BC-720 (0.087 mM; 0.37%, w/w). The CMC data were taken from Helenius & Simons (1975), Helenius *et al.* (1979) and Hjelmeland *et al.* (1983).

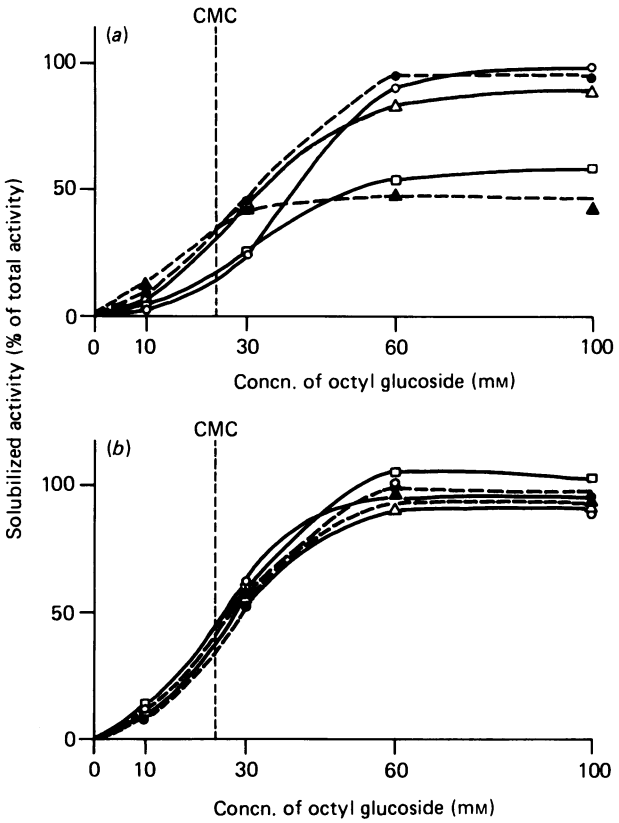

Fig. 2. Solubilization of pig kidney microvillar ectoenzymes by octyl glucoside

Pig kidney microvillar membrane fraction (approx. 2.0 mg of protein/ml) was incubated with octyl glucoside as described in the Materials and methods section. The results are the means for two separate incubations with detergent. (*a*) Proteins released from pig kidney microvillar membranes by PI-PLC (see Table 1): ○, alkaline phosphatase; △, renal dipeptidase; □, 5'-nucleotidase; ●, trehalase; ▲, total membrane protein released by octyl glucoside. (*b*) Proteins not released from pig kidney microvillar membranes by PI-PLC (see Table 1): ○, endopeptidase-24.11; △, aminopeptidase N; □, aminopeptidase A; ●, dipeptidyl peptidase IV; ▲, alkaline phosphodiesterase I.

The resultant solubilized activity was shown to be incorporated into liposomes (Chen *et al.*, 1987), consistent with the present results. In contrast, when the soluble fraction released by *B. thuringiensis* PI-PLC was subjected to phase separation in Triton X-114, the alkaline phosphatase, renal dipeptidase and trehalase activities partitioned predominantly into the detergent-poor phase (Table 2*c*), consistent with the loss of the hydrophobic membrane-anchoring domain.

Alkaline phosphodiesterase I

From the pattern of solubilization by detergents (Fig. 1) and its resistance to release by bacterial PI-PLC (Table 1), the present studies would predict that alkaline phosphodiesterase I is not anchored by a glycosyl-PI moiety, at least in the pig kidney microvillar membrane. In agreement with this conclusion, Low & Finean (1978) failed to observe any release of alkaline phosphodiesterase I from rat liver membranes or from pig

Table 2. Triton X-114 phase separation of pig kidney microvillar ectoenzymes

Pig kidney microvillar membranes (60 μg of protein) (*a*), and the 31000 *g* supernatant after treatment of pig kidney microvillar membranes either with 60 mM-octyl glucoside for 1 h at 4 °C (*b*) or with *B. thuringiensis* PI-PLC (1.0 unit/ml) for 1 h at 37 °C (*c*), were made up to 0.2 ml with 1.0% Triton X-114/150 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.4, and subjected to phase separation at 30 °C for 3 min as described by Bordier (1981). The detergent-rich and detergent-poor phases were separated through a sucrose cushion by centrifugation at 3000 *g* and assayed for enzyme activities. The results are the means ± S.E.M. for four phase separations. Activities recovered in the detergent-rich phase are expressed as percentages of the total activity. Abbreviation: N.D., not determined.

	Enzyme activity in detergent-rich phase (% of total activity)				
	Alkaline phosphatase	Renal dipeptidase	Trehalase	Endopeptidase-24.11	Dipeptidyl peptidase IV
(*a*) Untreated membranes	94.6±0.8	94.0±1.7	92.1±3.2	84.9±0.8	90.3±2.2
(*b*) Octyl glucoside-solubilized supernatant	80.0±10.3	92.6±6.3	82.4±5.9	72.8±6.1	81.9±3.0
(*c*) PI-PLC-released supernatant	1.9±3.9	4.3±2.7	12.5±5.9	N.D.	N.D.

lymphocytes with PI-PLC from *S. aureus*, although Nakabayashi & Ikezawa (1986) have reported the release of this ectoenzyme from various rat organs, including kidney, by the use of PI-PLC from *B. thuringiensis*. These variations may be due to species and tissue differences, but the status of alkaline phosphodiesterase I as a glycolipid-anchored enzyme (Nakabayashi & Ikezawa, 1986) requires reconsideration.

General conclusions

A useful criterion for detergent solubilization of membrane proteins is that the detergent should release the enzyme into a high-speed supernatant fraction in the smallest and least polydisperse form (see, e.g., Newby & Chrambach, 1979; Bailyes *et al.*, 1982). The present study has revealed that susceptibility to solubilization by a range of detergents appears to distinguish between microvillar ectoenzymes that are anchored by a glycosyl-PI moiety and those anchored by a sequence of hydrophobic amino acid residues. Whether this observation can be extended to other membrane preparations and other classes of glycolipid-anchored proteins awaits examination. In the case of glycolipid-anchored proteins it is likely that solubilization of the protein involves interactions between the detergent and the phospholipid anchor rather than a portion of the polypeptide chain. The differences observed between glycolipid-anchored and transmembrane proteins would therefore reflect the different parameters affecting the detergent solubilization of membrane phospholipids and membrane proteins respectively (Helenius & Simons, 1975; Lichtenberg *et al.*, 1983; Hjelmeland & Chrambach, 1984; Jones *et al.*, 1987).

The observation that octyl glucoside and CHAPS can solubilize efficiently glycosyl-PI-anchored proteins, with the resultant solubilized protein retaining the hydrophobic membrane anchor, should prove useful as an initial step in the purification of the amphipathic forms of such proteins.

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