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Solubilization of trehalase from rabbit renal and intestinal brush-border membranes by a phosphatidylinositol-specific phospholipase C

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Trehalase (EC 3.2.1.28) associated with renal and intestinal brush-border membranes was solubilized by highly purified phosphatidylinositol-specific phospholipase C (EC 3.1.4.10) from *Bacillus thuringiensis*, but not by phosphatidylcholine-hydrolyzing phospholipase C (EC 3.1.4.3) from *Clostridium welchii* or phospholipase D (EC 3.1.4.4) from cabbage. The solubilized trehalase was not adsorbed on phenyl-Sepharose, indicating that it was hydrophilic. Phosphatidylinositol-specific phospholipase C also converted Triton X-100-solubilized amphipathic trehalase into a hydrophilic form. These results suggest that trehalase is bound to the membrane through a direct and specific interaction with phosphatidylinositol.

Trehalase *Phosphatidylinositol specificity* *Phospholipase C* *Brush-border membrane* *Alkaline phosphatase* *Endopeptidase*

1. INTRODUCTION

Recent studies have demonstrated that phosphatidylinositol is involved in anchoring several ectoenzymes and proteins to the plasma membrane [1–5]. PIPLC (EC 3.1.4.10) can solubilize these enzymes, the first discovered one of which is alkaline phosphatase (EC 3.1.3.1) associated with the renal BBM [1]. The renal brush-borders are similar to the intestinal ones in function and morphology. Both have similar groups of membrane-bound enzymes, many of which can be solubilized by proteases such as papain and have been demonstrated to be anchored to the membrane through hydrophobic peptide segments located near the N-terminals of their polypeptides [6]. However, a few enzymes of both BBMs, including alkaline phosphatase, are resistant to solubilization by protease [6]. Therefore, it is possible that, like alkaline phosphatase, other protease-resistant enzymes such as trehalase (EC 3.2.1.28) and endopeptidase (EC 3.2.24.11) are also associated with the membrane via phosphatidylinositol bound to them. Here we have examined this possibility and found that PIPLC can solubilize trehalase but not endopeptidase.

Abbreviations: PIPLC, phosphatidylinositol-specific phospholipase C; BBM, brush-border membrane

2. EXPERIMENTAL

2.1. *Materials*

BBM vesicles were isolated from rabbit kidneys and small intestines as in [7] and [8], respectively. The isolated vesicles were suspended in buffer A (0.1 M NaCl, 10 mM sodium phosphate, pH 7.0) and stored at −80°C until use. Triton X-100 extracts were prepared by treating BBM vesicles (2 mg protein/ml) with 2% Triton X-100 at 4°C

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for 30 min, followed by centrifugation at 100000 × *g* for 30 min. PIPLC was purified from *Bacillus thuringiensis* [9] and aminopeptidase N (EC 3.4.11.2) from rabbit intestines [10]. Phosphatidylcholine-hydrolyzing phospholipase C (EC 3.1.4.3) (type XII) from *Clostridium welchii*, phospholipase D (EC 3.1.4.4) (type IV) from cabbage and proteinase K from *Tritirachium album* were purchased from Sigma, USA and amastatin, antipain, chymostatin, elastatinal, leupeptin, phosphoramidon and succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (substrate for endopeptidase) from Peptide Institute, Japan

2.2. *Treatment of trehalase samples with PIPLC*

BBM vesicles (2 mg protien/ml) were incubated with various amounts of purified PIPLC in buffer A at 37°C for 0–90 min. After portions of the mixtures were withdrawn, membrane vesicles were sedimented at 100000 × *g* for 30 or 60 min and the mixtures and supernatants were assayed for enzyme activities. Triton X-100 extracts were also incubated as above and 0.1-ml aliquots of the mixtures were applied to phenyl-Sepharose columns (bed volume, 0.4 ml) equilibrated with buffer A. The columns were then developed with 2.4 ml buffer A, followed by 2.5 ml buffer A containing 2% Triton X-100. The activities recovered in the first and the second 2.5-ml eluate were regarded as being due to hydrophilic and amphipathic forms of the enzymes, respectively.

2.3. *Other methods*

Enzyme activities were assayed as in [11,12]. Protein was determined according to Lowry et al. [13].

3. RESULTS AND DISCUSSION

When rabbit renal and intestinal BBM vesicles were incubated with *B. thuringiensis* PIPLC, none of the BBM enzymes examined was either activated or inactivated. PIPLC effectively solubilized trehalase and alkaline phosphatase from both BBM vesicles, depending on the incubation time and the amount of PIPLC used (fig.1). On the other hand, negligible amounts of the endopeptidase activity were recovered in the supernatants from PIPLC-treated samples compared with the nontreated ones. Aminopeptidase N, maltase (EC

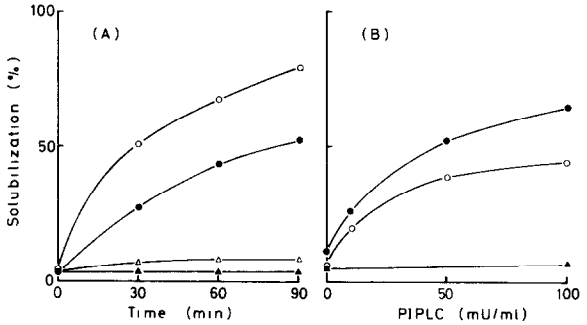

Fig.1. Solubilization of BBM enzymes by PIPLC. (A) Renal BBM vesicles were incubated with PIPLC (100 mU/ml) at 37°C for the indicated periods, after which the samples were centrifuged at 100000 × *g* for 30 min. (B) Intestinal BBM vesicles were incubated with the indicated concentrations of PIPLC at 37°C for 90 min, after which the samples were centrifuged as above. Enzyme activities recovered in the supernatants were represented relative to those of the total mixtures. (●) Trehalase; (○) alkaline phosphatase; (▲) endopeptidase, aminopeptidase N, maltase and (intestinal) sucrase; (△) protein.

3.2.1.20) or (intestinal) sucrase (EC 3.2.1.48) also were not solubilized by PIPLC. The amount of protein solubilized by PIPLC was only small fractions of the total protein even after 90-min incubation, during which, e.g., about 80 and 55% of alkaline phosphatase and trehalase, respectively, were solubilized from the renal BBM vesicles. This low level of solubilization of protein compared with alkaline phosphatase and trehalase suggests specific solubilization of these enzymes and is consistent with the findings that the contents of these two enzymes are very low in the renal and intestinal BBMs compared with the above-mentioned PIPLC-resistant enzymes [6,14,15]. Solubilization of trehalase by PIPLC was completely suppressed by 10 mM $CaCl_2$. EDTA (5 mM) showed no significant stimulatory or inhibitory effect on the process. Neither *Cl. welchii* phosphatidylcholine-hydrolyzing phospholipase C (2.4 U/ml, 10 mM $CaCl_2$; 37°C, 90 min) nor cabbage phospholipase D (40 U/ml, 50 mM sodium acetate, pH 6.2, 50 mM $CaCl_2$, 30 mM NaCl; 37°C, 90 min) solubilized trehalase and alkaline phosphatase.

Proteinase K (240 μg/ml; 37°C, 90 min), which can solubilize *Torpedo* electric organ ace-

tylcholinesterase having phosphatidylinositol as an anchoring factor [16], inactivated trehalase without any solubilization. A mixture of protease inhibitors (each 50 μM) amastatin, antipain, chymostatin, elastatinal, leupeptin and phosphoramidon did not inhibit trehalase solubilization by PIPLC. No protease activity was detected with the purified PIPLC preparation from *B. thuringiensis* [9]. These results indicate that the solubilization process by PIPLC was not mediated by any protease action.

Trehalase can be solubilized with detergents from renal and intestinal BBM vesicles. When Triton X-100- or PIPLC-solubilized trehalase was applied to a phenyl-Sepharose column equilibrated with buffer A, virtually all the activity of the former was adsorbed on the column and the adsorbed enzyme was eluted by buffer A containing Triton X-100, whereas the latter trehalase was scarcely adsorbed on phenyl-Sepharose (not shown), indicating that the Triton X-100- and PIPLC-solubilized trehalases are amphipathic and hydrophilic, respectively.

When the detergent-solubilized trehalase was incubated with PIPLC, it was converted into a hydrophilic form; the extent of conversion depended on the incubation time and the amount of PIPLC used (fig.2). Neither phosphatidylcholine-hydrolyzing phospholipase C nor phospholipase D induced such conversion. Triton-solubilized endopeptidase, as well as sucrase and aminopeptidase N, remained still amphipathic after the treatment with PIPLC. These results indicate that phosphatidylinositol is still bound to trehalase after solubilization with Triton X-100 and that splitting off of diacylglycerol from the phosphatidylinositol by PIPLC makes the enzyme hydrophilic. In contrast to the solubilization from the BBMs by PIPLC, the conversion of Triton X-100-solubilized amphipathic trehalase into a hydrophilic form was not inhibited by 10 mM $CaCl_2$, suggesting that the inhibitory effect of Ca^{2+} on the solubilization process is attributed to their interaction with BBMs, but not with PIPLC. Ca^{2+} may induce some structural changes in the membranes [17] so that the sensitive bond of phosphatidylinositol bound to trehalase becomes unaccessible to PIPLC.

The results presented above indicate that trehalase is associated with the renal and intestinal BBMs through phosphatidylinositol being tightly bound to it. The nature of association between trehalase and phosphatidylinositol is unknown but most likely covalent, as suggested for *Trypanosoma* variant surface glycoprotein [2] and *Torpedo* acetylcholinesterase [18]. On the other hand, endopeptidase is likely to be associated with the membrane through a hydrophobic anchor peptide segment and its resistance to papain solubilization may be due to the unaccessibility to papain, but not to the lack, of the susceptible peptide bond(s), as suggested by Kenny et al. [19].

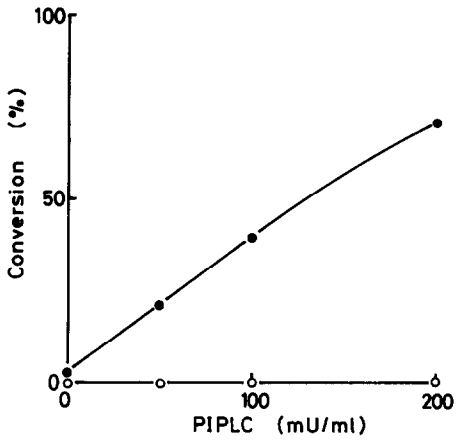

Fig.2. Conversion of Triton X-100-solubilized amphipathic trehalase into a hydrophobic form by PIPLC. Intestinal Triton X-100 extracts (equivalent to BBM vesicles containing 0.8 mg protein/ml) were incubated with the indicated concentrations of PIPLC at 37°C for 90 min, after which 0.1-ml aliquots of the mixtures were applied to phenyl-Sepharose columns to separate amphipathic and hydrophilic forms of the enzymes as described in the text. The extent of conversion of an amphipathic into a hydrophilic form of the enzyme was expressed in the terms of a ratio of the activity due to the hydrophilic form to that of the applied Triton X-100 extract. (●) Trehalase; (○) endopeptidase, aminopeptidase N and sucrase.

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