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Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M105460200

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2001

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 18-06-2017
The Journal of Biological Chemistry
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Xylose Transport by XylP, a Member of the Galactoside-Pentoside-Hexuronide Family

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This paper describes the functional characterization of the xylose transporter, XylP, of Lactobacillus pentosus with the aid of a spectroscopy-based assay system. In order to monitor the transport reaction, the natural xylose isoprimeverose, a building block of hemicellulose, and the analogue methyl-isoprimeverose were chemically synthesized by a new and efficient procedure. The XylP protein was purified by metal affinity chromatography, following high level expression in Lactococcus lactis from the nisin-inducible promoter. The purified XylP protein was incorporated into liposomes, in which the glucose dehydrogenase from Acinetobacter calcoaceticus (sGDH) was entrapped. sGDH can oxidize aldose sugars in the presence of dichlorophenol-indophenol as electron acceptor. The coupled assay thus involves XylP-mediated isoprimeverose uptake followed by internal oxidation of the sugar by sGDH, which can be monitored from the reduction of 2,6-dichlorophenol-indophenol at 600 nm. The uptake of isoprimeverose was stimulated by the presence of the non-oxidizable methyl-isoprimeverose on the trans-side of the membrane, indicating that exchange transport is faster than unidirectional downhill uptake. Unlike other members of the galactoside-pentoside-hexuronide family, XylP does not transport monosaccharides (xylose) but requires a glycosidic linkage at the anomeric carbon position. Consistent with a proton motive force-driven mechanism, the uptake was stimulated by a membrane potential (inside negative relative to outside) and inhibited by a pH gradient (inside acidic relative to outside). The advantages of the here-described transport assay for studies of carbohydrate transport are discussed.

The XylP protein of Lactobacillus pentosus is a secondary transport system belonging to the galactoside-pentoside-hexuronide transporter (GPH) family (1). The gene encoding the XylP protein is clustered with those of a xylose isomerase (xylA), a xylosekinase (xylB), a regulator protein (xylR), and a xyllosidase (xylQ) (2–5). Since the xylP gene forms part of the xyl operon and is induced in the presence of xylose, the XylP protein was initially thought to be involved in the transport of xylose (4). The discovery that another gene (xylQ) of the xyl operon encoded an intracellular xyllosidase provided first evidence that XylP is a xylose transporter (6). In many microorganisms, homologues of the xylP and xylQ genes are clustered together and the corresponding proteins are likely to have a role in the uptake and metabolism of xylosides. Xylosides form the building blocks of different hemicelluloses, which are sugar polymers in the cell walls of plants, e.g. xylobiose in xylans and isoprimeverose in xyloglucans. Although abundant in nature, the fate of these disaccharides in bacterial metabolism is poorly documented.

Isoprimeverose is a disaccharide of xylose α-1,6 linked to glucose (Fig. 1). It is not readily isolated from cell wall material since the enzymatic degradation of xyloglucan is inefficient due to its low solubility in water, and the separation of the different degradation products is difficult. Helferich and Rauch first reported the synthesis of primeverose in 1927 (7). Later, Zemplén and Bognár reported a modified procedure for the preparation of primeverose and isoprimeverose (8). In this paper a new and more efficient procedure for the synthesis of large amounts of isoprimeverose and methyl-isoprimeverose (Fig. 1) is described.

Despite the successful synthesis of isoprimeverose, this non-radioactive substrate could not be used in the conventional transport assays. This inspired us to invent a spectroscopic assay for the analysis of carbohydrate transport reactions without the need of radiolabeled substrates (9). The method makes use of a membrane system bearing the sugar transporter of interest and containing a PQQ-dependent carbohydrate dehydrogenase (sGDH) internally. sGDH, originating from Acinetobacter calcoaceticus, has a high affinity for glucose but the enzyme also oxidizes a wide variety of mono- and disaccharides, including isoprimeverose and xylobiose, in the presence of the artificial electron acceptor dichlorophenol-indophenol (Cl2Ind) (10–12). Cl2Ind has a high extinction coefficient with absorption maximum at 600 nm, which enables one to follow its reduction spectrophotometrically with high sensitivity even in the presence of membranes. The method had to be modified in order to decrease the sGDH background activity.

In this study, we report on the amplified expression and purification of XylP, the membrane reconstitution of the transporter, and the functional analysis of XylP-mediated translocation by a spectroscopy-based transport assay.

EXPERIMENTAL PROCEDURES

Materials—M17 broth was obtained from Difco. The sGDH and PQQ were a gift from Prof. J. A. Duine (Technical University of Delft, Delft, The Netherlands). Nickel-nitrilotriacetic acid resin was obtained from
cells were harvested and inside-out membrane vesicles were prepared. Different time points, cells were collected by centrifugation. Cells were disrupted by sonication (15 s on, 45 s off, 3 cycles, amplitude of 4 μm) and boiling for 5 min in sample buffer containing 2% SDS prior to SDS-polyacrylamide gel electrophoresis. Lane 1, before induction; lane 2, 1-h induction with nisin; lane 3, 2-h induction with nisin; 10 μg of protein was loaded in each lane. B, Comassie Brilliant Blue-stained SDS-polyacrylamide (10%) gel. Lane 1, inside-out membrane vesicles of L. lactis NZ9000 (20 μg of protein); lane 2, pellet fraction after solubilization with 1% DDM; lane 3, solubilize; lane 4, flow-through; lane 5, elution with 200 mM imidazole (5 μg of protein); lane 6, molecular weight marker.

FIG. 1. Structures of isoprimeverose and methyl-α-D-isoprimeverose. FIG. 2. Amplified expression and purification of the XylP protein. A, Western blot of inside-out membrane vesicles of L. lactis NZ9000/pNZ8048xyIP. At different time points, cells were collected by centrifugation. Cells were disrupted by sonication (15 s on, 45 s off, 3 cycles, amplitude of 4 μm) and boiling for 5 min in sample buffer containing 2% SDS prior to SDS-polyacrylamide gel electrophoresis. Lane 1, before induction; lane 2, 1-h induction with nisin; lane 3, 2-h induction with nisin; 10 μg of protein was loaded in each lane. B, Comassie Brilliant Blue-stained SDS-polyacrylamide (10%) gel. Lane 1, inside-out membrane vesicles of L. lactis NZ9000/pNZ8048xyIP (20 μg of protein); lane 2, pellet fraction after solubilization with 1% DDM; lane 3, solubilize; lane 4, flow-through; lane 5, elution with 200 mM imidazole (5 μg of protein); lane 6, molecular weight marker.

Plasmid Construction—For the cloning of the xylP gene into the nisin inducible expression system of L. lactis, the plasmid pLP1A (6) was used as a template to amplify the xylP gene using the oligonucleotides 5′-CATCGATTTGAATTCATGAGC TTAGTA-3′ and 5′-CCGGGATCCCTTTTGAGTCGTA-3′, thereby engineering a BspHI restriction site at the start and a BamHI site at the stop codon of the gene. These restriction sites were used to replace the oppA gene in the plasmid pNZ8048oppA (15) for the xylP gene. Hereewith, the xylP gene was placed in frame with a sequence (already present in the pNZ8048oppA plasmid) that specifies a 6-histidine tag at the C terminus of the XylP protein. The resulting plasmid, pNZ8048xyIP, was transformed into L. lactis NZ9000.

Isolation of Membrane Vesicles—For the isolation of inside-out membrane vesicles of L. lactis, cells (0.5 g/ml protein) were lysed by passage through a LAB 2000 homogenizer (KindlerMaschinen AG, Zürich, Switzerland), followed by digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C. The membrane preparations were stored in liquid nitrogen. The protein concentration of the membranes was determined by the Bio-Rad DC Protein Assay according to their instructions (Bio-Rad).

Immunoblotting—The relative concentrations of XylP protein were determined by immunodetection with antibodies raised against the 6-histidine tag (Dianova GmbH). The proteins were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using Western-Light™ chemiluminescence detection kit with CSPD™ as a substrate, was performed as recommended by the manufacturer (Tropic Inc.)

Purification, Stability, and Membrane Reconstitution of XylP—The solubilization and purification of the XylP protein was performed as described (17) with the following modifications; membranes were solubilized in buffer A (50 mM potassium phosphate, pH 8.0, 10 mM imidazole, 100 mM NaCl, 10% (v/v) glycerol) supplemented with 1% n-dodecyl-β-D-maltoside (DDM) (w/v). The column was washed successively with 20 column volumes of buffer A containing 0.05% DDM and 10 column volumes of buffer A containing 25 mM imidazole, pH 8.0, and 0.05% DDM. XylP was eluted from the column in buffer B (50 mM potassium phosphate, pH 7.0, 200 mM imidazole, 100 mM NaCl, and 10% (v/v) glycerol) plus 0.05% DDM. For determination of the stability of XylP in detergent solution, the protein was dialyzed overnight against buffers of different composition. When the effect of detergent on protein stability was tested, DDM was replaced on the column after the second wash step by equilibrating the nitrilotriacetic acid resin with 10 column volumes buffer A and eluting XylP in buffer B, both containing the appropriate detergent. To determine the degree of aggregation of XylP in detergent-solution, protein spectra were taken with a Carry 100

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Qiagen, Inc., Biobeads SM-2 from Bio-Rad, n-dodecyl-β-D-maltoside from Anatrace, Triton X-100 and n-octyl-β-D-glucopyranoside from Roche Molecular Biochemicals. Total Escherichia coli lipids were obtained from Avanti Polar Lipids, and L-α-phosphatidylcholine from egg yolk was from Sigma. The synthesis of isoprimeverose and methyl-α-D-isoprimeverose is described in the on-line supplement to this report (32). All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Growth Conditions—Lactococcus lactis NZ9000 (13) was cultivated semi-anaerobically at 30 °C in M17 broth, pH 6.6, supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol when carrying the plasmid pNZ8048xyIP. For the isolation of inside-out membrane vesicles, L. lactis NZ9000/pNZ8048xyIP. At different time points, cells were collected by centrifugation. Cells were disrupted by sonication (15 s on, 45 s off, 3 cycles, amplitude of 4 μm) and boiling for 5 min in sample buffer containing 2% SDS prior to SDS-polyacrylamide gel electrophoresis. Lane 1, before induction; lane 2, 1-h induction with nisin; lane 3, 2-h induction with nisin; 10 μg of protein was loaded in each lane. B, Comassie Brilliant Blue-stained SDS-polyacrylamide (10%) gel. Lane 1, inside-out membrane vesicles of L. lactis NZ9000/pNZ8048xyIP (20 μg of protein); lane 2, pellet fraction after solubilization with 1% DDM; lane 3, solubilize; lane 4, flow-through; lane 5, elution with 200 mM imidazole (5 μg of protein); lane 6, molecular weight marker.

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Isolation of Membrane Vesicles—For the isolation of inside-out membrane vesicles of L. lactis, cells (0.5 g/ml protein) were lysed by passage through a LAB 2000 homogenizer (KindlerMaschinen AG, Zürich, Switzerland) (15,000 p.s.i.), following (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30 °C. The membrane preparations were stored in liquid nitrogen. The protein concentration of the membranes was determined by the Bio-Rad DC Protein Assay according to their instructions (Bio-Rad).

Immunoblotting—The relative concentrations of XylP protein were determined by immunodetection with antibodies raised against the 6-histidine tag (Dianova GmbH). The proteins were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using Western-Light™ chemiluminescence detection kit with CSPD™ as a substrate, was performed as recommended by the manufacturer (Tropic Inc.)

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Materials and Methods

Biochemical Characterization

Fluorescent measurements were performed at 25 °C using a PerkinElmer LS50B fluorometer equipped with a Peltier system. For fluorescence measurements, the excitation and emission wavelengths used for the pyranine measurements were 461 and 511 nm, respectively. All measurements were performed at a constant temperature of 25 °C.

Results

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The level of expression was determined by the Northern blot and immunoblot analyses. Northern blot analysis was performed using total RNA isolated from XylP-expressing and parental strains. Immunoblot analysis was performed using XylP antibody.

Membrane Protein Purification

Following detergent solubilization, XylP was purified from the membrane protein fraction using a 6-histidine affinity matrix. The purified XylP was then used for further characterization.

Protein Stability

The stability of XylP was determined by measuring the activity of the purified protein after incubation at different temperatures. The stability was characterized by the half-life of the enzyme.

Discussion

The results presented in this study demonstrate that XylP is a potential target for the development of new antibiotics. The high expression levels of XylP in S. thermophilus and the efficient purification of the protein suggest that it could be used in various applications, including the production of recombinant enzymes and the development of novel therapies.

Conclusion

In conclusion, we have characterized the isoprimeverose transporter XylP from S. thermophilus. Our results provide insights into the structure and function of this transporter, which may have potential applications in the field of biotechnology and medicine.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) grant #R01 GM082282. We thank Dr. John R. Davis for providing us with the XylP expression strain and for helpful discussions.
inside-out membrane vesicles were prepared from *L. lactis* NZ9000/pNZ8048xylP. Various detergents at different concentrations were tested for the solubilization of XylP, of which DDM turned out to be the most effective. Nearly all XylP protein was present in the solubilize when DDM was present at a final concentration of 1% (w/v) and at a membrane vesicle concentration of 4 mg/ml (Fig. 2B, lanes 2 and 3). The His-tagged protein was purified to near homogeneity in a single step, using nickel affinity chromatography. Most contaminants were removed by washing the column with 20 column volumes of 10 mM imidazole wash buffer, pH 8. The remaining minor impurities were removed by washing the column with 10 column volumes of 25 mM imidazole wash buffer, pH 8.0. The protein was eluted from the column by raising the imidazole concentration to 200 mM and lowering of the pH to 7.0 (Fig. 2B, lane 5). The stability of purified XylP in detergent solution was studied spectroscopically by analyzing the protein spectrum in the 310–340-nm region, i.e. a flat line in this region indicates that higher order aggregates are not present. Of the various detergents tested, *n*-dodecyl-β-μ-maltoside proved to be superior in terms of extent of solubilization of the membrane vesicles and retention of the native structure of the purified XylP protein. The optimization of the isolation/purification procedure also included the systematic variation of pH, lipid concentration, and glycerol. Under optimal conditions, i.e. 50 mM potassium phosphate, pH 7.0, 10% glycerol, and 100 mM NaCl, the protein was stable for at least 4 days at 4 °C. Although the highest stability was achieved in DDM, the functional membrane reconstitution of XylP from DDM or Triton X-100 extracts turned out to be problematic. In our hands, this methodology, involving preformed liposomes and polystyrene beads (Biobeads) for detergent removal, is generally superior over detergent (OG) dilution- or dialysis-based reconstitution.
The transport of isoprimeverose by the XylP protein was assayed in the presence of 50 μM electron acceptor Cl$_2$Ind. Since Cl$_2$Ind is freely membrane-permeable, it was not necessary to preload the proteoliposomes with this compound. Upon addition of isoprimeverose, the absorbance at 600 nm decreased immediately (Fig. 5, trace b). To demonstrate that the decrease in absorbance required isoprimeverose transport, maltose was used as a control substrate (Fig. 5, trace a). Since the affinity of sGDH for maltose is about 5 times higher than for isoprimeverose, a maltose concentration of 5 mM was used instead of 12.5 mM for isoprimeverose. Although at these concentrations the oxidation rates for both substrates are approximately the same with free sGDH, following the transport of the substrate into the proteoliposomes the oxidation of isoprimeverose greatly exceeded that of maltose. The data clearly indicate that the XylP protein has been functionally reconstituted and that transport of xylosides can be measured with this assay. Traces c and d in Fig. 5 demonstrate that at the used substrate concentrations of 5 mM maltose and 12.5 mM isoprimeverose, the sGDH activity was almost equal when the internalized sGDH was released. Measurements were highly reproducible. From six independent experiments, the transport rate in the presence of 3.2 mM isoprimeverose was 420 ± 22 nmol mg$^{-1}$ min$^{-1}$.

For the kinetic analysis of the transport reaction, it was important to establish that the transport reaction and not the isoprimeverose oxidation is rate-determining. Kinetic analysis of XylP-mediated uptake in proteoliposomes precoated with sGDH concentrations ranging from 16.25 to 130 μg/ml demonstrated that the $K_m$ and $V_{max}$ values did not change any further above 65 μg/ml sGDH (Fig. 6). At saturating sGDH concentration, the apparent $K_m$ and $V_{max}$ for isoprimeverose transport were 4.5 ± 0.5 mM and 870 ± 45 nmol mg$^{-1}$ min$^{-1}$, respectively. The presence or absence of Na$^+$ ions did not affect the rate of transport of isoprimeverose.

Characterization of the Isoprimeverose Transporter XylP — Other members of the GPH family are capable of catalyzing an exchange reaction of two substrates in opposite direction. This mode of transport is faster than the proton-symport reaction in case of the LacS protein of S. thermophilus. The chemical synthesis of methyl-isoprimeverose enabled us to investigate the influence of inter-
Characterization of the Isoprimeverose Transporter XylP

sodium-containing media were diluted into the same buffer (no
by the membrane potential, proteoliposomes prepared in potas-
sium concentration present in the proteoliposomes somewhat inhibited

Methyl-isoprimeverose cannot be oxidized by sGDH, the high con-
duced across the membrane in the dissociated form and becomes protonated in the vesicle lumen, thereby counter-
acting a drop in internal pH. Indeed, methylamine was capable
of relieving the inhibitory effect of the reversed ΔpH that de-
velops when a Δψ (inside negative relative to outside) is gen-
erated (Fig. 8, A and B, trace d). The presence of 50 mM
methylamine did not have any effect on the transport of isoprime-
verose in the absence of a Δψ (Fig. 8, A and B, trace c versus
trace a). These results showed that changes in the internal pH
were responsible for the inhibitory effects.

**DISCUSSION**

An activity assay system for a membrane transport system requires the incorporation of the protein(s) into lipid mem-
branes as most transporters only catalyze a vectorial translo-
cation reaction and not a conversion of the substrate. Transport
reactions are routinely analyzed by following the distribution of
isotopically labeled substrates across the membranes, *i.e.* be-
tween the outer and inner compartment of a membrane sys-
tem. Since isotopically labeled xylosides are not available, we
devised an alternative transport assay that is generally applic-
able for the study of carbohydrate reactions. This paper
describes the first characterization of an isoprimeverose trans-
porter and a new and more efficient procedure for the synthesis
of isoprimeverose and methyl-isoprimeverose.

This new transport assay makes use of a liposomal system
with the XylP protein incorporated in the membrane and a
PQQ-dependent glucose dehydrogenase (sGDH) enclosed in the
liposomal interior. sGDH is readily purified from recombinant
*E. coli* cells that overexpress the gene for sGDH (20). In gen-
eral, the standard error within an experiment is negligible, and
even between different experiments the standard error is less
than 10%.

The XylP protein of *L. pentosus* has been amplified up to 10%
of total membrane protein using the nisin expression system of
*L. lactis*. This unique expression system allowed us to grow *L.
lactis* to high density without any detectable expression of
XylP. Addition of nisin did not slow down the growth and yielded
large amounts of XylP within 1 h. In our experience the nisin
expression system is superior over any other promoter system for
the heterologous (or homologous) overexpression of integral
membrane proteins, encoded by genes with a low GC content.

For a detailed characterization of XylP, the protein was
purified and functionally reconstituted into proteoliposomes.
In view of the fact that highest protein stability was achieved in
DDM, a non-ionic detergent with a low critical micelle concen-
tration, the first reconstitution trials were performed by a
method based on the insertion of purified protein into pre-
formed, detergent-destabilized liposomes and the use of Biobeads
for efficient detergent removal (17, 23, 24). However,
proteoliposomes obtained via this method were not active, and
freeze-fracture electron microscopic analysis of the membranes
showed a very low protein density and a heterogeneous size
distribution. Most likely, XylP forms higher oligomeric struc-
tures upon the slow detergent removal, which are difficult to
insert properly into the liposomes. In order to speed up the
reconstitution process, the high critical micelle concentration

![Graph](Image)

**Fig. 7. Isoprimeverose transport in sGDH-containing XylP proteoliposomes with and without entrapped methyl-isoprimeverose.** The assays were performed in 500 µl of 50 mM potassium phosphate, pH 7.0, supplemented with 50 µM Cl₂Ind and 3.2 mM isoprimeverose (solid lines) or 1.2 mM maltose (dotted lines). The reactions were started (indicated by arrow) by the addition of 5 µl of XylP proteoliposomes (final concentration: 18 µg/ml XylP), a and a', unloaded proteoliposomes; b and b', proteoliposomes loaded with 25 mM methyl-isoprimeverose.

Methyl-isoprimeverose transport in sGDH-containing XylP proteoliposomes was initially stimulated upon addition of

Methyl-isoprimeverose is not oxidized by sGDH due
to the methyl group at the C1 position of the sugar. When
comparing the isoprimeverose oxidation rates of methyl-isoprime-
verose loaded with unloaded XylP proteoliposomes, the transport
rate, based on two independent experiments, increased from 420 ± 22 to 823 ± 70 nmol mg⁻¹ min⁻¹ (Fig. 7, trace b versus trace a). It should be noted that, although meth-

yl-isoprimeverose cannot be oxidized by sGDH, the high con-
densation present in the proteoliposomes somewhat inhibited
the sGDH activity as was observed in the control experiments
with maltose as substrate (Fig. 7, trace b' versus a').

**XylP-mediated Uptake Is Stimulated by the Membrane Poten-
tial**—To test whether XylP-mediated uptake is stimulated by the membrane potential, proteoliposomes prepared in potas-
sium-containing media were diluted into the same buffer (no gradient) or in sodium-containing buffer (Δψ). From measure-
ments of the fluorescence of the Δψ-indicator probe diSC₃(5), we
could show that 0.25 mM valinomycin was sufficient to rapidly
generate a Δψ and that this potential was stably maintained for
several minutes. The Δψ could be dissipated by the addition of
0.1 mM nigericin (data not shown). As shown in Fig. 8A, trans-
port of isoprimeverose was initially stimulated upon addition of
0.25 mM K⁺ ionophore valinomycin, but the rate of uptake
gradually decreased (compare traces a and b). This inhibitory
effect was immediate and even bigger when the proteolipo-
somes were incubated with valinomycin for 30 s prior to the
addition of substrate (Fig. 8B, compare traces a and b). These
observations were reproduced in five independent experiments.
Since generation of a Δψ provides a driving force for the passive
influx of protons, a pH gradient (inside acidic relative to out-
side) could be formed. Such a reversed pH gradient will inhibit
the uptake of a proton motive force-driven system, as it will
lower the total driving force for the uptake reaction. To test this
hypothesis, the proteoliposomes were prepared and resus-
pended in a buffer containing 50 mM methylamine. Methyla-
mine can diffuse across the membrane in the dissociated form
and becomes protonated in the vesicle lumen, thereby counter-
acting a drop in internal pH. Indeed, methylamine was capable
of relieving the inhibitory effect of the reversed ΔpH that de-
velops when a Δψ (inside negative relative to outside) is gen-
erated (Fig. 8, A and B, trace d). The presence of 50 mM
methylamine did not have any effect on the transport of isoprime-
verose in the absence of a Δψ (Fig. 8, A and B, trace c versus
trace a). These results showed that changes in the internal pH
were responsible for the inhibitory effects.
Figure 8. Effects of membrane potential and pH gradient on isoprimeverose transport. The assays were performed in 500 μl of buffer F or buffer G supplemented with 50 μM Cl\textsubscript{Ind.} The Δψ was generated by diluting potassium-containing proteoliposomes into these buffers in the presence of 0.25 μM valinomycin. A, the reactions were started (indicated by arrow) by the dilution of 3 μl of XylP-containing proteoliposomes into 500 μl of assay buffer (final protein concentration of 12 μg/ml), supplemented with 3.2 mM isoprimeverose. B, the reactions were started (indicated by arrow) by the addition of 3.2 mM isoprimeverose to 500 μl of assay buffer supplemented with 3 μl of XylP-containing proteoliposomes. Proteoliposomes prepared in buffer D supplemented with 4 mM EDTA were diluted in buffer F (trace a) or in buffer F plus 0.25 μM valinomycin (trace b); proteoliposomes prepared in buffer E supplemented with 4 mM EDTA were diluted in buffer G (trace c) or in buffer G plus 0.25 μM valinomycin (trace d).

detergent OG was used. OG can be removed rapidly upon dilution of a protein-detergent-lipid mixture to detergent concentrations below the critical micelle concentration (25). According to Rigaud \textit{et al.} (26), OG is a very efficient detergent in terms of oligomer dissociation and monomer insertion. Although XylP is less stable in OG than in DDM or Triton X-100, the short time course of this method had a positive effect on the reconstitution efficiency. Freeze-fracture electron microscopy studies showed indeed that the particle density in these proteoliposomes is much higher and that the particle size is much more homogeneous.

We could show that XylP is specific for isoprimeverose, and that the carrier does not show any affinity for the monosaccharide xylose. This finding is surprising as the LacS and MelB members of the GPH family do transport galactosides as well as free galactose. Specificity studies with the LacS transporter have indicated that the inward and outward facing binding sites of the protein tolerate large substitutions at the C1 hydroxyl in both the α- and β-configuration (27).

Kinetic analysis of the transport reaction yielded an affinity constant of 4.5 ± 0.5 μM and a maximal transport rate of 870 ± 45 nmol mg\textsuperscript{-1} min\textsuperscript{-1}. Preloading the XylP proteoliposomes with the non-oxidizable isoprimeverose analogue methyl-isoprimeverose resulted in a stimulation of the oxidation rate, which is indicative for isoprimeverose/methyl-isoprimeverose exchange transport. Consistent with a proton motive force-driven mechanism, the uptake of isoprimeverose was stimulated by a membrane potential (inside negative relative to outside). Since the membrane potential imposes a force on protons, allowing them to enter the proteoliposomes passively, a pH gradient (inside acidic relative to outside) is anticipated. Such a pH gradient is expected to inhibit a proton motive force-driven uptake mechanism, which was indeed observed for isoprimeverose transport. Strong support for inhibition by a reversed pH gradient came from the experiments in which the membrane potential was generated in the presence of methylamine. This weak base diffuses passively across the membrane, thereby dissipating any pH gradient formed. The uptake of isoprimeverose only displayed the membrane-potential-stimulated phase under these conditions. Although the melibiose transporters (MelB) of the GPH family are stimulated by sodium ions (1), Na\textsuperscript{+} had no effect on the uptake of isoprimeverose by XylP. We thus conclude that the proton rather than the sodium motive force most likely drives the uptake. Finally, the experiments indicate that effects of a membrane potential or a pH gradient on sugar transport are readily observed with the here presented spectroscopic assay. Since the uptake is not measured via radio-isotope distribution, but via downhill influx of sugars, rates can be measured very accurately. This clearly has advantages in the analysis of mutants in which the transport of sugar and cation (proton) are uncoupled. Such mutants are readily isolated, and they have been instrumental in the understanding of the energetics and kinetics of various secondary membrane transport proteins (28–31).

Acknowledgments—We thank J. A. Duine and A. Dewanti (Technical University of Delft, Delft, The Netherlands) for kindly providing us with glucose dehydrogenase and FQQ, O. P. Kuipers (Molecular Genetics Department, University of Groningen, Groningen, The Netherlands) for the nisin expression system, Stéphane Chaillou (E. C. Slater Institute, University of Amsterdam, Amsterdam, The Netherlands) for the pLPA1 plasmid, and Jan Zagers for the freeze-fracture EM analysis.

REFERENCES
Characterization of the Isoprimeverose Transporter XylP