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Electrophysiological Characterization of Uncoupled Mutants of LacY

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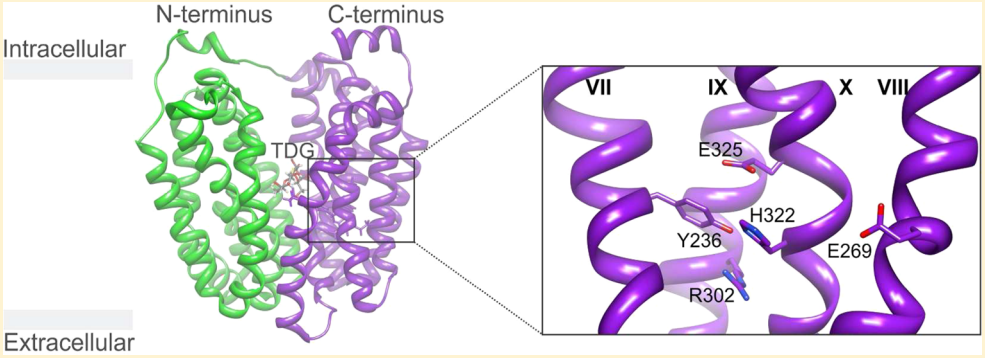

ABSTRACT: In this study of the lactose permease of *Escherichia coli* (LacY), five functionally irreplaceable residues involved specifically in H^+ translocation (Arg302 and Glu325) or in the coupling between protonation and sugar binding (Tyr236, Glu269, and His322) were mutated individually or together with mutant Glu325 → Ala. The wild type and each mutant were purified and reconstituted into proteoliposomes, which were then examined using solid-supported-membrane-based electrophysiology. Mutants Glu325 → Ala or Arg302 → Ala, in which H^+ symport is abolished, exhibit a weakly electrogenic rapid reaction triggered by sugar binding. The reaction is essentially absent in mutant Tyr236 → Phe, Glu269 → Ala, and His322 → Ala, and each of these mutations blocks the electrogenic reaction observed in the Glu325 → Ala mutant. The findings are consistent with the interpretation that the electrogenic reaction induced by sugar binding is due to rearrangement of charged residues in LacY and that this reaction is blocked by mutation of each member of the Tyr236/Glu269/His322 triad. In addition, further support is provided for the conclusion that deprotonation is rate limiting for downhill lactose/H^+ symport.

The major facilitator superfamily (MFS) is arguably the largest family of membrane transport proteins known at present.[1,2] The members are single-polypeptides with mostly 12 transmembrane helices that catalyze transport of small solutes into (uniport, symport) or out of (antiport) the cell. The lactose permease of *Escherichia coli* (LacY), a paradigm for the MFS, transduces free energy stored in an H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$; interior negative and/or alkaline) into a galactoside concentration gradient. However, because transport is obligatorily coupled (symport), LacY will also transduce free energy stored in an imposed sugar concentration gradient into a $\Delta\tilde{\mu}_{H^+}$, the polarity of which depends upon the direction of the sugar gradient.[3,4] LacY has been solubilized from the membrane and purified to homogeneity in a completely functional state,[5] and it is structurally,[6,7] as well as functionally,[8] a monomer.

X-ray crystal structures of LacY[9−12] and various independent biochemical and spectroscopic findings[13−20] provide converging evidence for an alternating access mechanism. By this means, H^+ and sugar binding induce coordinated opening and closing of periplasmic and cytoplasmic cavities, respectively, thereby allowing alternating accessibility of sugar- and H^+-binding sites to either side of the membrane (the alternating access model) (reviewed in refs 21 and 22). It is also likely that the alternating access model for LacY involves formation of an occluded intermediate(s),[23−25] which is consistent with the highly dynamic nature of the protein.[18,26−30]

Cys-scanning and site-directed mutagenesis of each residues in LacY demonstrate that only a few side chains that are located in a deep cavity in the middle of the molecule are irreplaceable for lactose/H^+ symport (Figure 1) (reviewed in refs 4 and 31). Thus, a carboxyl group at position 126 (helix IV), a guanidino group at position 144 (helix V), and an aromatic side chain at position 151 (helix V) are critical for sugar binding,[32−34] Tyr236 (helix VII), Glu269 (helix VIII), and His322 (helix X) are essential with respect to protonation of LacY and galactoside-binding affinity,[35−37] and Arg302 (helix IX) and Glu325 (helix X) are required for deprotonation.[38−40] Using site-directed alkylation, it was shown recently[41] that replacement of Tyr236 (helix VII), Glu269 (helix VIII), or His322 (helix X) causes spontaneous opening of the periplasmic cavity in the absence of sugar and decreased closing of the cytoplasmic cavity in the presence of a galactoside. In contrast, mutation of Arg302 (helix IX) or Glu325 (helix X) has no such

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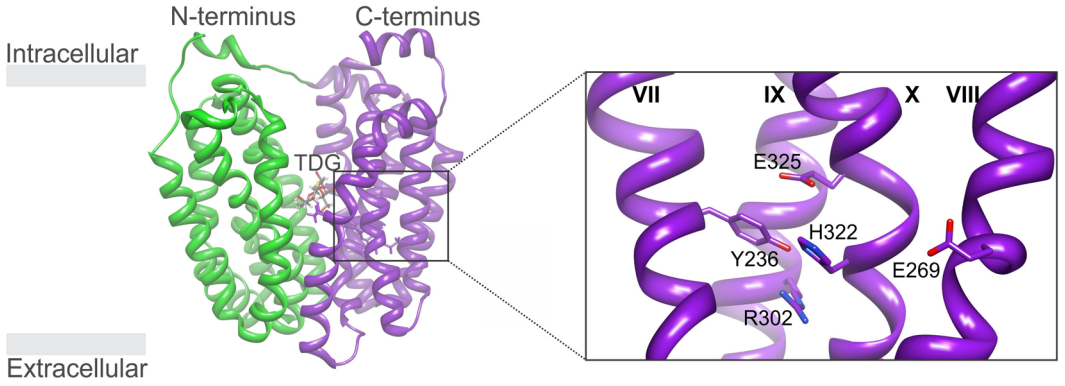

Figure 1. Structure of LacY showing residues in the H^+ binding site. The LacY monomer consists of 12 transmembrane domains with the N-terminus and C-terminus on the cytoplasmic side of the membrane. The transmembrane domains form two 6-helix bundles surrounding a deep hydrophilic cavity; the N-terminal helical bundle is colored green, and C-terminal bundle is purple. Residues Y236 (helix VII), E269 (helix VIII), R302 (helix IX), and H322 and E325 (helix X) are shown as sticks. The transmembrane helices are labeled with Roman numerals. The structural model was created using Chimera software with Protein Data Bank ID IPV7.

effect, and sugar binding induces normal opening and closing of periplasmic and cytoplasmic cavities. It was suggested that Glu269, His322, and Tyr236 act in concert, possibly as a triad, responsible for coordinating opening and closing of the cavities by binding water, which acts as a hydronium ion intermediate in H^+ translocation. Mutation of the triad results in loss of the bound water, which destabilizes LacY, and the cavities open and close in an uncoordinated manner. Thus, triad mutants exhibit very poor affinity for sugar, and galactoside/H^+ symport is abolished as well.

Here, we describe measurements using solid-supported-membrane (SSM)-based electrophysiology and proteoliposomes reconstituted with purified WT and mutant proteins in these key residues. The high resolution, with respect to time and sensitivity, of the technique[42] allows capacitive measurements of charge translocation originating either from the energetically downhill symport of lactose and H^+ or from charge rearrangements within LacY.[43,44] The findings provide strong support for the conclusion that Glu269, Tyr236, and His322 play a unique role in the symport mechanism and reinforce previous studies (reviewed in refs 3 and 4) that indicate that sugar binding involves rearrangement of charged residues in LacY.

MATERIALS AND METHODS

Materials. Protease inhibitor cocktail was obtained from Roche (Indianapolis, IN). Oligonuclectides were synthesized by Intergrated DNA Technologies (Coralville, CA). *p*-Nitrophenyl-α-D-galactopyranoside (NPG) was obtained from Sigma-Aldrich (St. Louis, MO). All other materials were reagent grade and purchased from commercial sources.

Construction and Purification of LacY Mutants. Plasmid pT7-5, encoding WT LacY with a 6× His-tag at the C-terminus, was used as a template for mutagenesis. LacY mutants were constructed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions except that the temperature of the extension reaction was lowered to 68 °C. All mutants were verified by sequencing the entire gene. LacY mutants were overexpressed in the *Escherichia coli* XL1-Blue strain and purified with Talon resin as described.[15] Purified protein−detergent complexes in 50 mM sodium phosphate (NaP_i)/0.01% *n*-dodecyl-β-D-maltoside (DDM) (pH 7.6) were rapidly frozen with liquid nitrogen and stored at −80 °C until use. The purified proteins electrophoresed as a single band by sodium dodecylsulfate/polyacrylamide electrophoresis followed by silver staining.

Preparation of Proteoliposomes. Reconstitution of purified proteins into liposomes was carried out with *E. coli* total lipid extract (Avanti Polar Lipids).[48,57] Briefly, 5 mg of lipid extract in chloroform was evaporated under a stream of argon and solubilized in 1 mL of 100 mM potassium phosphate (KPi, pH 7.6)/1.5% (final concentration) *n*-octyl-β-D-glucoside (OG) followed by sonification. Purified protein was mixed with liposomes at a lipid-to-protein ratio of 5:1 (w/w). Detergents were removed from protein/lipid mixture by rapid dilution in 100 mL of ice-cold 100 mM KP_i/200 mM NaCl/1 mM DTT (pH 7.6) followed by ultracentrifugation. The pellet was then resuspended in 100 mM KP_i/1 mM DTT (pH 7.6) to a final lipid concentration of 40 mg/mL and protein concentration of 8 mg/mL. Aliquots were frozen with liquid nitrogen and stored at −80 °C. For the SSM experiments, the samples were rapidly thawed and gently sonified for ∼10 s in a bath sonicator alternated by cooling on ice. This step was repeated until the sample was transparent. The sample was then kept at room temperature.

SSM Measurements. SSM measurements were performed as described previously.[43,45,46] The nonactivating (NA) and activating (A) solutions were prepared in 100 mM KP_i/1 mM DTT at pH 7.5. The NA solution always contained 100 mM glucose, and the A solution contained 100 mM lactose. When indicated, 5 mM NEM was added to both NA and A solutions without DTT. Concentration jump experiments at different pH values were carried out on the same sensor. Proteoliposomes immobilized on the SSM were equilibrated for 20 min at each new pH or with 5 mM NEM where indicated.

RESULTS

Transport Activity of WT LacY. Proteoliposomes containing purified WT LacY or a given mutant were immobilized on a solid-supported membrane.[45,46] Capacitive coupling between the proteoliposomes and the SSM enables measurement of electrogenic reactions as capacitive currents that are detected with an amplifier connected to the SSM and a reference electrode. Transport activity was initiated by rapid exchange (time constant 8 ms[44]) of a nonactivating solution with an activating solution containing 100 mM lactose at pH 7.5. To minimize ionic strength and osmotic effects, the

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nonactivating solution contained an equimolar concentration of glucose, which is not bound or transported by LacY instead of lactose. Upon exchange of 100 mM glucose to 100 mM lactose, capacitive currents induced either by downhill lactose/H^+ symport or by intermolecular charge rearrangements were detected, as shown previously.[43,44,46]

A typical electrical signal for WT LacY recorded after a lactose concentration jump (i.e., when LacY catalyzes downhill lactose/H^+ symport) is shown in Figure 2A. The sign of the current indicates displacement of positive charge (H^+) toward the SSM. The transient signal exhibits a rapid current rise to a maximal value of 733 ± 44 pA (average of peak currents, I_{peak}, from three experiments with three different electrodes) followed by decay toward the baseline.

To distinguish between actual electrical signals and artifacts, which can occur because of the differences in the composition of the solutions and their interaction with the SSM,[47] control experiments with N-ethylmaleimide (NEM) were carried out on the same electrode. NEM alkylates Cys148 in LacY[47,48] with inhibition of sugar binding and transport as well as electrogenic conformational changes.[43,44] After incubation of proteoliposomes containing WT LacY with NEM, no electrical signals were observed (Figure 2).

LacY Mutants. Relative to the WT, mutants E325A (Figure 2B) and R302A (Figure 2C) exhibit much smaller currents under the same experimental conditions, which are also abolished by treatment with NEM. The average I_{peak} values are 114 ± 22 (n = 4) and 125 ± 8 pA (n = 3) for E325A and R302A, respectively. Moreover, the signals with both E325A and R302A exhibit very rapid decays that fit a single-exponential function with τ values of 12 to 17 ms, respectively. These time constants are close to the time constant of the solution exchange, which indicates that the underlying electrogenic reaction is much faster. Indeed, a rate constant of ~180 s^{-1} has been determined for this reaction in E325A LacY.[44] Both mutants also exhibit the same signal over a pH range from 5.5 to 8.5 (see ref 44 and data not shown). The rapid monoexponential signal decay and the absence of pH dependence with the two mutants is consistent with the absence of significant steady-state charge transport across the proteoliposome membrane and indicate that the electrogenic reaction in these mutants does not result from lactose/H^+ symport.[43,44]

In marked contrast, no significant electrical signal is observed with mutants E269A (Figure 2D) or Y236F (Figure 2E) upon exposure to 100 mM lactose. The signal generated by mutant H322A (Figure 2F) is also about one-seventh of that observed with the WT (I_{peak} = 116 ± 11 pA; n = 4) and displays a slow decay like the WT signal (Figure 2A), indicating a low level of downhill lactose/H^+ symport activity.[49] The findings demonstrate that these three residues play a distinctly different role from Arg302 and Glu325 in the symport mechanism.

Mutations E269A, Y236F, and H322A Block the E325A Signal. We next tested whether the electrical signal observed with E325A LacY is altered by mutation E269A, Y236F, or H322A (Figure 3). Notably, the signal observed with mutant E325A is completely blocked in the double mutant (E269/E325A, Y236F/E325A, or H322A/E325A).

Effect of D_2O. Downhill lactose/H^+ symport by WT LacY is inhibited by D_2O,[51] which is consistent with the interpretation of SSM findings that indicate that deprotonation is the rate limiting-step for this reaction.[43,44] As shown in Figure 4A, the electrical signal generated by downhill lactose/H^+ symport is

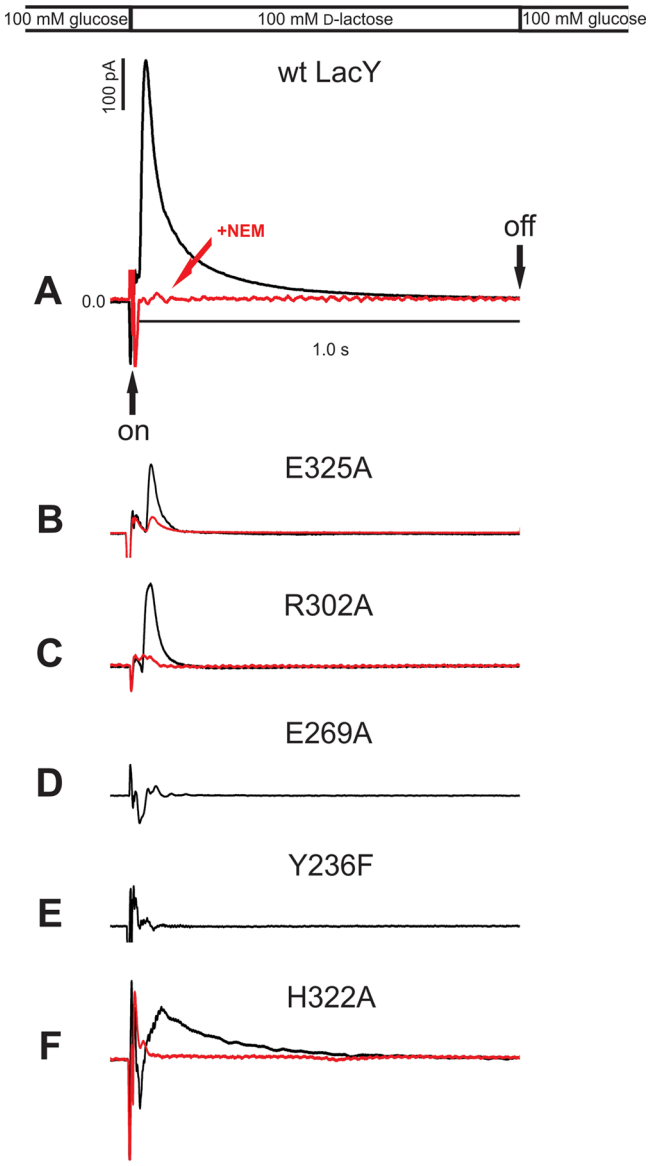

Figure 2. Representative transient currents because of the electrical activity of WT LacY and mutants measured by SSM-based electrophysiology. Proteins were activated after a rapid exchange of the nonactivating solution containing glucose to the activating solution containing lactose; "on" and "off" indicate time points at which the application of the activating solution was switched on and off, respectively. Currents were recorded using proteoliposomes containing (A) WT LacY or mutants (B) E325A, (C) R302A, (D) E269A, (E) Y236F, or (F) H322A. Where indicated, samples were incubated with 5 mM of NEM for 20 min (red trace) as described in the Materials and Methods. The traces shown represent the average of six traces recorded on at least three electrodes. The activating solution contained 100 mM lactose/100 mM KPi/1 mM DTT (pH 7.5). In the inhibition experiments with NEM, no DTT was added.

inhibited 2- to 3-fold when H_2O is replaced with D_2O, thereby providing direct evidence for this judgment. In contrast, no significant effect of D_2O is observed on the signal observed with mutant E325A (Figure 4B), providing further evidence that the signal is not due to H^+ translocation.

DISCUSSION

As shown previously,[43,44] the currents generated by downhill lactose/H^+ symport are specifically due to the activity of LacY.

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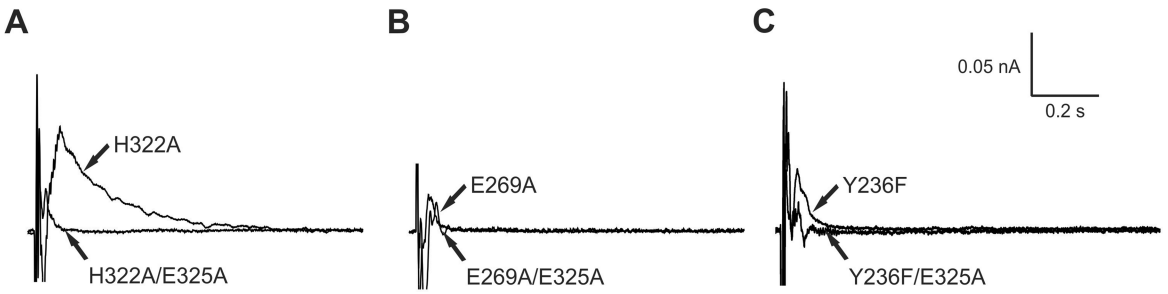

Figure 3. Activity of single and double mutants. Experiments were carried out as described in Figure 2 with (A) mutants H322A and H322A/E325A, (B) mutants E269A and E269A/E325A, and (C) mutants Y236F and Y236F/E325A. The activating solution contained 100 mM lactose/100 mM KP_i/1 mM DTT (pH 7.5).

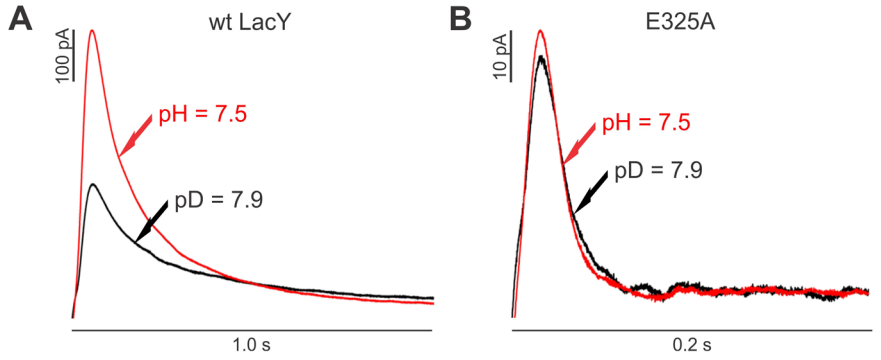

Figure 4. Effect of D2O. (A) WT LacY. Downhill lactose/H^+ symport was determined with reconstituted WT LacY as described in Figure 2A and the Materials and Methods except that H_2O was replaced with D_2O and the pD was adjusted to 7.9 (pH +0.4). (B) Mutant E325A. Measurements were made as described in panel A using reconstituted mutant E325A.

Furthermore, from analysis of the currents observed under different conditions and comparison with biochemical data, it is clear that the predominant electrogenic event in downhill sugar/H^+ symport is H^+ release inside of the proteoliposomes. LacY mutants E325A and R302A are severely defective with respect to lactose/H^+ symport, but they bind galactosides well[36] and catalyze exchange and counterflow.[38−40] In contrast to WT LacY, these mutants exhibit only weakly electrogenic events upon addition of LacY substrates, representing ~6% of the total charge displacement of the WT. This activity is also observed in the WT at acidic pH[44] and is due to either substrate binding per se or to a conformational transition following substrate binding and is not due to sugar/H^+ symport. Thus, it was proposed that turnover of LacY involves at least two electrogenic reactions:[44] (i) a minor electrogenic step that occurs upon sugar binding and is due to a conformational transition in LacY and (ii) a major electrogenic step because of deprotonation during downhill sugar/H^+ symport, which is the limiting step for downhill lactose/H^+ symport.

What type of conformational change resulting from galactoside binding would produce an electrical effect? Clearly, charge rearrangement resulting from sugar binding is a reasonable possibility. In this context and as examples, evidence has been presented indicating that sugar binding to LacY breaks the salt bridge between Glu126 (helix IV) and Arg144 (helix V)[10,32,50] and the salt bridge between Asp68 (helix II) and Lys131 (helix IV)[51] as well as the interaction between Asp240 (helix VII) and Lys319 (helix X).[52] These kinds of perturbations would clearly qualify as a potential explanation for the phenomenon described here. Moreover, LacY mutations in each residue comprising the triad (Glu269, His322, and Tyr236) do not exhibit significant electrical activity upon exposure to lactose, and these mutations block the activity observed with the E325A mutant. Each of these mutants binds a high-affinity lactose homologue with only marginal significance,[36] and the binding of lactose to the WT enzyme has a K_D of 1 to 2 mM.[53] Thus, a concentration of 100 mM lactose was used in all of the experiments presented to ensure saturation of a low-affinity binding site. However, in all likelihood, the total absence of an electrical signal in the triad mutants is due to insignificant binding of sugar.

Previous studies[54] show that D_2O decreases the rate of downhill lactose symport with no effect on the rate of $\Delta\tilde{\mu}_{H^+}$-driven uphill symport or equilibrium exchange. These and other observations (see refs 43−45 and 56) support the conclusion that the rate-limiting step for downhill lactose/H^+ symport is deprotonation and that this step is no longer rate-limiting either when there is a driving force on the H^+ in the form of $\Delta\tilde{\mu}_{H^+}$ or when sugar transfer across the membrane does not involve H^+ translocation (i.e., equilibrium exchange or counterflow). More recently, an inhibitory effect of D_2O on the rate of sugar binding was demonstrated under pre-steady-state conditions using stopped flow and Trp → NPG FRET.[37] Because it was suggested that this effect may be due to a H^+ transfer reaction within LacY related to the weak electrogenic reaction triggered by sugar binding to the protein, the effect of D_2O on downhill symport by WT LacY and on the signal triggered by sugar binding in the E325A mutant was investigated. Inhibition of the electrical signal generated during downhill lactose/H^+ symport is observed with WT LacY in D_2O, which provides direct support for the interpretation that deprotonation is rate-limiting for the reaction. However, a significant effect of D_2O is not observed on the signal induced by sugar binding with mutant E325A.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DDM, *n*-dodecyl-*β*-D-maltoside; NEM, N-ethylmaleimide; NPG, *p*-nitrophenyl-*α*,D-galactopyranoside; SSM, solid supported membrane; LacY, lactose permease; MFS, major facilitator superfamily; OG, *n*-octyl-*β*-D-glucoside; DTT, dithiothreitol

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