Proteolytic Mapping and Substrate Protection of the *Escherichia coli* Melibiose Permease†

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ABSTRACT: The topology and substrate-induced conformational change(s) of the Na^+ (Li^+ or H^+)-melibiose cotransporter (MelB) of *Escherichia coli* were investigated by limited protease digestion. To facilitate these analyses, MelB was epitope-tagged both at its carboxyl-terminus and at its amino-terminus. Limited digestion with different proteases indicates that the cytoplasmic loops connecting transmembrane domains 4−5, 6−7, and 10−11 together with the carboxyl-terminus of MelB are exposed in the cytoplasm. In contrast, periplasmic loops are highly resistant to all the proteases examined, including nonspecific proteases such as proteinase K and thermolysin. The effect of Na^+ or Li^+ and/or melibiose on the rate of protease digestion of the cytoplasmic loops was also analyzed. The rate of protease digestion of loop 4−5 is specifically reduced, by approximately 3-fold, by the presence of Na^+ or Li^+. These results suggest that loop 4−5 is near or part of the cation binding site. Moreover, the presence of both melibiose and either Na^+ or Li^+ further reduced the rate of protease digestion of this loop 4−5 by up to 9-fold, although no protection from protease digestion was observed when melibiose was added alone. The increase in resistance to proteases observed in the presence of the cation alone or the cation plus melibiose suggests that the interaction of the two cosubstrate with MelB results in change(s) of MelB conformation.

The melibiose permease (MelB)[1] of *Escherichia coli*, encoded by *melB* (Yazyu *et al.*, 1984), belongs to a superfamily of Na^+-solute symporters (Reizer *et al.*, 1994). MelB catalyzes the cotransport of Na^+, Li^+, or H^+ and α-galactoside (reviewed in Wilson *et al.*, 1982) and has been shown recently to be solely responsible for α-galactoside transport (Pourcher *et al.*, 1995). Extensive investigations of the kinetic properties of MelB demonstrate that MelB function depends on the nature of the coupling cation, Na^+ and Li^+ being better activators than H^+(reviewed in Leblanc *et al.*, 1988). In contrast, little is known with respect to MelB structure. A topological model proposed for MelB, consisting of 12 transmembrane domains with the carboxyl-terminus located in the cytoplasm, is supported by *melB*−*phoA* fusion analyses (Botfield & Wilson, 1992; Pourcher *et al.*, 1996) and anti-C-terminus polyclonal antibodies binding experiments (Botfield & Wilson, 1989). However, gene fusions analyses do not assess what regions of the physiological MelB are exposed in the periplasm or in the cytoplasm. For example, *phoA* fusion analyses suggest that loop 10−11 is cytoplasmic, yet functional data suggest that this loop may not protrude from the membrane (reviewed in Poolman *et al.*, 1996).

In the present study, *in situ* limited proteolysis is used as an independent and complementary assessement of the topology of MelB (Traxler *et al.*, 1993). In particular, the accessibility of various domains of the permease and the effect of substrates on this accessibility are investigated. Substrate protection to proteolysis is useful in implicating substrate binding domains. *In situ* proteolysis has been used successfully to identify membrane protein functional domains (Dohlman *et al.*, 1987; Shainskaya & Karlish, 1994). Alternatively, conformational changes occurring upon cosubstrate binding can be detected (Wyss *et al.*, 1993). Furthermore, MelB membrane insertion has been recently investigated *in vivo* (Bassilana & Gwizdek, 1996) but further analyses, such as *in vitro* studies, were awaiting an assay to determine the correct membrane insertion of MelB. The present study will provide the basis for such an assay. Indeed, proteolytic digestion has been used to assess the insertion of several membrane proteins (Zimmermann *et al.*, 1982; Wolfe *et al.*, 1985; Ahrem *et al.*, 1989; Traxler & Beckwith, 1992; Werner *et al.*, 1992).

In the present study MelB was epitope-tagged at its amino-terminus to facilitate immunodetection of the products of MelB digestion by several proteases. The tagged permease is functionally identical to the wild type MelB. Digestion of right side out (RSO) membrane vesicles with a number of proteases indicates that the accessibility of the periplasmic loops of MelB is low. In addition, proteolytic digestions of inverted membrane vesicles (IMV) show that, together with the carboxyl-terminus, loops connecting transmembrane domains 4−5, 6−7, and 10−11 of MelB protrude in the cytoplasm. Furthermore, the rate of protease digestion is specifically reduced by the presence of Na^+ or Li^+, approximately 3-fold, in loop 4−5 but not in loop 6−7 nor in the carboxyl-terminus tail of MelB, suggesting that loop 4−5 is near or part of the cation binding site. This Na^+ and Li^+ protection of loop 4−5 is further increased (to 9-fold) in the

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[1] Abbreviations: MelB, melibiose permease; PK, proteinase K; Th, thermolysin; Ti, trypsin; CTi, chymotrypsin; EGC, endoproteinase Glu-C; IMV, inverted membrane vesicles; RSO, right side out membrane vesicles; MW, molecular weight; TMD, transmembrane domain; LAPAO, 3-(laurylamido)-*N*,*N*′-(dimethylamino)propylamine oxide; α-NPG, α-nitrophenyl galactoside; EDTA, ethylenediaminetetraacetic acid.

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presence of melibiose, although no protection from protease digestion was observed with melibiose alone. This result suggests that binding of melibiose may result in a conformational change of MelB near or at the cation binding site.

MATERIALS AND METHODS

Materials. ^{3}H-melibiose (2.7 Ci/mmol) and ^{3}H-α-nitrophenyl galactoside (14 Ci/mmol) were from the Commissariat à l'Energie Atomique (France). Anti-MelB-C-terminus antibodies were polyclonal rabbit antibodies directed against the last 10 C-terminal residues of the permease (Botfield & Wilson, 1989). All other materials were from commercial sources. Methionine Assay medium (MAM) was from DIFCO. Anti-Flag (M2) antibodies were monoclonal mouse antibodies from International Biotechnologies (Kodak). IgG fraction of polyclonal rabbit antibodies raised against mouse IgG, trypsin (Ti), chymotrypsin (CTi), and trypsin−chymotrypsin-inhibitor were from Sigma. Proteinase K (PK), thermolysin (Th), endoproteinase-GluC (EGC), phenylmethanesulfonyl fluoride (PMSF) and α2-macroglobuline were from Boehringer. EN3HANCE, poly(vinylidene difluoride) (PVDF) membranes and Western blot chemioluminescence reagent Renaissance were from Dupont NEN. Horseradish Peroxidase (HRP) color development reagent was from BioRad. ^{35}S-Protein A was from Amersham.

Strain and Plasmid Constructs. DW2 (*lac* I^+ ΔZY, melA$^+$ΔB, strA) was obtained from T. Wilson (Botfield & Wilson, 1988). pK*melB*-6HisFLAG, derived from pK95ΔAHB (Mus-Veteau & Leblanc, 1996) allows constitutive expression of a C-terminal hexahistidine tagged and N-terminal FLAG tagged melibiose permease (MelB-6His-FLAG). Extension of MelB N-terminus with the FLAG epitope (MDYKDDDDK) was carried out by site-directed mutagenesis using the primer (5′-GCG ACC CGA TAC CCT ATG GAC TAC AAG GAC GAC GAT GAC AAG AGC ATT TCA ATG ACT ACA AAA CTC-3′) essentially as described in Zani *et al.* (1993), except that the uracil-labeled template was not degraded with Uracil-DNA glycosylase prior to DW2 transformation. The T7 RNA polymerase/promoter system composed of pGP1-2 and pT7*melB* (Pourcher *et al.*, 1990) was used to selectively label MelB.

^{35}S-Methionine Labeling and Purification of MelB. Mid-log phase DW2/pK*melB*-6His and DW2/pK*melB*-6HisFLAG, cultured in Luria Broth containing 0.1g/L ampicillin at 37 °C, were washed once and depleted of endogenous methionine by incubation 2 h in M9 medium containing MAM and 0.2% glucose. ^{35}S-Methionine labeling and subsequent purification of MelB-6His and MelB-6HisFLAG from radiolabeled bacteria were carried out as reported previously (Bassilana & Gwizdek, 1996). MelB was selectively radiolabeled in cells DW2/pT7*melB* pGP1-2 as described in Pourcher *et al.* (1990).

Preparation of Membrane Vesicles and Proteoliposomes. Membrane vesicles were derived from mid-log grown DW2/pK*melB*-6HisFLAG cells. Inverted membrane vesicles (IMV) were generated using French pressure cell (American Instrument Comp., 4500 psi), according to Reenstra *et al.* (1980), and right side out (RSO) membrane vesicles by osmotic shock (Kaback, 1971). IMV and RSO membrane vesicles were resuspended to a final concentration of 4 mg/mL in a 100 mM KH_2PO_4 buffer, pH 7.0, essentially free of Na^+ ions (measured contamination of 20 μM). Reconstitution in liposomes of purified MelB-6His (Pourcher *et al.*, 1995) was carried out by a procedure involving detergent adsorption on polystyrene beads (Rigaud *et al.*, 1988) according to Pourcher *et al.* (1995) and Mus-Veteau *et al.* (1995).

Protease Digestion Experiments. Protease digestions were carried out on inverted membrane vesicles (IMV) or right side out membrane vesicles (RSO). Membrane vesicles (2 mg/mL) were preincubated for 10 min at 25 °C in the presence or the absence of the cation or sugar substrates before addition of proteases (PK, Th, Ti, CTi, or EGC). The mixtures were further incubated for 30 min at 25 °C and the reactions quenched by addition of the appropriate protease inhibitor and rapid cooling at 4 °C. PK was inhibited with phenylmethanesulfonyl fluoride (PMSF, 1 μL of a fresh saturated solution in ethanol per 50 μL of membrane suspension), Th with K-EDTA pH 7.0 (20 mM), EGC with α_2-macroglobulin (2 mg/mL), and Ti and CTi with trypsin−chymotrypsin-inhibitor (2 mg/mL). Subsequently, one volume of 2X electrophoresis loading buffer was added and samples (40 μg of proteins/lane) were analyzed by 18% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Proteins were then transferred onto PVDF membranes and membranes probed either with anti-MelB C-terminus antibodies or with anti-Flag antibodies. Full length MelB-6HisFLAG and the protease digestion fragments were then visualized with an Enhance ChemiLuminescence kit using either protein A horseradish peroxidase linked or anti-mouse IgG horseradish peroxydase linked. Alternately, when substrate protection experiments were carried out, membranes were sequentially incubated with anti-Flag antibodies (1/5000), anti-mouse antibodies (1/3000), and ^{35}S-protein A (0.25 mCi/mL, 1.25−12.5 nM) before detection with the HRP color development kit. Immunodetected bands were then cut off and counted by liquid scintillation.

Other Procedures. ^{3}H-Melibiose (20 mCi/mmol) transport was measured by rapid filtration technique using Wathman GF/F glass fiber filters as previously described (Bassilana *et al.*, 1985). Binding of ^{3}H-α-nitrophenyl galactoside (α-NPG, 1.6 Ci/mmol) was measured by flow-dialysis, according to Damiano-Forano *et al.* (1986). For N-terminal amino acid sequencing, SDS-polyacrylamide gels were blotted onto PVDF membranes, and the band of interest was excised after coomassie blue staining of the blot. Sequencing was carried out on an ABI 477 using automated Edman degradation. Protein concentrations were determined (Lowry *et al.*, 1951), using bovine serum albumin as a standard.

RESULTS

Properties of the Epitope-Tagged MelB. For our studies, MelB has a C-terminal hexahistidine fusion (MelB-6His) thereby facilitating purification by Ni-NTA affinity chromatography (Pourcher *et al.*, 1995). In addition, in order to detect the MelB amino-terminus fragments resulting from protease digestion (see below), *melB* was epitope tagged with a MDYKDDDDK encoding sequence (FLAG) which is recognized by a commercial monoclonal antibody (see Materials and Methods). The activity of this double epitope-tagged MelB, referred to as MelB-6HisFLAG, was determined by cation-dependent ^{3}H-melibiose transport (Bassilana *et al.*, 1985) and ^{3}H-αNPG binding (Damiano-Forano *et al.*, 1986) measurements and compared to the activity of the

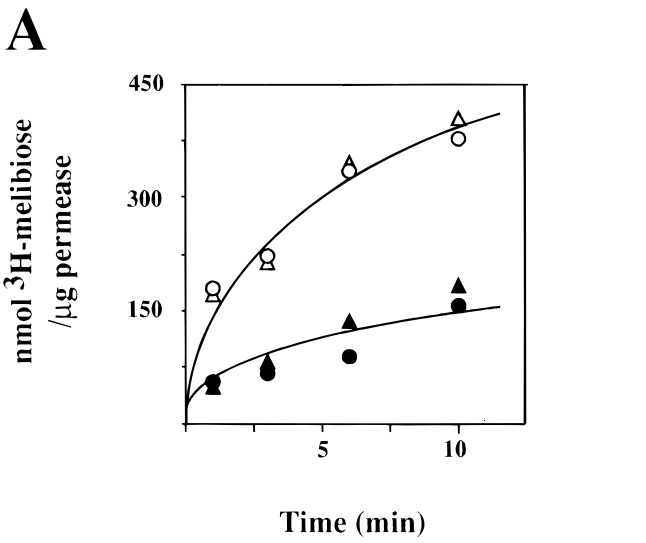

FIGURE 1: Functional and biochemical properties of the double epitope-tagged MelB. 1A: RSO derived from DW2/pK*melB* (circles) and DW2/pK*melB*-6HisFLAG (triangles) were resuspended (3 mg of membrane proteins/mL) in 0.1 M potassium phosphate (pH 7) in the absence (filled symbols) or the presence (open symbols) of 10 mM sodium chloride and assayed for ^{3}H-melibiose transport (0.8 mM; 20 mCi/mmol) by filtration, as previously described (Bassilana *et al.*, 1985). 1B: DW2/pK*melB*-6His and DW2/pK*melB*-6HisFLAG were grown at 30 °C in Luria Broth to OD_{600} = 0.5 and depleted 2 h of intracellular methionine. Cells (0.5 mL) were then pulse-labeled for 1 min with 50 μCi of ^{35}S-methionine, chased with an excess of cold methionine (10 mM) for 15 s (lanes 1 and 3) or 1 h (lanes 2 and 4), and immediately frozen in liquid nitrogen. After solubilization of the radiolabeled bacteria with LAPAO, MelB-6His (lanes 1 and 2) or MelB-6HisFLAG (lanes 3 and 4) were purified by Ni-NTA affinity chromatography (see Materials and Methods). Samples were separated by a 12% SDS-polyacrylamide gel electrophoresis, and radiolabeled permeases were visualized by fluorography.

wild-type MelB. As illustrated in Figure 1A, the time course of melibiose transport by MelB (circles) and MelB-6His-FLAG (triangles) is identical both in the presence of Na^+ (open symbols) or in its absence (filled symbols). Furthermore, the melibiose analog α-NPG binding constants are similar for MelB and MelB-6HisFLAG, with a maximal number of binding sites of approximately 0.15 nmol/mg protein and a K_d of 0.4 μM. These results demonstrate that MelB activity is not affected by the addition of the two tags.

Figure 1B illustrates MelB-6His (lanes 1, 2) and MelB-6HisFLAG (lanes 3, 4) purification from bacteria pulse-labeled for 1 min with ^{35}S-methionine followed by either a 15 s (lanes 1, 3) or 1 h (lanes 2, 4) chase with an excess of cold methionine, as previously described (Bassilana & Gwizdek, 1996). MelB-6HisFLAG migrates with a slightly higher apparent MW than MelB-6His (approximately of 40 kDa on a 12% SDS-PAGE), consistent with the presence of 8 additional residues. For both MelB-6His and MelB-6HisFLAG the amount of radiolabeled permease purified after a 1 h chase was identical to that purified after 15 s chase (compare Figure 1B lanes 1, 2 and 3, 4, respectively), indicating that MelB-6HisFLAG is as stable as MelB-6His once in the membrane. Thus, MelB-6HisFLAG exhibits the same biochemical and functional properties as MelB and was therefore used for the present topological analysis of the permease.

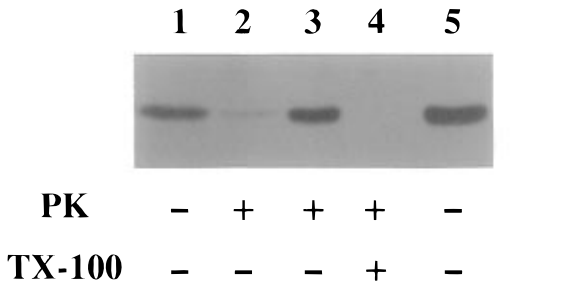

FIGURE 2: Orientation and protease impermeability of membrane vesicles preparations. IMV or RSO membrane vesicles were freshly prepared in order to avoid digestion by endogenous proteases (Akiyama and Ito, 1990). The membrane preparations (2 mg/mL in KH_2PO_4, pH 7.0) were digested by proteinase K (PK) at 10 μg/mL for 30 min at 25 °C as described in Materials and Methods. The digestion mixtures were loaded on a 12% SDS-PAGE gel (40 μg of protein/lane). The proteins were then transferred onto PVDF-membranes and the membranes probed with antibodies directed against the N-terminus of SecY (Lill et al., 1989; Watanabe & Blobel, 1989). SecY was visualized by enhanced chemiluminescence. PK digestion of IMV (lane 2) or RSO membrane vesicles (lane 3). Lane 4: RSO membrane vesicles were solubilized with 1% Triton X-100 (TX-100) prior to PK digestion. Lanes 1 and 5: untreated IMV and RSO membrane vesicles, respectively.

Accessibility of MelB Periplasmic and Cytoplasmic Loops to Protease Digestion. According to previous models based upon hydropathy profiling and PhoA-fusion analysis (Botfield & Wilson, 1992; Pourcher *et al.*, 1996), MelB has 12 transmembrane domains that are connected by hydrophilic loops. These loops are alternatively exposed on the cytoplasmic or the periplasmic faces of the membrane. Limited proteolysis of MelB in inverted (IMV) or right side out (RSO) membrane vesicles should result in a specific digestion of cytoplasmic or periplasmic loops, respectively. For these experiments the correct orientation of the membrane preparations used in the present study (IMV or RSO membrane vesicles) and their impermeability to protease, were probed by two independent experiments. First, as expected only RSO membrane vesicles and not IMV accumulate ^{3}H-melibiose, although IMV and RSO membrane vesicles contain the same number of active permeases, on the basis of α-NPG binding experiments (data not shown). Second, when the two membrane preparations were treated with proteinase K (PK), the digestion of the integral membrane protein SecY was assessed using antibodies directed against the cytoplasmic N-terminus of SecY (Lill *et al*, 1989; Watanabe & Blobel, 1989). As illustrated in Figure 2, in agreement with the cytoplasmic localization of its N-terminus (Akiyama & Ito, 1987; Watanabe & Blobel, 1989), SecY was digested in IMV (compare lanes 1 and 2) and not in RSO membrane vesicles (compare lanes 3 and 5). Furthermore, when RSO membrane vesicles were solubilized in the presence of Triton X-100 prior to PK digestion, both the periplasmic and the cytoplasmic faces being then accessible to the protease, SecY associated signal was no longer detected (lane 4). These data indicate that IMV and RSO membrane vesicles are correctly oriented and sealed to proteases.

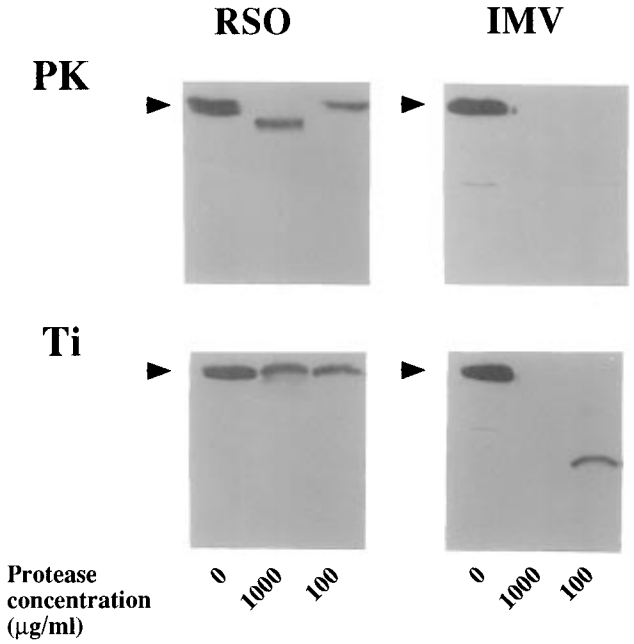

FIGURE 3: MelB fragments generated by proteinase K and trypsin digestion in IMV and RSO membrane vesicles. IMVs or RSO membrane vesicles (2 mg/mL) were digested by Proteinase K (PK) and Trypsin (Ti) at the indicated concentrations for 30 min at 25 °C as described in Materials and Methods. The digestion mixtures were loaded on a 18% SDS-PAGE gel (40 μg of protein/lane). The proteins were then transferred onto PVDF-membranes and the membranes probed with an anti-Flag monoclonal antibody. Full length MelB-6HisFLAG (indicated by the arrow) and MelB-6HisFLAG N-terminal fragments were visualized by enhanced chemiluminescence.

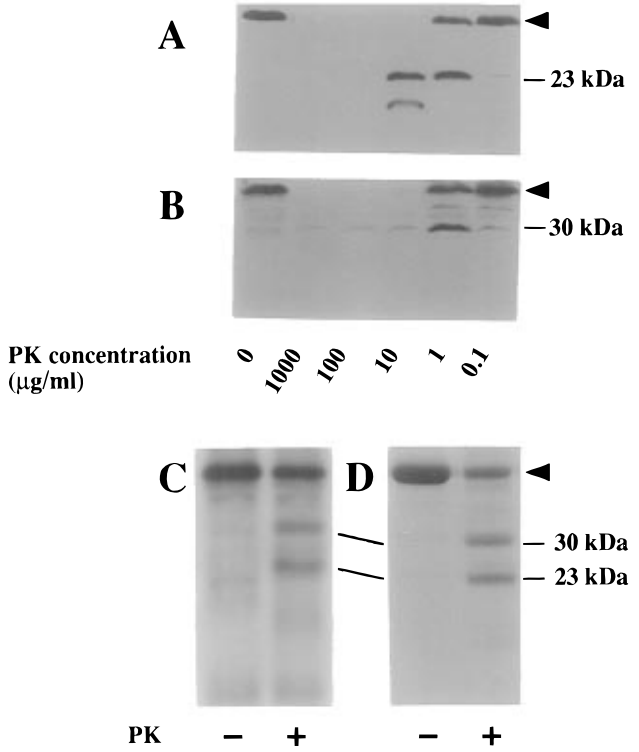

FIGURE 4: Limited PK digestion of MelB in IMV results in a single cut. 4A and 4B: IMVs (2 mg/mL) were digested with the indicated concentrations of PK for 30 min at 25 °C (Materials and Methods). The digestion mixtures were then treated as described in Figure 3. MelB-6HisFLAG fragments possessing either the N-terminus (4A) or the C-terminus (4B) were immunodetected with antibodies raised against the Flag epitope or the MelB C-terminus (Botfield and Wilson, 1989), respectively, and visualized by enhanced chemiluminescence. 4C: MelB was selectively labeled with ^{35}S-methionine *in vivo* using DW2 cells transformed with the pT7 polymerase-promoter system (see Materials and Methods). IMVs were prepared from these radiolabeled cells and treated with PK at a ratio protease/protein of 0.05%. Samples treated or not with PK were loaded on a 18% SDS-PAGE and the proteins detected by fluorography. 4D: Proteoliposomes containing purified MelB-6His were treated with PK at a ratio protease/protein of 0.15% . Peptides were visualized with coomassie blue.

Such IMV and RSO membrane vesicles were digested with either PK or trypsin (Ti) at high concentrations (100 and 1000 μg/mL), and MelB-6HisFLAG and the digestion products were detected with anti-FLAG antibodies. As illustrated in Figure 3, incubation of IMV with 100 μg/mL of either PK or Ti resulted in the total disappearance of the full-length MelB-6HisFLAG (migrating with a MW of 55 kDa on a 18% SDS-PAGE). In contrast, a concentration as high as 1000 μg/mL of either PK or Ti was required to digest MelB-6HisFLAG in RSO membrane vesicles. Under these conditions, the preferential PK digestion site appears to be the periplasmic loop 11−12 (based on the apparent molecular weight of the generated N-terminal fragment). Full length MelB-6HisFLAG or MelB-6HisFLAG fragments were no longer observed when RSO were solubilized in the presence of Triton X-100 prior to PK or Ti digestion (data not shown). These results indicate that MelB periplasmic loops are not highly accessible to PK or Ti digestion. In addition, digestion of MelB-6HisFLAG with either chymotrypsin (CTi) or thermolysin (Th) gave similar results and indicated that concentrations of 10 to 100 times more protease were necessary to digest MelB-6HisFLAG in RSO membrane vesicles compared to IMV (data not shown). Thus, MelB periplasmic loops are resistant to protease digestion, consistent with the recent model of secondary structure (Pourcher *et al.*, 1996 and Figure 5) predicting short periplasmic loops.

Limited Proteolysis in IMV. Figure 3 (right panel) shows that incubation of IMV with 100 μg/mL PK led to the total disappearance of full length MelB-6HisFLAG. This absence of full length MelB-6HisFLAG could result either from digestion of MelB-6HisFLAG into fragments too small to be detected on the 18% SDS-PAGE used (<6 kDa) or from digestion in (or removal of) the FLAG epitope. In order to map PK cleavage sites in MelB-6HisFLAG cytoplasmic loops and/or in the N-terminus FLAG epitope, digestion patterns of IMV were immunodetected using either the anti-FLAG monoclonal antibody or antibodies raised against the MelB C-terminus (Botfield & Wilson, 1989). Figure 4 illustrates the N-terminal (Figure 4A) and the C-terminal (Figure 4B) fragments generated by digestion of MelB-6HisFLAG in IMVs with PK concentrations ranging from 0.1 μg/mL to 1000 μg/mL. At concentrations lower than 1 μg/mL, PK did not digest MelB-6HisFLAG. When a concentration of 1 μg/mL of PK was used, detection of the digestion products with antibodies against either the carboxyl-terminus of MelB or the amino-terminus FLAG epitope revealed a single band, in addition to the full-length permease. The N-terminal fragment (Figure 4A) has a MW of approximately 23 kDa and the C-terminal fragment (Figure 4B) of approximately 30 kDa. Together, the MWs of these two fragments (30 + 23 kDa) approximately add up to full length MelB-6HisFLAG (56 kDa), suggesting that MelB-6HisFLAG is cut at a single site at this concentration of PK. To confirm this result, two independent approaches were used. PK digestion of MelB was analyzed using either IMVs

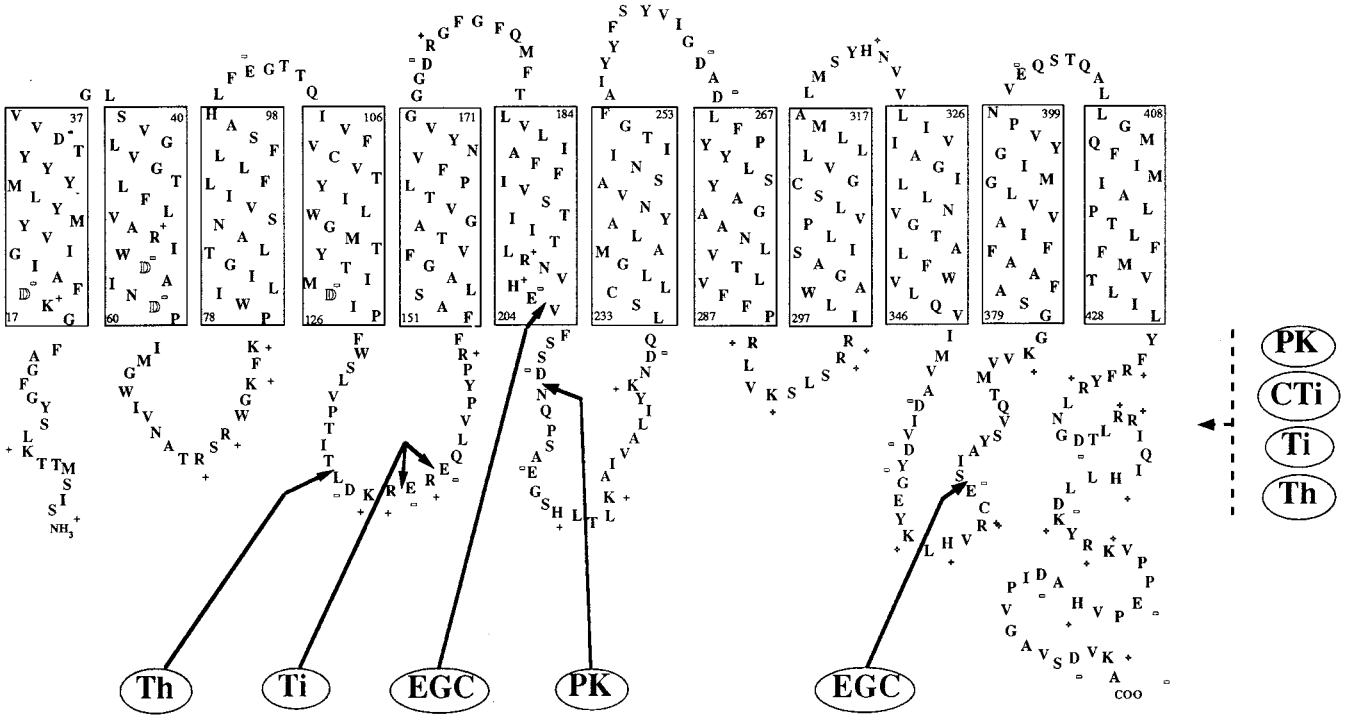

FIGURE 5: Localization of the protease cleavage sites in the secondary structure model of MelB. The secondary structure model of MelB is from Pourcher *et al.*, 1996. Solid lines indicate the cleavage sites identified by sequencing and dashed lines the putative cleavage sites as derived from the size of the apparent MWs of MelB-6HisFLAG amino-terminus digestion fragments. Proteinase K (PK), trypsin (Ti), chymotrypsin (CTi), thermolysin (Th), and endoproteinase GluC (EGC).

selectively radiolabeled for MelB with the T7 RNA-polymerase/promoter system (Pourcher *et al.*, 1990) or proteoliposomes reconstituted from purified MelB-6His (Pourcher *et al.*, 1995). The results are illustrated in Figures 4C and 4D. An autoradiogram of PK-digested radiolabeled IMVs shows two fragments of approximately 30 and 23 kDa in addition to the full length MelB (Figure 4C). A coomassie stain of the digested proteoliposomes indicates also two fragments with MW of approximately 30 and 23 kDa (Figure 4D), consistent with a single digestion site. It has to be mentioned that several proteases including PK digest greater than 50% of MelB-6His in proteoliposomes (see also below), indicating that more than half of the permeases is in inverted orientation in this reconstituted system (Pourcher *et al.*, 1995). The single PK cleavage site was identified by sequencing of the C-terminal fragment of MelB-6His generated from digestion of proteoliposomes and localized in the cytoplasmic loop 6−7 of the secondary structure model of MelB (Figure 5).

Proteolytic Mapping of MelB. Immunodetection of N- and C-terminal digested fragments combined with sequencing of the carboxyl-terminus products were used as described above to map the sites of proteolysis of MelB with several proteases, such as Ti, CTi, Th, and EGC. The fragments identified by immunodetection are listed in Table 1 and the sites of proteolysis identified by sequencing are mapped on the secondary structure model illustrated in Figure 5. Strikingly, nonspecific proteases such as PK and Th or proteases such as Ti, CTi, and EGC altogether generated only four N-terminal fragments with MW of approximately 45, 38, 23, and 16−17 kDa (Table 1). These results indicate that MelB-6HisFLAG is particularly exposed at four regions. Generation of the 45 kDa N-terminal fragments by Ti, CTi, or Th together with the concommitant absence of C-terminal fragments suggest that one of these four regions is the C-terminus cytoplasmic tail of MelB (dashed lines in Figure 5) . The three other regions were identified by sequencing (solid lines in Figure 5) and located in loops connecting transmembrane domains 4−5 (Th and Ti digestion), 6−7 (PK and EGC digestion), and 10−11 (EGC digestion). It was observed that the carboxyl-terminus is initially cleaved by Ti, Th, and CTi, but not by PK and EGC. In contrast, the initial cleavage for PK is loop 6−7 and then the carboxyl-terminus of MelB. No clear difference between the rate of digestion of loops 6−7 and 10−11 with EGC could be detected.

Table 1: MelB N-Terminal and C-Terminal Fragments Resulting from Protease Digestion in IMV[a]

protease	concentration (μg/mL)	C-terminal digestion fragments (kDa)	N-terminal digestion fragments (kDa)
proteinase K	1	30	23
	10	not detected	23; 16
thermolysin	100	not detected	45; 17
	1000		17
trypsin	10	not detected	45; 17
	100		17
chymotrypsin	10	not detected	45; 16
	100		16
endoproteinase	100	18; 30	38; 23
Glu-C	1000	not detected	23

[a] Protease digestion in IMV and identification of MelB-6HisFLAG N-terminal and C-terminal fragments were performed as described in Figure 4A.

Substrate Protection Analyses. MelB is a Na^+ or Li^+ melibiose cotransporter, in which Na^+ or Li^+ activate melibiose transport and binding. Altogether, analyses of the properties of sugar binding (Damiano-Forano *et al.*, 1986), facilitated diffusion (Bassilana *et al.*, 1987), and intrinsic fluorescence (Mus-Veteau *et al.*, 1995) suggest that binding of the cosubstrates induce conformational change(s) of the

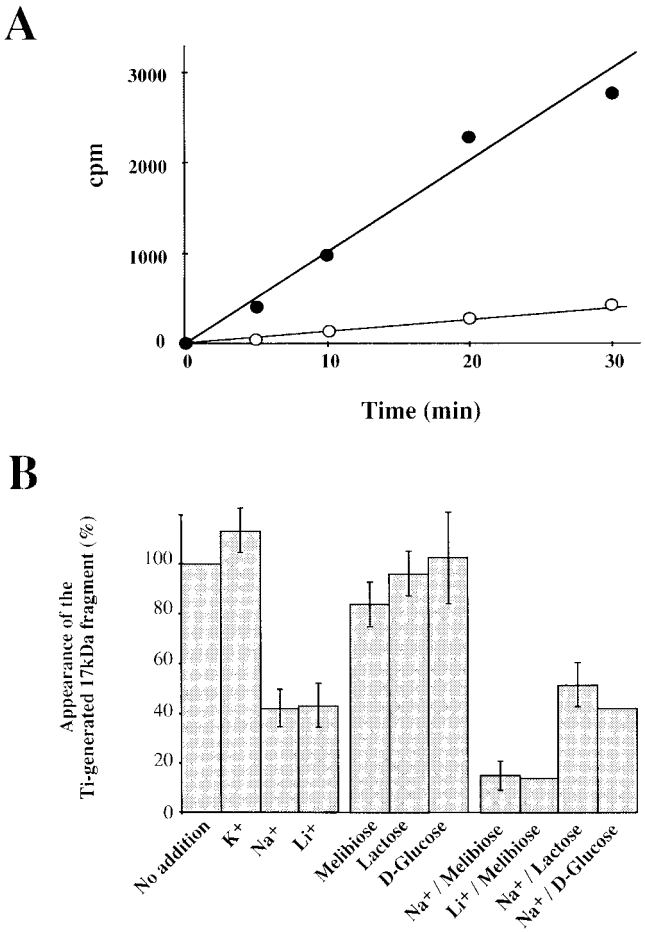

FIGURE 6: Effect of MelB cosubstrates on Ti digestion in IMV. 6A: Time course of appearance of the MelB-6HisFLAG 17 kDa amino-terminus fragment generated in the absence (filled circles) or the presence (open circles) of the two cosubstrates, Na^+ and melibiose. IMV (2 mg/mL in 100 mM KH_2PO_4, 7.0) were incubated for 10 min with or without 10 mM NaCl and 20 mM melibiose prior to Ti digestion (100 μg/mL) for the indicated times at 25 °C. Proteins (76 μg/lane) were separated on a 18% SDS-PAGE and transferred on PVDF membranes, and the membranes were incubated successively with anti-Flag antibodies (1/5000), anti-mouse antibodies (1/3000), ^{35}S protein A (0.25 mCi/mL) and Horseradish peroxidase-linked anti-rabbit antibodies (1/3000). The 17 kDa amino-terminus fragments were visualized using a HRP color reagent, and the bands were excised and counted by liquid scintillation. The rate of appearance of the 17kDa fragment is calculated from the slopes. 6B: Effect of Na^+, Li^+, melibiose, lactose, and/or D-glucose on the rate of appearance of the MelB-6HisFLAG 17 kDa amino-terminus fragment. Time course of appearance of the 17 kDa fragment was determined as described in Figure 6A in the presence or the absence of KCl (10 mM), NaCl (10 mM), LiCl (10 mM), melibiose (20 mM), lactose (20 mM), and/or D-glucose (20 mM). The results of three independent experiments are presented for each condition in percent of the control rate (100%) measured in the absence of either of the substrates, i.e when IMV were resuspended only in 100 mM KH_2PO_4, pH 7.

permease. In order to obtain additional insights into this substrate-induced conformational change(s), the effect of Na^+ or Li^+ and melibiose on the rate of MelB-6HisFLAG digestion was analyzed by measuring the relative rate of appearance of the N-terminal fragments, generated by Ti, Th, or PK digestion. Figure 6A shows the time course of appearance of the 17 kDa N-terminal fragment of MelB-6HisFLAG generated by Ti digestion in the presence (open circles) or the absence (filled circles) of both Na^+ and melibiose at saturating concentrations. The rate of appearance of this Ti-generated 17 kDa fragment was reduced by 9-fold in the presence of the two cosubstrates, indicative of substrate protection. A similar reduction of the rate of appearance was observed when Li^+ instead of Na^+ was added with melibiose (not shown). To investigate the contribution of each substrate separately, similar experiments were carried out in the presence of either Na^+ or melibiose. The data presented in Figure 6B are the average of three independent experiments and are expressed as percent of the control rate of digestion measured in the absence of substrate. Addition of 10 mM of either NaCl or LiCl (but not KCl) reduced the relative rate of appearance of the 17 kDa fragment by more than 2-fold, whereas melibiose had no significant effect. Thus, the 9-fold protection observed in the presence of both melibiose and Na^+ or Li^+ cannot be accounted for by the individual or added effects of the two cosubstrates. Importantly, lactose, which is a poor substrate of MelB or D-glucose which is not a substrate of MelB, had no effect by itself or when added together with Na^+. Similar results were obtained when MelB-6HisFLAG was digested with thermolysin to generate the 17 kDa fragment. The relative rate of appearance of this Th-generated 17 kDa fragment was reduced by 3-fold in the presence of Na^+ or Li^+ alone and was further reduced in the presence of either cation plus melibiose (by 9-fold). Overall, these results indicate that binding of the cation alone protects MelB loop 4−5 from protease digestion and that this cation protection is increased in the presence of the physiological substrate melibiose. Two additional findings indicate that the cosubstrate protection from protease digestion on loop 4−5 is specific for this region. First, the rate of appearance of the 45 kDa band generated by either Ti or Th was not affected by the presence of Na^+ or Li^+ and melibiose. Second, the relative rate of appearance of the 23 kDa band generated by PK digestion was not affected by the presence of either Na^+ or Li^+. Altogether, these results are consistent with change(s) in the conformation of MelB upon binding of the cosubstrate cation and sugar.

DISCUSSION

The topology and substrate-induced conformational change(s) of the *E. coli* MelB sugar cotransporter was examined by limited digestion of IMV, RSO membrane vesicles, and proteoliposomes with a number of proteases. Our results show that the accessibility to protease digestion of an epitope-tagged MelB (MelB-6HisFLAG) is asymmetrical. MelB periplasmic domains are extremely resistant to protease digestion, while several cytoplasmic domains are the targets of different proteases. The proteolytic sites were mapped to the cytoplasmic loops 4−5, 6−7, 10−11 and the carboxyl-terminus of MelB, indicating that these four regions are exposed. Moreover, the effect of MelB cosubstrates (Na^+, Li^+, and melibiose) on the rate of protease digestion of MelB-6HisFLAG indicates that Na^+ and Li^+ selectively reduce the rate of protease digestion of loop 4−5 and that this cation protection is specifically increased by the presence of melibiose. These results suggest that MelB undergoes conformational change(s) upon binding of its cosubstrate.

Secondary structure models of MelB, based on hydropathy profiling (Yazyu *et al.*, 1984), on the constraints imposed by the "positive inside rule" (von Heijne, 1989) and on *phoA*-fusion analyses (Botfield & Wilson, 1992; Pourcher *et al.*, 1996), indicate that MelB is composed of 12 transmembrane domains (TMD). *PhoA*-fusions analysis involves construc-

tion of chimeras of the alkaline phosphatase gene fused to carboxyl-termini of MelB-encoding sequences of various lengths. PhoA is only active in the periplasm, and therefore a chimera which exhibits PhoA activity indicates that the carboxyl-terminus of the MelB fusion is either in the periplasm or in the outer half of a transmembrane domain (Manoil & Beckwith, 1986; Calamia & Manoil, 1990; Pourcher *et al.*, 1996). As a result, *phoA*-fusion analysis is an appropriate method to localize the domains of MelB close to or in the periplasm. On the other hand, cytoplasmic loops and the cytoplasmic half of a transmembrane domain are typically identified by the combination of fusions which result in little to no PhoA activity, the constraints imposed by the "positive inside rule", and the theoritical length of transmembrane domains (approximately 21 amino acids). Using the above criteria, regions close to (or in) the periplasm and those close to (or in) the cytoplasm can be discriminated and used to predict the transmembrane domains. However, this approach is unable to localize the amino acids residues which are close to the membrane/cytoplasm or membrane/periplasm interface.

In this respect, proteolytic digestion can directly identify regions of membrane proteins which are exposed to the cytoplasm or to the periplasm, and therefore this approach is complementary to gene fusion analysis, in membrane topology determination. Our studies indicate that MelB periplasmic loops are resistant to protease digestion. MelB-6HisFLAG fragments were only observed when PK at high concentration (1 mg/mL) was used to digest RSO. Such a resistance of the periplasmic loops to protease digestion is consistent with the most recent secondary structure model (Figure 5), in which periplasmic loops are short (less than 15 amino acids). Short periplasmic loops appear to be a common feature to secondary transport proteins in prokaryotes (reviewed in Poolman & Konings, 1993). Protease digestion of IMV indicates that four domains of MelB are exposed in the cytoplasm. These domains of MelB correspond to the carboxyl-terminus and to predicted loops 4−5, 6−7, and 10−11 on the secondary structure model illustrated in Figure 5. The two proteolytic digestion sites identified in loop 4−5 and the one identified in loop 10−11 are located in the middle of these cytoplasmic loops, in highly charged regions. In contrast, the two proteolytic digestion sites identified in predicted loop 6−7 are located near the membrane interface: the PK site, D208, was predicted to be in the cytoplasmic loop 6−7 while the EGC site, E203, was predicted to be within the cytoplasmic end of TMD 6 (Pourcher *et al.*, 1996). Our results on proteolytic digestion indicate that this region of loop 6−7, including E203, is exposed to the cytoplasm. Furthermore, the two charged residues E203 and R199 can be replaced by uncharged residues (E203Q and R199A) without modification of MelB activity (unpublished observations), indicating that these two residues do not have an important role in function, in contrast to the previous suggestion (Pourcher *et al.*, 1996).

The second important finding reported in this study is the observation indicating that MelB cosubstrates protect specifically loop 4−5 against proteolysis. Alone, the cation (Na^+ or Li^+) reduces by 2-fold the rate of digestion of this loop 4−5. In addition, while the sugar has no significant effect in itself, it amplifies (up to 9-fold) the protective effect of the coupling ion. As protection against proteolysis most likely reflects structural rearrangements of the transporter, these data indicate that the cosubstrates promote at least two different conformational changes of MelB: one occurring upon interaction of the coupling ion with the transporter and one triggered by sugar binding on the binary complex Na^+-permease. These data also provide some insights into the topological organization of the functional domains of the transporter and into their possible interactions. Thus, loop 4−5 is specifically protected against proteolysis by Na^+ or Li^+, suggesting that this cytoplasmic loop is near or part of the cation binding site. This interpretation is consistent with analyses of the functional properties of site-directed mutants (Pourcher *et al.*, 1993; Zani *et al.*, 1993) and of chimeras (Hama & Wilson, 1993) which suggest that residues in the amino-terminal domain of MelB are involved in the cation recognition. On the other hand, melibiose binding enhances the cation protection of Na^+ or Li^+ up to 9-fold, suggesting that the sugar triggers a conformational change near or at the cation binding site. This result is consistent with tryptophan fluorescence studies of MelB reconstituted in proteoliposomes (Mus-Veteau & Leblanc, 1996), which suggested that binding of one cosubstrate affects the conformation of the other cosubstrate binding site. An interaction between two separate (or overlapping) substrate binding sites would readily explain that single site mutations alter concommitantly the cationic and sugar selectivity properties of MelB (Botfield & Wilson, 1988; Zani *et al.*, 1993). In summary, the results on substrate protection converge with the results from previous functional and structural approaches to localize the cation binding site in the amino-terminus domain of MelB and to suggest conformational interactions between the cation and the sugar binding sites. Furthermore, the proteolytic mapping of MelB and associated substrate protection will be a useful criteria to assess the functional membrane insertion of MelB in *in vitro* studies.

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