Melibiose Permease of *Escherichia coli*: Large Scale Purification and Evidence That H^+, Na^+, and Li^+ Sugar Symport Is Catalyzed by a Single Polypeptide†

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ABSTRACT: As much as 20−30 mg of functional recombinant melibiose permease (Mel-6His permease) of *Escherichia coli,* carrying a carboxy-terminal affinity tag for metallic ions (six successive histidines), can be routinely purified from 10 g of cells (dry weight) by combining nickel chelate affinity chromatography and ion exchange chromatography. Mel-6His permease was constructed by modifying the permease gene (*melB*) *in vitro* and then overproduced in cells transformed with multicopy plasmids. The tagged permease was efficiently solubilized in the presence of 3-(laurylamido)-*N,N'*-dimethylaminopropylamine oxide (LAPAO) and high sodium salt concentration and then selectively adsorbed on a nickel nitrilotriacetic acid (Ni-NTA) affinity resin. After the replacement of LAPAO by *n*-dodecyl β-D-maltoside to maintain the activity of the soluble permease in low ionic strength media, the permease-enriched fraction (>90%) was eluted with 0.1 M imidazole and finally purified to homogeneity (>99%) using ion exchange chromatography. Determination of the permease N-terminal sequence shows that an initiating methionine is missing and that a Ser-Ile-Ser stretch precedes the postulated primary amino acid sequence. Purified permeases, reconstituted in liposomes, display H^+-, Na^+-, or Li^+-dependent sugar binding and active transport activities similar to those of the native permease in its natural environment, proving that all three modes of symport activity are mediated by one and the same polypeptide.

The melibiose (Mel)[1] permease of *Escherichia coli*, encoded by the *melB* gene, transports the α-galactoside melibiose against its chemical gradient according to a symport or cotransport mechanism (Lopilato et al., 1978; Tsuchiya & Wilson, 1978). It uses, alternatively, Na^+, H^+, or Li^+ as the coupling ion species, depending on the ionic conditions and configuration of the sugar substrate (Bassilana et al., 1985). This unique property has been used extensively to describe the Mel permease function in kinetic terms (Wilson & Wilson, 1987) and in particular to establish that one essential role of the coupling ion is to enhance the transporter affinity for its sugar substrate (Pourcher et al., 1990a).

On the basis of cloning and sequencing of the *melB* gene, recombinant DNA technology and molecular biology strategies have been used for the study of this symporter and its mechanism of action. The *melB* nucleotide sequence suggests that Mel permease consists of 469 amino acids, 70% of which are apolar residues, and that it has a theoretical molecular weight of 52 000 (Yazyu et al., 1984). The *melB* gene product was identified as a cytoplasmic membrane protein with an M_r of 39 000 by means of *in vivo* labeling techniques (Pourcher et al., 1990b) and immunological studies with antibodies directed against the permease C-terminus (Botfield & Wilson, 1989). Examination of the hydropathy profile of Mel permease and analysis of its topology using a gene fusion strategy suggest that the permease contains 12 membrane-spanning segments in a helical configuration (Botfield et al., 1992). Site-directed mutagenesis studies on several acidic and polar residues located in the N-terminal hydrophobic domains of the permease suggest that three aspartic acid residues, distributed on helices II and IV, act as a network involved in coordination of the coupling cation (Pourcher et al., 1991, 1993; Wilson & Wilson, 1992, 1994; Zani et al., 1993, 1994). Also, analysis of the ionic properties of hybrid permeases constructed from homologous *mel* permease of *Escherichia coli* and *Klebsiella pneumoniae* further emphasizes the importance of the permease N-terminal domains for cation recognition (Hama & Wilson, 1993). Determination of the validity of these conclusions, however, must await further knowledge of the symporter structure.

Analysis of the structure of Mel permease using biophysical techniques depends on its purification in large amounts. Although this membrane transporter can be solubilized using nonionic detergents (e.g., *n*-octyl glucoside) and remains functional once reconstituted into liposomes (Tsuchiya et al., 1982; Wilson et al., 1985), there is as yet no known procedure for its purification. A very attractive strategy has

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[1] Abbreviations: Mel, melibiose; IMV, inverted membrane vesicle; His, histidine; Mel-6His permease, melibiose permease carrying a carboxy-terminal affinity tag of six successive histidines; TMG, methyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DDAO, *N,N*-dimethyldodecylamine oxide; LAPAO, 3-(laurylamido)-*N,N'*-dimethylaminopropylamine oxide; SB 12, 3-(*N*-dodecyl-*N,N*-dimethylammonio)-1-propanesulfonate (Zwittergent 3-12); $C_{12}E_8$, octaethylene glycolmono-*n*-dodecyl ether; TX100, poly(ethylene glycol) *p*-isooctylphenyl ether (Triton X-100); MEGA 8, octanoyl-*N*-methylglucamide; OG, *n*-octyl glucoside; DM, *n*-dodecyl β-D-maltoside.

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been developed by Hochuli and colleagues (Hochuli et al., 1987, 1988) for protein purification. These authors reported that the attachment of a polyhistidine peptide (His tag) to the N- or C-terminal extremity of a protein confers high affinity to the recombinant protein for metallic ions. The tagged protein is subsequently purified by immobilized metal ion affinity chromatography using an affinity nickel nitrilotriacetic acid (Ni-NTA) resin. This strategy has been successfully applied to purify a large number of soluble recombinant proteins (Stüber et al., 1990), and its utilization recently extended to the purification of detergent-solubilized membrane proteins involved in solute transport: the chloroplast triose phosphate translocator expressed in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Loddenkötter et al., 1993), the $IIBC^{GLC}$ subunit of the *E. coli* glucose phosphotransferase system (Waeber et al., 1993), and the Na^+-dependent citrate carrier (CitS) of *Klebsiella pneumoniae* (Pos et al., 1994).

In the present paper, we show that the combination of overproduction of an His-tagged Mel permease, selection of an efficient detergent for its solubilization, and immobilized metal ion affinity chromatography can be used for the routine purification of large amounts of Mel permease in a functional state.

MATERIALS AND METHODS

Materials. [^{3}H]Melibiose (2.7 Ci/mmol) and DDAO were purchased from the Commissariat à l'Energie Atomique (France). *p*-Nitrophenyl α-D-[6-^{3}H]galactopyranoside (α-NPG) was synthesized under the direction of Dr. B. Rousseau in our department (Dept. Biol. Cell. Mol. du CEA), and LAPAO was synthesized as described previously (Brandolin et al., 1980). All other materials were obtained from commercial sources. CHAPS, SB 12, TX100, MEGA 8, OG, and DM are from Boehringer Mannheim, $C_{12}E_8$ is from Calbiochem, Inc., Ni-NTA resin is from Qiagen, Inc., anion exchange resin (Macro-Prep Q support) and Bio-Beads SM-2 are from Bio-Rad Lab., Inc., and total *E. coli* lipids are from Avanti Polar Lipids, Inc. (acetone/ether precipitated). High purity grade chemicals (Suprapur, Merck) were used to prepare media virtually devoid of sodium salts, which will be referred to hereafter as Na^+-free solutions. Flame photometry measurements indicated that the level of contaminating sodium salts in these solutions was generally below 20 μM.

Bacterial Strains and Plasmids. E. coli DW2-R, a *recA*$^-$ derivative of strain DW2 (*melA*$^+$, Δ*melB*, Δ*lacZY*) was used throughout this study (Botfield & Wilson, 1988). *E. coli* CJ236 (*dut, ung, thi, relA, pCJ105 [Cm^r]*), used for site-directed mutagenesis, was from Bio-Rad. Plasmids pK31 (Pourcher et al., 1990c) and pK40 (Pourcher et al., 1991) are pKK223-3 recombinant vectors carrying *melA* and *melB* genes under the control of the tac promoter. pB40 plasmid is a BlueScriptII KS$^+$ vector previously constructed for mutagenesis of the *melB* gene (Zani et al., 1994). A T7 RNA polymerase/promoter expression system, comprising pGP1-2 and a recombinant pT7-6 plasmid (pT40), was used to label the permease *in vivo*. pT40 plasmid, carrying the *melB* gene flanked at its 5′- and 3′-extremities by *Eco*RI and *Hin*dIII sites, respectively, was constructed as follows: The starting vector was pT(SD/B), containing *melB* flanked by two *Eco*RI sites (Pourcher et al., 1990b). First, all sites except the *Eco*RI site of the multicloning sequence present in pT(SD/B) were removed by restricting the plasmid with *Bam*HI and *Hin*dIII enzymes, digesting the extremities with Mung bean nuclease, and ligating the resulting blunt ends. The resulting plasmid was then linearized by partial digestion with *Eco*RI, digested with Mung bean nuclease, and finally digested to completion with *Eco*RI. The largest fragment (vector) was purified and mixed with an *Eco*RI−*Ssp*I DNA fragment prepared from pK40 containing the *melB* gene, and the mixture was incubated with T4 DNA ligase. Plasmid (pT40) carrying the *melB* gene with the correct orientation was screened by complementation.

Addition of a Ni Affinity Site to Mel Permease Using Site-Directed Mutagenesis. Adjunction of six successive histidine residues to the C-terminal extremity of the Mel permease was carried out by modifying the *melB* gene sequence in pK31 or pT40 plasmid using uracil-labeled pB40 DNA as a template and the oligonucleotide primer (5′-G AGT GAT GTG AAA GCC CAT CAT CAT CAT CAT CAT TGA GAG CTC GGA CAA TAG CTA ACG-3′). Following primer annealing, enzymatic filling in, and ligation, the mutated strand was selected by degrading the uracil-labeled template with uracil-DNA glycosylase and used to transform *E. coli* DW2-R. pB40 DNAs were isolated, and an *Eco*RV−*Hin*dIII DNA fragment (435 bp), encoding for the C-terminal amino acid sequence of Mel permease, was restricted and inserted into pK31 or pT40 plasmid in place of the analogous DNA fragment of the original *melB* sequence. Clones containing the six successive histidine codons were screened using the *Sac*I restriction site (GAGCTC) introduced by the mutagenic primer. The exchanged *Eco*RV−*Hin*dIII DNA fragments in pK31 or pT40 were verified by sequencing, and the modified plasmids were termed pK31HB and pT40HB, respectively. Finally, the first one-third of *melA* in pK31HB (or pK31) plasmid was deleted using *Sma*I and *Hpa*I restriction sites to give pK31ΔAHB (or pK31ΔA) plasmid.

In Vivo Specific Labeling of the Mel Permease. DW2-R cells, transformed with pGP1-2 and pT40HB plasmids, were depleted of internal methionine reserves in 250 mL of M9 medium, poisoned with rifampicin, and finally labeled as described previously (Pourcher et al., 1990b). Cells (OD_{600} = 0.5) were labeled with radioactive methionine and cysteine (250 μCi, express ^{35}S-labeling mix (DuPont-NEM)) and washed with M9 salt medium. The specificity of Mel permease [^{35}S]methionine labeling was controlled by autoradiographic analysis of cell proteins resolved by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Preparation of Inverted Membrane Vesicles (IMVs). Labeled or unlabeled cells were resuspended at 0−4 °C in 50 mM Tris-HCl (pH 8), 50 mM NaCl, and 5 mM β-mercaptoethanol. IMVs were prepared using a French pressure cell (American Instrument Comp., 18 000 psi) at 0−4 °C and were finally washed three times with the same buffered solution.

Purification of the Mel Permease. Freshly transformed DW2-R/pKE3ΔAHB cells were grown to OD_{600} = 2 in 200 L of M9 medium containing 2 g/L casamino acid (Difco Co.), 5 g/L glycerol, and 0.1 g/L ampicillin at 30 °C. IMVs were prepared using 50 g (wet weight) of paste cell as described earlier. All subsequent steps were performed at 0−4 °C. IMVs were washed once and resuspended at about 5 mg/mL in a medium containing 50 mM sodium phosphate

(pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, and 10 mM imidazole. Solubilization was carried out in the same medium supplemented with 1% LAPAO. After 10 min of incubation, the samples were centrifuged (280000*g*, 30 min). The supernatant was then incubated for 1 h with gentle shaking with Ni-NTA resin (25 mL of resin/g of membrane vesicle protein) preequilibrated with 50 mM sodium phosphate (pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, 10 mM imidazole, 0.2 mg/mL *E. coli* lipid, and 0.2% LAPAO. The resin with adsorbed material was centrifuged, washed once with the same medium, and used to load a FPLC column (Waters 650). Detergent exchange was carried out on the column by progressively replacing the medium containing 0.2% LAPAO by one of similar composition, but instead containing 0.2% DM, by means of a linear gradient (50 mL) and terminated by an additional wash with 0.2% DM medium (50 mL). NaCl was then progressively eliminated by means of a linear gradient (50 mL) using a buffered solution containing 50 mM sodium phosphate (pH 8), 10 mM Tris-HCl (pH 8), 20% glycerol, 5 mM β-mercaptoethanol, 10 mM imidazole, 0.2 mg/mL *E. coli* lipids, and 0.2% DM. After an additional 50 mL wash, the adsorbed proteins were eluted by adding 0.1 M imidazole to the buffer. The few contaminant proteins eluting together with the permease (see Results) were eliminated by applying the Ni-NTA pooled eluate to a Macro-Prep Q anion exchange support preequilibrated with the elution buffer used during Ni-NTA chromatography. The flow-through containing the purified permease was collected.

Reconstitution. All steps were performed at 0−4 °C. Reconstitution was carried out by a procedure involving detergent adsorption on polystyrene beads (Rigaud et al., 1988). The pooled fractions containing the purified permease (about 60 mL) were mixed with 1 vol of 0.2 M potassium phosphate solution (pH 7). *E. coli* lipids (180 mg) were added and the sample was incubated under gentle agitation. Then, 15−20 g of Bio-Beads SM-2 was added progressively (four additions at 10 min intervals) and the sample was left overnight in the cold. The proteoliposomes formed were washed twice in 0.1 M potassium phosphate solution (pH 7). When desired, sodium salts trapped within the vesicles were eliminated by repeated freeze−thaw−sonication−wash cycles in Na^+-free, 0.1 M potassium phosphate solution (pH 7).

Sugar Binding and Transport Assays. [^{3}H]NPG (0.8 Ci/mmol) binding to IMV or proteoliposomes was measured at 20 °C using a flow dialysis technique as previously described (Damiano-Forano et al., 1986). [^{3}H]NPG binding to solubilized permease was carried out at 10 °C. In all of the experiments, the lower and upper dialysis chambers contained media with similar salt compositions and, when needed, similar detergent concentrations. [^{3}H]Melibiose (40 mCi/mmol) transport was assayed on freshly transformed cells grown at 30 °C in LB medium to mid-log phase (OD_{600} = 1) and incubated at 20 °C. [^{3}H]Melibiose uptake by the reconstituted permease was measured in proteoliposomes (5 mg of protein/mL) previously loaded with Na^+-free, 0.1 M potassium phosphate solution (pH 7). Aliquots were diluted (1/250, v/v) in 0.1 M sodium (or lithium or choline) phosphate (pH 7) or 0.1 M choline phosphate (pH 6) medium devoid of potassium and containing valinomycin (0.2 μM) and [^{3}H]melibiose (0.4 or 0.8 mM, 8 mCi/mmol). Sugar uptake by cells or proteoliposomes was monitored by a rapid filtration technique using GF/F Whatman glass fiber filters.

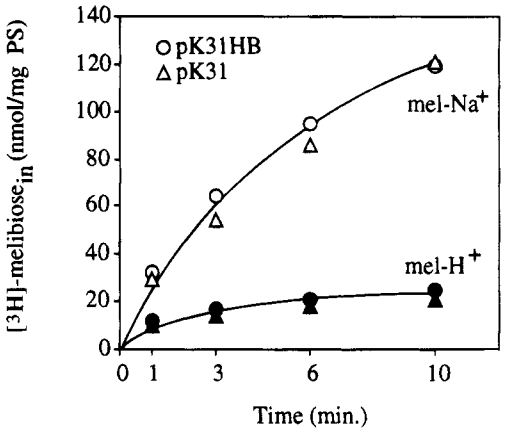

FIGURE 1: H^+- or Na^+-coupled [^{3}H]melibiose transport in *E. coli* cells expressing the wild-type or Mel-6His permease. *E. coli* DW2-R cells (*melA*$^+$, Δ*melB*, Δ*lacZY*, *RecA*$^-$) were transformed with plasmid pK31 (▲, △) or pK31HB (●, ○) encoding the wild-type or Mel-6 His permease, respectively. Cells grown in LB medium at 30 °C were washed, resuspended at 2 mg of cell protein/mL either in 0.1 M potassium phosphate (pH 6.6) medium containing less than 20 μM NaCl (●, ▲) or in the same medium supplemented with 10 mM sodium chloride (○, △). Transport reactions (50 μL) were initiated by the addition of [^{3}H]melibiose (40 mCi/mmol) to a final concentration of 0.2 mM and terminated by dilution and immediate filtration of the samples. Assays were carried out at 20 °C.

Analytical Methods. Cell and IMV proteins were assayed according to Lowry et al. (1951) using bovine serum albumin as a standard. Proteins solubilized in the presence of LAPAO were assayed by a modified Lowry protocol (Wessel & Flügge, 1984). Proteins were separated by SDS−PAGE (12% gels) (Laemmli, 1970). Silver staining was performed using a Bio-Rad silver stain kit. The N-terminal amino acid sequence of the purified permease was determined by D. Moinier (Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, France) using an Applied Biosystems Model 477A protein sequencer.

RESULTS

Properties and Overexpression of the Mel-6His Permease. As can be deduced from its accessibility to polyclonal antipeptide antibodies directed against the last 10 amino acids of Mel permease, the C-terminal extremity of the carrier is exposed to the aqueous medium (Botfield & Wilson, 1989). A nickel affinity site was therefore added to this extremity of the permease by extending the *melB* gene sequence by six consecutive triplets encoding histidine, using oligonucleotide-directed, site-specific mutagenesis. The gene encoding the tagged permease (Mel-6His permease) was cloned in pKK223-type vectors (pK31HB or pK31ΔAHB) that enable constitutive and moderate (or enhanced) permease expression and in the pT7-6 vector of the T7 RNA polymerase/promoter system, which can be used for selective *in vivo* [^{35}S]-methionine labeling of the Mel permease.

Extension of the C-terminal end of Mel permease with six consecutive histidines has no effect on its H^+- or Na^+-dependent melibiose transport properties. Figure 1 shows that *E. coli* DW2-R (Δ*melB*, Δ*lacZY*, *RecA*$^-$) cells, expressing to similar levels the wild-type and Mel-6His permeases,

Table 1: Purification and Reconstitution of Mel-6His Permease[a]

step	protein total (mg)	protein yield (%)	NPG binding activity specific (nmol/mg of protein)	NPG binding activity total (nmol)	NPG binding activity yield (%)
membrane	760	100	1.45	1100	100
LAPAO extract[b]	290	38	3	860	79
Ni-NTA[c]	37.5	5	13.4	503	46
Mono-Q[d]	31.4	4.2	14.2	446	41
proteoliposomes[e]	26.2	3.5	11.7	306	28

[a] Inverted membrane vesicles (IMVs) were prepared from 50 g (wet weight) of *E. coli* DW2-R/pK31ΔAHB cells by passage through a French press. At each stage of the solubilization/purification/reconstitution procedure, the sample proteins were estimated using a modified Lowry procedure (Lowry et al., 1951) and their respective sugar binding activities assessed from [^{3}H]NPG binding monitored by a flow dialysis technique (Damiano-Forano et al., 1986). [^{3}H]NPG binding to solubilized proteins or IMVs (and proteoliposomes) was measured at 10 or 20 °C, respectively. Complete deenergization of the IMVs or proteoliposomes was achieved by adding FCCP (10 μM) and monensin (1 μM). Eadie−Hofstee representation of [^{3}H]NPG binding activity plotted as a function of free NPG concentration (0.2−10 μM) was used to estimate graphically the maximal number of [^{3}H]NPG binding sites. [b] Protein extracted from IMV solubilized in the presence of 1% LAPAO. [c] DM-solubilized proteins eluted from the Ni-NTA column by raising the imidazole concentration to 0.1 M. [d] DM-solubilized proteins of the flow-through from the Mono-Q column. [e] Purified Mel-6 His permease reconstituted into proteoliposomes using detergent adsorption on polystyrene beads.

accumulate melibiose at the same rate and to the same extent when incubated in salt medium containing 0.8 mM [^{3}H]-melibiose. This is observed in both the presence and absence of NaCl (10 mM). Also, NaCl has similar stimulatory effects on the transport activities of the two permeases. As will be detailed later in this paper (Tables 1 and 2), Mel-6His permease binds sugars in a Na^+-dependent fashion; the apparent sugar binding affinity constants and apparent Na^+ and Li^+ activation constants are identical to those previously reported for the wild-type permease (Damiano-Forano et al., 1986; Pourcher et al., 1992).

In preliminary experiments, we observed that deletion of the first one-third of the *melA* gene located immediately upstream of the *melB* gene in pK31HB enhances expression of the tagged permease, probably by suppressing a polarity effect. SDS−PAGE analysis of membrane proteins prepared from cells transformed with the modified plasmid (pK31ΔAHB) shows that Mel-6His permease is easily identified as an intense diffuse band with an M_r of about 40 000 (see, for example, Figure 3, lane Mb). A scan of these gels indicates that the membrane permease content reaches values of up to 15% of the total membrane protein level. This broad band, which is absent when the permease is not expressed, specifically reacts with polyclonal antipeptide antibodies directed against the modified C-terminal extremity of the permease (not shown). Finally, the maximal number of sugar binding sites detected with the high-affinity ligand NPG is 1.5 nmol/mg of membrane protein (Table 1), i.e., 7 times higher than the value generally found in physiological conditions (Damiano-Forano et al., 1986). Membranes from cells expressing the tagged permease from pK31ΔAHB plasmid were therefore used for purification of the permease.

Detergent Solubilization. Efficient solubilization of the tagged permease in a functional state is one of the important requisites for large scale purification. To select an effective

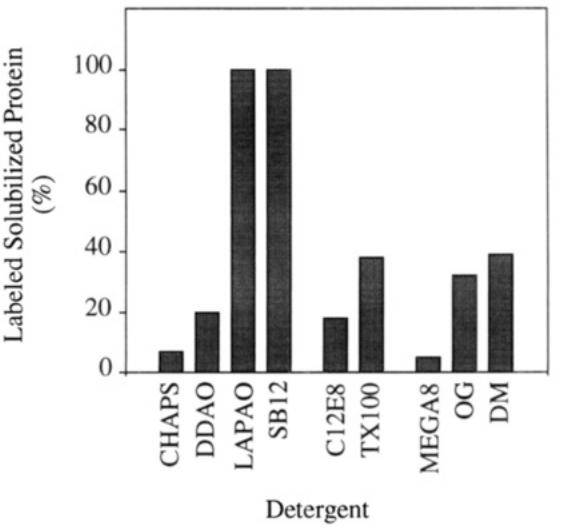

FIGURE 2: Solubilization of ^{35}S-labeled Mel-6His permease using different detergents. *E. coli* DW2-R cells were transformed with plasmids pGP1-2 and pT40HB. *In vivo* Mel-6His permease labeling and subsequent preparation of inverted membrane vesicles (IMVs) were carried out as described in Materials and Methods. Labeled IMVs (1 mg/mL) were equilibrated in 50 mM sodium phosphate (pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, and 5 mM β-mercaptoethanol at 0−4 °C. Aliquots (50 μL) were solubilized at 4 °C for 10 min in the presence of 1% detergent (1.5% for octyl glucoside), and the samples were centrifuged (340000*g*, 15 min). Radioactivity in the samples was measured before and after centrifugation, and the ratio was used to evaluate the extent of solubilized material.

detergent meeting this criterion, cells were labeled with [^{35}S]-methionine under conditions in which up to 70% of the label incorporated in the membrane fraction is specifically associated with Mel-6His permease. Inverted membrane vesicles (IMVs) were prepared from the labeled cells and incubated in the presence of different detergents at final concentrations exceeding their critical micellar concentrations (cmc) (generally 1%, but 1.5% for OG). Judging from the fraction of membrane radioactivity extracted after 10 min at 4 °C, it was concluded that the zwitterionic detergents SB 12 (cmc 0.12%) and LAPAO (cmc 0.02%; Brandolin et al., 1980) completely solubilize the permease (Figure 2). On the other hand, several nonionic detergents (OG, DM, or MEGA 8), detergents of the nonionic polyoxyethylene family ($C_{12}E_8$ and TX100), or finally zwitterionic ones such as CHAPS or DDAO have limited solubilization efficiency, even at reduced membrane protein concentrations (1 mg/mL, Figure 2). In samples containing 5 mg of protein/mL, LAPAO still solubilizes 80% of the permeases (Table 1).

In order to verify whether Mel-6His permease solubilized with SB 12 or LAPAO remains active, NPG binding to the permease in the solubilized state or after reconstitution of the crude material in liposomes was measured. Preliminary studies indicated that permeases solubilized in LAPAO retained sugar binding activity only when solubilization was performed in media containing a high concentration of NaCl (or LiCl). Half-maximal protection against permease inactivation is observed at 50 mM NaCl (or LiCl) and is complete in the presence of 0.6 M salts (see Tables 1 and 2). Neither an equimolar concentration of KCl nor the addition of magnesium or calcium salts at 1 mM efficiently replaces NaCl (or LiCl). These data suggest that the protective effect is not related to an increase in the ionic strength of the medium and instead is specific for Na^+ (or Li^+) ions. In

Table 2: Comparison of the [^{3}H]NPG Binding Constants of the Purified Mel-6His Permease in Proteoliposomes and of the Wild-Type Mel Permease in Membrane Vesicles[a]

	K_D (μM)	K_i^{Mel} (mM)	K_{Na^+} (mM)	K_{Li^+} (mM)
melibiose permease in RSO vesicles[b]	0.9	1	0.5	0.6
purified Mel-6His permease in proteoliposomes	0.8	1.2	0.3	1.2

[a] Purified Mel-6His permease solubilized in DM was mixed with total *E. coli* lipids and reconstituted in liposomes using detergent adsorption on polystyrene beads, as described in Materials and Methods. Proteoliposomes were washed twice in Na-free 0.1 M potassium phosphate (pH 7), submitted to repeated cycles of wash/freeze/thaw/sonication, and resuspended at 0.4 mg/mL in the same buffer in the presence of FCCP (10 μM) and monensin (1 μM). [^{3}H]NPG (0.8 Ci/mmol) binding to the proteoliposomes was monitored by a flow dialysis technique. The apparent [^{3}H]NPG binding constant was measured on proteoliposomes equilibrated in a salt medium containing 10 mM NaCl in the presence of increasing concentrations of NPG (0.2-10 μM). The [^{3}H]NPG apparent binding affinity (K_D) was estimated graphically from an Eadie−Hofstee plot of the binding data. The experiment was repeated in the presence of increasing concentrations of melibiose (0.1−20 mM) to estimate the apparent inhibition constant of melibiose on NPG binding (K_i^{Mel}). The apparent Na^+ or Li^+ activation constants of NPG binding (K_{Na^+}, K_{Li^+}) were determined by varying the concentration of sodium or lithium chloride in the buffer (0.2−10 mM) and calculated from the plot of the apparent K_D for NPG as a function of the reciprocal of the ion concentration. [b] [^{3}H]NPG binding activity measured on right side out membrane vesicles [data taken from Pourcher et al. (1992)].

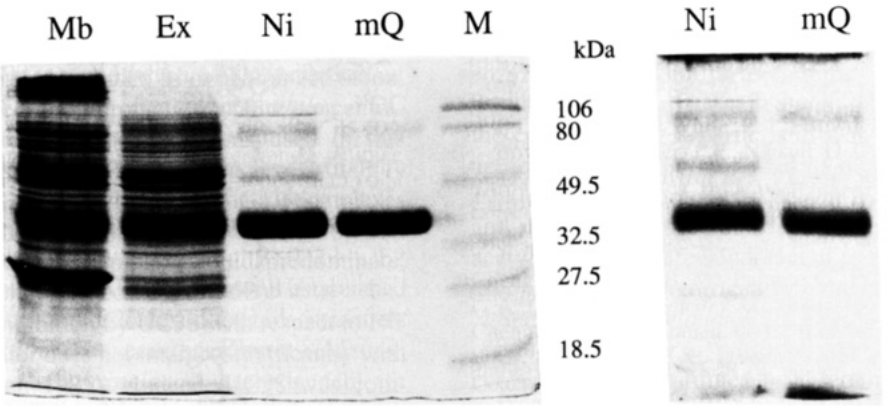

FIGURE 3: SDS−PAGE analysis of samples from different steps of the solubilization/purification procedure. At each step of the solubilization/purification procedure described in Table 1, aliquots were solubilized in 1% SDS and incubated for 20 min at 42 °C. Proteins were resolved using 12% acrylamide gels. (Left panel) Coomassie Blue R-250 staining: lane Mb, IMV proteins (70 μg); lane Ex, LAPAO-solubilized proteins (70 μg); lane Ni, DM-solubilized proteins eluted from the Ni-NTA column with 0.1 M imidazole (8 μg); lane mQ, DM-solubilized proteins (8 μg) of the flow-through from the Mono-Q columm; lane M, molecular size markers. (Right panel) Silver staining: lane Ni, DM-solubilized proteins eluted from the Ni-NTA column with 0.1 M imidazole (4 μg); lane mQ, DM-solubilized proteins (4 μg) of the flow-through from the Mono-Q column.

contrast, SB 12 completely and irreversibly inactivates the permease under all conditions tested. Incidentally, and as reported for other hydrophobic membrane proteins (Ambudkar & Maloney, 1984; Newman & Wilson, 1980), we noticed that the addition of glycerol and total *E. coli* lipids significantly protects the solubilized permease against inactivation. Finally, it was observed that Mel-6His permease remains active when solubilized with DM (Table 1) and OG (not shown) in both high and low ionic strength media (not shown). This observation will be used as a basis for the final purification of Mel-6His permease.

Large Scale Purification of the Mel-6His Permease. The protocol used for the purification of large amounts of Mel-6His permease comprises three major steps: solubilization of Mel-6His permease using the efficient detergent LAPAO, separation of the solubilized permease from most of the other solubilized membrane proteins by Ni chelate affinity chromatography, and finally separation of the permease from the remaining contaminants using ion exchange chromatography. Experiments were generally carried out starting from about 1 g of IMVs. Progress in the purification of the permease in an active state was judged by measuring, at each stage, the protein yield and sugar binding capacity of the pooled fractions (Table 1). Membrane proteins present in each of these fractions were identified by SDS−PAGE analysis (Figures 3 and 4).

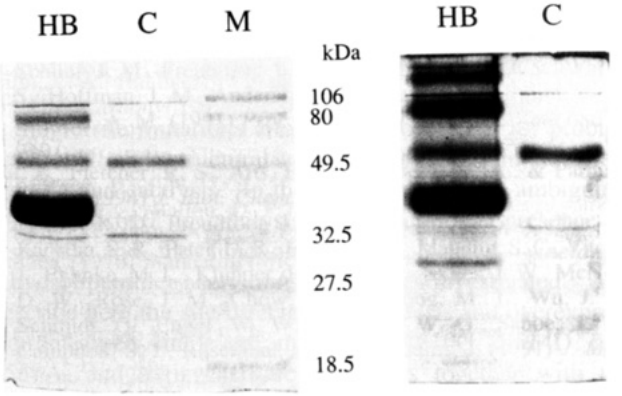

FIGURE 4: SDS−PAGE analysis of the LAPAO-solubilized proteins eluted from the Ni-NTA column with 0.1 M imidazole. IMVproteins solubilized in the presence of LAPAO (1%) were loaded on the Ni-NTA column, and the column was washed extensively to remove the unadsorbed material. Adsorbed proteins were eluted by raising the imidazole concentration to 0.1 M. Proteins contained in the pooled fractions were separated on 12% acrylamide gels. (Left panel) Coomassie Blue R-250 staining: proteins purified from IMV carrying either Mel-6His permease (lane HB, 15 μg) or untagged permease (lane C, 1.5 μg); lane M, molecular size markers. (Right panel) Silver staining: proteins purified from IMV carrying either Mel-6His permease (lane HB, 7.5 μg) or untagged permease (lane C, 0.75 μg).

In the particular experiment illustrated, inverted membrane vesicles (5 mg/mL) were solubilized in the presence of 1%

LAPAO in a medium containing 50 mM sodium phosphate (pH 8), 10 mM Tris-HCl (pH 8), 0.6 M NaCl, 20% glycerol, and 5 mM β-mercaptoethanol. Table 1 shows that 80% of the total NPG binding capacity of the starting material is retained in the extract, while only 38% of the total membrane proteins are solubilized, indicating a 2-fold purification of the permease. Consistently, and except for proteins with a high M_r and the β-lactamase (M_r around 27 500), little difference in the protein composition of the extract compared to that of initial IMV was noted (compare lanes Mb and Ex in Figure 3).

The Ni^+ chelate affinity chromatography protocol that gives the highest degree of Mel-6His permease purification and the highest yield of active material is based on the following considerations. The amount of tagged permease that binds to the Ni-NTA column is significantly enhanced when the LAPAO extract is equilibrated in batch conditions with the resin prior to column filling in. Secondly, the addition of a low concentration of imidazole (10 mM) during the wash out of untagged proteins further enhances the retention selectivity of the Ni-NTA resin [see also Janknecht et al. (1991)]. Thirdly, the material strongly adsorbed on the resin is eluted by 0.1 M imidazole rather than by lowering the medium pH (pH 5), as this latter procedure partially inactivates Mel-6His permease. Finally, lowering of the medium LAPAO concentration to 0.2%, i.e., about 10 times the cmc of LAPAO, and addition of total lipid extract from *E. coli* (0.2 mg/mL) and glycerol (20%, v/v) afford protection against inactivation of the permease.

Examination of the electrophoretic analysis of the fraction eluted with 0.1 M imidazole shows the presence of a major diffuse band at about 40 kDa and several additional polypeptide components (120, 100, 80, 55, 37, and 29 kDa), some of which are most visible on silver-stained gels (Figure 3, lane Ni). The 40, 80, and 120 kDa bands react with anti-Mel permease antibodies (not shown), indicating that they correspond to the tagged permease in the monomeric, dimeric, or polymeric state, respectively. The 29 kDa band is only detected on silver-stained gels. These contaminant proteins are membrane-associated since they can be co-reconstituted with Mel-6His permease in proteoliposomes (not shown). They bind directly on the resin, rather than indirectly by interaction with Mel-6His permease, and can indeed be purified from DW2-R/pK31ΔA membrane protein extracts carrying untagged Mel permease by Ni-NTA chromatography (Figure 4, lane C). Variation in neither the imidazole concentration nor the pH of the elution buffer efficiently separates these contaminant proteins from Mel-6His permease during Ni-NTA affinity chromatography.

Ion exchange chromatography was used as a last step to separate Mel-6His permease from its contaminants. For this, the permease-enriched fraction as such could not be used since it contained a high NaCl concentration essential to keeping the LAPAO-solubilized permease in an active state. To overcome this problem, we took advantage of the fact that DM-solubilized permeases remain fully active in both high and low ionic strength media (see above). Once the untagged proteins solubilized in LAPAO were washed out from the Ni-NTA column, LAPAO (0.2%) in the column buffer was exchanged stepwise for DM (0.2%), and the ionic strength was then progressively lowered by elimination of the NaCl salt in the buffer. In the experiments illustrated here, 13% of the DM-solubilized material (i.e., 5% of the initial IMV proteins) was eluted with 0.1 M imidazole (Table 1). Comparison of Figures 3 (lane Ni) and 4 (lane HB) indicates that the protein compositions of the DM-solubilized and LAPAO-solubilized proteins eluted with 0.1 M imidazole are similar. Scanning the SDS−PAGE gels shows that Mel-6His permease (monomer + dimer) accounts for 93% (Coomassie Blue-stained, lane Ni in Figure 3) or 75% (silver-stained, lane Ni in Figure 3) of the total protein of the pooled Ni-NTA fraction. The pooled fractions of DM-solubilized material, eluted from the Ni-NTA column with imidazole, were then subjected to ion exchange chromatography on a Mono-Q column. In these ionic conditions, Mel-6His permease is not adsorbed on the Mono-Q column and is eluted immediately after the void volume of the column, whereas the contaminants are retained and can be eluted in the presence of at least 300 mM NaCl (not shown). Figure 3 (Coomassie Blue-stained, lane mQ and silver-stained, lane mQ) shows that the permease fraction is free of contaminants and that the permease is essentially in the monomeric form (40 kDa, 96%), with a small amount of the dimeric form (80 kDa, 4%). As shown in Table 1, this fraction represents only 11% of the proteins initially solubilized in LAPAO and retains 52% of NPG binding activity.

N-Terminal Sequence of the Purified Mel-6His Permease. Analysis of the N-terminal extremity of the 40 kDa polypeptide shows, within the limits of the technique, that only one polypeptide is present in the sample, confirming the purity of the purified material. The determined N-terminal sequence is S-I-S-M-T-T-K (Table 3). This amino acid sequence has three extra residues (S-I-S) that precedes the N-terminal M-T-T-K sequence predicted by Yazyu et al. (1984) and also lacks an initial methionine residue. Such a sequence is best explained by first considering that the codon initiating the *melB* gene is not the ATG initially postulated by Yazyu et al., but rather a second ATG located 10−12 bp upstream from the postulated one (Yazyu et al., 1984). Accordingly, the predicted primary amino acid sequence of the permease becomes M-S-I-S-M-T-T-K. We then suggest that the observed N-terminal sequence is generated by subsequent excision of the initiator Met residue of the M-S-I-S-M-T-T-K sequence by cellular Met-specific amino peptidases (Miller, 1987). It is known that such posttranslational modification frequently occurs in proteins with Ala, Ser (as in the case of the permease), Gly, Pro, Thr, or Val as the second amino acid (Tsunasawa et al., 1985). In any instance, this result indicates that the Mel permease of *E. coli* consists of 472 amino acids and not 469. These results stress the need for revision of the proposed *melB* -encoding sequence. Finally, our results suggest that the N-terminal sequences of the Mel permease from *E. coli*, *Salmonella thyphimurium* (M-S-I-S-L-T-T-K), and *Klebsiella pneumoniae* (M-S-I-S-M-T-T-K) show a greater degree of homology than previously thought (Mizushima et al., 1992; Hama & Wilson, 1992).

Properties of the Purified Mel-6His Permease in Proteoliposomes. Mel-6His permease was reconstituted into liposomes by overnight incubation with total lipid extract from *E. coli* and hydrophobic Bio-Beads SM-2 to remove detergents (Rigaud et al., 1988). Detergent removal was accelerated by reducing the glycerol concentration to 10%. In the experiment described in Table 1, the protein to lipid ratio is about 1/500. Efficiency of the permease reconstitution and sugar binding activity was 83% and 70%, respectively,

Table 3: Amino-Terminal Sequence of the Mel-6His Permease

-30		-20		-12				1						
ACAGC	GACCC	GATAC	CCT	ATG	AGC	ATT	TCA	ATG	ACT	ACA	AAA	CTC	AGT.....	*melB DNA* [a]
								Met	Thr	Thr	Lys	Leu	Ser.....	*Predicted 1* [b]
				Met	Ser	Ile	Ser	Met	Thr	Thr	Lys	Leu	Ser.....	*Predicted 2* [c]
					Ser	Ile	Ser	Met	Thr	Thr				*Identified* [d]

[a] The DNA sequence shows the initiation ATG codon (position 1, underlined) of *melB* suggested by Yazyu and colleagues (Yazyu et al., 1984), preceded by an upstream sequence of 30 nucleotides and followed by nucleotides of the encoding sequence (Yazyu et al., 1984). [b] The corresponding predicted permease sequence (Yazyu et al., 1984). [c] Protein sequence corresponding to an alternative ATG codon (double underlined, row 1). [d] Identified protein sequence.

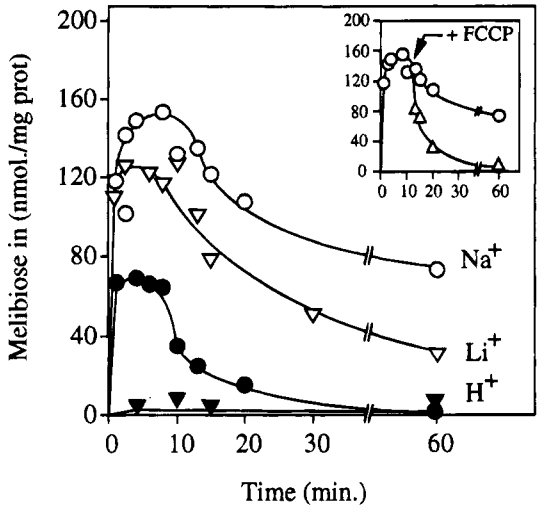

FIGURE 5: H^+-, Na^+-, and Li^+-coupled [^{3}H]melibiose uptake by purified Mel-6His permease in proteoliposomes driven by artificially imposed membrane potential and ion gradients. Proteoliposomes were washed and equilibrated in Na^+-free, 0.1 M potassium phosphate (pH 7) by a combination of wash/freeze/thaw/sonication cycles. Proteoliposomes were concentrated (5 mg of protein/mL) and incubated in the presence of valinomycin at a final concentration of 0.2 μM. Aliquots (50 μL) were diluted 250-fold into K^+-free medium containing valinomycin (0.2 μM), [^{3}H]melibiose (0.4 (○, ▽, ▼) or 0.8 mM (●), 8 mCi/mmol), and one of the following phosphate salts: 0.1 M choline phosphate (imposed $\Delta\mu_{H^+}$, ●), 0.1 M sodium phosphate (imposed $\Delta\mu_{Na^+}$, ○), 0.1 M lithium phosphate (imposed $\Delta\mu_{Li^+}$, ▽). Control experiments were carried out in a medium containing 0.1 M potassium phosphate (control, ▼). All dilution media were at pH 7 except for the choline phosphate (pH 6). Inset: Samples were diluted in 0.1 M sodium phosphate (pH 7). As indicated by the arrow, FCCP was added at a final concentration of 10 μM (△). Transport reactions at 20 °C were stopped by dilution of the samples and immediate filtration.

suggesting that no major permease inactivation occurred during the reconstitution procedure (Table 1).

The Na^+-dependent sugar binding and transport properties of the reconstituted Mel-6His permease were measured in proteoliposomes and compared with those of the untagged Mel permease in its natural environment (Table 2 and Figure 5). In the presence of a NaCl concentration (10 mM) that produces a maximal increase in permease affinity for the sugar substrates, reconstituted Mel-6His permeases exhibit an affinity for [^{3}H]NPG similar to that of untagged permeases (Pourcher et al., 1992). Moreover, competition experiments of melibiose on NPG binding to the carriers in inverted membrane vesicles and proteoliposomes demonstrate that the inhibition constant K_i^{Mel} is again similar for both, suggesting that the affinity of the permease for the physiological sugar is not altered during its purification. Finally, NPG binding studies carried out in the presence of Na^+ or Li^+concentrations ranging from 0.02 to 10 mM do not indicate significant differences in the apparent activation constants (K_{Na^+} or K_{Li^+}) in proteoliposomes and membrane vesicles. The 2-fold difference observed between the K_{Li^+} values of tagged or untagged permeases is of doubtful significance, as all values are within the variability of the ion activation constant measured on different membrane preparations harboring untagged permeases.

It should be briefly mentioned that the B_{max} for [^{3}H]NPG binding on washed, frozen, thawed, and sonicated proteoliposomes is slightly higher (16 nmol/mg of protein) than that measured immediately after reconstitution (12 nmol/mg of protein, Table 1). Provided that protein determination of the solubilized samples is fully reliable, this result suggests that the washing procedure removes some permeases that were trapped in the liposomes during reconstitution. In any case, using a molecular mass of about 53.4 kDa for the tagged permease, one can calculate that 83% of the permeases in the washed proteoliposomes is still active.

Cation-coupled symport activity of the reconstituted Mel-6His permease was analyzed by measuring [^{3}H]melibiose accumulation by proteoliposomes loaded with 0.1 M potassium phosphate (pH 7) in response to the generation of an electrochemical potential gradient favorable for the entry of coupling ions via the permease. The membrane potential ($\Delta\psi$, interior negative) and desired ionic gradients were imposed across the proteoliposome membrane by creating an outward K^+ diffusion gradient in the presence of valinomycin and changing the ionic composition and/or pH of the dilution buffer, respectively. Figure 5 shows that a significant amount of [^{3}H]melibiose is transiently accumulated when the K^+-loaded proteoliposomes are diluted in 0.1 M choline phosphate buffer (pH 6) (generation of $\Delta\mu_{H^+}$), 0.1 M sodium phosphate buffer (pH 7) (generation of $\Delta\mu_{Na^+}$)

or 0.1 M lithium phosphate buffer (pH 7) (generation of $\Delta\mu_{Li^+}$). The level of sugar taken up is, in all cases, higher than that measured under conditions in which [^{3}H]melibiose only equilibrates with the liposomal compartment (dilution in 0.1 M potassium phosphate buffer). Moreover, the amount of sugar accumulated depends on the coupling ion, varying in an order ($Na^+ > Li^+ > H^+$) that is characteristic of the sugar transport selectivity of Mel permease (Bassilana et al., 1985). Finally, dissipation of $\Delta\mu_{Na^+}$ ($\Delta\mu_{H^+}$ or $\Delta\mu_{Li^+}$, not shown) by the addition of the protonophore FCCP leads to an immediate decrease in the previously established sugar concentration gradient (Figure 5, inset).

DISCUSSION

The experiments presented in the current study describe a procedure for the purification of functional Mel permease from *E. coli* in large amounts. The data show that adjunction of an affinity tag to the membrane transporter and use of metal chelate affinity chromatography provide an efficient and simple means to separate the permease from most of the other membrane proteins. When this strategy is associated with the efficient solubilization of membrane vesicles and the use of ion exchange chromatography for final purification, we found that as much as 30 mg of purified, tagged melibiose permease was routinely recovered from 50 g (wet weight) of *E. coli* cells overexpressing the transporter (Table 1). This yield could certainly be increased by improving the solubilization efficiency and/or by optimizing the protein to resin ratio during the affinity chromatography step. Examination of the purified material on SDS−PAGE gels together with N-terminal sequencing indicates that the permease has been purified to homogeneity (>99%). Finally, calculation of the specific sugar binding activity of the purified permease suggests that 80% of it is functional. A more accurate estimate must await a more direct determination of the permease concentration than the one relative to BSA, for example, determination of the extinction coefficient of the pure melibiose permease.

The second important conclusion to be drawn from the results described here is that pure Mel-6His permease, reconstituted in liposomes, catalyzes H^+-, Na^+-, or Li^+-dependent sugar binding and active transport activities, with characteristics similar to those of the permease in its natural environment. These observations conclusively demonstrate that one and the same polypeptide catalyzes all three modes of symport activity. They also invalidate a previously considered hypothesis that the functional melibiose transporter comprises more than one polypeptide and in particular shares a Na^+-specific subunit with other Na^+-coupled symporters (Guffanti et al., 1981; Zilberstein et al., 1982). Thirdly, the absence of any detectable effect of addition of the His tag on melibiose permease properties suggests that there is no need, at present, to consider removal of the His tag.

Finally, purification of a recombinant melibiose permease in a functional form and large quantity opens large perspectives of an analysis of the structural properties of the transporter by physical techniques, including its 2D and 3D crystallization. Analysis of the influence of the cation and sugar substrates on the intrinsic tryptophan fluorescence properties of Mel-6His permease reconstituted in proteoliposomes is currently being investigated.

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REFERENCES

Ambudkar, S. V., & Maloney, P. C. (1984) *J. Biol. Chem. 261*, 10079−10086.
Bassilana, M., Damiano-Forano, E., & Leblanc, G. (1985) *Biochem. Biophys. Res. Commun. 129*, 626−631.
Botfield, M. C., & Wilson, T. H. (1988) *J. Biol. Chem. 263*, 12909−12915.
Botfield, M. C., & Wilson, T. H. (1989) *J. Biol. Chem. 264*, 11649−11652.
Botfield, M. C., Naguchi, K., Tsuchiya, T., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 1818−1822.
Brandolin, G., Doussière, J., Gulik, A., Gulik-Krzywicki, T., Lauquin, G. J.-M., & Vignais, P. V. (1980) *Biochim. Biophys. Acta 592*, 592−614.
Damiano-Forano, E., Bassilana, M., & Leblanc, G. (1986) *J. Biol. Chem. 261*, 6893−6899.
Guffanti, A. A., Cohn, D. E., Kaback, H. R., & Kruwlich, T. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1481−1485.
Hama, H., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 18371−18376.
Hama, H., & Wilson, T. H. (1993) *J. Biol. Chem. 268*, 10060−10065.
Hochuli, E., Döbeli, H., & Schacher, A (1987) *J. Chromatogr. 411*, 177−184.
Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R., & Stüber, D. (1988) *Bio/Technology 6*, 1321−1325.
Janknecht, R., de Martynoff, G., Lou, J., Hiskind, R. A., Nordheim, A., & Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8972−8976.
Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
Loddenkötter, B., Kammerer, B., Fischer, K., & Flügge, U. I. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2155−2159.
Lopilato, J., Tsuchiya, T., & Wilson, T. H. (1978) *J. Bacteriol. 134*, 147−156.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Miller, C. G. (1987) in *Escherichia coli and Salmonella typhimurium*, pp 680−691, American Society for Microbiology, Washington, DC.
Mizushima, K., Awakihara, S., Kuroda, M., Ishikawa, T., Tsuda, M., & Tsuchiya, T. (1992) *Mol. Gen. Genet. 234*, 74−80.
Newman, M. J., & Wilson, T. H. (1980) *J. Biol. Chem. 255*, 10583−10586.
Pos, M. K., Bott, M., & Dimroth, P. (1994) *FEBS Lett. 347*, 37−41.
Pourcher, T., Bassilana, M., Sarkar, H. R., Kaback, H. R., & Leblanc, G. (1990a) *Philos. Trans. R. Soc. London B 326*, 411−423.
Pourcher, T., Bassilana, M., Sarkar, H. K., Kaback, H. R., & Leblanc, G. (1990b) *Biochemistry 29*, 690−696.
Pourcher, T., Sarkar, H. K., Bassilana, M., Kaback, H. R., & Leblanc, G. (1990c) *Proc. Natl. Acad. Sci. U.S.A. 87*, 468−472.
Pourcher, T., Deckert, M., Bassilana, M., & Leblanc, G. (1991) *Biochem. Biophys. Res. Commun. 178*, 1176−1181.
Pourcher, T., Bassilana, M., Sarkar, H. K., Kaback, H. R., & Leblanc, G. (1992) *Biochemistry 31*, 5225−5231.
Pourcher, T., Zani, M. L., & Leblanc, G. (1993) *J. Biol. Chem. 268*, 3209−3215.
Rigaud, J. L, Pasternostre, M. T., & Bluzat, A. (1988) *Biochemistry 27*, 2677−2688.
Stüber, D., Matile, H., & Garotta, G. (1990) *Immunol. Methods* (Lefkovits, I., & Pernis, B., Eds.) *4*, 121−152.
Tsuchiya, T., & Wilson, T. H. (1978) *Membr. Biochem. 2*, 63−79.

Tsuchiya, T., Ottina, K., Moriyama, Y., Newman, M. J., & Wilson, T. H. (1982) *J. Biol. Chem. 257*, 5125−5128.

Tsunasawa, S., Stewart, J. W., & Sherman, F. (1985) *J. Biol. Chem. 260,* 5382−5391.

Waeber, U., Buhr, A., Schunk, T., & Erni, B. (1993) *FEBS Lett. 324*, 109−112.

Wessel, D., & Flügge, U. I. (1984) *Anal. Biochem. 138*, 141−143.

Wilson, D. M., & Wilson, T. H. (1987) *Biochim. Biophys. Acta 904*, 191−200.

Wilson, D. M., & Wilson, T. H. (1992) *J. Bacteriol. 174*, 3083−3085.

Wilson, D. M., & Wilson, T. H. (1994) *Biochim. Biophys. Acta 1190*, 225−230.

Wilson, D. M., Ottina, K., Newman, M. J., Tsuchiya, T., Ito, S., & Wilson, T. H. (1985) *Membr. Biochem. 5*, 269−290.

Yazyu, H., Shiota-Niiya, T., Shimamoto, T., Kanazawa, H., Futai, M., & Tsuchiya, T. (1984) *J. Biol. Chem. 259*, 4320−4326.

Zani, M. L., Pourcher, T., & Leblanc, G. (1993) *J. Biol. Chem. 268*, 3216−3221.

Zani, M. L., Pourcher, T., & Leblanc, G. (1994) *J. Biol. Chem. 269*, 24883−24889.

Zilberstein, D., Ophir, I. J., Padan, E., & Schuldiner, S. (1982) *J. Biol. Chem. 257*, 3692−3696.

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