Melibiose Permease of *Escherichia coli*: Mutation of Histidine-94 Alters Expression and Stability Rather than Catalytic Activity†

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ABSTRACT: Previous studies utilizing site-directed mutagenesis [Pourcher et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 468–472] indicate that out of seven histidinyl residues in the melibiose (*mel*) permease of *Escherichia coli*, only His94 is important. The role of His94 has now been investigated by replacing the residue with Asn, Gln, or Arg. Cells expressing *mel* permease with Asn94 or Gln94 retain 30% or 20% of wild-type activity, respectively, and surprisingly, immunological assays demonstrate that diminished transport activity is due to a proportional reduction in the amount of permease in the membrane. Moreover, kinetic analyses of transport and ligand binding studies with right-side-out membrane vesicles indicate that both substrate recognition and turnover (k_{cat}) are comparable in the mutant permeases and the wild-type. *Mel* permease with Arg in place of His94 also binds ligand and catalyzes sugar accumulation, but only when the cells are grown at 30 °C, and evidence is presented that Arg94 permease is inactivated at 37 °C. Finally, labeling studies demonstrate that expression and/or insertion of the permease, but not degradation, is strongly dependent on the amino acid present at position 94 and temperature. The findings indicate that an imidazole group at position 94 is required for proper insertion and stability of *mel* permease, but not for transport activity per se. Since replacement of the other six histidinyl residues in *mel* permease with Arg has little or no effect on transport activity, it is concluded that histidinyl residues do not play a direct role in the mechanism of this secondary transport protein.

α-Galactoside accumulation in *Escherichia coli* occurs via the melibiose (*mel*)[1] permease which catalyzes cation/substrate symport (i.e., cotransport; for reviews, see Wilson et al. (1982), Pourcher et al. (1990a), and Leblanc et al. (1990b)]. In contrast to other bacterial symporters, *mel* permease utilizes H^+, Na^+, or Li^+ as the coupling cation depending on the specific sugar transported (Tsuchiya & Wilson, 1978; Wilson & Wilson, 1987). Transport and binding studies in right-side-out (RSO) membrane vesicles are consistent with the idea that the coupling cation enhances the affinity of *mel* permease for the cotransported sugar and also reduces permease turnover by stabilizing the ternary complex between the permease, the cation, and the sugar (Cohn & Kaback, 1980; Bassilana et al., 1985, 1987; Damiano-Forano et al., 1986). Other experimental observations (Leblanc et al., 1988) suggest that the membrane potential (interior negative) reduces the cation-induced conformational stability of the ternary complexes and hence increases the rate of release of the cotransported species into the cytoplasm.

The *melB* gene has been cloned (Hanatani et al., 1984) and sequenced (Yazyu et al., 1984) and shown to encode a protein with 469 amino acid residues and a calculated molecular mass of 52 029 Da. The gene product has been identified as a cytoplasmic membrane protein with a molecular mass of 39 kDa by in vivo labeling studies (Pourcher et al., 1990b) and immunodotblot analysis with antibodies directed against the C-terminus (anti-MBct10; Botfield & Wilson, 1989; Leblanc et al., 1990b). Hydropathy profiling of the primary amino acid sequence (Pourcher et al., 1990c) and analysis of a series of *melB–phoA* fusions (Botfield, 1989; Botfield et al., 1992) suggest that the *mel* transport protein contains 12 hydrophobic membrane-spanning segments.

Recently, oligonucleotide-directed, site-specific mutagenesis was used to replace each of the seven histidinyl residues of *mel* permease at positions 94, 198, 213, 318, 357, 442, and 456 with Arg (Pourcher et al., 1990c). Replacement of His94 (putative helix III) leads to a drastic decrease in ligand binding and transport activity, while replacement of each of the other histidinyl residues has little or no effect. Thus, there is an interesting analogy between *mel* and *lac* permeases, since mutagenesis of His322 (putative helix X) in the latter also specifically impairs ligand binding and active lactose transport (Padan et al., 1985; Putner et al., 1989; King & Wilson, 1989, 1990; Brooker, 1990; Franco & Brooker, 1991). As pointed out, however, the analogy may be superficial, as H322R[2] *lac* permease exhibits an uncoupled phenotype, while H94R *mel* permease does not.

In order to investigate further the role of His94 in the mechanism of *mel* permease, we have now replaced the residue with Asn and Gln, in addition to Arg, and studied the ligand binding and transport properties of the mutants, as well as the

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[1] Abbreviations: *mel*, melibiose; RSO, right-side-out; TMG, methyl 1-thio-β-D-galactopyranoside, NPG, *p*-nitrophenyl α-D-galactopyranoside.

[2] Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in wild-type *mel* permease. The sequence is followed by a second letter denoting the amino acid replacement at this position.

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concentration of the proteins in the membrane. The results suggest that His94 is important for expression and/or insertion of *mel* permease into the membrane but that an imidazole moiety at this position is not required for cation/sugar symport.

Materials and Methods

Materials

[^{14}C]Methyl 1-thio-β-D-galactopyranoside ([^{14}C]TMG; 30–40 mCi/mmol) and [^{3}H]melibiose (2.7 Ci/mmol) were from the Commissariat â l'Energie Atomique (France). *p*-Nitro[2-^{3}H]phenyl α-D-galactopyranoside (NPG) was synthesized by Yu-Ying Liu under the Direction of Arnold Liebman (Isotope Synthesis Group at Hoffmann–La Roche, Inc.). All other materials were reagent grade and obtained from commercial sources.

Methods

Bacterial Strains and Plasmids. *E. coli* DW2-R, which is a *recA*$^-$ derivative of strain DW2 (*melA*$^+$, Δ*melB*, Δ*lacZY*), was transformed with given plasmids and used for transport studies. *E. coli* DW2 and RA11 strains were obtained from T. H. Wilson (Harvard Medical School). *E. coli* BMH71-18 mutL [*sup E, thi,* Δ(*lac-pro*), *mutL:Tn10/traD36, pro A*$^+$ *B*$^+$, *lac I*^{q}, *lac Z* Δ *M15*] and JM-101 [*sup E, thi,* Δ(*lac-pro*)/*traD36, pro A*$^+$ *B*$^+$, *lac I*^{q}, *lac Z* Δ *M15*] were used for site-directed mutagenesis (Pourcher et al., 1990c). Plasmid pK32 is recombinant pKK223-3 vector (Pharmacia) which contains *mel A*, *mel B*, and putative *mel C* genes (Pourcher et al., 1990c). In this plasmid, the *mel B* gene sequence is bordered by two *Eco*RI sites which enables selective excision and replacement of the entire *mel B* gene in the recombinant vector. Plasmids pT7-6 and pGP1-2, which comprise the T7 RNA polymerase expression system (Tabor & Richardson, 1985) were a gift from S. Tabor (Harvard Medical School).

Site-Directed Mutagenesis, Verification of Mutations by DNA Sequencing, and Complementation Analysis. Site-directed mutagenesis was performed as described (Sarkar et al., 1985) using single-stranded (ss) M13mp18 DNA containing the sense strand of the *mel B* gene (Pourcher et al., 1990b). The mutagenic primers used to replace His94 with Asn or Gln, respectively, were as follows: 5′-TTT-AGT-GCG-CAA*-CTG-TTT-GAA-G-3′ or 5′-C-TTT-AGT-GCG-A*AT-CTG-TTT-GAA-G-3′, where a single mismatch (asterisk) was introduced into the underlined codons. Subsequently, ss phage DNA containing the mutated *mel B* genes was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with an appropriate primer complementary to the region of *mel B* 120 bases upstream from codon 94 to verify the presence of the mutations. The *Eco*RI–*Eco*RI DNA fragments containing each mutated *mel B* gene were then restricted from the respective M13mp18 replicative form (RF) DNA and inserted into the *Eco*RI site of pK32 in place of the analogous DNA fragment containing wild-type *mel B*. The presence of undesired mutations in the 3′ two-thirds part of the mutated *mel B* genes was eliminated by ligating a *Bgl*II–*Kpn*I DNA fragment, which includes the 5′ third of the mutated *mel B* containing codon 94 and *mel A* (Pourcher et al., 1990c), to the large *Bgl*II–*Kpn*I from pK32 harboring the complementary portion of *mel B*. Finally, the 5′ third of the DNA sequence of the mutated *mel B* genes was verified by dideoxynucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978). Complementation analyses were carried out on MacConkey plates containing 10 mM melibiose.

Transport and Binding Assays. Transport of [^{14}C]TMG (5 mCi/mmol) or [^{3}H]melibiose (40 mCi/mmol) was monitored in freshly transformed cells or right-side-out (RSO) membrane vesicles by rapid filtration (Kaback, 1971). Binding of [^{3}H]NPG (800 mCi/mmol) was assayed in RSO membrane vesicles under nonenergized conditions by using flow dialysis (Damiano-Forano et al., 1986).

Permease Labeling and Immunoassays. In vivo [^{35}S]-methionine labeling experiments were carried out with the wild-type or mutated *mel B* genes expressed from the T7 RNA polymerase/promoter system (Pourcher et al., 1990b). Western blots were carried out on RSO membrane vesicles using a protocol similar to that described by Botfield and Wilson (1989). Aliquots of membrane vesicle suspensions containing about 50 μg of protein were solubilized in sodium dodecyl ($NaDodSO_4$), subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970) using 12% polyacrylamide gels, and electroblotted onto an Immobilon P membrane (Amincon). Immunoblotting was carried out with a rabbit antiserum (anti-MBct10; 1/1000 dilution) directed against the C-terminus of *mel* permease (Botfield & Wilson, 1989). Immune complexes were visualized by incubating the Immobilon membrane successively with biotinylated goat anti-rabbit immunoglobulin (1/400 dilution) and streptavidin/biotinylated peroxidase, followed by color development in the presence of 4-chloro-1-naphthol and H_2O_2 (Amersham). Molecular weight markers (Rainbow) were from Amersham.

Immunodotblots were performed as described by Loklema et al. (1988) with anti-MBct10 and [^{35}S]-protein A. Values were corrected for nonspecific adsorption (usually <5% of the total counts) by subtracting the signal obtained with DW2-R membrane vesicles which do not contain *mel* permease.

Protein Determinations. Whole cell, membrane, or cytoplasmic protein was assayed according to Lowry et al. (1951) with bovine serum albumin as standard.

Results

H94Q and H94N Mel Permeases

Transport. *E. coli* DW2-R/pK32, which expresses wild-type *mel* permease, grows at 37 °C as dark red colonies on MacConkey agar containing melibiose. In contrast, DW2-R/pH94Q or DW2-R/pH94N, which express *mel* permease with Gln or Asn in place of His94, respectively, give rise to pink colonies, suggesting that the cells have diminished transport activity. In order to extend the qualitative observations, transport was measured directly with TMG in cells grown at 37 °C (Figure 1). As shown previously (Pourcher et al., 1990c), in the presence of 10 mM Na^+ which produces maximal stimulation (Lopilato et al., 1978), DW2-R expressing wild-type permease transports TMG at a relatively high rate and achieves a steady-state level of accumulation in ca. 6 min, while cells transformed with the vector alone or pH94R exhibit little or no TMG transport. In contrast, DW2-R/pH94Q or DW2-R/pH94N transports the nonmetabolized analogue about 30% or 20%, respectively, as well as wild-type to levels that are significantly greater than that observed in cells transformed with the vector alone or with pH94R.

Kinetic studies of Na^+-dependent melibiose (Table I) or TMG transport (not shown) in membrane vesicles from DW2-R/H94N and DW2-R/H94Q demonstrate that diminished transport activity is due primarily to reduction in the maximum rate of transport. Thus, double-reciprocal plots of the initial rate of Na^+/melibiose transport in energized vesicles as a function of melibiose concentration show that V_{max} is decreased by 75% or 85%, respectively, with H94Q or H94N *mel* permease relative to wild-type permease while the apparent

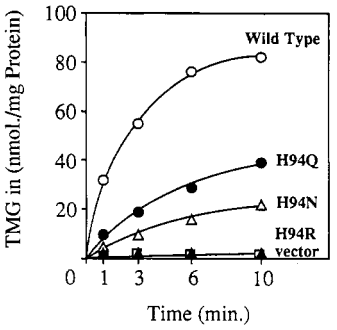

FIGURE 1: Na^+-dependent TMG transport in *E. coli* cells expressing *mel* permease with Asn, Gln, or Arg in place of His94. *E. coli* DW2-R cells (*melA*$^+$, Δ*melB*, Δ*lacZY*, *RecA*$^-$) were transformed with plasmids pH94Q (●), pH94N (△), or pH94R (▲), respectively. Cells transformed with pKK223-3 (□, no insert) or pK32 (○, wild-type) were used as control. Cells grown in LB medium at 37 °C were washed and resuspended (2 mg/mL) in 100 mM potassium phosphate (pH 6.6)/10 mM magnesium sulfate/10 mM sodium chloride. Transport reactions were carried out at 20 °C and initiated by adding [^{14}C]TMG (3 mCi/mmol) to a final concentration of 0.2 mM. Reactions were terminated by diluting the samples with 4 mL of resuspending medium followed by immediate filtration as described (Kaback, 1971).

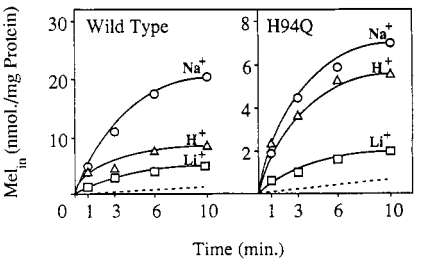

FIGURE 2: H^+, Na^+, or Li^+-coupled melibiose transport in wild-type and H94Q RSO membrane vesicles. RSO membrane vesicles carrying wild-type (left panel) or H94Q (right panel) permease were prepared from DW2-R/pK32 and DW2-R/pH94Q cells grown in LB medium at 37 °C, respectively, by an osmotic lysis procedure (Kaback, 1971) and resuspended in 100 mM potassium phosphate (pH 6.6)/10 mM magnesium sulfate containing less than 20 μM sodium chloride. H^+-coupled melibiose transport (△) was assayed in this medium at 20 °C under oxygen and in the presence of reduced phenazine methosulfate and 0.8 mM [^{3}H]melibiose (40 mCi/mmol). Na^+- or Li^+-coupled transport reactions were assayed in media supplemented with either 10 mM sodium (○) or 10 mM lithium (□) chloride and in the presence of 0.2 mM [^{3}H]melibiose. Dashed lines, Na^+- or Li^+-coupled melibiose uptake in deenergized membrane vesicles. Notice the difference in the ordinate scales.

Table I: Melibiose Transport and NPG Binding Constants of Wild-Type, H94Q, or H94N Permease Right-Side-Out Membrane Vesicles[a]

		WT	H94Q	H94N
melibiose transport	V_{max} [nmol (mg of protein)$^{-1}$ min^{-1}]	26	5.7	3.1
	K_T (mM)	0.08	0.09	0.15
α-NPG binding	B_{max} (nmol/mg of protein)	0.19	0.06	0.04
	K_D (μM)	0.9	0.8	1.1
	K_i^{mel} (mM)	1	0.9	nd
	K_{Na^+} (mM)	0.5	0.25	nd
	K_{Li^+} (mM)	0.6	1.1	nd
permease turnover	K_{cat} (s^{-1})	2.3	1.6	1.3

[a] RSO membrane vesicles containing wild-type permease or permease with Asn (H94N) or Gln (H94Q) in place of His94 were prepared by osmotic shock (Kaback, 1971) from DW2-R/pK32, DW2-R/pH94N, or DW2-R/pH94Q cells, respectively, and resuspended in 100 mM potassium phosphate (pH 6.6)/10 mM magnesium sulfate. Initial rates of melibiose transport (10 s) were measured by rapid filtration (Kaback, 1971) using membrane vesicles (100 μg of protein) incubated in the presence of 10 mM sodium chloride reduced phenazine methosulfate and [^{3}H]melibiose (40 mCi/mmol) at final concentrations varying from 0.05 to 2.4 mM. V_{max} and apparent K_t were calculated graphically from Eaddie–Hofstee representations of the data. [^{3}H]NPG (800 mCi/mmol) binding to membrane vesicles (15–40 mg of protein/mL) incubated in the presence of 10 mM sodium chloride, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 μM), and monensin (0.75 μM) was assayed by flow dialysis (Damiano-Forano et al., 1986). B_{max} and apparent K_D were calculated graphically from Scatchard plots of the data. Activation constants for NPG binding by Na^+ (K_{Na^+}) or Li^+(K_{Li^+}) were determined from plots of the apparent K_D for NPG as a function of the reciprocal concentration of sodium (or lithium) chloride in the range 0.2–10 mM. The apparent inhibitory constant for NPG binding by melibiose (K_i^{mel}) was estimated from the variation in the K_D for NPG as a function of melibiose concentration in the range 0.1–20 mM. Permease turnover number (k_{cat}) was calculated as V_{max}/B_{max}. nd, not determined; WT, wild-type.

K_t is not significantly different from wild-type in either case (ca. 0.1 mM).

NPG Binding with H94N and H94Q Membrane Vesicles. NPG is a high-affinity ligand for *mel* permease that binds in a Na^+-dependent fashion (Cohn & Kaback, 1980; Damiano-Forano et al., 1986). Scatchard analysis of [^{3}H]NPG binding in H94Q and H94N membrane vesicles in the presence of 10 mM sodium chloride shows that the maximal number of binding sites (B_{max}) is 32% or 26%, respectively, of that detected in wild-type membrane vesicles (Table I). In contrast, the K_D values for NPG binding in wild-type, H94Q, and H94N membrane vesicles are similar (ca. 1.0 μM).

Additional studies indicate that other NPG binding properties of H94Q or H94N permease are also comparable to those of wild-type *mel* permease (Table I). Progressive displacement of NPG bound to H94Q membrane vesicles by increasing melibiose concentration demonstrates that the K_i^{mel} is similar to that observed in wild-type vesicles (ca. 1.0 mM). In addition, NPG binding studies carried out in the presence of Na^+ or Li^+ concentrations ranging from 0.02 to 10 mM yield comparable apparent activation constants (K_{Na^+} or K_{Li^+}) in H94Q and wild-type membrane vesicles. Thus, replacement of His94 with Asn (or Gln) has little or no effect on substrate recognition or activation of binding by Na^+ or Li^+. Finally, H94Q permease retains the ability to catalyze energy-driven transport of melibiose with H^+, Na^+, or Li^+ as coupling ions, although the activities are significantly diminished relative to wild-type permease (Figure 2). Although data are not shown, it should be emphasized that H94N permease also catalyzes H^+- or Na^+-coupled melibiose accumulation and that a low level of Li^+-coupled TMG transport is also observed. The reduction in the number of NPG binding sites (i.e., of functional permeases molecules) in H94N and H94Q membrane vesicles is in close proportion to the diminished V_{max} values observed transport. As a result, the calculated turnover number (k_{cat}) for H94N or H94Q permease is comparable to that of wild-type permease (Table I). Taken together, therefore, the data suggest that *mel* permease with Asn or Gln in place of His94 catalyzes cation-coupled melibiose accumulation in a manner comparable to wild-type permease and that the diminished activity observed may be due to a decrease in the amount of permease in the membrane rather than a defect in intrinsic activity.

Immunological Assays. Western blotting with anti-C-terminal antibody (Botfield & Wilson, 1989) and [^{35}S]-protein A was used to estimate the permease content of H94N and H94Q membranes prepared from cells grown at 37 °C (Figure 3). As shown previously (Pourcher et al., 1990b), wild-type membranes exhibit a selective and intense band at 39 kDa which has been identified as the *mel B* gene product (slot 1). In contrast, the intensity of the 39-kDa band with H94Q (slot 2) or H94N (slot 3) membranes is significantly and progressively decreased. Qualitatively, therefore, it appears that

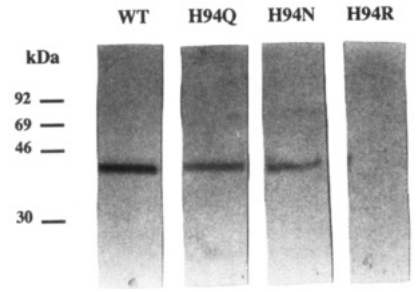

FIGURE 3: Western immunoblot of wild-type, H94Q, H94N, or H94R cytoplasmic membrane vesicles. *E. coli* DW2-R cells, transformed with plasmids pH94Q, pH94N, pH94R, or pK32, respectively, were grown in LB medium at 37 °C and membrane vesicles prepared as described in Figure 2. An aliquot of each membrane vesicle preparation (50 μg of protein) was solubilized in $NaDodSO_4$, subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, and electroblotted on an Immobilon P membrane. The Immobilon membrane was successively incubated with anti-MBct10 serum (1/1000 dilution), streptavidin/biotinylated anti-rabbit immunoglobin, and a biotinylated peroxidase system, and color development was followed in the presence of chloro-1-naphthol and H_2O_2.

Table II: Immunoblot Analyses of Wild-Type, H94Q, H94N, or H94R Membranes Vesicles[a]

permease content	WT	H94Q	H94N	H94R
% of wild-type	100	45	24	0
nmol/mg of protein[b]	0.21	0.09	0.5	0
immunoassay/NPG binding[c]	1.1	1.5	1.25	

[a] Membrane vesicles were prepared from cells grown at 37 °C as described in Figure 2. Membrane vesicle aliquots (<5 μg of protein) were spotted on nitrocellulose filters and successively solubilized in alkaline $NaDodSO_4$ buffer, incubated overnight with rabbit serum containing antibody MBct10 (1/1000 dilution), and finally incubated with [^{35}S]-protein A (34 Ci/mmol, 5 pmol/filter). Results were expressed as the percent of signal recorded in wild-type membrane vesicles. [b] Counts retained on the washed filters were converted into the amount of membrane permease (nmol/mg of protein) using a linear relationship existing between the [^{35}S]-protein A signal and the amount of permease molecules in wild-type membrane vesicles. [c] Immunoassay/NPG binding: ratio between the amount of permease molecules calculated from the immunoassay and the maximal number of NPG binding sites determined on the same membranes by flow dialysis (data from Table I).

mel permease with Asn or Gln in place of His94 is present to a diminished extent relative to wild-type.

The specific permease content of wild-type, H94Q, or H94N membranes was determined more quantitatively by using immunodotblot analysis (Loklema et al., 1988) with anti-MBct10 and [^{35}S]-protein A (Table II). In this assay, low concentrations of membrane vesicles were used in order to ensure linearity between the protein A signal and the amount of permease molecules present in the samples. Table II shows that the amount of permease in H94Q or H94N membranes is 45, 24, and less than 1%, respectively, of that measured in wild-type membranes. This table finally shows that, for each membrane vesicle preparation, there is good agreement between the permease content calculated from the immunodotblot assay and those estimated from the values of the B_{max} for NPG binding determined independently by flow dialysis (Table II). This excellent correlation indicates that the decreased rate of transport in cells expressing the modified *mel* permease is due to a reduction in the total amount of permease molecules in the membrane rather than inactivation of a fraction of the molecules.

H94R Mel Permease

E. coli DW2-R transformed with pH94R does not catalyze Na^+-dependent TMG accumulation when grown at 37 °C (see Figure 1), and NPG binding is not observed in membrane vesicles (Pourcher et al., 1990c). In addition, [^{35}S]methionine labeling experiments carried out with the overproducing T7 RNA polymerase/promoter system suggest that wild-type or H94R permease is present in the membrane in comparable amounts. Therefore, it was inferred that the lack of transport activity in DW2-R/pH94R is due to a defect in the transport mechanism rather than a reduction in the amount of *mel* permease in the membrane. In view of the results obtained with H94Q and H94N *mel* permeases, the findings were reassessed.

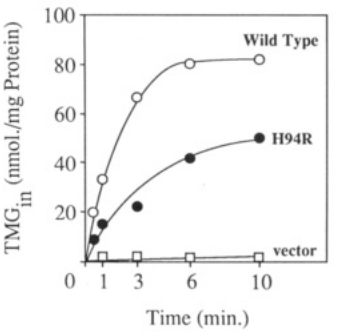

FIGURE 4: TMG transport in *E. coli* cells expressing H94R permease. *E. coli* DW2-R cells were cotransformed with plasmid pGP12 and pT7-6 recombinant plasmids (Pourcher et al., 1990c) encoding for wild-type permease (○) or permease with Arg in place of His94 (●), or plasmid devoid of insert (□), respectively. Cells were grown at 30 °C in Luria broth, washed and assayed for [^{14}C]TMG transport at 20 °C as described in Figure 1.

Table III: NPG Binding Properties of H94R RSO Membrane Vesicles Prepared from Cells Grown at 30 °C[a]

NPG binding constants	WT	H94R
K_D (μM)	0.4	0.3
K_i^{mel} (mM)	0.5	0.3
K_{Na^+} (mM)	0.5	0.4

[a] *E. coli* DW2-R cells cotransformed with plasmid pGP1-2 and pT7-6 recombinant plasmids encoding for wild-type permease (WT) or H94R *mel* permease were grown at 30 °C in Luria broth, washed, and used to prepare RSO membrane vesicles by osmotic lysis (Kaback, 1971). Measurement of [^{3}H]NPG binding to RSO membrane vesicles and calculation of the apparent K_D for NPG, K_{Na^+} and K_i^{mel} were as described in Table I.

TMG Transport and NPG Binding. Although transport is not observed, the *mel B* gene encoding the H94R mutation is clearly expressed from the T7 promoter (Pourcher et al., 1990c). Experimental conditions were therefore sought under which DW2-R/pT7-5(H94R)-pGP12 might exhibit transport activity, and it was found that TMG accumulation in the mutant, but not the wild-type, is drastically dependent on growth temperature. While TMG transport is not observed in DW2/pT7-5(H94R)-pGP1-2 grown at 37 °C, the same cells grown at 30 °C transport TMG at over 50% the wild-type rate and accumulate up to 50 nmol of TMG/mg of cell protein of sugar in 10 min (Figure 4).

Cells grown at 30 °C were then used to characterize the transport and binding characteristics of H94R permease more precisely (Table III). Kinetic analyses of TMG transport in the presence of 10 mM Na^+ demonstrate that the K_t for transport with wild-type or H94R permease is identical (ca. 0.25 mM) while the V_{max} is reduced by only 30%. Scatchard analyses of NPG binding in wild-type or H94R membrane vesicles in the presence of 10 mM Na^+ yield similar apparent K_Ds (0.3 and 0.4 μM, respectively). Finally, the K_{Na^+} and the apparent K_i for melibiose (K_i^{mel}) with respect to NPG binding are identical in membrane vesicles containing wild-type or H94R *mel* permease.

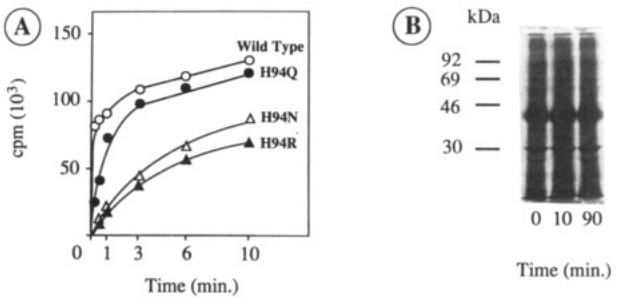

FIGURE 5: Time course of [^{35}S]methionine incorporation into DW2-R cells expressing *mel* permease with His, Asn, Gln, or Arg at position 94 (A) and stability of H94R permease labeling during pulse–chase (B). *E. coli* DW2-R cells cotransformed with plasmid pGP1-2 and pT7-6 recombinant plasmids encoding wild-type (○), H94Q (●), H94N (△), or H94R (▲) *mel* permease were grown to an OD_{600} of 0.5 at 30 °C. Washed cell suspensions (1 mg of cell protein/mL) were firt incubated at 42 °C to allow expression of T7 RNA polymerase from pGP1-2 plasmid for 20 min and then in the presence of rifampicin (200 μg/mL) at the same temperature for 15 min. (A) Labeling of *mel* permease was initiated at 30 °C by adding 20 μCi of [^{35}S]methionine (1000 Ci/mmol) (Pourcher et al., 1990c). At a given time, incorporation was terminated by precipitating cell aliquots (200 μg) with cold 10% TCA, followed by filtration on Whatman glass filters and measurement of the radioactivity retained on the filters. (B) Pulse–chase experiments were carried out at 30 °C. DW2-R/pH94R cells were incubated for 5 min with [^{35}S]methionine, and incorportion was terminated by adding unlabeled methionine (10 mM). Cell samples (1 mg) were removed at zero time, 10 or 90 min after addition of cold methionine, extensively washed, and lysed for purification of cytoplasmic membranes vesicles. Membranes were solubilized in $NaDodSO_4$ and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography.

A similar temperature dependence is observed in DW2-R cells transformed with the less efficient pH94R expression vector. TMG transport assays at 20 °C demonstrate that DW2-R/pH94R cells grown at 30 °C accumulate up to 18 nmol of TMG/mg of cell protein in 10 min while cells grown at 37 °C take up only 2 nmol/mg of cell protein (see Figure 1). Importantly, immunoblots with anti-C-terminal antibody show that membranes from DW2-R/pH94R grown at 30 °C contain about 0.05 nmol of H94R permease/mg of membrane protein while no permease is detected by the same technique (Table II) or by Western blot (Figure 3, slot 4) in membranes from cells grown at 37 °C.

Labeling and Pulse–Chase Experiments. Replacement of His94 by either Asn, Gln, or Arg leads to systematic reduction in the amount of *mel* permease present in the membrane (Asn > Gln ≫ Arg; Figure 3 and Table II). In order to determine whether the decreased expression results from reduced rates of synthesis and/or insertion, enhanced rate of proteolysis after the permease is inserted into the membrane, or both, time courses of [^{35}S]methionine incorporation into wild-type or mutant permeases expressed from the T7 expression system (Pourcher et al., 1990c) were studied, and the stability of the proteins was analyzed by chase experiments with unlabeled methionine.

Cells were first incubated at 42 °C for 20 min to allow expression of the T7 RNA polymerase from plasmid pGP1-2 and facilitate penetration of rifampicin and then cooled to 30 °C. [^{35}S]Methionine incorporation into wild-type, H94R, H94N, or H94Q cells was then monitored by precipitating cell aliquots with trichloroacetic acid at given times after addition of the labeled amino acid. Pourcher et al. (1990b) have shown previously that 80% of the label associated with the precipitated material is incorporated into the *mel B* gene product. Figure 5A shows that cells expressing H94Q, H94N, or H94R permease incorporate [^{35}S]methionine at diminished rates relative to cells expressing wild-type permease. The rate of

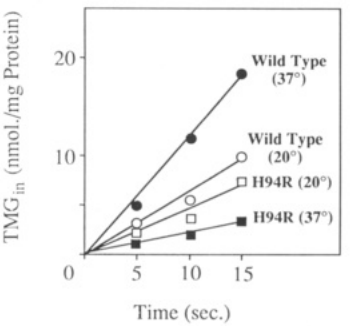

FIGURE 6: Effect of temperature on Na^+-dependent TMG transport. *E. coli* DW2-R cells were cotransformed with plasmid pGP12 and pT7-6 encoding either wild-type permease (○, ●) or H94R permease (□, ■). Cells were grown at 30 °C in LB medium, washed, incubated at 37 or 20 °C for 5 min, and assayed for TMG transport at the same temperature (closed symbols, 37 °C; open symbols, 20 °C).

permease labeling decreases in the order wild-type > H94Q > H94N > H94R, i.e., an order comparable to that of expression of the mutated permeases in DW2-R cells transformed with the corresponding pKK223-3 recombinant plasmids (Table II and Figure 3).

The susceptibility of inserted wild-type and mutant permeases to proteolysis after insertion was analyzed by pulse–chase experiments on cells previously labeled for 5 min with [^{35}S]methionine at 30 °C. Unlabeled methionine was then added, and aliquots were withdrawn at 10 or 90 min. Membranes were isolated, solubilized in $NaDodSO_4$, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography. As shown in Figure 5B, the intensity of labeling of the 39-kDa band corresponding to *mel* permease does not diminish for at least 90 min after addition of unlabeled methionine. Although data are not shown, similar results were obtained with the wild-type, H94N, or H94Q *mel* permease. Moreover, raising the temperature to 37 °C during the chase experiment had no effect on the intensity of the labeled 39-kDa band. The results demonstrate that once the mutated permeases are inserted into the membrane, they are as stable as the wild-type.

Effect of Temperature on the Catalytic Activity of H94R Permease. In addition to reducing the level of H94R permease expression, elevated temperature has an effect on the catalytic activity of H94R permease. This is shown in Figure 6 where TMG transport in DW2-R/pT7-5(H94R)-pGP1-2 cells grown at 30 °C was assayed at either 20 or 37 °C. The initial rate of transport in cells with H94R permease decreases by 50% at 37 °C relative to 20 °C, while transport in cells with wild-type permease increases by about 2-fold. Although data are not shown, inactivation of H94R permease at 37 °C is time-dependent, irreversible, and unaffected by the presence of chloramphenicol. Immunodotblot analyses indicate that the amount of H94R permease detected in the membrane is not significantly reduced by raising the temperature. Thus, in addition to altering the insertion of H94R *mel* permease, incubation at 37 °C reduces the functional stability of the protein.

Discussion

The experiments reported in the present study provide strong evidence that *mel* permease with Gln, Asn, or Arg in place of His94 catalyzes cation-coupled melibiose symport in a manner identical to wild-type permease. On the other hand, the variability in the concentration of the mutated permeases in the membrane and in their activities as a function of temperature suggests that a His residue at position 94 is important for expression and/or insertion of the permease in the mem-

brane as well as for functional stability of the transport protein.

Significant TMG accumulation is observed with H94Q, H94N, or H94R permeases in cells grown at relatively low temperature, thereby demonstrating that *mel* permease with Asn, Gln, or Arg in place His94 retains the capacity to promote active sugar transport. Moreover, the excellent correlation between the reduction in transport activity, the B_{max} for NPG, and the permease concentration per se in RSO vesicles (H94N and H94Q) or cells (H94R) and the similarity of turnover numbers (Table I) provide a strong indication that the diminished rate of transport in cells with the mutant permeases is due specifically to reduction in the concentration of modified permease in the membrane rather than a defective symport mechanism. Finally, the constants for cosubstrate interaction with the modified *mel* permeases (i.e., K_d, K_{Na^+} or K_{Li^+} and K_i^{mel} for NPG binding) are all comparable to those of wild-type permease. Taken together, therefore, the data demonstrate that neither cosubstrate recognition nor the translocation mechanism of *mel* permease is modified by replacement of His94 with Asn, Gln, or Arg. Since replacement of histidinyl residues at positions 198, 213, 318, 357, 442, and 456 with Arg has also been shown to induce little or no change in *mel* permease activity (Pourcher et al., 1990c; Leblanc et al., 1990), it is concluded that none of the seven histidinyl residues are critical for transport activity by *mel* permease.

These findings contradict a previous conclusion (Pourcher et al., 1990c) that His94 is essential for *mel* permease activity which was based on replacement of His94 with Arg. In the earlier work, it was shown that DW2-R cells transformed with a low-expression plasmid encoding H94R permease and grown at 37 °C are devoid of transport and sugar binding activities. The immunodotblot analyses presented here provide a straightforward explanation for the transport defect, as H94R membrane vesicles prepared from these cells virtually exhibit no permease (Table II and Figure 3). It is noteworthy that transport and binding activities of membrane vesicles containing H94Q or H94N also decrease in a manner commensurate with the specific permease content of the membranes. Strikingly, the severe defect in expression of H94R permease is very significantly ameliored by reducing the growth temperature of the cells. As this effect is not observed with wild-type permease, it is apparent that expression of *mel* permease, its insertion into the membrane, or both become temperature-sensitive when His94 is mutated and the effect is most pronounced with H94R.

Additional support for the erroneous conclusion that His94 is essential for *mel* permease activity came also from labeling experiments demonstrating that H94R or wild-type *mel* permease is synthesized from the T7 promoter to about the same extent and from the observation that cells grown at 37 °C are defective in transport activity (Pourcher et al., 1990c). These results are confirmed in Figure 5 which shows that H94R permease expression in *E. coli* DW2-R/pT7H94R approximates 50% of wild-type permease and by the observation that, under the condition of labeling, cells have negligible transport activity. H94R permeases synthesized at higher temperature from the overproducing T7 RNA polymerase system described by Tabor and Richardson (1985) are clearly inactive. It should be recalled, however, that enhanced permease synthesis from the T7 promoter requires incubation of the host cells at elevated temperature in order to derepress the structural gene for T7 polymerase. Two experiments strongly suggest that the H94R transport defect might result from inactivation of the mutant permease by heat: (i) *E. coli* DW2-R/pT7H94R grown at 30 °C, rather than 37 °C, exhibits highly significant transport activity (Figures 4 and 6); (ii) in contrast to wild-type permease, H94R permease in DW2-R/pT7H94R cells grown at 30 °C is inactivated when the cells are incubated at 37 °C for a short time (Figure 6). The heat lability of H94R permease is reminiscent of the properties of TMG permease II described by Prestidge and Pardee (1965) in several *E. coli* K12 strains.[3] Thus, although significant expression of H94R permease can be achieved in cells grown at 37 °C by using the overexpression T7 RNA polymerase system, transport activity is impaired as a consequence of functional inactivation of the mutant permease at this high temperature. The observed functional unstability of H94R *mel* permease further resolves the apparent contradiction between the conclusions of Pourcher et al. (1990c) regarding H94R *mel* permease and those presented here.

The dual effect of temperature on the functional stability and expression of H94R *mel* is remarkable. If the proposed secondary structure model of the transport protein (Pourcher et al., 1990c) is correct, His94 is located in a putative membrane-spanning segment of the NH_2-terminal domain of *mel* permease (helix III). Introduction of a bulky and positively charged residue into a hydrophobic segment of the protein might decrease the conformational stability of *mel* permease, particularly at higher temperature. Since recent evidence (Pourcher et al., 1991) suggests that the NH_2-terminal domain of *mel* permease is important for recognition of the coupling ion, the defect in conformational stability might even be restricted to this domain. The second consequence of His94→R substitution is that expression and/or insertion of *mel* permease becomes temperature-sensitive. Pulse–chase experiments on labeled H94R permease suggest that proteolysis of the permease in the membrane does not account for the reduced level of permease detected in the membrane as inserted H94R permease is, in a manner similar to wild-type permease, stable at temperatures as high as 37 °C (Figure 5B). Therefore, elevated temperature must adversely affect synthesis and/or insertion of H94R permease. In any case, the data taken as a whole demonstrate that none of the histidinyl residues in *mel* permease play an important role in the transport mechanism.

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[3] In contrast, *mel* permease referred to as wild-type in this study is from *E. coli* CS520 and is fairly stable at temperatures up to 37 °C (Hanatani et al., 1984).

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