Carboxyl-terminal Truncations of the Melibiose Carrier of Escherichia coli*

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The melibiose carrier of Escherichia coli is a cytoplasmic membrane protein which mediates the cotransport of α- and β-galactosides with monovalent cations (see Ref. 1 for a review). Although most cotransport systems of bacteria utilize H+ gradients (2), the melibiose carrier is unusual in its ability to use H+, Na+, or even Li+ as the coupled cations (3-7). From an evolutionary perspective, this carrier may thus represent a descendant of the class of transport proteins that bridged the gap between the "H" economy of primitive cells and the "Na" economy of animal cells (8,9). Insights into the structure/function relationships of the melibiose carrier may help to elucidate motifs that are essential to the molecular mechanism of transmembrane helix 11 (truncations of 41 or more residues), the carrier was no longer found in the membrane following proteolytic scavening. A new plasmid that expresses the temperature-resistant isoform of the melibiose carrier within the membrane following insertion, or protection of the inserted carrier from proteolytic scavenging. A new plasmid that expresses the temperature-resistant isoform of the melibiose carrier under inducible control of a tac promoter, designated(pkKMB), is also described.

The melibiose carrier of Escherichia coli is predicted to possess a short NH2 terminus, 11 transmembrane segments joined by short hydrophilic regions, and a 40-residue hydrophilic carboxyl terminus of unknown function. This paper describes truncations of the carboxyl-terminal region using site-specific mutagenesis to introduce stop codons. Measurement of sugar transport and cation-coupling characteristics indicate that the carboxyl tail plays no direct role in substrate recognition or energy transduction. Thirty-six amino acids could be removed from the hydrophilic carboxyl domain without the loss of sugar specificity, facilitated diffusion, uphill transport, H+-coupling or Na+-coupling characteristics. These results are consistent with the hypothesis that the sugar/cation binding site is formed by the interaction of the transmembrane helices 3, 4, 6, 9, and 19 and does not involve the carboxyl-terminal portion of the protein. When truncations were made within the hydrophobic domain of transmembrane helix 11 (truncations of 41 or more residues), the carrier was no longer found in the membrane. This suggests that the carboxyl terminus may be involved in the membrane insertion process, stabilization of the carrier within the membrane following insertion, or protection of the inserted carrier from proteolytic scavenging. A new plasmid that expresses the temperature-resistant isoform of the melibiose carrier under inducible control of a tac promoter, designated(pkKMB), is also described.

The protein is deduced to be composed of 469 amino acids and have a molecular mass of 52,215 daltons (11). Hydrophathy analysis predicts a short NH2 terminus, 11 transmembrane segments joined by short hydrophilic regions, and a long hydrophilic COOH terminus (13). Amino acid substitutions that alter sugar and/or cation specificity are predominantly found in four clusters on the periplasmic side of transmembrane segments 3, 6, 9, and 10 (8, 12-15) and may define a common sugar/cation binding site when folded in the native conformation (13). The function, if any, of the long COOH tail has not previously been investigated.

This paper describes mutations of the melB gene that result in the truncation of the COOH tail at eight positions. Measurement of transport and cation coupling characteristics indicate that the hydrophilic domain of the tail has no direct role in substrate recognition or energy transduction but may play a role in stabilizing the carrier in the membrane.

MATERIALS AND METHODS

Reagents—Melibiose and methyl-β-D-thiogalactopyranoside (TMG) were purchased from Sigma. [Methyl-14C]TMG and [3H]raffinose were from Du Pont-New England Nuclear. [PH]Melibiose was generously provided by Dr. Gerard Leblanc of the Department de Biologie du Commissariat à l'Energie Atomique, Villefranche-sur-Mer, France. Radiolabeled sugars were purified by paper chromatography on Whatman No. 3 MM chromatography paper using a mixed solvent phase of 3 parts of 1-propanol to 1 part of H2O. α- and [3H]dATP (>600 mCi/mmole) was purchased from Amersham Corp. DNA sequencing reagents and restriction enzymes were purchased from New England Biolabs (Beverly, MA). All other chemicals were of reagent grade.

Construction of the melB Expression Plasmid pKKB—EcoRI and HindIII restriction sites were introduced into the melB DNA fragment of pSTY-91 (10) by oligonucleotide-directed site-specific mutagenesis as described below. The EcoRI site was introduced at nucleotide 246 (numbering scheme of Ref. 11) by changing the native sequence 5'-GAAGGC-3' to 5'-GAATTC-3'. The HindIII site was introduced at nucleotide 1473 by changing the sequence 5'-ATGCTT-3' to 5'-AAAGCT-3'. The HindIII site was introduced at nucleotide 1473 by changing the sequence 5'-ATGCTT-3' to 5'-AAAGCT-3'. This produced a 1518-base pair EcoRI/HindIII DNA fragment containing the entire melB gene and Shine-Dalgarno sequence with little unessential DNA 3' or 5' of the structural gene. The fragment was ligated into the corresponding restriction sites of the expression vector pKK223-3 (Pharmacia LKB Biotechnology Inc.). The resulting plasmid, designated(pKKB), is schematically outlined in Fig. 1.

Site-specific Mutagenesis—Stop codons were introduced into the melB gene of pKKB via oligonucleotide-directed site-specific mutagenesis according to the method of Eckstein (16, 17). Mutagenic oligonucleotides were synthesized using a Codex 300 DNA synthesizer (Du Pont Biotechnology Systems, Wilmington, DE) using standard phosphoramidite chemistry. Oligonucleotides were cleaved from the resin support according to the manufacturer's specifications, extracted three times with H2O-saturated butanol, and used without further purification.
DNA Sequencing—A single-stranded template for DNA sequencing was produced in M13 according to standard methods (18). DNA sequencing was via the dideoxy chain termination method of Sanger et al. (19) using the Klenow fragment of DNA polymerase I and $a$ -dATP. Elongation and chase reactions were carried out at 50 °C to eliminate the sequencing artifacts resulting from temperature-sensitive secondary structure characteristics of the DNA template. The entire melB gene and flanking regions were sequenced to ensure that only the desired mutation(s) had been incorporated. Areas of ambiguous interpretation were repeatedly sequenced until all ambiguities were resolved.

Uphill Sugar Transport—Transport was performed as described in the figure legends. Samples were rapidly filtered through a 0.65-μm pore size nitrocellulose membrane filter (Sartorius Filters Inc., Hayward, CA). Accumulated label was quantified by liquid scintillation counting using Liquiscint (National Diagnostics, Somerville, NJ).

α-Galactosidase Activity—α-Galactosidase activity was estimated by a colorimetric assay based on the hydrolysis of α-nitrophenyl α-galactopyranoside using a modification of the method of Burstein and Kepes (20). Early log phase cells were washed twice and resuspended in M63 (100 mM potassium phosphate buffer, pH 7.2, 15 mM (NH₄)₂SO₄, 1 mM MgSO₄) to a density of $5 \times 10^8$ cells/ml. Cells were sonicated to clarity with a probe sonicator and centrifuged for 10 min at 30,000 × g at 4 °C in a Sorvall SA-600 rotor to produce a clear supernatant containing the enzyme activity. α-Galactosidase activity was measured at 25 °C in a reaction mixture containing 3 mM α-nitrophenyl α-galactopyranoside, 3 mM MgSO₄, 100 mM β-mercaptoethanol, 50 mM Tris-HCl (pH 8.1), and 1 μM NAD°. Reactions were stopped by the addition of 250 mM Na₂CO₃ and 40 mM EDTA and the absorbance measured at 420 nm. Enzyme activity was estimated by comparison to an α-nitrophenol standard curve.

pH Electrode Studies—Host strain DW1 (ΔmelAB ΔlacZ2: Ref. 21) was used to measure H⁺ movement by the truncated carriers. Strain RVSmXΔlacZ2A1 (Ref. 22), which was used for all other physiological studies, could not be used for the measurement of H⁺ movement due to a small but significant number of lysed cells present following growth at 40 °C. The proteins released from these cells
was calibrated following each assay by adding a known amount of anaerobic KOH. The response to the addition of melibiose (5 mM final concentration) was vigorous shaking until early log phase (approximately four doublings) and preloaded with 20 mM TMG by incubating in azide buffer containing 20 mM TMG at 4°C for 20 min. Approximately 5 x 10^6 cells (2.5 x 10^9 cells/ml) were used per recording. H' movement in the course of RVSmX containing pKKMB-ct30 was followed using a Radiometer (Copenhagen) GK231-C combined pH electrode. A stock melibiose solution (500 mg/ml) was prepared in Tricine buffer and made “Na+-free” by chromatography through Dowex 50W (hydrogen form) pre-equilibrated with 2 volumes of 500 mM melibiose. Electrode response was calibrated after each assay by adding known quantities of anaerobic NaCl in Tricine buffer.

**Sodium Electrode Studies**—RVSmX containing pKKMB or a truncated derivative were grown in 1% Tryptone medium 63 at 37°C with vigorous shaking until early log phase (approximately four doublings) and preloaded with 20 mM TMG by incubating in azide buffer containing 20 mM TMG at 4°C for 20 min. Approximately 5 x 10^6 cells (2.5 x 10^9 cells/ml) were used per recording. H' movement in the course of RVSmX containing pKKMB-ct30 was followed using a Radiometer (Copenhagen) GK231-C combined pH electrode. A stock melibiose solution (500 mg/ml) was prepared in Tricine buffer and made “Na+-free” by chromatography through Dowex 50W (hydrogen form) pre-equilibrated with 2 volumes of 500 mM melibiose. Electrode response was calibrated after each assay by adding known quantities of anaerobic NaCl in Tricine buffer.

**Radionabelling of Cells and Preparation of Membranes**—Cells of strain RVSmX containing pKKMB, pKKMB-ct14, pKKMB-ct41, or pKKMB-ct56 were grown in 40 ml of 0.5% glycerol M63 containing 100 µg of ampicillin/ml and thiamine at 40°C to early log phase. H-Labeled amino acids (125 µCi, Amersham mixture TRK 440) were added and growth continued for 2.5 doublings. Cultures of RVSmX containing pKK223-3 (parent plasmid lacking the melB gene) were grown and labeled as above using 14C-labeled amino acids (25 µCi, Amersham mixture CFB.104). Equal volumes of H- and 14C-labeled cells were mixed and used to prepare cytoplasmic membranes as described by Osborn et al. (22). Membrane samples were dissolved in sample buffer (24) at 25°C for 30 min and electrophoresed through a 12.5% acrylamide SDS gel (24). The gel was fixed in methanol:acetic acid:water (50:10:40), stained with Coomassie Brilliant Blue, de-stained, and cut into 1-mm sections. Radioactivity was extracted from the gel slices by treatment with 0.5 ml 90% Protosol (Du Pont-New England Nuclear), 10% H2O for 18 h at room temperature. Samples were mixed with 5 ml of Liquiscint, briefly vortexed, held overnight at room temperature to dissipate chemiluminescence, and radioactivity was quantitated by liquid scintillation spectroscopy.

**Carboxyl Truncations of the Melibiose Carrier**

### TABLE I

| Plasmid Designation | Amino acids | Codon Position | Original Codon | Stop Codon | Sugar Transport |
|---------------------|-------------|----------------|----------------|------------|----------------|
| pKKMB-ct2           | 2           | 468            | UAA            | AAA        | +              |
| pKKMB-ct10          | 10          | 460            | UAG            | CCG        | +              |
| pKKMB-ct14          | 14          | 456            | UAA            | CAU        | +              |
| pKKMB-ct24          | 24          | 446            | UAA            | AAA        | +              |
| pKKMB-ct30          | 30          | 440            | UAG            | CAG        | +              |
| pKKMB-ct26          | 36          | 434            | UAA            | GAC        | +              |
| pKKMB-ct41          | 41          | 429            | UAA            | UAU        | -              |
| pKKMB-ct56          | 56          | 414            | UGA            | CCA        | -              |

**Fig. 4. TMG counterflow.** A, RVSmX containing pKKMB-ct2 ( ), pKKMB-ct10 ( ), and pKKMB-ct14 ( ), B, RVSmX containing pKKMB ( ), pKKMB-ct14 ( ), pKKMB-ct30 or pKKMB-ct36 ( ), and pKKMB-ct41, pKKMB-ct26, or pKKMB-56-3 ( ). C, extended time course of RVSmX containing pKKMB-ct30 ( ), pKKMB-ct36 ( ), pKKMB-ct41 ( ), pKKMB-ct56 ( ), or pKKMB-23-3 ( ).

**Fig. 5. Uphill TMG transport by COOH-truncated melibiose carriers.** A, RVSmX containing pKKMB ( ), pKKMB-ct30 ( ), pKKMB-ct41 ( ), pKKMB-ct24 ( ), or pKKMB-ct36, pKKMB-ct30, pKKMB-ct36, pKKMB-ct41, pKKMB-ct56, or pKKB-23-3 ( ). B, RVSmX containing pKKMB-ct30 ( ), pKKMB-ct36 ( ), pKKMB-ct41 ( ), pKKMB-ct56 ( ), and pKKB-22-3 ( ). Cells were grown at 40°C in 1% Tryptone M63 containing 100 µg of ampicillin/ml and thiamine. Cells were washed and TMG transport determined as described in Fig. 2. Points represent the mean of duplicate samples.
pKKMB is known, and the insert contains less than pKKMB (Fig. 1), contains the melB gene from pSTY91 (10) biose carrier as discussed below. The melB expression plasmid pKKMB-A  new plasmid that expresses the temperature-resistant isoform of the melibiose carrier (4, 25) was constructed. The plasmid, designated pKKMB (Fig. 1), contains the melB gene from pSTY91 (10) under inducible control of the strong tac promoter (26). Transcription of the melB RNA is terminated by the strong rnb transcription terminator (27, 28). This helps stabilize the host-vector system by inhibiting detrimental overexpression (29, 30, 31). When transformed into a lacI+ background, melB expression is repressed unless induced by isopropyl-1-thio-β-D-galactoside (data not shown). In a lacI+ host such as DW1, expression of the carrier is only partially repressed. Uninduced DW1/pKKMB accumulates TMG 120-fold, melibiose 76-fold, and raffinose 76-fold (Fig. 2). Unlike previous melB expression plasmids, the entire nucleotide sequence of pKKMB is known, and the insert contains less than 5% nonessential DNA.

The RVSmX Assay System—Measurement of plasmid-encoded melibiose carrier activity requires that the host strain be both lactose carrier and melibiose carrier negative. Two such strains of E. coli are available: DW1 and DW2 (21). Unfortunately, these strains could not be used in the current study due to amber suppression by DW1 and DW2. Therefore transport characteristics of the truncated carriers were determined in RVSmX (com strA11, Ref. 22) grown at 40 °C by exploiting the heat lability of the chromosome-encoded melibiose carrier as discussed below.

Two isoforms of the melibiose carrier that differ in temperature sensitivity are known (4, 25). The wild-type carrier exhibits full activity when grown at 30 °C but is irreversibly inactivated when grown at elevated temperatures; the temperature-resistant isoform is fully active throughout the temperature range that permits growth. The chromosomal melB gene (Fig. 1) contains the melB gene product is detectable. To determine if this loss of transport activity was due to a direct perturbation of protein structure by the truncation or indirectly due to poor membrane insertion or postinsertional proteolytic scavenging, we checked cytoplasmic membrane preparations. To determine if the melibiose carrier was present in the cytoplasmic membrane RVSmX containing pKKMB (A), pKKMB-ct14 (B), pKKMB-ct41 (C), or pKKMB-ct56 (D) were grown in the presence of 3H-labeled amino acids as described under “Materials and Methods” and mixed with an equal number of RVSmX containing pKK223-3 (vector without melB gene) grown in the presence of 14C-labeled amino acids. Cytoplasmic membranes were prepared from the pooled cells and electrophoresed through a 12% polyacrylamide SDS gel. The gel was fixed, cut into 1-nm slices, and 3H and 14C activity determined. Total counts for each isotope were normalized to 100% and a 3H:14C ratio calculated. The two profiles coincide except in the area of 3H excess corresponding to the melibiose carrier protein (A and B). Approximate correspondence between gel slice and apparent molecular weight is indicated.

RESULTS

MelB Expression Plasmid pKKMB—A new plasmid that expresses the temperature-resistant isoform of the melibiose carrier (4, 25) was constructed. The plasmid, designated pKKMB (Fig. 1), contains the melB gene from pSTY91 (10) under inducible control of the strong tac promoter (26). Transcription of the melB RNA is terminated by the strong rnb transcription terminator (27, 28). This helps stabilize the host-vector system by inhibiting detrimental overexpression (29, 30, 31). When transformed into a lacI+ background, melB expression is repressed unless induced by isopropyl-1-thio-β-D-galactopyranoside (data not shown). In a lacI+ host such as DW1, expression of the carrier is only partially repressed. Uninduced DW1/pKKMB accumulates TMG 120-fold, melibiose 76-fold, and raffinose 76-fold (Fig. 2). Unlike previous melB expression plasmids, the entire nucleotide sequence of pKKMB is known, and the insert contains less than 5% nonessential DNA.

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COOH-terminal Truncations—The COOH end of the melibiose carrier was truncated at eight locations by insertion of stop codons into the melB gene of pKKMB by site-specific mutagenesis. The location of the truncations and the nucleotide changes are listed in Table I.

Thirty-six amino acids could be removed from the COOH terminus of the carrier without the complete loss of facilitated diffusion (Fig. 4) or uphill transport (Figs. 5 and 6). When 41 or more residues were removed, no transport of any type was detectable. To determine if this loss of transport activity was due to a direct perturbation of protein structure by the truncation or indirectly due to poor membrane insertion or postinsertional proteolytic scavenging, we checked cytoplasmic membranes for the presence of the carrier protein using differential labeling of cells either expressing or not expressing the melB gene (Fig. 7). In both cases (41 and 56 amino acid truncations) the carriers were absent from the cytoplasmic membrane. Wild-type and the 14-amino acid truncated carriers could be clearly detected by this technique. Sugar specificity did not appear to be affected by truncation.

FIG. 6. Uphill raffinose and melibiose transport. A, raffinose transport. B, melibiose transport. RVSmX containing pKKMB (C), pKKMB-ct24 (D), or pKK223-3 (A) were grown, washed, and transport rates determined as described in Fig. 2. Points represent the mean of duplicate samples.

FIG. 7. Distribution of radioactivity in SDS-polyacrylamide gels of cytoplasmic membrane preparations. To determine if the melibiose carrier was present in the cytoplasmic membrane RVSmX containing pKKMB (A), pKKMB-ct14 (B), pKKMB-ct41 (C), or pKKMB-ct56 (D) were grown in the presence of 3H-labeled amino acids as described under “Materials and Methods” and mixed with an equal number of RVSmX containing pKK223-3 (vector without melB gene) grown in the presence of 14C-labeled amino acids. Cytoplasmic membranes were prepared from the pooled cells and electrophoresed through a 12% polyacrylamide SDS gel. The gel was fixed, cut into 1-nm slices, and 3H and 14C activity determined. Total counts for each isotope were normalized to 100% and a 3H:14C ratio calculated. The two profiles coincide except in the area of 3H excess corresponding to the melibiose carrier protein (A and B). Approximate correspondence between gel slice and apparent molecular weight is indicated.
Arrows indicate the addition of melibiose to give a final concentration of 5 mM. Upward deflections correspond to the uptake of cation by the cells. Recordings are corrected for the gradual background acidification observed prior to the addition of substrate.

Monosaccharide, disaccharide, and trisaccharide substrates (TMG, melibiose, and raffinose, respectively) were all transported. It is interesting to note that TMG showed significantly enhanced accumulation when 2, 10, or 14 amino acids were truncated from the carrier (Fig. 5). However, melibiose and raffinose accumulation remained comparable to that of the untruncated carrier (Fig. 6).

The ability of the truncated proteins to participate in H⁺-coupled and Na⁺-coupled cotransport was measured using pH or sodium electrodes as described under "Materials and Methods." Arrows indicate the addition of melibiose in comparison to the other truncated tail of the melibiose carrier plays no direct role in substrate gradients remained qualitatively intact. These results are consistent with the hypothesis that the sugar/cation binding site is formed by the interaction of the transmembrane helices 3, 4, 6, 9, and 10 and does not involve the COOH-terminal portion of the protein (13).

Although significant transport by the truncated carriers could be demonstrated, the rate of transport was less than normal when 24, 30, or 36 amino acids were removed. This could be due to impairment of the translocation process at the level of the individual carrier or to the presence of fewer carriers within the membrane. Due to the lack of an antibody specific for the melibiose carrier, we were unable to distinguish between the possibilities. However, the counterflow data suggest that the decreased rates may be largely accounted for by a decrease in the number of carriers. As more of the COOH terminus was truncated, the initial rates and peak intracellular concentrations were progressively decreased and the time required to achieve peak intracellular concentration was progressively lengthened. Identical counterflow behavior was observed with the lactose carrier when the number of active transporters incorporated in the membrane was varied (32). This decrease in carrier number with progressive COOH truncation may be the result of progressively increased proteolytic scavenging of the membrane-inserted carriers, as discussed below.

When truncations were made within the hydrophobic domain of transmembrane helix 11 (pKKMB-ct41 and pKKMB-ct56, Fig. 9), the carrier could no longer be found in the membrane. This suggests that the COOH terminus may be involved in the membrane insertion process, stabilization of the carrier within the membrane following insertion, or protection of the inserted carrier from proteolytic scavenging. The recent finding that the 417-amino acid lactose carrier of E. coli can be truncated by as much as 366 amino acids (88% of the carrier being deleted) without impairment of insertion into minicell membranes (33) suggests that the truncated melibiose carriers were removed or degraded following initial insertion into the membrane. Lack of the hydrophilic tail to anchor helix 11 within the membrane or the failure of helix 11 to properly insert in the membrane in the absence of the anchor may target the protein for proteolytic removal. In contrast, truncations originating in the hydrophilic domain, all of which retained a COOH tail with at least four strongly hydrophilic residues (including a minimum of two charged residues), adequately anchored the terminal transmembrane segment and protected the carrier from degradation.

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