Resistance of the Melibiose Carrier to Inhibition by the Phosphotransferase System Due to Substitutions of Amino Acid Residues in the Carrier of Salmonella typhimurium*

(Received for publication, March 30, 1992)

Masayuki Kuroda, Sjaak de Waard†, Kaori Mizushima, Masaaki Tsuda, Pieter Postma†, and Tomofusa Tsujiyā

From the Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan and the †E. C. Slater Institute for Biochemical Research, University of Amsterdam, Amsterdam, The Netherlands

The melibiose carrier of Salmonella typhimurium is under the control of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). We isolated mutants of the melibiose carrier that showed resistance to inhibition via the PTS. Growth of the mutants on melibiose was not inhibited by 2-deoxyglucose, a non-metabolizable substrate of the PTS, although growth of the parent strain was inhibited. Transport activity of the melibiose carrier in the mutants was fairly resistant to inhibition by 2-deoxyglucose, although the activity in the parent was sensitive to inhibition. We cloned the mutated melB gene that encodes the melibiose carrier, determined the nucleotide sequences, and identified replaced nucleotides. The mutations resulted in substitutions of Asp-438 with Tyr, Arg-441 with Ser, or Ile-445 with Asn. All of these residues are in the COOH-terminal region of the carrier. The secondary structure of this region is predicted to be an α-helix, and the mutated residues were on the same side of the helix. This region showed sequence similarity to a region of the MalK protein, in which substitution of amino acid residues also resulted in PTS-resistant mutants. Thus the COOH-terminal portion of the melibiose carrier is important for the interaction of dephosphorylated IIIIC, which is an entity causing reversible inactivation of the carrier.

When cells of Escherichia coli or Salmonella typhimurium are grown in the presence of glucose and melibiose, the growth curve exhibits two successive growth cycles separated by a lag period (1, 2). This type of growth behavior has been termed diauxie. Glucose is utilized in the first growth cycle and melibiose in the second. In other words, the utilization of melibiose is inhibited by glucose. The two mechanisms responsible for this phenomenon are catabolite repression and inducer exclusion (2, 3). Catabolite repression is a control mechanism for gene expression, and inducer exclusion is a

* This research was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to T. T.) and by a grant from the Netherlands Organization for Scientific Research (NWO) under the auspices of the Netherlands Foundation for Chemical Research (SON) (to P. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed. Fax: 81-862-51-7957.

† The abbreviations used are: PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system; IIIIC, glucose factor III; TMG, methyl-β-d-thiogalactopyranoside; kbp, kilobase pair(s).

EXPERIMENTAL PROCEDURES

Bacteria and Growth—S. typhimurium LT2 (wild type) and PP2098, a leaky ptsI derivative of LT2, were used. PP2102, PP2103, PP2104, PP2106, PP2107, PP2155, and PP2158 are 2-deoxyglucose-resistant derivatives of PP2098. E. coli RE16r (melB, recA, Δlac) (17), DW2 (ΔmelB, Δlac) (18), PPA172 (ptsI-leaky, Δlac), JM83 (19), and GM33 (dam) were used. PPA172 was isolated after diethyl sulfate mutagenesis of W3133-2 (6) and penicillin selection as a strain that could not grow on mannitol but was still able to grow on maltose and glycerol but not in the presence of 2-deoxyglucose.
Results

Properties of the Mutants—S. typhimurium PP2098 is a leaky ptsA mutant and is hypersensitive to PTS sugars, such as 2-deoxyglucose or methyl-α-glucoside. PP2102 is a mutant that is resistant to such PTS sugars. We tested the effects of the nonmetabolizable PTS sugars on the growth of the mutants. In fact, PP2098 was unable to grow on melibiose as a sole source of carbon in the presence of 2-deoxyglucose (Fig. 1) or methyl-α-glucoside (data not shown). On the other hand, mutant PP2102 grew on melibiose even in the presence of 2-deoxyglucose or methyl-α-glucoside. It is noteworthy that the growth of both PP2098 and PP2102 on maltose or glycerol was strongly inhibited by both 2-deoxyglucose and methyl-α-glucoside (data not shown). We obtained similar results with other mutants, PP2103, PP2104, PP2105, PP2106, PP2107, PP2108, and PP2158.

Although our results indicate that mutations in the mutant PP2102 exist in the melibiose operon, as described above, two types of mutations in the operon seem to be possible. One type is a mutation in the regulatory region of the operon. If overexpression of the operon takes place, a large amount of the melibiose carrier could be synthesized, and the limited amount of IIIIC present in cells would not be able to inhibit all of the melibiose carrier. In this case, we should detect elevated activities of α-galactosidase (mlaA product) and the melibiose carrier (mlbB product). In fact, we obtained a number of these mutations in E. coli. The second type of mutation is in the melB gene. In this case, normal levels of α-galactosidase activity and melibiose transport activity and PTS-resistant transport would be observed. We are interested in the latter type of mutants.

We measured the α-galactosidase activity of the mutants and found that the activity in all of the mutants was almost the same as that of the parent (PP2098) (data not shown). Thus it seemed that the mutations were not in the regulatory region of the operon, but in the melB gene.

We then tested the effects of the nonmetabolizable PTS sugars on transport activity of the melibiose carrier by two methods. In the first method, we measured the uptake of [14C]TMG (Table I). The uptake of TMG was inhibited about 70% by 5 mM 2-deoxyglucose in PP2098 (parent), whereas the TMG uptake in the mutants (PP2102–PP2106, PP2115, and PP2158) was very resistant (0–15% inhibition). In the second method, we measured uptake of Na+, elicited by the addition of 5 mM 2-deoxyglucose at 37°C under aerobic conditions. The growth was monitored turbidimetrically at 650 nm.
of melibiose to a cell suspension, using an Na⁺-selective electrode. Since the melibiose carrier mediates cotransport of Na⁺ and melibiose, influx of melibiose into cells elicits the Na⁺ uptake (35). The Na⁺ uptake was inhibited 75% by 2-deoxyglucose under aerobic conditions at 25 °C. The assay was initiated by adding [3H]TMG (final concentration, 1 mM). Samples were taken at 5 min.

Thus, all of the mutants we tested seem to possess mutated melB gene, including any environmental conditions (data not shown).

We tested the effects of 2-deoxyglucose on the activity of the melibiose carrier derived from the pTKK2102. Because PPA172 possesses its own melibiose carrier, pTKK2102 was introduced into E. coli strain DW2 that lacks the melibiose carrier. The carrier activity was tested by measuring Na⁺ uptake elicited by melibiose influx. The Na⁺ uptake in DW2/pTKK2102 was not inhibited significantly, whereas that in DW2/pTKK1 was strongly inhibited (85% inhibition). Thus we confirmed that the 2-deoxyglucose-resistant growth of PPA172/pTKK2102 was due to the melB gene carried by the plasmid pTKK2102. Similar results were obtained with cells harboring a plasmid carrying the other mutated melB gene (data not shown).

Next we tried to do intracistronic mapping of the mutations in the melB gene of the mutants. Three types of plasmids (for example, pTKK11-2102, pTKK12-2102, and pTKK13-2102), each carrying a different segment of the mutant melB gene, were constructed (Fig. 4). Competent cells of PPA172 were transformed with the plasmids, and growth of PPA172/pTKK11-2102, PPA172/pTKK12-2102, and PPA172/pTKK13-2102 was measured in modified Tanaka medium supplemented with 5 mM melibiose in the absence or presence of 2-deoxyglucose. The growth was monitored turbidimetrically at 650 nm.

![Fig. 2. Cloning strategy of the mutated melB gene. For details, see "Experimental Procedures." EV, EcoRV site; S, SmaI site; BgII site; 2DG, 2-deoxyglucose.](image-url)
PTS-resistant Melibiose Carrier of S. typhimurium

Fig. 4. Intracistronic mapping of mutations. Plasmids carrying various portions (thick black bar) of the mutant melB gene were constructed. Growth of cells of E. coli PPA172 harboring each plasmid was tested on a plate containing modified Tanaka medium supplemented with 5 mM melibiose in the presence of 10 mM 2-deoxyglucose (2DG) at 37 °C for 24 h. + indicates growth, and − indicates no growth. P, promoter; S, Smal site; EV, EcoRV site, N, NruI site; K, KpnI site.

Fig. 5. Southern blot analysis of chromosomal DNA of wild type and mutants. Chromosomal DNA of parent and mutants was digested with a restriction endonuclease and electrophoresed in an agarose gel. After electrophoresis, Southern blot analysis was performed using a DNA probe. Lane 1, PPA172; lane 2, PP2102; lane 3, PP2103; lane 4, PP2104; lane 5, PP2105; lane 6, PP2106; lane 7, PP2107; lane 8, PP2155; lane 9, PP2158.

pTKK13-2102 on melb was tested in the absence or presence of 2-deoxyglucose. Only PPA172 cells harboring pTKK12-2102 grew in the presence of 2-deoxyglucose (Fig. 4). Thus the mutation of PP2102, responsible for the resistance to the PTS sugar, is localized in the NruI-NruI fragment of the melB region, which corresponds to the COOH-terminal portion of the melibiose carrier. We obtained the same result with all other mutants tested (data not shown).

Sequence Analyses—We determined the nucleotide sequences of the NruI-NruI DNA fragment derived from mutated melB of PP2102. We found only one nucleotide replacement in the DNA region. The replacement was T with A at position 1,334 starting from A of initiation ATG of the melB gene (16). This replacement results in the amino acid substitution of Ile-445 with Asn in the melibiose carrier.

The nucleotide sequence of the region where the mutation was found is AGATCC in the wild type and AGAACC in the mutant. A restriction endonuclease BstYI cleaves the AGATCC site, but not the AGAACC site. To test whether or not we can detect a difference in the digestion pattern between the melb region of wild type cells and the mutant cells, the chromosomal DNA from each strain was prepared, digested with BstYI, and analyzed by Southern blot hybridization. In fact, we detected cleavage of wild type melB at the expected site (Fig. 5) and DNA bands of 1.4 and 3.35 kbp. But we could not detect a difference in the digestion pattern between the parental cells and the mutant cells harboring melB of PP2102. We found only one nucleotide replacement in the DNA region. The replacement was T with A at position 1,312 in PP2155 and C with A at position 1,321 in PP2158. Thus we identified amino acid substitutions Asp-438 with Tyr and Arg-441 with Ser, in addition to Ile-445 with Asn (Table II).

DISCUSSION

E. coli and S. typhimurium are closely related microorganisms (36). We introduced the melB gene of S. typhimurium into E. coli cells; and the melB gene was functional in E. coli cells. The melibiose carrier of S. typhimurium synthesized in E. coli showed properties similar to those of the carrier synthesized in S. typhimurium cells. Furthermore, the wild type melibiose carrier of S. typhimurium was sensitive to the PTS of E. coli. Roseman and co-workers (37) demonstrated that the lactose carrier of E. coli reconstituted into vesicles of S. typhimurium was sensitive to the PTS of S. typhimurium. In other words, III" of S. typhimurium is able to regulate the lactose carrier of E. coli. It is reported that the sequence homology in III" of E. coli and S. typhimurium is 98% (38, 39).

We isolated mutants of S. typhimurium carrying the melibiose carrier that became resistant to inhibition via the PTS. The mutant cells grew on melibiose in the presence of 2-deoxyglucose, a nonmetabolizable PTS sugar, although the parental cells did not. This means that the transport process for melibiose is crucial for cell growth in those cells under such conditions.

Since the melB genes, encoding the mutant melibiose carriers, were subsequently cloned on a multicopy plasmid, the resulting strains might become resistant to inhibition by PTS sugars due to overproduction of the melibiose carrier (40). However, from Fig. 3 it is clear that cells containing the pTKK1 plasmid (encoding the wild type melibiose carrier) are still sensitive to inhibition by 2-deoxyglucose. In cells containing this plasmid, the activity of the melibiose carrier is about three times higher compared with control cells (data not shown). Clearly, this level of the melibiose carrier is not sufficient to escape from inhibition via III".

Recently, we determined the nucleotide sequences of the wild type melB gene of S. typhimurium (16). Thus it became possible to determine altered nucleotides in the melB gene of mutants. As a result of cloning and sequencing, we identified the replaced nucleotides in the melB gene of the mutants that became resistant to PTS sugars. The deduced amino acid substitutions in the melibiose carrier of the mutants were Asp-438 with Tyr, Arg-441 with Ser, and Ile-445 with Asn.

**Table II**

<table>
<thead>
<tr>
<th>Replacement</th>
<th>Nucleotide</th>
<th>Mutant</th>
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<tbody>
<tr>
<td>G-1312 with T</td>
<td>Asp-438 with Tyr</td>
<td>PP2155</td>
</tr>
<tr>
<td>C-1321 with A</td>
<td>Arg-441 with Ser</td>
<td>PP2158</td>
</tr>
<tr>
<td>T-1334 with A</td>
<td>Ile-445 with Asn</td>
<td>PP2102–PP2107</td>
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*Six mutants.*
All of these residues are in the COOH-terminal region of the carrier. Surprisingly, we found the same amino acid substitution (Ile-445 with Asn) in six mutants that were isolated independently. This suggests that Ile-445 is very important for the regulation of the carrier by III^Glc. Since Asp-438, Arg-441, and Ile-445 are all located in the COOH-terminal region of the carrier, it is very likely that this COOH-terminal region is involved in III^Glc binding. Judging from the positions of the identified residues (438, 441, and 445), it seemed that these residues could be present on the same side if this region is in an α-helix form. In fact, calculation by the method of Chou and Fasman (41) predicted an α-helix structure in this region (data not shown). A vertical view of the α-helix of this region showed that Asp-438, Arg-441, and Ile-445 are really on the same side of the α-helix (Fig. 6). Therefore, it is likely that these 3 residues are involved in interaction with III^Glc. It would be interesting to substitute Arg-452, which is on the same side of the α-helix as Asp-438, Arg-441, and Ile-445 (Fig. 6), with another residue by site-directed mutagenesis. Among the 3 identified residues, 2 residues possess a charge (Asp and Arg) that might be important for binding (or interaction) with III^Glc.

We proposed a topological model of the melibiose carrier of E. coli based on several types of analysis (42). Since the sequence homology between the melibiose carriers of E. coli and of S. typhimurium is very high (16), the topology of the two carriers would be very similar. Fig. 7 shows the topological model of S. typhimurium melibiose carrier. According to this model, the COOH-terminal portion of the melibiose carrier of S. typhimurium is on the cytoplasmic side. Since III^Glc is a soluble cytoplasmic protein, this model is convenient for understanding the interaction between the III^Glc and the carrier.

Saier and co-workers (14) identified substituted amino acid residues in the MalK protein of PTS-resistant mutants. Two of the substitutions are Gly-278 and Gly-284. They pointed out that this region of the MalK protein showed some similarity of amino acid sequence to the central loop portion of the melibiose carrier of E. coli. The amino acid sequences of the melibiose carriers of S. typhimurium and of E. coli are highly homologous, and the sequences of the central loop portions of the two melibiose carriers are very similar (92% homology including conservative changes) (16). Thus, it is likely that the central loop portion of the melibiose carrier of S. typhimurium is involved in the binding of III^Glc. The central loop portion is on the cytoplasmic side according to our topological model (Fig. 7). It has been reported that a central loop portion of the E. coli lactose carrier is important for binding of III^Glc (13). Perhaps the COOH-terminal portion and the central loop portion of the melibiose carrier are in close contact and form a binding site for III^Glc. In the case of the lactose carrier, it seems that the NH2-terminal portion is also involved in the III^Glc binding (43). Analysis of more mutants will clarify domains that are involved in III^Glc binding.

We also found sequence similarity between the COOH-terminal region that contained Ile-445 of the melibiose carrier and a region of PTS-resistant MalK in which amino acid substitution (Ala-124 with Thr) was found (14) (Fig. 8). Thus it seems reasonable to assume that these regions are really involved in regulation by the PTS, namely binding of III^Glc.

Both the melibiose carrier and the lactose carrier mediate the cotransport of H+ and melibiose (and other galactosides). The two carriers are similarly regulated by the PTS. However, no significant sequence similarity was found between the two carriers (44, 45). Interestingly, hydropathy patterns and topological models of the two carriers are similar and suggest 12 transmembrane segments (42, 46). Although the sequence similarity is low, the three-dimensional structure of the binding site for III^Glc in the two carriers could be similar.

The hydrophilic COOH-terminal part of the melibiose carrier of S. typhimurium (16) and E. coli (44) is almost 30 residues longer than that of the lactose carrier (45). It was not clear whether this long COOH-terminal portion of the melibiose carrier is really necessary for the function of the carrier. It has been reported that introduction of a termination codon in position 434, 440, or 446 of the melibiose carrier of E. coli (original length is 469 residues) resulted in a reduction of activity of 25% of that of the intact carrier (47). However, truncation at position 460 or 456 caused no significant reduction in transport activity. These results indicate that the 14 COOH-terminal residues of the carrier are not necessary for carrier activity and that the residues at positions 15–36 from

**Fig. 6. Vertical view of a putative α-helix of the COOH-terminal region of the melibiose carrier.** A region from Gly-437 to Thr-464 is shown. Substituted residues in the mutant carriers are boxed.

**Fig. 7. Location of substituted amino acid residues in topological model of the melibiose carrier of S. typhimurium.** Numbers from 1 to 12 indicate the 12 putative membrane-spanning regions of the melibiose carrier. The substituted residues identified in PTS-resistant melibiose carrier are in the COOH-terminal portion, which is on the cytoplasmic side of the membrane. Two regions (I and II) surrounded by the dotted line are regions that showed sequence similarity to suggested sequences for III^Glc binding in MalK, LacY, and GlpK (region I) (14) or in MalK (region II) (see Fig. 8).

**Fig. 8. Sequence similarity between the COOH-terminal region of MelB and a portion of MalK.** Amino acid sequences of the COOH-terminal region of S. typhimurium MelB (S.t.) and E. coli MelB (E.c.) and a central region of E. coli MalK (E.c.) are shown. Identical residues (+) and conservative changes (•) are indicated.
the COOH terminus are fairly important. The Ile-445 is at position 28 from the COOH terminus. Thus this region is important not only for the binding of III^ct but also for the total function of the carrier.

REFERENCES