Enzymatic and Biochemical Probes of Residues External to the Translocation Pathway of UhpT, the Sugar Phosphate Carrier of Escherichia coli*

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Part of the substrate translocation pathway through UhpT, the Escherichia coli sugar phosphate carrier, has been assigned to a transmembrane helix extending between residues 260 and 282. To set limits on the external portion of the pathway, we identified nearby residues fully exposed to the periplasm. In one case, we used Western blots to evaluate cleavage by extracellular trypsin. The protease cleaved UhpT variants retaining lysine 294, but not those lacking lysine 294, indicating that trypsin acts at a single extracellular site, lysine 294. In other work we labeled single-cysteine variants with 3-(N-maleimidylpropiony1)biocytin and scored accessibility to extracellular streptavidin by shift of SDS-polyacrylamide gel electrophoresis mobility. Positions 283 and 284 were fully exposed to the periplasm, since the modified residue was bound by streptavidin in the native protein; by contrast, although the biotin-linked probe modified position 276, streptavidin decoration was not achieved without protein denaturation. We conclude that a 12-residue stretch (283–294) of UhpT is sufficiently exposed to be accessible to large probes (trypsin, streptavidin), while position 276 and more proximal residues are more deeply buried or otherwise shielded from the external phase.

The Escherichia coli phosphate/sugar phosphate antiport carrier, UhpT, is a polytopic membrane protein resembling others within the Major Facilitator Superfamily (1) in having 12 likely transmembrane segments (2–4). Recent studies have identified part of the translocation pathway by monitoring accessibility to pCMBS1 in single-cysteine variants (5, 6). That work suggested that the seventh transmembrane segment, TM7, extending roughly between residues 260 and 282, is an amphiphilic α-helix whose hydrophilic face lines the transport pathway. In studies reported here, we sought to determine which residues within and beyond TM7 are available to probes whose sizes limit their sites of action to the external phase. For this purpose, we exploited the protease, trypsin, as well as a biotin-linked maleimide, MPB, whose presence was detected by decoration with streptavidin. We find that residues 283–294 are fully exposed to the bacterial periplasm and provide for the docking and binding of trypsin or streptavidin. By contrast, residue 276 and the more proximal positions of TM7 are more deeply buried or otherwise shielded from the external phase, since they are either inaccessible to MPB, or, if accessible, require cell lysis and protein denaturation before a decoration by streptavidin. This work supports early conclusions regarding UhpT topology and offers physical evidence consistent with the assignment of residues lining the translocation pathway.

EXPERIMENTAL PROCEDURES

Cdls and Plasmids—Phagemid p261 (Ref. 5) was constructed by inserting a 1.8-kilobase Nsi-I BamHI fragment encoding UhpT into plasmid pBluescript II K S+ (Stratagene) in which the multiple cloning region was modified to remove restriction sites duplicated within UhpT. In this construct, UhpT expression is controlled by the lac promoter. Earlier work (5) had described a UhpT variant in which the six natural cysteine residues are replaced by serine residues; this cysteine-less UhpT served as parent to most of the cysteine-substitution mutants used here (6). New mutants (K293C and K294C in the cysteine-less background; R9C, R448C, and a K293N-K294C double mutant, all in wild type background) were obtained by the method of Kunkel (7), as described (5), and were confirmed by sequencing (8). Plasmids were housed in strain RK5000 (9), where chromosomal lac and uhp deletions allow constitutive expression of plasmid-encoded UhpT. Overnight broth cultures were diluted 100-fold in LB medium with ampicillin and harvested after 4–5 h growth at 33 °C.

Trypsin Treatment of Osmotically Sensitized Cells—Freshly grown cells were washed and resuspended in one-fifth volume of 0.5 mM sucrose, 50 mM Tris/HCl (pH 8.0) and placed on ice for 30 min to provide access to external trypsin in strain RK5000; neither EDTA nor lysozyme (10) was required. Freshly prepared trypsin was then added, and incubation was extended an additional 30 min at 30 °C; we normally used trypsin in 25-fold excess (500 μg/ml) of the amount required to give about 50% cleavage of UhpT. The reaction was stopped by adding an equal volume of twice-concentrated acidified SDS-PAGE sample buffer (11).

Labeling with 3-(N-Maleimidylpropiony1)biocytin—Cells were harvested, washed, and resuspended in 20 mM MOPS/KOH, 250 mM KCl, 1 mM MgSO4 (pH 7) (Buffer A) at 1 or 3 μg of protein/ml. For labeling with MPB ( Molecular Probes, Inc.), an aliquot (50 μg of protein) was removed, and cells were pelleted in a microcentrifuge and then resuspended in 60 μl of Buffer A containing 3.3 mM EDTA and MPB at the concentrations specified (solvents were present as 1.8% dimethyl sulfoxide and 1.2% dimethylformamide). After 10 min at 23 °C, 1 ml of a 0.15 M Tris/HCl/1 mM MgSO4 (pH 6.8) buffer A was added, with mixing, and cells were pelleted and resuspended once again in 1 ml of Buffer A. When labeling was to be followed by streptavidin (SA) (Pierce), 0.4–1 μl aliquots were transferred to a fresh tube, and cells were pelleted and resuspended in 20 μl of 50 mM Tris/HCl (pH 6.8) to which was added 20 μg of SA in 2.5 μl of 50 mM Tris/HCl (pH 6.8), or Tris/HCl alone, before incubation at 23 °C for 30 min (a 10-min incubation was sometimes used). The reaction was stopped by adding SDS-PAGE sample buffer with SDS at a final concentration of 0.1% to avoid disruption of the biotin-streptavidin complex (12). Biotin was added from a 0.1% stock prepared in 0.1% sodium phosphate (pH 7.2).

Gel Electrophoresis and Western Blots—SDS-PAGE using 12% or 12.5% acrylamide (13) was done without preheating samples, and for these conditions UhpT migrated at 40–45 kDa. Protein was transferred.
to nitrocellulose membranes as described (14, 15), and UhpT was visualized using antiserum (12, 500) prepared in rabbits by coinmunizion with bovine serum albumin-conjugated peptides corresponding to UhpT residues 141-155 (GSCSYSTIKWTPT) and to the C-terminal 14 residues (RKIREKKIQQLTV). Only reactivity to the C-terminal epitope was evident in Western blots (not shown). To facilitate visualization of the UhpT-SA complex, SA was removed after electrophoresis by immersing the gel, with shaking, in transfer solution (50 mM Tris, 40 mM glycine, 0.08% SDS, in 20% methanol) supplemented with 5 mM biotin. Without this intervention, steric factors prevented reaction of antibody with the UhpT-SA complex, and formation of the complex was reported only by disappearance of immunoreactivity at 40 kDa. Western blots were developed using chemiluminescence (Amersham).

**RESULTS**

A study of the responses of UhpT single-cysteine variants to the impermeant SH-reactive agent, pCMBS, showed that part of TM7 lines the substrate translocation pathway (5, 6). It therefore seemed important to provide direct evidence regarding surface topology in this region. To place limits on the external boundaries of the pathway, we examined the susceptibility of residues in this vicinity to cleavage by protease (trypsin) and to decoration by streptavidin after the modification of single-cysteine variants with a biotin-linked maleimide, MPB.

**Cleavage by Extracellular Trypsin**—The presumed topology of UhpT in the neighborhood of TM7 predicts that the lysines at positions 291 and 294 might be exposed to the periplasm (Fig. 1). This suggestion was tested experimentally by exposing osmotically sensitized cells to external trypsin and following the fate of UhpT by Western blots using an antibody selective for the corresponding single-cysteine mutant; SA, decoration by streptavidin (SA) after modification by MPB; trypsin, cleavage by external trypsin.

**Modification by External 3-(N-Maleimidylpropionyl)biocytin**—To evaluate the accessibility of other residues in the loop connecting TM7 and TM8, shaded residues (Cys-265, Asn-268, Ile-269, Leu-271, Val-273, and Ile-276) were assigned to the translocation pathway in earlier work (5, 6). The notation at the bottom of the figure identifies four residues (Ile-276, Thr-283, Val-284, and Lys-294) that interact with external probes as noted in the text. Key: MPB, modification by 3-(N-maleimidylpropionyl)biocytin of the corresponding single-cysteine mutant; SA, decoration by streptavidin (SA) after modification by MPB; trypsin, cleavage by external trypsin.

**Immobilization of UhpT on Western Blots**—The experimental approach to modify UhpT with biotin and visualize biotin on Western blots was developed using chemiluminescence (Amersham). Cells of cysteine-less UhpT (Parent) and its K291C and K294C derivatives were osmotically sensitized and exposed to buffer (−) or to buffer with trypsin (+) as described under "Experimental Procedures"; the K291C/K294N double mutant (in wild type background) was examined in the same way. Each lane contained 20 μg of cell protein except the rightmost lane, which was loaded with trypsin (29 kDa) equivalent in amount to that used in treatment of cells. Arrows indicate the positions of uncleaved UhpT (40 kDa) and the C-terminal UhpT fragment (about 18 kDa).

**Modification by External 3-(N-Maleimidylpropionyl)biocytin**—To evaluate the accessibility of other residues in the loop connecting TM7 and TM8 (Fig. 1), we probed single-cysteine variants with MPB, a hydrophilic SH-reactive compound of moderate size (600 daltons). Because the association between MPB and a 47-kDa streptavidin derivative (SA) is stable to

**Probes of Residues External to the Transport Pathway in UhpT**

![Fig. 1. Topology in the vicinity of UhpT TM7. The schematic gives the predicted composition of UhpT TM7 and the extracellular loop connecting TM7 and TM8. Shaded residues (Cys-265, Asn-268, Ile-269, Leu-271, Val-273, and Ile-276) were assigned to the translocation pathway in earlier work (5, 6). The notation at the bottom of the figure identifies four residues (Ile-276, Thr-283, Val-284, and Lys-294) that interact with external probes as noted in the text. Key: MPB, modification by 3-(N-maleimidylpropionyl)biocytin of the corresponding single-cysteine mutant; SA, decoration by streptavidin (SA) after modification by MPB; trypsin, cleavage by external trypsin.](image-url)
Probes of Residues External to the Transport Pathway in UhpT

| Variant | Relative expression | Relative activity | Activity remaining after pCMBS |
|---------|--------------------|------------------|-------------------------------|
| Parent  | 1.00               | 1.00             | 0.92 ± 0.05                   |
| K291C   | 0.58               | 0.46             | 0.93 ± 0.03                   |
| K294C   | 0.42               | 0.22             | 0.33 ± 0.04                   |

**TABLE I**

Effect of pCMBS on single-cysteine variants of UhpT

Cells in Buffer A were incubated for 10 min at 23 °C with 200 μM pCMBS before assays of glucose 6-phosphate transport; mean values ± S.E. from three experiments are shown. The relative expression of K291C and K294C mutants was estimated from Fig. 2.

SDS-PAGE (12), residues modified by MPB, if sufficiently exposed, could be detected directly by treating intact cells with SA and using a UhpT-directed antibody to monitor appearance of the UhpT-SA complex.

Initially, we examined MPB reactivity at position 283, since the pCMBS sensitivity of the T283C mutant suggested an accessibility to external probes (6). Accordingly, EDTA-treated cells of the single-cysteine mutant, T283C, were exposed to 100 μM MPB for 10 min at 23 °C. After removing residual MPB, cells were next exposed in varying sequence to each of the following: (i) to SA, to decorate any available MPB; (ii) to excess biotin, to prevent any subsequent reaction between MPB and SA; and (iii) to 0.1% SDS, to lyse cells and denature UhpT. Later examination of Western blots (Fig. 3) showed that when performed in the order noted (first SA, then biotin, finally SDS) there was loss of UhpT reactivity at 40 kDa and appearance of a newly reactive species at 80–90 kDa, the position expected of a 1:1 UhpT-SA complex (Fig. 3, lane 1). At lower yield, a second newly reactive band appeared at about 180 kDa, and this was taken to be a dimer of the UhpT-SA complex. There was no mobility shift when SA was omitted (see later), nor was there a shift when cysteine-less UhpT was examined (not shown). It is of special significance that the reaction between UhpT and SA (via MPB) was not seen when biotin preceded SA (Fig. 3, lanes 3, 4, and 6), as one should expect. Since addition of biotin after SA, but before cell lysis, had no effect on formation of the UhpT-SA complex (Fig. 3, compare lanes 1 and 2), we conclude this complex arises in the intact cell, without UhpT denaturation. It is also evident the UhpT-SA complex can form after protein denaturation (e.g. Fig. 3, lane 5).

In related studies, we confirmed the specificity of MPB’s action at position 283 by showing that MPB modification was blocked by prior exposure to pCMBS (Fig. 4A), with 50% protection at a level (about 10 μM pCMBS) comparable to that giving 50% inhibition of UhpT function (6). We also found that formation of the UhpT-SA complex had an MPB concentration dependence resembling that of MPB inhibition of UhpT activity. For example, assays of transport showed a 50% reduced activity after exposure to 3–10 μM MPB (Fig. 5); Western blots confirmed this same concentration range converted roughly half the UhpT population to its higher molecular weight form (Fig. 4B). Together, such observations (Figs. 3 and 4) lead us to infer that the SA-induced shift in UhpT mobility is a direct reflection of the binding of SA to MPB-modified UhpT.

That SA reports MPB bound to position 283 without UhpT denaturation (Fig. 3) shows that the biotin moiety of MPB extends sufficiently from the modified residue to be enclosed by streptavidin. Since positions closer to the membrane surface might be accessible to the smaller MPB (0.6 kDa), but not the larger SA (47 kDa), we examined other single cysteine variants within and near TM7, focusing on those known to be pCMBS-sensitive (5, 6). For example, on the basis of accessibility to pCMBS from both internal and external surfaces, five positions within TM7 (positions 265, 268, 271, 273, and 276) had been assigned to the translocation pathway (Fig. 1). Four, more distal positions (282, 283, 284, and 286), were classified as external to the pathway, since they responded to only external pCMBS (5, 6). Along with these nine variants, we studied wild type UhpT, whose six cysteines include the two that confer sensitivity to the lipid-soluble probe, p-chloromercuribenzoic acid (Cys-143 and Cys-265) (5), two cysteines presumed to be within the hydrophobic sector (Cys-108 and Cys-331), and a cysteine pair near the C terminus (Cys-436, Cys-438). As well, we tested cysteine substitution mutants in which the targeted position was clearly intracellular, at either the N or C terminus (R9C, R449C). In all these tests we used MPB at concentrations ranging from 5 to 1000 μM, with exposure times of up to 60 min, conditions favorable to modification of position 283 (above), and
to maximize the chance of binding SA to a modified residue, we delayed addition of the reporter protein until after cell lysis (as in Fig. 3, lane 5).

Of the 16 positions examined in this way, only three, 276, 283, and 284, were modified by MPB as judged by inhibition of UhpT function; the parallel tests of decoration by SA (after cell lysis) also identified these positions as the only responsive ones. Each case reflects action of a relatively low probe concentration (50% effects at \( \approx 50 \mu M \) MPB) (Fig. 5, legend), and the order of sensitivity, 284 > 283 > 276, is consistent with the idea that position 276 is the most deeply buried of the targets. We also note that the failure of high MPB concentrations (1 mM) to modify cysteines at the N or C terminus (e.g. R9C, R449C) supports the generally impermeant nature of this probe (and see Ref. 20), since construction of functional UhpT derivatives bearing epitope tags at these termini suggests these regions are not buried.

New experiments showed that while positions 276, 283, and 284 were each readily modified by MPB (e.g. Fig. 5), they were not equivalently accessible to the larger SA (Fig. 6). Position 284 behaved as did 283, in that decoration by SA could be observed in the intact cell. Thus, for both these positions, addition of biotin after SA, but before UhpT denaturation, did not block generation of the UhpT-SA complex (Figs. 3 and 6). Such findings are of significance, since highly expressed membrane proteins need not be uniformly accessible where in the present work we used probes whose accessibility can be visualized directly. This latter strategy has specific benefits. In particular, because both wild type and cysteine-less UhpT were cleaved by extracellular trypsin at Lys-294 (Fig. 2), it is apparent that both forms of the protein have the same general topology and that both fully integrate into the membrane in our overexpression system. A similar conclusion is driven by the observation that after modification by MPB in the intact cell the entire UhpT population is decorated by SA (e.g. Figs. 3 and 4). Such findings are of significance, since highly expressed membrane proteins need not be uniformly accessible at the plasma membrane (17, 18).

**DISCUSSION**

General Conclusions—Part of the translocation pathway through UhpT has been defined by studies of PCMBS inhibition using a collection of single-cysteine variants in and around TM7 (5, 6). Such an approach relies on assays of activity, without reference to unambiguous physical parameters, whereas in the present work we used probes whose accessibility can be visualized directly. This latter strategy has specific benefits. In particular, because both wild type and cysteine-less UhpT were cleaved by extracellular trypsin at Lys-294 (Fig. 2), it is apparent that both forms of the protein have the same general topology and that both fully integrate into the membrane in our overexpression system. A similar conclusion is driven by the observation that after modification by MPB in the intact cell the entire UhpT population is decorated by SA (e.g. Figs. 3 and 4). Such findings are of significance, since highly expressed membrane proteins need not be uniformly accessible at the plasma membrane (17, 18).

MPB Modification and Decoration with Streptavidin—The presence of an external trypsin cleavage site (Lys-294) gives concrete information regarding UhpT topology, a conclusion strengthened by the generation of new cleavage sites when new lysine residues were placed in this vicinity. In principle, one might extend such work to further test UhpT topology, but introduction of basic residues seems improbable as a general method. By contrast, cysteine substitutions are well tolerated in UhpT (6), in the E. coli lactose carrier LacY (19), and in several other membrane proteins (12, 20–24). This, along with the availability of single-cysteine mutants in the neighborhood of UhpT TM7 (6), led us to choose a cystein-directed agent as an additional probe of this region. In turn, the biotin-linked compound, MPB, was selected for its generally impermeant...
nature (Ref. 20 and see above) and because the stability of the bond between biotin and streptavidin makes it possible to detect MPB modification by shifts of SDS-PAGE mobility. Normally, one requires purified materials to detect these mobility shifts (e.g. Refs. 12 and 20), but we avoided this constraint by including excess biotin in the transfer buffer to chase SA from MPB before immunoblotting. Without this intervention, the proximity of SA prevented reaction of UhpT with its antibody, formation of the UhpT-SA complex was marked only by the disappearance of reactivity at 40 kDa. This simplification of technique should make it easy to use MPB as a general probe of other exposed positions on UhpT, or other membrane proteins, without regard to the functional consequence of MPB modification.

Surface Topography in the Neighborhood of TM 7—Four positions (276, 283, 284, and 294) responded to the probes used here (Fig. 1), and these responses allow one to extract information about surface topography. For example, during cleavage by trypsin the enzyme-substrate complex is stabilized by hydrogen bonds in the immediate neighborhood of the scissile bond by trypsin the enzyme-substrate complex is stabilized by hydrogen bonds in the immediate neighborhood of the scissile bond (that is, i ± 2 residues) (25, 26), so at least this short stretch in the target must be sufficiently exposed to interact with protease. Given that sensitivity to extracellular trypsin arises when lysine is placed at UhpT positions 292, 293, or 294, we have modeled these residues as external to the membrane surface (Fig. 1).

In the same way, knowledge of the interaction between biotin and streptavidin supports rational speculation about the exposure of residues available for modification by MPB and decoration by SA. When streptavidin binds biotin, the ligand becomes completely enclosed within a 9Å cleft in the protein (27, 28). As a result, because MPB has a maximum length of 26 Å, if a MPB-modified residue is decorated by SA, there must be no more than 15 Å distance separating the two protein surfaces (e.g. SA and UhpT). Since positions 283 and 284, when modified by MPB, were decorated by SA without protein denaturation (Figs. 3 and 5), these positions, too, must be external to the membrane surface (strictly speaking, within 15 Å of its border with the periplasm). In turn, this supports the view developed by functional studies, which had designated these positions as external to the pathway since they were modified by pCMBS from the external but not the internal surface (6). By contrast, pCMBS modified position 276 from both surfaces, so the behavioral tests identified position 276 as lining the transport pathway. This assignment, too, is consistent with the physical properties revealed here. Thus, the behavior of position 276 was intermediate between that of positions 283 and 284, whose modification by MPB was directly reported by SA, and that of its N-terminal partners along the pathway (265, 268, 271, and 273), none of whom were modified by MPB (or SA). In line with the arguments given earlier, then, the simplest explanation for the accessibility of position 276 to small (MPB, pCMBS) but not to large (SA) probes is that position 276 lies more deeply buried within the protein, effectively 15 Å or more removed from the external phase. If MPB projects at an angle, or if it is not in its linear form, position 276 would be closer to the surface. For this reason, position 276 is modeled as within the hydrophobic sector, compatible with the earlier assessment by functional criteria (6) (Fig. 1). In the aggregate, these experiments offer physical evidence supporting the idea that along TM 7 the external portion of the transport pathway emerges between residues 276 and 282.

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