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The First Cytoplasmic Loop of the Mannitol Permease from *Escherichia coli* is Accessible for Sulfhydryl Reagents from the Periplasmic Side of the Membrane

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The mannitol permease (EIIMtl) from *Escherichia coli* couples mannitol transport to phosphorylation of the substrate. Renewed topology prediction of the membrane-embedded C domain suggested that EIIMtl contains more membrane-embedded segments than the six proposed previously on the basis of a PhoA fusion study. Cysteine accessibility was used to confirm this notion. Since cysteine 384 in the cytoplasmic B domain is crucial for the phosphorylation activity of EIIMtl, all cysteine mutants contained this activity-linked cysteine residue in addition to those introduced for probing the membrane topology of the protein. To distinguish between the activity-linked cysteine and the probed cysteine, either trypsin was used to specifically digest the two cytoplasmic domains (A and B), thereby removing Cys384, or Cys384 was protected by phosphorylation from alkylation by N-ethylmaleimide (NEM). Our data show that upon phosphorylation EIIMtl undergoes major conformational changes, whereby residues in the putative first cytoplasmic loop become accessible to NEM. Other residues in this loop were accessible to NEM in intact cells and inside-out membrane vesicles, but cysteine residues at these positions only reacted with the membrane-impermeable sulfhydryl reagent from the periplasmic side of the protein. These and other results suggest that the predicted loop between TM2 and TM3 may fold back into the membrane and form part of the translocation path.

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Abbreviations used: PTS, phosphoenolpyruvate-dependent transport system; EIIMtl, the mannitol-specific transporter, enzyme IIIMtl; IIAMtl, IIBMtl, IICMtl, A, B and C domains, respectively, of EIIMtl; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; PEP, phosphoenolpyruvate; NEM, N-ethylmaleimide; AMdiS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; PCMB, 4-(chloromercuri)benzenesulfonic acid; dPEG, decyl-polyethylene glycol; APMSE, 4-(amidino-phenyl)-methane-sulfonyl fluoride; DTT, dithiothreitol; TM, transmembrane; CBB, Coomassie brilliant blue; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate bromide; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate.

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Introduction

Enzyme II proteins (EIIis) encompass the substrate-specific, membrane-embedded part of the phosphoenolpyruvate-dependent transport system (PTS). The PTS acts by a mechanism that couples the translocation and phosphorylation of substrates. EIIis usually consist of three functional units, a membrane-embedded substrate translocating domain (IIC) and two cytoplasmic domains (IIA and IIB), which are either subunits of a complex or domains in a single polypeptide. The driving force for the transport is supplied by phosphoenolpyruvate (PEP), which donates its phosphoryl group in a number of steps to the substrate. The phosphoryl transfer is mediated by two general energy coupling proteins, enzyme I (EI) and HPr, and two system-specific proteins or protein domains, IIA and IIB.1,2
Enzyme II\textsuperscript{Mannitol} (EI\textsuperscript{Mtl}) from \textit{Escherichia coli} is a multidomain polypeptide, composed of IIA, IIB and IIC domains. EI\textsuperscript{Mtl} is active as a dimer with the dimer interface situated between the membrane-embedded C domains (Figure 1(a)).\textsuperscript{3,4} The X-ray structure of the IIA domain and the solution structure of the IIB domain of EI\textsuperscript{Mtl} have been solved,\textsuperscript{5,6} and a 5 Å resolution 2D electron microscopy (EM) structure is available for the IIC domain.\textsuperscript{7} The membrane-embedded domain of EI\textsuperscript{Mtl} has been subjected to topological analysis, from which it was concluded that EI\textsuperscript{Mtl} is composed of six transmembrane (TM) segments coupled by short periplasmic loops and two large cytoplasmic loops (Figure 1(b)).\textsuperscript{8–10} On the basis of these studies, the six major densities in the EM structure of IIC\textsuperscript{Mtl} were assigned to the six TM helices. However, there are indications that IIC\textsuperscript{Mtl} might have more than six TM segments, because the surface area of the IIC dimer in the EM structure is larger than typical for a 12 TM \(\alpha\)-helical transport protein.\textsuperscript{11,12} Furthermore, the IIC hydrophobicity plots of different PTS families are similar\textsuperscript{1,13} and most other PTS systems are predicted to span the membrane at least eight times.\textsuperscript{1,13–16}

Here, IIC\textsuperscript{Mtl} was first subjected to renewed hydropathy profiling, and on the basis of the newly predicted models and previous PhoA fusion studies, several cysteine mutants were constructed and used for topological analysis of EI\textsuperscript{Mtl}. The cysteine mutants were subjected to labeling with sulfhydryl-reactive agents and their accessibility and membrane-sidedness were determined.

## Results

### Membrane Protein Topology Prediction

For the initial topology model (Figure 2(a), top), the results were examined manually by inspecting the distribution of positively charged residues.\textsuperscript{19}
fixing the B and A-domain at the cytoplasmic side of the membrane, and assuming that the predicted segments are TM α-helical structures. All programs predicted at least one TM helix more than the original six helix model proposed by Sugiyama et al. (Figure 2(a), bottom). TMHMM, TMpred, and DAS gave two additional TM helices in the former second cytoplasmic loop, HMMTOP and TOPPRED2 only one and SOSUI none. In all cases, one TM helix was predicted in the first cytoplasmic loop (2a in Figure 2(a)). The hydrophobic part of this region is too short to traverse the membrane twice as an α-helix, but, in line with the positive-inside rule for the N and C-terminal flanking sequences, this region could also fold back in the membrane and form a re-entrant loop (Figure 2(b)). To investigate the correctness of the new model, several cysteine mutations were introduced in the formerly predicted first cytoplasmic loop (residues 70–134; helix 2a in Figure 2(a)).

Cysteine mutants

The following residues were chosen for cysteine replacement: Thr85, Val90, Gly91, Ala92, Asp93, and Ser100, which are all part of the predicted helix 2a (Figure 2(b)). Residues 91–93 had intermediate PhoA activity in the Sugiyama study. The positions were chosen to clarify the difference between the old and new topology model. The cysteine mutations were introduced into a mutant of EII\textsuperscript{Mt}, EII SSCS, in which the native cysteine residues were replaced by serine residues and only the activity-linked cysteine 384 was still present. This mutant had wild-type mannitol binding and phosphorylation activity.

McConkey base indicator plates supplemented with 1% (w/v) mannitol were used to check the ability of the mutants to ferment mannitol, cysteine-less EII (EII SSSS) was used as a negative control (white colonies) and wild-type EII\textsuperscript{Mt} was used as a positive control (red) for fermentation. Cysteine-less EII can still bind mannitol, but is unable to phosphorylate and transport mannitol. The phosphorylation activity normalized to the expression levels and the mannitol binding constants of the mutants are presented in Table 1. The phosphorylation activity of most mutants was at least 40% of wild-type except EII SSCS 85C, which only had 24% of wild-type activity; this mutant also had a fivefold lower expression level compared to the other mutants. One of the mutants, although red on McConkey, EII SSCS 100C, had no measurable mannitol binding or phosphorylation activity, but apparently was able to ferment mannitol. This mutant was not used for further analysis. Since most of the other cysteine mutants were not much affected in their binding affinity or phosphorylation activity, we conclude that they must have an overall conformation and membrane topology very similar to SSCS and thus also to the wild-type protein.

<table>
<thead>
<tr>
<th>Enzyme II species</th>
<th>K_D (nM)</th>
<th>S.A. (%)</th>
<th>Mannitol fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>92±15</td>
<td>100±9</td>
<td>Red</td>
</tr>
<tr>
<td>SSSS</td>
<td>137±20</td>
<td>n.a.</td>
<td>White</td>
</tr>
<tr>
<td>CSCS</td>
<td>152±12</td>
<td>98±10</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 85C</td>
<td>&gt; 500</td>
<td>24±5</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 90C</td>
<td>&gt; 500</td>
<td>94±23</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 91C</td>
<td>307±9</td>
<td>79±14</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 92C</td>
<td>48±15</td>
<td>46±5</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 93C</td>
<td>129±18</td>
<td>48±6</td>
<td>Red</td>
</tr>
<tr>
<td>SSCS 100C</td>
<td>–</td>
<td>&lt; 1</td>
<td>Red</td>
</tr>
</tbody>
</table>

The mannitol-dissociation constants (K_D values) were determined with the flow dialysis technique by titration of EII\textsuperscript{Mt} in inside-out membrane vesicles with small quantities of [\textsuperscript{3}H]mannitol. The specific phosphorylation activity was determined by measuring the amount of mannitol-phosphate formed in time, and taking into account the expression levels of the various mutants. The expression level was calculated from the n_{max} of mannitol binding in the flow dialysis experiments. For the phosphorylation activity, [\textsuperscript{3}P]mannitol was used at a concentration of 1 mM; 100% corresponds to a specific activity of 1500 min\(^{-1}\) (± values indicate the standard deviation in triple experiments); n.a., not applicable.

Phosphoprotection and trypsin digestion of EII\textsuperscript{Mt}.

In order to distinguish between labeling of the activity-linked cysteine 384 in the B domain and the target cysteine residues in the C domain, two different strategies were used. Phosphorylation of EII\textsuperscript{Mt} was used to specifically protect cysteine 384 against labeling by sulfhydryl reactive agents. The basis for the protection is the continuous phosphorylation of EII\textsuperscript{Mt} by a high concentration of histidine-containing phosphocarrier protein (HPr) and PEP in the presence of EII. In this way cysteine 384 could be protected from N-ethylmaleimide (NEM) labeling for at least one hour (Figure 3). After labeling, the phosphoryl group can be removed by the addition of mannitol, which is then converted to mannitol-1-phosphate.

To determine the accessibility of the cysteine residues in the unphosphorylated or mannitol-bound protein, a second strategy was used. The resistance against trypsinolysis of IIC\textsuperscript{Mt} was used to distinguish between labeling of cysteine 384 in the B domain and target cysteine residues in the C domain. Ten minutes of trypsin (2 µg/ml) digestion was sufficient to completely remove the A and B domain of EII\textsuperscript{Mt} (2 µM), that is, when the enzyme was in the unphosphorylated or in the mannitol-bound form (Figure 4(a)). Phosphorylation of purified EII\textsuperscript{Mt}, on the contrary, stabilized EII\textsuperscript{Mt} (Figure 4(b)) and the A and B domains were largely protected from proteolytic breakdown, suggesting that the structure of P-EII\textsuperscript{Mt} is more compact than that of the unphosphorylated enzyme.

In inside-out membrane vesicles or whole cells, it was not possible to completely phosphoprotect cysteine 384 under the labeling conditions used.
Therefore, labeling of inside-out membrane vesicles and whole cells was only performed with dephosphorylated EIIMtl and labeling of the C domain was examined after trypsin digestion.

Boer et al.22 showed that IICMtl migrates on SDS/polyacrylamide gels as a double band, one with high and one with low intensity, around 30 kDa. This double band most likely represents two different conformations of IICMtl. The IIC domain obtained after trypsin digestion showed the same behavior. On SDS-PAGE there was always a sharp and a fuzzy band present (Figures 4–6), but the relative amounts varied from experiment to experiment.

\[ {\text{[14C]NEM}} \text{labeling of the purified cysteine mutants} \]

Wild-type EIIMtl has four cysteine residues per subunit, two in the C domain (110, 320), one in the B domain (the activity-linked cysteine 384) and one in the A domain (571). Roossien et al.23 and Pas et al.21 showed that labeling of dephosphorylated EIIMtl with the thiol-specific reagent NEM resulted in incorporation of one NEM-label per monomer (cysteine 384) and in loss of catalytic activity. They also showed that phosphorylation of EIIMtl fully protected the activity, but one NEM-label per polypeptide chain was still incorporated under these conditions. Using single-cysteine mutants of a His-tagged C domain, we determined that, of the two cysteine residues present in the C domain, only cysteine 110 became labeled by NEM (data not shown). It is this cysteine 110 that is also accessible for NEM when EIIMtl is phosphorylated. Cysteine 110 was not labeled by NEM in the presence of mannitol (Table 2, Figure 5(a)), indicating that the accessibility of this residue is not determined by conformational changes caused by substrate binding.

Alkylation with \[ {\text{[14C]NEM}} \] of a cysteine-less EIIMtl, EI-SSSS, did not result in incorporation of \[ {\text{[14C]NEM}} \], implying that there was no aspecific labeling (results not shown). \[ {\text{[14C]NEM}} \] treatment of EI SSCS, which only has the activity-linked cysteine 384 in the B domain, resulted in \[ {\text{[14C]NEM}} \] labeling of the cysteine under unphosphorylated and under mannitol-bound conditions. As expected, after phosphoprotection cysteine 384 could no longer be labeled and no alkylation was found in the C domain (Figure 5(a)).

The C domain of wild-type EIIMtl and EI-SCCS, the latter with only the native cysteine 110 present
in the C domain, were not labeled in the unphosphorylated or in the substrate-bound state under the conditions used. The C domain was labeled when the proteins were phosphorylated or the IIBA domains were removed (Table 2, Figure 5(a)). In the unphosphorylated or in the substrate-bound protein residues 85 and 90 could not be labeled by [14C]NEM. After phosphorylation of Cys384, these positions, like 110, became readily accessible for [14C]NEM. Removal of the A and B domains by trypsinolysis also made the cysteine residues at postions 85 and 90 accessible for [14C]NEM (Table 2, Figure 5(b)). The positions 85, 90 and 110 were also checked for their accessibility towards MTSEA, MTSET and MTSES, since these reagents are known to react faster than maleimides and do not require the sulfhydryl to be deprotonated. The mutants were also not labeled by any of these reagents (results not shown), indicating that the positions 85, 90 and 110 are buried in the unphosphorylated protein. The three residues with intermediate PhoA activity (91–93) were always accessible for [14C]NEM. These results indicate that the unphosphorylated B domain hinders labeling of residues 85, 90 and 110, but not of residues 91–93. Furthermore, upon phosphorylation of the A and B domain, EIIMtl undergoes conformational changes that alter the accessibility of residues 85, 90 and 110.

**Sulfhydryl labeling of EIIMtl mutants in inside-out membrane vesicles and whole cells**

The accessibility of the cysteine mutants towards membrane-permeable and impermeable sulphydryl-reactive labels were tested in inside-out membrane vesicles and whole cells. NEM was used as a membrane-permeable, sulphydryl-reactive label to determine the accessibility of the residues in the membrane, and the impermeable maleimide, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AmdiS), and mercury derivative, 4-(chloromercuri)-benzenesulfonic acid (PCMBS), were used to determine the sidedness of the residues. Labeling with these agents was visualized after purification of the proteins and subsequent treatment with [14C]NEM. It is important to keep in mind that cysteine residues labeled by the permeable or impermeable reagents do not give a signal on the
phosphorimager screen, whereas non-reacted cysteine residues give a signal after $^{14}$C]NEM labeling.

The results of these experiments are shown in Figures 6 and 7, and are summarized in Table 3. In inside-out membrane vesicles the activity-linked cysteine 384 was labeled by both the permeable and impermeable reagents, whereas it could not be labeled by AMdiS or PCMBs in whole cells, in accordance with its cytoplasmic position. Under the conditions used, residue 110 in the wild-type EIIMtl protein could still react with $^{14}$C]NEM after purification and thus was not labeled by NEM in inside-out vesicles (Table 3 and Figure 6). However, Cys110 was labeled by NEM in whole cells (Table 3 and Figure 7), most likely because EIIMtl was kept (at least part of the time) in the phosphorylated state. The phosphorylation potential in intact cells was insufficient to protect Cys384 completely from labeling by NEM.

In inside-out membrane vesicles only residues 85 and 90 were not accessible for NEM, in line with the results obtained with the purified proteins. Furthermore, residues 91–93 were inaccessible for both membrane-impermeable reagents (Table 3 and Figure 6). In whole cells, residue 85 was only accessible for NEM, while residue 90 was accessible for NEM and PCMBs. Thus, also in this experiment, the accessibilities of cysteine residues 85 and 90 seem to increase when the A and B domain are phosphorylated. Due to the low expression level of mutant EII SSCS 85C, relatively little protein was isolated in the whole cell labeling experiment and the SDS-PAGE gel had to be silver-stained to visualize the protein. This is also the cause for the low intensity of the radioactive protein bands on the phosphorimager plate. However, multiple ($n \approx 4$) independent labeling experiments with this mutant consistently indicated that a cysteine residue at position 85 is only accessible to NEM. Of the three residues with intermediate PhoA activity, only residue 93 was accessible for AMdiS and PCMBs in whole cells. The other two, 91 and 92, were only accessible for the smaller impermeable reagent, PCMBs (Table 3 and Figure 7).

### Discussion

Hydropathy profiling of EIIMtl, using the latest algorithms available on the internet, showed that the membrane-embedded part of EIIMtl may comprise more than six TM segments. Other indications also point in this direction. The 5 Å resolution 2D EM structure of IICMtl revealed a dimer with axes of about 40 Å and 90 Å. Other electron microscopy studies indicate that this size is considerably larger than expected for a 12 TM helical protein. These comparisons suggest that the membrane-embedded part of EIIMtl could be composed of more...
than six putative z-helices. Furthermore, IIC\textsuperscript{Mt} has a hydropathy profile similar to that of IIC\textsuperscript{Glc} \cite{1,13} and a reporter fusion study by Buhr and Erni \cite{13} suggested that IIC\textsuperscript{Glc} contains eight membrane spanning segments. The possible explanation given by Buhr and Erni for the discrepancy between the membrane topologies of EIIM\textsuperscript{Mt} and EII\textsuperscript{Glc} is that the medium PhoA activity of the fusion at residue 92 of EIIM\textsuperscript{Mt} reflects a truly periplasmic location and not an artifact as was proposed by Sugiyama \textit{et al.} \cite{10}. However, this explanation does not take into account that then either the N or C-terminal helices would change orientation. This would conflict with the positive-inside rule. It would also result in residues with high PhoA activity moving to the cytoplasmic side and residues with low PhoA activity to the periplasmic side. An alternative explanation would be the presence of a turn region around residues 91–93, allowing the predicted TM a-helix, we propose.

The labeling results of this study show that residues 91–93 are accessible from the periplasmic side of the membrane. Van Montfort \textit{et al.} \cite{25} showed that residue 124 is in close proximity to cysteine 384 of the B domain and thus close to the cytoplasmic face of the protein. Given the constraint of the most likely cytoplasmic location of cysteine 124 and the fact that the hydrophobic segment in the first cytoplasmic loop is not large enough to traverse the membrane twice as a TM a-helix, we propose that the first cytoplasmic loop folds back into the membrane. The sequence around proline 95 could form the turn structure. \textit{(Figure 2(b)).}

The prediction programs also suggested two TM segments in the second cytoplasmic loop of EIIM\textsuperscript{Mt}. In the PhoA fusion study this loop yielded low PhoA activities, indicating a cytoplasmic orientation. However, a low fusion activity is not always a clear argument for a cytoplasmic position, because sequences around the fusion point may determine the proper localization of the TM segment. \cite{27–29} In the topology prediction of IIC\textsuperscript{Glc}, the region equivalent to the second cytoplasmic loop of EIIM\textsuperscript{Mt} is localized in the membrane. \cite{13} Experiments with the cysteine 238 mutant show that this residue is labeled by impermeable agents from both sides of the membrane. These data suggest that the predicted second cytoplasmic loop of EIIM\textsuperscript{Mt} may form a re-entrant loop or be part of an aqueous channel that allows residue 238 to be accessible from the cytoplasmic and the periplasmic side of the membrane.

The resistance of the C domain towards trypsin digestion also argues against large and accessible cytoplasmic loops. There are several tryptic sites on either side of the putative membrane-embedded parts of the predicted first and second cytoplasmic loop, but none of these is cleaved in our experiments. The increased trypsin resistance of EIIM\textsuperscript{Mt} in the phosphorylated state suggests strong interactions between the B and C domain, when B is phosphorylated. Also the fact that residues 85, 90 and 110 in the C domain are only accessible when the B domain is phosphorylated points to interactions between the two domains. These observations are in line with previous studies. Firstly, Lolkema \textit{et al.} \cite{30} showed that phosphorylation of the B domain leads to an increase in the rate of mannitol transport at the C domain. Secondly, a differential scanning calorimetry study by Meijberg \textit{et al.} \cite{31} showed that the stability of IIC is decreased in the absence of IIB. Thirdly, isothermal titration calorimetry (ITC) studies showed that up to 60 residues in the B and C domain become buried upon mannitol binding or phosphorylation. \cite{32} These results, together with the changing accessibility of residues 85, 90 and 110, indicate that the C domain undergoes conformational changes upon phosphorylation of the B domain.

This study has contributed to a refined membrane topology model for EIIM\textsuperscript{Mt}, which we believe is also relevant for other PTSs. Without high-resolution structural data it is difficult to determine the exact structure of the regions that previously were
thought to be cytoplasmic loops. We propose that at least the region between TM2 and TM3 folds back in the membrane.

Materials and Methods

Chemicals

Decyl-polyethylene glycol (dPEG) was synthesized by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). Ni2+-nitrilotriacetic acid agarose (Ni-NTA-agarose) was from Qiagen. NEM was from Fluka and [14C]NEM (1.3 GBq/mmol) from NEN. AMdiS was from Molecular Probes and PCMBs from Toronto Research Chemicals Inc. Trypsin and the trypsin inhibitor (4-amidino-phenyl)-methane-sulfonyl fluoride (APMSF) were obtained from Sigma. [3H]mannitol (629 GBq/mmole) was from PerkinElmer (Boston, USA) and [14C]mannitol (59 mCi/mmole) from Amersham Biosciences (Uppsala, Sweden). All of the chemicals used were analytical grade.

Site-directed mutagenesis

Mutations in EIIMtl were generated by PCR with the QuikChange site-directed mutagenesis kit from Stratagene. A single cysteine-containing mannitol transporter with a C-terminal six His-tag, designated EIIMtlAPr-SSCS-6H, was generated by replacing cysteine 110, 320 and 571 for serine in the plasmid pMamntlAPr-6H, which codes C-terminal His-tagged wild-type EIIMtl (E. P. P. Vos, unpublished results). The single cysteine-containing plasmid, pMamntlAPr-SSCS-6H was used as template for additional cysteine mutations. The introduced mutations were identified by restriction analysis of silent sites and confirmed by DNA sequence analysis. The cysteine-less mutant of EIIMtl is denoted EII SSSS, whereas all of the various cysteine mutations in EIIMtl are indicated as EIIMtl followed by the residue number of the mutation site.

Characterization of the cysteine mutants

Fermentation, indicative of mannitol uptake via EIIMtl, was measured in vivo by plating the cells on McConkey base indicator plates supplemented with 1% (w/v) mannitol and 100 µg/ml of ampicillin. Wild-type EIIMtl and the inactive EIIMtl SSSS were used as a positive and negative control, respectively, for the red/white screening of the colonies on a plate. The phosphorylation activity of the cysteine mutants was determined in inside-out membrane vesicles with and without dPEG as described.35 The mannitol-binding constant and the ElIMtl concentration in the vesicles were determined by flowdialysis as described by Veldhuis et al.34

Preparation of inside-out membrane vesicles

E. coli LGS322 (F- thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, gatG6, gatR49, gatA50, Δ(mtl2A)p, mtlD2, Δ(gutK’MDBA-recA))35 cells carrying pMaMtlAPr-SSCS 6H derivatives were grown at 30 °C in LB medium (10 g/l of tryptone, 10 g/l of NaCl, 5 g/l of yeast extract), supplemented with 100 µg/ml of ampicillin. Protein expression was started at an absorbance of 0.6 at 300 nm of 0.6 by raising the temperature to 42 °C and the cells were harvested by centrifugation after two hours of induction.36 Inside-out membrane vesicles were prepared as described.37

Labeling of EIIMtl mutants in whole cells

Cells were washed and resuspended in 50 mM KPi (pH 7.5), to an absorbance of 600 nm of 100. Aliquots of 0.5 ml were used for labeling with either 5 mM NEM, AMdiS or PCMBs (30 minutes, 30 °C). Unlabeled cells were used as a control. Unreacted label was removed by centrifugation and the labeled cells were resuspended in 1 ml of 50 mM KPi (pH 7.5), plus 1 mM PMSF. The cells were kept overnight on ice and the following day 1 mM MgCl2, and approximately 0.1 mg/ml each of DNase and RNase was added.

Cell extracts were prepared by lysing the cell suspension with a glass bead beater (FastPrep®m, power 6 for 20 seconds using 300 mg of glass beads (<100 µm, Sigma)). Glass beads and broken cells were removed by low-speed centrifugation (23,000g; ten minutes at 4 °C). The membrane vesicles were spun down by high-speed centrifugation (393,000g; 30 minutes at 4 °C) and solubilized in 1.5 ml of solubilization buffer (25 mM Tris–HCl (pH 7.5), 2% (w/v) dPEG, 50 mM NaCl, 5 mM imidazole). EIIMtl was purified in the absence of β-mercaptoethanol and labeled with [14C]NEM as described in the following paragraphs.

Labeling of EIIMtl mutants in inside-out membrane vesicles

Inside-out membrane vesicles containing EIIMtl mutants were washed with 25 mM Tris–HCl (pH 7.5), 5 mM DTT, 20 mM Na-EDTA and resuspended in 1 ml of the same buffer. The membrane vesicles were incubated for ten minutes at 30 °C, and excess DTT was removed via an NAP™ 10 column (Amersham Biosciences). To the vesicles (300 µl aliquots), 5 mM NEM, AMdiS or PCMBs or the same volume of water was added and the membrane vesicles were labeled for 30 minutes at 30 °C. After the labeling the unreacted reagent was removed on a Sephadex G50 column.

Next, EIIMtl was purified from the membrane vesicles as described in the following paragraph, except that β-mercaptoethanol was omitted from all buffers. To minimize oxidation of non-labeled cysteine residues, the purified proteins were immediately subjected to labeling with 0.2 mM [14C]NEM for 20 minutes at 30 °C in the absence and presence of 5 µg/ml of trypsin. Trypsin digestion was stopped after ten minutes at 30 °C by the addition of 1 mM trypsin inhibitor (APMSF). Finally, 5 mM β-mercaptoethanol was added to reduce unreacted [14C]NEM and the samples were analyzed on SDS-PAGE.

Purification of EIIMtl

EIIMtl was purified from inside-out membrane vesicles after fivefold dilution with solubilization buffer (25 mM Tris–HCl (pH 7.5), 2% dPEG, 1 mM β-mercaptoethanol, 50 mM NaCl, 5 mM imidazole) and incubation at room temperature for ten minutes after which the non-solubilized fraction was spun down (393,000g; 15 minutes at 4 °C). The solubilized EIIMtl was mixed with Ni-NTA-agarose (150 µl per 1 ml of solubilization buffer) and allowed to bind for one hour at 4 °C under mild rotation. The Ni-NTA-agarose was washed with ten column volumes of buffer, containing 50 mM Tris–HCl (pH 7.5), 0.5% dPEG, 1 mM β-mercaptoethanol, 150 mM NaCl, 20 mM imidazole, and subsequently ten volumes of wash...
buffer without imidazole. EIIMtl was eluted in 50 mM Tris (pH 7.5), containing 0.25% dPEG, 1 mM β-mercaptoethanol, 200 mM imidazole. The fractions containing the enzyme were pooled and NaCl was added to a final concentration of 150 mM. The protein concentration was determined by absorption at 280 nm, using a molar extinction coefficient of 31,190 M⁻¹ cm⁻¹.

**Labeling of EIIMtl with NEM**

Prior to labeling, purified EIIMtl was diluted to 2 µM and subsequently treated with 1 mM β-mercaptoethanol for ten minutes at 30 °C. β-Mercaptoethanol was removed by passage of the EIIMtl-containing mixture over a Biospin P-6 column (Biorad) equilibrated with 25 mM NaPi (pH 7.5), 0.25% dPEG. Samples were labeled with 0.2 mM [¹⁴C]NEM for 20 minutes at 30 °C, and the labeling was stopped by adding 5 mM β-mercaptoethanol. In some experiments the labeling was preceded by passage of the EIIMtl-containing mixture over a Biospin P-6 column for ten minutes at 30 °C. The labeling was preceded by passage of the EIIMtl-containing mixture over a Biospin P-6 column (Biorad) equilibrated with 25 mM NaPi (pH 7.5), 0.25% dPEG. Samples were labeled with 0.2 mM [¹⁴C]NEM for 20 minutes at 30 °C, and the labeling was stopped by adding 5 mM β-mercaptoethanol. In some experiments the labeling was preceded by incubation of EIIMtl with 1 mM mannitol for ten minutes at 30 °C, protection of cysteine 384 by phosphorylation, or digestion of the enzyme by trypsin (see below).

**Labeling of EIIMtl with MTS-reagents**

After purification, EIIMtl was diluted to 2 µM in 50 mM Tris–HCl (pH 7.5), containing 0.25% dPEG and 150 mM NaCl. β-Mercaptoethanol carried over from the elution buffer was removed by passage of the mixture over a Biospin P-6 column (Biorad). Samples were labeled for 20 minutes at 30 °C with 1 mM MTSEA, MTSET or MTSES. Unreacted label was removed by a second passage over a Biospin P-6 column. Subsequently, part of the samples were treated with trypsin (0.02 mg/ml; ten minutes at 30 °C). The digestion was stopped by addition of 1 mM APMSF (ten minutes at 30 °C). To all samples, 0.2 mM [¹⁴C]NEM was added and after 20 minutes at 30 °C, 10 mM β-mercaptoethanol was added to reduce the free label.

**Phosphoprotection of EIIMtl**

Before purification, EIIMtl was diluted to 2 µM in 50 mM Tris–HCl (pH 7.5), containing 0.25% dPEG and 150 mM NaCl. β-Mercaptoethanol was removed by passage of the mixture over a Biospin P-6 column (Biorad). Samples were labeled for 20 minutes at 30 °C with 1 mM MTSEA, MTSET or MTSES. Unreacted label was removed by a second passage over a Biospin P-6 column. Subsequently, part of the samples were treated with trypsin (0.02 mg/ml; ten minutes at 30 °C). The digestion was stopped by addition of 1 mM APMSF (ten minutes at 30 °C). To all samples, 0.2 mM [¹⁴C]NEM was added and after 20 minutes at 30 °C, 10 mM β-mercaptoethanol was added to reduce the free label.

**Tris-protection of EIIMtl**

Prior to the Biospin column, EI (0.5 µM) and EIIMtl (2 µM) were mixed and treated with 1 mM β-mercaptoethanol. The β-mercaptoethanol was removed on a Biospin P-6 column. Subsequently, EIIMtl was phosphorylated by the addition of 10 µM HPr, 1 mM MgCl₂, 10 mM PEP and incubation for ten minutes at 30 °C.

**Trypsin digestion of EIIMtl**

Trypsin digestion was performed on purified EIIMtl (wild-type or mutants) to obtain the corresponding IIC protein. Trypsin (2.0 µg/ml, final concentration) from a frozen stock (0.2 mg/ml) was added to the purified EIIMtl and incubated for ten minutes at 30 °C. The digestion was stopped by addition of 1 mM APMSF.

**Visualization of labeled protein**

Purified protein was run on an SDS/12.5% (w/v) polyacrylamide gel and protein bands were visualized with either Coomassie brilliant blue or by silver-staining. After staining, the gels were dried and autoradiograms were made with a Phosphorimagery (Molecular Dynamics). The autoradiograms were analyzed with the Imagequant Software from Molecular Dynamics.

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**References**


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