The Mannose Transporter of *Escherichia coli*

STRUCTURE AND FUNCTION OF THE IIAB\textsuperscript{Mac} SUBUNIT*

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The bacterial phosphotransferase system (PTS)\(^1\) comprises a group of proteins which mediate uptake of carbohydrates and in response to it regulate activities of the intermediate metabolism (for reviews, see Postma and Lengeler (1985) and Meadow *et al.* (1990)). The vectorial transport of the substrates is coupled to their phosphorylation (group translocation). Phosphoryl donor to the PTS is P-enolpyruvate, an intermediate formed during the glycolytic breakdown of carbohydrates. Phosphoryl groups are sequentially transferred from P-enolpyruvate to the different transporters via two cytoplasmic proteins enzyme I and HPr. *Escherichia coli* expresses between five and 8 PTS transporters of different substrate specificity and subunit composition (for review, see Erni (1992)). The bacterial PTS transporters can be grouped into families according to amino acid sequence, subunit composition, and the chemical nature of the phosphorylation site (histidine or cysteine). The transporters for mannone of *E. coli*, for sorbose of *Klebsiella pneumoniae*\(^2\) and for fructose of *Bacillus subtilis* (Martin-Verstraete *et al.*, 1990) form one such family. They are approximately 70% similar in amino acid sequence.

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The mannose transporter of the bacterial phosphotransferase system consists of two transmembrane subunits (IIIC\textsuperscript{Man} and IID\textsuperscript{Man}) and a hydrophilic subunit (IIAB\textsuperscript{Man}). IIAB\textsuperscript{Man} has two flexibly linked domains containing one phosphorylation site each and occurs as a dimer. Substrate transport is coupled to phosphorylation. The phosphoryl group is transferred from a phosphoryl carrier protein to His\textsuperscript{10} on IIA, hence to His\textsuperscript{176} on IIB and finally to the substrate. IIAB\textsuperscript{Man} mutants were analyzed *in vitro* for complementation, negative dominance, cysteine cross-linking and reactivity. Conclusions: (i) His\textsuperscript{10}, Trp\textsuperscript{12}, Lys\textsuperscript{18}, and Ser\textsuperscript{72} form a functional unit (phosphorylation site 1); (ii) His\textsuperscript{66} on the IIA domain and His\textsuperscript{176} on the IIB domain of the same subunit form a functional unit (phosphorylation site 2); (iii) phosphoryl transfer can occur between His\textsuperscript{10} and His\textsuperscript{176} of the same as well as different subunits and His\textsuperscript{66} is necessary for this transfer; (iv) the subunits in the dimer are interdependent; (v) The phosphorylation site mutant H10C is highly reactive toward thiol reagents and it forms extensive homodimers and heterocross-links with other surface-exposed cysteines. The phosphorylation site mutant H10C is 1000-fold less reactive. The two residues might be in complementary locations, His\textsuperscript{10} buried in a concave, His\textsuperscript{176} exposed on a convex surface.

The Abbreviations used are: PTS, phosphoenolpyruvate-sugar phosphotransferase system; P-enolpyruvate, phosphoenolpyruvate; IIAB\textsuperscript{Man}, hydrophilic subunit of the mannose transporter; IIIC\textsuperscript{Man}, amino-terminal domain of IIAB\textsuperscript{Man}; IID\textsuperscript{Man}, carboxy-terminal domain of IIAB\textsuperscript{Man}; IIC\textsuperscript{Man} and IID\textsuperscript{Man}, transmembrane subunits of the mannose transporter; IIA, cytoplasmic subunit of the glucose transporter; IIBC\textsuperscript{Man}, transmembrane subunit of the glucose transporter; HPr, histidine-containing phosphoryl carrier protein of the PTS; DTNB, 5,5'-dithiobis(nitrobenzoic acid); manXYZ, genes encoding IIAB\textsuperscript{Man}, IIC\textsuperscript{Man}, and IID\textsuperscript{Man}; MOPS, 4-morpholinepropanesulfonic acid, kb, kilobase; bp, base pair.

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2 J. Lengeler, personal communication.
the subject of this communication.

IIAB\textsuperscript{mas} consists of two domains, IIA\textsuperscript{man} and IIB\textsuperscript{man}, which are linked by an Ala-Pro-rich hinge peptide. The IIA and IIB domains each contain one phosphorylation site. In the cascade phosphorylation reactions, IIA\textsuperscript{mas} is phosphorylated on His\textsuperscript{10} by the cytoplasmic phosphoril carrier protein phospho-protein. The phosphoryl group is then transferred from His\textsuperscript{10} to His\textsuperscript{170} of the IIB\textsuperscript{man} domain and hence to the transported sugar. IIB\textsuperscript{mas} forms a dimer in solution which is stabilized by contacts between the IIA domains (Erni et al., 1989). The IIB domain which does not dimerize by itself binds to the transmembrane subunits (Fig. 1). IIB\textsuperscript{mas} can be split into its two domains, in vitro by limited trypic digestion, and in vivo by gene reconstruction (Erni et al., 1989). The separated domains are stable. They functionally complement each other in vitro and in vivo. Crystals of IIB\textsuperscript{mas} suitable for x-ray diffraction analysis have been obtained (Génévois-Taverne et al., 1990), and the secondary structure of the IIB\textsuperscript{mas} dimer has been determined by heteronuclear three-dimensional NMR spectroscopy.\textsuperscript{2}

Here we show that phosphorylation sites on different subunits in the dimer interact, that the subunits function cooperatively and that phosphorylation affects the stability of the dimer. Negative dominance as well as interdomainal complementation between a number of site-specific IIB\textsuperscript{mas} mutants was tested in vitro and in vivo. Cysteines were introduced in the two phosphorylation sites of IIB\textsuperscript{mas} and in other locations presumed to be close to the surface of the protein. They were chosen based on algorithms that predict antigenicity, hydrophilicity, and flexibility of peptide segments (GGC package, version 7, 1991, from the Genetics Computer Group, Wisconsin, Madison, WI). The cysteine mutants were also used to probe the accessibility of the mutated residues using 5',5'-dithiobis(nitrobenzoic acid) (DTNB) and to map the distance between sulphydryl groups on different subunits by oxidative cross-linking (Falke and Koshland, 1987). Where appropriate additional mutations of other residues were also considered.

MATERIALS AND METHODS

Media, Strains, and Plasmids—E. coli K12 strains UT580 and WA2127 have been described (Erni et al., 1987). UT580 is wild type with respect to the pis genes, and WA2127 is manX Y Z. In strain WA2127AH, the genes ptaHI and ccr encoding enzymes I, II, and IIIa are deleted. Plasmids pMa5-8 and pMC5-8 are described in Stanssens et al. (1988). Plasmid pTSMP6 is a pACYC177 derivative encoding manY and manZ (Erni et al., 1987). The expression plasmid pF119E\textsuperscript{man} contains lac\textsubscript{I}, pIB, and a polylinker (Fürst et al., 1986). Cells were grown at 37°C in LB medium containing appropriate antibiotics.

Plasmid Construction—Standard procedures (Sambrook et al., 1989) were used for restriction analysis, ligations, and the preparation of plasmid and single-stranded DNA. Plasmid pMal, which was used for screened-duplex mutagenesis was constructed as follows. Plasmid pTSLM4 (Erni et al., 1987) which contains manX, manY, and manZ was linearized with BamHI, restricted ends were filled using Klenow polymerase, and the product was cut with EcoR1. The 1.9-kb fragment containing manX and a fragment of manY was inserted into the pMal vector opened at EcoR1 and HindIII (blunted) in the polylinker. The inserted fragment contained 500 bp of 5'-non-coding sequence. A 820-bp EcoR1/HindIII fragment containing these 500 and 320 bp of the manX coding sequence was exchanged against a 490-bp fragment from plasmid pTacL293 (DeMeyer, 1992) containing only 93 bp of 5'-non-coding sequence. The 93-bp upstream sequence, manX and part of manY were flanked by two unique restriction sites, EcoR1 and Xbal, respectively, which subsequently were used for cloning of the mutated manX genes back into the expression vector pF119E\textsuperscript{man}.

Site-directed Mutagenesis—The gapped duplex procedure was applied as described by Stanssens et al. (1988). Mutant clones were identified by DNA sequencing. Dideoxy sequencing was carried out as specified by the manufacturer (U. S. Biochemical Corp.).

Overproduction and Purification of Proteins—E. coli WA2127AHIC was transformed with the expression vectors pF119E\textsuperscript{man} encoding the mutated IIB\textsuperscript{mas}. Cells from overnight cultures were diluted 1:100 into fresh LB medium, and when the culture had reached 0.5opt, isopropyl-β-D-galactopyranoside was added to 100 μM. Incubation was continued for 16 h. Cells from 6 liters of culture were collected by centrifugation, resuspended in a buffer containing 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, 0.5 mM dithiorethiol, and 100 μg/ml phenylmethylsulfonyl fluoride, and ruptured by passing through a French pressure cell. Cell debris were sedimented by low speed centrifugation, and the membrane fraction was removed by ultracentrifugation. Protamine sulfate was added to the supernatant (final concentration of 0.33%). The clarified supernatant was applied to a phosphocellulose column (P11, Whatman Biosystems Ltd.) and eluted with a 0-2 M NaCl gradient. The IIB\textsuperscript{mas}-containing fractions were pooled and concentrated by amomionic salt precipitation (80%), and IIB\textsuperscript{mas} was further purified by gel filtration with Sephacryl S-100 (Pharmacia LKP Biotechnol-
TABLE I

**In vitro phosphorylation activities of IIAB\textsuperscript{Man}**

The assay conditions are described under "Materials and Methods." Phosphorylation activities are in (nmol of dGlc/pmol of protein/30 min). The colony appearance on McConkey mannose plates is indicated as red, white or white with a red center (r.c.).

<table>
<thead>
<tr>
<th>Mean ±S.E. of mean</th>
<th>No. of assays</th>
<th>Fermentation/colony type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IIAB\textsuperscript{Man} w.t.</strong></td>
<td>16.6 ± 0.9</td>
<td>6</td>
</tr>
<tr>
<td>H10C</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>H86N</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>H175C</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>H219Q</td>
<td>0.7</td>
<td>1</td>
</tr>
<tr>
<td>H10,175C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K48C</td>
<td>8.9</td>
<td>1.6</td>
</tr>
<tr>
<td>S72C</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>S110C</td>
<td>11.5</td>
<td>1.7</td>
</tr>
<tr>
<td>W12F</td>
<td>0.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

mutants H10Q, H86N, H175N, and H219Q where the His is replaced by Gln and Asn (Erni et al., 1989).\(^1\) His\(^{66}\) and His\(^{75}\) are the phosphorylated residues in the active sites of the IIA and IIB domains (Erni et al., 1989). His\(^{66}\) and His\(^{75}\) are not phosphorylated. TrpL2 is close to one active site. Lys\(^{66}\), Ser\(^{72}\) and Ser\(^{110}\) are supposed to be on the surface of the IIA domain.

All IIAB\textsuperscript{Man} mutants could be overexpressed like the wild-type protein as judged from Coomassie Blue-stained polyacrylamide gels (not shown). This indicates that the mutations do not disturb the folding or the stability of the protein. CD spectra of H10C, W12F, K48C, S72C, S110C, and H175C do not differ from the CD spectrum of wild-type protein. Moreover, the purified mutants H10C, H175C, H10C/H175C, and wild-type protein showed identical biphasic change in ellipticity during unfolding in guanidinium hydrochloride.\(^5\)

Four mutants, however, behaved atypically during purification. In contrast to wild-type IIAB\textsuperscript{Man}, H175C, H175N, H86N, and H10C/H175C did not bind well to phosphocellulose at pH 7.0. Because binding to phosphocellulose is a property of the IIB domain (Erni et al., 1989) it is surprising that the H86N mutation in the IIA domain can interfere with binding. Together with the fact that binding of another His mutant (H219Q) of IIB is normal, this suggests that binding of IIB to phosphocellulose is phosphate-specific rather than electrostatic and that His\(^{66}\) and His\(^{75}\) interact as part of a phosphate binding site. This conclusion is supported by the observation that IIAB\textsuperscript{Man} does not bind to the cation exchanger carboxymethylcellulose.\(^4\)

**Phosphotransferase Activity**—The phosphotransferase activity of the mutant proteins was tested in vivo and in vitro. The in vivo activity was estimated from the fermentation behavior on McConkey mannose indicator plates of E. coli WA2127 (pTSPM6) expressing plasmid encoded IIAB\textsuperscript{Man} mutants under the control of the tac promoter. The in vitro phosphotransferase activity was measured as IIAB\textsuperscript{Man}-dependent phosphorylation of dGlc. Results are summarized in Table I. H10C, H86N, H175C, and H10C/H175C are inactive; S72C, W12F, and H219Q have less than 7% of wild-type activity. Loss and reduction of activity are reflected by reduced fermentation in vivo. K48C and S110C retain more than 50% activity.

**Protein Phosphorylation**—The IIAB\textsuperscript{Man} mutants were phosphorylated in vitro with \(^{32}\)P-P-enolpyruvate, and the domains were separated by limited trypsinolysis and analyzed by gel electrophoresis. Wild-type and partly inactive mutants are phosphorylated on both domains (Fig. 2). In contrast, the H175C and the H86N mutants are phosphorylated on the IIA domain only, thus confirming the notion that His\(^{66}\) of the IIA domain and His\(^{75}\) of the IIB domain together are required for phosphorylation of the IIB domain. It appears that His\(^{66}\) is involved in the phosphoryl transfer between His\(^{110}\) and His\(^{175}\) but does not become phosphorylated in this process. Unexpectedly the H10C mutant which is not phosphorylated on the IIA domain is nevertheless slowly phosphorylated on the IIB domain. The isolated IIB domain is also slowly phosphorylated by HPr and dephosphorylated in the presence of IIC\textsuperscript{Man}/IID\textsuperscript{Man} and mannose, suggesting that IIB\textsuperscript{Man} is phosphorylated at His\(^{75}\) (results not shown). By contrast the H86N mutation which is phosphorylated on His\(^{66}\) is not phosphorylated on His\(^{75}\), which suggests once more that H86N can have a negative dominant effect on His\(^{75}\). However, the reaction bypassing His\(^{66}\) as an obligatory phosphorylation intermediate is slow (not shown) and the H10C and H10Q mutants have no phosphotransferase activity in vivo and in vitro (Table I). It appears that His\(^{75}\) as part of a phosphate binding site slowly reacts with non-cognate phospho-donors. The bypass phosphorylation of His\(^{75}\) is reminiscent of the in vitro phosphorylation of the bacterial chemotaxis proteins CheY and CheB by low molecular weight phospho-donors (Lukat et al., 1992). Fig. 2 shows yet another property of IIAB\textsuperscript{Man} namely to form dimers which are partly resistant to dissociation by sodium dodecyl sulfate. Of all the mutants only H10C, W12F, and S72C do not dimerize at all. These residues appear to stabilize the dimer. They are also part of the phosphorylation site as demonstrated by non-complementation (see below).

**Interallelic Complementation and Negative Dominance**—To test for functional interdependence between the two subunits in the IIAB\textsuperscript{Man} dimer, inactive and partly inactive mutants were mixed in pairs and the phosphorylation activity was compared with the activity of wild-type homodimeric IIAB\textsuperscript{Man}. The results (Fig. 3) allow a division of the mutants into two complementation groups. H10C, W12F, and S72C are muta-
tions in the IIA domain which do not complement, suggesting that these residues are components of a functional unit comprising the phosphorylation site of the IIA domain. H68N and H175C do not complement. This confirms previous results which indicate that the two residues constitute a cooperative unit and they indicate that this unit can be formed only between domains of the same subunit. Good complementation between H10C and H68N on the other hand confirm that His10 and His46 belong to different functional units, although these residues are on the same protein domain.

Complementation activities of less than 5% suggest that intersubunit phosphoryl transfer should be of minor importance and that phosphoryl transfer mainly occurs between domains on the same subunit. Therefore it is expected that the phosphotransferase activity of wild-type IIA[Met] in heterodimers with an inactive mutant should not be affected. To test this proposition, increasing concentrations of inactive H10C, H68N, H175C, and H10C/H175C were added to a constant concentration of wild-type IIA[Met]. Assuming that the dimer distribution is binomial, over 90% of the wild-type subunits should be in heterodimers at a mutant:wild-type ratio of 16:1. Fig. 4 shows that single site mutants have little effect but that a 16-fold excess of H10C/H175C reduces wild-type activity to 40% and thus has a negative dominant effect. These experiments were done in the presence of an excess of IIC[Met]/IID[Met], if the concentration of IIC[Met]/IID[Met] is rate-limiting, the inactive mutants act as competitive inhibitors of wild-type IIA[Met] by binding to the IIC/IID complex (results not shown).

Mixed results were obtained, when partly active mutants were combined with inactive mutants. W12F and S72C in combination with inactive mutants showed either interallelic complementation or unaltered activity (see Fig. 3). H219Q, in contrast, was strongly inhibited by H10Q, H68N, H175N, and H10Q/H175N. Inhibition occurs already at mutant:H219Q molar ratios of less than 1. It appears from this that the heterodimers between an inactive mutant and H219Q are almost inactive.

Surface Accessibility of Cysteine Residues—Unique cysteines were introduced at different positions predicted to be close to the surface of the protein. The second order rate constant of the reaction between the protein sulfhydryl and DTNB was taken as measure of accessibility. For each mutant the reaction rates of native and guanidinium HCl unfolded IIA[Met] were compared (Table II). Cysteines in unfolded IIA[Met] react with similar rates (6 \times 10^{-3} \text{ to } 3 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}). Cys^{75} in native H175C reacts as fast as the cysteines of unfolded proteins. In contrast Cys^{10} of H10C is 1000 times less reactive. It is the least reactive of all cysteines. The accessibility of cysteine 48 is intermediate, Cys^{72} and Cys^{110} appear to be exposed. The difference in reactivity of Cys^{10} and Cys^{75} is noteworthy because the cysteines replace histidines which are phosphorylated and therefore are expected to be exposed on the protein surface.

Oxidative Cross-linking of Cysteine-containing Mutants—The extent of disulfide bond formation between cysteines in different positions of a protein depends on their reactivity, accessibility, and distance. It might also reflect structural fluctuations of the protein and segmental flexibility (hinge bending). Disulfide formation between IIA[Met] monomers was induced with H_2O_2 or Cu(II)(1,10-phenanthroline). Disulfide cross-linking was fully reversible upon treatment of the oxidized proteins with dithiothreitol. It was concentration independent as tested for H10C and S72C in the concentration range between 0.3 and 3.0 mg/ml (not shown). The different cross-linked homodimers and heterodimers could be distinguished by their different electrophoretic mobility. The results of cross-linking between identical as well as pairwise different mutants are shown in Fig. 5 and summarized in Table II. Oxidative cross-linking was in no case complete. All mutants form homocross-links but only 2 out of 10 pairwise combinations afford heterocross-links. Of the four cysteines in IIA[Met] Cys^{72} yields the largest fraction of homocross-links (62%). Cys^{75} is likely to be not only accessible from the surface but also close to the interface between the IIA subunits in the dimer. Cys^{110} which is equally accessible must be further from the interface (22% cross-links). Cys^{46}, Cys^{10} show homo- and heterocross-linking. Heterocross-linking is slightly better (Cys^{10}-Cys^{46}, 15%) than homocross-linking (Cys^{46}-Cys^{46}, 5%). Cys^{110} and Cys^{72} do not cross-link, although His^{10} and His^{175} are expected to closely approach each other during phosphoryl transfer. In contrast Cys^{75} on the IIB[Met] domain of one subunit can reach Cys^{75} and Cys^{72} on the opposite subunit with about equal probability (175-175, 9%; 175-72, 18%). The two kinds of cross-linking reflect the high reactivity of Cys^{75} and the segmental flexibility of the IIB domain which does not dimerize but is flexibly attached to IIA via an Ala-Pro-rich linker.

**FIG. 3.** Interallelic complementation (A–C). The mutants indicated below the x axis were mixed with the mutants indicated above the curves in the indicated mole ratios. The total IIA[Met] concentration was (80 ng/ml). The phosphotransferase activity is given in percent of a control containing 80 ng/ml wild-type IIA[Met]. 100% corresponds to 19.3 nmol of dGlc-6P/pmol of wild-type IIA[Met]/30 min.
The indicated molar ratios to a constant concentration of wild-type HlOC homodimers and 5% were K48C homodimers. Transferase activity corresponds to 17.3 nmol of dGlc-6P/pmol of protein. Diagonal: cross-links between homologous pairs. Off diagonal: intersubunit cross-linking efficiencies are in percentage of total.

**DISCUSSION**

IIABMan is the hydrophilic subunit of the mannose transporter of the phosphotransferase system. It consists of two domains, IIAMan and IIBMan. Complexed with the transmembrane IIC and IID subunits, it sequentially transfers a phosphoryl group from the phosphoryl carrier protein phospho-

**FIG. 5. Oxidative cross-linking of cysteine containing IIABMan mutants.** Sulfhydryl groups were oxidized by incubation with H2O2 or Cu(II)(1,10-phenanthroline) as described under “Materials and Methods.” A, Cys9 unoxidized (1) and oxidized by H2O2 (2) or Cu(II)(1,10-phenanthroline) (Cupe; 3); Cys19 combined with Cys45 unoxidized (4) and oxidized (5); Cys45 unoxidized (6) and oxidized with H2O2 (7). B, Cys72 unoxidized (1) and oxidized with H2O2 (2) and Cupe (3); Cys72 combined with Cys175 unoxidized (4) and oxidized with H2O2 (5) or Cupe (6); Cys175 oxidized (7) and oxidized with H2O2 (8). Solid arrows indicate the position of heterodimers. The thin arrow in lane A5 indicates the position of the Cys45 homodimer.

**TABLE II**

Reactivity of mutant cysteine residues and cysteine cross-linking efficiencies

Second-order rate constants for the reaction of equimolar amounts of DTNB with cysteine mutants (m-1 s-1). Homologous and heterologous intersubunit cross-linking efficiencies are in percentage of total protein. Diagonal: cross-links between homologous pairs. Off diagonal: heterologous disulfide cross-linking products.

<table>
<thead>
<tr>
<th>DTNB reactivity</th>
<th>Cross-linking efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>H10C H48C S110C S72C H175C</td>
</tr>
<tr>
<td>H10C</td>
<td>3.5 x 10^3  1.9 x 10^4</td>
</tr>
<tr>
<td>K48C</td>
<td>5.0 x 10^2  1.0 x 10^4</td>
</tr>
<tr>
<td>S110C</td>
<td>5.0 x 10^3  6.0 x 10^4</td>
</tr>
<tr>
<td>S72C</td>
<td>5.0 x 10^3  1.0 x 10^4</td>
</tr>
<tr>
<td>H175C</td>
<td>4.0 x 10^4  3.0 x 10^4</td>
</tr>
</tbody>
</table>

a In addition to the 15% heterodimer, 26% of the total IIABMan were H10C homodimers and 5% were K48C homodimers.

b No significant amounts.

In addition to the 15% heterodimer, 41% of the total IIABMan were S72C homodimers and 9% were H175C homodimers.

HPr to the transported sugar (see Fig. 1). The two domains of IIABMan are presumed to be functionally analogous to the IIA and IIB domains of other PTS transporters. But there are some striking differences. Both domains are phosphorylated on histidines, whereas in the other transporters only IIA is phosphorylated on a histidine while the IIB domains are phosphorylated on a cysteine. There is also no amino acid sequence similarity and the secondary structures are different (comparison of unpublished results with Pelton et al. (1991)). Finally, IIABMan is a dimer, which is stabilized by contacts between the IIA domains. No dimerization between the isolated IIA and IIB domains of other PTS transporters has been observed (Lolkema et al., 1993).

In this report IIABMan point mutants are described. The two active site histidines as well as a number of presumably surface exposed residues on the IIABMan domain were replaced by cysteines. Cysteines were chosen because wild-type IIABMan does not contain cysteines, and the newly introduced sulfhydryl groups can serve as probes for the study of surface accessibility, of subunit interaction in a protein oligomer, and for heavy metal binding in protein crystals. All four histidines of IIABMan were also replaced by asparagine and glutamine, residues which can hydrogen bond and assume conformations close to isosteric with histidine.

Replacement of His19 or His175 by Cys or Asn and Gln completely abolished phosphotransferase activity in vivo and in vitro. Cysteine cannot substitute for His in the IIB phosphorylation site, although a Cys is the phosphorylated residue in the IIB domains of other PTS transporters (Pas and Robillard, 1988; Meins et al., 1993). This result together with a reciprocal experiment where C384 of the mannitol transporter was exchanged against His (Weng et al., 1992) confirms that His and Cys are not equivalent in functionally homologous phosphorylation sites.

The H10C and the H175C mutants have very different reactivities toward the sulfhydryl reagent DTNB. Cys175 of native and unfolded IIABMan react with equal rates suggesting that Cys175 is fully exposed on the IIB surface. In contrast, Cys19 of native IIABMan is 1000-fold less reactive, which indicates that the sulfhydryl group is partially buried in the IIA domain. However, it has to be kept in mind, that the sulfhydryl reactivity does not depend solely on accessibility, but might also be modulated by environmental factors, e.g. charge and hydrophobicity (e.g. see Kalli and Holmgren (1980)).
Cys$^{10}$ and Cys$^{175}$ also do not cross-link even though the two sites are expected to approach each other during phosphoryl transfer. The inaccessibility of Cys$^{10}$ in the IIA phosphorylation site appears to reflect a characteristic feature of the IIA$^{CIC}$ subunit of the glucose transporter. Based on the known structures, an atomic model of the interactions between IIA$^{CIC}$ and phospho-HPr of $R. subtilis$ has been proposed (Herzberg, 1992). According to this model, His$^{86}$, the phosphor acceptor of IIA$^{CIC}$ which is functionally homologous to His$^{10}$ of IIA$^{Man}$ is located in a depression surrounded by hydrophobic amino acids. Only N3 of His$^{86}$ is accessible to solvent, while NI is hydrogen bonded to a carbonyl oxygen of the amide backbone. This concave surface of IIA$^{CIC}$ is complementary to the convex surface of HPr carrying His$^{15}$. Provided that the docking mechanisms between HPr and the different IIA domains are similar, the binding site on IIA$^{Man}$ is likely to be concave, too. And considering that a sulfhydryl group is significantly smaller than an imidazole ring, Cys$^{10}$ might not be accessible from the surface. In contrast, the high reactivity of Cys$^{175}$ suggests that His$^{175}$ of the IIB domain sticks out from a convex surface like His$^{15}$ of HPr. This parallels the similarly high reactivity of the functionally homologous Cys$^{20}$ of IIB$^{CIC}$ (Meins et al., 1998).

His$^{86}$ is the third functionally important His of IIA$^{Man}$. This is not without precedent either (Presper et al., 1989). His$^{86}$ of IIA$^{Man}$, like His$^{9}$ of the $E. coli$ IIA$^{CIC}$, is important for the transfer of phosphoryl groups from the IIA to the IIB domain but not for the phosphorylation of the IIA domain by HPr. His$^{86}$ and His$^{175}$ form a functional unit, but only if they are on the same IIA$^{Man}$ subunit. The negative dominant effect of the H86N mutation on two properties of the (isolated) IIA domain, namely binding to phosphocellulose and phosphorylation of His$^{175}$ by the non-cognate phosphoryl donor phospho-HPr, suggests that a mutation of His$^{86}$ on the IIA domain can induce a structural change in the IIB domain.

Complementation between the IIA$^{Man}$ mutants is weak which, however, does not mean that they are functionally independent. Wild type as well as the H219Q mutant of IIA$^{Man}$ are subject to negative dominance. Wild-type IIA$^{Man}$ in a heterodimer with the inactive H10C/H175C mutant is less active than in the homodimer. Identical results were obtained with the H10Q/H175N double mutant (not shown) while the H10C and the H175C mutants alone did not strongly inhibit. In contrast, the partly active H219Q mutant is strongly inhibited by all inactive mutants. The reason for this is not known at present. Taken together the results, however, provide evidence for cooperative subunit interaction in the IIA$^{Man}$ dimer.

A model of the IIA$^{Man}$ dimer is shown in Fig. 1. It is assumed that in the intact mannose transporter the IIA domains are in permanent contact with the membrane bound IIC and IID complex, and that the IIA dimer is flexibly attached to the IIB/IIC/IID complex. Phosphoryl transfer occurs between domains on the same subunit as well as between different subunits. Oligomerization between PTS transporters and interallelic complementation appear to be a general phenomenon. It has been shown for mannitol transporter mutants with defective phosphorylation sites (Weng et al., 1992). However, phosphoryl transfer does not only occur between mutant subunits belonging to the same transporter. It also occurs between the homologous subunits of different transporters as demonstrated by intergenic complementation between the transporters for glucose and $\beta$-glucosides (Vogler and Lengeler, 1988; Vogler et al., 1988; Schnetz et al., 1990). In these cases the IIA domains of one transporter acted as phosphohydroydinol to the IIB domains of the other.

The biological significance of intersubunit phosphoryl transfer between subunits is not yet known. It might be a mechanism to channel phosphoryl groups away from one transporter toward another and thus increase the activity at one of the expense of the other (Schötte and Postma, 1981). We can only speculate on the biological significance of intersubunit cooperativity. Binding of the sugar substrate to the IIA/IIC/IID complex could increase the "affinity" of IIA to take up phosphoryl groups from phospho-HP$^r$. This would protect the system from wastefully transferring high energy phosphates to the transporters for which substrates are not available. It is also possible that IIA$^{Man}$ regulates other processes in response to the changing rate of mannose uptake. However, the target system of such regulation has yet to be discovered. One possibility is feed back regulation of transcription, in analogy to the regulatory circuit which has been described for the $\beta$-glucoside transporter (Amster-Choder et al., 1989; Schnetz and Rak, 1990; Amster-Choder and Wright, 1992). With the mutants at hand, some of these open questions will now be addressed using operon fusion techniques.

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REFERENCES


