Mechanism of Phosphoryl Transfer in the Dimeric IIAB\textsuperscript{Man} Subunit of the \textit{Escherichia coli} Mannose Transporter\textsuperscript{*}

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The mannose transporter of \textit{Escherichia coli} is a membrane-bound hexokinase. It catalyzes the phosphorylation of mannose to mannose-1-phosphate and is a member of the phosphoenolpyruvate (PEP)-dependent carbohydrate transporters. These transporters utilize the energy of the ATP-ADP exchange for the active transport of nutrients into cells. The mannose transporter is a heterotetramer consisting of two A subunits (IIAMan) and two B subunits (IIDMan). The A subunits carry out the bulk of the transport activity and are responsible for the selectivity of the transporter. The B subunits, on the other hand, are responsible for the phosphorylation and dephosphorylation of the substrate.

TheIIAMan subunit is a homodimer (see Fig. 1A). Each monomer comprises two independently folding domains, the A domain (residues 1–133) and the B domain (residues 156–323) connected by a 23-residue long alanine-proline-rich linker. The B domain contains a five-stranded parallel\(\beta\)-sheet (strand order 4\(\beta\)-A\(\beta\)-A\(\beta\)-3\(\beta\)) covered by helices on either face ((\(\beta\)A),\(\beta\)B). Four strands are parallel, and the fifth antiparallel strand which forms one edge of the sheet is swapped between the subunits in the dimer. This domain is phosphorylated during phosphoryl transfer from HPr to IID, and is located at the topological switchpoint of the fold. Its imidazole ring is hydrogen bonded to Asp-67, which acts as a general base increasing the nucleophilicity of the imidazole ring (19). The domain

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**Mannose Transporter**

It has been shown previously (34) that the active site mutants of IIAB\textsuperscript{Man}, H10C, and H175C, are completely inactive when assayed alone, but that approximately 3% of wild-type activity is recovered when the purified proteins are mixed in a 1:1 ratio. Here we show, that much higher activity is recovered when the purified mutants are mixed, completely unfolded with GuHCl, and then renatured. True heterodimers form only under these drastic conditions. Phosphoryl transfer between subunits within the dimer is very efficient, whereas transfer between different dimers is possible but inefficient.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Overproduction, and Purification of Proteins—**E. coli W2127ΔHIC (manXYZ ptsHtrr (42)) was transformed with derivatives of pfI encoding wild-type and mutant IIAB\textsuperscript{Man} (34). IIAB\textsuperscript{Man} was overexpressed and purified as described (34). Enzyme I and HPr were purified, and membranes containing IIC\textsuperscript{Man}-IID\textsuperscript{Man} were prepared as described (42, 43).

**GuHCl Unfolding and Renaturation of IIAB\textsuperscript{Man}**—Stock solution of purified wild-type and mutant IIAB\textsuperscript{Man} were adjusted to a protein concentration of 5 mg/ml. Volumes from the different stocks were mixed to achieve the desired molar ratios or molar fractions. The mixtures were then split in two aliquots. One aliquot was diluted with 8 M GuHCl to a final concentration of 4 M GuHCl (37), and to the other aliquot, the same volume of buffer A (10 mM MOPS, pH 7.0, 50 mM NaCl, 0.5 mM dithiothreitol) was added. Both samples were incubated for 2 h at room temperature. Both samples were then diluted 20–60-fold with buffer A to the desired IIC\textsuperscript{Man} concentration (3–125 μg/ml) and incubated for another 2 h at 4 °C.

**Assay for Phosphotransferase Activity—**In vitro phosphorylation of \[^{14}C\]Glc was assayed by ion-exchange chromatography as described (34). 100 μl of incubation mixture contained 0.5 μg of enzyme I, 2.5 μg of HPr, and 0.5 μl of crude membranes (~4 μg of protein) containing the IIC\textsuperscript{Man}-IID\textsuperscript{Man} complex. The final concentration of renatured IIAB\textsuperscript{Man} varied between 3 ng and 130 ng/100 μl of incubation mixture. The exact values are indicated in the figure legends. The specific activity of \[^{14}C\]Glc was 1000 cpm/nmol.

**Assay for Protein Phosphorylation—**The rate and the extent of protein phosphorylation was measured as described (45). The incubation mixture (50 mM NaP, pH 7.4, 5 mM MgCl\textsubscript{2}, 2.5 mM NaF, 2.5 mM dithiothreitol) contained, per 250 μl, 1.5 μg of enzyme I, 2.5 μg of HPr, and 85 μg of IIAB\textsuperscript{Man}. The phosphorylation reaction was started by adding to the incubation mixture at 24 °C \[^{33}P\]PEP to a final concentration of 80 μM. Aliquots of 40 μl were withdrawn at the indicated time points and diluted into 1 ml of 80% ammonium sulfate solution at 4 °C. The protein precipitates were collected on glass microfibre filters (GF/F, Whatman) under suction, washed, and counted in a liquid scintillation counter. The background counts because of enzyme I and HPr (less than 10%) were subtracted from the counts of the complete system. Phosphorylated proteins were analyzed on 17.5% polyacrylamide gels as described (21). 20-μl incubation mixtures contained 134 μM \[^{33}P\]PEP, 0.15 μg of enzyme I, 0.46 μg of HPr, 10 μg of IIAB\textsuperscript{Man}, and 0.3 μl of IIC\textsuperscript{Man}-IID\textsuperscript{Man}-containing membranes.

**RESULTS**

**Functional Interaction of Subunits in IIAB\textsuperscript{Man} Dimers—**Wild-type IIAB\textsuperscript{Man}, H10C, H175C, and H10C/H175C double mutant were purified by phosphocellulose chromatography and gel filtration. A 1:1 mixture of purified H10C and H175C has about 5% of the specific activity of wild-type IIAB\textsuperscript{Man}. The activity increases nonlinearly at low concentration, and the concentration activity profile does not change after 24 h of preincubation (Fig. 2). These results suggest that the activity is because of transient association between two different inactive heterodimers (second order reaction) and that most monomers do not exchange to form heterodimers. However, when mixtures of H10C and H175C were denatured in GuHCl and then refolded by rapid dilution, a 20-fold higher specific phosphotransferase activity was obtained (Fig. 3A). When H10C and H175C were mixed in different proportions, the activity profile was bell-shaped with a maximum at a 1:1 molar ratio (Fig. 3B), as

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**Fig. 1. Hypothetical model of the mannose transporter complex.** A. HPr and the two monomers of the IIAB\textsuperscript{Man} complex are in different shades of gray. The orientation of the IIB (PDB code 1BLE) and HPr (1POH, (55)) in the complex with the IIA dimer (1PDO) are taken from the model proposed by Schauder et al. (23). The active site histidines H10, H175, and H15 are shown in ball and stick representation. IIC\textsuperscript{Man} and IID\textsuperscript{Man} span the membrane. The cartoon of IIAB\textsuperscript{Man} was produced using MOLSCRIPT (56). B, backbone representation of IIAB\textsuperscript{Man} with the alanine-proline-rich linkers in a fully extended conformation. C, schematic representation of cis and trans orientations of the IIA dimer relative to the IIB domains with monomers. Active site contacts are indicated in black.

contains a 180° twisted seven-stranded β-sheet (strand order 3241567, 1–6 are parallel and 7 is antiparallel) covered by helices on both faces, as deduced from the IIB-Lev subunit which is 47% identical to the IIB\textsuperscript{Man} domain. His-175, which accepts the phosphorolysis from H10 and transfers it to the sugar, is located on an exposed loop between the first β-strand and α-helix (23).

Only the A domain participates in the dimer interface. The monomer-monomer interaction occurs through the interlocked β-strands and an extensive contact area of 1700 Å² composed mainly of hydrophobic residues. This confers high stability, and the IIAB\textsuperscript{Man} dimer can be dissociated only concomitant with complete denaturation (37). The B domain interacts with the transmembrane IIC\textsuperscript{Man}-IID\textsuperscript{Man} complex of the mannose transporter. The IIAB\textsuperscript{Man}-IIC\textsuperscript{Man}-IID\textsuperscript{Man} complex, which can be purified intact, has a stoichiometry closest to 2:1:2 (38–40). The IIAB\textsuperscript{Man} dimer can also be purified as a soluble protein. Dissociated from the transmembrane IIC\textsuperscript{Man}-IID\textsuperscript{Man} complex, IIAB\textsuperscript{Man} has an elongated form. Ratios \(f/f_0\) of 1.81 and 1.72 were calculated from the sedimentation coefficient \((s_{20,w} = 3.7 S)\) determined by analytical ultracentrifugation (37) and the diffusion coefficient \((D = 4.73 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1})\) determined by dynamic light scattering, respectively. The axial ratio of >10:1 derived from \(f/f_0\) (41) is compatible with a fully extended dimer (Fig. 1B) composed of the central A dimer (50 Å along the major axis), the two linkers (66 Å when fully elongated), and the two B domains (35 Å average diameter).
expected for a binomial distribution of active heterodimers and inactive homodimers. The activities of heterodimers between wild-type and mutated subunits was characterized in the following experiments. Constant amounts of wild-type IIAB\textsuperscript{Man} were mixed with increasing amounts of either H10C or H175C. One-half of the mixture was denatured with GuHCl and then renatured by dilution, the other was diluted only. The phosphotransferase activity remained approximately constant at all concentrations of H10C and H175C (Fig. 3C) independently of whether 100% of wild-type IIAB\textsuperscript{Man} occurs as homodimer (no GuHCl) or whether only 11% of IIAB\textsuperscript{Man} was in homodimers and the rest in heterodimers with an inactive subunit. The activity was linearly dependent upon the concentration of wild-type IIAB\textsuperscript{Man} when wild-type and H10C or H175C were mixed in different molar ratios, denatured, and then renatured (Fig. 3D). This suggests that the presence of a subunit with only one inactive domain in a heterodimer has no effect on the overall phosphotransferase activity of the wild-type subunit. Mixtures between wild-type IIAB\textsuperscript{Man} and an excess of the H10C/H175C double mutant were prepared to characterize the phosphoryl transfer between A and B domains on the same subunit. The concentration of wild-type IIAB\textsuperscript{Man} was kept constant, and the concentration of the double mutant increased to a maximum of 16:1 (Fig. 3, E and F). At a concentration ratio of 16:1, when only 6% of the wild-type protein is in homodimers and 94% in heterodimers with the double mutant, the activity is still 60% of the control and identical to the activity of the non-denatured mixture. The 40% decrease of activity is because of competition of the excess of inactive homodimers (8-fold over active homodimers) for the II\textsuperscript{C}\textsuperscript{Man}-II\textsuperscript{D}\textsuperscript{Man} complex. Competitive inhibition becomes more pronounced when the concentration of II\textsuperscript{C}\textsuperscript{Man}-II\textsuperscript{D}\textsuperscript{Man} is rate-limiting. Under these conditions, the phosphotransferase activity is reduced to 50% when the concentration of wild-type homodimer plus heterodimer equals the concentration of the H10C/H175C homodimer (Fig. 3F).

With each experiment, a control with pure wild-type IIAB\textsuperscript{Man} was carried along as a reference for 100% activity and as control of refolding yield. The activity recovered after rapid dilution of wild-type IIAB\textsuperscript{Man} was 80 ± 30% (Table I, column IIAB\textsuperscript{Man} homodimer). The specific activity of heterodimers was calculated as follows. The activity contributed by IIAB\textsuperscript{Man} wild-type homodimers was subtracted from the total phosphotransferase activity of a mixture of all dimers. The resulting difference was then divided by the concentration of heterodimers in the mixture. The concentrations of homo- and heterodimers were calculated from the binomial distribution. The specific activities of the different dimers are summarized in Table I. The turn-over number of wild-type IIAB\textsuperscript{Man} from experiment to experiment varies between 2500 min\(^{-1}\) and 1200 min\(^{-1}\). The H10C-H175C heterodimer has a turnover of 370 min\(^{-1}\). This is 37% of the activity of wild-type IIAB\textsuperscript{Man} measured under the same conditions. The turn-over numbers of heterodimers between a wild-type subunit and either H10C or H175C are 50 and 45% of wild-type homodimer, and the turnover-number of a heterodimer between a wild-type subunit and a H10C/H175C double mutant is 30%.

**Protein Phosphorylation—IIAB\textsuperscript{Man} is phosphorylated with \[^{32}\text{P}\]PEP in the presence of enzyme I and HPr and is dephosphorylated in the presence of II\textsuperscript{C}\textsuperscript{Man}-II\textsuperscript{D}\textsuperscript{Man} complex.** Competitive inhibition becomes more pronounced when the concentration of II\textsuperscript{C}\textsuperscript{Man}-II\textsuperscript{D}\textsuperscript{Man} is rate-limiting. Under these conditions, the phosphotransferase activity is reduced to 50% when the concentration of wild-type homodimer plus heterodimer equals the concentration of the H10C/H175C homodimer (Fig. 3F).
Mannose Transporter

The specific activities were calculated from Fig. 3, A-E and the control experiments with pure wild-type IIA\textsuperscript{Man}. The specific activities (in bold) of the homo- and heterodimers were calculated from the measured activity of the mixtures of dimers, the measured specific activity of pure wild-type IIA\textsuperscript{Man} homodimers (in bold), and the concentrations of hetero- and homodimers in the mixtures derived from the binomial distribution (in italics).

### Table I

|                  | Measured for mixture of all dimers | Calculated for |
|------------------|-----------------------------------|----------------|
|                  |                                   | IIA\textsuperscript{Man} homodimer | Active heterodimer | Inactive homodimer |
|                  |                                   | nmol Glc 6-phosphate/nm dimer/min |
| IIA\textsuperscript{Man} . IIA\textsuperscript{Man} | Mixed\textsuperscript{a} | 2550 |
|                  | GuHCl\textsuperscript{b}          | 1586 |
|                  | Mixed                             | 1230 |
|                  | GuHCl                             | 996  |
| H10C . H175C (1:1)| Mixed                             | 10\textsuperscript{c} |
|                  | GuHCl                             | 186  |
| IIA\textsuperscript{Man} . IIA\textsuperscript{Man} | Mixed                             | 2656 |
|                  | GuHCl                             | 1805 |
|                  | Mixed                             | 1813 |
|                  | GuHCl                             | 1787 |
| IIA\textsuperscript{Man} . IIA\textsuperscript{Man} | Mixed                             | 2246 |
|                  | GuHCl                             | 1387 |
| IIA\textsuperscript{Man} . H175C (1:8) | Mixed                             | 1580 |
|                  | GuHCl                             | 1432 |
| IIA\textsuperscript{Man} . IIA\textsuperscript{Man} | Mixed                             | 1154 |
|                  | GuHCl                             | 1496 |
| IIA\textsuperscript{Man} . H10C/H175C (1:16)| Mixed                             | 834  |
|                  | GuHCl                             | 921  |

\textsuperscript{a} IIA\textsuperscript{Man} components mixed and diluted.
\textsuperscript{b} IIA\textsuperscript{Man} components mixed, denatured and refolded by dilution.
\textsuperscript{c} % recovered wild-type IIA\textsuperscript{Man} activity after denaturation and refolding.
\textsuperscript{d} Activity of heterodimer in % of wild-type dimer.
\textsuperscript{*} Activity due to phosphoryltransfer between dimers.

IIA function, can be excluded because H10C was isolated from an E. coli strain with a chromosomal deletion of the manXYZ operon. It is likely, that phosphorylation of IIB is a consequence of high local concentration of HPr which binds to mutated IIA and then nonspecifically delivers the phosphoryl group to a nearby His-175. Phosphorylation at His-10, whether in wild-type IIAB\textsuperscript{Man} or in H10C results in an increased stabilization of the IIAB\textsuperscript{Man} dimer against dissociation by sodium dodecyl sulfate, and this effect is not reversed as a consequence of dephosphorylation by IIC\textsuperscript{Man}-IID\textsuperscript{Man} and mannose (Fig. 4B).

**DISCUSSION**

IIA\textsuperscript{Man} consists of two domains, IIA and IIB, that sequentially transfer a phosphoryl group from the phosphoryl carrier protein HPr to the transported sugar. IIA\textsuperscript{Man} is a homodimer. The subunits are tightly linked through mutual exchange of α-helices (in *cis*) and one trans), and interruption of one of the two pathways results in a reduction of the activity by 20% to 30% of the control.

The results confirm our previous observation of interallelic complementation (34) and similar observations by others (26, 31, 46, 47). But in the case of IIA\textsuperscript{Man}, the interpretation has changed. The weak complementation was because of phosphoryl transfer between randomly colliding homodimers. IIAB\textsuperscript{Man} dimers do not exchange, as evident from the structure of the IIAB\textsuperscript{Man} dimer (19). However, the linker (Fig. 1B) allows sterically constrained interaction between IIA and IIB domains on different dimers. The linker allows the IIA dimer to dock on the IIAB\textsuperscript{Man}-IIC\textsuperscript{Man}-IID\textsuperscript{Man} complex in either of two orientations (Fig. 1C). The *cis* orientation is presented in Fig. 1A.

A IIA\textsuperscript{Man} mutant with His-86 on the IIA domain replaced by Asn was described to have the same properties as H175C mutant with an inactive IIB domain (34, 36). However, the x-ray structure of IIA showed that His-86 is in a surface-exposed loop and far from the active site. In addition, His-86 is not conserved in any of the homologous proteins (see below). Both observations make His-86 an unlikely target for mutagenesis.

**Bacillus subtilis**, *Klebsiella pneumoniae*, *Vibrio furnissii*, and *Lactobacillus casei* express transporters homologous to the mannose transporter of E. coli except that IIA and IIB are expressed as separate proteins subunits and not as two domains connected by an alanine-proline-rich linker (48–51). Using the Basic Local Alignment Search Tool (BLAST) program, IIA homologs with alanine-proline-rich or Q-linkers (52, 53) were found in bacterial genomes\textsuperscript{2} (complete and in progress) of: *Yersinia pestis*, *Actinobacillus actinomycetemcomitans*, *Enterobacteriaceae*.

\textsuperscript{2} http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html.
IIABMan-IIICMan complex must be much stronger than binding of a monomeric IIB subunit because the dimer forms two contacts per molecule, whereas a IIB monomer forms only one (54). Although not covalent in the chemical sense, binding might become very strong, and IIAB remain membrane-bounded for most of the time. Uniting of IIB from the IIC-IIID complex is necessary whenever IIB has a regulatory function and must diffuse to other targets. For example, monomeric IIBLev of B. subtilis is not only a subunit of the fructose transport complex, but it also can phosphorylate and thereby inactivate the transcriptional activator LevR (16–18). An analogous situation is observed in E. coli. The transporter for Glc and GlcNAc (IIICB\textsuperscript{Glc}IIAGlc and IIICB\textsuperscript{GlcNAc}) are homologous, but whereas IIICB\textsuperscript{GlcNAc} is a three-domain protein, IIAGlc and IIICB\textsuperscript{Glc} are independent subunits. IIAGlc plays a pivotal role in regulation of catabolite repression and inducer inclusion, and it has been shown to interact with glycerol kinase, the transporters for lactose and maltose, and adenylate cyclase (5–12). These interactions with soluble and membrane-bound target proteins require that IIAGlc can freely diffuse through the cell.

The structural stability of the IIAB dimers and their mechanism of phosphoryl transfer might be unique among the different families of dimeric PTS transporters. Nevertheless, it indicates that interactions between different subunits within a dimer (first order reaction) as well as interactions between different dimers (second order reaction) have to be taken into consideration when weak interallelic complementation is observed. The ease with which stable heterodimers can be generated by reversible unfolding will facilitate the characterization by fluorescence energy transfer of domain motions that might occur during phosphorylation and transport of mannose.

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