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Expression, Purification, and Kinetic Characterization of the Mannitol Transport Domain of the Phosphoenolpyruvate-dependent Mannitol Phosphotransferase System of *Escherichia coli*

KINETIC EVIDENCE THAT THE *E. COLI* MANNITOL TRANSPORT PROTEIN IS A FUNCTIONAL DIMER*

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The overexpression of the membrane-bound C domain of the mannitol transport protein EII^{Mtl} of *Escherichia coli* has been achieved. This protein, IIC^{Mtl}, consisting of the first 346 amino acids, was purified from membrane vesicles and still bound mannitol with a high affinity. Gel filtration experiments showed that purified IIC^{Mtl} was a dimer, confirming that the interaction within the EII^{Mtl} dimer occurs between the membrane-bound portions of the protein. IIC^{Mtl} in combination with a chimeric protein consisting of the membrane-bound EII^{Glc} C domain and the cytoplasmic EII^{Mtl} BA domain could restore both phosphoenolpyruvate-dependent phosphorylation and mannitol/mannitol-P exchange activity. The interaction in this complex was comparable to that of IIC^{Mtl} with soluble $IIBA^{Mtl}$ in as much as there appeared to be no specific interaction between IIC^{Mtl} and the membrane-bound EII^{Glc} C domain; the K_m of IIC^{Mtl} for the chimer was so low that saturation could not be achieved. In contrast, a very high affinity with a K_m of 2 nM was measured between purified IIC^{Mtl} and purified EII^{Mtl}. This interaction was manifested in a IIC^{Mtl}-dependent stimulation of the EII^{Mtl} catalyzed phosphoenolpyruvate-dependent mannitol phosphorylation reaction and the mannitol/mannitol-P exchange reaction. The high affinity of IIC^{Mtl} for the wild type enzyme can be explained by the formation of heterodimers consisting of a IIC^{Mtl} monomer and an EII^{Mtl} monomer which interact at the level of the membrane-bound domains. The 2-fold increase in mannitol phosphorylation activity of the hetero- *versus* homodimer is an indication that the individual subunits in the homodimer are functionally coupled and work at only half their maximum rate.

It is known that the EII^{Mtl} dimer, but not the monomer, catalyzes the mannitol/mannitol-P exchange reaction. Since the heterodimer also catalyzes this reaction, it appears that only one functional B domain is required per dimer.

The mannitol transport protein, Enzyme II^{Mtl},[1] is a three-domain protein responsible for the uptake of mannitol in *Escherichia coli*. It is a member of the phosphoenolpyruvate-dependent phosphotransferase system proteins, whose main characteristic is the phosphorylation of their carbohydrate substrates during transport (1, 2).

The three domains of EII^{Mtl} have separate enzymatic functions. The two cytoplasmic domains, A and B, each possess a phosphorylation site. The A domain is phosphorylated at His-554 by a soluble cytoplasmic protein, P-HPr, and then transfers its phosphoryl group to the second phosphorylation site, Cys-384, on the B domain. The C domain is an integral cytoplasmic membrane component which spans the membrane six times (3). Several studies indicate that this domain is responsible for binding of mannitol and its subsequent translocation (4, 5).

The association state of Enzyme II^{Mtl} has been the subject of gel filtration studies which have shown that the enzyme forms dimers (6–8) and kinetic studies which have demonstrated that the interaction between the subunits within the dimer is a functional one. The most recent kinetic study revealed that the dimer is the form with the highest activity in both the PEP-dependent mannitol phosphorylation reaction and the mannitol/mannitol 1-phosphate exchange reaction (9). The mannitol/mannitol 1-phosphate exchange activity almost completely disappeared under conditions where monomers existed, while the PEP-dependent mannitol phosphorylation activity was still present but strongly reduced. Gel filtration experiments with subcloned domains of EII^{Mtl} show that IIC^{Mtl}, extracted from membranes, forms dimers comparable with the intact enzyme while the isolated $IIBA^{Mtl}$ protein is monomeric under the same conditions indicating that the interaction within the dimer in the intact protein occurs at the level of the membrane-bound C domains (8).

Subcloning and expression experiments have shown that the cytoplasmic domains of EII^{Mtl} can exist as separate soluble cytoplasmic proteins which can still be phosphorylated (10, 11). The protein, IIA^{Mtl}, has been crystallized and the three-dimensional structure is being determined by x-ray diffraction and NMR (12, 13). Attempts to crystalize intact EII^{Mtl} have been unsuccessful possibly due to the mobility associated with the

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[1] The abbreviations used are: EII nomenclature, when referring to domains which are covalently attached, we use the terminology "A domain, B domain, C domain, BA domain, etc". When referring to the domains which have been subcloned and expressed separately we use the nomenclature: IIA^{Mtl}, domain A of the mannitol-specific Enzyme II; IIB^{Mtl}, domain B of the mannitol-specific Enzyme II; IIC^{Mtl}, domain C of the mannitol-specific Enzyme II; $IIBA^{Mtl}$, domain BA of the mannitol-specific Enzyme II; IIB^{Glc}, domain B of the glucose-specific Enzyme II; IIC^{Glc}, domain C of the glucose-specific Enzyme II; $IIC^{Glc}BA^{Mtl}$, the chimeric protein consisting of the C domain of the glucose-specific Enzyme II covalently attached to the BA domain of the mannitol-specific Enzyme II; Mtl, mannitol; Glc, glucose; HPr, histidine-containing protein; EI, Enzyme I of the phosphoenolpyruvate-dependent carbohydrate transport system; DTT, dithiothreitol; decyl-PEG, decylpolyethylene glycol 300; PEP, phosphoenolpyruvate.

flexibly linked cytoplasmic domains. Removal of these domains might assist in the crystallization of the membrane-bound portion of the protein.

This article reports on the overexpression, purification, and kinetic characterization of IIC^{Mtl}. Kinetic evidence will be presented that IIC^{Mtl} can interact with wild type enzyme probably via the same interaction that exists in the wild type EII^{Mtl} dimer. The data support earlier results indicating that EII^{Mtl} is a dimer and that the two monomers within a dimer might functionally interact during phosphorylation and transport.

EXPERIMENTAL PROCEDURES

Materials

The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer by Eurosequence bv. Groningen. M13K07 helper phage and the DNA sequencing kit were obtained from Pharmacia Biotech Inc. Klenow enzyme, restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from Boehringer Mannheim. Decyl-PEG was synthesized by B. Kwant at the Department of Chemistry, University of Groningen. D-[1-^{14}C]Mannitol (2.04 GBq/mmol) and D-[1-^{3}H]glucose (2.1 GBq/mmol) were obtained from Amersham, D-[1-^{3}H]mannitol (976.8 GBq/mmol) was from DuPont NEN, and mannitol 1-phosphate was purchased from Sigma. Hexyl-agarose was obtained from Sigma and S-Sepharose from Pharmacia. The TSK-250 column was from Bio-Rad. EII^{Mtl}, Enzyme I, HPr, and $IIBA^{Mtl}$ were purified as described previously (11, 14–16).

Bacterial Strains, Plasmids, and Growth Conditions

The *E. coli* bacterial strain which contains a chromosomal deletion in the wild type *mtlA* gene LGS-322 F$^-$ *thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gatR49, gatA50,* Δ(*mtlA'p*), *mtlD*^{c}, Δ(*gutR'MDBA-recA*) (4) was used for expression of the domains of EII^{Mtl}. ASL-1 F$^-$, *lacY1, galT6, xyl-7, thi-1, hisG1, argG6, metB1, rpsL104, mtlA2, recA* is an *E. coli* strain that was selected for a *mtlA*$^-$ phenotype (10). *E. coli* strain ZSC112 *ptsM, ptsG, glk, lacZ, rha, rpsL relA* (17) was used to prepare membranes containing the $IIC^{Glc}IIBA^{Mtl}$ chimer. *E. coli* strain CJ236 *dut1, ung1, thi-1, relA1* / pCJ105(cam^r F′) (18) was used to prepare single-stranded template DNA that contains uracil for site-directed mutagenesis. JM101 Δ(*lac-proAB*), *supE, thi,* [F′, *traD36, proA*$^+$*B*$^+$, *lacI*^{q}*ZΔM15*] was used for various DNA techniques (19).

Plasmid pMamtlA is the expression vector used to produce wild type EII^{Mtl} (20). Special attention was paid to the region encoding Gly-222 during sequencing of the pMamtlA plasmid since an unwanted mutation had been found in some of our earlier pWAMa constructs. This mutation has only a slight influence on the kinetic properties of the enzyme but manifested itself during the course of protein purification by an enhanced sensitivity to inactivation in the presence of detergent. The plasmids pJRD187, pMcCIII, and pMc5–8 were described previously (16). Plasmid pTSG4 containing the *ptsG* gene was a gift from P. Postma, University of Amsterdam (21). All *E. coli* strains were grown on LB medium (10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl per liter) containing 25 μg/ml chloramphenicol or 100 μg/ml ampicillin depending on the plasmid used.

Construction of the IIC^{Mtl} Overexpression System

An amber stop codon in combination with a BamHI restriction site were created at position 1039 in the wild type *mtlA* gene with the primer 5′-ACGACGAGGATCCTATTCAATATC-3′. The plasmid, pMamtlA, containing this gene behind the natural P_{Mtl} promoter was used in this site-directed mutagenesis procedure according to the method described by Kunkel (22). The choice of position 1039 was based on the stable expression of the cytoplasmic domains and the membrane topology of the enzyme (3). Restriction analysis confirmed the creation of an extra *Bam*HI site in the pMamtlA(am) plasmid (Fig. 1). The new *Bam*HI site was used to delete the part of the *mtlA* gene that encodes for the cytoplasmic IIBA domain which has been expressed previously (16). The resulting pMaIIC consists of the P_{Mtl} promoter followed by an open reading frame coding for the first 346 amino acids of the N-terminal membrane-bound portion of EII^{Mtl}. The overexpression system was made by the insertion of the λ-P_r promoter with the cI857 λ-repressor gene into the pMaIIC plasmid (20). For this purpose an *Eco*RI-*Sal*I fragment containing the P_r promoter and repressor gene was excised from pJRD187 and ligated into the corresponding restriction sites in the plasmid, pMaIIC. The resulting overexpression vector, pMaIICP$_r$, containing IIC^{Mtl} behind a tandem promoter, was sequenced by the method of Sanger *et al.* (23) and was identical to the previously published sequence of this portion of the gene (24).

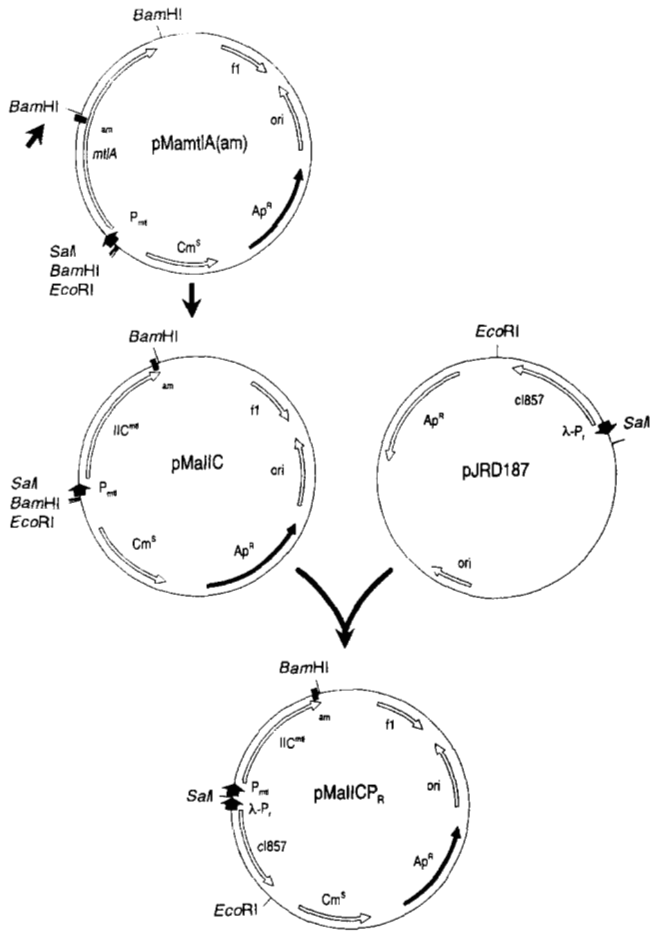

FIG. 1. **Construction of the IIC^{Mtl} overexpression system.** Plasmid pMamtlA(am) contains the wild type *mtlA* gene with an amber stop codon (indicated with an *arrow*) in combination with a *Bam*HI restriction site, which makes it possible to subclone the IIC^{Mtl} domain. The *Bam*HI sites are used to construct vector pMaIIC. Insertion of a *Eco*RI-*Sal*I fragment with the λ-P_r promoter and its repressor from plasmid pJRD187 in plasmid pMaIIC results in vector pMaIICP$_r$ used for the overexpression of IIC^{Mtl}.

Construction of the $IIC^{Glc}IIBA^{Mtl}$ Chimeric Protein Expression System

The chimeric protein is a fusion between the membrane-bound C domain of EII^{Glc} and the cytoplasmic BA^{Mtl} domain of EII^{Mtl}. For the production of single-stranded uracil template DNA, an *Eco*RI-*Eco*RI fragment of plasmid pTSG4 containing the *ptsG* gene and its promoter P_{Glc} was cloned into the vector, pMc5–8, resulting in the plasmid, pMcptsG. A *Bam*HI restriction site was created at position 1162 in the *pts*G gene in this plasmid with the 5′-CTTCAGTCGGATCCTCAC-GACC-3′ primer (Fig. 2). This position is predicted to be in a flexible region between the C and B domains at the end of the membrane-bound portion of EII^{Glc}. The fusion protein was constructed by restriction of the pMcptsG plasmid with *Bam*HI to remove the part of the *pts*G gene that encodes for the cytoplasmic B^{Glc} domain and then ligating it with a *Bam*HI-*Bam*HI fragment from the vector, pMcCIII, encoding $IIBA^{Mtl}$. The construction of the fusion in the resulting plasmid, pMcIIC^{Glc}BA^{Mtl}, was confirmed by restriction analysis and DNA sequencing. The construct was sequenced starting in the $IIBA^{Mtl}$ encoding part and an in-frame fusion with the IIC^{Glc} encoding part was found.

Preparation of Chimer Containing Membranes

For measurements with IIC^{Mtl}, the $IIC^{Glc}BA^{Mtl}$ chimer was expressed in the *mtlA*$^-$ *E. coli* strain ASL-1 containing plasmid pMcIIC^{Glc}BA^{Mtl}. Cells were grown in LB medium until an A_{600} of 1 at 37 °C, harvested, washed, and used to prepare membrane vesicles by the same method described below for IIC^{Mtl}. Western blot analyses of these vesicles, using

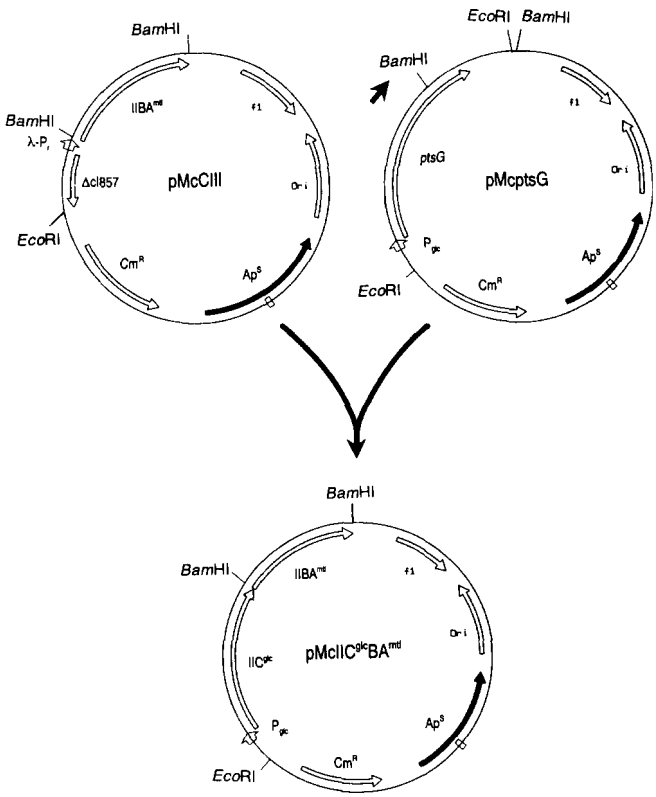

FIG. 2. **Construction of the IIC^{Glc}BA^{Mtl} chimer expression system.** Plasmid pMcptsG contains the *Eco*RI-*Eco*RI fragment with the *ptsG* gene on it from vector pTSG4. The *arrow* indicates the *Bam*HI restriction site constructed with site-directed mutagenesis. The fusion on plasmid pMcIIC^{Glc}BA^{Mtl} is made by replacing the *Bam*HI-*Bam*HI fragment of plasmid pMcptsG with the *Bam*HI-*Bam*HI fragment of plasmid pMcCIII encoding for the IIBA^{Mtl} protein.

polyclonal antibodies against EII^{Mtl}, showed a band on the blot of about 71 kDa. This mass is in good agreement with the expected mass of the chimer.

For flow dialysis and [^{3}H]glucose binding measurements, the chimer was expressed in *E. coli* strain ZSC112 containing the pMcIIC^{Glc}BA^{Mtl} plasmid. Cells were grown in LB medium at 37 °C.

Growth and Expression of IIC^{Mtl}

A 6-liter culture of LB medium inoculated with *E. coli* LGS322 containing pMaIICP$_r$ was grown at 30 °C until an A_{600} of 0.7 was reached. At this point the temperature of the culture was raised to 42 °C to induce expression of the protein under control of the temperature-sensitive cI857 repressor. The cells were grown for another 2 h, harvested by centrifugation for 15 min at 8000 × *g* at 4 °C, washed with 25 mM Tris-HCl, pH 7.5, and recentrifuged. This yielded 26 g of cells, wet weight, which were kept on ice and used to produce membrane vesicles as described by Lolkema *et al.* (9). The membrane vesicles were suspended in 15 ml of 25 mM Tris-HCl, pH 7.5, 1 mM DTT and stored in liquid nitrogen.

Purification of IIC^{Mtl}

Deoxycholate Extraction—The membrane vesicles (3 ml of vesicles from 5.2 g of cells) were added dropwise over a period of 5 min to 75 ml of extraction buffer (20 mM Tris-HCl, pH 8.4, 500 mM NaCl, 1 mM NaN_3, 1 mM DTT, and 0.5% deoxycholate) and stirred for 30 min at 25 °C. The solution was then centrifuged for 45 min at 150,000 × *g*. This and all further purification steps were carried out at 4 °C. The supernatant containing the extracted membrane protein was dialyzed against the extraction buffer without NaCl.

Hexyl-Agarose Chromatography—A hexyl-agarose column (2.5 × 20 cm) equilibrated with buffer containing 20 mM Tris-HCl, pH 8.4, 1 mM NaN_3, 1 mM DTT, and 0.5% deoxycholate was loaded with the dialysate and washed with the equilibration buffer at a flow rate of 1.25 ml/min until the A_{280} of the effluent reached the value of the equilibration buffer. The column was then washed with at least 1 column volume of 20 mM Tris-HCl, pH 8.4, 1 mM NaN_3, 1 mM DTT, and 0.1% decyl-PEG to change the detergent and eluted with a linear gradient (2 × 200 ml) of 0.1–2% decyl-PEG in the wash buffer. Fractions were collected and screened for IIC^{Mtl} by measuring the mannitol phosphorylation activity in a complementation assay with the IIC^{Glc}IIBA^{Mtl} chimer.

S-Sepharose Chromatography—The fractions containing IIC^{Mtl} were pooled and dialyzed against 50 mM sodium acetate, pH 5.2, 1 mM DTT, 1 mM NaN_3, and 0.35% decyl-PEG for 2 h and then overnight against fresh buffer. The dialysate was loaded onto a S-Sepharose column (1 × 40 cm) equilibrated with 50 mM sodium acetate, pH 5.2, 1 mM DTT, 1 mM NaN_3, 0.35% decyl-PEG. After washing with 100 ml of this buffer, the protein was eluted with a gradient (2 × 200 ml) of 0–400 mM NaCl. Fractions were collected and again screened for IIC^{Mtl}. The fractions that contained IIC^{Mtl} were pooled and dialyzed against 25 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.35% decyl-PEG and concentrated with an Amicon filtration system using a 30-kDa cut-off filter.

Phosphorylation Assays

The PEP-dependent phosphorylation kinetics of EII^{Mtl}, IIC^{Mtl}, and combinations of these proteins were measured in 25 mM Tris, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG at 30 °C. The concentration of Enzyme I, HPr, and labeled mannitol depended on the experiment. Details are given in the figure legends and the text. The volume of the assay mixture was 100 μl. Four 20-μl samples were taken at various times and loaded onto Dowex columns. A sample of 10 μl was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (25).

Chimer Complementation Assay

PEP-dependent mannitol phosphorylation was measured with the IIC^{Glc}BA^{Mtl} chimer and different concentrations of IIC^{Mtl}. A 100-μl assay mixture contained: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 100 nM EI, 17.6 μM HPr, 22 μg of chimer-containing membrane protein, and different amounts of IIC^{Mtl}. After incubation for 10 min at 30 °C the reaction was started with 10 μl of 600 μM [^{3}H]mannitol. At given time intervals, 4 samples of 20 μl were taken and loaded onto a Dowex column to determine the amount of mannitol 1-phosphate formed. The rates were calculated from this data. A 10-μl sample was used to determine the total amount of mannitol in the assay mixture. This assay procedure has been described in detail by Robillard and Blaauw (25).

Mannitol/Mannitol 1-Phosphate Exchange Assays

The assays were done at 30 °C in 25 mM Tris, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.25% decyl-PEG and a given concentration mannitol 1-phosphate. The exchange reaction was started with D-[1-^{3}H]mannitol. The assay volume and the sample size were the same as for PEP-dependent phosphorylation assays. The assay procedure has been described by Lolkema *et al.* (9). The pH and buffer were chosen to be the same as for the phosphorylation reaction, even though a pH of 7.6 is not the pH optimum of the exchange reaction, in order to be able to compare directly the kinetics of the enzyme in both reactions.

Binding Experiments with Flow Dialysis

The binding of tritium-labeled mannitol and glucose was measured by flow dialysis as described by Lolkema *et al.* (5). The buffer conditions were 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 0.5% decyl-PEG. The measurements were done at 25 °C.

Size Exclusion Chromatography

The association state of purified IIC^{Mtl} was measured by injecting samples onto a TSK-250 size exclusion high performance liquid chromatography column. The procedure was essentially the same as that used previously for the separation of EII^{Mtl} from membrane extracts (8). The buffer was 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.5% decyl-PEG. Five globular proteins, thyroglobulin, bovine γ-globulin, chicken ovalglobulin, bovine myoglobin, and cyanocobalamin, ranging in molecular mass from 670 to 1.35 kDa were used as references. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min to measure phosphorylation activity in combination with the chimer. A fluorescence detector was also used with an excitation wavelength of 290 nm and an emission wavelength of 340 nm.

Protein Determinations

Protein concentrations were determined by the method of Bradford (26) with bovine serum albumin as the standard. The purity of the protein was checked by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27). The gels were stained with Coo-

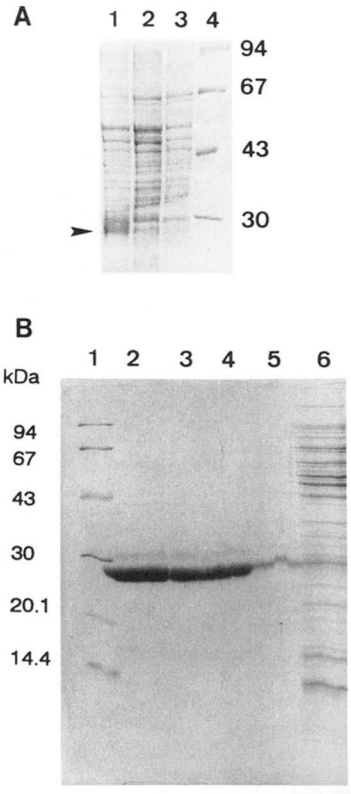

FIG. 3. **SDS-polyacrylamide gels showing the expression and purification of IIC^{Mtl}.** *A*, 10% SDS-polyacrylamide gel stained with Coomassie Blue. *Lane 1*, membranes of *E. coli* LGS322 containing plasmid $pMaIICP_r$; *lane 2*, membranes of *E. coli* LGS322 containing plasmid pMaIIC; *lane 3*, membranes of *E. coli* LGS322 without a plasmid; *lane 4*, molecular mass marker proteins (mass is indicated in kilodaltons). *B*, 15% SDS-polyacrylamide gel stained with Coomassie Blue. *Lane 1*, molecular mass marker proteins (mass is indicated in kilodaltons); *lanes 2–4*, show 1.2, 0.8, and 0.4 μg of concentrated IIC^{Mtl}, respectively, after S-Sepharose chromatography; *lane 5*, pooled fractions after hexyl-agarose chromatography; *lane 6*, soluble fraction after the deoxycholate extraction. The purity of the protein in *lanes 2–4* was determined by digitizing the photo of the gel using a CDC camera and determining the intensities with IMAGIC, software for electron microscopy image processing. Processing involved averaging 10 tracks in the lengthwise direction across a lane and then integrating. The intensities of the two IIC^{Mtl} bands was more that 99% of the total intensity in the lane.

massie Blue. The EII^{Mtl} concentration was determined by the pyruvate burst method (25). The N-terminal amino acid sequence was determined with an Applied Biosystems model 477A protein sequenator (pulse-liquid sequenator), connected on-line with a 120A phenylthiohydantoin analyzer (28). The molecular weight of IIC^{Mtl} was determined using matrix-assisted laser desorption ionization mass spectroscopy.

RESULTS

Overproduction and Purification of IIC^{Mtl}

Membranes from LGS322 cells containing $pMaIICP_r$ gave an extra band on SDS-polyacrylamide gels when compared with membranes from LGS322 with pMcIIC which lacks the λ-P_r promoter. Fig. 3*A*, *lane 1*, shows membranes of the IIC^{Mtl} overproducer; *lane 2* shows membranes of the nonoverproducer and *lane 3* is a control with membranes of LGS322 without a plasmid. The extra band has a molecular mass of approximately 28 kDa. IIC^{Mtl} has a mass of 36.3 kDa based on its primary sequence. The anomalous position is probably due to the hydrophobic nature of the protein; similar deviations are observed for native EII^{Mtl} and other membrane proteins (29). The expression of IIC^{Mtl} was also confirmed by measuring the mannitol binding with flow dialysis. Only membranes from LGS322 cells containing $pMaIICP_r$ or pMaIIC bound [^{3}H]mannitol. The level of expression of IIC^{Mtl} from both constructs was determined from these binding experiments. The construct with the λ-P_r promoter (1.6 nmol of binding sites/mg of membrane protein) gave a 16-fold higher expression compared with the pMaIIC construct (0.1 nmol of binding sites/mg of membrane protein) without this promoter. As found previously, the PEP-dependent phosphorylation activity could be restored by adding $IIBA^{Mtl}$ to membranes from cells expressing either of the IIC^{Mtl} plasmids (16).

The deoxycholate extraction used to solubilize the membrane-bound domain results in a complex mixture of proteins (Fig. 3*B*, *lane 6*), however, after a single hexyl-agarose column, IIC^{Mtl} remains as the main band at a molecular mass of 28 kDa (*lane 5*). Some other minor protein bands are still visible in this fraction which are removed by the S-Sepharose column yielding protein that was more than 99% pure as judged from scans of *lanes 2, 3*, and *4* in Fig. 3*B*. One point concerning IIC^{Mtl}s behavior on SDS-polyacrylamide gels is noteworthy. The protein position varies, running mostly at 28 kDa with a minor band at 32 kDa or *vice versa*. This has been observed on different gels with enzyme from the same pool and with enzyme from different pools. Since the same protein could be found in the low mass form one day and the high mass form the following day, proteolysis cannot be the cause. We suspect that the enzyme converts between two forms under conditions which we have not yet learned to control; these forms probably bind different amounts of SDS.

The purification was followed by measuring PEP-dependent phosphorylation activity in the assay with the $IIC^{Glc}IIBA^{Mtl}$ chimer. A 37% yield was observed starting from membrane vesicles, before extraction, and going through to the end of the S-Sepharose step. A 35% yield was determined by measuring mannitol binding of membrane vesicles *versus* the purified protein by flow dialysis. Purification resulted in 0.4 mg of IIC^{Mtl} from 5.2 g of cells, wet weight.

Amino acid analysis of the N-terminal peptide gave the expected sequence, except that the N-terminal methionine was missing. The sequence determined was Ser-Ser-Asp-Ile-Lys-Ile. No second sequence was detectable indicating that the protein was homogeneous as far as its N-terminal composition was concerned.

Mass Spectroscopy

The molecular mass found for IIC^{Mtl} using matrix-assisted laser desorption ionization mass spectroscopy was 35,493 ± 300. The expected molecular mass, based on the amino acid sequence, minus the N-terminal methionine is 36,184. This is in reasonable agreement with the expected mass, considering the difficulties that are involved in determining the mass of a membrane protein in detergent by this technique.

Binding Experiments

Mannitol binding to purified IIC^{Mtl} was monitored by flow dialysis. The Scatchard plot in Fig. 4 shows a single binding site with a dissociation constant of 295 nM. This value is 2–3 times higher than the affinity determined for EII^{Mtl} in solubilized membrane vesicles and for the purified enzyme (5). The difference could be due to removal of the cytoplasmic domains. Nevertheless it is clear that the isolated purified protein still binds mannitol with a high affinity.

Physical Size Measurements

The association state of IIC^{Mtl} was determined by gel filtration. Concentrations of the protein ranging from 108 nM to 4.3 μM were injected onto a TSK-250 column (Fig. 5). Mannitol phosphorylation activity measurements and fluorescence detection showed a single peak at a position corresponding to a globular protein with a molecular mass of 103 kDa. The con-

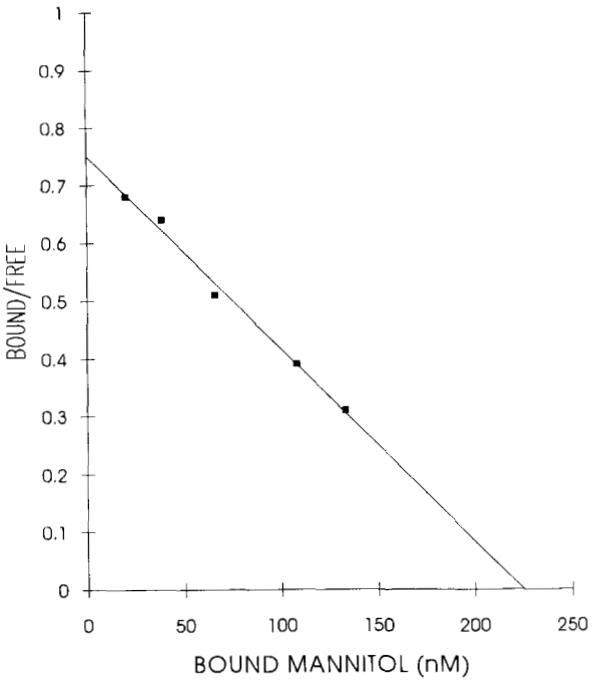

FIG. 4. **Scatchard plot showing the binding of [^{3}H]mannitol to purified IIC^{Mtl}.** The binding of [^{3}H]mannitol to the enzyme was determined by adding 50, 99, 196, 385, and 566 nM labeled mannitol to the upper compartment of the flow dialysis cell containing 400 μl of IIC^{Mtl} in 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 0.5% decyl-PEG. The temperature was 25 °C. The amount of free mannitol in the upper compartment was measured at the indicated mannitol concentrations, by collecting samples of the flow and determining the amount of labeled mannitol in the samples.

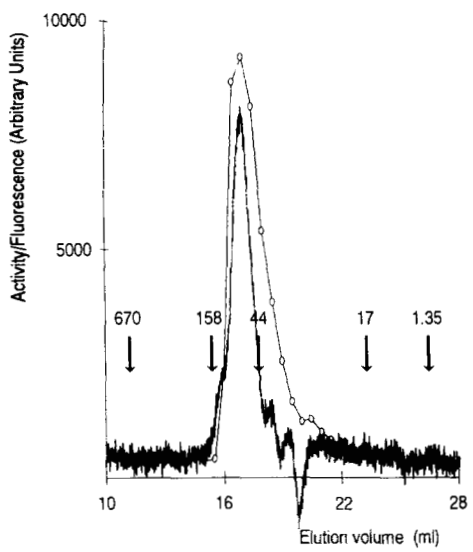

FIG. 5. **Elution profile of IIC^{Mtl} on a TSK-250 column.** Activity and fluorescence profile of 4.3 μM IIC^{Mtl} and 215 nM IIC^{Mtl}, respectively. Samples of 20 μl were injected onto the column. The experiments were done at room temperature and the buffer used was 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, and 0.5% decyl-PEG. The 4.3 μM sample was detected with the chimer complementation assay (○), which is described in the legend of Fig. 6*A*. For the 215 nM sample, fluorescence detection was used. The *arrows* indicate the elution position of the set of five reference proteins: thyroglobulin, bovine γ-globulin, chicken ovalglobulin, bovine myoglobin, and cyanocobalamin ranging in molecular mass from 670 to 1.35 kDa.

tribution of the decyl-PEG detergent micelle to the total mass is approximately 35 kDa (38), therefore, the protein contributes approximately 70 kDa. Since the mass of a monomer based on the amino acid sequence is 36.3 kDa, we conclude the purified IIC^{Mtl} is a dimer at the concentrations investigated.

Kinetic Characterization

The IIC^{Glc}-IIBA^{Mtl} Chimer and IIC^{Mtl}—IIC^{Mtl} is not active in PEP-dependent phosphorylation because it lacks the His-554 and Cys-384 phosphoryl group-transferring sites which are located on the A and B domains, respectively. With this in mind and realizing that IIC^{Mtl} does not saturate until very high concentrations of subcloned IIBA^{Mtl} in the mannitol phosphorylation reaction (16), we constructed a chimer consisting of cytoplasmic IIBA^{Mtl} fused to the membrane-bound IIC^{Glc}. The intention was to use the chimer as a source of IIBA^{Mtl} and, at the same time, provide IIC^{Mtl} with a hydrophobic counterpart with which it might interact. In this way we would hopefully lower the concentration of BA^{Mtl} domain necessary to achieve high mannitol phosphorylation rates.

The IIC^{Glc}-IIBA^{Mtl} chimer binds glucose with a high affinity. This has been determined using detergent solubilized inside-out cytoplasmic membrane vesicles of *E. coli* ZSC112 containing the plasmid, pMcIIC^{Glc}IIBA^{Mtl}. Glucose concentration-dependent binding measurements have been done with flow dialysis. Scatchard plots reveal a K_d of 2.9 μM for [^{3}H]glucose. This value is approximately a factor of 2 higher than the 1.5 μM K_d of wild type EII^{Glc} (30). However, the chimeric enzyme does not catalyze PEP-dependent glucose phosphorylation even though the IIBA^{Mtl} portion can be phosphorylated by PEP in the presence of HPr and EI and the IIC^{Glc} portion can bind glucose. The inability to phosphorylate glucose is apparently due to a lack of interaction between the mannitol-specific and glucose-specific portions of the chimer. Nevertheless the chimeric protein, together with IIC^{Mtl}, does restore PEP-dependent mannitol phosphorylation and mannitol/mannitol-P exchange. The IIC^{Mtl} concentration dependence of the PEP-dependent mannitol phosphorylation activity in combination with the chimer is shown in Fig. 6*A*. The 60 μM mannitol concentration used in this experiment is high compared with the K_d of mannitol for IIC^{Mtl}. Thus, essentially all mannitol binding sites are occupied in the experiment. An increase in substrate concentration in the form of IIC^{Mtl}:mtl leads to an increase in phosphorylation activity. At the concentrations used, the rate increases linearly with the IIC^{Mtl} concentration. The IIC^{Mtl} concentration dependence of the mannitol/mannitol-P exchange reaction gives the same result, a linear increase in rate with increasing IIC^{Mtl} concentrations (Fig. 6*B*). These data show that, while the BA^{Mtl} domain of the chimer is unable to catalyze the phosphorylation of glucose bound to a covalently linked C^{Glc} domain, it is able to catalyze phosphorylation of mannitol bound to isolated IIC^{Mtl} and do so using either P-HPr or mannitol-P as the phosphoryl group donating substrate. The linear increase in both the phosphorylation and exchange rates seen over the 0–340 nM IIC^{Mtl} concentration range indicates that there is no strong interaction between the chimer and the mannitol-IIC^{Mtl} complex; the rate does not saturate at mannitol-IIC^{Mtl} substrate concentrations up to 340 nM. The lack of saturation is reminiscent of the kinetic behavior of isolated IIBA^{Mtl} with IIC^{Mtl} reported earlier (16). Apparently, there is no strong, specific interaction of IIC^{Mtl} with the C^{Glc} domain in the chimer.

Native EII^{Mtl} and IIC^{Mtl}—The interaction between the chimer and IIC^{Mtl} and the dimeric nature of both EII^{Mtl} and IIC^{Mtl} leads to the question of whether IIC^{Mtl} can functionally interact with native EII^{Mtl}. The kinetic characteristics of this interaction have been examined both in the PEP-dependent mannitol phosphorylation reaction and the mannitol/mannitol-P exchange reaction.

The PEP-dependent mannitol phosphorylation kinetics over the 0–200 nM IIC^{Mtl} concentration range are presented in Fig.

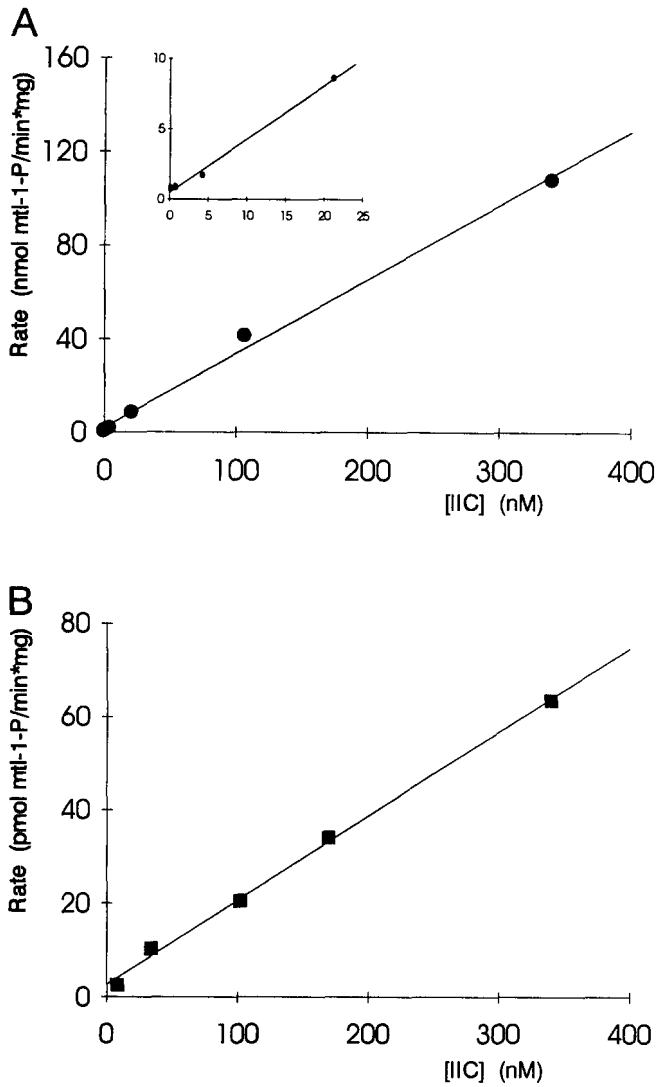

FIG. 6. **The IIC^{Mtl} concentration dependent phosphorylation kinetics in combination with the $IIC^{Glc}BA^{Mtl}$ chimer.** *A*, PEP-dependent mannitol phosphorylation rates of the $IIC^{Glc}BA^{Mtl}$ chimer with different concentrations of purified IIC^{Mtl}. The 100-μl assay mixture contained: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 100 nM EI, 17.6 μM HPr, 22 μg of chimer-containing membrane protein, and 0.17–340 nM IIC^{Mtl}. After incubation for 10 min at 30 °C the reaction was started with 10 μl of 600 μM [^{3}H]mannitol. At given time intervals, 4 samples of 20 μl were taken and loaded onto a Dowex column to determine the amount of mannitol 1-phosphate formed. The rates were calculated from this data. A 10-μl sample was used to determine the total amount of mannitol in the assay mixture. This assay procedure has been described in detail by Robillard and Blaauw (25). *Inset*, an expanded portion of the plot covering the range from 0 to 25 nM IIC. *B*, mannitol/mannitol-P exchange rates of the $IIC^{Glc}BA^{Mtl}$ chimer with different concentrations of purified IIC^{Mtl}. The 100-μl assay mixture contained: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.25% decyl-PEG, 200 nM [^{3}H]mannitol, and 250 μM mannitol 1-phosphate. After incubation for 10 min at 30 °C the reaction was started by the addition of 22 μg of chimer-containing membrane protein. The further assay procedure was identical to that described under *A*.

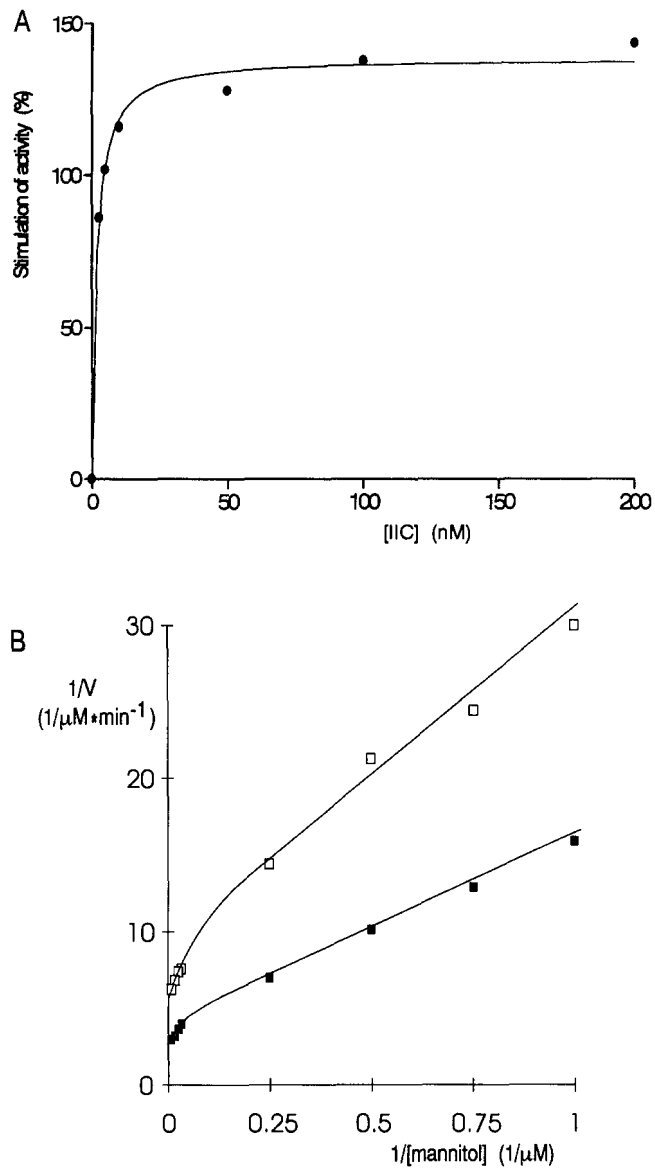

FIG. 7. **The effect on EII^{Mtl} PEP-dependent phosphorylation kinetics of the addition of IIC^{Mtl}.** *A*, IIC^{Mtl} (0–200 nM) was incubated with reaction mixture containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 100 nM EI, 17.6 μM HPr, and 0.39 nM EII^{Mtl}. After incubation for 10 min at 30 °C the reaction was started with 10 μl of 600 μM [^{3}H]mannitol. The final volume of the assay mixture was 100 μl in each case. The further assay procedure was identical to the procedure described under Fig. 6*A*. The experiment without IIC^{Mtl} was taken as 0% stimulation, and corresponds with an activity of 422 nmol of Mtl-P min^{-1} nmol^{-1} of EII^{Mtl}. The *line* is a fit of the data points to the Michaelis-Menten equation using the Origin data analysis software from MicroCal Software, Inc. *B*, Lineweaver-Burk plot of the mannitol concentration dependence in the presence and absence of IIC^{Mtl}. Assay mixtures of 100 μl containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 100 nM EI, 17.6 μM HPr, 0.39 nM EII^{Mtl} with (■) and without (□) 43 nM IIC^{Mtl} were incubated for 10 min at 30 °C. The reaction was started with different concentrations of labeled mannitol resulting in mannitol concentrations in the assay mixture of 1, 1.33, 2, 4, 30, 40, 60, and 120 μM. The further assay procedure was identical to the procedure described under Fig. 6*A*.

7*A*. The mannitol concentration in these measurements is 60 μM and the activity in the absence of IIC^{Mtl} is taken as 0% stimulation. Addition of IIC^{Mtl} to EII^{Mtl} gives an increase in phosphorylation rate which appears to have a half-saturation point of 2 nM, suggesting a very specific interaction between EII^{Mtl} and IIC^{Mtl}. Under the experimental conditions used, most of the IIC^{Mtl} will be in the IIC^{Mtl}:Mtl form. The stimulation appears to saturate at approximately 100%. The mannitol concentration dependence at a fixed IIC^{Mtl} concentration was next examined to determine whether this stimulation occurs over the entire mannitol concentration range.

The mannitol concentration-dependent steady-state kinetics of EII^{Mtl} have been well characterized. They do not follow simple Michaelis-Menten kinetics; instead, biphasic Lineweaver-Burk

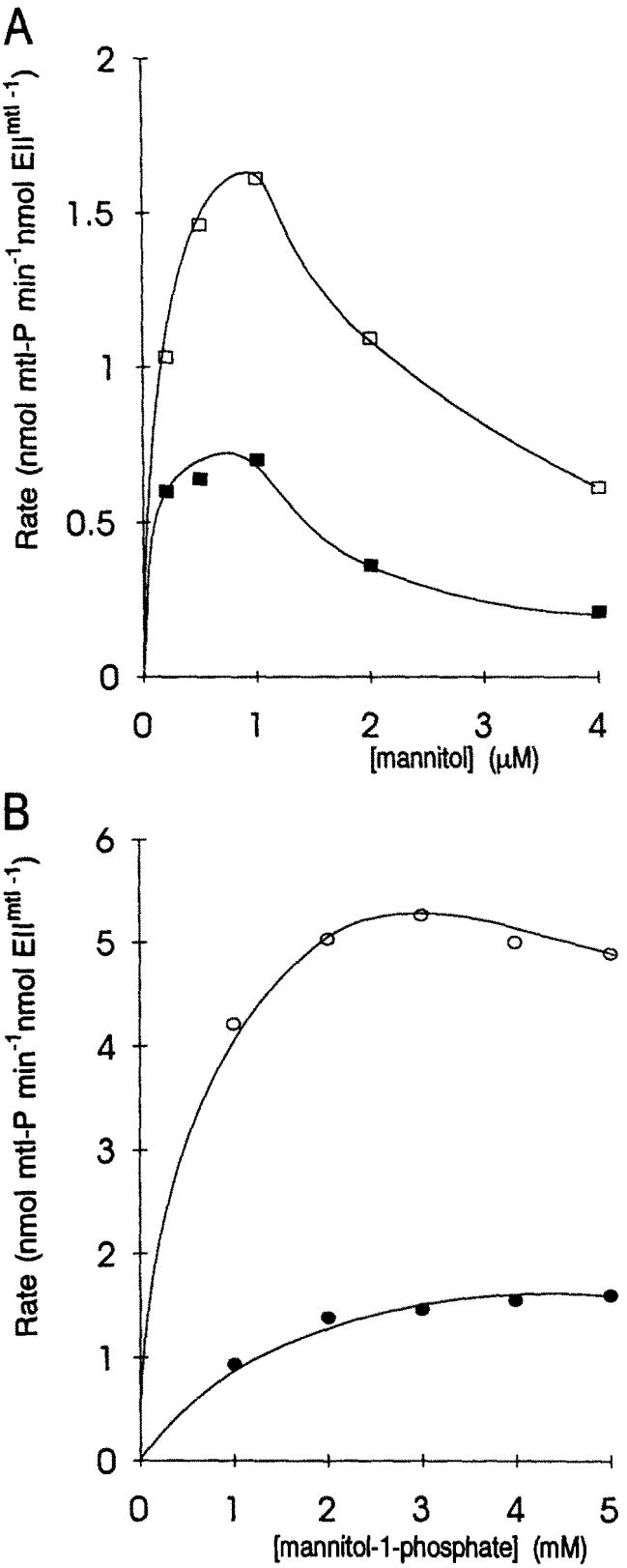

FIG. 8. **The effect of the addition of IIC^{Mtl} on EII^{Mtl} mannitol/mannitol-P exchange kinetics.** *A*, the mannitol concentration dependence of the exchange reaction and the effect of addition of IIC^{Mtl} on this reaction. The exchange rate was measured with a 100-μl reaction mixture containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.25% decyl-PEG, 250 μM mannitol 1-phosphate, 10 nM EII^{Mtl} at different concentrations of [^{3}H]mannitol (100 nM to 4 μM). The reaction was started after incubation at 30 °C for 10 min by the addition of [^{3}H]mannitol to the mixture and the formation of mannitol 1-phosphate was measured as described in the legend to Fig. 6*A*. The ■ symbols show the experiment without IIC^{Mtl} in the mixture and the □ symbols indicate the experiment with 330 nM IIC^{Mtl} present. *B*, the mannitol 1-phosphate concentration dependence of the mannitol/mannitol-P exchange reaction and the effect of addition of IIC^{Mtl} on this reaction. The exchange rate was measured with a 100-μl reaction mixture containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.25% decyl-PEG, 200 nM [^{3}H]mannitol, 0.83 nM EII^{Mtl} at different concentrations mannitol 1-phosphate (1–5 mM). The reaction was started after incubation at 30 °C for 10 min by the addition of [^{3}H]mannitol to the mixture and the formation of mannitol 1-phosphate was measured as described in the legend to Fig. 6*A*. The ● symbols show the experiment without IIC^{Mtl} in the mixture and the ○ symbols indicate the experiment with 330 nM IIC^{Mtl} present.

plots are observed. This has been interpreted by Lolkema *et al.* (31) as two kinetic regimes: a high affinity regime at low mannitol concentrations and a low affinity regime at higher mannitol concentrations. Further analysis showed that the second regime could not simply be described as a second Michaelis-Menten term, but was due to a different mechanistic route for mannitol phosphorylation which manifests itself only at high mannitol concentrations. The Lineweaver-Burk plot in Fig. 7*B* presents the mannitol concentration dependence of the phosphorylation rate at a saturated concentration of HPr and EI, with and without added IIC^{Mtl}. The second regime reported earlier is seen here, in both plots, as a deviation from a straight line. The presence of 43 nM IIC^{Mtl} results in a stimulation in the rate of a factor of 2 over the whole mannitol concentration range from 1 to 120 μM mannitol. While the K_m for mannitol in the high affinity regime is not changed by the presence of IIC^{Mtl} (K_m = 2.3 μM without IIC^{Mtl}, K_m = 3.0 μM with IIC^{Mtl}), there is a clear effect on the V_{max} in the high affinity regime. This rate is increased by approximately a factor of 2 compared with the experiment without IIC^{Mtl} (V_{max} = 109 nM mannitol-P/min without IIC^{Mtl}, V_{max} = 249 nM mannitol-P/min with IIC^{Mtl}). Similar observations apply to the low affinity regime.

Mannitol/mannitol-P exchange kinetics *versus* EII^{Mtl} concentration have, in the past, suggested that the EII^{mtl} dimer is essential for this reaction (9, 33, 34). Since the PEP-dependent mannitol phosphorylation is affected by the addition of IIC^{Mtl}, the question arises of whether the exchange reaction will be affected in a similar manner. Measurements of the exchange rates were carried out over the same IIC^{Mtl} concentration range as used for the PEP-dependent mannitol phosphorylation kinetics. The data for the highest IIC^{Mtl} concentration used in the phosphorylation, 330 nM, are presented in Fig. 8. The exchange kinetics of EII^{Mtl} are clearly influenced by IIC^{Mtl}; both Fig. 8, *A* and *B*, show that IIC^{Mtl} increases the exchange activity. Since both substrates, mannitol and mannitol 1-phosphate, have been reported to inhibit exchange (29), Fig. 8, *A* and *B*, examine the mannitol and mannitol 1-phosphate dependence of the stimulation by IIC^{Mtl}. Mannitol (Fig. 8*A*, ■) causes inhibition at concentrations higher than 1 μM; addition of IIC^{Mtl} (□) increases the rate but has no effect on the inhibition by mannitol. Mannitol 1-phosphate (Fig. 8*B*) does not cause the inhibition reported previously (29), instead, a saturation is observed. As in Fig. 8*A*, the presence of IIC^{Mtl} affects the rate but not the form of the curve.

Since previous kinetic studies have shown that the rates of both phosphorylation and exchange are dependent on the association state of EII^{Mtl}, we could ask whether the stimulation of the phosphorylation and exchange rates reported above is a direct or indirect effect of the presence of IIC^{Mtl}. For instance, the stimulation could be due to the occurrence of a EII^{Mtl}-IIC^{Mtl} heterodimeric complex, or it could be due to a population of the EII^{Mtl} monomers present in the reaction mixtures which are driven into the dimeric form by mass action in the presence of IIC^{Mtl}. If EII^{Mtl} is partially dissociated under our experimental conditions, it will be manifested as a change in EII^{Mtl} specific

TABLE I
Activity as function of the EII^{Mtl} concentration for the PEP-dependent phosphorylation and mannitol/mannitol-P exchange

EII^{Mtl} concentration	Specific activity PEP-dependent phosphorylation[a]
nM	*nmol Mtl-P min^{-1} nmol EII^{Mtl-1}*
0.125	312
0.25	374
0.375	407
0.75	410
1.5	426

EII^{Mtl} concentration	Specific activity mannitol/mannitol-P exchange[b]
nM	*nmol Mtl-P min^{-1} nmol EII^{Mtl-1}*
2.5	0.39
3.3	0.42
5	0.54
10	0.64

[a] The PEP-dependent phosphorylation was measured with a 100-μl reaction mixture containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 60 μM [^{3}H]mannitol, 100 nM EI, and 17.6 μM HPr at 30 °C. The assay procedure is described in the legend of Fig. 6A.

[b] The mannitol/mannitol-P exchange was measured with a 100-μl reaction mixture containing: 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 0.25% decyl-PEG, 250 μM mannitol 1-phosphate, and 200 nM [^{3}H]mannitol at 30 °C. The assay procedure is described in the legend of Fig. 6A.

activity as a function of the enzyme concentration. Table I lists the specific activities for PEP-dependent mannitol phosphorylation and mannitol/mannitol-P exchange as a function of EII^{Mtl} concentration in the concentration range used in the current experiments. The reaction conditions, pH, buffer, and temperature were the same as those used throughout this study. In Table I the specific activity found for the PEP-dependent phosphorylation increased only 4.5% upon a 4-fold increase of the EII^{Mtl} concentration from 0.375 to 1.5 nM, while in Fig. 7*A* where 0.39 nM EII^{Mtl} was used a stimulation of more then 100% was observed. This stimulation cannot, therefore, be attributed to a IIC^{Mtl}-dependent mass action effect on the association of EII^{Mtl} monomers. Similar arguments apply to the exchange reaction in Fig. 8*A*. Table I shows only a 20% increase in the mannitol/mannitol-P exchange rate on doubling the EII^{Mtl} concentration from 5 to 10 nM while, in Fig. 8*A*, where 10 nM EII^{Mtl} was used, a IIC^{Mtl}-dependent stimulation of more than 100% was observed.

The situation is different, however, in Fig. 8*B* where a 400% stimulation is observed due to the presence of IIC^{Mtl}. These measurements were done at 0.83 nM EII^{Mtl} where, judging from the rate increase in Table I, EII^{Mtl} is partially dissociated. Thus the higher degree of stimulation by IIC^{Mtl} could be due to a fraction of EII^{Mtl} present as monomers, which are inactive in the exchange process in the absence of IIC^{Mtl}. Both the inactive monomers and active homodimers would form active heterodimers in the presence of IIC^{Mtl} leading to more than a doubling of the exchange rate.

DISCUSSION

The plasmid $pMaIICP_r$ overexpresses IIC^{Mtl} to such an extent that the protein is visible on a Coomassie-stained SDS-polyacrylamide gel. The insertion of the λ-P_r promoter is necessary for this improved expression which is also manifested as a 16-fold increase in mannitol binding. These binding data confirm earlier reports (4, 5) that the membrane-bound portion of EII^{Mtl} contains the mannitol-binding site; it also confirms that the two cytoplasmic domains are not directly involved in binding. The affinity of mannitol for IIC^{Mtl} is only 2–3-fold lower than its affinity for the intact enzyme. EII^{Mtl}, in solubilized membranes or the purified form, has a K_d of 96 nM for mannitol (5), whereas IIC^{Mtl} has a K_d of 142 nM in solubilized membranes but 295 nM in the purified form. These changes could reflect some subtle change in the enzyme resulting from removal of the A and B domains or some difference in bound phospholipid. The binding data for the wild type enzyme indicated one high affinity binding site per dimer and a second site with a lower affinity in the order of 10 μM (32). With the mannitol concentrations used in the flow dialyses measurements in this study it is only possible to detect the high affinity site and not the second, low affinity site.

Association State

The association state of transport proteins could play an important role in their function (9, 33–36). There is much evidence that intact EII^{Mtl} is a dimer (6–8, 37) and the molecular mass of 103 kDa found for the purified IIC^{Mtl} suggests that this protein is also a dimer. After correction for the 35-kDa mass of a decyl-PEG micelle (38), the value comes very close to the expected mass of a IIC^{Mtl} dimer based upon the amino acid sequence. The value found after purification is substantially lower than the 175 kDa found for solubilized membrane extracts containing IIC^{Mtl} (8). This might be due to lipids or other components bound to the protein, which are removed by the purification procedure. The mass found for the smaller IIC^{Mtl} protein corresponds better with the expected mass of a dimer than that determined for EII^{Mtl} by gel filtration. The reason might be a more globular form compared with the multidomain EII^{Mtl}. The IIC^{Mtl} concentrations used in our kinetic measurements varied from 0.1 to 340 nM. The concentrations applied to the TSK column varied from 108 nM to 4.3 μM. Since a 100–150-fold dilution occurs on these columns and we still observe dimers, we conclude that the membrane-bound domain is predominately dimeric in the concentration range used in most of the kinetic experiments.

IIC^{Mtl}

Chimer Kinetics—When the chimer and IIC^{Mtl} were used separately to measure phosphorylation activity, it was not possible to detect PEP-dependent phosphorylation or mannitol/mannitol-P exchange activity. The combination of IIC^{Mtl} and the chimer, however, did catalyze both reactions. This shows that an active $IIBA^{Mtl}$ domain is essential for both reaction types, although a covalent link between IIC^{Mtl} and $IIBA^{Mtl}$ is not necessary. This result is comparable with what was found for the complementation of IIC^{Mtl} and purified $IIBA^{Mtl}$ where activity was also observed but, in those experiments, the apparent affinity of $IIBA^{Mtl}$ for IIC^{Mtl} was so low that saturation could not be observed at $IIBA^{Mtl}$ concentrations up to 80 μM (16). A similar low affinity was observed in the present experiments with IIC^{Mtl} and the chimer. When we compare this with the very high affinity of IIC^{Mtl} for native EII^{Mtl} (K_m = 2 nM) we are forced to conclude that there is very little if any specific interaction between IIC^{Mtl} and IIC^{Glc} in the chimer. The activity observed suggest that IIC^{Mtl} gains direct access to the $IIBA^{Mtl}$ in the chimer.

EII^{Mtl} Phosphorylation Kinetics—Evidence from size exclusion experiments of Lolkema *et al.* (8) shows that the interaction within an EII^{Mtl} dimer occurs between membrane-bound regions of the protein. The size exclusion data presented in this report confirm these observations. The functional nature of the interactions between the membrane-bound domains can be seen in the effect of IIC^{Mtl} on the activity of the wild type enzyme. Adding IIC^{Mtl} to EII^{Mtl} gives a maximum increase of approximately a factor of 2 in the rate of PEP-dependent phosphorylation, which could be explained by the formation of a heterodimer consisting of a IIC^{Mtl} monomer and an EII^{Mtl}

monomer. The situation is analogous to that described earlier for heterodimers consisting of phosphorylation site mutants (39). There it was shown that C384S EII^{Mtl}, which was inactive in mannitol/mannitol-P exchange due to the lack of its B domain phosphorylation site, caused nearly a doubling of the exchange rate of H554A EII^{Mtl} upon heterodimer formation.

The EII^{Mtl} monomer itself is active in PEP-dependent phosphorylation (9). The doubling of the phosphorylation rate by formation of a IIC^{Mtl}:EII^{Mtl} heterodimer seen in this report must mean that the monomers in the EII^{Mtl} homodimer are not maximally active. If we assume that the monomers in the homodimer functionally interact such that they operate sequentially rather than simultaneously, then they can only operate at half their maximum rate. Placing each EII^{Mtl} monomer in a heterodimer allows them to operate independently and accounts for the observed doubling of the phosphorylation rate. The sequential rather than simultaneous operation of each monomer in the dimer may be necessary to integrate both catalytic functions of the enzyme, phosphorylation and transport of mannitol. While one subunit is phosphorylating mannitol bound to the cytoplasmic site, the other is transporting mannitol from the periplasmic to the cytoplasmic site. A kinetic model that tries to explain this integration between transport and phosphorylation by functional dimers with coupled sites was described by Lolkema (40).

EII^{Mtl} Mannitol/Mannitol-P Exchange Kinetics—The interaction between IIC^{Mtl} and EII^{Mtl} also affects the mannitol/mannitol-P exchange reaction. There is evidence that the EII^{Mtl} dimer is essential for catalyzing mannitol/mannitol-P exchange (9, 33, 34), meaning that both subunits are involved in the reaction. Inhibition of exchange by both mannitol and mannitol 1-phosphate has also been reported (29). We observe similar inhibition by mannitol both in the presence and absence of IIC^{Mtl} but we have not observed inhibition by mannitol 1-phosphate; instead saturation is observed. The earlier experiments were done in the presence of P_i, F^-, Mg^{2+}, and mannitol 1-phosphate. We have shown that the combination of P_i or mannitol-1-P plus Mg^{2+} and NaF leads to a complex which inhibits the mannitol/mannitol-P exchange reaction (9). In the present experiment, NaF was not used, therefore, the inhibition did not occur.

The addition of IIC^{Mtl} causes an increase in mannitol/mannitol-P exchange activity, following the curve that is found without the addition of IIC^{Mtl}. In contrast to the stimulation found for the PEP-dependent phosphorylation, an increase of more than a factor of 2 is possible for the mannitol/mannitol-P exchange reaction. From the dependence of the specific activity on the enzyme concentration one cannot exclude the possibility that part of this stimulation might be explained by the presence of inactive EII^{Mtl} monomers in solution; these could be activated in mannitol/mannitol-P exchange by heterodimer formation with IIC^{Mtl}. The mannitol dependence of the IIC^{Mtl} stimulation was measured at higher EII^{Mtl} concentrations than the mannitol-phosphate dependence, resulting in a lower stimulation. At higher EII^{Mtl} concentrations there is only a small change in specific activity; nevertheless, the IIC^{Mtl} stimulation is still observed, meaning that this protein directly affects the exchange kinetics of EII^{Mtl}, probably by the formation of heterodimers. If both subunits are involved in catalyzing the mannitol/mannitol-P exchange reaction, the heterodimer data indicate that only one functional B domain per dimer is necessary for catalysis. The same conclusion was derived from the heterodimer studies of van Weeghel *et al.* (39).

Conclusion

The rate increase in both the PEP-dependent and mannitol/mannitol-P exchange reaction caused by a specific interaction between the mannitol-binding domain and the wild type enzyme gives experimental evidence that EII^{Mtl} is a functional dimer. The observed rate increase might indicate that both subunits in the wild type dimer do not work simultaneously. The interactions between the mannitol-binding domains might be essential for the integration of the transport and phosphorylation functions of the enzyme.

Acknowledgment—The mass spectroscopy determination of the IIC^{Mtl} molecular weight was performed in the laboratory of P. Roepstorff at the University of Odense, Denmark.

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