Regulated high-level expression of the mannitol permease of the phospho*enol*pyruvate-dependent sugar phosphotransferase system in *Escherichia coli*

(expression vectors/enzyme reactivation)

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ABSTRACT   The structural gene (*mtlA*) of the *Escherichia coli* phospho*enol*pyruvate-dependent mannitol-transport protein (EII^{mtl}) and its upstream promoter region (P_{mtl}) were subcloned ≈150 base pairs downstream of a λ P_R promoter on a multicopy mutagenesis/expression vector and used to transform a mutant (MtlA$^-$) *E. coli* strain. Induction at 42°C led to 50- to 100-fold overproduction of EII^{mtl} (5–10 mg/g of cell wet weight) relative to mannitol-induced levels in a wild-type (Mtl$^+$) strain. Most of the overproduced protein was sequestered as an inactive form in inclusion bodies and cytoplasmic membranous structures. The protein could be extracted in an active form by rupturing the cells with lysozyme and sonication or with a passage through a French pressure cell and incubating the inclusion bodies and membranous structures with detergent (Lubrol PX or deoxycholate) in the presence of Q or S Sepharose ion-exchange resin for several hours. This procedure resulted in a 20- to 25-fold overproduction of active EII^{mtl} compared with mannitol-induced wild-type levels.

The mannitol-specific enzyme II (EII^{mtl}) of the phospho*enol*pyruvate-dependent sugar phosphotransferase system (PTS) in *Escherichia coli* catalyzes, in combination with heat-stable protein (HPr) and enzyme I (EI), the uptake and concomitant phosphorylation of D-mannitol (1, 2). It was the first PTS EII to be purified (3), functionally reconstituted in liposomes (4, 5), and physicochemically analyzed (6–11). It has been the subject of several kinetic studies (12, 13). In addition, the structural gene (*mtlA*) has been sequenced (14) and genetically analyzed (15, 16). EII^{mtl}, therefore, represents an attractive and important model for studying structure–function relationships of the mechanism of sugar transport. The availability of a regulated expression system to produce large amounts of protein would contribute significantly to these investigations. We report here on the high-level expression and recovery of active EII^{mtl} from a mutagenesis/expression phagemid system in *E. coli*.

MATERIALS AND METHODS

Materials. Q and S Sepharose Fast Flow, hexylagarose, Lubrol PX, sodium deoxycholate, and phospho*enol*pyruvate were purchased from Sigma. Goat anti-rabbit IgG–horseradish peroxidase conjugate and reagents for color development were from Bio-Rad. D-[^{14}C]Mannitol (56 mCi/mmol; 1 mCi = 37 MBq) was from Amersham. The nonionic detergent decyl-PEG was obtained from B. Kwant (Department of Chemistry, University of Groningen). The EI and HPr components of the PTS were purified as described (17). Restriction enzymes and phage T4 DNA ligase were obtained from

Table 1.   *E. coli* strains and plasmids

Name	Genotype or phenotype	Source or ref.
Strains		
ML308-225	MtlA$^+$	*
LJ1008	C600; F$^-$ *thr-1 leu-6 thi-1 supE44 lacY tonA21 mtlA29*	18
LJ1008-113	LJ1008; *recA*	†
L146-1	F$^-$ *lacY1 galT6 xyl-7 thi-1 hisG1 argG6 metB1 rpsL104 mtlA2 gutA50 gatA50* (Mal$^+$)	19
ASL-1	L146-1; *recA*	†
Plasmids		
pASL	pACYC184, Cm^R, *mtlA$^+$*	†
pJRD187	Amp^R, *cI857–P_R*	20
pMa5-8	Amp^R, Cm^R, f1 *ori*	21
pWAMa	pMa5-8; *mtlA$^+$*	‡
pWAMaPr	pWAMa; *cI857–P_R*	‡
pJRDA	pJRD187; *mtlA$^+$*	‡

*Our laboratory collection.
†J. Lengeler and A. Scholle (Department of Biology, University of Osnabruck, Osnabruck, F.R.G.).
‡This study.

Boehringer Mannheim or Bethesda Research Laboratories and were used as recommended by the supplier. All other biochemicals were of analytical grade.

Strains and Plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strain LJ1008 had a chromosomal deletion in the *mtlA* gene. *E. coli* strains ASL-1 and LJ1008-113 were used for plasmid propagation and expression. *E. coli* strain ML308-225 was a wild-type (MtlA$^+$) strain from our laboratory collection. Plasmid pJRD187, containing the *cI857* repressor mutation and P_R promoter of bacteriophage λ (20), was used for vector constructions. Plasmid pMa5-8, a mutagenesis vector, was obtained from H. J. Fritz and W. Kramer (21). Plasmid pASL was a pACYC184 derivative containing the *mtlA* structural gene and its promoter (P_{mtl}). Small- and large-scale plasmid isolations, restriction and ligation of DNA, and transformations of bacteria were carried out as described (22).

SDS/Polyacrylamide Gel Electrophoresis and Immunoblotting. Protein samples were denatured in SDS-containing buffer for 5 min at 60°C. Electrophoresis was carried out in 12.5% gels according to the method of Laemmli (23). The gels were either stained with Coomassie brilliant blue or used for transfer of proteins to nitrocellulose filters (Western blots). EII^{mtl} on the Western blots was detected immunochemically by polyclonal antibodies raised in rabbit against purified EII^{mtl}, in combination with an IgG–horseradish peroxidase

conjugate, as described (10). Protein was determined by the method of Bradford (24) with bovine serum albumin as a standard.

Growth and Selection Conditions for EII^{mtl} Expression. After transformation of the recombinant plasmids into various host strains, the bacteria were selected on plates for the antibiotic-resistance markers (ampicillin, 100 μg/ml, and chloramphenicol, 25 μg/ml) and for sugar fermentation on McConkey base indicator plates (Difco) containing 0.2% mannitol. TY (Bacto-tryptone-yeast, with NaCl) supplemented with ampicillin (100 μg/ml) was used as the complex medium. For overexpression, 250-ml cultures were grown aerobically at 30°C to mid- or late-exponential phase (OD$_{600}$ of 0.6–1.0). The inactivation of the temperature-sensitive cI857 repressor and the concomitant induction of the λ P_R promoter was achieved by addition of an equal volume of fresh 53°C medium followed by growth at 42°C. At the appropriate time after induction, the culture was harvested (14,000 $\times$ g for 10 min) and washed in buffer A (50 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride) and pelleted.

Preparation of Cell Fractions. The pelleted cells (5 g, wet weight) were resuspended in 50 ml of buffer A and passed through a French pressure cell at 8000–10,000 psi (1 psi = 6.89 kPa). The cell debris, containing the inclusion bodies, was removed by centrifugation at 24,000 $\times$ g for 10 min and resuspended in 5 ml of buffer A. The membrane fraction was collected by centrifugation of the resulting supernatant at 200,000 $\times$ g for 60 min and resuspended in 5 ml of buffer A. The supernatant from this step and the above-mentioned fractions were stored in small aliquots in liquid nitrogen until used. In the second method, the cells were resuspended in 50 ml of buffer A with lysozyme (1 mg/ml), kept on ice for 30 min, and ruptured by sonication (six times, 20 sec each; Vibrocell, Sonics and Materials, Danbury, CT; output control setting: g at maximum output). The broken-cell suspension was then centrifuged at 20,000 $\times$ g for 5 min. The supernatant was collected and the pelleted cell debris was resuspended in 25 ml of buffer A. Both fractions were stored in liquid nitrogen until used.

Extraction of EII^{mtl}. Solubilization or extraction of EII^{mtl} from the above fractions was achieved by mixing 0.5-ml volumes with an equal bed-volume of Q or S Sepharose Fast Flow or hexylagarose equilibrated in buffer A supplemented with 0.5% deoxycholate, Lubrol PX, or decyl-PEG and shaking the suspensions gently at 4°C overnight (25). The suspensions were then centrifuged to remove the resins. The pelleted resins were resuspended in buffer A containing the same detergents and 0.5 M NaCl and were shaken overnight at 4°C for elution of adsorbed EII^{mtl}. All supernatant fractions were measured for phosphorylation activity.

EII Activity Assay. Mannitol phosphorylation activity was measured as the amount of [^{14}C]mannitol 1-phosphate formed per minute per milligram of protein, as described (9). Activities were measured as a function of time at various enzyme concentrations and were linear with respect to both parameters.

Electron Microscopy. Whole cells were fixed in 1.5% (wt/vol) KMnO$_4$ for 20 min at room temperature and 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C. Postfixation was performed in a solution of 1% (wt/vol) OsO$_4$ and 2.5% (wt/vol) K$_2$CrO$_7$ in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at room temperature. After dehydration in a graded ethanol series the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips (Eindhoven, The Netherlands) EM 300 without further staining (26).
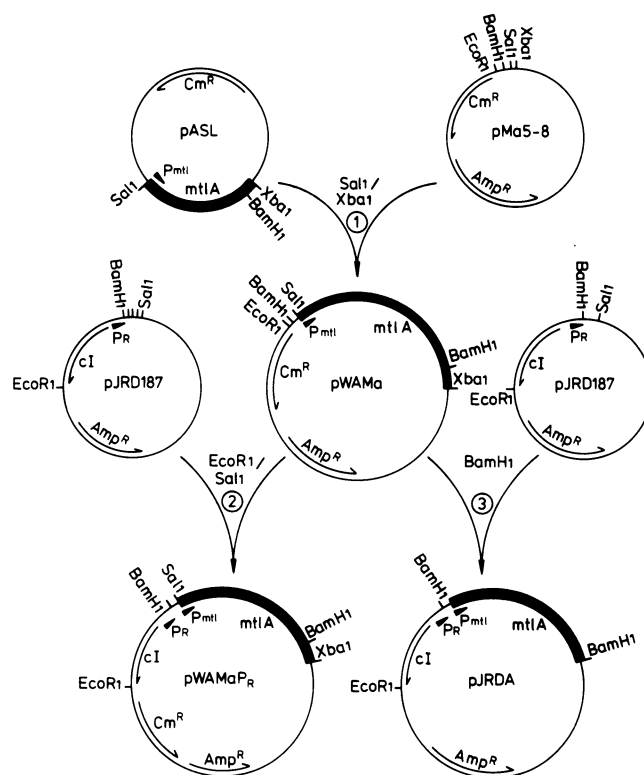

FIG. 1. Plasmid construction. (Step 1) pWAMa was constructed by insertion of a *Sal* I–*Xba* I fragment containing the *mtlA* and promoter sequences from pASL into the *Sal* I–*Xba* I sites of pMa5-8. The recombinant plasmid was identified by EII^{mtl} activity and ampicillin resistance (Amp^R) on indicator plates. (Step 2) pWAMaPr was obtained by insertion of an *Eco*RI–*Sal* I fragment from pJRD187 containing the *cI857–P$_R$* cassette into the *Eco*RI–*Sal* I sites of pWAMa and selection for chloramphenicol resistance (Cm^R). (Step 3) pJRDA was constructed by insertion of an isolated *Bam*HI fragment from pWAMa into the unique *Bam*HI site of pJRD187 and selection for EII^{mtl} activity on ampicillin-containing indicator plates. The *mtlA* gene sequences are represented as black bars. Promoter sequences (P_R and P_{mtl}) are indicated by arrowheads. Direction of transcription is indicated by arrows.

RESULTS

Overexpression of EII^{mtl}. Two plasmids containing the *mtlA* gene were constructed for use in this study (Fig. 1). The plasmid pWAMa was constructed by subcloning the *mtlA* gene from pASL into the mutagenesis vector pMa5-8. In spite of the multiple copy number of pWAMa, there was no increased production of EII^{mtl} compared to the mannitol-induced wild-type level of ML308-225 as judged by Western blots and activity assays. To improve enzyme production, the λ temperature-sensitive repressor–promoter cassette, *cI857–P$_R$*, from the expression vector pJRD187, was inserted 150 base pairs upstream of the mannitol promoter (P_{mtl}) in pWAMa, resulting in the plasmid pWAMaPr. A similar expression vector, pJRDA, was constructed by introducing the *mtlA* gene into the expression vector pJRD187, just behind the λ P_R promoter.

The expression levels of EII^{mtl} in mannitol-induced wild-type cells and in induced and uninduced cells harboring the plasmid pWAMaPr were compared by SDS/polyacrylamide gel electrophoresis and immunoblotting (Figs. 2 and 3). Both plasmids containing a tandem promoter arrangement constitutively expressed EII^{mtl} from the P_{mtl} promoter when the cells were grown at 30°C (Fig. 3; pJRDA data not shown). When the growth temperature of the cells harboring pWAMaPr was shifted to 42°C, induction of the P_R promoter led to a concomitant 50- to 100-fold overproduction of EII^{mtl}

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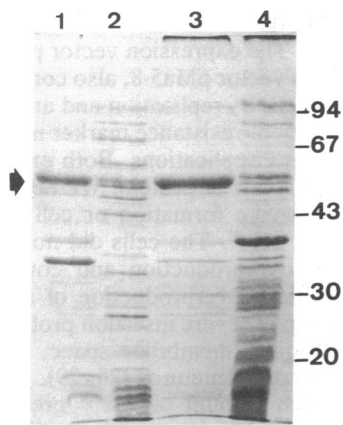Proc. Natl. Acad. Sci. USA 87 (1990)   2615

FIG. 2. Comparison by SDS electrophoresis of EII^{mtl} expression levels in mannitol-induced wild-type cells versus induced cells harboring the plasmid pWAMaPr. Five micrograms of protein was loaded per lane. Lanes 1–3, debris pellet, cytoplasm, and membrane fractions, respectively, from induced cells harboring pWAMaPr; lane 4, membrane fraction from D-mannitol-induced ML308-225. Positions of size markers (kDa) are at right: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. Arrow indicates EII^{mtl} band. The gel was stained with Coomassie brilliant blue.

(5–10 mg/g of cell, wet weight) compared to the uninduced cells harboring pWAMaPr and to the mannitol-induced wild-type strain ML308-225. The enzyme production was measured by serial dilution on Western blots and by EII^{mtl} activity assays. In spite of the relatively high overexpression, the level of EII^{mtl} phosphorylation activity was not always increased. The discrepancy observed between enzyme levels and enzyme activity could indicate that much of the enzyme was sequestered in an inactive form in the intracellular aggregates seen in Fig. 4A. This would be in agreement with
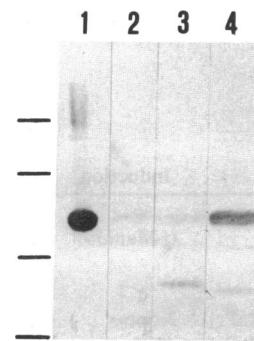

FIG. 3. Immunoblot comparison of EII^{mtl} expression levels in total cell extract of mannitol-induced wild-type cells versus cells harboring the plasmid pWAMaPr. Lane 1, purified EII^{mtl} (78 ng); lane 2, mannitol-induced ML308-225 (5 μg of protein); lane 3, uninduced LJ1008-113 harboring pWAMaPr (5 μg); lane 4, induced LJ1008-113 harboring pWAMaPr (5 μg). Markers indicate (from top) 94, 68, 43, and 30 kDa.

the relatively high EII^{mtl} content in the cell debris pellet after a low-speed centrifugation step (Fig. 2, lane 1). The enzyme could also be sequestered in incorrectly folded inactive protein patches in the cytoplasmic membrane or in the intracellular membranous structures seen in Fig. 4B. This could explain the high enzyme content in the membrane fraction (Fig. 2, lane 3). We cannot rule out the possibility that the membrane fraction was contaminated by small aggregates after the second high-speed centrifugation step. A dramatic increase of intracellular membranous structures (Fig. 4B) always accompanied the induction and expression of the enzyme; this was never noticed in the control cells harboring the parental plasmids pMa5-8 or pJRD187.

Recovery of Active EII^{mtl}. Inactive EII^{mtl} was recovered in an active form by the procedure of Hoess et al. (25). Both ion-exchange resins, Q and S Sepharose Fast Flow, were able
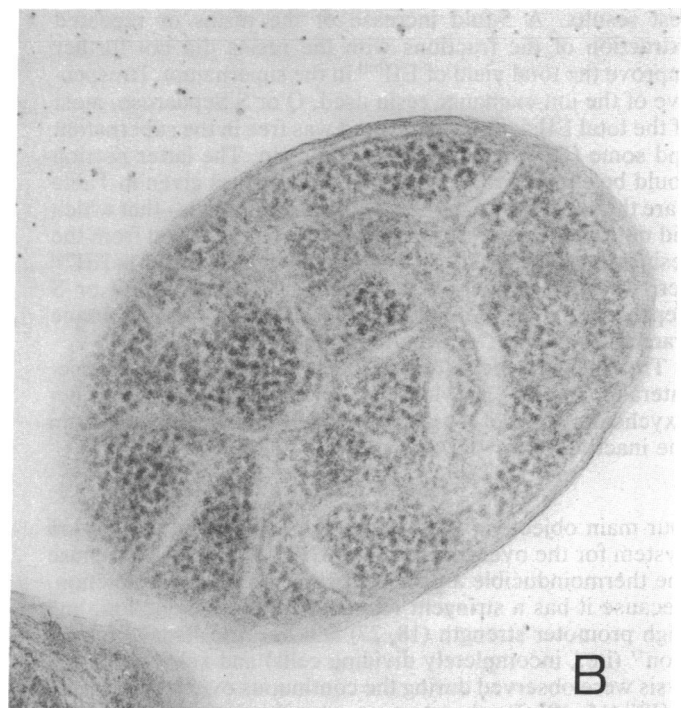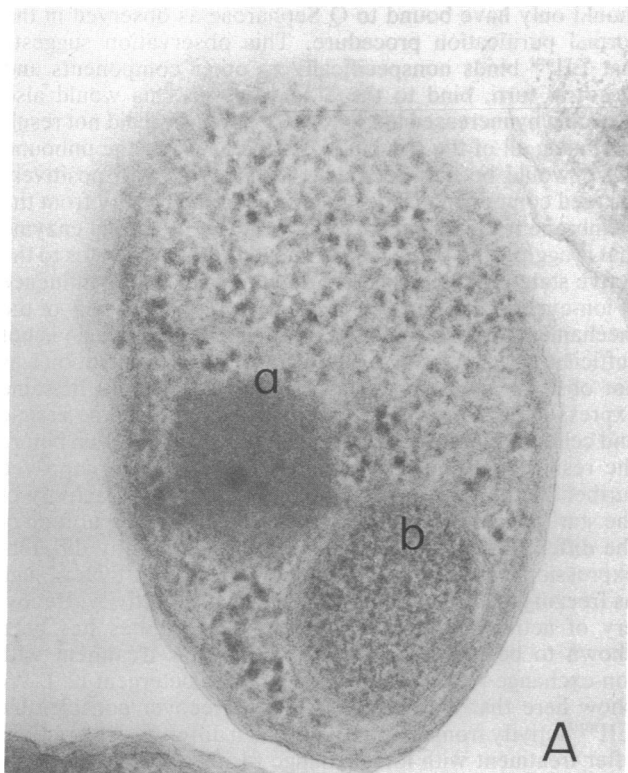

FIG. 4. Electron micrographs of E. coli carrying the plasmid pWAMaPr after induction of the λ P_R promoter at 42°C. Induced E. coli cells were fixed, thin-sectioned, and examined in a Philips EM 300. (A) A vesicle-like structure (a) and a possible inclusion body (b). (B) Intracytoplasmic membranous structures. (×60,000.)

Table 2. Extraction, recovery, and comparison of EII^{mtl} activity

Method, strain, and fractions	Induction	EII^{mtl} activity, nmol/min per mg of protein	
		Before extraction	After extraction
French press			
ML308-225	D-Mannitol		
Vesicles		250	350
Debris pellet		ND	ND
LJ1008-113(pWAMaPr)	None	130	130
LJ1008-113(pWAMaPr)	42°C		
Vesicles		300	3000
Debris pellet		200	1400
Lysozyme/sonication			
LJ1008-113(pWAMaPr)	42°C		
Supernatant		ND	2400
Debris pellet		100	2700

*Mean activity from two independent expression and extraction experiments with S Sepharose in combination with Lubrol-containing buffer A. The values represent the sum of the activities of the supenatants collected before and after elution of the resin. Previous measurements have shown that no EII^{mtl} activity is found in the supernatant after the 200,000 × g step to remove cytoplasmic membranes from a crude cell extract. ND, not determined in this series of experiments.

to enhance the recovery to approximately the same extent in the presence of the detergent deoxycholate or Lubrol PX. Table 2 presents the data for recovery from S Sepharose. A 10-fold increase of active EII from the membrane vesicle fraction was achieved compared to the starting material, while from the cell debris fraction of the lysozyme/sonication preparation a 27-fold increase of active EII could be obtained by exposure to either Q or S Sepharose in buffer containing 0.5% deoxycholate or 0.5% Lubrol, but not by the detergent-containing buffer alone. Incubation periods of 4, 16, and 48 hr were checked to improve the yield of active enzyme in the supernatants; the overnight incubation period, 16 hr, gave the best results. A 5-fold increase of the resins or repeated extraction of the fractions with the resins did not further improve the total yield of EII^{mtl} in the supernatant. Irrespective of the ion-exchange resin used, Q or S Sepharose, most of the total EII activity recovered was free in the supernatant and some (10%) was bound to the resin. The latter portion could be eluted with 0.5 M NaCl. The values given in Table 2 are the sum of the activities of the supernatants—that which did not bind to the resin plus that which was eluted from the resin with 0.5 M NaCl. All fractions gave an increase in EII^{mtl} activity after extraction with buffer A containing Q or S Sepharose and 0.5% deoxycholate or Lubrol. The membrane fraction, however, showed the highest increase.

Treatment of the different fractions with a hydrophobic-interaction resin, hexylagarose, in buffer A containing deoxycholate, Lubrol, or decyl-PEG left the enzyme mostly in the inactive form.

DISCUSSION

Our main objective was to establish a regulated expression system for the overproduction of EII^{mtl} in *E. coli*. We chose the thermoinducible λ promoter system for overproduction, because it has a stringent regulation, simple induction, and high promoter strength (18, 27) and because "snake formation" (i.e., incompletely dividing cells) and subsequent cell lysis were observed during the continuous overproduction of EII^{mtl} (15, 18). Furthermore, overproduction of β-glucoside-specific EII under the control of a tightly regulated λ promoter was reported to occur without severe cell-lysis problems (28). We constructed two expression vectors, pWA-

MaPr and pJRDA, that contained the λ cI857 repressor–P_R promoter cassette. The expression vector pWAMaPr, based on the mutagenesis vector pMa5-8, also contains an f1 origin for single-stranded DNA replication and an amber mutation in the chloramphenicol-resistance marker necessary for site-directed mutagenesis applications. Both expression vectors yield equally high levels of protein. We never observed the above-mentioned snake formation or cell lysis during the overproduction of EII^{mtl}. The cells did not show abnormal phenotypes after overproduction and could be grown to normal cell densities. Overproduction of an integral membrane protein can give severe insertion problems, because of a possible limitation in membrane space, inactivation, and increase in membrane structures (28, 29). This may be one reason for the high amount of EII^{mtl} present in inclusion bodies and intracytoplasmic membranous material. The inactivity of EII^{mtl} is most likely due to the occurrence of insoluble aggregates formed with other proteins in the cytoplasm or to incorrect folding of the enzyme in the membrane (30).

If incorrect folding is the explanation for the occurrence of inactive EII^{mtl}, it is difficult to rationalize how the relatively mild treatment with ion-exchange resins is able to regenerate activity, since these resins do not induce folding changes in active, soluble EII^{mtl}. This leaves the possibility that EII^{mtl} is rendered insoluble and inactive by virtue of nonspecific complexation with other cellular material. The role of the ion-exchange resin as activator, in this case, may be explained in terms of the forces they exert on the binding interactions between the various components in the complexes. These complexes are stabilized by ionic and nonionic interactions. The resin could compete for some of the components in the complex, thereby freeing coprecipitated protein. EII^{mtl} was recovered by using either Q or S Sepharose, indicating that both positively and negatively charged components participate in the complexes. Surprisingly, EII^{mtl} activity bound to both resins even though, at the pH used, it should only have bound to Q Sepharose as observed in the normal purification procedure. This observation suggests that EII^{mtl} binds nonspecifically to other components and these, in turn, bind to the S Sepharose. This would also explain why increased amounts of Q Sepharose did not result in binding all of the solubilized EII^{mtl} activity; the unbound EII^{mtl} would be nonspecifically complexed with positively charged components. The large increase of activity from the membrane fraction could be due to correctly folded enzyme that is aggregated in the membrane but that dissociates to the active state in the presence of detergent under the influence of ion-exchange resins. The presence of detergent only or the mechanical forces of agitation in the presence of resin is not sufficient to account for the activation, since reactivation was not observed using hexylagarose and detergents. In some expression experiments the activity in the membrane vesicle and cell debris pellet was increased 10- to 25-fold even before the resin extraction treatment and could not be improved further by the resin procedure. This variation in activity of the starting material after overproduction and preparation of the different cell fractions may be due to slightly different expression levels, time of expression, or storage factors such as freezing and thawing before assaying the activity. Recovery of active enzyme from inactive aggregates has been shown to be possible in some cases after treatment with ion-exchange resins in combination with detergent (25). We show here that it is also possible to recover considerable EII^{mtl} activity from inactive material in different cell fractions after treatment with ion-exchange resins and detergents.

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