Phosphoenolpyruvate-Dependent Mannitol Phosphotransferase System of *Escherichia coli*: Overexpression, Purification, and Characterization of the Enzymatically Active C-Terminal Domain of Enzyme II^{mtl} Equivalent to Enzyme III^{mtl} †

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ABSTRACT: The extreme C-terminus (Ser-490 to Lys-637) of the *Escherichia coli* EII^{mtl} was subcloned to test structural and mechanistic proposals about the existence of an EIII-like domain in this enzyme. Oligonucleotide-directed mutagenesis was used to produce a unique *Nco*I restriction site and, at the same time, to change Ser-490 into methionine in a flexible region in front of the proposed EIII-like domain. The 16-kDa C-terminal domain (CI) was overexpressed in *Escherichia coli*, purified, and analyzed in vitro for catalytic activity in the presence of an EII^{mtl} mutated at its first phosphorylation site, His-554 (EII-H554A). The results presented show that this domain can be expressed as a structurally stable, enzymatically active entity which is able to restore the PEP-dependent phosphorylation activity of the mutant EII^{mtl}-H554A to 25% of wild-type levels. To demonstrate the EIII activity of the CI domain in a more direct way, we also substituted it for $EIII^{mtl}$ in the *Staphylococcus carnosus* system. The CI domain was active in transferring the phosphoryl group to *Staph. carnosus* EII; however, it was 6.5 times less active compared to *Staph. carnosus* $EIII^{mtl}$ itself. $EIII^{mtl}$ from *Staph. carnosus*, on the other hand, was able to substitute for the isolated C-terminal domain in the *E. coli* mannitol phosphorylation assay; however, it appeared to be 2 or 3 times less effective.

The mannitol-specific phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system (PTS)[1] in *Escherichia coli* catalyzes the translocation and concomitant phosphorylation of its sugar D-mannitol (Postma & Lengeler, 1985; Robillard & Lolkema, 1988). This process requires the participation of two general, cytoplasmic phosphoproteins of the PTS, EI and HPr, as shown in Scheme I.

Scheme I

$$\text{PEP} + \text{EI} \leftrightarrow \text{P-EI} + \text{pyruvate} \quad (1)$$

$$\text{P-EI} + \text{HPr} \leftrightarrow \text{P-HPr} + \text{EI} \quad (2)$$

$$\text{P-HPr} + \text{EII}^{mtl} \leftrightarrow \text{P-EII}^{mtl} + \text{HPr} \quad (3)$$

$$\text{P-EII}^{mtl} + \text{mtl}_{out} \leftrightarrow \text{P-mtl}_{in} + \text{EII}^{mtl} \quad (4)$$

The mannitol-specific transport protein (EII^{mtl}) has been extensively characterized (Jacobson et al., 1983; Stephan & Jacobson, 1986; Stephan et al., 1989; Pas et al., 1988; Roossien et al., 1984; Pas & Robillard, 1988a,b; Grisafi et al., 1989; White & Jacobson, 1990), and its gene, *mtl*A, has been cloned and sequenced (Lee et al., 1983). These studies have revealed that the protein is a single polypeptide chain of 637 amino acid residues and consists of a hydrophobic N-terminal domain and a hydrophilic C-terminal domain which protrudes into the cytoplasm. It was speculated that a portion of this hydrophilic region of EII^{mtl} serves as a covalently bound enzyme III ($EIII^{mtl}$) component (Saier et al., 1988). Recently, Pas et al. (1988) have shown that EII^{mtl} contains two phosphorylation sites per monomer and that both sites are located exclusively in the cytoplasmic domain (Pas & Robillard, 1988b).

The uptake of mannitol in *Staphylococcus aureus* and *Staphylococcus carnosus* cells is also achieved by the PTS (Friedman & Hays, 1977). It differs from the *E. coli* phosphorylation scheme by the fact that it possesses a soluble mannitol-specific EIII ($EIII^{mtl}$). In *E. coli*, the phosphoryl group is transferred directly from P-HPr to EII^{mtl} as shown in the reaction scheme above. Recently, nucleotide and peptide sequence information about $EIII^{mtl}$ of *Staph. aureus* and *Staph. carnosus* was published which showed a 38% homology to the hydrophilic portion of EII^{mtl} of *E. coli* (Reiche et al., 1988; Fischer et al., 1989). These results together with the data on deletion mutants of EII^{mtl} produced by Grisafi et al. (1989) and the complementation data of a truncated EII^{mtl} with a large C-terminal fragment of EII^{mtl} (Stephan et al., 1989; White et al., 1990) strongly support the idea that EII^{mtl} of *E. coli* is a fusion protein of an $EIII^{mtl}$ with an EII^{mtl} protein located in the membrane. Oligonucleotide-directed mutagenesis provides a convenient method for producing gene fragments corresponding to known and suggested structural domains for studies concerning structure–function relationships. In the present study, this method has been applied to EII^{mtl}, a protein with two or more domains. The mutagenesis,

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PEP, phosphenolpyruvate; EI, enzyme I; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; CI-EII^{mtl}, cytoplasmic domain residues 490–637; CII-EII^{mtl}, cytoplasmic domain residues 348–488; CIII-EII^{mtl}, cytoplasmic domain residues 348–637; NIII-EII^{mtl}, membrane domain residues 1–347; ISO, inside-out.

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overproduction, and purification of a genetically engineered EIII-like C-terminal polypeptide are described. Furthermore, we report kinetic evidence that this domain is able to restore the transport activity of an *E. coli* EII^{mtl} mutated at the first phosphorylation site, His-554, and to substitute for an $EIII^{mtl}$ in the *Staph. carnosus* system, by complementation in vitro.

Materials and Methods

Materials

Restriction endonucleases, DNA polymerase (Klenow fragment), T_4 DNA ligase, T_4 polynucleotide kinase, and isopropyl β-D-thiogalactoside were obtained from Boehringer. Nitrocellulose filters were from Schleicher & Schuell, and the goat anti-rabbit IgG–HRP conjugate was purchased from Bio-Rad. Polyclonal antibodies against EII^{mtl} were raised in rabbits with purified EII^{mtl} as described by Pas et al. (1987). Sephadex G-75 and Q-Sepharose Fast Flow resins were obtained from Pharmacia. The oligonucleotide R2, 5′-pCAGGTTAGCC*A*TGGAATCGTC-3′, obtained from Eurosequence BV Groningen, was prepared on an Applied Biosystems Model 380B DNA synthesizer, completely deprotected, and used unpurified. Enzyme I and HPr were prepared as previously described (Dooyewaard et al., 1979). D-[1-^{14}C]Mannitol (59 mCi/mmol) was purchased from Amersham.

Methods

Mutagenesis and Subcloning of CI. Oligonucleotide-directed mutagenesis with amber selection was performed according to the procedure of Stanssens et al. (1989). See also the previous paper for detailed information about this protocol (van Weeghel et al., 1990).

The oligonucleotide primer, R2, was synthesized to direct the mutation that introduced a new *Nco*I restriction site upstream of the region thought to encode the EIII-like domain starting at residue Leu-493. Positive mutants were identified by preparing double-stranded plasmid DNA (dsDNA) from randomly picked transformants and cutting them with *Nco*I restriction enzyme. Mutant plasmid pWAMc2 gave, by restriction analysis, two fragments of 4.95 and 1.2 kb, respectively, whereas the parental vector pWAMa gave one fragment of 6.15 kb only.

The recombinant plasmid pWAMc2 was constructed to obtain a convenient *Nco*I site, with an internal ATG initiation codon, for easy subcloning of the *Nco*I–*Hin*dIII gene fragment encoding the C-terminal domain (CI, amino acids 490–637). Subcloning was done in the expression vector pKK233-2 (Amann & Brosius, 1985) for controlled overexpression of the domain behind the *trc* promoter. The pKK233-2 and pWAMc2 vectors were cut with *Nco*I and *Hin*dIII, mixed, and religated. The ligation mixture was first cut with *Pst*I, to eliminate the parental vector pKK233-2, transformed into BMH71-18 MutL, and plated on 2×TY plates containing ampicillin (100 μg/mL). The resulting recombinant plasmid, pKKCI, now contained a strong regulated promoter, *trc*, the *lac*Z ribosome binding site, and an ATG start codon within the *Nco*I site (Figure 1). To maintain the facility for oligonucleotide-directed mutagenesis and manipulation of CI, pKKCI was digested with *Eco*RI and *Bam*HI. The resulting *Eco*RI–*Bam*HI DNA fragment, containing the complete CI promoter region, was recloned in the mutagenesis vector pMc5-8. After selection on 2×TY plates containing chloramphenicol (25 μg/mL), plasmid DNA was prepared from several colonies and tested for the right *Eco*RI–*Bam*HI insert of 1.2 kb. The recombinant plasmid was named pMcCI (Figure 1).

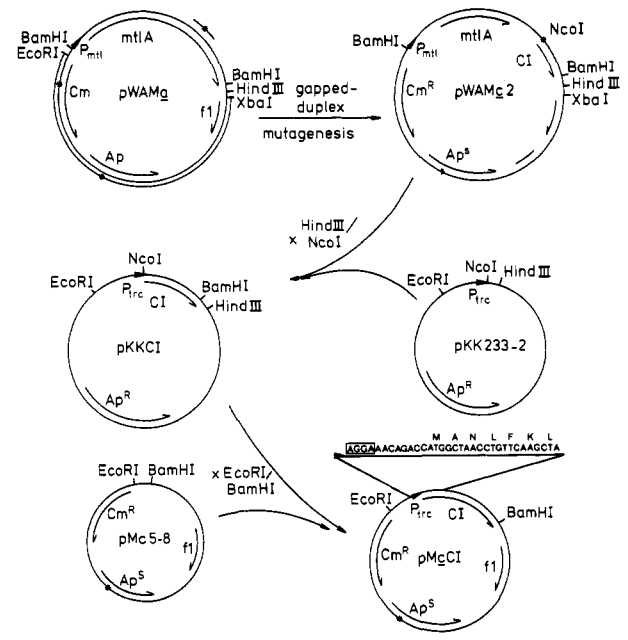

FIGURE 1: Construction of an expression/mutagenesis vector system for the C-terminal domain CI. A *Nco*I site was introduced into pWAMa by oligonucleotide-directed mutagenesis using R2 as a mismatch primer. A *Nco*I–*Hin*dIII fragment from the resulting plasmid, pWAMc2, containing amino acids 491–637, was used to construct the expression plasmid, pKKCI. Plasmid pMcCI was constructed by cloning the *Eco*RI–*Bam*HI fragment, now containing the CI domain fused to the *trp-lac* promoter, into pMc5-8 DNA linearized with *Eco*RI and *Bam*HI. The expression vector pMcCI can also be used in the gapped-duplex mutagenesis method as described under Materials and Methods.

The entire gene fragment encoding the CI domain was sequenced by the dideoxynucleotide method (Sanger et al., 1977), using two synthetic primers.

Overexpression of the CI-Terminal Domain. The expression/mutagenesis vector pMcCI was transformed by the calcium chloride procedure (Maniatis et al., 1982) into the *E. coli* strains ASL-1 [F⁻, *lac*Y1, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, *gut*A50, *gat*A50 (MAL⁺)] and JM101, Δ(*lac-pro*AB), thi,[F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15] for expression. In contrast to the JM101 strain, ASL-1 cells did not contain a lac repressor for repression of the *trc* promoter during growth of the cells; therefore, there would be a continuous expression of CI. JM101 cells had to be induced with IPTG prior to expression.

A single colony of both strains harboring pMcCI was picked to set up an overnight culture in 2×TY medium. One hundredth of the volume was used to inoculate 1 L of 2×TY medium in the presence of chloramphenicol (25 μg/mL). ASL-1 was grown for 24 h. JM101 cells were grown to an OD_{600} of 0.7 at which point IPTG was added to a final concentration of 1 mM. The induced JM101 culture was incubated for 24 h; to follow the course of the induction, 1-mL samples were taken at different times and centrifuged, and the pellets were frozen at −20 °C until used for protein characterization. The cultures were harvested after 24 h (20000*g* for 10 min). The cell pellets were resuspended at 10 mL/g wet weight of cells in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT, RNase, and DNase) and passed 2 times through a French pressure cell at 8000–10000 psi. The suspension was then centrifuged at 200000*g* for 60 min to remove cell debris and membrane fragments. The pellet was discarded, and the supernatant was dialyzed overnight against buffer B (20 mM Tris-HCl, pH 9.5, and 1 mM DTT) and stored in liquid nitrogen if not used immediately.

Purification of the CI Terminal. The supernatant (60 mL) containing the soluble CI domain was applied to a Q-Sepharose Fast Flow column (10 × 2.5 cm) equilibrated with buffer B (20 mM Tris-HCl, pH 9.5, and 1 mM DTT). After the sample was loaded, the column was washed with 100 mL of buffer B containing 150 mM NaCl. Soluble proteins were eluted with a gradient of 150–300 mM NaCl in buffer B (120 mL/h, total volume 300 mL). The CI-containing fractions eluted at 250 mM NaCl. Because of the high amount of CI expressed by these overproducing cells, fractions containing CI could be identified directly by SDS–PAGE (15%) after being stained with Coomassie brilliant blue. The fractions were pooled, dialyzed overnight against buffer C (50 mM KP_i, pH 8.0), and concentrated by pressure dialysis (Amicon UM-10 membrane) to a final volume of 5 mL. The resulting protein suspension was chromatographed on a 150 × 2 cm Sephadex G-75 with buffer C. The resulting fractions were analyzed by SDS–PAGE, and those containing CI were pooled and lyophilized.

Protein Characterization. One-milliliter samples of cultures grown in 2×TY medium were harvested by centrifugation, and the cell pellets were lysed in 100 μL of Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol, 0.05% bromophenol blue, and 4% β-mercaptoethanol). Samples were boiled for 5 min prior to being loaded, and 10–20 μL of lysate per well was analyzed by electrophoresis on 15% SDS–polyacrylamide gels (SDS–PAGE) and stained with Coomassie brilliant blue (Laemmli, 1970).

The purity of the CI domain was tested by SDS–PAGE (15%) stained with Coomassie brilliant blue or silver by the method of Wray et al. (1981). Protein determination was done according to the procedure of Bradford (1976), with bovine serum albumin (BSA) as a standard. Isoelectrofocusing (IEF) of the CI domain was done on a Pharmacia Phast System for gel electrophoresis with silver staining.

The N-terminal amino acid sequence was determined with an Applied Biosystems Model 477A protein sequencer (pulse-liquid sequenator), connected on-line with a 120A PTH analyzer (Hewick et al., 1981).

Immunodetection of CI was done by a standard dot-blotting procedure as described by Harlow and Lane (1988).

Preparation of Membrane Vesicles. E. coli ASL-1 cells containing a chromosomal mutation for EII^{mtl} were transformed with the vector pWAMc7, which encodes a mutant EII^{mtl} (EII-H554A),[2] or pWAMa, which encodes wild-type EII. Measurement of EII concentrations and preparation of ISO vesicles are described in more detail in the previous paper (van Weeghel et al., 1991).

Complementation Assay for CI. Mannitol phosphorylation activity was measured according to the procedure of Robillard and Blaauw (1987). An assay volume of 100 μL contained 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM PEP, 5 mM DTT, 0.35% decyl-PEG, 0.6 μM EI, and 3 μM HPr, and 1.7 nM wild-type EII^{mtl} or 5.6 nM EII-H554A inside-out (ISO) vesicles. To complement the mutated phosphorylation site (P1) in the EII-H554A mutant, purified CI domain was added to the mixture in different amounts (10–120 μM). The reaction was started by addition of [^{14}C]mannitol (59 mCi/mmol), and activities were measured as a function of time at various enzyme concentrations. They were linear with respect to both parameters. The mannitol phosphorylation activity of CI was measured in a complementation assay with the *Staph. carnosus* system as described by Reiche et al. (1988).

Results

Gene Construction and Subcloning of the CI Domain. The gene fragment encoding the CI-terminal domain was constructed by oligonucleotide mutagenesis using a synthetic 21-oligomer. By introducing two mismatches into the gene, a unique *Nco*I restriction site was generated at position 1603 of the *mtl*A nucleotide sequence. Because of the high mutation frequency with the gapped-duplex mutagenesis method as previously reported (Stanssens et al., 1989), it is possible to find the mutated gene by picking randomly a few colonies for restriction analysis. After confirmation of the *Nco*I mutation by restriction analysis of plasmid DNA, the actual efficiency of the mutagenesis was 30%. The vector containing the new *Nco*I site was designated pWAMc2.

To express the CI domain at high levels in *E. coli*, we used the expression vector pKK233-2 which carries the strongly regulated *trp-lac* fusion promoter (*trc*) and the *lac*Z ribosome binding site (RBS) followed by an ATG translation initiation codon located within a *Nco*I restriction site. A *Nco*I–*Hin*dIII fragment encoding the CI domain was excised from pWAMc2 and recloned into the vector pKK233-2 (Figure 1). The position of the new *Nco*I site was such that simple ligation of the complementary *Nco*I and *Hin*dIII sticky ends maintained the correct translational reading frame. The translation product would have the amino acid sequence Met-Ala-Asn-Leu-Phe-... (Figure 1), where the Met residue corresponds to the position of the substituted serine-490 of the *mtl*A sequence. The N-terminal methionine is now located in a predicted flexible loop of the protein, just before position 493 where the amino acid homology with $EIII^{mtl}$ of *S. carnosus* begins (Fischer et al., 1989). To obtain again a coupled mutagenesis–expression system for the manipulation of the domain, the resulting expression plasmid, pKKCI, was cut with *Eco*RI and *Bam*HI, followed by insertion of the fragment containing the whole promoter trc-ATG-CI fusion into the mutagenesis vector pMc5-8 (Figure 1). The resulting plasmid, pMcCI, carried a chloramphenicol and ampicillin resistance marker, containing an amber mutation, and an fl filamentous phage origin for ssDNA(+) preparation, which allows the use of the gapped-duplex mutagenesis method.

Expression of the CI Domain. To identify the predicted polypeptide after overproduction, ASL-1 and JM101 cells were lysed in Laemmli sample buffer. Gel electrophoresis of total cell lysates showed, after staining with Coomassie, a band of the predicted size, 16 000 daltons, amounting to 5–15% of the total cell protein (Figures 2 and 3). A thick band of 26 000 daltons was also visible after staining and is most likely the chloramphenicol acetyltransferase (CAT) which confers resistance to chloramphenicol. The 16-kDa protein band was not present in control lysates from ASL-1 and JM101 (lane 1), or lysates from cells containing the parental vector pMc5-8 (Figure 2, lane 2). The expression level of the CI domain as illustrated in Figure 3 (left panel) showed that CI was overproduced stably even after 20 h in ASL-1 cells with constitutive expression. Stable overproduction was also observed in JM101 cells after induction of the trc promoter with IPTG (Figure 3, right panel).

Purification of the CI Domain. A 1-L culture of ASL-1/pMcCI was grown overnight for 16–20 h and yielded 12 g wet weight of cells. The CI protein from this overproduction approach was present mostly in a soluble form. Some CI could be detected in the cell debris, pelleted after passage of the cells through a French press. The first Q-Sepharose column was

[2] Site-directed mutants are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type permease and then by a second letter denoting the amino acid replacement at this position.

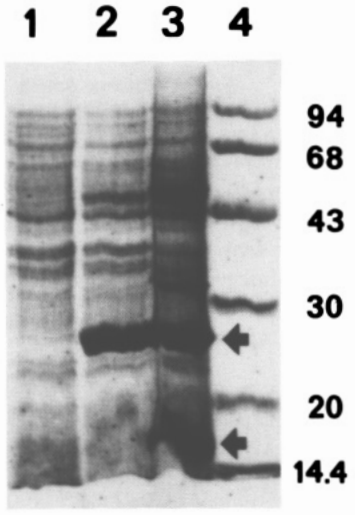

FIGURE 2: SDS–PAGE analysis of total cell extracts of *E. coli* cells harboring different plasmids. Lane 1, ASL-1 host strain containing no plasmid; lane 2, ASL-1 containing the parental plasmid; lane 3, ASL-1 containing the recombinant plasmid pMcCI. Extracts of ASL-1 cells were denatured in sample buffer and loaded on a standard 15% polyacrylamide gel and stained with Coomassie brilliant blue. Positions of protein size standard are given in kilodaltons: phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20.1; α-lactalbumin, 14.4. The arrow at position 26 kDa indicates, most likely, chloramphenicol acetyltransferase, while the arrow at position 16 kDa indicates the CI domain.

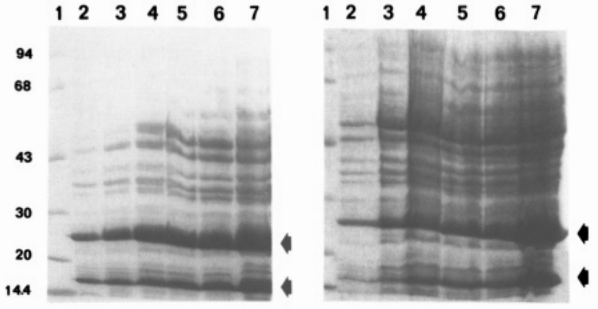

FIGURE 3: pMcCI-directed C-terminal domain synthesis following constitutive expression (left) versus induction (right). In both experiments, 1-mL samples were collected at various time intervals after expression and boiled in Laemmli sample buffer before SDS–PAGE as described under Materials and Methods. In the constitutive experiment, ASL-1 was used as the recipient host. The IPTG-induced synthesis was monitored in JM101 cells which contain the *lacI* repressor. Cell cultures were grown to OD_{660} = 0.7. At that point, IPTG was added to the JM101 cells; nothing was added to the ASL-1 cells. The first samples were taken 30 min later, lane 2; lane 3, 60 min; lane 4, 120 min; lane 5, 180 min; lane 6, 240 min; lane 7, after 20 h. Lane 1 in each panel contains the protein size markers. An arrow indicates the position of the CI domain of 16000 daltons. The protein markers are given in kilodaltons as described in Figure 2.

very important because, in this step, we could remove most of the CAT protein content present in the supernatant (Figure 4A). It was observed that at a pH of 8.5–9.5 the CAT protein strongly bound to the resin and did not elute during the 150–300 mM NaCl gradient. The CAT protein could be washed from the column with buffer containing 0.5–1.0 M NaCl. The CI domain eluted with 250 mM NaCl from the Q-Sepharose column and was 90% pure, as shown in Figure 4A. The G-75 column step was necessary to eliminate some of the high molecular weight proteins (Figure 4B), which remain after the first column. The purification resulted in 55 mg of CI protein starting from 12 g wet weight of cells, which showed a high grade of purity as demonstrated by SDS–PAGE (Figure 4B). Gas-phase sequence determination of the first

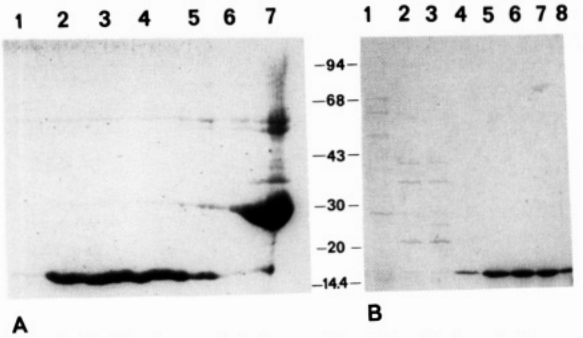

FIGURE 4: Purification and elution profile of the CI domain illustrated by 15% SDS–PAGE of fractions after a Q-Sepharose Fast Flow column (A) and after a G-75 Sephadex column (B) during purification of the CI domain. The gels were stained with Coomassie brilliant blue. (Panel A) Lane 1, fraction with proteins that eluted during the wash step; lanes 2–6, fractions that eluted within the 250–300 mM NaCl gradient; lane 7, proteins which eluted during the wash with 0.5–1.0 M NaCl. Protein size markers are given in kilodaltons as listed in Figure 2. (Panel B) Lanes 1–8, different fractions of proteins which eluted during the G-Sephadex column. Lanes 4–8 contained the purified CI domain, running at 16000 daltons.

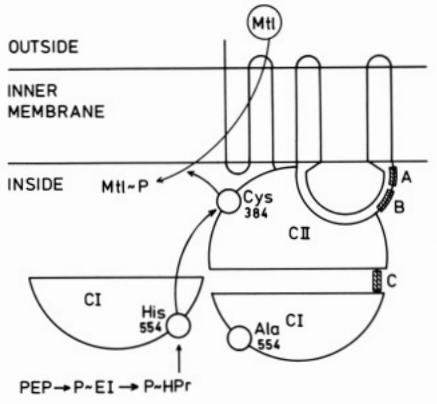

FIGURE 5: Schematic representation of the interaction of the CI domain and the PI phosphorylation site mutant H554A. The route of the phosphoryl group is from PEP, via the general cytoplasmic components EI and HPr, to the His-554 on the CI domain. The CI domain donates its phosphoryl group via an intermolecular transfer to the second phosphorylation site (P2), Cys-384, from which the sugar is phosphorylated. It is also possible to substitute $EIII^{mtl}$ from *Staph. carnosus* for the CI domain in this particular reaction scheme. The short amino acid stretches which are boxed and indicated by A, B, and C are the predicted flexible loops between the various domains.

10 amino acid residues, Met-Ala-Asn-Leu-Phe-Lys-Leu-Gly-Ala-Glu-, confirmed the purity and correct translation start of the CI as deduced from the constructed DNA sequence (Figure 1). Antibodies raised against native *E. coli* EII^{mtl} reacted strongly with purified CI in the dot-blotting procedure but not with BSA which was used as a control. The protein had a p*I* of 4.5 as determined by isoelectrofocusing.

Complementation of EII-H554A Mutant with CI. If the CI domain fulfills the function of $EIII^{mtl}$ and can be produced as a stable structural entity, it should possess an EIII-like enzymatic activity. The function of EIII is to catalyze the phosphorylation of the active-site cysteine on EII^{mtl} starting from phospho-HPr. An intermediate phospho-EIII involving an active-site phosphohistidine plays an obligatory role in the reaction as shown in Figure 5. We have examined the ability of CI to catalyze the phosphorylation of the EII^{mtl} active-site cysteine in a mutant, in which the EIII-like phosphorylation site His-554 has been replaced by alanine so that it cannot be directly phosphorylated by phospho-HPr. This mutant protein, by itself, is incapable of mannitol phosphorylation (van Weeghel et al., 1991); however, mannitol phosphorylation should be able to occur via CI, as shown in Figure 5.

Table I: In Vitro Complementation of the EII-H554A Mutant with the CI Domain of *E. coli* or $EIII^{mtl}$ of *Staph. carnosus*

EII^{mtl} in ISO vesicles[a]	plasmid[b]	$[EIII^{mtl}]$[d] (μM)	[CI][d] (μM)	PEP-dependent phosphorylation act.[c] [nmol of mtl-P min^{-1} (nmol of EII)$^{-1}$]
no EII^{mtl}				0
no EII^{mtl}			250	0
EII-H554A	pWAMc7			0.07
EII-wild-type	pWAMa			855
EII-H554A	pWAMc7		120	205
EII-H554A	pWAMc7		100	205
EII-H554A	pWAMc7		80	178
EII-H554A	pWAMc7		40	111
EII-H554A	pWAMc7		20	87
EII-H554A	pWAMc7		10	60
EII-H554A	pWAMc7	25μM		40

[a] Membrane fragments were prepared as described under Materials and Methods using ASL-1 cells grown with or without plasmid. [b] Wild-type EII^{mtl} or EII-H554A were expressed constitutively from the *trc* promoter in ASL-1. [c] Assay conditions and protein concentrations used in these experiments are specified under Materials and Methods. [d] Purified $EIII^{mtl}$ from *Staph. carnosus* or purified CI from *E. coli* were used.

The data of the in vitro PEP-dependent phosphorylation experiments shown in Table I demonstrate that ASL-1 ISO vesicles containing EII^{mtl}-H554A or the purified CI domain alone are not capable of phosphorylating mannitol in the presence of PEP and HPr and EI from *E. coli*. The substitution of the essential His-554 for Ala abolished the first phosphorylation site. Therefore, we had the correct EII mutant to test whether the intact His-554 of the purified CI domain could substitute for the essential mutated His-554 of EII^{mtl}-H554A in phosphorylating mannitol.

The results listed in Table I show that the CI domain is active in the overall phosphorylation scheme. In this process, phospho-HPr has to donate its phospho group to the active-site residue His-554, located on the CI domain. The essential binding site for phospho-HPr is, therefore, still present and functional on the isolated CI domain. In order to determine the efficacy of CI in restoring the activity of the EII-H554A mutant, the CI concentration dependence of mannitol phosphorylation was measured in the presence 20 μM mannitol, 3 μM HPr, 0.6 μM EI, and 10–120 μM CI. Increasing the HPr concentration up to 25 μM did not result in a further increase in the phosphorylation activity, suggesting that all CI is present in the phospho-CI form. The data were plotted in a double-reciprocal plot yielding a K_m for phospho-CI on the H554A EII^{mtl} mutant of 26.4 μM and a V_{max} of 212 nmol min^{-1} (nmol of EII-H554A)$^{-1}$. These data were compared with the specific mannitol phosphorylation activity of wild-type EII^{mtl} [855 nmol min^{-1} (nmol of EII^{mtl})$^{-1}$] measured under the same conditions of HPr, EI, and mannitol concentration. The data clearly show that there is a saturable binding site for CI on EII-H554A. CI is not able to restore the phosphorylation activity of EII-H554A to more than 25% of the wild-type EII^{mtl} activity (Table I). This difference in activity is not due to unphosphorylated CI molecules, because elevated levels of phospho-HPr did not further stimulate the phosphorylation activity.

Considering the level of amino acid homology between the two proteins, we investigated whether $EIII^{mtl}$ from *Staph. carnosus* could substitute for CI in the previous complementation assay. We also investigated whether CI could substitute for $EIII^{mtl}$ in a *Staph. carnosus* phosphorylation assay. According to the data of Table I, $EIII^{mtl}$ is able to complement the H554A mutant, at comparable CI concentrations, however, with lower activity. CI was also able to catalyze mannitol phosphorylation in combination with *Staph. carnosus* EII^{mtl}, HPr, and EI, although 6.5 times less effective (data not shown). The activity could be raised by a factor of 2, when *Staph. carnosus* EI and HPr were substituted with the same components from *E. coli*. Since extensive concentration dependence kinetics were not measured in both cases, it is not possible to tell whether the decreased activity is a result of an altered K_m or V_{max}. From these results, we can conclude that CI is an intact structural and functional domain equivalent to EII^{mtl}. It is recognized by phospho-HPr and is active in the transfer of the phosphoryl group to the second phosphorylation site on EII-H554A, cysteine-384 (Pas et al., 1988).

Discussion

Mannitol-specific EII lacks a soluble $EIII^{mtl}$ in contrast to some other systems like EII^{glc} and EII^{scr} which need a specific EIII for activity. The high molecular weight of these single EII species and their sequence homology with EII/EIII pairs led Saier to propose that such single EII's are probably fusion products, an EII with a covalently attached EIII domain (Lee & Saier, 1983; Saier et al., 1988). In support of this proposal, Vogler et al. (1988) reported that EII^{nag} and EII^{bgl}, whose C-terminal domains show considerable homology with $EIII^{glc}$, could replace the $EIII^{glc}$ in EII^{glc}-dependent glucose transport and phosphorylation. Furthermore, addition of $EIII^{glc}$ to a truncated and inactive EII^{nag} restored the activity.

A hydropathy plot of the amino acid sequence (Lee & Saier, 1983) and the work of Stephan and Jacobson (1986) showed that EII^{mtl} consists of a hydrophobic N-terminal domain and a hydrophilic C-terminal domain. The latter is a cytoplasmic domain which contains two chemically identified phosphorylation sites, His-554 and Cys-384, both of which appear to be essential in the overall phosphorylation process (Pas et al., 1988). Recently, Fischer et al. (1989) reported the cloning, sequencing, and overproduction of the mannitol-specific EIII of *Staph. carnosus*. Amino acid sequence comparison showed a 38% homology to this EIII to the hydrophilic C-terminal domain of *E. coli* EII^{mtl}. The sequence at the first phosphorylation site of *E. coli* EII^{mtl}, Pro-His-Gly-Thr (Pas et al., 1988), was also present in $EIII^{mtl}$ of *Staph. carnosus* and *Staph. aureus* (Reiche et al., 1988). Computer predictions using the method of Karplus and Schulz (1985) indicated several flexible loops in *E. coli* EII^{mtl}. Two loops, 334–340 and 355–365, were located in an amino acid stretch between the hydrophobic and hydrophilic domains. One other loop, 480–495, was located in the middle of the C-terminal domain precisely before the predicted EIII part of *E. coli* EII^{mtl}. This flexible hinge region separates the C-terminal into two smaller domains. Its position is comparable to a loop found in $EIII^{man}$ which connects two structural domains, both of which become transiently phosphorylated during mannose phosphorylation (Erni et al., 1989).

The results presented in this paper demonstrate the expression of a functional 16 000-dalton domain which is able to restore the in vitro phosphorylation activity of the H554A mutant. It is phosphorylated by phospho-HPr and is able to donate its phosphoryl group to the second phosphorylation site, Cys-384, on the H554A mutant. Whether the precise route of phosphoryl group transfer between sites 1 and 2 is intra- or intermolecular has not been established. Vogler et al. (1988) and Vogler and Lengeler (1988) reported that phosphoryl group transfer could occur between different EII's. Stephan et al. (1989) and White and Jacobson (1990) demonstrated intermolecular phosphotransfer between a truncated EII^{mtl}, missing the extreme C-terminal part, and an inactive EII or a partially purified C-terminal domain. Transfer only

took place if a free Cys-384 was available.

Our data support and quantify these observations and demonstrate that the expressed and purified CI domain is capable of functioning as an isolated $EIII^{mtl}$. When we replaced the CI domain by soluble $EIII^{mtl}$ of *Staph. carnosus*, we found significant phosphorylation activity, although 2.5 times lower compared to the CI domain.

We also substituted CI for the $EIII^{mtl}$ of *Staph. carnosus* in the overall phosphorylation reaction with *Staph. carnosus* EII^{mtl}. The less effective complementation in this system could be expected since the reversed experiment also gave 2.5 times lower activity.

The available data lead to the conclusion that the active sites of phosphorylation of EII^{mtl} are positioned on different functional domains, one of which is an $EIII^{mtl}$, and that there is a binding site at which the domains associate before transferring the phosphoryl group.

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Registry No. PTS II, 37278-09-4; PTS III, 97162-87-3; L-Cys, 52-90-4; L-His, 71-00-1; mannitol, 69-65-8.

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