Identification of domain interfaces in the E. coli mannitol permease

van Montfort, Bart

Chapter 5

Cysteine cross-linking defines part of the dimer and B/C domain interface of the *E. coli* mannitol permease

Bart A. van Montfort
Gea K. Schuurman-Wolters
Ria H. Duurkens
Robert Mensen
Bert Poolman
George T. Robillard

Accepted for publication in The Journal of Biological Chemistry

Abstract

Part of the dimer and B/C domain interface of the *E. coli* mannitol permease (EII^{mtl}) has been identified by the generation of disulfide bridges in a single-cysteine EII^{mtl}, with only the activity-linked Cys^{384} in the B domain, and in a double-cysteine EII^{mtl} with cysteines at 384 and position 124 in the first cytoplasmic loop of the C domain. The disulfide bridges were formed in the enzyme in inside-out membrane vesicles and in the purified enzyme by oxidation with Cu(II)-(1,10-phenanthroline)$_3$, and they were visualized by SDS-PAGE. Discrimination between possible disulfide bridges in the dimeric double-cysteine EII^{mtl} was done by partial digestion of the protein and the formation of heterodimers, in which the cysteines were located either on different subunits or on one subunit. The disulfide bridges that were identified are an intersubunit Cys^{384}-Cys^{384}, an intersubunit Cys^{124}-Cys^{124}, an intersubunit Cys^{384}-Cys^{124}, and an intrasubunit Cys^{384}-Cys^{124}. The disulfide bridges between the B and C domain were observed with purified enzyme and confirmed by MALDI-TOF MS. Mannitol did not influence the formation of the disulfide between Cys^{384} and Cys^{124}. The close proximity of the two cysteines 124 was further confirmed with a separate C domain by oxidation with Cu(II)-(1,10-phenanthroline)$_3$ or by reactions with dimaleimides of different length. The data in combination with other work show that the first cytoplasmic loop around residue 124 is located at the dimer interface and involved in the interaction between the B and C domain.

Introduction

The uptake and concomitant phosphorylation of a wide variety of carbohydrates into bacterial cells is, in many cases, accomplished by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (*3*). In a cascade of phosphorylation reactions (Fig. 1), the phosphoryl group is transferred from the energy donor phosphoenolpyruvate (PEP) via the two general components EI and HPr to the carbohydrate-specific components (Enzyme II's), which transport and phosphorylate the carbohydrates (*11*). All EII's have a similar architecture and consist of the cytoplasmic A and B domains and a membrane-embedded C domain. This article deals with the mannitol-specific EII (EII^{mtl}) of *E. coli*, in which the three domains are covalently linked. HPr phosphorylates the A domain of EII^{mtl} on His^{554}, which subsequently phosphorylates Cys^{384} in the B domain. Mannitol in the periplasm is bound by the C domain, transported into the cell via C and, while bound at the cytoplasmic site of C, phosphorylated by the B domain. EII^{mtl} is most likely a dimeric protein and the subunit interactions occur in the C domain (*14*, *26*, *27*, *29*, *30*, *32*).

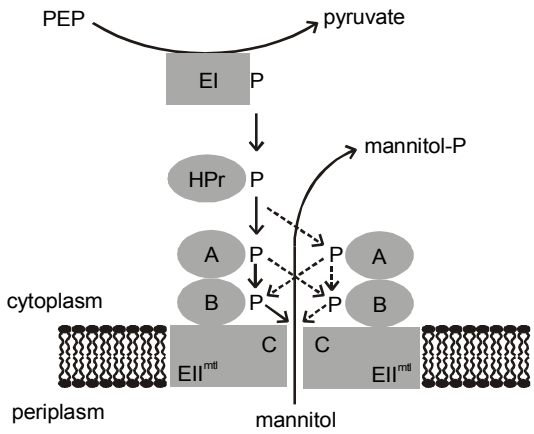

Figure 1. Schematic representation of the mannitol-specific phosphoenolpyruvate-dependent phosphotransferase system of *E. coli*. Dotted arrows indicate that the phosphoryl group transfer from HPr can proceed to each of the EII^{mtl} subunits and that inter and intradomain phosphoryl group transfer is possible as well.

Domain interactions and in particular the B/C domain interface play an important role in the catalytic cycle of EII^{mtl}. The energy coupling mechanism involves conformational interaction between the B and C domain. The evidence for this notion is manifold: 1) Phosphorylation of the B domain increases the rate of transport two to three orders of magnitude (*48*, *49*). 2) Modification or mutagenesis of the phosphorylation site in the B domain as well as removal of the cytoplasmic domains changes the mannitol binding kinetics of the C domain (*50*, *51*). 3) Time-resolved

fluorescence and phosphorescence spectroscopy showed that, upon phosphorylation of the B domain, Trp^{109} in the C domain becomes immobilized whereas Trp^{30} in the C domain becomes more flexible (*40, 52*). 4) Differential scanning calorimetry showed that the thermal stability of the C domain is higher in the presence of the B domain (*53*). 5) Isothermal titration calorimetry experiments indicated that a significant part of the structural changes upon the binding of mannitol to the C domain reside in the B domain. Approximately 50-60 residues are removed from the bulk water upon binding of mannitol, which was much less when the same measurements were done after removal of the B domain (*54*). 6) Close proximity of the B and C domain has been suggested for another PTS transporter, that is the BglF system of *E. coli* (Amster-Choder, personal communication).

To date, there is no structural information about the B/C domain or dimer interface of EII^{mtl} or any other EII. The topological model of the C domain predicts 6 membrane-spanning α-helices and two large cytoplasmic loops (*36*), which is in accordance with the recently resolved projection map of the C domain (*32*). The location of the loops, however, is not known. A photocross-linking approach to identify the B/C domain interface suggested that the end of the first cytoplasmic C domain loop is located in the vicinity of Cys^{384} in the B domain (van Montfort *et al.*, unpublished results). Here we describe the generation of disulfide bridges of cysteines within and between B and C domains. This work demonstrates that the first cytoplasmic loop of the C domain is in close proximity to the B domain active site and near the dimer interface.

Materials and Methods

Chemicals

Decyl-polyethylene glycol (dPEG) was synthesized by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). Bovine pancreas TPCK-treated trypsin was from Sigma and endoproteinase Glu-C of Staphylococcus aureus V8 was from Fluka. The bimaleimides o-PDM and p-PDM were from Aldrich and BMH was obtained from Pierce. EI and HPr were purified as described previously (*140, 141*). All chemicals used were analytical grade.

Construction of plasmids for expression of SSCS, SSCS-S124C, and IIChis-S124C

Site-directed mutagenesis was performed with the Stratagene Quickchange mutagenesis kit. His-tagged EII^{mtl} with cysteines at positions 110, 320, and 571 replaced by serine (SSCS) was constructed in pMaHisMtlAPr, which carries the gene for N-terminally His-tagged EII^{mtl} (E. Vos, personal communication). Subsequently, SSCS-S124C was generated by replacing Ser^{124} with a cysteine. IIChis-CL was generated by replacing Cys^{110} and Cys^{320} by serine in pMaMtlIICHis, which carries the gene for the C domain with a C-terminal His-tag (*54*). IIChis-S124C was then

generated by replacing Ser^{124} by a cysteine as described above. Mutations were identified by introducing silent restriction sites and confirmed by DNA sequence analysis.

Generation of ISO vesicles and purification of SSCS, SSCS-S124C, and IIChis-S124C

Growth of *E. coli* LGS322 [F^- *thi-1*, *hisG1*, *argG6*, *metB1*, *tonA2*, *supE44*, *rpsL104*, *lacY1*, *galT6*, *gatR49*, *gatR50*, *Δ(mtlA'p)*, *mtlD*^{c}, *Δ(gutR'MDBA-recA)*] carrying the various plasmids, and procedures to overexpress the mutant proteins were identical to those described for wild type EII^{mtl} (*13*). Inside-out (ISO) membrane vesicles containing the mutant proteins were obtained as described (*142*). SSCS and SSCS-S124C were purified by Ni-NTA agarose affinity chromatography as described for $6HEII^{mtl}$ (*52*), except that dPEG was used as the detergent. IIChis-CL and IIChis-S124C were purified as described (*54*).

EII^{mtl} concentration determination and mannitol binding and phosphorylation activities

The dissociation constant for mannitol binding and EII or IIC concentration in vesicles was determined by flow dialysis after solubilisation with 0.25% dPEG (*43*) with some modifications as will be described elsewhere (E. Vos et al.). The mannitol phosphorylation activity was determined at 1 mM mannitol (*143*). The concentration of purified SSCS and SSCS-S124C was determined with the pyruvate burst assay, which determines the amount of PTS phosphorylation sites (*144*). The activities of IIChis-CL and IIChis-S124C were determined after formation of a heterodimer with EII^{mtl}-G196D, using 33 μM mannitol as described (*145*).

Disulfide cross-linking

A solution of approximately 6 μM of enzyme in ISO vesicles or 1 μM of purified protein was brought to a final concentration of 5 mM DTT and 20 mM EDTA from stocks of 0.1 and 0.5 M, respectively. If appropriate, 90 nM EI was added. After 15 minutes of incubation at 30 °C, DTT and EDTA were removed on a Bio Micro-spin 6 column (Biorad), equilibrated with 50 mM NaPi pH 7.5, 0.1 mM EDTA, with (purified enzyme) or without (ISO vesicles) 0.25% dPEG. The buffer was deaerated with helium before addition of the detergent. Subsequently, HPr, $MgCl_2$, dPEG, and PEP were added to phosphorylate the purified enzyme, provided EI was present; these additions increased the final volume by 50%. The same mixture without PEP was used to represent conditions in which the enzyme was not phosphorylated. The final concentrations of the components were 2 μM HPr, 5 mM PEP, and 5 mM $MgCl_2$. After 5 minutes at 30 °C, disulfide bridge formation was initiated by oxidation with 0.1 volume of 3 mM Cu(II)-(1,10-phenanthroline)$_3$ (CuPhe), followed by incubation at

30 °C for 30 minutes. The reaction was quenched by the addition of 65 mM EDTA from a stock of 0.5 M NaEDTA, pH 8.

Partial digestion and reduction

The protein was partially digested with 20 μg trypsin or 100 μg endoproteinase Glu-C per ml of reaction mixture for 1 hour at room temperature. The digestion of the vesicles with endoproteinase Glu-C was done in the presence or absence of 0.4% dPEG. The digestion was stopped by the addition of SDS-PAGE denaturation buffer without β-mercaptoethanol. If appropriate, reduction was accomplished by the addition of 10 mM DTT after digestion.

Heterodimer formation

Heterodimers between 3 μM of SSCS and 1 μM of IIChis-S124C or 0.2 μM of SSCS-S124C and 3 μM of IIChis-CL were formed by mixing purified proteins, followed by an incubation at 30 °C for 30 minutes. To promote heterodimer formation between SSCS-S124C and IIChis-CL, 170 mM Na_3PO_4 was added from a 1 M stock solution at pH 7.6. This lowers the cloudpoint of the detergent (dPEG), in which the protein is solubilized. This treatment results in dissociation of the initially homodimeric enzymes and thereby facilitates the mixing of the species (*23*). Subsequently, the heterodimers were treated as described above for the *disulfide cross-linking* procedure except that EI, HPr, $MgCl_2$, and PEP were omitted from the mixture.

Cross-linking with dimaleimides of varying length

1 μM of purified IIChis-S124C was reduced and demetalated as described under disulfide cross-linking. Cross-linking was initiated by adding to the protein, in 50 mM NaPi pH 7.5, 0.1 mM EDTA plus 0.25% dPEG, 5 μM *o*-PDM, *p*-PDM, or BMH from a 10-times concentrated stock solution in DMF. The reaction was stopped with 10 mM DTT after incubation at 30 °C for 30 minutes.

SDS-PAGE analysis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was done with 10% acrylamide gels as described (*117*). A denaturation buffer without β-mercaptoethanol was used. The samples were not boiled in denaturation buffer, because this leads to aggregation. The proteins were visualized either by silver-staining (*119*) or by immuno-detection after the proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using the Western-Light™ chemiluminescence detection kit with CSPD™ as the substrate, was performed as recommended by the

manufacturer (Tropix Inc.). The first antibody was an anti-His antibody from Amersham Pharmacia Biotech or Boehringer Mannheim, and the second antibody was an anti-mouse IgG alkaline phosphatase conjugate (Sigma).

MALDI-TOF mass spectrometry

A coomassie-stained band containing the C domain, generated by tryptic digestion, was excised from an SDS-polyacrylamide gel and completely destained with 50 mM NH_4HCO_3 in 40% ethanol. Subsequently, the gel piece was washed three times with 200 µl of 25 mM NH_4HCO_3 and cut into pieces of approximately 1 mm^3. A 200 µl volume of 50 mM β-mercaptoethanol in 25 mM NH_4HCO_3 was added and, after 2 hours of mixing at room temperature, the peptides were extracted with two times 200 µl of 60% acetonitrile, 0.1% TFA by 5 minutes of sonication in a bath sonicator. The β-mercaptoethanol solution and the extracts were pooled and dried in a SpeedVac. The last traces of ammonium bicarbonate were removed by adding 10 µl of 1% TFA and subsequent drying in the SpeedVac. The dried samples were dissolved in 5 µL of 50% acetonitrile, 0.1% TFA and sonicated for 5 minutes. Aliquots of 0.75 µl were applied onto the MALDI target and allowed to air dry. Subsequently, 0.75 µl of 10 mg/ml α-cyano-4-hydroxysuccinnamic acid in 50% acetonitrile, 0.1% (v/v) TFA was applied to the dried samples, which was then allowed to dry again. MALDI mass spectra were recorded with a Micromass Tofspec E MALDI time-of-flight mass spectrometer operated in reflectron mode. Spectra were calibrated externally.

Results

Generation and characterization of SSCS, SSCS-S124C, IIChis-CL and IIChis-S124C

Two His-tagged EII^{mtl} mutants were constructed. SSCS is a single-cysteine enzyme with only Cys^{384} in the B domain. SSCS-S124C contains Cys^{124} in the C domain in addition to Cys^{384}. The phenotype of *E. coli* LGS322 expressing these mutants was analyzed on MacConkey agar plates with 1% D-mannitol. Both strains formed purple-red colonies, indicating that the mutants transport and ferment mannitol. Inside-out (ISO) membrane vesicles from LGS322 cells expressing both mutants were solubilized with 0.25% dPEG and analyzed for mannitol binding and phosphorylation activities. The dissociation constants for mannitol were 25 and 45 nM and the turnover values for phosphorylation of mannitol of these mutants were 5200 and 4900 min^{-1} for SSCS and SSCS-S124C, respectively. The turnover values for mannitol phosphorylation of purified SSCS and SSCS-S124C were 3200 and 2700 per minute, respectively. These activities are similar to that of wild-type EII^{mtl} (*39, 43*). ISO membrane vesicles bearing IIChis-CL or IIChis-S124C, solubilized with 0.25% dPEG and analyzed for mannitol binding, displayed dissociation constants for mannitol of 32 and 70 nM, respectively. These dissociation constants are similar to previously

determined values of the wild type C domain generated by tryptic digestion of the complete protein (*43*) or of the separately expressed IIC (*14*). In addition, IIChis-CL and IIChis-S124C could both complement the mannitol binding-defective EII^{mtl}-G196D for phosphorylation activity in an in vitro assay, a result similar to that described for IIC (*14*). Overall, these kinetic data clearly indicate that SSCS and SSCS-S124C are fully functional enzymes and that both mutant C domains can bind mannitol and form functional heterodimers with EII^{mtl}.

Disulfide bridge formation in ISO vesicles and purified enzyme

Figure 2A shows the results of CuPhe-induced oxidation of SSCS and SSCS-S124C in ISO membrane vesicles, as visualized by immunoblotting with an antibody raised against the N-terminal His-tag. The untreated proteins migrated as a major band at approximately 60 kDa and are indicated as EII. The band at 36 kDa is a degradation product, whereas the one at 116 kDa (band 1) is most likely the dimer. This dimer has been observed previously upon extraction of the enzyme from the membrane (*26*) and is most likely not held together by a disulfide bond, since it is resistant to reduction with DTT. The amount of this band 1 is the same in all lanes. The oxidation by CuPhe of both mutants resulted in the appearance of a higher molecular weight band (band 2). The molecular weight was approximately 200 kDa but varied depending on the concentration of acrylamide that was used (not shown). The formation of this band was almost completely reversed by reduction of the sample with DTT, suggesting that it is stabilized by a disulfide bridge. The reduction with DTT is not complete in the particular sample in lane 5. However, in lane 11 of figure 2A, lane 1 of figure 2B, and in duplo experiments complete reduction was observed. Both high molecular weight bands (1 and 2) have been observed previously and both were denoted as dimeric species (*27-29*). Also in this paper we refer to band 2 as a dimeric species, but we can not fully exclude the possibility that it represents another oligomeric state as will be discussed below. Since Cys^{384} is the only candidate for disulfide formation in SSCS, the enzyme is thus capable of forming a disulfide between the two Cys^{384} residues. This is an important observation, because the dimer contacts are between the C domains (*14*).
Partial digestion of EII^{mtl} with endoproteinase Glu-C generated a band at 33 kDa, which corresponds to the C domain without the A and B domains. Endoproteinase Glu-C instead of trypsin was used, because it did not cleave off the N-terminal His-tag within the 1 hour incubation period. The cleavage pattern of digested SSCS was the same irrespective of whether the disulfide was formed or not. In addition, the cleavage pattern was unchanged upon reduction. This shows that the disulfide bridge resides in the domains that were degraded, which is consistent with the location of Cys^{384} in the B domain. However, if the same oxidation and digestion procedure was followed with oxidized SSCS-S124C, a new band at 50 kDa appeared (Fig. 2A; indicated by the arrow), irrespective of whether the digestion was done in the

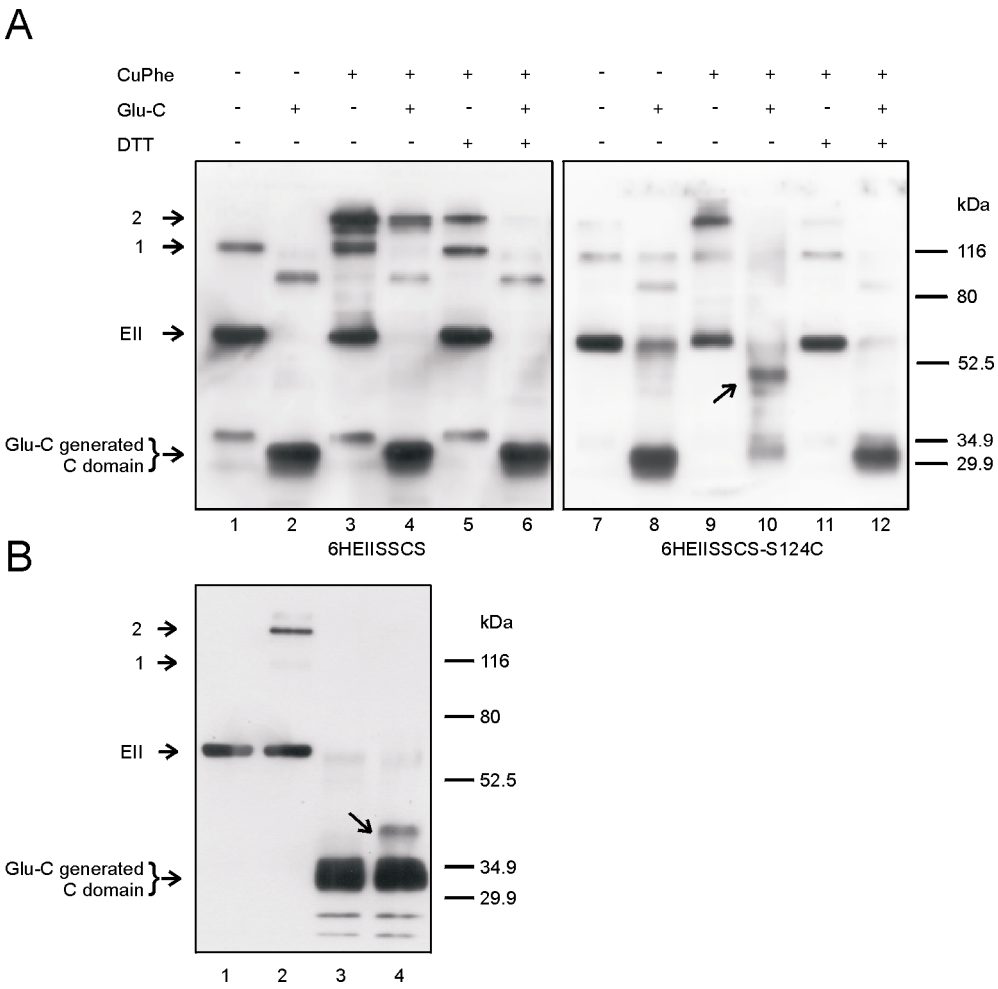

Figure 2. Immunoblot analysis of CuPhe-induced disulfide bridge formation in SSCS and SSCS-S124C in inside-out membrane vesicles (A) and in purified SSCS-S124C (B). (A) After oxidation, the samples were left untreated, digested with 100 μg/ml endoproteinase Glu-C, reduced with 10 mM DTT, or digested and subsequently reduced as described in the materials and methods section. The conditions are depicted in the figure. The left panel (lanes 1-6) depicts the data with SSCS and the right panel (lanes 7-12) that of SSCS-S124C. The arrow indicates the location of the 50 kDa band, which was formed in SSCS-S124C only. The bands denoted 1 and 2 represent different conformers of EII (see text). Not shown are both mutants with added DTT but not oxidized, because these samples were identical to those that were DTT-reduced after CuPhe oxidation. (B) Approximately 1 μM of SSCS-S124C was oxidized with CuPhe and, subsequently reduced with DTT (lane 1), left untreated (lane 2), endoproteinase Glu-C digested and, subsequently, reduced with DTT (lane 3), or endoproteinase Glu-C digested (lane 4). The arrow indicates the 42 kDa band. Approximately 0.5 μg (A) or 0.08 μg (B) of EII^{mtl} was loaded per lane.

presence or absence of the detergent dPEG. This band disappeared upon reduction of the sample with DTT. This excludes the possibility that the protein was only partially cleaved under the oxidizing conditions. Based on its size, this band is probably the result of a disulfide bridge between Cys^{124} of both monomers, indicating that residue 124 is at the dimer interface. Very vaguely, some other products, which could include a disulfide bridge between Cys^{384} and Cys^{124} (see below) might be visible as well.

To further examine the nature of the Cys^{124}-Cys^{124} disulfide bridge, the CuPhe-induced oxidation was repeated with purified SSCS-S124C. Figure 2B shows the result of this experiment, visualized by immunoblotting. The same observations were made when the gel was silver-stained (not shown). The oxidation of purified SSCS-S124C also yields the reducible dimer band 2. Almost no band 1 was observed after purification, which probably indicates that the affinity between two monomers is decreased. Upon endoproteinase Glu-C digestion, the 50 kDa fragment was not observed. Instead, a His-tagged fragment of 42 kDa was visible in addition to the 33 kDa C domain band. This 42 kDa band disappeared upon reduction of the sample with DTT (compare lane 3 and 4). Endoproteinase Glu-C digestion of the B domain will generate a 7.9 kDa fragment containing Cys^{384}. The size of the 42 kDa band thus suggests that a disulfide bond is formed between the 33 kDa C domain harboring Cys^{124} and the 7.9 kDa B domain fragment with Cys^{384}. In conclusion, the data in Fig. 2A and 2B point to the formation of two different disulfides, one intersubunit Cys^{124}-Cys^{124} and one interdomain Cys^{124}-Cys^{384}. Further evidence for both disulfides will be supplied in the following sections.

Requirements for B/C domain disulfide bridge formation

To elucidate the composition of the disulfide bridges, the CuPhe-induced oxidation was performed with purified SSCS-S124C and SSCS (Fig. 3). The two higher molecular weight bands (1 and 2) were observed again with both proteins (lanes 2 and 14). These bands were not present upon reduction with DTT and were much less intense or absent upon phosphorylation of the protein at His^{554} and Cys^{384}, confirming that these bands, at least in SSCS, arise from a disulfide bridge between the two Cys^{384}'s in the dimeric complex. Instead of endoproteinase Glu-C, trypsin was used to define the cross-links. Trypsin first cleaves in the linker between the C and B domain and, subsequently, digests the A and B domain completely but leaves the C domain intact except for the N-terminal His-tag (*43, 146*). The proteolytically generated C domain can be observed on SDS-PAGE at 30 kDa, which is somewhat smaller than the endoproteinase Glu-C generated C domain. This is consistent with the removal of the His-tag and a different cleavage site in the linker between the B and C domain. The tryptic B domain fragment with Cys^{384} has a calculated mass of approximately 1.9 kDa. Tryptic digestion of CuPhe-oxidized SSCS-S124C also showed an approximately 30 kDa band, which was significantly broadened upwards when the sample was not

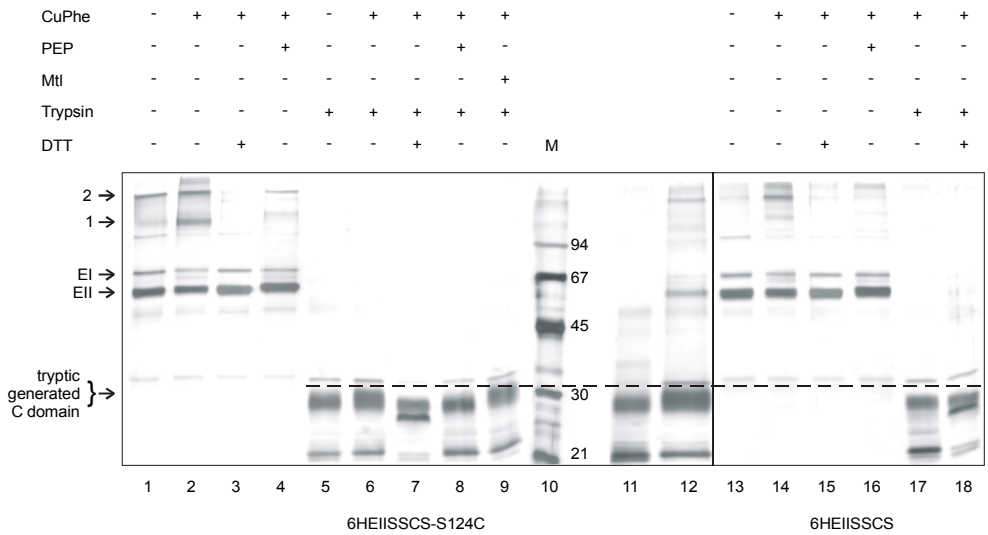

Figure 3. SDS-PAGE analysis of the effect of phosphorylation and mannitol binding on CuPhe-induced cross-linking of purified SSCS-S124C and SSCS. The left panel (lanes 1-12) depicts the data with SSCS-S124C and the right panel (lanes 13-18) that of SSCS. The addition of PEP, EI plus HPr is indicated by "PEP". After oxidation, the samples were left untreated, digested with 20 μg/ml trypsin, reduced with 10 mM DTT, or digested and subsequently reduced as described in the materials and methods. Conditions are depicted in the figure. The dashed line makes the difference in height between oxidized and reduced IIC in SSCS-S124C more clearly visible. The bands denoted 1 and 2 both represent dimeric EII. Lane 10 contains the molecular weight marker. Lanes 11 and 12 show additional control experiments with SSCS-S124C. Lane 11: trypsin was added to the reduced enzyme and, after 1 hour at 30 °C, oxidation was induced, which after 30 minutes was quenched with EDTA. Lane 12: the reduced protein was left at 30 °C for 60 minutes, after which CuPhe was added. After 30 minutes, EDTA and trypsin were added and incubated for another 60 minutes at room temperature. Approximately 0.1 μg EII^{mtl} was loaded per lane. In addition, shorter oxidation times (10 min. instead of 30) yields the same broadening.

treated with DTT (compare lanes 6 and 7). This suggests that the broadened 30 kDa band comprises the C domain plus the 1.9 kDa fragment of the B domain, analogous with the 42 kDa band in figure 2B. Consistent with this conclusion are the following observations: (i) the broadening is not observed with SSCS (Fig. 3: compare lanes 17and 18); (ii) Phosphorylation prior to oxidation prevented the occurrence of the broadening (compare lanes 6 and 8); and (iii) The broadening was not observed in wild-type EII^{mtl} or in mutants of SSCS with cysteines at positions 158 or 199 (not shown). A tryptic-generated C domain of SSCS-S124C, not treated with CuPhe, also led to some broadening, which is probably due to spontaneous oxidation (compare lanes 5 and 6). Another point to note is that the addition of 100 μM mannitol did not have an effect on the occurrence of the broadening (lane 9). Finally, dimeric C domain was not observed in the tryptic digest of oxidized SSCS-S124C.

Taken together, the broadening must be the result of a disulfide bridge between Cys^{384} and Cys^{124}. To exclude the possibility that this disulfide bridge is an aspecific reaction between two accessible cysteines, another control experiment was performed, (Fig. 3; lanes 11 and 12). With the sample in lane 11, oxidation was carried out after trypsin treatment, whereas in lane 12 it was carried out before trypsinolysis. Clearly, the broadening of the C domain band is no longer observed when the trypsin digestion preceded the oxidation. Thus, Cys^{124} is not capable of reacting with just any cysteine-containing peptide, present at the same concentration.

MALDI-TOF mass analysis

To demonstrate that the fragment, which caused the broadening of the tryptic-generated C domain, originated from the B domain, the protein was excised from the SDS-polyacrylamide gel, reduced with β-mercaptoethanol to cleave the disulfide bond between the C domain and the B domain peptide, the peptides were extracted with organic solvents, and the extract was analyzed with MALDI-TOF MS. Figure 4 shows the mass spectrum of the extracted peptides. The spectrum only contained 4 peaks, all of which could be assigned to tryptic B domain fragments that contain Cys^{384}. The peak at m/z 1879.05 is the fully cleaved peptide with residues 380-399 (expected m/z 1878.92), and the peak at m/z 2007.05 represents the partially cleaved fragments of residues 379-399 and/or 380-400 (expected m/z 2007.02). This partial cleavage is due to the presence of the RK and RKK sequences at the N- and C-terminus of these peptides, respectively, which cannot be fully cleaved by trypsin. The peaks at m/z 1954.79 and 2083.05 represent the same peptides but with a β-mercaptoethanol adduct, which gives a mass increase of 76 Da. If the same procedure was applied to the C domain band that was reduced prior to SDS-PAGE and excised from the gel following the same procedure no peptides were observed.

The B/C disulfide bridge can cross the dimer interface

To this point, we have provided evidence for a B/C interdomain cross-link between Cys^{384} and Cys^{124} in purified SSCS-S124C, and a cross-link between the Cys^{124} of both monomers in the enzyme in ISO membrane vesicles. In addition, the data on SSCS provide unequivocal evidence for a disulfide across the dimer interface between the Cys^{384}'s on each B domain. To establish whether the disulfide between Cys^{384} in the B domain and Cys^{124} in the C domain could be formed between different subunits, heterodimers consisting of IIChis-S124C and SSCS were subjected to CuPhe-induced oxidation. Each subunit in a heterodimer provides only one of the cysteines that participate in the disulfide bridge. Figure 5A (lane 2) shows that when a 3:1 mixture of SSCS and IIChis-S124C was oxidized, the same homodimer as observed with purified SSCS alone (lane 4 and Fig. 2 and 3) was formed. In addition, a new band at

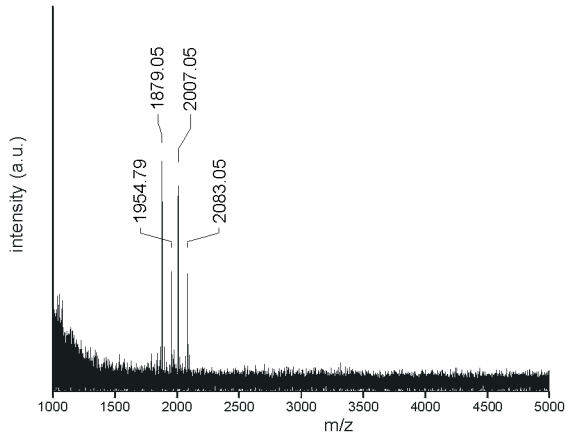

Figure 4. MALDI-TOF mass spectrum of the B domain peptides extracted from a gel piece with the larger C domain. The peptides have been isolated as described in the materials and methods.

approximately 90 kDa appeared, whereas the IIChis-S124C band became less intense (lane 2).This 90 kDa band can only be the heterodimer between SSCS and IIChis-S124C. After reduction, both the SSCS dimer and the heterodimer disappeared. This shows that the B/C domain disulfide bridge can cross the dimer interface.

The B/C disulfide bridge can be formed intramolecularly

Next, we addressed the question whether or not the disulfide bridge between Cys^{384} and Cys^{124} can also be formed within one monomer. For this purpose, heterodimers of SSCS-S124C and a cysteine-less IIChis (IIChis-CL) were formed. It is crucial for this experiment that all the SSCS-S124C monomers are forming a heterodimer with a IIChis-CL monomer, such that only the intrasubunit disulfide bridge is possible. Therefore, an approximately 15-fold excess IIChis-CL was added and the heterodimer formation was facilitated by the addition of 170 mM Na_3PO_4, pH 7.6, which lowered the cloud point of the solution (see materials and methods). Na_3PO_4 alone did not have an influence on the cross-linking behavior of SSCS-S124C (not shown). As can be seen in figure 5B, homodimers were no longer present upon oxidation with CuPhe, which is indicative of complete heterodimer formation (compare Fig. 2B, lane 2 and Fig. 5B, lane 2). Endoproteinase Glu-C digestion of the oxidized sample generated the 33 kDa C domain and the additional 42 kDa band (arrow in lane 4 of Fig. 5B); the latter is absent upon reduction of the sample with

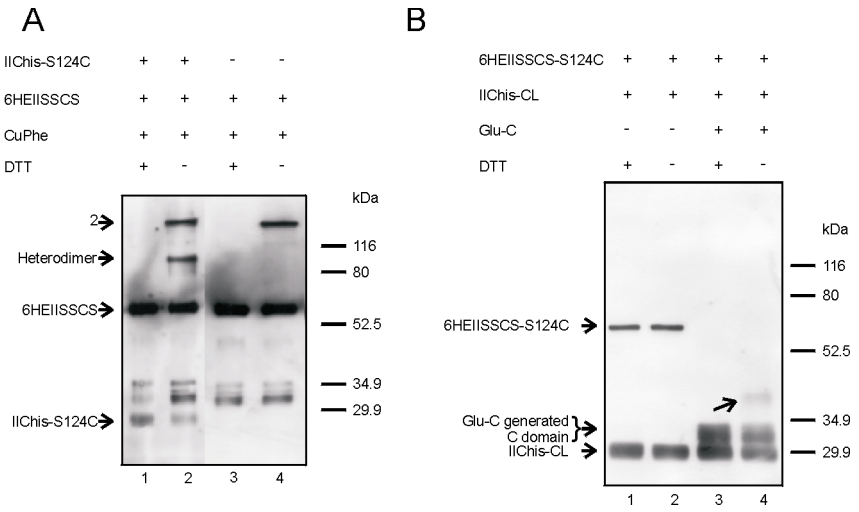

Figure 5. Heterodimer experiments to determine if the Cys^{384}-Cys^{124} disulfide can be formed intra- and intermolecularly. **(A)** Immunoblot of CuPhe-induced oxidation of homo- and heterodimers of purified SSCS (3 μM) and IIChis-S124C (1 μM). After CuPhe-induced oxidation, the samples were split and either reduced with 10 mM DTT or left untreated. Due to a poor blotting efficiency of the C domain alone, the apparent concentrations on the PVDF of EII^{mtl} and the C domain differ. **(B)** Immunoblot of CuPhe-induced oxidation of heterodimers formed between purified SSCS-S124C (0.2 μM) and IIChis-CL (3 μM) in the presence of 170 mM Na_3PO_4, pH 7.6. After CuPhe-induced oxidation, the samples were reduced with DTT (lane 1), left untreated (lane 2), endoproteinase Glu-C digested and, subsequently, reduced with DTT (lane 3), or endoproteinase Glu-C digested (lane 4). The arrow indicates the 42 kDa band. Approximately 0.15 μg **(A)** and 0.075 μg **(B)** of EII^{mtl} was loaded per lane.

DTT. This experiment, therefore, proves that the B/C disulfide bridge can also be formed within one subunit.

Residue 124 is located near the dimer interface

The CuPhe-induced cross-linking of SSCS-S124C in ISO membrane vesicles, corresponds most likely to a disulfide bridge between Cys^{124} of both monomers. The CuPhe-induced oxidation was repeated with purified IIChis-S124C (Fig. 6). IIChis-S124C migrates as a monomer with an apparent molecular weight of 28 kDa in the presence of DTT. Upon oxidation, a dimeric IIChis-S124C with an apparent molecular weight of 50 kDa was observed. To confirm the close proximity of both Cys^{124}'s in one dimer, reduced IIChis-S124C was subjected to cross-linking with dimaleimides of different lengths, ranging from 7.7 to 15.1 Å. Figure 6 shows that all three dimaleimides also yielded the formation of the 50 kDa dimer. These data indicate that the residue at position 124 is located at the dimer interface in purified IIChis-S124C.

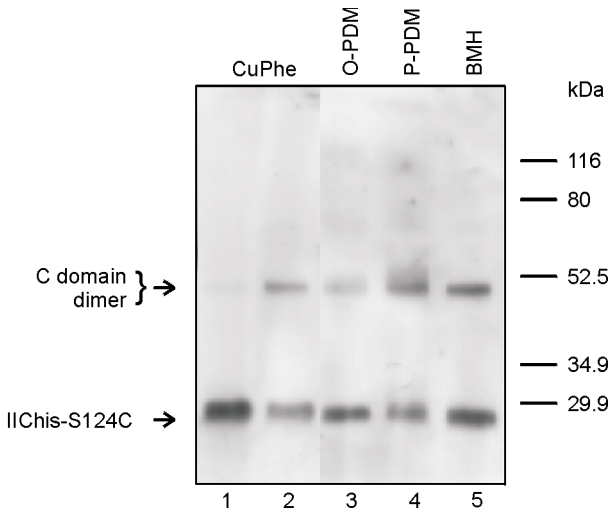

Figure 6. Immunoblot of cross-linking experiments with purified IIChis-S124C. IIChis-S124C (1 μM) was reduced and subjected to cross-linking by oxidation with CuPhe (lanes 1 and 2) or with *o*-PDM, *p*-PDM and BMH, which can span 7.7, 12.0, and 15.1 Å, respectively. Lane 1 represents the sample in which DTT after CuPhe oxidation. Approximately 0.1 μg IIChis-S124C was loaded per lane.

Discussion

In this article we describe the generation of several disulfide bridges, indicative for close proximity, between two cysteines in the dimeric EII^{mtl}. In the enzyme in ISO membrane vesicles, intersubunit disulfide bridges between both Cys^{384}'s and between both Cys^{124}'s are formed. Upon purification in the detergent dPEG, the intersubunit disulfide between both Cys^{384}'s is still formed. In addition, an interdomain cross-link between Cys^{384} and Cys^{124} is observed, which either can be formed as an intrasubunit or an intersubunit disulfide. The intersubunit disulfide between both Cys^{124}'s is not formed in purified EII, but it is formed in purified C domain harbouring Cys^{124}. It is important to stress that both the detergent-solubilized EII and IIC retain full binding capacity, and the soluble EII retains full mannitol phosphorylation activity. The observations made with the detergent-solubilized enzymes thus reveal structural information of functionally relevant conformations of the protein.

To form a disulfide bond, the C_β atoms of the cysteines have to come within 3.8-4.5 Å of each other (*147*). The two Cys^{384}'s and two Cys^{124}'s are thus in very close proximity both at the B/C domain interface and the dimer interface of EII^{mtl}. The multiplicity of possible disulfides is easy to understand in the light of the interdomain dynamics that are essential for the entire phosphorylation and transport cycle. The architecture and functioning of EII^{mtl} necessitates an interdomain flexibility in which the B domain interacts with various domains at different stages in the

catalytic cycle. i) The active sites of the A and B domains, each of which are proteins of approximately 15 kDa, must approach one another in order to transfer the phosphoryl group from His^{554} on the A domain to Cys^{384} on the B domain, then ii) this same region of the B domain, containing a phosphorylated Cys^{384}, must interact with the C domain to effect the conformational energy coupling which enables the translocation and subsequent phosphorylation of mannitol. iii) The phosphoryl group can cross the dimer interface from the A domain of one monomer to the B domain of the other monomer (illustrated in fig. 1) (*18, 24*) or from the B domain of one monomer to the mannitol bound by the C domain of another monomer (*25, 145*). It is, therefore, logical that the active site Cys^{384} in the B domain is in close proximity with different regions of the enzyme at different steps in the catalytic cycle and that, during the cross-linking process, these various domain interactions transiently occur and lead to the cross-links observed. Upon purification in detergent, a different pattern of the disulfide bridges is observed, which is additional evidence for this dynamic situation.

The observation of different higher aggregated forms of EII^{mtl}, all denoted as dimeric forms, is not new. Band 1 was observed upon extraction of the enzyme from the membrane, whereas band 2 appeared upon cross-linking via disulfides, dimaleimides, or lysine-specific cross-linkers, (*26-29*). Band 1 is insensitive to reduction suggesting that it is stabilized by non-covalent interactions rather than disulfides. In this regard, it is noteworthy that most observations of band 1stem from measurements on protein extracted from the membrane but not purified. It is likely that its aggregation state is stabilized by lipids complexed to the protein. Band 2's position and, thus, its estimated mass varies with the degree of cross-linking in the polyacrylamide gel, but it is close to that of a tetramer. In a single-cysteine EII^{mtl}, the tetramer can be generated when a single disulfide bond cross-links two dimers whose subunits are held together by native-like interactions, as in the case of band 1. The tetramer could also arise from two disulfide-bonded dimers that come together to form a tetramer. The band in between 1 and 2 (lane 3, fig 2A) could represent the loss of a monomer from a tetramer, which is only possible in the interdimer cross-linked tetramer. A possible tetrameric state of the enzyme, however, does not lead to other conclusions for the close proximity of the cysteines at positions 384 and 124. In the future we will examine the possible tetrameric nature of EII^{mtl} in more detail with analytical ultracentrifugation as has been done for the lactose carrier of *Streptococcus thermophilus* (*148*).

As summarized in the introduction, there is a lot of kinetic and thermodynamic evidence for conformational coupling at the B/C domain interface. However, there was no direct structural information about the location of this interface. Here, we present for the first time the location of at least part of the B/C domain interface, which is formed by the first cytoplasmic loop in the C domain and the region around the active-site cysteine in the B domain. The first evidence for the importance of this loop in the interaction with the B domain came from time-resolved fluorescence spectroscopy studies on a series of single-tryptophan containing mutants (*40*).

Notably, a change in the time-resolved anisotropy of tryptophan 109 was observed upon phosphorylation of the B domain. The location around residue 124 is in a region, which is conserved among mannitol-specific EII's of different origin (*149*). Close proximity of this region of the C domain and the B domain active site was already suggested by photocross-linking experiments (van Montfort *et al.*, unpublished results). Recent kinetic data on a series of phenylalanine to tryptophan replacements in this first cytoplasmic loop suggest that residues 126 and 133 are critical for the phosphorylation of mannitol but not for mannitol binding. The data suggest that these residues are located at or near the dimer interface and involved in the B/C domain interaction (Vos *et al.*, to be published), which is consistent with the data presented here.

The location of the interface in the first cytoplasmic loop is very interesting, because all previously described mutations that have an influence on the functioning of EII^{mtl} are located in the predicted second cytoplasmic loop. This is also the location of the GIXE motif (residues 254-257), which is highly conserved in all EII's and speculated to be involved in substrate binding (*38*). Replacement of E257 in this motif led to enzymes with no or only low affinity mannitol binding, depending on the substituent, as well as defective transport (*150*). Another region in the same loop is also important for activity. For instance, replacement of G196 or H195 led to enzymes, which exhibited no or low affinity binding for mannitol (*24, 145, 151*). Interestingly, a heterodimer of the inactive mutants E257A and H195A was significantly active in transport and phosphorylation of mannitol, suggesting that these residues are in close proximity (*25*). Also data on the glucose transporter of *E. coli* point to a role for the second cytoplasmic loop in substrate binding (*152*).

In conclusion, the data presented here suggests that residues 124 and 384 of both subunits can come within 5 Å of each other and are located at the B/C domain and dimer interface. An intriguing question is what happens at the B/C domain interface upon phosphorylation. Phosphorescence data of single tryptophan mutants in the C domain show that a conformational change takes place upon phosphorylation of Cys^{384}, different from that upon mannitol binding (*52*). With the current pairs of cysteines, however, it is not possible to figure out what is happening precisely, because phosphorylation prevents Cys^{384} from forming a disulfide. In the future, we will screen several cysteine mutants in these regions of the protein to determine the exact borders of the B/C domain and dimer interface and changes therein upon mannitol binding and/or B domain phosphorylation. Eventually this will lead to a more detailed understanding of the energy coupling mechanism at the B/C domain interface in EII^{mtl}.