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Interaction between the Cytoplasmic and Membrane-Bound Domains of Enzyme II^{mtl} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System†

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ABSTRACT: Sulfhydryl reagents affected the binding properties of the translocator domain, NIII, of enzyme II^{mtl} in two ways: (i) the affinity for mannitol was reduced, and (ii) the exchange rate of bound and free mannitol was increased. The effect on the affinity was very much reduced after solubilization of enzyme II^{mtl} in the detergent decylPEG. The effects were caused exclusively by reaction of the sulfhydryl reagents with the cysteine residue at position 384 in the primary sequence. Interaction between two domains is involved, since Cys384 is located in the cytoplasmic domain, CII. When Cys384 was mutated to serine, the enzyme exhibited the same binding properties as the chemically modified enzyme. The data support our proposal that phosphorylation of enzyme II^{mtl} drastically reduces the activation energy for the translocation step through interaction between domains CII and NIII [Lolkema J. S., ten Hoeve-Duurkens, R. H., Swaving Dijkstra, D., & Robillard, G. T. (1991) *Biochemistry* (preceding paper in this issue)]. Functional interaction between the translocator domain, NIII, and domain CI was investigated by phosphorylation of His554, located in domain CI, in the C384S mutant. No effect on the binding properties was observed. In addition, the binding properties were insensitive to the presence of the soluble phosphotransferase components enzyme I and HPr.

Enzyme II^{mtl} [1] of the bacterial P-enolpyruvate-dependent phosphotransferase system is responsible for the transport of mannitol into the cell. It combines the transport activity with the phosphorylation of mannitol at the expense of P-enolpyruvate. Mannitol appears as mannitol-P in the cytoplasm [for recent reviews, see Postma and Lengeler (1985) and Robillard and Lolkema (1988)]. The primary phosphoryl group donor is P-HPr that itself is phosphorylated by P-enolpyruvate in a reaction catalyzed by enzyme I. Phosphorylated enzyme II^{mtl} is a catalytic intermediate in the phosphoryl group transfer from P-HPr to mannitol.

The primary sequence of enzyme II^{mtl} from *Escherichia coli* was deduced from the base sequence of the cloned MtlA gene (Lee & Saier, 1983). Studies in which fragments of the gene were subcloned or the gene was partially digested from the C-terminal end suggest that the enzyme consists of three domains (Grisafi et al., 1989; White & Jacobson, 1990; van Weeghel et al., 1991b,c). These have been called CI, CII, and NIII from the C-terminal to the N-terminal of the protein, respectively. About half of the protein constitutes the N-terminal domain NIII. It is very hydrophobic and presumably completely membrane-bound. NIII and the complementary C-terminal half of the protein, termed CIII, were expressed separately in *E. coli*. Mannitol phosphorylation was restored after bringing the two together. CIII was shown to protrude into the cytoplasm (Stephan & Jacobson, 1986). It has been isolated as a soluble protein which consists of the two domains CI and CII. CI, CII, and CIII have been expressed separately in *E. coli* and shown to be functional proteins.

Enzyme II^{mtl} is phosphorylated at two distinct sites during turnover, a histidine residue at position 554 in the primary sequence and a cysteine residue at position 384, both located in the cytoplasmic domains (Pas & Robillard, 1988b). His554 in domain CI accepts the phosphoryl group from P-HPr after which transfer takes place to Cys384 in domain CII. From

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[1] Abbreviations: ISO, inside out; RSO, right side out; DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol; pts, phosphotransferase system; NEM, *N*-ethylmaleimide; diamide, 1,1'-azobis(*N*,*N*-dimethylformamide).

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here, the phosphoryl group is transferred to mannitol. The membrane-bound domain constitutes the mannitol translocator. In the absence of phosphorylation, it catalyzes mannitol transport at a low rate (Lolkema et al., 1990).

Experiments described in the preceding paper (Lolkema et al., 1991) led us to conclude that transport and phosphorylation of mannitol are not obligatorily coupled events. In fact, less than one out of two mannitol molecules transported were phosphorylated under the experimental conditions investigated. A model was proposed for the coupling of the transport and phosphorylation activity in which the main effect of the phosphorylation of enzyme II was to lower the activation energy for the translocation step. The state of phosphorylation of enzyme II^{mtl} modulates the activity of the translocator domain. This requires conformational interaction between the membrane-bound domain and one or both of the cytoplasmic domains. In the present study, we will demonstrate this interaction by the effect of modifications in the cytoplasmic domains on the mannitol binding and translocation activities of the membrane-bound domain. The cytoplasmic domain, CII, will be modified by chemical modification of the phosphoryl group binding site using sulfhydryl reagents. The effect of phosphorylation of domain CI will be studied in a mutant enzyme in which the phosphoryl group cannot be transferred to mannitol.

Materials and Methods

Materials. D-[1-^{3}H(N)]Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. *N*-Ethylmaleimide was purchased from Jansen. Diamide was from Aldrich. Decylpoly(ethylene glycol) 300 (decylPEG) was synthesized by B. Kwant in our laboratory. The *E. coli* phosphotransferase enzymes E_I and HPr were purified as described (Robillard et al., 1979; van Dijk et al., 1990).

Cell Growth. Escherichia coli strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing 0.5% mannitol as the carbon source. *Escherichia coli* strain ASL-1 (see below) was grown on LB medium with the appropriate antibiotic. Cells were grown in 1-L serum bottles and aerated by blowing in sterile air near the bottom of the bottle. Cells were harvested at an OD_{650} of 1.0.

Membrane Preparations. ISO membrane vesicles and RSO membrane vesicles were prepared according to Reenstra et al. (1980) and Kaback (1971), respectively. RSO membranes were purified on a sucrose gradient (Lolkema et al., 1990). Membranes were stored in 25 mM Tris, pH 7.6, 1 mM DTT, and 1 mM NaN_3 in liquid nitrogen. The aliquots of 200 μL contained 1–4 mg/mL membrane protein (Lowry et al., 1951). Samples were thawed rapidly before use. Each sample was used only 1 time.

Mutant C384S. Plasmid pWAMa carrying the MtlA gene and plasmid pWAMc5 carrying the MtlA gene in which codon 384 was mutated to Ser have been described by van Weeghel et al. (1991a). The plasmids were transformed into *E. coli* strain ASL-1 [F$^-$, *lac*Y1, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, recA] (a gift from J. Lengeler and A. Scholle). The strain was checked for a mannitol negative phenotype on McKonkey indicator plates (Difco bacto agar base, Difco Lab.) with 0.2% mannitol.

P-enolpyruvate-dependent mannitol phosphorylation catalyzed by ISO membranes prepared from ML308-225 cells or ASL-1 cells harboring plasmid pSer384 coding for the C384S mutant of enzyme II^{mtl} was measured as described (Robillard & Blaauw, 1986). The assay mixture contained 5 mM P-enolpyruvate, 3 μM HPr, 0.11 μM enzyme I, and 100 nM [^{3}H]mannitol. The buffer contained 25 mM Tris, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, and 0.25% decylPEG. Membrane protein concentrations were 62 ng/mL and 0.24 mg/mL for the ML308-225 and ASL-1 vesicles, respectively. The ML308-225 membrane preparation contained 4 times more mannitol binding sites per milligram of membrane protein than the ASL-1 preparation as determined by binding assays.

Binding Assays. Binding of [^{3}H]mannitol to enzyme II^{mtl} was measured with flow dialysis as described (Lolkema et al., 1990a). Technical improvements of the system allowed for a half-time of the system response of 10 s. All experiments were performed in a volume of 400 μL and at room temperature. Bound [^{3}H]mannitol was displaced from the binding sites by addition of 100 μM unlabeled mannitol. The exchange rate was analyzed as described (Lolkema et al., 1990a). Routinely, the total [^{3}H]mannitol concentrations used in the Scatchard analyses of the binding were 50, 100, 200, and 400 nM. Measurement of the binding to intact membranes as well as to membranes solubilized with decylPEG was done in the same run by adding decylPEG to the upper compartment after enough samples had been collected to measure the binding to the intact membranes.

Inactivation of Enzyme II^{mtl}. The inactivation of enzyme II^{mtl} was measured in the mannitol/mannitol-P exchange reaction. In this way, potential effects on the other pts components, HPr and enzyme I, could be excluded. Routinely, exchange was measured between 200 nM [^{3}H]mannitol and 250 μM mannitol-P in 25 mM Tris, pH 7.6, 5 mM $MgSO_4$, 5 mM DTT, and 0.25% decylPEG. Typically, a membrane protein concentration of 4 μg/mL resulted in an exchange rate of 1.3 nM/min.

Oxidation of enzyme II^{mtl} by molecular oxygen in the presence of 100 μM DTT was measured in the following way. The exchange reaction was started by diluting membranes in 1 mM DTT 10-fold into the assay mixture that contained all the other components except for DTT. In a control experiment, 5 mM DTT was included. The formation of [^{3}H]-mannitol-P was measured at intervals of 5 min as described (Robillard & Blaauw, 1986).

The inactivation of enzyme II^{mtl} by the sulfhydryl reagents was measured in the same way, but the assay mixture contained, in addition, 2.5 mM NEM, 5 mM diamide, or 250 μM $HgCl_2$. No formation of [^{3}H]mannitol could be detected after treatment with these three reagents, indicating instantaneous inactivation of enzyme II^{mtl}.

Results

Binding of [^{3}H]mannitol to enzyme II^{mtl} was assayed by measuring binding to RSO and ISO membrane vesicles derived from cells induced for the mannitol transport protein. The technique used was flow dialysis (Colowick & Womack, 1969), which measures the free concentration of [^{3}H]mannitol in equilibrium with the [^{3}H]mannitol–enzyme complex. It was shown before that all binding to the membranes could be accounted for by binding specifically to enzyme II^{mtl} (Lolkema et al., 1990). This paper focuses on two properties of the binding to these membranes: (i) the affinity constant and (ii) the rate of exchange of bound [^{3}H]mannitol with excess unlabeled mannitol. The latter is rapid in the case of binding to RSO vesicles; the response of the flow dialysis system is identical with the response to a stepwise change in the free concentration [Figure 1A (○)]. With ISO membranes, the exchange is slower than the response time of the system [Figure 1B (□) or Figure 2 (○); see also Lolkema et al. (1990)].

Effect of Sulfhydryl Reagents on the Binding Properties. RSO membranes were equilibrated with 100 nM [^{3}H]mannitol

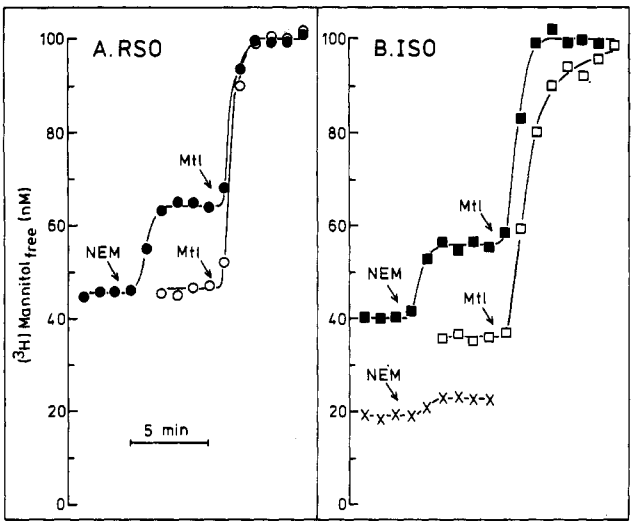

FIGURE 1: Effect of NEM on the binding properties of mannitol to RSO and ISO membranes. RSO membranes (A) at a membrane protein concentration of 0.17 mg/mL were incubated with 100 nM [^{3}H]mannitol in the presence of 0.18 (●) or 5 mM DTT (○). ISO membranes (B) [0.3 mg/mL] were incubated with 100 nM [^{3}H]-mannitol in the presence of 90 (■) or 5 mM DTT (□). The mixtures were loaded in the upper compartment of a flow dialysis chamber, and the free concentration was measured. NEM was added at a concentration of 2.5 mM at the arrow marked NEM (closed symbols). At the arrow marked Mtl, excess unlabeled mannitol was added. Experiment B: (×) ISO membranes (0.6 mg/mL) were incubated with 100 nM [^{3}H]mannitol in the presence of 100 μM DTT and 0.5% decylPEG and treated identically with NEM as above. The ISO preparation was different from the one used in the other experiments (■ and □). The half-time of the system response in these experiments was 31 s.

in the presence of 0.18 or 5 mM DTT [Figure 1A, (●) and (○), respectively]. In both cases, the concentration of free [^{3}H]mannitol was 45 nM, and, therefore, the concentration of mannitol bound to enzyme II^{mtl} was 55 nM. Addition of 3 mM *N*-ethylmaleimide (NEM) resulted in an increase of the free concentration to 65 nM or a decrease of the bound concentration to 35 nM. NEM is a known inhibitor of enzyme II^{mtl} (Roossien & Robillard, 1984), and control experiments showed that addition of 3 mM NEM to the membranes in 0.18 mM DTT caused an instantaneous inactivation of over 99% of the enzyme II^{mtl}-catalyzed mannitol/mannitol-P exchange activity. Addition of excess unlabeled mannitol to the NEM-treated membranes (●) or the untreated membranes (○) resulted in a rapid release of all bound [^{3}H]mannitol, indicating rapid exchange of bound and free mannitol in both cases.

In contrast to RSO membranes, the binding of [^{3}H]mannitol to ISO membranes in the presence of low concentrations of DTT decreased slowly in time. The decrease in mannitol binding was paralleled by a lowering of the mannitol/mannitol-P exchange activity catalyzed by enzyme II^{mtl}, indicating that oxidation of the enzyme had occurred. In the presence of 5 mM DTT, there was only 36 nM free [^{3}H]-mannitol at the start of the experiment in Figure 1B while in the presence of 90 μM DTT there was 40 nM free [^{3}H]-mannitol. The activity decreased 40% in 15 min under the same conditions. Figure 1B shows that rapid sample preparation could not prevent some reduction in the binding when ISO membranes were incubated with 100 nM [^{3}H]mannitol in the presence of 90 μM DTT (■) compared to 5 mM DTT (□). Addition of NEM led to a reduced binding as was observed with the RSO membranes. Scatchard analysis of NEM-treated ISO membranes and untreated membranes demonstrated that the reduced binding was due to an increase of the affinity constant from 35 to 80 nM (Table I) upon NEM

Table I: Affinity Constants and Rate of Exchange of Bound and Free Mannitol for Mannitol Binding to ISO Membranes[a]

enzyme II^{mtl}	K_D (nM)	$t_{1/2}$ (s)
wild type	35	65
wild type + decylPEG	90	
wild type + NEM	80	7
wild type + Hg^{2+}	110	16
wild type + diamide	nd[b]	5
C384S	69	15
C384S + decylPEG	89	
C384S + NEM	id[c]	14

[a] The affinity constants K_D were determined from a Scatchard analysis of the binding of [^{3}H]mannitol to ISO membranes containing wild-type enzyme II^{mtl} or the C384S mutant. The rate of exchange was measured as described in the legend of Figure 2. ISO membranes at protein concentrations of 0.5 or 1.6 mg/mL for the wild type and the C384S mutant, respectively, were incubated with 100 nM [^{3}H]-mannitol. The indicated half-times were corrected for the response time of the system which had a half-time of 14 s. DecylPEG was added at a concentration of 0.5%. The inactivations with the sulfhydryl reagents were performed as described under Materials and Methods. [b] nd is not determined. [c] id (identical) indicates that no complete Scatchard analysis was performed but that the addition had no effect on the level of binding in a single experiment.

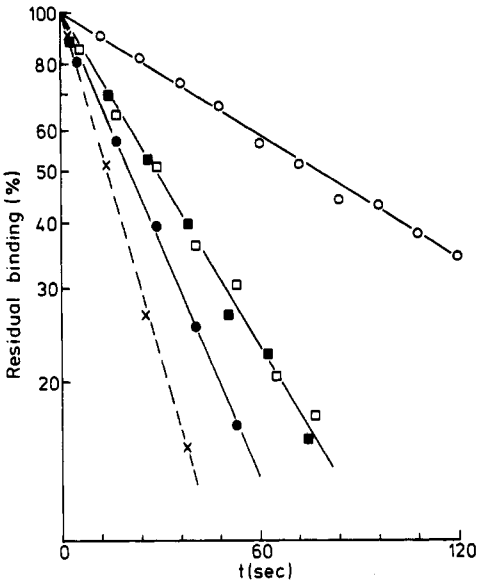

FIGURE 2: Effect of NEM on the rate of exchange of free mannitol and mannitol bound to ISO membranes containing wild-type enzyme II^{mtl} or the C384S mutant. ISO vesicles at a membrane protein concentration of 0.5 or 1.6 mg/mL for wild-type (○ and ●) or mutant enzyme (□ and ■), respectively, were incubated with 100 nM [^{3}H]mannitol. In the experiments indicated by the closed symbols (● and ■), the membranes were pretreated with NEM as described under Materials and Methods. The residual binding after addition of 100 μM unlabeled mannitol to the upper compartment was plotted on an exponential scale. The dashed line (×) indicates the response of the system to a stepwise change of the free label concentration in the upper compartment ($t_{1/2}$ = 14 s). The half-times for the exchange processes after correction for the response of the system are listed in Table I.

treatment. Addition of excess unlabeled mannitol to the NEM-treated ISO membranes showed that NEM treatment resulted in a 10-fold increase in the rate of release of bound [^{3}H]mannitol over that observed with the untreated membranes [Figure 2 (○ and ●)]. The exchange rates of bound and free mannitol yielded half-times of 7 and 65 s for the treated and untreated membranes, respectively (Table I).

Oxidation of enzyme II^{mtl} by incubating ISO membranes for prolonged periods of time in the presence of only low concentrations of DTT resulted in levels of binding comparable to those observed after NEM treatment. Addition of NEM

under these conditions did not decrease the binding any further (not shown), indicating that oxidation by molecular oxygen or alkylation by NEM affects the binding by the same mechanism.

Two other sulfhydryl reagents were tested for their effects on the extent of binding and their potency to enhance the rate of exchange of [^{3}H]mannitol bound to ISO membranes with excess unlabeled mannitol. The oxidant diamide has been shown to protect enzyme II^{mtl} from inactivation with NEM (Pas & Robillard, 1988a). In this study, diamide was used at a concentration where it instantaneously inhibited all mannitol/mannitol-P exchange activity catalyzed by enzyme II^{mtl}. In addition, $HgCl_2$ was used. Addition of $HgCl_2$ at a concentration slightly in excess of the DTT sulfhydryl groups immediately abolished all enzyme II^{mtl} activity (see Materials and Methods). Both diamide and $HgCl_2$ reduced the binding of [^{3}H]mannitol to enzyme II^{mtl} when used instead of NEM in the experiment described in Figure 1B (■). In the case of $HgCl_2$-treated membranes, the mannitol binding was titrated, resulting in a K_D of 110 nM (Table I). Both diamide and Hg^{2+} increased the exchange rates of free mannitol and [^{3}H]-mannitol bound to ISO membranes. The increase by Hg^{2+} was somewhat smaller than that observed with NEM or diamide (Table I).

Remarkably, the binding of mannitol to enzyme II^{mtl} solubilized in the detergent decylPEG was much less sensitive to the sulfhydryl reagents [Figure 1B (×)]. ISO membranes at a high protein concentration were equilibrated with 100 nM [^{3}H]mannitol, resulting in a binding of 81 nM [^{3}H]mannitol. Inactivation of enzyme II^{mtl} by NEM reduced the binding by only 5% to 77 nM, which is much less than observed with the intact membranes [compare (×) and (■) in Figure 1B]. Addition of $HgCl_2$ instead of NEM had a comparably small effect (not shown).

Binding Properties of the C384S Mutant. The inhibition of enzyme II^{mtl} catalyzed phosphorylation activity by sulfhydryl reagents has been shown to be due to reaction with the cysteine residue at position 384 in the primary sequence (Cys384) which is the second phosphoryl group binding site in the cytoplasmic domain (Pas & Robillard, 1988b). A mutant in which residue Cys384 is replaced by Ser was constructed by site-directed mutagenesis. The catalytic activities of the mutant in the mannitol phosphorylation activities have been reported (van Weeghel et al., 1991a). The mutant was expressed in *E. coli* strain ASL-1, which does not express chromosomally encoded enzyme II^{mtl} due to a mutation in the promoter region (Lengeler and Scholle, personal communication). ISO membranes were prepared from the host cell ASL-1 without the plasmid. No binding of mannitol could be detected when these membranes were incubated at a membrane protein concentration of 1.5 mg/mL with 100 nM [^{3}H]mannitol. Therefore, no correction for aspecific binding was necessary. Wild-type enzyme II^{mtl} expressed from plasmid pWAMa in strain ASL-1 exhibited binding properties similar to those observed with the ML308-225 membranes. The maximal number of binding sites per milligram of membrane protein indicated that, on average, the level of expression of enzyme II^{mtl} was about 4-fold lower in ASL-1 versus ML 308-225. The mannitol binding properties of the C384S mutant were investigated after isolation of ISO membranes from ASL-1 cells harboring plasmid pWAMc5, which carries the gene coding for the mutant. Scatchard analysis of the binding revealed a K_D for mannitol of 69 nM (Table I). The total number of binding sites per milligram of membrane protein was 116 pmol/mg. Solubilization of the C384S membranes with decylPEG seemed to have very little effect on the binding properties. Scatchard analysis of the binding to the mutant in the presence of decylPEG indicated only a slightly elevated K_D of 89 nM and an increase in the total number of sites to 133 pmol/mg. In contrast, solubilization of membranes containing wild-type enzyme II^{mtl} leads to an almost 3-fold increase in the affinity constant (see Table I). The rate of exchange of [^{3}H]mannitol bound to the ISO membranes with excess unlabeled mannitol was considerably faster for the C384S mutant than observed for the wild-type enzyme, 15 s versus 65 s [Figure 2, (○) versus (□)].

The effect of sulfhydryl reagents on the binding in this mutant enzyme II^{mtl} was investigated by treating the membranes with NEM. C384S membranes at a concentration of 0.34 mg/mL were equilibrated with 100 nM [^{3}H]mannitol in 0.2 mM DTT. The membranes bound 29 nM [^{3}H]mannitol under these conditions. No change in the amount of bound mannitol could be detected upon the addition of 2.5 mM NEM, in contrast to the effect observed with the wild-type enzyme [Figure 1B (□)]. In addition, the rate of exchange between bound and free mannitol was not affected after treatment of the membranes with NEM [Figure 2 (□ and ■)]. The insensitivity of the binding and the exchange rate in the C384S mutant strongly indicates that modification of Cys384 in the wild-type enzyme is responsible for the changed binding properties.

Binding to Enzyme II^{mtl} Phosphorylated at His554. The first phosphorylation site in enzyme II^{mtl}, His554, can be phosphorylated in the C384S mutant by P-HPr without transfer of the phosphoryl group to mannitol (van Weeghel et al., 1991a). This allows for the measurement of the binding of mannitol to enzyme II^{mtl} phosphorylated at the phosphorylation site in domain CI. However, first a number of control experiments were performed.

Phosphorylation of enzyme II^{mtl} by P-enolpyruvate requires the presence of the phosphotransferase components enzyme I and HPr. The effects of these components on the binding of mannitol to enzyme II^{mtl} were tested by equilibration of 400 μL of ISO membranes containing wild-type enzyme II^{mtl} with 100 nM [^{3}H]mannitol in the upper compartment of the flow dialysis system. The free concentration of mannitol was 44 nM. The addition of 4 μL of a 300 μM HPr or a 22 μM enzyme I solution had no significant effect on the free concentration, indicating no effect of enzyme I or HPr on the level of binding. In addition, there was no effect on the rate of exchange between bound and free mannitol (not shown).

Binding is routinely measured with very high concentrations of enzyme II^{mtl} relative to concentrations necessary to measure phosphorylation activity. Consequently, high mannitol phosphorylation capacities are present in the binding assay, and even very low levels of activity of a particular mutant could easily disturb the binding measurement by phosphorylation of the substrate. The P-enolpyruvate-dependent mannitol phosphorylation activity of the membranes containing the C384S mutant was measured by using high membrane concentrations. The phosphorylation rate catalyzed by 0.24 mg/mL membrane protein in the presence of 100 nM [^{3}H]-mannitol and 3 μM HPr was 0.5 nM/min at 30 °C. The specific activity was a factor of 1.6×10^{-4} less than that observed with a membrane preparation containing the wild-type enzyme under identical conditions. The low activity in the C384S membranes is not due to residual activity of the mutated enzyme, because (i) ISO membranes prepared from the host cell ASL-1 exhibited the same activity per milligram of membrane protein and (ii) NEM completely abolished all

activity. The residual activity could be due to a very low level of expression of the chromosomally encoded enzyme II^{mtl} of the host cell or to some other enzyme II species with low affinity for mannitol.

The residual mannitol phosphorylation activity of the C384S membranes was low enough to allow us to measure mannitol binding to the C384S mutant phosphorylated at His554 in domain CI without interference from conversion of mannitol to mannitol-P. C384S membranes at a concentration of 0.39 mg/mL membrane protein were equilibrated with 100 nM [^{3}H]mannitol in the presence of 0.22 μM enzyme I and 3.4 μM HPr. The concentration of bound mannitol was measured to be 38 nM. Subsequently, 5 mM P-enolpyruvate was added, leading to phosphorylation of His554 in the mutant enzyme. No significant effect on the level of binding nor on the rate of exchange between bound and free mannitol could be detected (not shown).

Discussion

Domain/Domain Interaction. Sulfhydryl reagents are known inhibitors of enzymes II of the bacterial phosphotransferase system (Hagenauer-Tsapis & Kepes, 1980; Robillad & Konings, 1981; Roossien & Robillard, 1984; Lolkema et al., 1985; Grenier et al., 1985). The base sequence of the MtlA gene coding for enzyme II^{mtl} indicates that the primary sequence contains four cysteine residues (Lee & Saier, 1983). Cysteines at positions 100 and 320 in the primary sequence are located in the membrane-bound domain and the cysteines at positions 384 and 571 in the cytoplasmic domains CII and CI, respectively. Reaction of radioactively labeled NEM with purified enzyme II^{mtl} demonstrated that one molecule of label per molecule of enzyme was incorporated (Roossien & Robillard, 1984). The labeled cysteine was shown to be the residue at position 384 in domain CII (Pas & Robillard, 1988a). Later, Cys384 turned out to be one of the phosphorylation sites on enzyme II^{mtl}, which provided a perfect rationale for the inhibition of mannitol phosphorylation by the sulfhydryl reagents (Pas & Robillard, 1988b). The present data support the above conclusions. Reaction of wild-type enzyme II^{mtl} with NEM had significant effects on the binding properties for mannitol, whereas a mutant enzyme in which Cys384 was replaced by a serine residue was insensitive to NEM in this respect. The same conclusion was reached when $HgCl_2$ was used. This indicates that reaction with Cys384, the second phosphorylation site, is solely responsible for the changed binding properties of the translocator domain. Therefore, changes in cytoplasmic domain CII affect both the binding properties and the translocation activity of the membrane-bound domain NIII.

Modification of Cys384. In this study, Cys384 was chemically modified by alkylation with NEM, by oxidation with molecular oxygen and diamide, and by complexation with Hg^{2+}. All these modifications had the same two effects on the activity of the translocator: (i) the affinity for mannitol was reduced, and (ii) the rate of exchange of free mannitol with mannitol bound to ISO membranes was increased (Table I). In addition, the replacement of the thiol group by a hydroxyl group in the C384S mutant by site-directed mutagenesis exhibited the same two effects.

Mannitol bound to enzyme II^{mtl} in ISO vesicles was shown to be situated at the periplasmic side of the membrane which is at the inner face of the vesicle. The slow exchange with excess unlabeled mannitol has been interpreted as the isomerization of the loaded site to the cytoplasmic face of the membrane, followed by dissociation into the cytoplasmic volume. Modification of Cys384 increases the rate of exchange of free and bound mannitol with ISO membranes, while the exchange with RSO membranes remains fast, indicating that, with the modified enzymes, bound mannitol is still situated at the periplasmic side of the membrane (Figure 1). Apparently, modification of Cys384 speeds up the overall rate constant for the transition to the cytoplasmic volume of mannitol bound at the periplasmic-facing binding site. The sulfhydryl reagents appear to stimulate the translocation activity of the translocator domain NIII.

Phosphorylation of Cys384 can also be considered a chemical modification like those mentioned above. In the preceding paper (Lolkema et al., 1991), the coupling between transport and phosphorylation was studied. It was concluded that the coupling is at the level of the transport of mannitol, rather than the phosphorylation of mannitol, since phosphorylated enzyme II^{mtl} could catalyze facilitated diffusion of mannitol 2–3 orders of magnitude faster than the unphosphorylated enzyme. In other words, the energy barrier for the translocation would be much lower in the phosphorylated versus the unphosphorylated enzyme. The present results strongly support this conclusion, since modifications of Cys384 other than phosphorylation seem to have the same effect. Apparently, any modification of Cys384 in the CII domain induces, to some extent, the conformational changes in this domain that lead to the activation of the translocator domain. At present, we are in the process of making a number of other amino acid substitutions at position 384 by site-directed mutagenesis which will allow us to establish the parameters at this position in the primary sequence that are responsible for the activation of the translocator domain.

Effect of DecylPEG. Pas et al. (1988) have reported identical affinity constants for the binding of mannitol to purified enzyme II^{mtl} of enzyme treated with NEM or diamide. The present data support this notion but, in addition, indicate that the insensitivity of the binding to these sulfhydryl reagents was due to the presence of detergent. DecylPEG raises the K_D for mannitol binding to wild-type enzyme II^{mtl} by a factor of 2–3. NEM, Hg^{2+}, and diamide have the same effect. No additional increase in K_D was observed when NEM-, Hg^{2+}, or diamide-treated membranes were solubilized. The same observations were made with the C384S mutant (Table I). It appears that the modification or replacement of Cys384 has the same effect on the affinity for mannitol as does the solubilization with decylPEG. Assuming that the affinity and exchange rate are modulated through specific interactions between the CIII and NIII domains, modification of Cys384 or solubilization of the enzyme could alter these parameters by weakening or fully disrupting the interaction between the two domains. On the other hand, decylPEG could exert its effect on the affinity by a mechanism different from the one associated with Cys384 modifications. The elevated K_D could simply be due to the binding of detergent to the hydrophobic translocator domain. If so, decylPEG would, in addition, inhibit the interaction with the CIII domain, since no additional effect of modification of Cys384 is observed.

Interactions of the Translocator Domain with Other pts Components. Several lines of evidence suggest that the pts enzymes II form functional complexes with the soluble components HPr and enzyme I [see Ghosh et al. (1989) and references cited therein]. We have looked for structural interaction of the translocator domain of enzyme II^{mtl} with CI and the soluble components HPr and enzyme I by testing for functional interaction. The mannitol binding properties were not affected by the presence of enzyme I or HPr. No indication for a functional interaction between enzyme II^{mtl} and

enzyme I was found. HPr does interact with enzyme II^{mtl} since P-HPr is the substrate in the phosphorylation of domain CI. Apparently, binding of HPr to its binding site on CI has no effect on the activities of the translocator. The insensitivity of NIII to changes in CI is best demonstrated by the phosphorylation of His554 in the C384S mutant, which did not change the binding properties. The experiment shows that the stimulation of the translocation rate by phosphorylation of the enzyme is solely due to phosphorylation of Cys384 in domain CII. In conclusion, no functional interaction of the translocator other than with domain CII could be demonstrated.

Registry No. PEP-dependent phosphotransferase, 56941-29-8; mannitol, 69-65-8; cysteine, 52-90-4.

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