Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System: Mannitol-Specific EII Contains Two Phosphoryl Binding Sites per Monomer and One High-Affinity Mannitol Binding Site per Dimer†

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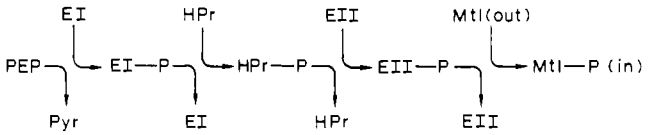
ABSTRACT: The amino acid composition and sequence of EII^{Mtl} is known [Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767]. This information was combined, in the present study, with quantitative amino acid analysis to determine the molar concentration of the enzyme. The stoichiometry of phosphoryl group incorporation was then determined by phosphorylation of enzyme II from [^{14}C]-phosphoenolpyruvate (pyruvate burst procedure). The native, reduced enzyme incorporated two phosphoryl groups per monomer. Both phosphoryl groups were shown to be transferred to mannitol. Oxidation or *N*-ethylmaleimide (NEM) labeling of Cys-384 resulted in incorporation of only one phosphoryl group per monomer, which was unable to be transferred to mannitol. The number of mannitol binding sites on enzyme II was determined by centrifugation using Amicon Centricon microconcentrators. The reduced unphosphorylated enzyme contained one high-affinity binding site (K_D = 0.1 μM) per dimer and a second site with a K_D in the micromolar range. Oxidation or NEM labeling did not change the number of binding sites.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] is responsible for uptake of a number of hexoses and hexitols in both Gram-negative and Gram-positive organisms [for a review see Postma and Lengeler (1985)]. The mannitol PTS of *Escherichia coli*, the subject of this study, consists of two general proteins, EI and HPr, which are cytoplasmic, and a mannitol-specific EII, which is membrane bound and responsible for the actual translocation of mannitol across the cytoplasmic membrane. The driving force for mannitol uptake is phosphoenolpyruvate (PEP). The reaction scheme for uptake is



During turnover both the cytoplasmic proteins as well as EII become phosphorylated (Misset & Robillard, 1982; Weigel et al., 1982; Roossien et al., 1984). Phosphorylated EII is responsible for mannitol translocation; during transport a phosphoryl group is transferred to mannitol, which enters the cytoplasm as mannitol phosphate. A sulfhydryl group is essential for EII^{Mtl} activity. The enzyme is active only in the reduced form. Oxidation results in a catalytically inactive enzyme (Roossien & Robillard, 1984a; Grenier et al., 1985). NEM alkylation or reaction with phenylarsine oxide also inactivates. Prior phosphorylation or oxidation prevents this inactivation (Roossien & Robillard, 1984a). Labeling of a single cysteine, Cys-384, is responsible for the loss of activity (Pas & Robillard, 1988a). Several lines of evidence suggest that EII^{Mtl} may contain more than one phosphorylation site per peptide chain: (i) Hydropathy analysis of the sequenced Mtl A gene showed that the enzyme possessed two distinct domains. The N-terminal half was hydrophobic, containing 7 stretches of 20 or more residues, which could potentially span the membrane. The C-terminus was hydrophilic and appeared to be a typical globular soluble protein (Lee & Saier, 1983). (ii) The hydrophylic domain was on the cytoplasmic side of the membrane (Stephan & Jacobson, 1986a). (iii) The molecular weight of EII^{Mtl} is equal to the combined molecular weights of the other EII–EIII pairs (Saier et al., 1985). Since EII^{Mtl} functions without an $EIII^{Mtl}$, it has been proposed that the C-terminal domain is a covalently attached EIII. Since all EIII's examined are phosphorylated by P-HPr and many EII's are phosphorylated by their respective P-EIII, one would expect EII^{Mtl} to contain two phosphorylation sites, one on the EIII domain and another on the hydrophobic domain (Saier et al., 1985). No chemical data have yet been presented quantitating these sites or examining whether they can be phosphorylated at the same time.

The number of sugar binding sites per EII is also unknown. Several purified EII species have been found to occur as dimers (Roossien & Robillard, 1984a; Leonard & Saier, 1983; Roossien et al., 1984; Erni, 1986; Robillard & Blaauw, 1987), and a dimeric EII has been extracted from membrane preparations (Roossien & Robillard, 1984b; Lolkema et al., 1985; Stephan & Jacobson, 1986b). Radiation inactivation analysis on EII^{Mtl}-containing membranes indicates that the functional form of the enzyme is at least a dimer (Pas et al., 1987). Subunit interactions may play a critical role in the structure and/or mechanism of the carrier. At the mechanistic level, they might be essential for substrate binding.

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; Pyr, pyruvate; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; EII^{Mtl}, mannitol-specific enzyme II; DTT, dithiothreitol; Tris, tris(hydroxymethyl)-aminomethane; NaP_i, sodium phosphate; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); decyl-PEG, decylpoly(ethylene glycol) 300; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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Table I: Amino Acid Composition of Purified EII^{Mtl} [a]

	amino acid analysis	gene sequence		amino acid analysis	gene sequence
Asx	50	50	Pro	nd	26
Glx	55	49	Gly	62	67
Arg	28	23	Phe		29
Lys	33	31	Trp	nd	4
His	11	13	Ala	65	64
Ser	34	40	Val	46	50
Thr	29	30	Ile	44	54
Tyr	10	11	Leu	68	67
Met	25	25	Cys	nd	4

[a] nd, not determined. A phenylalanine value is not given because hydrolysis products of decyl-PEG interfered with the phenylalanine peak. The amino acid ratio was based on a value of 50 nmol of Asx/nmol of EII^{Mtl} (gene sequence). The gene sequence ratio was derived from Lee and Saier (1983).

This paper examines the number of phosphoryl groups and mannitol binding sites on EII and the effect of oxidation and NEM labeling on these binding sites. The reduced enzyme appears to contain two phosphorylation sites per monomer. Oxidation of EII with diamide reduces the number of sites to one per monomer. Labeling of Cys-384 with *N*-ethylmaleimide also results in halving of the number of sites. Both phosphoryl groups on EII appear to be transferred to mannitol; thus, both sites are part of the catalytic mechanism. In the second part of the paper we show that only one high-affinity mannitol binding site (K_D = 0.1 μM) is present per EII dimer. Oxidation or NEM labeling does not affect the number of mannitol binding sites. A second binding site with a higher dissociation constant can also be observed.

Materials and Methods

N-Ethylmaleimide was from Jansen. Diamide was from Aldrich. ^{14}C-labeled phosphoenolpyruvate and mannitol were from Amersham. Centricon-30 microconcentrators were from Amicon and had a cutoff of 30 kDa.

EI and HPr. EI and HPr were purified as described previously (Dooijewaard et al., 1979; Robillard et al., 1979). The concentrations were determined by the pyruvate burst method (Robillard & Blaauw, 1987).

Purification of EII^{Mtl}. EII^{Mtl} was purified by use of the nonionic detergent decyl-PEG as described by Robillard and Blaauw (1987). The purity of the preparation was confirmed by SDS–PAGE. The concentration of the preparation was determined (in sixfold) by quantitative amino acid analysis after lyophilization and hydrolysis at 110 °C in 6 N HCl (Table I). The concentration was computed to be 3.8–4.1 μM on the basis of the amino acid composition derived from the gene sequence (Lee & Saier, 1983).

Determination of Phosphoryl Binding Sites by the Pyruvate Burst Method. Assay conditions were as stated in the figure legends. The [^{14}C]Pyr formed was separated from [^{14}C]PEP exactly as described by Robillard and Blaauw (1987). To compare the number of phosphorylation sites on the oxidized versus reduced enzyme, EII was incubated for 5 min with diamide or DTT, respectively, in the reaction mixture before [^{14}C]PEP was added. The final concentrations of diamide and DTT in the assays were 5 mM. NEM-labeled EII was prepared as described by Pas and Robillard (1988). EII labeled this way was inactivated to 3% of its initial activity. The pyruvate burst determination of the NEM-labeled enzyme was performed under the same conditions as used for the reduced unlabeled enzyme. Pyruvate burst measurements have been done with a variety of experimental conditions including different detergent, HPr, and NaCl concentrations. Therefore, control experiments were performed with decyl-PEG concentrations between 0.05 and 0.4%, with 15 and 100 mM NaCl, and with 3 times the amount of HPr stated in the figure legends. No alteration in the number of phosphoryl group binding sites was observed.

PEP-Dependent Mannitol Phosphorylation Activity. PEP-dependent mannitol phosphorylation activity was measured as described by Robillard and Blaauw (1987).

Mannitol Binding Studies. EII^{Mtl} (75 μL) was added to a total volume of 1 mL of buffer containing 20 mM Tris-HCl, pH 7.6, 5 mM DTT, 1 mM NaN_3, and 0.1% decyl-PEG. [^{14}C]Mannitol (specific activity 55 Ci/mol) was present at concentrations ranging from 0.05 to 5 μM. The mixture was incubated for 20 min at room temperature and was then centrifuged in a Centricon microconcentrator for 45 min at 5000*g* (room temperature). EII was concentrated in this way to a final volume of ~50 μL. The filtrate containing free substrate was collected. The exact volume of the concentrated enzyme fraction was determined by weighing. The amount of mannitol was then determined by counting, in triplicate, 15-μL samples of EII and the filtrate. The smallest ratio between total mannitol (bound plus free) and free mannitol in these experiments was 1.75. In the binding studies with 0.05 and 0.1 μM mannitol, 300 μL of filtrate was counted instead of 15 μL, so that no collected sample contained less than 1000 cpm. Binding to oxidized enzyme was measured in the presence of 5 mM diamide. Binding to NEM-labeled EII was measured with the same procedure used to measure binding to the reduced enzyme.

Controls of EII Recovery during Binding Studies. Control runs showed that all EII, as determined by PEP-dependent mannitol phosphorylation and [^{14}C]pyruvate burst measurements, was recovered in the retentate. No inactivation or loss of EII was observed due to binding to the membrane of the Centricon concentrators.

Specificity of Mannitol Binding. Controls without enzyme showed that no label concentrated in the retentate. Addition of 100 μM cold mannitol in a 1μM [^{14}C]mannitol binding experiment abolished [^{14}C]mannitol binding, whereas addition of 100 μM methyl α-glucopyranoside did not decrease the binding of labeled mannitol; both experiments show that the binding is specific for mannitol.

Results

Number of Phosphorylation Sites on Enzyme II. The number of phosphorylation sites on EII^{Mtl} was determined by quantitative phosphorylation of purified EII from [^{14}C]PEP. The conversion of [^{14}C]PEP to [^{14}C]Pyr was measured in a assay volume of 210 μL containing 0.9 μM EI, 2.9 μM HPr, and 1.7 μM EII. The concentration of EII was determined by quantitative amino acid analysis (see Materials and Methods). In Figure 1 the stoichiometry of phosphorylation was measured both under reduced conditions in the presence of 5 mM DTT (□, ■) and under oxidized conditions in the presence of 5 mM diamide (○, ●). EII (1.7 μM) in the reduced form incorporated in this experiment 3.2 μM phosphoryl groups, which equals 1.9 phosphoryl groups per monomer. Similar experiments always resulted in values between 1.7 and 2.0 phosphoryl groups per mole of EII. Enzyme II concentration-dependent measurements as well as control experiments in the presence of increased concentrations of HPr, decyl-Peg, or NaCl did not influence this stoichiometry. These experiments show that the active form of the enzyme (i.e., reduced) contains two phosphorylation sites per EII^{Mtl} monomer. Oxidation renders EII completely inactive, both in the PEP-dependent reaction and in the mannitol/mannitol-P ex-

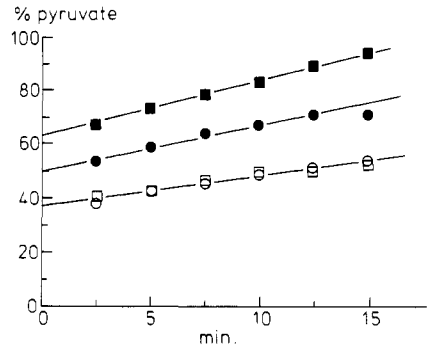

FIGURE 1: Determination of number of phosphorylation sites on reduced and oxidized EII^{Mtl}. The 210-μL assay volume contained 50 mM NaP_i, pH 7.0, 5 mM $MgCl_2$, 2.5 mM NaF, 0.15% decyl-PEG, 0.9 μM EI, 2.9 μM HPr, no EII (open symbols) or 1.7 μM EII (closed symbols), and either 5 mM DTT (□, ■) or 5 mM diamide (○, ●). The reaction was started after 5-min preincubation at 30 °C by adding 11.9 μM [^{14}C]PEP. The incubation was continued at 30 °C, and 30-μL samples were taken at the indicated times and processed as indicated under Materials and Methods. The background of the [^{14}C]Pyr/[^{14}C]PEP column separation was 3% pyruvate.

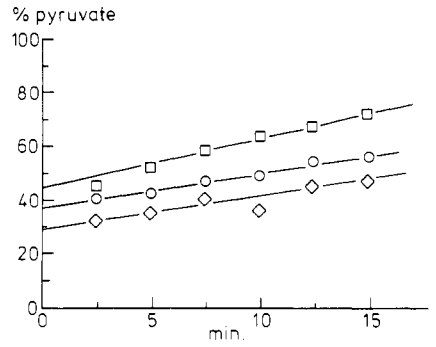

FIGURE 2: Determination of number of phosphorylation sites after reaction of cysteine 384 with NEM. The 200-μL assay mixture contained 50 mM NaP_i, pH 7.0, 5 mM $MgCl_2$, 2.5 mM NaF, 2.5 mM DTT, 0.18% decyl-PEG, 0.9 μM EI, 3.0 μM HPr, and no EII (◇), 1.3 μM NEM-labeled EII (○), or 1.3 μM unlabeled EII (□). After 5-min preincubation at 30 °C, the reaction was started by adding 15.6 μM [^{14}C]PEP. The incubation was continued at 30 °C, and 30-μL samples were taken at the indicated times and processed as indicated under Materials and Methods. The background of the [^{14}C]Pyr/[^{14}C]PEP column separation was 3% pyruvate.

change reaction. The effect of oxidation on phosphoryl group incorporation is also shown in figure 1. As can be seen, oxidation does not affect the phosphorylation of EI and HPr (○); thus, the lower pyruvate burst with oxidized enzyme (●) versus the reduced form (■) must be attributed solely to the effect of oxidation on EII. The number of phosphoryl groups incorporated by the oxidized enzyme was 1.0 per monomer, half of that incorporated by the reduced enzyme. Apparently, oxidation prevents phosphorylation of the second site on EII^{Mtl}.

Number of Phosphorylation Sites on Enzyme II after NEM Treatment. Treatment of the reduced enzyme with the sulfhydryl reagent *N*-ethylmaleimide (NEM) results in labeling of Cys-384 (Pas & Robillard, 1988a) and inhibition of both the phosphorylation and exchange activity. Figure 2 shows the effect of NEM on phosphoryl group stoichiometry. The NEM-labeled enzyme (○) incorporated 0.9 phosphoryl groups per monomer while the control enzyme incorporated 1.7 (□), indicating an incorporation of only one phosphoryl group per monomer after alkylation of the activity-linked cysteine.

Both Phosphoryl Groups on Enzyme II Are Transferred to Mannitol. When PEP-dependent phosphorylation of mannitol was measured under limiting PEP concentrations and a catalytic concentration of EII, almost all (96%) phosphoryl

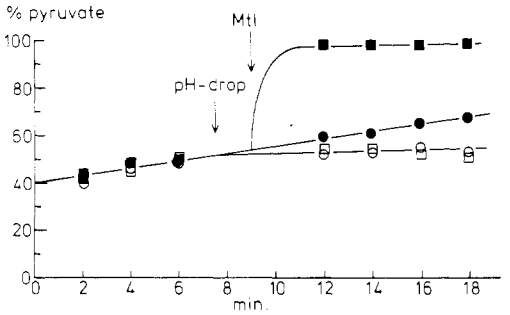

FIGURE 3: Inhibition of the PTS reaction by a pH drop. The assay mixture (210 μL) contained 25 mM NaP_i, pH 7.0, 5 mM $MgCl_2$, 5 mM DTT, 0.1% decyl-PEG, 1.1 μM EI, 3.6 μM HPr, and 1.3 μM EII. At t = 0, 40 μL of 125 μM [^{14}C]PEP was added to give a final PEP concentration of 20 μM. At t = 2, 4, and 6 min, 30-μL samples were taken. At t = 7.5 min, the pH was either dropped to 5 (○, □) by adding 53 μL of 250 mM NaAc, pH 4.5, or kept constant (●, ■) by adding H_2O. At t = 9 min, 24 μL of either 100 μM mannitol (□, ■) or H_2O (○, ●) was added to the reaction mixture. The incubation was prolonged, and 44.5-μL samples were taken at 12, 14, 16, and 18 min. The temperature during the assay was 30 °C. The background of the [^{14}C]Pyr/[^{14}C]PEP column separation (see Materials and Methods) was 3% pyruvate.

groups of PEP were recovered as mannitol phosphate. However, due to the low EII:PEP ratio in these experiments it is not possible to determine whether both phosphoryl groups on EII are transferred to mannitol or whether one phosphoryl group remains on EII, for instance, on a hypothetical effector site. Such a distinction requires that comparable amounts of P-HPr and EII are used and that all phosphoryl groups are shown to be passed on to mannitol. The experiments in Figures 3 and 4 are designed to address this issue.

Figure 3 shows that phosphoryl group transfer from PEP can be inhibited by dropping the pH to 5. A mixture of EI, HPr, and EII were phosphorylated from [^{14}C]PEP, resulting in a burst of [^{14}C]Pyr. The slow increase in Pyr is due to hydrolysis of the phosphorylated enzymes and subsequent rephosphorylation. A drop in the pH of the reaction mixture from pH 7 to pH 5 completely abolished further conversion of PEP to Pyr (○), while the control, to which H_2O was added instead of NaAc, still hydrolyzed PEP with the same rate (●). All PEP could quickly be converted to Pyr by adding excess mannitol to the pH 7 reaction mixture (■), while addition of mannitol at pH 5 produced no further increase in Pyr (□). The pH drop procedure allows us to prepare P-HPr in situ and, after dropping pH, measure the phosphoryl transfer to mannitol. The rephosphorylation of HPr from PEP is prevented by the low pH.

Figure 4A shows the formation of Mtl-1-P after the PEP consumption was stopped. EI and increasing amounts of HPr were preincubated with nonradioactive PEP in the presence of [^{14}C]mannitol. No mannitol phosphate could be formed since EII was absent. After preincubation, the pH was dropped by adding NaAc, and after 2 min, 0.8 μM EII was added and samples were withdrawn at the indicated times. Addition of EII resulted in a burst of mannitol phosphate, followed by a slight increase and a leveling off of Mtl-P production. The mannitol phosphate produced can only originate from HPr and EI, since EII was added after the pH drop that abolished PEP consumption (see Figure 3). The concentration of mannitol phosphate formed at the end of the incubation period is plotted, in Figure 4B, versus the concentration of HPr. Extrapolation of the straight line in Figure 4B gives a value of 0.6 μM Mtl-P formed at zero HPr concentration. Two conclusions can be drawn from this experiment. First, the pH drop stops the PTS reaction at the level

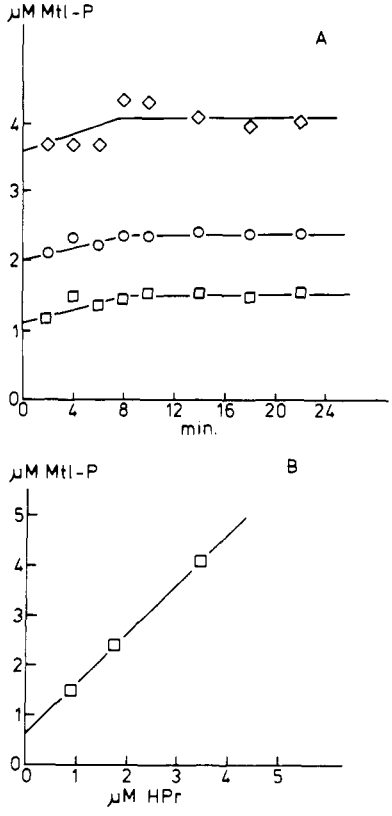

FIGURE 4: (A) HPr concentration-dependent phosphorylation of mannitol after pH drop. The assay mixture contained 30 mM NaP_i, pH 7.0, 6 mM $MgCl_2$, 6 mM DTT, 3 mM NaF, 1.1 μM EI, 26 μM PEP, 15.2 μM [^{14}C]Mtl and 1.5 (□), 3.0 (○), or 6.0 μM (◇) HPr in a volume of 164.5 μL. After 5-min preincubation at 30 °C the pH was dropped to 5 by addition of 71.5 μL of 250 mM NaAc, pH 4.5. Two minutes later EII was added; the incubation was continued at 30 °C, and samples were taken at the indicated times. The final concentrations of the enzymes were 0.6 μM EI, 0.8 μM EII, and 0.8 (□), 1.7 (○), or 3.4 μM (◇) HPr. The final decyl-PEG concentration was 0.07%. All concentrations of enzymes were previously determined by pyruvate burst measurements. (B) HPr concentration plotted versus the concentration of Mtl-P formed. The amount of Mtl-P formed in 22 min in A was plotted versus the final HPr concentration.

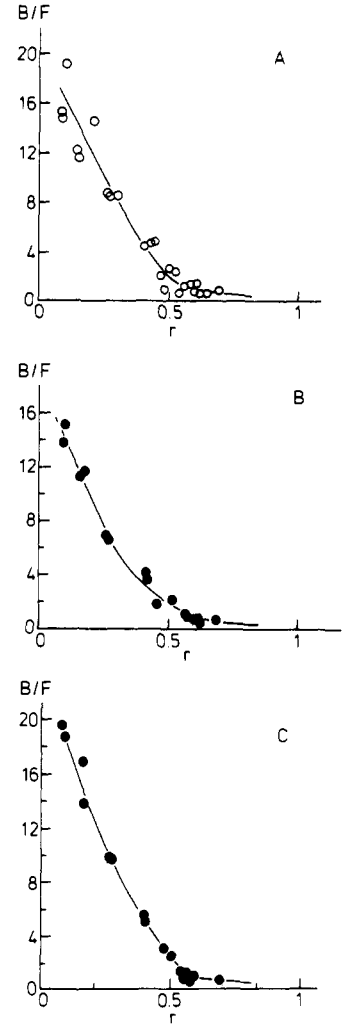

FIGURE 5: Scatchard plots of the number of mannitol binding sites. (A) reduced enzyme: 0.3 μM EII^{Mtl} in 20 mM Tris-HCl, pH 7.6, 5 mM DTT, and 1 mM NaN_3 was incubated with [^{14}C]Mtl concentrations from 0.05 to 5 μM at room temperature in a volume of 1 mL. After 15 min, free substrate was separated from the enzyme by centrifugation in Centricon microconcentrators. The 50-μL retentate, containing enzyme-bound mannitol plus free mannitol, and the filtrate, containing only free mannitol, were counted as described under Materials and Methods. (B) oxidized enzyme: the experimental conditions were identical with those in A except that in the incubation buffer DTT was replaced by the same concentration diamide. (C) NEM-labeled enzyme: the conditions are identical with those in A. The data are plotted as B/F versus r, where r equals the moles of substrate bound per mole of enzyme.

of phosphorylation of EI (The value of the y-axis intercept is 0.6. This is the same as the amount of EI in the mixture). The phosphorylation of EII from P-HPr still occurs, although nothing can be said about the rates of phosphorylation of EII or mannitol at this pH. Second, if one phosphoryl group were to remain on EII, then all points would be 0.8 μM lower. The y-axis intercept would be at −0.2 μM, which is not the case. Therefore, *all* the label present on the phosphorylated proteins after the pH drop, i.e. on EI and HPr, is transferred to mannitol phosphate; no label remains on EII.

Mannitol Binding Sites on Enzyme II. Equilibrium binding studies were performed with microconcentrators as described under Materials and Methods. The binding of mannitol was measured at room temperature in 20 mM Tris-HCl, pH 7.6, containing 5 mM DTT, 1 mM NaN_3, and 0.1 % decyl-PEG. Enzyme II (75 μL) was incubated in a total volume of 1 mL for 15 min with concentrations of [^{14}C]mannitol ranging from 0.05 to 5 μM. The enzyme was then separated from buffer by centrifugation, and the amount of mannitol bound was determined. Control experiments in the presence of 100 μM unlabeled mannitol or 100 μM methyl α-glucopyranoside showed the binding to be absolutely specific. As stated in the methods, the occurrence of nonspecific binding of EII^{Mtl} to the microconcentrators was controlled by measuring recovery of both mannitol phosphorylation activity and [^{14}C]pyruvate burst measurements. No loss of EII or phosphorylation activity was observed under the conditions of the binding experiment. Mannitol binding to active EII^{Mtl} is reported in the Scatchard plot in Figure 5A. The binding of mannitol to oxidized enzyme was measured by repeating the above experiments in the presence of 5 mM diamide instead of 5 mM DTT (Figure 5B). Binding to enzyme alkylated with NEM at cysteine 384 is shown in Figure 5C. The curves are in all cases, biphasic. A total of seven binding experiments, three on the native enzyme, and two each on oxidized and alkylated enzyme were fitted by the method of least squares. The data did not fit a single site per EII^{Mtl} dimer but rather two sites per dimer. There is a high-affinity site with a K_D of 0.1 ± 0.02 μM and a lower affinity site with a K_D of 9 ± 5 μM.

DISCUSSION

Phosphorylation Sites. The present results show that, under reduced conditions, two phosphorylation sites occur on every

EII^{Mtl} monomer and both phosphoryl groups can be transferred to mannitol. Thus, both sites participate in the catalytic mechanism. Saier et al. (1985) proposed, on the basis of similar sizes of various EII's and EIII–EII pairs and on the basis of amino acid sequence homology, that EII–EIII pairs and EII's that function without a separate EIII originate from a common ancestor. Thus, there should be two phosphorylation sites on the EII's that lack a separate EIII partner. If indeed the catalytic mechanism of the mannitol-specific EII is similar to, for instance, that of the glucose-specific EIII–EII pair, then a phosphoryl transfer should occur within the mannitol-specific EII. If we number the sites P_1 and P_2, then HPr is assumed to phosphorylate P_1 and P_1 phosphorylates P_2. Oxidized EII is completely inactive (Roossien & Robillard, 1984a; Grenier et al., 1985). Our results show that, under these conditions, only one site is phosphorylated from HPr (P_1). Oxidation or alkylation of Cys-384 blocks the subsequent step, indicating that a reduced unmodified Cys-384 is a prerequisite for a step leading to the phosphorylation of P_2. This second site P_2 is probably involved in phosphorylation of mannitol since, when phosphorylation of this site is blocked, no phosphorylation of mannitol is observed. The results obtained by Sutrina et al. (1987) for EII^{Mtl}-catalyzed exchange between HPr-P and HPr fit in very well with our data. They showed that NEM labeling of EII did not abolish the phosphoryl exchange between HPr and HPr-P. Clearly P_1, still being phosphorylated, was responsible for this exchange. Roossien and Robillard (1984a) showed that phosphorylated EII was protected against NEM labeling and oxidation. This study shows that the doubly phosphorylated enzyme is the protected species. Since the NEM-labeled enzyme can still be phosphorylated on P_1, the simplest conclusion would be that an alkylated or oxidized Cys-384 and phosphorylation at P_2 are incompatible. It is, however, possible that phosphorylation of P_1 protects Cys-384 indirectly, by a conformational change. Conformational changes resulting from P_1 phosphorylation have been detected by fluorescence quenching (Gage and Robillard, unpublished data).

Roossien and Robillard (1984a) studied the stoichiometry of NEM labeling and the effects of NEM and oxidation on phosphorylation of EII. Effects similar to those reported above were observed, but they were quantitatively different. Fe^{3+} or NEM inactivation led to only a 30% reduction in the level of phosphorylation in place of the 50% reduction reported here. This can be attributed to a low pyruvate burst value for the active enzyme due to a too low concentration of HPr (0.24 μM versus 2.9 μM) and to a partly oxidized enzyme preparation. Storage in 1 mM DTT can lead to a partly oxidized enzyme by oxidation of DTT. We observed that stored EII often is partly protected to NEM. Complete inactivation can be achieved in such cases only by preincubation with a freshly made solution of 5 mM DTT.

Mannitol Binding Sites. Previous kinetic experiments have shown that mannitol binds to unphosphorylated EII; it inhibits the mannitol/mannitol phosphate exchange reaction (Jacobson et al., 1983; Roossien et al., 1984), apparently by binding at the mannitol phosphate binding site and inhibiting mannitol phosphate binding. Since, in the exchange reaction, EII is not phosphorylated in the steady state, this binding site is present on the unphosphorylated enzyme.

We measure one high-affinity site per dimer. Its K_D value of 0.1 μM is lower then the kinetically measured K_M's for the PEP-dependent phosphorylation and the exchange reaction (0.5–11 μM; Jacobson et al., 1983; Roossien et al., 1984; Grenier et al., 1986); it is also lower than the K_I value of 1.5 μM for mannitol inhibition of the exchange reaction (Roossien et al., 1984). A second site with lower affinity has been measured. Whether it is a distinct site or the same site with a higher K_D (negative cooperativity) cannot be specified.

The occurrence of only one high-affinity site per two EII^{Mtl} molecules implies that, under these conditions, the enzyme is at least present as a dimer. Kinetic evidence for both the PEP-dependent phosphorylation reaction and the mannitol-exchange reaction indicated a concentration-dependent association of EII (Saier, 1980; Leonard & Saier, 1983; Robillard & Blaauw, 1987; Roossien et al., 1984). The final concentration of EII used in our studies far exceeds the concentration range where monomerization occurs. The specific activity of the labeled mannitol prohibited us from measuring at lower enzyme concentration to determine whether the binding stoichiometries or affinities would be different with the monomeric enzyme. Stephan and Jacobson (1986b) extracted radiolabeled unphosphorylated EII from minicells and found both monomeric and dimeric enzymes on SDS–PAGE. Mannitol increased the ratio of monomer to dimer leading them to propose that mannitol binding led to monomerization. However, their experiments used 40 mM mannitol, while our studies were performed with 0.05–5 μM mannitol. If concentrations lower than 40 mM were used during minicell extraction, the monomerization effect decreased (G. R. Jacobson, personal communication).

Binding of mannitol to oxidized or NEM-labeled enzyme or active enzyme resulted in the same binding curves. This supports the evidence that cysteine 384 is only important in the phosphorylation of P_2 from P_1 and not in substrate binding. Studies on the localization of the two phosphorylation sites and their relationship with cysteine 384 are in progress.

ACKNOWLEDGMENTS

We thank P. Jehel for performing the amino acid analyses.

ADDED IN PROOF

P_2 has been identified as *S*-phosphocysteine-384 (Pas & Robillard, 1988b).

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Tyrosyl-tRNA Synthetase Acts as an Asymmetric Dimer in Charging tRNA. A Rationale for Half-of-the-Sites Activity†

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ABSTRACT: Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is a classical example of an enzyme with half-of-the-sites activity. The enzyme crystallizes as a symmetrical dimer that is composed of identical subunits, each having a complete active site. In solution, however, tyrosyl-tRNA synthetase binds tightly, and activates rapidly, only 1 mol of Tyr/mol of dimer. It has recently been shown that the half-of-the-sites activity results from an inherent asymmetry of the enzyme. Only one subunit catalyzes formation of Tyr-AMP, and interchange of activity between subunits is not detectable over a long time scale. Paradoxically, however, the kinetics of tRNA charging are biphasic with respect to [Tyr], suggesting that both subunits of the dimer are catalytically active. This paradox has now been resolved by kinetic analysis of heterodimeric enzymes containing different mutations in each subunit. Biphasic kinetics with unchanged values of K_M for Tyr are maintained when one of the two tRNA-binding domains is removed and also when the affinity of the "inactive" site for Tyr is reduced by 2–58-fold. The biphasic kinetics do not result from catalysis at both active sites, but instead appear to result from two molecules of Tyr binding sequentially to the same site. A second molecule of Tyr perhaps aids the dissociation of Tyr-tRNA by displacing the tyrosyl moiety from its binding site. A monomer of the enzyme is probably too small to allow both recognition and aminoacylation of a tRNA molecule. This could explain the requirement for the enzyme to function as an asymmetric dimer.

Tyrosyl-tRNA synthetase (YTS/YTS)[1] from *Bacillus stearothermophilus* catalyzes aminoacylation of tRNA as a two-step reaction (eq 1 and 2). The enzyme comprises two

$$E + Tyr + ATP \rightleftarrows E{\cdot}Tyr\text{-}AMP + PP_i \quad (1)$$

$$E{\cdot}Tyr\text{-}AMP + tRNA \rightarrow E + Tyr\text{-}tRNA + AMP \quad (2)$$

subunits of identical composition and crystallizes as a symmetrical dimer (Blow & Brick, 1985). Each monomer has a complete active site, but YTS/YTS exhibits half-of-the-sites activity in that only 1 mol of Tyr is bound tightly, and 1 mol of Tyr-AMP formed rapidly per mol of dimer (Fersht, 1975; Fersht et al., 1975). Paradoxically, the Tyr dependence of tRNA charging kinetics has two phases, implying that 2 mol of Tyr bind during each turnover (Jakes & Fersht, 1975). Further, each enzyme dimer behaves asymmetrically as the same subunit is used for every turnover in the steady state (Ward & Fersht, 1988). The second subunit does not have sufficient catalytic activity in formation of E·Tyr-AMP to produce a second phase in tRNA charging kinetics. This raises two questions. First, does binding of 2 mol of tRNA cause both subunits to act catalytically? Second, if not, does the second mole of Tyr bind to the subunit that forms Tyr-AMP or to the "inactive" subunit?

We now answer these questions using simple and direct experiments that are based upon kinetic analysis of heterodimers that have been engineered by using the known domain structure of the enzyme (Carter et al., 1986; Ward & Fersht, 1988). Each subunit of YTS/YTS comprises two functional domains (Waye et al., 1983). The subunits interact through the N-terminal domains, which catalyze activation of Tyr (eq 1). The C-terminal domains are required for binding of tRNA but are not involved in contacts between the monomers. Deletion of the C-terminal domains produces ΔYTS/ΔYTS

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[1] Abbreviations: (for subunits of tyrosyl-tRNA synthetase) YTS, wild-type; ΔYTS, truncated wild-type [see Waye et al. (1983)]; YTS-(Asn-45), His → Asn-45 mutation; YTS(Ala-173), Gln → Ala-173 mutation; YTS(Gly-195), Gln → Gly-195 mutation.

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