Stereochemical Course of the Reactions Catalyzed by the Bacterial Phosphoenolpyruvate:Mannitol Phosphotransferase System†

Eugene G. Mueller,‡ Sanjay S. Khandekar,§,‖ Jeremy R. Knowles,‡ and Gary R. Jacobson*,§

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and Department of Biology, Boston University, Boston, Massachusetts 02215

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ABSTRACT: We have determined the overall stereochemical course of the reactions leading to the phosphorylation of D-mannitol by mannitol-specific enzyme II (EII^{Mtl}) of the *Escherichia coli* phosphoenolpyruvate- (PEP) dependent phosphotransferase system (PTS). In the presence of enzyme I and HPr of the PTS, and of membranes containing EII^{Mtl}, the phospho group from [(*R*)-^{16}O,^{17}O,^{18}O]PEP was transferred to D-mannitol to form mannitol 1-phosphate with *overall inversion* of the configuration at phosphorus with respect to that of PEP. Since in the course of these reactions enzyme I and HPr are each covalently phosphorylated at a single site and inversion of the chiral phospho group from PEP indicates an odd number of transfer steps overall, transfer from phospho–HPr to mannitol via EII^{Mtl} must also occur in an odd number of steps. Taken together with the fact that catalytically important phospho–EII^{Mtl} intermediates have been demonstrated biochemically, our results imply that EII^{Mtl} is *sequentially* phosphorylated at *two different sites* during phospho transfer from phospho–HPr to mannitol. This conclusion is consistent with the available evidence on phospho–EII^{Mtl} intermediates and in particular with the recent report that two different phospho peptides can be isolated from the fully phosphorylated protein [Pas, H. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5835–5839].

The bacterial phosphoenolpyruvate- (PEP) dependent carbohydrate phosphotransferase system (PTS) catalyzes the concomitant transmembrane transport and phosphorylation of its carbohydrate substrates by way of two or three soluble proteins and a sugar-specific transmembrane protein, an enzyme II (EII) of the system, as follows [for reviews, see Postma and Lengeler (1985), Saier (1985), and Robillard and Lolkema (1988)]:

PEP + enzyme I ⇌
phospho∼(*N*-3-histidyl)–enzyme I + pyruvate

phospho–enzyme I + HPr ⇌
phospho∼(*N*-1-histidyl)–HPr + enzyme I

phospho–HPr + enzyme III ⇌
phospho∼(*N*-3-histidyl)–enzyme III + HPr

phospho–enzyme III + $sugar_{(out)}$ $\xrightarrow{\text{enzyme II}}$
enzyme III + $sugar\text{–}P_{(in)}$

Enzyme I and HPr are nonspecific, soluble proteins of the PTS, and each is phosphorylated during phospho transfer at a single histidyl residue as indicated above. For some PTS substrates (e.g., glucose) a third soluble protein, enzyme III, is required which is also phosphorylated at a single histidyl residue. In contrast, for other substrates (e.g., mannitol) the function of the enzyme III is apparently incorporated into a hydrophilic domain of the membrane-bound enzyme II (Ebner & Lengeler, 1988; Saier et al., 1988).

In 1982, we showed that the glucose PTS catalyzes phospho transfer from PEP, chiral at phosphorus, to methyl α-D-glucopyranoside with overall inversion of the configuration with respect to that of PEP (Begley et al., 1982). Since the glucose PTS requires a separate enzyme III and inversion of the configuration of the chiral phospho group implied an odd number of phospho-transfer steps overall (Knowles, 1980, 1982), we concluded that a covalent phospho–EII^{Glc} was an intermediate during phospho transfer from phospho–enzyme III to the sugar (Begley et al., 1982). Subsequently, Erni (1986) demonstrated covalent phosphorylation of purified EII^{Glc}, probably at a single site on the protein, and showed that the phospho group could indeed be transferred from the enzyme to glucose.

Among the PTS EII's that do not require a separate enzyme III for their function are EII^{Mtl} and the EII's specific for *N*-acetylglucosamine and β-glucosides, all from *Escherichia coli*, as well as the sucrose-specific enzyme II from *Streptococcus mutans*. The amino acid sequences of all of these proteins have been deduced from the nucleotide sequences of their genes (Lee & Saier, 1983b; Peri & Waygood, 1988; Rodgers et al., 1988; Bramley & Kornberg, 1987; Schnetz et al., 1987; Sato et al., 1989). In the cases of the EII's specific for *N*-acetylglucosamine, β-glucosides, and sucrose, the hydrophobic domains exhibit significant amino acid sequence similarity to EII^{Glc}, and the hydrophilic C-terminal domains of these proteins are very similar to the glucose-specific enzyme III including a region around the known phosphorylated histidine of the latter (Saier et al., 1988; Ebner & Lengeler, 1988; Sato et al., 1989). For EII^{Mtl}, significant amino acid sequence similarities between residues 500–637 of this protein and the separate, soluble, enzymes III specific for mannitol from *Staphylococcus carnosus* and for fructose from *Salmonella typhimurium* have also been observed (Reiche et al., 1988; Geerse et al., 1989). These similarities include a region around His-554 of EII^{Mtl}, a residue that is believed to be phosphorylated in this protein (Pas & Robillard, 1988). It therefore appears that those EII's that do not require an enzyme III contain an enzyme III-like domain at their C-termini.

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‡ Department of Chemistry, Harvard University.
§ Department of Biology, Boston University.
‖ Present address: Procept, Inc., Cambridge, MA 02139.

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Given the above information, it would seem likely that the overall phospho-transfer mechanisms of the independent EII's and of the EII/enzyme III pairs would be similar. In particular, it would be predicted that an enzyme III independent EII such as EII^{Mtl} would be phosphorylated at two different sites in sequence by phospho–HPr: the enzyme III-like site and the site analogous to the phospho-acceptor site in EII^{Glc}. Although the current biochemical evidence for EII^{Mtl} favors the existence of two phosphorylation sites on this protein (Pas & Robillard, 1988), there is as yet no proof that both of these sites are bona fide sequential catalytic intermediates during phospho transfer to mannitol. For example, one or the other site could be artifactually phosphorylated, or one of the sites could constitute a regulatory locus. Moreover, biochemical experiments, and isolation of phospho peptides, may not detect phospho–enzyme intermediates that are very short-lived. In view of these considerations and to test the hypothesis that EII^{Mtl} contains two different phosphorylated sites that function sequentially, we have determined the overall stereochemical course of the reactions catalyzed by the mannitol PTS using chiral PEP as the initial phospho donor and employing methods analogous to those that we used previously for the glucose PTS (Begley et al., 1982).

Materials and Methods

Materials. D-[^{14}C]Mannitol (52 mCi/mmol) was purchased from Dupont/New England Nuclear Co. (Boston, MA). Unlabeled D-mannitol, PEP (tricyclohexylammonium salt), ATP (sodium salt), AMP (sodium salt), NAD^+ (grade III-C from yeast), mannitol 1-phosphate (barium salt), glucose 6-phosphate (sodium salt), pyruvate (sodium salt), phenylmethanesulfonyl fluoride, ethylenediaminetetraacetate, tris(hydroxymethyl)aminomethane (Tris), and yeast enolase were products of Sigma Chemical Co. (St. Louis, MO). $NADP^+$ (disodium salt), pyruvate kinase (rabbit muscle), phosphofructokinase (rabbit muscle), and phosphoglucose isomerase (yeast) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Mannitol-1-phosphate dehydrogenase was isolated from *E. coli* K12 strain C600 harboring plasmid pCD7.5 (see below) following the procedure of Novotny et al. (1984). 2-[(*R*)-^{16}O,^{17}O,^{18}O]Phospho-D-glycerate was prepared by the method of Blättler and Knowles (1980). The isotopic composition of the water used in the synthesis was as follows: $H_2{}^{17}O$, 11.6% ^{16}O, 59.9% ^{17}O, and 28.5% ^{18}O; $H_2{}^{18}O$, 1.9% ^{16}O, 1.8% ^{17}O, and 96.3% ^{18}O. All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Bacterial Strains, Plasmids, Cell Growth, and Preparation of Cell-Free Fractions. The bacterial strains and plasmids used in this study are listed, along with their genotypes, in Table I. *E. coli* K12, strain LGS322, harboring plasmid pGJ9 was used as the source of membranes containing EII^{Mtl}. Plasmid pGJ9 contains the *mtlA* gene encoding EII^{Mtl}, while strain LGS322 has chromosomal deletions in *mtlA* and the glucitol (Gut) operon to remove the only others PTS EII (EII^{Gut}) that can phosphorylate mannitol (Lengeler, 1975; Grisafi et al., 1989). *S. typhimurium* strain LJ144, containing the PTS operon on an *E. coli* episome, was used as the source of enzyme I and HPr. *E. coli* K12, strain C600, harboring plasmid pCD7.5, which encodes the mannitol operon and overproduces mannitol-1-phosphate dehydrogenase approximately 10-fold (Novotny et al., 1984), was used to purify this enzyme (see above).

E. coli strain LGS322/pGJ9 was grown on medium 63 (Saier et al., 1976) containing chloramphenicol (40 μg/mL), 0.5% mannitol as the carbon source, and 50 μg/mL each of thiamin, histidine, arginine, and methionine to satisfy auxotrophic requirements (see Table I). Otherwise, cell growth conditions and the preparation of membranes from this strain were as previously described for strain ML308 (Begley et al., 1982). *S. typhimurium*, strain LJ144, was grown, and a cytoplasmic fraction was prepared, also as previously described (Begley et al., 1982). *E. coli* strain C600/pCD7.5 was grown, and cell-free fractions were prepared, as described by Novotny et al. (1984).

Spectroscopy and Chromatography. ^{1}H NMR spectra were recorded on either a Bruker AM 250 or a Bruker AM 300 instrument. ^{31}P NMR spectra were recorded on a Bruker WM 300 instrument; chemical shifts are referenced to external 85% phosphoric acid (downfield is positive). Ion-exchange chromatography was performed with AG1-X8 (200–400 mesh) and AG501-X8 (20–40 mesh) from Bio-Rad Laboratories (Richmond, CA), Dowex 50 (100–200 mesh, 8% cross-linked) from Sigma Chemical Co., and DE-52 (microgranular) from Whatman Paper Co. (Maidstone, England).

Assays. Assays of mannitol 1-phosphate, glucose 6-phosphate, and 2-phosphoglycerate were done according to Bergmeyer (1974).

Mannitol PTS Reaction Conditions. 2-[(*R*)-^{16}O,^{17}O,^{18}O]Phosphoglycerate was converted to chiral PEP of the same configuration by inclusion of enolase in the PTS reaction mixture. This mixture (160 mL) consisted of 25 mM Tris-HCl buffer, pH 8.0, containing $MgCl_2$ (5 mM), KF (10 mM), dithiothreitol (1 mM), 2-[(*R*)-^{16}O,^{17}O,^{18}O]phosphoglycerate (1 mM), enolase (1000 units), D-mannitol (1 mM), *S. typhimurium* strain LJ144 cytoplasm (17 mL), and *E. coli* strain LGS322/pGJ9 membrane suspension (80 mL). To monitor the time course of the reaction, a reaction mixture was prepared of identical composition but containing, in addition, D-[^{14}C]mannitol (final specific radioactivity = 0.25 mCi/mmol). Each of these reaction mixtures was incubated with shaking at 37 °C. After 90 min, 65% of the 2-phosphoglycerate and mannitol substrates had been converted into mannitol 1-phosphate and pyruvate as judged by analysis of the radioactive reaction mixture by the DEAE filter disc technique (Jacobson et al., 1979). At this time, the reaction mixture containing unlabeled mannitol was rapidly cooled in an ice bath and then centrifuged at 100000*g* for 120 min at 4 °C. Over 65% of the mannitol 1-phosphate product remained in the supernatant as judged by analysis of the mannitol 1-phosphate concentration using a spectrophotometric assay employing mannitol-1-phosphate dehydrogenase (total yield = 105 μmol of mannitol 1-phosphate in 160 mL). This supernatant was stored at −70 °C.

Purification and Analysis of Mannitol 1-[^{16}O,^{17}O,^{18}O]-Phosphate. The supernatant from the 160-mL reaction mixture was chromatographed on a column (7 mL) of AG1-X8 (HCO_3^- form) that had been equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 7.6, elution being with a linear gradient (800 mL + 800 mL, 10–150 mM) of the same buffer. Fractions containing mannitol 1-phosphate were combined, and buffer salt was removed by repeated evaporation of added water under reduced pressure. Analysis of the resulting sample by ^{31}P NMR (100 mM Tris-HCl buffer, pH 9.0; D_2O lock) revealed the presence of mannitol 1-phosphate, inorganic phosphate, glucose 6-phosphate, and an unknown phospho species. The sample was therefore chromatographed over a column (35 mL) of DE-52 (HCO_3^- form) that had been equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 7.9, elution being with a linear gradient (1 L + 1 L, 10–75 mM) of the same buffer. To remove the contaminating glucose 6-phosphate, the sample

(containing 49 μmol of mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate) was incubated with pyruvate kinase (3 units), phosphoglucose isomerase (3.5 units), and phosphofructokinase (3 units) in 94 mM Tris-HCl buffer, pH 9.0 (3.2 mL), that contained KCl (4.6 mM), $MgSO_4$ (1.4 mM), ATP (2.8 mM), PEP (17 mM), and AMP (6.9 mM). After 40 min at room temperature, ethylenediaminetetraacetate was added to quench the reaction. ^{31}P NMR analysis of the reaction mixture showed the complete conversion of glucose 6-phosphate to fructose 1,6-bisphosphate. The solution was then passed over a short column of Dowex 50 (H^+ form) to remove protein; the eluate was adjusted to pH 7.6; and this solution was subjected to chromatography on a column (4 mL) of AG1-X8 (HCO_3^- form) that had been equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 7.8, elution being with a linear gradient (1 L + 1 L, 10–75 mM) of the same buffer. Fractions containing mannitol 1-phosphate were combined, and buffer ions were removed by repeated evaporation of added water and 2-propanol under reduced pressure. The yield of mannitol 1-phosphate was 48 μmol (98%). Analysis by ^{31}P NMR revealed the presence of the unknown phospho compound (9%) and inorganic phosphate (<1%).

Conversion of mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate to glucose 6-[^{16}O,^{17}O,^{18}O]phosphate for stereochemical analysis was effected enzymatically. Mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate (48 μmol) was dissolved in 100 mM Tris-HCl buffer, pH 9.0 (2.0 mL), containing pyruvate (97 mM) and NAD^+ (0.24 mM). Mannitol-1-phosphate dehydrogenase (19 units) and lactate dehydrogenase (20 units) were then added; after 5 min at room temperature, phosphoglucose isomerase (21 units) was added. After a further 15 min, the enzymes were removed by passage of the solution through a short column of Dowex 50 (H^+ form). The eluate was adjusted to pH 9.3 and then lyophilized. Analysis by ^{31}P NMR revealed the quantitative conversion of mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate to a mixture of glucose 6-[^{16}O,^{17}O,^{18}O]phosphate and fructose 6-[^{16}O,^{17}O,^{18}O]phosphate in a ratio of 7.9:1. This mixture was then incubated with pyruvate kinase (20 units) and phosphofructokinase (30 units) in 75 mM Tris-HCl buffer, pH 9.0 (4.0 mL), which contained KCl (4.6 mM), $MgSO_4$ (1.4 mM), ATP (4 mM), and PEP (21 mM). After 25 min at room temperature, the solution was passed through a short column of Dowex 50 (H^+ form) to remove the enzymes. The eluate was adjusted to pH 9.6, and analysis of this solution by ^{31}P NMR confirmed the complete conversion of fructose 6-phosphate into fructose 1,6-bisphosphate. The mixture was subjected to chromatography on a column (4 mL) of AG1-X8 (HCO_3^- form) that had been equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 8.0, elution being with a linear gradient (1 L + 1 L, 10–75 mM) of the same buffer. Analysis of the isolated glucose 6-[^{16}O,^{17}O,^{18}O]phosphate (28 μmol) by ^{31}P NMR revealed that the product was contaminated with the unknown phospho compound (7%). No inorganic phosphate was detected.

The glucose 6-[^{16}O,^{17}O,^{18}O]phosphate was converted to glucose cyclic 4,6-[^{16}O,^{17}O,^{18}O]phosphate with the method for the cyclization of 1-phospho-(3*S*)-butane-1,3-diol (Friedman et al., 1988). The yield of the cyclic diester was 45% as estimated by ^{31}P NMR analysis. The reaction mixture was subjected to chromatography over a column (4 mL) of AG1-X8 (HCO_3^- form) equilibrated with 10 mM triethylammonium bicarbonate buffer, pH 8.0, and eluted with 10, 15, 20, 25, and 50 mM steps of 100 mL each of the same buffer. The cyclic diester was converted to the potassium salt by passage down a short column of Dowex 50 (K^+ form), and the eluate was evaporated to dryness under reduced pressure. The cyclic ether 18-crown-6 (30 mg) was added, and the mixture was dried by the addition and removal (under reduced pressure at 0 °C) of five portions (2 mL) of dry dioxane. The resulting white residue was dissolved in dry dimethyl sulfoxide (1 mL), and iodomethane (100 μL, after passage over basic alumina) was added. The solution was stirred for 21 h under argon. Dry [$^2H_6$]dimethyl sulfoxide (1 mL) was added; analysis by ^{31}P NMR showed the complete methylation of the cyclic diester. Dry methanol (1 mL) was added to the solution of the cyclic triester, and the resulting solution was passed over a short column of AG501-X8 to remove ions. The eluate from this column was then analyzed by ^{31}P NMR.

Table I: Bacterial Strains and Plasmids Used in This Study

strain or plasmid	genotype	source
E. coli LGS322	F⁻ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rpsL104 lacY1 galT6 gatR49 gatA50* Δ(*mtlA'p*) *mtlD*^{c} Δ(*gutR'-MDBAp – recA*)	Grisafi et al., 1989
E. coli C600	F⁻ *thr-1 leu-6 thi-1 supE44 lacY tonA21*	M. Saier
S. typhimurium LJ144	*cpd-401 cysA1150*/F'198 (pts⁺ on *cysA*⁺)	M. Saier
pGJ9	Cm *mtlAp* on pACYC184	Grisafi et al., 1989
pCD7.5	Tc *mtlCAD*⁺ on pBR322	Lee & Saier, 1983a

Results and Discussion

Several control experiments established that phospho transfer from 2-[(*R*)-^{16}O,^{17}O,^{18}O]phosphoglycerate to D-mannitol takes place by the expected pathway via EII^{Mtl}. No phosphorylation of mannitol was observed if 2-phosphoglycerate (the in situ precursor of PEP), *Salmonella* cytoplasm, or *E. coli* membranes were not present in the reaction mixture. Moreover, membranes from *E. coli* strain LGS322 containing various derivatives of pGJ9 from which parts of the *mtlA* gene have been deleted showed no PEP-dependent mannitol phosphorylation activity (Grisafi et al., 1989), demonstrating that no protein in these membranes other than EII^{Mtl} could carry out this reaction. Indeed, the only other protein in *E. coli* that is known to carry this activity at a low level is the glucitol-specific EII (Lengeler, 1975), which is also absent from strain LGS322 by virtue of a deletion of the glucitol operon (Table I).

The primary substrate, 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-D-glycerate, was synthesized as described earlier (Blättler & Knowles, 1980) and converted in situ to [(*R*)-^{16}O,^{17}O,^{18}O]-phosphoenolpyruvate by inclusion of enolase in the reaction mixture. The chiral phospho group was then transferred to mannitol via the mannitol-specific PTS, and the resulting mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate was isolated. Determination of the absolute configuration at phosphorus in the product required conversion of mannitol 1-[^{16}O,^{17}O,^{18}O]-phosphate to glucose 6-[^{16}O,^{17}O,^{18}O]phosphate. This transformation was achieved by using mannitol-1-phosphate dehydrogenase and NAD^+ to produce fructose 6-phosphate and phosphoglucose isomerase to afford the equilibrium mixture of glucose 6-phosphate and fructose 6-phosphate. The latter was removed by using phosphofructokinase and ATP to convert it into fructose 1,6-bisphosphate, and the desired product,

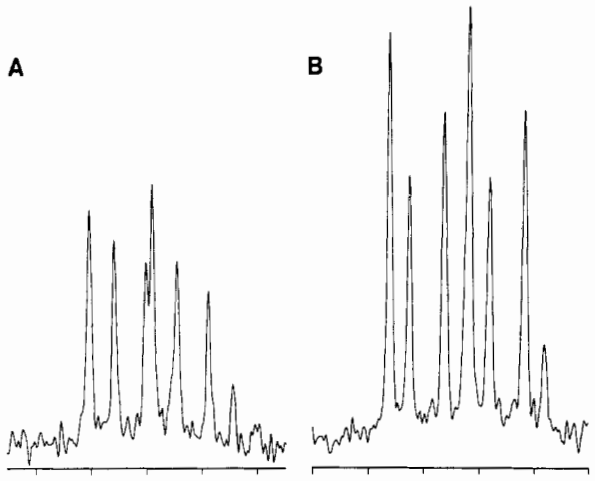

FIGURE 1: ^{31}P NMR spectra of equatorial and axial methyl esters of D-glucose cyclic 4,6-phosphate derived from stereochemical analysis of the D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate obtained in the overall PTS^{Mtl} reaction. The spectra were run on a Bruker WM-300 instrument at 121.50 MHz with a deuterium field lock and broad-band decoupling: spectral width 500 Hz, acquisition time 8.1 s, pulse width 15.5 μs, number of transients 4904, Gaussian multiplication in 8K (Gaussian broadening 0.07 Hz; line broadening −0.10 Hz), and Fourier transform in 32K. The chemical shifts for the equatorial resonances (A) are −0.4925, −0.5109, −0.5343, −0.5390, −0.5575, −0.5808, and −0.5992 ppm and for the axial resonances (B) are −2.3608, −2.3757, −2.4018, −2.4202, −2.4354, −2.4616, and −2.4762 ppm, upfield from external 85% phosphoric acid. The scale is 5 Hz per division.

glucose 6-phosphate, was purified by ion-exchange chromatography. No step in the conversion of mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate to glucose 6-[^{16}O,^{17}O,^{18}O]phosphate affects the configuration at phosphorus. The glucose 6-[^{16}O,^{17}O,^{18}O]phosphate was then analyzed by the method of Jarvest et al. (1981).

The sample of glucose 6-[^{16}O,^{17}O,^{18}O]phosphate was first cyclized to give the three isotopomers of glucose 4,6-cyclic phosphate. Methylation of these cyclic diesters then yielded six triesters, one with an axial methoxy group and one with an equatorial methoxy group from each diester. Analysis of this mixture by high-resolution ^{31}P NMR (Buchwald et al., 1982) then established the absolute configuration at phosphorus in the sample of glucose 6-[^{16}O,^{17}O,^{18}O]phosphate. The ^{31}P NMR spectra of the cyclic triester isomers having equatorial and axial methoxy groups is shown in Figure 1, panels A and B, respectively (eight resonances arise because of the existence of α- and β-anomers). The stereochemical information is contained in the relative intensities of the two middle peaks of each group of four resonances from a particular anomer of the cyclic triesters having either an axial or an equatorial methoxy group. The anomer quartets overlap in each case, and the stereochemically informative peaks are numbers 2 and 3 and numbers 5 and 6 of the seven resonances seen for each of the axial and the equatorial triesters (Figure 1). Cyclization of D-glucose 6-[(*S*)-^{16}O,^{17}O,^{18}O]phosphate puts ^{18}O in the equatorial position of the resulting cyclic diester. In the spectrum of the equatorial methyl esters of glucose cyclic 4,6-phosphate derived from the *S* isotopomer, therefore, the more downfield peaks are more intense: peak 2 > peak 3 and peak 5 > peak 6, as observed. The converse obtains for the axial methyl esters [also cf. Begley et al. (1982)]. It is thus clear from Figure 1 that the product mannitol 1-[^{16}O,^{17}O,^{18}O]phosphate contained a phospho group of the *S* configuration. The phospho group therefore suffered overall *inversion* of configuration in its transfer from phosphoenolpyruvate to mannitol.

The substrates of EII^{Mtl} are phospho–HPr and D-mannitol, and the products are mannitol 1-phosphate and HPr. EII^{Mtl} is known not to require a separate enzyme III for this activity (Jacobson et al., 1979). Therefore, there are two phospho-transfer steps from PEP, one to enzyme I and a second to HPr, that occur before those steps catalyzed by EII^{Mtl} which result in the phosphorylation of mannitol to mannitol 1-phosphate. Since the results of the stereochemical analysis indicate an odd number of phospho-transfer steps overall, there must be an odd number of such steps in the reaction catalyzed by EII^{Mtl} (phospho transfer from phospho–HPr to mannitol). [There is, of course, no consequence at phosphorus in the conversion of 2-phosphoglycerate (the primary source of chiral phospho groups) to PEP.] A single phospho-transfer step would occur only if EII^{Mtl} catalyzed a direct phospho group transfer from phospho–HPr to mannitol without the participation of a covalent phospho–EII^{Mtl} intermediate. There is ample kinetic and biochemical evidence, however, that such an intermediate does participate in this reaction (Roossien et al., 1984; Grenier et al., 1986; Pas et al., 1988; Pas & Robillard, 1988; Stephan et al., 1989). A single phospho-transfer step catalyzed by EII^{Mtl} can therefore be ruled out. On the other hand, three phospho-transfer steps would imply the existence of two catalytically necessary phosphorylation sites in EII^{Mtl} that are phosphorylated in sequence before the final phospho-transfer step to mannitol. As discussed below, this interpretation of our results is consistent with all available biochemical evidence concerning phospho–EII^{Mtl} intermediates. The possibility of there being five or seven phospho-transfer steps involving the enzyme is exceedingly unlikely.

The recent demonstration of considerable sequence similarity of residues 500–637 of EII^{Mtl} and the mannitol-specific enzyme III from *S. carnosus* (Reiche et al., 1988), as well as the fructose-specific protein FPr from *S. typhimurium* that combines an enzyme III-like domain with an HPr-like domain (Geerse et al., 1989), strongly suggests that this C-terminal subdomain of EII^{Mtl} comprises a "fused" enzyme III. Indeed, the longest region of uninterrupted homology between the three proteins mentioned occurs in a region that includes His-554 of EII^{Mtl} (Reiche et al., 1988; Geerse et al., 1989), which is almost certainly the phospho acceptor from phospho–HPr (Pas & Robillard, 1988). In addition to a phospho peptide containing His-554, Pas and Robillard (1988) also isolated a phospho peptide containing Cys-384 from EII^{Mtl} that had been phosphorylated with enzyme I, HPr, and [^{32}P]PEP. On the basis of the pH dependence of hydrolysis of the latter phospho peptide and other considerations, these workers concluded that Cys-384 was the phosphorylated residue. Moreover, recent experiments employing C-terminal deletion mutants of EII^{Mtl} are consistent with two consecutively phosphorylated sites and with the assignments of Pas and Robillard (1988), as well as with His-554 being the phospho acceptor from phospho–HPr and Cys-384 the phospho donor to mannitol (Grisafi et al., 1989; Stephan et al., 1989).

The results of our stereochemical analysis of the mannitol PTS are therefore entirely consistent with the biochemical evidence and eliminate the possibility that the observation of two phosphorylated sites in EII^{Mtl} was artifactual, that the sites were alternate phosphorylation loci, or that one of the sites was regulatory. Our data also eliminate the possibility of a third phosphorylation site too unstable to have been detected biochemically (Pas & Robillard, 1988; Stephan et al., 1989). We cannot, of course, provide any additional evidence regarding the identity of the two phosphorylation sites. As discussed above, the first such site is almost surely His-554,

while the second site has been identified as Cys-384. Although sequence similarity and evolutionary arguments have been made for phosphohistidine as the phospho donor from the PTS EII's to their substrates (Saier et al., 1988), and at least one such residue in EII^{Mtl} in addition to His-554 does appear to be required for this process on the basis of chemical modification studies (S. S. Khandekar and G. R. Jacobson, unpublished observations), there is every reason to suspect that the assignment of Cys-384 in EII^{Mtl} as the direct phospho donor to mannitol is correct. In fact, Alpert et al. (1989) have recently concluded from site-directed mutagenesis studies that none of the histidine residues is required for sugar phosphorylation activity of the lactose-specific EII from *Lactobacillus casei*, an EII that requires a separate enzyme III for PEP-dependent lactose phosphorylation. Interestingly, however, a single cysteine residue in a hydrophilic region of this protein does appear to be essential for its activity (C.-A. Alpert and B. M. Chassy, personal communication).

Even if additional work (including site-directed mutagenesis studies) will be necessary to prove the identities of the catalytically important phosphorylation sites in EII^{Mtl}, the conclusion that there are two such sites in this protein now seems firm. How phosphorylation of these sites and the subsequent phospho transfer to mannitol result in the vectorial translocation of this hexitol across the membrane remains to be determined. Conformational changes in the protein are undoubtedly involved: indeed, a conformational change that is responsible for the interconversion of two forms of EII^{Mtl} that differ in the stability of a dimeric form of the protein has been observed in detergent solution (Khandekar & Jacobson, 1989). A dimer of EII^{Mtl} has been implicated as necessary for both transport and phosphorylation activities (Robillard & Blaauw, 1987; Pas et al., 1987), and catalytically productive phospho transfer between different sites in normal and mutant subunits of an EII^{Mtl} heterodimer has been observed (Stephan et al., 1989). Why the two phosphorylation sites of EII^{Mtl} have been maintained in the same protein, while in some other PTS's these sites are found on separate proteins, remains a matter for further work.

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