Communication

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Direct Transfer of the Phosphoryl Moiety of Mannitol 1-Phosphate to [^{14}C]Mannitol Catalyzed by the Enzyme II Complexes of the Phosphoenolpyruvate:Mannitol Phosphotransferase Systems in *Spirochaeta aurantia* and *Salmonella typhimurium**

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SUMMARY

***Spirochaeta aurantia* possesses a phosphoenolpyruvate:mannitol phosphotransferase system which catalyzes the transmembrane transport and phosphorylation of mannitol. *In vitro* studies showed that both phosphoenolpyruvate and mannitol 1-phosphate could serve as phosphate donors. The phosphoenolpyruvate-dependent reaction required two soluble proteins, Enzyme SI and HPr, and an integral membrane complex, Enzyme SII. Only Enzyme SII was required for the mannitol 1-phosphate-dependent reaction. Enzyme II-dependent transphosphorylation of sugars was also demonstrated in eubacterial extracts. The results lead to the suggestion that the Enzyme II complexes of bacterial phosphotransferase systems possess nonoverlapping binding sites for sugar and sugar phosphate.**

The eubacterial phosphoenolpyruvate-dependent phosphotransferase system catalyzes the concomitant transmembrane transport and phosphorylation of sugars (1, 2). This complex enzyme system consists of two "general" proteins, Enzyme I and a small heat-stable protein, HPr, and several pairs of sugar-specific proteins, each pair which consists of an Enzyme II and an Enzyme III (3-5). The phosphorylation of a particular sugar involves the sequential transfer of the phosphoryl moiety of phosphoenolpyruvate first to Enzyme I, second to HPr, third to the Enzyme III specific for that sugar, and finally, in the presence of the corresponding membrane-bound Enzyme II complex, phosphate is transferred to sugar (6). The sugar-specific Enzyme III may either be cytoplasmic (6, 7) or membrane-associated as a component of the Enzyme II complex (4). Although the β-galactoside Enzyme II complex of *Staphylococcus aureus* has been shown to bind both sugar and Enzyme III in a ternary complex prior to phosphoryl transfer, the mechanism by which this process is coupled to transmembrane sugar transport is unknown (7).

In this communication evidence is presented which supports the suggestion that the membrane-associated enzyme complex of a mannitol-specific phosphotransferase system in *Spirochaeta aurantia* can catalyze the direct transfer of the phosphoryl moiety from mannitol 1-phosphate to [^{14}C]mannitol. Employing our standard assay conditions it is shown that the sugar specificity for this reaction is more restricted than that for the *in vitro* phosphorylation of sugar with phosphoenolpyruvate and corresponds to the transport specificity of the system *in vivo*. The results lead to the suggestion that the Enzyme II complex possesses two topologically distinct binding sites, one on the external membrane surface which normally binds sugar, the other on the cytoplasmic surface which can bind sugar phosphate.

Materials used in this study were obtained commercially with the following exceptions. Mannitol 1-phosphate and sorbitol 6-phosphate were synthesized by reduction of mannose 6-phosphate and glucose 6-phosphate respectively with sodium borohydride (8). The reaction was monitored employing the phenol sulfuric acid reaction (9). Boric acid was removed as described (8). The tricyclohexylammonium salt of phosphoenolpyruvate was synthesized by the method of Clark and Kirby (10) and twice recrystallized from aqueous ethanol. Procedures used to estimate the rates of sugar uptake by intact bacteria and sugar phosphorylation *in vitro* have been described (11, 12). Sugars were of the D configuration. Protein was measured by the biuret procedure (13).

Spirochaeta aurantia strain J1 (ATCC 25082) was obtained from Dr. E. Canale-Parola, Department of Microbiology, University of Massachusetts, Amherst. Morphological, physiological, and metabolic characteristics of this organism have been reported (14-17). The growth medium consisted of 0.4% Bacto-yeast extract, 0.2% Bacto-peptone, and 0.2% sugar in 10 mM potassium phosphate buffer, pH 7.4. Cells were grown aerobically at 30° in Erlenmeyer flasks filled to less than 50% of capacity. The generation time was 3 to 4 hours. It was confirmed that *S. aurantia* transported, utilized, and fermented maltose, glucose, mannitol, fructose, and galactose, but not sorbitol. The purity of bacterial cultures was routinely verified by phase contrast microscopy.

Assay of cell free extracts of *S. aurantia* cells revealed that mannitol and sorbitol (and to a lesser extent fructose) were phosphorylated by a phosphoenolpyruvate-dependent mechanism (Table I)[1] but that glucose, galactose, *N*-acetylglucosamine, fructose, and metabolites of maltose were preferentially phosphorylated by ATP-dependent kinases. The properties of the mannitol phosphotransferase system will be described in detail in a separate communication[1] but are briefly summarized below. Phosphoenolpyruvate-dependent phosphorylation of mannitol, sorbitol, and fructose required the presence of three protein fractions: Enzyme SI, Enzyme SII, and HPr which were quantitatively separated. Enzyme SI ($M_r \approx$ 80,000) is a soluble protein which is inactivated by both heat and *N*-ethylmaleimide. Enzyme SII is an integral membrane constituent which is heat-labile, but partially resistant to *N*-ethylmaleimide inactivation.[2] HPr ($M_r \approx$ 10,000) is a soluble protein which is heat-stable but inactivated by high concentrations of *N*-ethylmaleimide. Synthesis of these three enzymes and of mannitol 1-phosphate dehydrogenase was induced about 100-fold by growth of *S. aurantia* cells in media containing mannitol, sorbitol, or fructose. The phosphotransferase

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[1] Since mannitol is a symmetrical molecule, mannitol 1-phosphate is identical with mannitol 6-phosphate. The products of the phosphoenolpyruvate and mannitol 1-phosphate-dependent [^{14}C]mannitol phosphorylation reactions were characterized as follows. They co-chromatographed with authentic mannitol 1-phosphate in two solvent systems, co-electrophoresed with this compound in two buffer systems, and were cleaved by crystalline alkaline phosphatase to an uncharged ^{14}C-labeled compound which co-chromatographed and co-electrophoresed with mannitol (M. J. Newman, A. W. Rephaeli, and M. H. Saier, Jr., manuscript in preparation).

[2] Enzyme SII activity was maximally inhibited when 0.1 mM *N*-ethylmaleimide was employed, and a 50-fold higher concentration was without further effect.

TABLE I

Characteristics of the phosphoenolpyruvate:sugar phosphotransferase system in Spirochaeta aurantia

Uptake experiments, cells were grown as described in the text, harvested during exponential growth by centrifugation, washed twice with Medium 63 (11), and resuspended to a cell density of 0.18 mg dry weight/ml in Medium 63 containing 0.04% yeast extract and 0.02% Bacto-peptone as exogenous energy sources. Aliquots of the cell suspension (5 ml) were equilibrated to 30° with shaking at 200 rpm before initiation of the uptake experiment by addition of radioactive substrate (20 μM, final concentration). One-milliliter aliquots were periodically removed and filtered. Uptake rates were linear with time and cell density. *Sugar phosphorylation*, phosphorylation assays were conducted essentially as described previously (12) with excess amounts of Enzyme SI and HPr, a rate-limiting amount of the membrane-associated Enzyme SII complex, the phosphate donor at a concentration of 5 mM, and the radioactive sugar at a concentration of 50 μM. The temperature of incubation was 30°. Under these conditions, rates of phosphorylation were proportional to the amount of membrane protein added. *N-Ethylmaleimide inactivation*, Enzyme SII (7.3 mg of membrane protein/ml) was exposed to 1.0 mM *N*-ethylmaleimide for 5 min at 30°. Subsequently dithiothreitol was added to 5 mM. After a 5-min incubation, the samples were assayed for phosphorylation activity. The control sample was treated similarly except that *N*-ethylmaleimide was omitted. *Heat inactivation*, an aliquot of the Enzyme SII preparation was maintained at 56° for 5 min prior to assay.

Carbon source for growth (0.2%)	^{14}C-sugar substrate	Sugar uptake *in vivo* (% of control)	Alteration in the *in vitro* phosphorylation assay procedure	*In vitro* sugar phosphorylation with	
				Phosphoenolpyruvate (% of control)	Mannitol 1-phosphate (% of control)
Experiment 1					
Mannitol	Mannitol	100[a]		100[b]	100[c]
Fructose	Mannitol	90		70	85
Glucose	Mannitol	<5		1	1
Maltose	Mannitol	<5		0.5	0.5
Mannitol	Mannitol		Minus Enzyme SII	<1	<1
Mannitol	Mannitol		*N*-Ethylmaleimide-treated Enzyme SII	25	28
Mannitol	Mannitol		Partially heat-inactivated Enzyme SII	31	33
Experiment 2					
Mannitol	Mannitol	100[a]		100[b]	100[c]
Mannitol	Sorbitol	<5		120	<1
Mannitol	Fructose	<5		6	<1
Mannitol	Mannitol		Minus Enzyme SI and HPr	<1	96
Mannitol	Mannitol		$-Mg^{2+}$; +EDTA (5 mM)	22[d]	90
Mannitol	Mannitol		[^{14}C]Mannitol concentration increased to 250 μM	90	40

[a] 100% corresponds to a rate of uptake of radioactive sugar of 1.3 μmol/min/g dry wt of cells.
[b] 100% corresponds to 0.95 μmol of sugar phosphorylated/min/g of membrane protein.
[c] 100% corresponds to 0.38 μmol of sugar phosphorylated/min/g of membrane protein.
[d] The inhibitory effect of EDTA was completely reversed by addition of excess $MgCl_2$.

components from *S. aurantia* exhibited poor enzymatic cross-reactivity with those from *Salmonella typhimurium*, but purified HPr from *S. typhimurium* could replace the Spirochete HPr when used in 100-fold excess of that required to saturate the homologous system. The simplicity of the Spirochete phosphotransferase system (as compared with those in eubacteria) greatly facilitated interpretation of experimental results (see below).

Several experiments suggested that the phosphoenolpyruvate-dependent phosphorylation of mannitol, sorbitol, and fructose *in vitro* was catalyzed by an enzyme system, the membrane component of which was a single Enzyme II complex. *(a)* Phosphorylation of each sugar required both the soluble and membraneous fractions. *(b)* The inducer specificities for the phosphorylation of mannitol, sorbitol, and fructose were the same (see Table I for induction of mannitol phosphorylation activity). *(c)* Phosphorylation of all three sugars was most strongly inhibited by mannitol, less by sorbitol, and only weakly by fructose. *(d)* Partial inactivation of Enzyme SII by exposure to *N*-ethylmaleimide[2] or heat depressed the rates of phosphorylation of all three sugars to a similar degree.

Extracts of mannitol-grown *S. aurantia* cells could catalyze the phosphorylation of [^{14}C]mannitol in the presence of either phosphoenolpyruvate or mannitol 1-phosphate, but other phosphorylated compounds tested were less than one-tenth as effective. These compounds included ATP, GTP, the 1- and 6-phosphate esters of glucose, mannose, and fructose, sorbitol 6-phosphate, 2- and 3-phosphoglycerate,and a dozen other potential phosphate donors. The ratio of activities with phosphoenolpyruvate and mannitol 1-phosphate was highly dependent on the specific experimental conditions.

Rates of mannitol 1-phosphate-dependent phosphorylation of radioactive sugars are compared with the phosphoenolpyruvate-dependent rates and with transport rates in Table I. Several lines of evidence suggested that a single enzyme complex in the membrane (Enzyme SII) was responsible for the two *in vitro* activities (Experiment 1 in Table I). *(a)* Transfer of phosphate from either phosphoenolpyruvate or mannitol 1-phosphate to [^{14}C]mannitol required the membrane fraction, and no activity was detected in the cytoplasmic fraction. *(b)* Both phosphoryl transfer reactions exhibited the same inducer specificities. *(c)* Both reactions were strongly inhibited by nonradioactive mannitol, while sorbitol and fructose were less inhibitory. *(d)* Partial inactivation of Enzyme SII by exposure to *N*-ethylmaleimide or heat depressed the rates of both phosphoryl transfer reactions to the same extent.

Although the above evidence suggested that a single Enzyme SII complex in the membrane catalyzed phosphoryl transfer from both mannitol 1-phosphate and phosphoenolpyruvate to [^{14}C]mannitol, differences between these two reactions were noted (Experiment 2 in Table I). *(a)* While phosphate transfer from phosphoenolpyruvate to [^{14}C]mannitol required Enzyme SI, Enzyme SII, and HPr, only the Enzyme SII complex was required for phosphate transfer from mannitol 1-phosphate. *(b)* Employing the assay conditions described in Table I, mannitol, sorbitol, and fructose all served as phosphate acceptors when phosphoenolpyruvate was the phosphate donor, but transfer of the phosphoryl moiety of mannitol 1-phosphate to radioactive sugar was observed only when [^{14}C]mannitol was the phosphate acceptor.[3] The specificity of the latter reaction corresponds to the transport specificity of the system (Table I). *(c)* In the absence of added divalent

[3] The apparent discrepancy between the broad sugar phosphorylating capacity of the *Spirochaeta* phosphotransferase system and its more restricted transport specificity is also a characteristic of the eubacterial phosphotransferase system. Thus, the phosphotransferase system of *Salmonella typhimurium* catalyzed the transfer of phosphate from [^{32}P]phosphoenolpyruvate to a large number of sugars of the D configuration (hexoses, hexitols, pentoses, pentitols, etc.), although many of these sugars were not transported into intact cells. L Sugars were not phosphorylated. (M. H. Saier, Jr. and S. Roseman, unpublished observations.)

cation, EDTA strongly inhibited phosphoenolpyruvate-dependent phosphorylation of [^{14}C]mannitol, but was not inhibitory when mannitol 1-phosphate served as the phosphate donor.[4] *(d)* Increasing the concentration of [^{14}C]mannitol from 50 μM to 250 μM did not depress the rate of phosphate transfer from phosphoenolpyruvate to [^{14}C]mannitol, but was inhibitory when mannitol 1-phosphate served as the phosphate donor.[5]

If the observations noted above are reflective of a general transport/phosphorylation mechanism catalyzed by all bacterial phosphoenolpyruvate:sugar phosphotransferase systems, it should be possible to demonstrate the Enzyme II-dependent transfer of phosphate from sugar phosphate to [^{14}C]sugars in cell free extracts of eubacteria. Although optimal conditions differed from those employed with the Spirochete system, this reaction could be demonstrated in extracts of *Salmonella typhimurium, Escherichia coli,* and *Staphylococcus aureus.* As for the *S. aurantia* phosphotransferase system, a high degree of donor specificity was observed. Mannitol 1-phosphate was the only effective phosphate donor for [^{14}C]mannitol transphosphorylation catalyzed by the mannitol Enzyme II complex of *S. typhimurium;* sorbitol 6-phosphate specifically served as phosphoryl donor for the phosphorylation of [^{14}C]sorbitol, catalyzed by the sorbitol Enzyme II of *S. typhimurium;* glucose 6-phosphate was the preferred phosphate donor when glucose, methyl α-glucoside, or mannose phosphorylation catalyzed by either of the two glucose Enzyme II complexes of *S. typhimurium* was studied, and galactose 6-phosphate was the best of several phosphate donors tested when [^{14}C]methyl β-thiogalactoside phosphorylation, catalyzed by the galactoside phosphotransferase system of *S. aureus,* was examined. The sugar phosphate-dependent transphosphorylation reactions (but not the phosphoenolpyruvate dependent reactions) occurred at wild type rates in a *Salmonella* mutant which was deleted for the *pts* and *crr* genes and therefore lacked Enzyme I, HPr, and soluble Enzyme III^{Glc} (18). Mutational loss of the relevant Enzyme II complexes in the membrane resulted in complete loss of activity.

Representative data which illustrate these points for the mannitol phosphotransferase system of *S. typhimurium* are included in Table II. It can be seen that of several sugar phosphates tested, only mannitol 1-phosphate served as a phosphoryl donor under the conditions employed. The *∇trzAptsHIcrr-49* mutation (18), which abolished phosphoenolpyruvate-dependent mannitol phosphorylation, did not depress the rate of the mannitol 1-phosphate-dependent phosphoryl transfer reaction (Table II). Furthermore, extraction of soluble and peripheral proteins from the membrane with butanol and urea (4) did not diminish activity (data not shown).[6] By contrast, three independently isolated mutants which lacked appreciable phosphoenolpyruvate-dependent mannitol Enzyme II activity exhibited negligible mannitol 1-phosphate:mannitol transphosphorylase activity (Table II).

TABLE II

Transfer of phosphoryl moiety of mannitol 1-phosphate to [^{14}C]mannitol catalyzed by the mannitol-specific Enzyme II of Salmonella typhimurium phosphotransferase system

Salmonella strains were grown in Medium 63 (11) with 1% DL-lactate and 0.2% mannitol as sources of carbon and inducer. The washed cells were ruptured by passage through a French pressure cell at 10,000 p.s.i., and the membrane fractions were prepared and washed by differential centrifugation. The membrane preparations, in the absence of the soluble protein fraction, were assayed for sugar phosphorylation as described in Table I with the concentration of sugar phosphate and [^{14}C]mannitol at 10 mM and 50 μM, respectively. Rates of phosphoryl transfer were linear with time and protein concentration. Strains *mtl-61, mtl-64,* and *mtl-65* are three independently isolated mutants which have been shown to lack detectable phosphoenolpyruvate-dependent mannitol Enzyme II activity. The *∇trzAptsHIcrr-49* mutation has been shown to result in deletion of the genes which code for Enzyme I and HPr (18). All strains were isogenic except for the mutations indicated below.

Strain (genotype)	Phosphoryl donor	[^{14}C]mannitol phosphorylation (nmol/min/g of membrane protein)
LT2 (wild type)	None	<2
LT2	Mannitol 1-phosphate	120
LT2	Sorbitol 6-phosphate	<2
LT2	Fructose 1-phosphate	<2
LT2	Fructose 6-phosphate	<2
LT2	Mannose 6-phosphate	<2
∇trzAptsHIcrr-49	Mannitol 1-phosphate	140
∇trzAptsHIcrr-49	Phosphoenolpyruvate	<2
mtl-61	Mannitol 1-phosphate	4
mtl-64	Mannitol 1-phosphate	3
mtl-65	Mannitol 1-phosphate	<2

We have additionally found that under appropriate conditions, the rates and extent of uptake of [^{14}C]methyl α-glucoside and [^{14}C]mannitol into isolated membrane vesicles of *E. coli* (19) can be stimulated about 5-fold by intravesicular (but not extravesicular) glucose 6-phosphate and mannitol 1-phosphate, respectively.[7] These results suggest a transport/phosphorylation mechanism which appears to explain several previously anomalous observations.[8] We envisage the Enzyme II complex as an integral membrane constituent which penetrates the phospholipid bilayer structure and possesses a stereo-specific transmembrane "pore" (27) as well as nonoverlapping binding sites for sugar and sugar phosphate on the external and cytoplasmic sides of the membrane, respectively. This speculation serves as a working hypothesis for the design of future experiments.

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[4] Although phosphoenolpyruvate-dependent sugar phosphorylation catalyzed by the phosphotransferase system of *Escherichia coli* is dependent on Mg^{2+}, only the first phosphoryl transfer reaction (phosphorylation of Enzyme I) appears to require this divalent cation (1). The same may be true of the Spirochete phosphotransferase system.

[5] The concentration of [^{14}C]mannitol used in Table I (50 μM) was sufficiently high to exert appreciable inhibition when mannitol 1-phosphate (5 mM) was the phosphoryl donor. Employing conditions which were optimal for this reaction and with Enzyme SII rate limiting for the phosphoenolpyruvate-dependent reaction, the rates of the two phosphoryl transfer reactions were comparable.

[6] Extraction of the membrane fraction of *S. typhimurium* strain *∇trzAptsHIcrr-49* with butanol and urea according to the procedure of Kundig and Roseman (4) did not diminish activity employing as phosphoryl donor either *phosphoenolpyruvate (in the presence of the requisite soluble proteins of the* phosphotransferase system) or sugar phosphate (measured in the absence of the soluble proteins). Enzyme II specific activity, measured by either reaction, increased about 2-fold due to the removal of soluble and peripheral membrane proteins. By contrast, greater than 95% of the membrane associated glucose-6-phosphatase activity in the preparation was removed by this extraction procedure. Employing a butanol-urea extracted membrane preparation, the rate of transphosphorylation from [^{14}C]glucose 6-phosphate to either [^{14}C]methyl α-glucoside or [^{3}H]glucose was about five times greater than the rate of [^{14}C]glucose 6-phosphate hydrolysis.

[7] E. G. Moczydlowski and M. H. Saier, Jr., unpublished results.

[8] If the phosphoryl moiety can be transferred from an intracellular sugar phosphate (bound to the inner sugar phosphate binding site of the Enzyme II complex) to extracellular sugar (bound to the outer sugar binding site) with concomitant translocation of extracellular sugar into the cell, then a mechanism is available to explain the partial insensitivity of glucose and methyl α-glucoside uptake to arsenate inhibition in *E. coli* and *S. typhimurium* cells (20, 21). Such a mechanism could serve the physiological function of protecting the cell against toxic intracellular nonmetabolizable sugar phosphates. If nonsubstrate sugar phosphates can bind to the inner sugar phosphate binding site, the accumulation of intracellular sugar phosphate should inhibit transport *in vivo* in an apparently allosteric fashion (19, 22–24). The binding of free sugar to the inner sugar phosphate binding site might be expected to inhibit sugar phosphorylation, particularly when sugar phosphate serves as the phosphoryl donor (Table I). Additionally, the binding of free sugar at this site might be a prerequisite for phosphotransferase system-mediated efflux of the sugar from the cell (25, 26).

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