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The Bacterial Phosphotransferase System: Kinetic Characterization of the Glucose, Mannitol, Glucitol, and N-Acetylglucosamine Systems

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The kinetic mechanisms by which the glucose, glucitol, N-acetylglucosamine, and mannitol enzymes II catalyze sugar phosphorylation have been investigated in vitro. Lineweaver-Burk analyses indicate that the glucose and glucitol enzymes II catalyze sugar phosphorylation by a sequential mechanism when the two substrates are phospho-enzyme III and sugar. The N-acetylglucosamine and mannitol enzymes II, which do not function with an enzyme III, catalyze sugar phosphorylation by a ping-pong mechanism when the two substrates are phospho-HPr and sugar. These results, as well as previously published kinetic characterizations, suggest a common kinetic mechanism for all enzymes II of the system. It is suggested that all enzymes II and enzyme II-III pairs arose from a single (fused) gene product containing two sites of phosphorylation and that phosphoryl transfer from the second phosphorylation site to sugar can only occur when the enzyme II-III pair is present in the associated state.

Key words: phosphotransferase system, enzyme II, kinetics

The bacterial phosphotransferase system (PTS) catalyzes the transmembrane translocation and concomitant phosphorylation of a variety of sugars (Saier, 1985). Sugar-specific, integral membrane transport proteins, termed enzymes II, catalyze phosphorylation of the incoming sugar. While phosphoenolpyruvate (PEP) is the ultimate PTS phosphoryl donor, enzymes II interact directly with either phospho-heat-stable phosphocarrier (phospho-HPr), a non-sugar-specific phosphocarrier pro-

Abbreviations used: PTS, phosphotransferase system; HPr, heat-stable phosphocarrier protein of the PTS; Enzyme II^{glc}, glucose Enzyme II; Enzyme II^{gut}, glucitol Enzyme II; Enzyme II^{nag}, N-acetylglucosamine Enzyme II; III^{glc}, glucose-specific phosphocarrier protein; III^{gut}, glucitol-specific phosphocarrier protein; PEP, phosphoenolpyruvate.

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tein of the PTS, or with a phospho-III, a sugar-specific phosphocarrier protein of the system.

The kinetic mechanisms by which various enzymes II catalyze PEP-dependent sugar phosphorylation have been investigated in a number of laboratories. Some of these investigators studied phosphoryl transfer from phospho-HPr to sugar and concluded that sugar phosphorylation followed a ping-pong mechanism [Rose and Fox, 1971; Marquet et al, 1978; Misset et al, 1983; Roossien et al, 1984]. On the other hand, Simoni et al [1973] studied phosphoryl transfer from phospho-III^{lac} to sugar and concluded that a sequential mechanism was followed. We suggest in this report that there is no actual inconsistency in the literature as regards the kinetic mechanism. The characteristics of the particular enzyme II being investigated, as well as the experimental design of the kinetic measurements, can account for the discrepancies. We hypothesize that for those enzymes II which require an enzyme III, sequential kinetics are always followed, and the reaction proceeds via a ternary III/phosphoenzyme II/sugar complex. Enzymes II which do not require III, for instance, the mannitol and N-acetylglucosamine enzymes II, probably proceed by a similar mechanism. In these cases, however, III is a covalently attached region of the enzyme II [Saier et al, 1985].

MATERIALS AND METHODS

Chemicals

Methyl α-D-[^{14}C(U)]glucopyranoside (279 mCi/mmol), D-[^{14}C(U)]glucitol (302 mCi/mmol), and N-acetyl-D-[^{14}C(1)]glucosamine (58 mCi/mmol) were obtained from Amersham. D-[^{14}C(U)]Mannitol (45 mCi/mmol) was obtained from New England Nuclear. All other reagents were of reagent grade or better.

Preparation of Vesicles

Escherichia coli strains ML308 and LJ246 (enzyme II^{man}-negative) and *Salmonella typhimurium* strain LJ409 (enzyme II^{mtl}-negative) were grown aerobically to the late logarithmic phase in Luria broth containing 0.5% glucose, N-acetylglucosamine, or glucitol, respectively. The LJ246 cells were harvested, and inverted vesicles were prepared as described by Reenstra et al [1980]. Butanol/urea extracted membranes were prepared from ML308 and LJ409 as described by Kundig and Roseman [1971b]. Membrane preparations were kept at −20°C.

Purification of Enzymes

Enzyme I and HPr were purified as described by Waygood and Steeves [1980]. Enzyme III^{glc} (III^{glc}) was purified essentially as described by Meadow and Roseman [1982]. The III^{gut} was purified as described by Grenier et al [1985]. Enzyme II^{mtl} was purified as described by Jacobson et al [1983].

Enzyme II Assays

Phosphoenolpyruvate-dependent [^{14}C]sugar phosphorylation reactions for enzyme II^{glc}, enzyme II^{gut}, and enzyme II^{nag} were carried out in 0.1 ml volumes containing 50 mM potassium phosphate, pH 7.5, 10 mM $MgSO_4$, 10 mM KF, and 10 mM phosphoenolpyruvate. When enzyme II^{mtl} was assayed, 0.5% Lubrol-PX was

also included in the assay. Nonradioactive methyl-α-glucoside used in these assays was purified free of glucose and recrystallized as described by Roseman et al [1952]. The concentrations of enzyme I, HPr, III^{glc}, and III^{gut} varied and are given in the figure legends. In all cases, enzyme II was the rate-limiting component. The initial velocity was proportional to the amount of membrane preparation or purified enzyme II^{mtl} at all concentrations of substrate assayed. Incubations were for 20–30 min at 37°C and were stopped by the addition of ice-cold water (3 ml). The [^{14}C]sugar phosphate was separated from free sugar using Dowex (AG1-X2) columns and quantitated as described [Kundig and Roseman, 1971a].

RESULTS

Glucose Phosphotransferase System

In *Escherichia coli* and *Salmonella typhimurium*, glucose and methyl α-glucoside are phosphorylated by an enzyme II^{glc} which functions in conjunction with a sugar-specific enzyme III^{glc} (III^{glc}). Misset et al (1983) demonstrated that enzyme II^{glc} follows ping-pong kinetics when the two substrates whose concentrations were varied were methyl α-glucoside and phospho-HPr. This result is predicted because phospho-HPr transfers its phosphoryl group to III^{glc} as a necessary step leading to sugar phosphorylation. Phospho-III^{glc} has been isolated as a stable intermediate and will transfer its phosphoryl moiety either back to HPr or forward to sugar via the enzyme II^{glc} [Meadow and Roseman, 1982].

In the experiments shown in Figure 1, we measured the initial velocity of methyl-α-glucoside phosphorylation while varying both the methyl-α-glucoside and phospho-III^{glc} concentrations. The III^{glc} was maintained in the phosphorylated state by inclusion of high levels of phosphoenolpyruvate, enzyme I, and HPr in the assay. The source of enzyme II^{glc} was butanol/urea-extracted membranes of glucose-grown *E coli* ML308 cells. The extraction procedure removed greater than 95% of the peripherally associated III^{glc}. Under these conditions, the reaction clearly proceeds

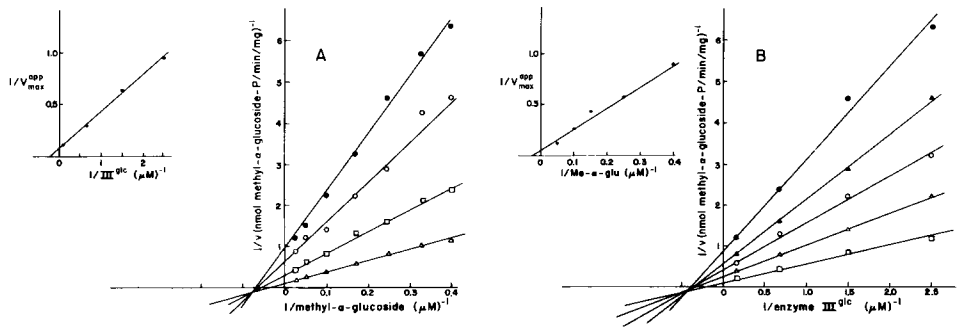

Fig. 1. Lineweaver-Burk plots of methyl-α-glucoside phosphorylation. Reciprocal initial velocities are plotted as a function of either the reciprocal methyl-α-glucoside (A) or phospho-III^{glc} (B) concentration. Replots of the data are shown as inserts. The enzyme I and HPr concentrations used for these assays were 1 and 10 μM, respectively. A) The four lines show data for four different concentrations of III^{glc} as shown in the insert. B) The five lines show data for five different concentrations of methyl α-glucoside as shown in the insert.

by a sequential mechanism. Plots of 1/v versus 1/methyl α-glucoside at different constant concentrations of phospho-III^{glc} or of 1/v versus 1/phospho-III^{glc} at different constant concentrations of methyl α-glucoside yielded intersecting lines [Segal, 1975]. The K_m and V_{max} values determined from replots of the $1/V_{max}^{app}$ values versus 1/methyl α-glucoside and $1/III^{glc}$ are given in Table I. The V_{max} value (12 nmole sugar phosphate formed/min/mg) is lower than some, yet higher than other specific activities reported by Stock et al [1982]. In general, the specific activities reported in the literature vary considerably. The K_m value for III^{glc} (5 μM) is in close agreement with the previously reported value of 3 μM [Meadow and Roseman, 1982]. The K_m value for methyl-α-glucoside (28 μM) is approximately four-fold higher than previously reported [Stock et al, 1982]. The reason for this discrepancy is not known.

Glucitol Phosphotransferase System

In *E coli* and *S typhimurium*, glucitol, like glucose, is phosphorylated by an enzyme II which functions in conjunction with an enzyme III. The III^{gut} has recently been purified (Grenier et al, 1985), and experiments analogous to those conducted with the glucose PTS (Fig. 1) could therefore be conducted with the glucitol system (Fig. 2). The source of enzyme II^{gut} in these experiments was butanol/urea extracted membranes from glucitol-grown *S typhimurium* LJ409, a mannitol enzyme II-negative mutant. This mutant strain was used because glucitol can be phosphorylated by the

TABLE I. Kinetic Constants*

System	K_m(sugar)[a]	K_m(phospho-HPr)	K_m(phospho-III^{sugar})	V_{max}[b]
Glucose	28[c]	—	5	12
Glucitol	1	—	1	2
Mannitol	6	7	—	4
N-acetylglucosamine	4	9	—	70

*Enzyme II kinetics were performed as described under "Materials and Methods." Kinetic constants were determined from the replots of Figures 1, 2, 3, and 4.
[a]All K_m values are given in μM.
[b]V_{max} values are given in nmoles sugar phosphate formed/min/mg except for the mannitol system in which case it is given in μmoles sugar phosphate formed/min/mg.
[c]The substrate used in this experiment was methyl α-glucoside.

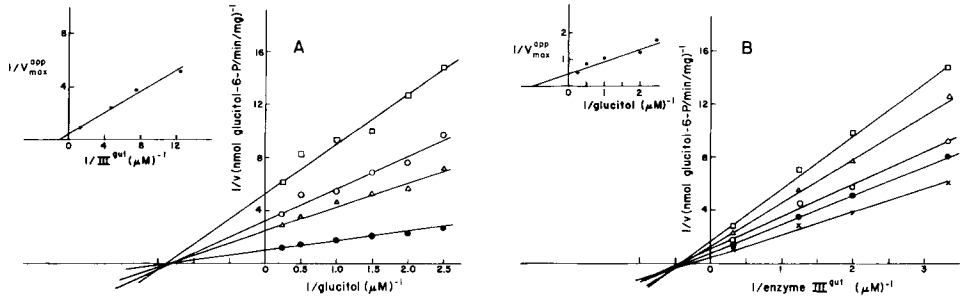

Fig. 2. Lineweaver-Burk plots of glucitol phosphorylation. Reciprocal initial velocities are plotted as a function of either the reciprocal glucitol (A) or the phospho-III^{gut} (B) concentration. Replots of the data are shown as inserts. Enzyme I and HPr concentrations were as in Figure 1. The data points of the same symbol represent values obtained with a constant concentration of the second substrate as for Figure 1.

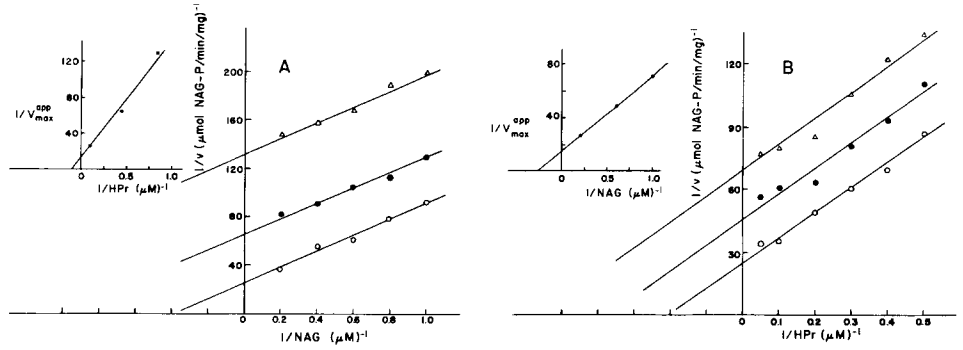

Fig. 3. Lineweaver-Burk plots of mannitol phosphorylation. Reciprocal initial velocities are plotted as a function of either the reciprocal mannitol (A) or phospho-HPr (B) concentration at different but constant concentrations of the second substrate. Replots of the data are shown as inserts. The enzyme I concentration was 1 μM.

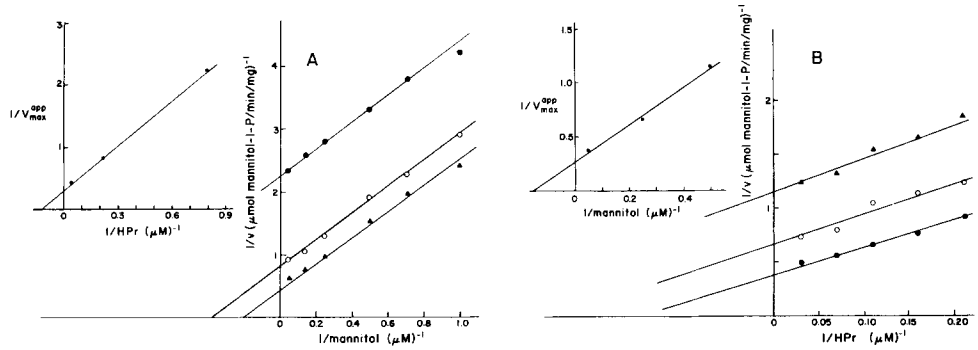

Fig. 4. Lineweaver-Burk plots of N-acetylglucosamine phosphorylation. Reciprocal initial velocities are plotted as a function of either the reciprocal N-acetylglucosamine (A) or the phospho-HPr (B) concentration at constant concentrations of the second substrate. Replots of the data are shown as inserts. The enzyme I concentration was 1 μM.

mannitol system in the wild-type bacterium. Butanol/urea extraction removed greater than 95% of the peripherally membrane associated III^{gut}. As with the glucose system, plots of 1/v versus 1/glucitol and 1/phospho-III^{gut} yielded intersecting lines indicating a sequential mechanism. The K_m and V_{max} values determined from replots of the $1/V_{max}^{app}$ values are given in Table I. The V_{max} value (2 nmole sugar phosphate/min/mg) is in close agreement with the value reported by Lengeler [1975] yet is low compared to the specific activity of most other enzymes II. The K_m value for III^{gut} (1 μM) has not been reported previously, but is in the expected range. The K_m value for glucitol (1 μM) is more than an order of magnitude smaller than the value reported by Lengeler [1975]. It is, however, quite near the enzyme II^{mtl} K_m value for mannitol.

Mannitol Phosphotransferase System

The mannitol enzyme II, unlike the glucitol and glucose enzymes II, does not function in conjunction with an enzyme III [Jacobson et al, 1979; Saier et al, 1985]. Enzyme II^{mtl} activity was therefore assayed as a function of the phospho-HPr and mannitol concentrations. The source of enzyme II^{mtl} for these experiments was

purified enzyme II^{mtl}. Plots of 1/v versus 1/mannitol or 1/phospho-HPr yielded parallel lines (Fig. 3). This pattern is diagnostic of ping-pong kinetics [Segal, 1975] and is consistent with the results of Rephaeli and Saier [1980] and Roossien et al [1984]. The K_m and V_{max} values determined from replots of apparent $1/V_{max}$ values are given in Table I. The V_{max} value (4 μmole sugar phosphate/min/mg) is in the expected range. The K_m value for mannitol (6μM) is intermediate between two previously published values [Jacobson et al, 1983; Roossien et al, 1984]. The K_m value for phospho-HPr (7μM) is higher than the previously published value of 1 μM [Roossien et al, 1984].

N-Acetylglucosamine Phosphotransferase System

In 1970, it was shown that there are two distinct phosphotransferase systems in *E coli* which transport N-acetylglucosamine [White, 1970]. Subsequently, Jones-Mortimer and Kornberg [1980] demonstrated that the mannose and N-acetylglucosamine systems were the two responsible systems. Waygood et al [1984] have provided evidence that the N-acetylglucosamine enzyme II, like the mannitol enzyme II, does not require an enzyme III. In experiments analogous to those conducted with the mannitol system, we measured N-acetylglucosamine phosphorylation as a function of the N-acetylglucosamine and phospho-HPr concentrations. The source of enzyme II^{nag} for these experiments was membranes from an N-acetylglucosamine grown *E coli manA* mutant. Plots of 1/v versus 1/NAG or 1/phospho-HPr yielded parallel lines (Fig. 4). The K_m and V_{max} values determined from replots of the $1/V_{max}^{app}$ values are given in Table I. The V_{max} value (70 nmole sugar phosphate/min/mg) for this system has not been reported under comparable conditions. The K_m values for phospho-HPr (9 μM) and N-acetylglucosamine (4 μM) also have not been reported previously but are in the expected range.

DISCUSSION

The results reported here as well as those previously published suggest that the kinetic analysis of enzyme II catalyzed reactions follow a common pattern. In those experiments in which kinetic reaction mechanisms were determined under conditions where sugar and phospho-enzyme III concentrations were varied, sequential kinetics have always been observed. These systems include the *E coli* glucitol and glucose enzymes II, investigated in this report, and the *Staphylococcus aureus* lactose enzyme II investigated by Simoni et al [1973]. In contrast, when the reaction mechanisms were determined under conditions of varying sugar and phospho-HPr concentrations, ping-pong kinetics have always been observed. These systems include the *E coli* N-acetylglucosamine enzyme II investigated in this report, the *E coli* glucose enzyme II-III pair [Misset et al, 1983], the β-glucoside enzyme II [Rose and Fox, 1971], the mannitol enzyme II investigated here and described by Roossien et al [1984] and the *Bacillus subtilis* enzyme II [Marquet et al, 1978].

In some cases, this apparent discrepancy has a straightforward explanation. Enzymes II which function in conjunction with an enzyme III, but which were analyzed by varying the HPr concentration at a constant enzyme III concentration always yielded ping-pong kinetics as expected. Enzymes III exist in a stable phosphorylated form, and phospho-enzymes III can donate their phosphoryl group either to HPr or to a sugar via the enzyme II. Thus, the *E coli* glucose enzyme II yielded ping-pong kinetics when reaction rate was studied as a function of phospho-HPr and

sugar concentrations but sequential kinetics when reaction rate was studied as a function of the phospho-III^{glc} and sugar concentrations. A similar situation may also have existed when the *B subtilis* glucose enzyme II was studied. There is some evidence that an enzyme III exists for this system [Reizer et al, 1984]. The kinetic behavior observed in this case resulted from the use of bacterial membranes together with partially purified HPr and enzyme I. Small amounts of contaminating enzyme III would have been sufficient to ensure activity.

Unlike these systems, however, the mannitol and N-acetylglucosamine enzymes II do not require an enzyme III. Nevertheless, ping-pong kinetics were observed in both these systems when the two substrates were phospho-HPr and sugar (Figs. 3 and 4). These observations suggest either (1) that enzymes II which interact directly with phospho-HPr function by a fundamentally distinct reaction mechanism from enzymes II which interact with a phospho-III, or (2) that a III-like region exists within those enzymes II which interact directly with phospho-HPr. Evidence has been presented suggesting that the latter possibility pertains to the mannitol enzyme II [Saier et al, 1985]. Waygood and coworkers have obtained evidence for two distinct phosphorylation sites within the mannitol and N-acetylglucosamine enzymes II [unpublished results]. An investigation of the stereochemical course of mannitol phosphorylation using purified protein components and chiral phosphoenolpyruvate [Begley et al, 1982] could resolve this issue.

Sequential reaction mechanisms are often considered to be evidence for the lack of a stable enzyme reaction intermediate (in this case, phospho-enzyme II). There is, however, no theoretical requirement for such an assumption. Considerable evidence suggests that phosphorylated enzyme II intermediates exist. The stereochemical experiments [Begley et al, 1982], the phosphorylation experiments [Peri et al, 1984; Waygood et al, 1984; Roossien et al, 1984; and B. Erni (personal communication)] and the pyruvate burst experiments [Misset et al, 1983] all suggest the existence of such an intermediate. Stoichiometric phosphorylation, however, remains to be demonstrated. We propose that the sequential kinetics reported in this paper are consistent with the notion that the formation of a ternary complex of III/phospho-enzyme II/sugar is required for sugar phosphorylation (Fig. 5). Direct evidence for such a complex has recently been obtained (B. Erni, personal communication). Purified phospho-enzyme II^{glc} from *E coli* was not readily dephosphorylated in the presence of glucose unless purified, unphosphorylated III^{glc} was added. Addition of III^{glc} resulted in the rapid formation of glucose-6-phosphate. The mechanism by which III^{glc} promotes the transfer of phosphate from phospho-enzyme II^{glc} to sugar has yet to be investigated. Further studies will be required to provide a physicochemical basis for the kinetic generalizations revealed in this study.

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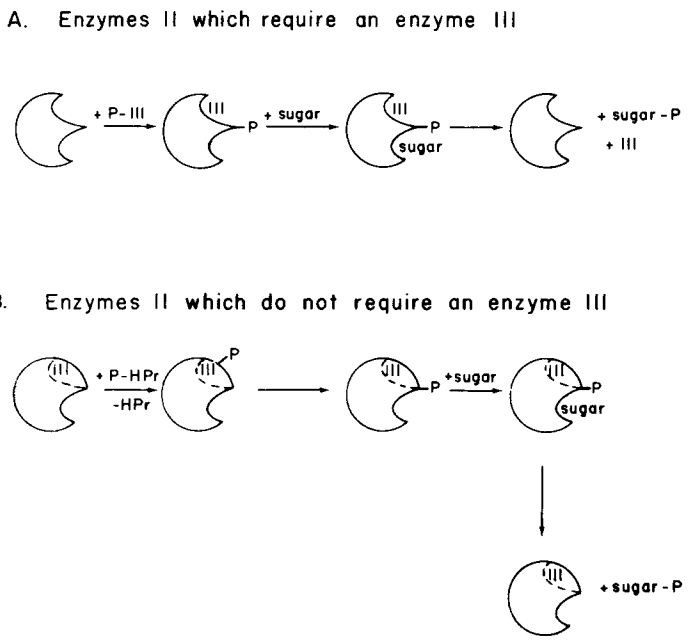

Fig. 5. Kinetic mechanisms accounting for the phosphoenolpyruvate-dependent phosphorylation of sugar by the enzymes II of the phosphotransferase system. The region enclosed by the dashed line in panel B represents the covalently attached enzyme III - like portion of an enzyme II. The presence of two distinct phosphorylation sites on some enzymes II (eg, enzyme II^{mtl}) is conjecture and remains to be rigorously established.

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