Bacterial Phosphotransferase System: Regulation of Mannitol Enzyme II Activity by Sulfhydryl Oxidation†

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ABSTRACT: The mechanism by which the oxidation–reduction potential regulates the bacterial phosphotransferase system in *Escherichia coli* has been investigated. Transphosphorylation experiments verified that the oxidizing agent, potassium ferricyanide, directly inhibits mannitol enzyme II activity. Phosphorylation of enzyme II^{mtl} with enzyme I, heat-stable phosphocarrier protein of the phosphotransferase system, and phosphoenolpyruvate partially protects the enzyme from ferricyanide inhibition. The enzyme is even less sensitive to inhibition during catalytic turnover. Preincubation of unphosphorylated enzyme with ferricyanide, however, reversibly inactivates it even at high mannitol concentrations. The results are inconsistent with a regulatory mechanism in which sulfhydryl oxidation influences the affinity of the enzyme for the substrate. Instead, it is concluded that the oxidized enzyme is inactive.

The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS)[1] of *Escherichia coli* catalyzes the transport and concomitant phosphorylation of a number of sugars (Dills et al., 1980). Several proteins are required for the transfer of a phosphoryl moiety from phosphoenolpyruvate to the incoming sugar. The general reaction scheme is (Saier, 1984)

$$\text{PEP} \xrightarrow[\text{pyruvate}]{\text{EI}} \text{EI-P} \xrightarrow[\text{EI}]{\text{HPr}} \text{HPr-P} \xrightarrow[\text{HPr}]{\text{III}} \text{III-P} \xrightarrow[\text{III}]{\text{II}}$$

$$\text{II-P} \xrightarrow[\text{II}]{\text{sugar}} \text{sugar-P} \quad (1)$$

Enzyme I (EI) and HPr are general cytoplasmic components of the *E. coli* PTS. The enzymes II (II), which in some cases act in conjunction with an enzyme III (III), confer sugar specificity upon the system. Enzymes II are integral membrane proteins that catalyze concomitant transport and phosphorylation of the sugar. These enzymes also catalyze vectorial "transphosphorylation" reactions, which in intact cells occur vectorially as follows (Saier & Newman, 1976; Saier et al., 1977):

$$*\text{sugar}_{out} + \text{sugar-P}_{in} \overset{II}{\rightleftharpoons} \text{sugar}_{out} + *\text{sugar-P}_{in} \quad (2)$$

This reaction requires only enzyme II, and being independent of the other components of the PTS, it provides a useful assay for investigating enzyme II function.

A number of different studies have shown that the activity of the PTS is regulated by energy. Studies with whole cells and with membrane vesicles have shown that the proton electrochemical gradient inhibits methyl α-glucoside transport by the PTS (Hernandez-Asensio et al., 1975; del Campo et al., 1975; Reider et al., 1979). Recently, it was also shown that both the protonmotive force and a variety of oxidizing agents can inhibit PTS-mediated methyl α-glucoside phosphorylation in membrane vesicles. On the basis of the results reported, a mechanism for this inhibitory effect was postulated (Robillard & Konings, 1981; Robillard, 1982). The enzyme II was presumed to exist in two forms: a low-affinity oxidized form and a high-affinity reduced form. Elevated sugar concentrations were reported to relieve the inhibitory effects of oxidizing agents. Oxidation was therefore presumed to alter the affinity of the enzyme for its sugar substrate.

In this paper we report our studies employing homogeneous preparations of the mannitol enzyme II (enzyme II^{mtl}). It was possible to verify as shown by Roossien & Robillard (1984) that oxidizing agents such as ferricyanide inhibit the activity of the enzyme. However, inhibition was not overcome by high substrate concentrations although it was diminished by a phosphorylation-dependent mechanism. We conclude that the oxidized form of the enzyme is inactive.

Materials and Methods

Chemicals. D-[1-^{14}C]Mannitol (45 mCi/mmol) was obtained from New England Nuclear. [U-^{14}C]Phosphoenolpyruvate was obtained from Amersham. Phenazine methosulfate, plumbagin, Lubrol-PX, and mannitol 1-phosphate were from Sigma. All other reagents were of reagent grade or better.

Purification of Enzymes. Enzyme II^{mtl} was purified as described by Jacobson et al. (1979, 1983). Following the last dialysis step in 1 mM dithiothreitol, the enzyme was dialyzed against 0.2 mM dithiothreitol. The enzyme was kept frozen at −70 °C until used. The specific activities of our pure enzyme II^{mtl} preparations varied over a 3–4-fold range. The experiments presented herein were conducted with at least two different preparations of enzyme II^{mtl}. Milligram quantities of *E. coli* HPr were provided by Dr. H. Kornberg. Enzyme I was purified as described by Waygood & Steeves (1980). The studies reported were conducted with homogeneous en-

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[1] Abbreviations: PTS, phosphotransferase system; EI, enzyme I; HPr, heat-stable phosphocarrier protein of the PTS; III, enzyme III; II, enzyme II; Fe^{3+}, potassium ferricyanide; PEP, phosphoenolpyruvate.

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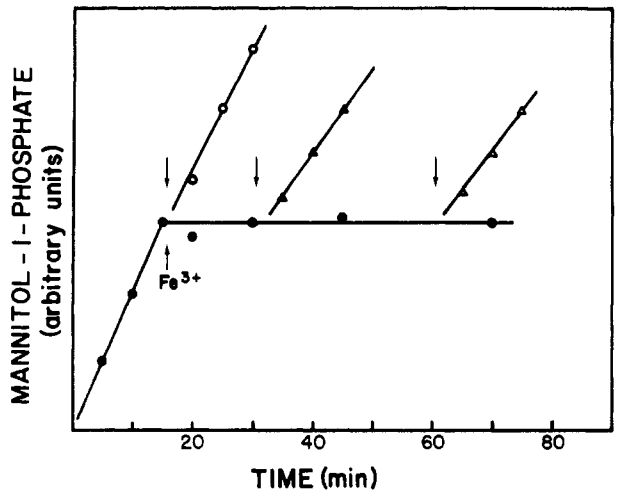

FIGURE 1: Effect of potassium ferricyanide on mannitol-1-P/[^{14}C]-mannitol transphosphorylation catalyzed by purified enzyme II^{mtl}. Ferricyanide (1.5 mM) was added to the reaction mixture 16 min after the reaction was initiated by addition of 2.5 μg of enzyme II^{mtl}. Dithiothreitol (3 mM) was added to aliquots of the reaction mixture at the times indicated by the upper arrows. The initial rate of transphosphorylation was 0.43 pmol min^{-1} (μg of enzyme II^{mtl})$^{-1}$.

zymes unless otherwise stated. Protein determinations were performed as described by Lowry et al. (1951).

Preparation of Vesicles. Escherichia coli strain ML 308/225 was grown aerobically in Luria broth containing 0.5% mannitol to the late logarithmic growth phase. The cells were harvested, and inverted vesicles were prepared as described by Reenstra et al. (1980). Membrane preparations were kept at −70 °C until used.

Enzyme II^{mtl} Assays. Phosphoenolpyruvate-dependent reactions were carried out in a 0.1-mL final volume containing 50 mM potassium phosphate buffer, pH 7.5, 10 mM $MgSO_4$, 10 mM KF, and 10 mM phosphoenolpyruvate. When purified enzyme II^{mtl} was used, 0.5% Lubrol-PX was also included. The dithiothreitol concentration in the assay mixture varied from 0 (vesicle experiments) to 0.2 mM in the experiments with purified enzyme II^{mtl}. Dithiothreitol at a low concentration was provided in the assay mixture to ensure reasonable rates of activity prior to addition of an excessive quantity of an oxidizing reagent. Purified enzyme II^{mtl} has extremely low activity in assay mixtures lacking a sulfhydryl reducing agent.

The concentrations of enzyme I and HPr used were 0.5–2.5 μM and 0.83–83 μM, respectively. In all cases, the enzyme II^{mtl} was the rate-limiting component. For transphosphorylation experiments, 1–10 mM mannitol 1-phosphate replaced phosphoenolpyruvate. Incubations were at 37 °C for 20–80 min and were stopped by addition of ice-cold water (3 mL). The [^{14}C]sugar–phosphate formed was separated from free sugar on Dowex (AG 1-X2) columns as previously described (Kundig & Roseman, 1971) and quantitated. In all cases, less than 20% of the [^{14}C]mannitol present was converted to mannitol 1-phosphate.

Enzyme I Assay. Enzyme I was assayed independently of enzyme II^{mtl} by measuring enzyme I catalyzed conversion of phosphoenolpyruvate to pyruvate during HPr phosphorylation. The general approach was as described by Waygood et al. (1979). However, [^{14}C]pyruvate production was followed with [^{14}C]phosphoenolpyruvate (Saier et al., 1980). These reactions were carried out in a 0.1-mL final volume containing 50 mM potassium phosphate buffer, pH 7.5, 10 mM $MgSO_4$, 10 mM KF, 6.5 μM HPr, and 0.1 mM phosphoenolpyruvate (0.1 μCi). Enzyme I (50 pmol) was preincubated in the assay mixture (without phosphoenolpyruvate and HPr) for 10 min prior to starting the reaction by addition of phosphoenolpyruvate and HPr.

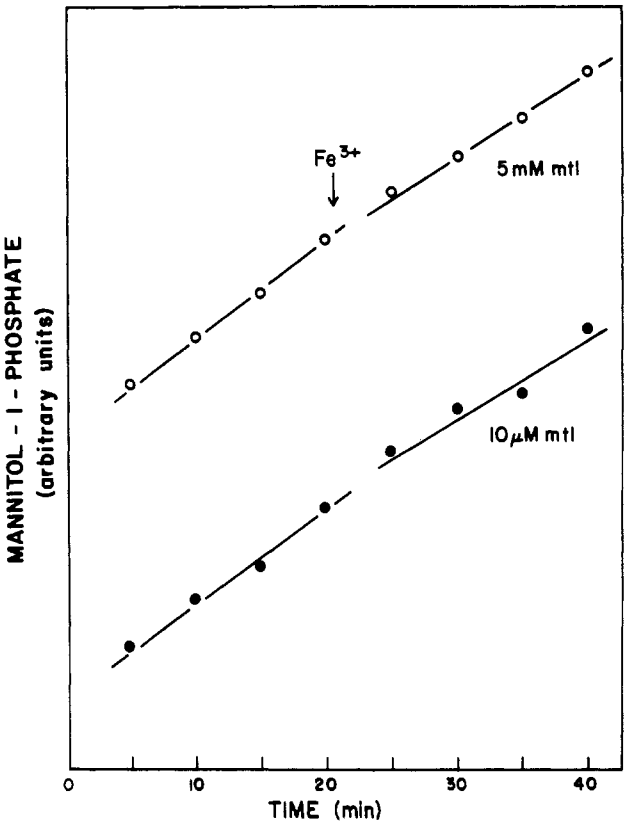

FIGURE 2: Effect of potassium ferricyanide on phosphoenolpyruvate-dependent enzyme II^{mtl} activity. The reactions at 5 mM and 10 μM mannitol were initiated by the addition of 1.2 μg and 6.0 ng of purified enzyme II^{mtl}, respectively. Enzyme I and HPr concentrations for these two reactions were 0.5 and 20 μM, respectively. Ferricyanide (1.5 mM) was added at the time indicated by the arrow. The initial rates of the two reactions were 0.3 nmol min^{-1} μg^{-1} (10 μM) and 0.75 nmol min^{-1} μg^{-1} (5 mM). Assay conditions were as described under Materials and Methods.

RESULTS

Effect of Potassium Ferricyanide on Enzyme II^{mtl} Transphosphorylation Activity. Figure 1 illustrates the effect of potassium ferricyanide on mannitol 1-P/[^{14}C]mannitol transphosphorylation activity catalyzed by purified enzyme II^{mtl}. Ferricyanide caused essentially complete inhibition of the activity. This inhibition was observed when either 1 or 10 mM mannitol 1-phosphate was used in the reaction mixture. Potassium ferrocyanide inhibited less than 10%. Inhibition by ferricyanide was relieved by dithiothreitol, but the extent of relief correlated with the duration of exposure to the oxidizing agent (Figure 1). Apparently, the oxidized enzyme is slowly converted to an irreversibly oxidized form. These experiments establish that ferricyanide acts directly on enzyme II^{mtl}.

Effect of Potassium Ferricyanide on Phosphoenolpyruvate-Dependent Enzyme II^{mtl} Activity. Figure 2 illustrates the effect of ferricyanide on phosphoenolpyruvate-dependent enzyme II^{mtl} activity. In these experiments, homogeneous enzyme II^{mtl}, enzyme I, and HPr were used. The concentrations of enzyme I and HPr were high (0.5 and 20 μM, respectively) relative to that of enzyme II^{mtl} (1 nM or 0.2 μM). On the basis of results reported with enzyme II^{glc} (Robillard & Konings, 1981), we expected to observe inhibition of the mannitol PTS activity by ferricyanide at low but not high mannitol concentrations. As the figure illustrates, only slight inhibition was observed upon addition of 1.5 mM ferricyanide

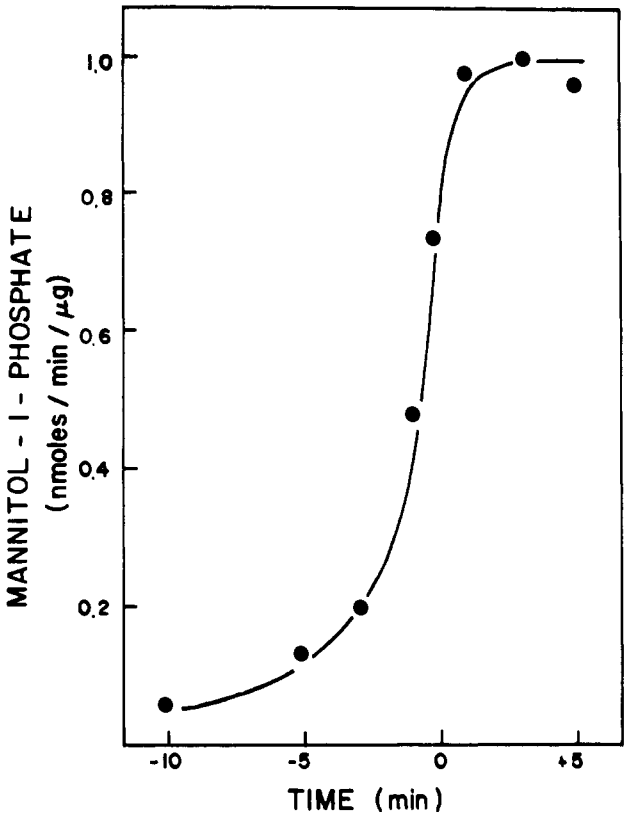

FIGURE 3: Effect of preincubation with potassium ferricyanide on the phosphoenolpyruvate-dependent enzyme II^{mtl} activity. Substrate and enzyme concentrations were as follows: mannitol, 5 mM; HPr, 40 μM; enzyme I, 1.5 μM. To 0.1-mL aliquots of purified enzyme II^{mtl} (0.8 μg) was added ferricyanide (1.5 mM) either before (−10 to 0 min) or after (0 to 5 min) initiation of the reaction. The reaction was started by addition of enzyme I, HPr, and [^{14}C]mannitol to the assay mixture as described under Materials and Methods. After a 30-min incubation period at 37 °C, the reactions were terminated, and the mannitol 1-phosphate formed was determined.

at either 10 μM or 5 mM mannitol. Even decreasing the concentration of mannitol to 2.5 μM did not result in any increased inhibition. Increasing the concentration of ferricyanide to 10 mM caused only a slight (10–15%) increase in inhibition. Control experiments with potassium ferrocyanide indicated that inhibition was not due to a nonspecific effect. Enzyme II^{mtl} was evidently more sensitive to oxidizing conditions when catalyzing transphosphorylation (Figure 1) than when catalyzing the phosphoenolpyruvate-dependent reaction (Figure 2).

The principle difference between the phosphoenolpyruvate-dependent and transphosphorylation reactions is the means of phosphorylating enzyme II^{mtl}, and the different phosphorylation mechanisms are most likely responsible for the different responses to ferricyanide. We have previously shown that the rate of transphosphorylation is very slow relative to the rate of the phosphoenolpyruyvate-dependent reaction (Saier et al., 1976, 1977). This slow rate presumably reflects the high activation energy for enzyme II^{mtl} phosphorylation by mannitol 1-phosphate. A much greater percentage of the enzyme II^{mtl} is presumably phosphorylated in the presence of phosphoenolpyruvate and saturating concentrations of the energy-coupling enzymes of the PTS. Phosphorylated enzyme II^{mtl} may be much less sensitive to ferricyanide oxidation, explaining the different degrees of inhibition observed in Figures 1 and 2.

To test this possibility, ferricyanide was added to the assay mixtures either before or after addition of the other PTS proteins. In this way, enzyme II^{mtl} was exposed to ferricyanide either in its unphosphorylated form or under conditions of rapid turnover. The results of such an experiment are reproduced in Figure 3. Preincubation of enzyme II^{mtl} with ferricyanide prior to addition of enzyme I rendered the enzyme inactive. At 1.5 mM ferricyanide, a preincubation period of 10 min was required to observe 95% inhibition of the activity. Phenazine methosulfate (300 μM) and plumbagin (300 μM) also caused approximately 90% inhibition under the same conditions. Addition of ferricyanide during active turnover of the enzyme caused only slight inhibition (Figure 3). The degree of inhibition observed following preincubation with ferricyanide was the same at mannitol concentrations of 1 μM, 2.5 μM, 5 μM, 10 μM, 25 μM, 5 mM, and 25 mM. Thus, high mannitol concentrations did not relieve the inhibitory effect of ferricyanide. Dithiothreitol (5 mM), however, completely reversed this effect. Qualitatively similar results were obtained when the source of enzyme II^{mtl} was membrane vesicles. These findings do not support a mechanism in which oxidized enzyme II exhibits lower affinity for its substrate than the reduced form. Oxidized enzyme II^{mtl} is apparently inactive under the conditions employed.

Table I: Protection of Enzyme II^{mtl} by Phosphorylation[a]

incubation order	enzyme II^{mtl} activity (%) 5-min intervals	1-min intervals
nothing/EI/mannitol	100	100
nothing/EI/mannitol/Fe^{3+}	87	89
EI/Fe^{3+}/mannitol	58	86
mannitol/Fe^{3+}/EI	5	37
Fe^{3+}/EI/mannitol	4	34

[a] Purified enzyme II^{mtl} (0.8–1.5 μg) was mixed with HPr (40 μM) and phosphoenolpyruvate (10 mM) in 50 mM potassium phosphate, pH 7.5, 10 mM $MgSO_4$, 10 mM KF, and 0.5% Lubrol-PX. To this mixture, enzyme I (1.5 μM), potassium ferricyanide (1.5 mM), and mannitol (5 mM) were added in the sequences shown. The assay mixture was incubated for 5 or 1 min prior to the addition of the next component as indicated. The reaction commenced upon addition of the last component except in the sequence EI/mannitol/Fe^{3+} in which ferricyanide was added 5 or 1 min after the start of the reaction. All reactions were stopped after a 30-min incubation period. Mannitol 1-phosphate formation is expressed as a percentage of the control rate, which was 0.70–1.5 nmol min^{-1} (μg of enzyme $II^{mtl})^{-1}$.

Protective Effect of Enzyme II^{mtl} Phosphorylation. The results of the experiment shown in Figure 3 suggested that phosphorylated enzyme II^{mtl} is insensitive to ferricyanide inhibition. In order to establish this fact, enzyme II^{mtl} was incubated with ferricyanide after preincubation with enzyme I, HPr, and phosphoenolpyruvate or before exposure to these agents. The concentrations of enzyme I and HPr were very high (1.5 and 40 μM, respectively; as in Figure 3). The possible effect of preincubation of enzyme II^{mtl} with mannitol (5 mM) was also examined. The results of these experiments are presented in Table I. After phosphorylation, ferricyanide inactivated approximately 40% (column 2, line 3) of the enzyme II^{mtl} activity under the conditions employed. The control experiment in which ferricyanide was added after the reaction was started by the addition of mannitol showed only 13% inhibition (column 2, line 2). These results indicate that while phosphorylating conditions partially protect the enzyme from oxidation, the presence of mannitol increases this protective effect, although mannitol alone does not protect the enzyme. Thus, the presence of mannitol can enhance the protective effect of phosphorylation.

The ratio of unphosphorylated to phosphorylated enzyme II^{mtl} can be varied in situ by varying the HPr concentration. In the presence of excess enzyme I, essentially all of the HPr is maintained as phospho-HPr. The overall rate of mannitol 1-phosphate formation under these conditions is dependent on

Table II: Inhibition of Enzyme II^{mtl} by Ferricyanide at Varying HPr Concentrations[a]

HPr (μM)	$-Fe^{3+}$	$+Fe^{3+}$	% inhibition
83	0.95	0.77	19
8.3	0.74	0.46	38
0.83	0.20	0.07	65

[a] Purified enzyme II^{mtl} (0.6–0.9 μg) was mixed with enzyme I (1.5 μM), mannitol (4 mM), and varying concentrations of HPr. The buffer and salt concentrations were as in Table I. The reactions were started by the addition of enzyme II and proceeded for 30 min at 37 °C. Three minutes after the initiation of the reaction, potassium ferricyanide (3 mM) was added to the appropriate samples. Mannitol 1-phosphate formation is expressed in nmol min^{-1} (μg of enzyme $II^{mtl})^{-1}$.

the rate of (1) phosphorylation of enzyme II^{mtl} by phospho-HPr and (2) dephosphorylation of phosphoenzyme II^{mtl} by mannitol. If, as is believed, enzyme II^{mtl} is phosphorylated on two distinct histidyl residues (E. B. Waygood, unpublished results), then the position(s) of phosphorylation in enzyme II^{mtl} may also influence reaction rate. At a high mannitol concentration (5 mM, 1000 times the K_m value for mannitol), the latter reaction proceeds at maximal velocity. As the HPr concentration decreases, however, the overall reaction rate will decrease because the phosphorylation of enzyme II becomes rate limiting. Decreasing the HPr concentration under these conditions also has the effect of increasing the unphosphorylated to phosphorylated enzyme II^{mtl} ratio. Thus, at lower HPr concentrations, one would expect increased inhibition by ferricyanide if unphosphorylated enzyme is more sensitive to oxidation than phosphorylated enzyme. The results summarized in Table II confirm this prediction. Decreasing the HPr concentration 100-fold (83–0.83 μM) caused an additional 46% inhibition of enzyme II^{mtl} activity.

The results shown in Figures 1 and 2 indicated that mannitol 1-phosphate was not an efficient phosphorylating agent. To confirm this, we determined whether preincubation with mannitol 1-phosphate (at 1 and 10 mM) protected the enzyme against oxidation. As expected, no protective effect was observed (data not shown).

Effect of Potassium Ferricyanide on Enzyme I Activity. The results in Figures 1 and 3 indicate that ferricyanide does not significantly inhibit enzyme I activity. Measuring enzyme I activity independently of enzyme II^{mtl} by assaying pyruvate production during HPr phosphorylation confirmed this conclusion. The rate of pyruvate production by enzyme I under the conditions described under Materials and Methods was 49 nmol min^{-1} (mg of enzyme I)$^{-1}$. Preincubation of enzyme I for 10 min with potassium ferricyanide (1.5 mM), plumbagin (300 μM), phenazine methosulfate (300 μM), or dithiothreitol (1 mM) gave rates that were ±10% of the control rate. Thus, under the conditions employed there is no evidence that enzyme I is inhibited by oxidizing conditions.

Discussion

The experiments presented in this paper were performed to determine if enzyme II^{mtl} is regulated by the oxidation–reduction potential as proposed for enzyme II^{glc} (Robillard & Konings, 1981). These investigators suggested that oxidized enzyme II^{glc} possesses low affinity for its sugar substrate while the reduced form of the enzyme possesses high affinity for its substrate. Our experiments with enzyme II^{mtl} indicate that this enzyme does not follow this pattern of regulation. In experiments with purified enzyme II^{mtl} or with membrane vesicles, we observed that potassium ferricyanide, phenazine methosulfate, and plumbagin inhibited the phosphorylation activity of the enzyme. Mannitol, at a concentration 5000-fold in excess of the K_m value, did not overcome these inhibitory effects, but dithiothreitol completely reversed inhibition. The simplest explanation for these observations is that oxidized enzyme II^{mtl} is not a low-affinity form of the enzyme but is inactive.

Under appropriate conditions, enzyme II^{mtl} could be rendered insensitive to inhibition by ferricyanide. Our results indicate that phosphorylated enzyme II^{mtl} is much less sensitive to inhibition than the unphosphorylated enzyme and that rapid turnover of the enzyme, promoted by the presence of mannitol, enhances this protective effect. The mechanism by which mannitol causes this additional protection may involve the formation of a phosphoenzyme II^{mtl}–mannitol reaction intermediate. The protective effect of mannitol in the presence of excess phospho-HPr provides the first evidence for such an intermediate. Because the enzyme II^{mtl} may be phosphorylated on two separate histidyl residues (E. B. Waygood, unpublished results), several possibilities for this intermediate state clearly exist.

The recently published work of Neuhaus & Wright (1984) appears to be relevant to the present studies. Previous studies had indicated that modifying reagents and thiol reagents inhibit the lactose permease by a K_m-type mechanism analagous to that proposed for enzyme II^{glc}. Neuhaus and Wright, however, obtained no evidence for distinct high- and low-affinity forms of the lactose permease. In agreement with the results reported here for enzyme II^{mtl}, these workers concluded that the modified permease was completely inactive. It should be noted, however, that Neuhaus and Wright measured binding by flow dialysis, which might not detect low-affinity binding of substrate to the lactose permease.

On the basis of results obtained with purified proteins of the PTS, we feel that it is necessary to reevaluate the results of Robillard & Konings (1981) obtained with crude enzyme II^{glc} preparations. The apparent K_m shift observed by these workers in some experiments may have been due in part to conditions in which elevated levels of substrate or phosphorylation potential prevented oxidation of enzyme II^{glc}. We have observed that both HPr and sugar concentrations effect the degree of inhibition observed after ferricyanide treatment. Nevertheless, the results presented here cannot adequately explain all of the data of Robillard and Konings. It is, of couse, possible that enzyme II^{glc} and enzyme II^{mtl} are simply regulated differently by sulfhydryl oxidation. Further studies will be required to establish the mechanistic and physiological significance of sulfhydryl oxidation in the enzymes II of the PTS as well as in other permeases.

Added in Proof

We have recently established that the glucose enzyme II (like enzyme II^{mtl}) is oxidized to a fully inactive form. The low-affinity methyl α-glucoside phosphorylation activity is attributable to the mannose enzyme II (unpublished results).

Registry No. Enzyme II, 37278-09-4; phosphoenolpyruvate-sugarphosphotransferase, 56941-29-8; potassium ferricyanide, 13746-66-2.

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Phosphoenolpyruvate Synthetase and Pyruvate, Orthophosphate Dikinase: Stereochemical Consequences at both the β-Phospho and γ-Phospho Groups of ATP†

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ABSTRACT: [(*R*)-^{16}O,^{17}O,^{18}O]Phosphoenolpyruvate and adenosine 5′-*O*-[(γS)-$\beta\gamma$-^{17}O,γ-^{17}O,^{18}O](3-thiotriphosphate) have been synthesized and used to determine the stereochemical course of the several displacements at phosphorus catalyzed by phosphoenolpyruvate synthetase and by pyruvate, orthophosphate dikinase, two enzymes that catalyze the formation of phosphoenolpyruvate from pyruvate and ATP. The catalytic mechanisms for each of these enzymes are believed to involve both phospho- and pyrophospho-enzyme intermediates. The stereochemical results are entirely in accord with these pathways: the β-phospho group of ATP suffers overall retention of configuration that is presumably the consequence of two displacements with inversion, and the γ-phospho group of ATPγS suffers inversion of configuration that is most probably the consequence of a single displacement at this center.

The energetically favorable synthesis of phosphoenolpyruvate (PEP)[1] from pyruvate and ATP in extracts of *Escherichia coli* was first reported by Cooper & Kornberg (1965). The reaction yielded AMP and P_i as well as PEP, and the enzyme

$$\text{ATP} + \text{pyruvate} + H_2O \rightleftarrows \text{AMP} + \text{PEP} + P_i \quad (1)$$

involved has been named phosphoenolpyruvate synthetase (EC 2.7.9.2). In 1968, three groups (Hatch & Slack, 1968; Reeves, 1968; Reeves et al., 1968; Evans & Wood, 1968) reported a reaction that occurs in various microorganisms and several

$$\text{ATP} + \text{pyruvate} + P_i \rightleftarrows \text{AMP} + \text{PEP} + PP_i \quad (2)$$

plants that yields PEP, AMP, and PP_i. The enzyme catalyzing this reaction was named pyruvate, orthophosphate dikinase (EC 2.7.9.1) (Wood et al., 1977).

The initial proposals for the reaction sequence of PEP synthetase from *E. coli* and *Salmonella typhimurium* (Cooper & Kornberg, 1967a-c) and of pyruvate, orthophosphate dikinase from *Propionibacteria shermanii* (Evans & Wood, 1968) suggested three partial reactions for each of these two enzymes: eq 3, 4a, and 5 and eq 3, 4b, and 5, respectively.

$$\text{ATP} + \text{enzyme} \rightleftarrows \text{AMP} + \text{enzyme}\text{–}P_\beta P_\gamma \quad (3)$$

$$H_2O + \text{enzyme}\text{–}P_\beta P_\gamma \rightleftarrows P_{\gamma i} + \text{enzyme}\text{–}P_\beta \quad (4a)$$

$$P_i + \text{enzyme}\text{–}P_\beta P_\gamma \rightleftarrows P_\gamma P_i + \text{enzyme}\text{–}P_\beta \quad (4b)$$

$$\text{pyruvate} + \text{enzyme}\text{–}P_\beta \rightleftarrows \text{PEP} + \text{enzyme} \quad (5)$$

These mechanistic schemes were proposed primarily on the basis of investigations of the following exchange reactions: [^{14}C]AMP/ATP; $H_2{}^{18}O/P_i$; [^{32}P]P_i/PP_i; [^{32}P]PEP/ATP; [^{32}P]P_i/ATP; [^{32}P]PP_i/ATP; [^{14}C]pyruvate/PEP (Cooper &

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[1] Abbreviations: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$; PEP, phosphoenolpyruvate; P_i, orthophosphate; PP_i, pyrophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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