hydrodynamic radius determined from the diffusion coefficient and Stokes' law.

The area of the reactive patch, assumed equal for heads and tails, is

$$A_P = R_i^2 \int_0^{2\pi} d\phi \int_0^{\theta_i} \sin\theta \, d\theta = 2\pi R_i^2(1 - \cos\theta_i) \quad \text{(A1)}$$

where i = H or T. Thus the reactive fraction of the total surface area of each sphere is

$$F_i = A_P/4\pi R_i^2 = (1 - \cos\theta_i)/2 \quad \text{(A2)}$$

and the probability that the two patches will be opposite each other is

$$F = F_H F_T = (1 - \cos\theta_H)(1 - \cos\theta_T)/4 \quad \text{(A3)}$$

The overall deviation of the observed bimolecular rate constant from the value for k predicted by eq 8 is the product of three factors:

$$k_{obsd} = kf\delta F \quad \text{(A4)}$$

The first of these, the electrostatic factor f, is evaluated by using eq 9–11. At ionic strength 0.08 M, 293 K, and for the charge product $z_1z_2 = -60$, which gives the best fit to the data in Figure 6, we obtain an acceleration of $f = 9.1$. The second factor, the enhancement of the reaction rate due to rotational diffusion of the reactants, was estimated as $\delta = 2$ from Table I of Schmitz & Schurr (1972).

Since under these conditions of ionic strength and temperature, $k_{obsd}/k = {}^1/_{675}$, we find $F = {}^1/_{12\,300}$. With $R_H = 595$ Å and $R_T = 357$ Å and noting that $R_H^2(1 - \cos\theta_H) = R_T^2(1 - \cos\theta_T)$, we find $\theta_H = 8.5°$ and $\theta_T = 14.2°$. These give a reactive patch area A_P of 2.45×10^4 Å^2.

Reference

Schmitz, K. S.,* & Schurr, J. M. (1972) *J. Phys. Chem. 76*, 534–545.

Physical Mechanism for Regulation of Phosphoenolpyruvate-Dependent Glucose Transport Activity in *Escherichia coli*†

G. T. Robillard* and W. N. Konings

ABSTRACT: The activity of the phosphoenolpyruvate-dependent glucose phosphotransferase system (PTS) in *Escherichia coli* is coupled to the oxidation–reduction potential. It is inhibited when the redox potential is increased above −300 mV either via substrate oxidation or via direct addition of oxidizing agents. Depending on the point of addition, dithiothreitol either blocks or reverses these effects. Inhibition occurs at the level of sugar binding to E_{II}. A sulfhydryl group associated with E_{II} activity undergoes reversible oxidation to, presumably, a disulfide, resulting in the conversion of E_{II} from a reduced, high-affinity form to an oxidized, low-affinity form which has a 10^2–10^3 times lower affinity for the sugar. An identical change in affinity occurs as the result of the generation of a $\Delta\bar{\mu}_{H^+}$ during the oxidation of reduced *N*-methylphenazonium methosulfate or nicotinamide adenine dinucleotide. In this case, uncouplers and ionophores reverse the change. A mechanism is proposed in which the electrical potential difference across the membrane regulates the glucose PTS by shifting the midpoint potential of the E_{II}-associated redox transition to more negative values. As a result, E_{II} is converted to the oxidized, low-affinity state in the presence of a $\Delta\bar{\mu}_{H^+}$.

The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) of *Escherichia coli* couples the transport of glucose and certain other hexoses to the hydrolysis of phosphoenolpyruvate (PEP)[1] via a series of phosphoryl-group transfer reactions

$$\text{PEP} + \text{HPr} \underset{Mg^{2+}}{\overset{E_I}{\rightleftharpoons}} \text{P-HPr} + \text{Pyr}$$

$$\text{P-HPr} + \text{hexose}_{out} \underset{Mg^{2+}}{\overset{F_{III},\,E_{II}}{\rightleftharpoons}} \text{HPr} + \text{hexose-P}_{in}$$

[for a review, see Postma + Roseman (1976)]. This system has been implicated in the control of transport of non-PTS substrates by a variety of mechanisms. It exerts control either by inhibiting synthesis of enzymes required for the transport and metabolism of non-PTS substrates (catabolite repression) (Magasanik, 1970) or by regulating the activity of already existing transport systems. McGinnis & Paigen (1969) have shown, for instance, that addition of glucose to cells growing on various carbon sources such as glycerol or lactose resulted in inhibition of the utilization of these solutes. Even though the activity of these systems is somehow controlled by the PTS, the activity of the PTS itself can be modulated when transport of some of these same solutes occurs via secondary transport systems. The physical mechanism for the mutual regulation of these various transport activities will be treated in this report.

Early studies (Englesberg et al., 1961; Hoffee & Englesberg, 1962; Hoffee et al., 1964; Hagihira et al., 1963) of αMGlc

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[1] Abbreviations used: αMGlc, methyl α-glucoside; αMG-P, methyl α-glucoside 6-phosphate; Asc, ascorbate; PMS, *N*-methylphenazonium methosulfate; NEM, *N*-ethylmaleimide; DCPIP, 2,6-dichlorophenolindophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, *p*-(trifluoromethoxy)phenylhydrazone; DTT, dithiothreitol; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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transport by *E. coli* or *Salmonella typhimurium* demonstrated that the *steady-state levels* of αMGlc accumulation reached in intact cells could be suppressed by addition of an exogenous energy source capable of being metabolized by the cell. The decreased steady-state levels were attributed to an increased rate of exit of αMGlc. Since this effect could be reversed by addition of the respiratory chain inhibitor azide and the uncoupler 2,4-dinitrophenol or by anaerobiosis, the concept was advanced that there was an energy-dependent reaction for the exit of αMGlc. More recent studies have focused on the effect of exogenous energy sources on the *initial rates* of αMGlc transport in intact cells (Hernandez-Asensio et al., 1975; del Campo et al., 1975). These investigations showed that substrates which stimulated respiration inhibited αMGlc uptake. The measured exit rates, however, were too slow to attribute inhibition of αMGlc uptake to increased exit rates. Rather, the inhibition was found to originate from an increased apparent K_m for αMGlc binding. The inhibition induced by respirable substrates was abolished, with a concomitant drop in the K_m, by the uncoupler CCCP or the inhibitor azide, supporting the concept that respiration somehow inhibits PTS activity, possibly by bringing the membrane into an energized state. Subsequently Singh & Bragg (1976), using cytochrome-deficient mutants, demonstrated that when substrate oxidation was blocked by the absence of cytochromes, αMGlc transport could still be inhibited if an energized membrane state was generated by ATP hydrolysis via the membrane-bound ATPase complex. ATPase inhibitors or uncouplers prevented the inhibition.

Reider et al. (1979) attempted to simplify the problems surrounding the regulation of PTS activity by working with membrane vesicles rather than whole cells. Employing right-side-out membrane vesicles, they demonstrated that oxidation of D-lactate via the respiratory chain inhibited αMGlc uptake. This inhibition was reversed by KCN, a respiratory chain inhibitor. Similar effects were measured for other PTS sugars. One can safely conclude from this catalog of data that sugar transport by the PTS is somehow modulated by the activity of the respiratory chain, possibly via a proton motive force generated by substrate oxidation.

All studies so far have been executed on intact cells or membrane vesicles where one is confronted with the complicated scenario of competing uptake and exit reactions which restrict experimental design and complicate data interpretation. A more direct and flexible approach is possible. The PTS phosphorylates as well as transports its sugars. While transport without phosphorylation does not occur (Postma & Stock, 1980), phosphorylation in the absence of transport can be monitored (Kundig & Roseman, 1971) by using solubilized membrane fragments or inside-out membrane vesicles. This provides a simple, readily controllable system for detailed kinetic studies of the E_{II}-catalyzed reaction. When inside-out vesicles (Reenstra et al., 1980) in the presence of excess pure HPr and E_I (Dooyewaard et al., 1979; Robillard et al., 1979) are used, we can determine which step, phosphorylation or transport, is modulated by the oxidation of respiratory chain substrates. If only transport is modulated, no inhibition of sugar phosphorylation will be observed with inside-out vesicles. Conversely, if phosphorylation is sensitive, inhibition will be observed. Furthermore, if inhibition is observed then, with the help of ionophores and uncouplers, we should be able to decide whether inhibition stems from a $\Delta\bar{\mu}_{H^+}$ or one of its components, the pH gradient across the membrane (ΔpH) or the transmembrane electrical potential (ΔΨ) (Mitchell, 1968; Konings & Michels, 1980).

The data presented below show that the activity of the PTS is directly modulated by a change in the oxidation state of the membrane-bound E_{II} which can be effected either by direct addition of oxidizing and reducing agents or by a transmembrane electrochemical potential resulting from substrate oxidation.

Material and Methods

HPr and E_I were purified from *E. coli* P 650 as described previously (Dooyewaard et al., 1979; Robillard et al., 1979).

E_{II}. Inside-out membrane vesicles containing E_{II} were prepared from *E. coli* ML 308-225 as described by Reenstra et al. (1980). The carbon source was 0.4% glucose. The membrane preparation was kept in liquid N_2 until used.

Rates of Sugar Phosphorylation. In preliminary experiments (not shown), we determined the dependence of the phosphorylation rate on the concentration of each PTS component, HPr, E_I, or E_{II}, at fixed concentrations of the other two components. All rates reported below were measured in the range where E_{II} was rate limiting and at saturating HPr and E_I concentrations. To be specific, HPr ranged from 3 to 20 μM, E_I from 0.05 to 0.4 μM, and E_{II} from 5 to 80 μg of membrane protein/mL of reaction volume. E_I is unstable in the absence of DTT. Nevertheless, for reasons which will become obvious, it was necessary to measure phosphorylation rates in the absence of DTT. Therefore, E_I levels at least 10 times higher than the measured saturation levels were employed in all experiments. Even if partial inactivation of E_I occurred during the course of the reaction, sufficient active E_I remained to be in excess over E_{II}. Since all E_{II} limited rate measurements were linear with time, we can conclude that active E_I was in excess throughout the measurements.

Reactions were carried out in 50-mL beakers agitated on a rotary shaker in a thermostated water bath when oxygenation was required; otherwise, they were carried out in test tubes in a thermostated water bath. At the stated time intervals, aliquots were withdrawn from the reaction mixture, pipetted directly onto 1-mL columns of Dowex AG 1-X2, and washed with 20 mL of water. The sugar phosphate was then eluted into scintillation vials with 12 mL of 0.1 N HCl and counted in a liquid scintillation counter after the addition of 8 mL of Packard emulsifier–scintillator. The counting efficiency was 60%. Unless otherwise stated, the reactions were carried out at pH 7.5, 25 °C, in 50 mM potassium phosphate buffer containing 10 mM $MgSO_4$, 10 mM KF, and 5 mM PEP (potassium salt).

Protein concentrations were determined by the method of Lowry et al. (1951). Radioactive sugars were purchased from the Radiochemical Centre, Amersham. Phosphoenolpyruvate (potassium salt) was purchased from Sigma. 1,2-Naphthoquinone was a gift of the Department of Organic Chemistry, University of Groningen. All other chemicals were analytical grade and obtained from commercial sources.

Results

Effect of Respiratory Chain Substrates on the Phosphorylation Activity of E_{II}. Reduced PMS, D-lactate, NADH, and succinate can be enzymatically oxidized by both inside-out and right-side-out bacterial membrane vesicles (Konings et al., 1971; Reenstra et al., 1980; Ramos & Kaback, 1977). Under proper conditions, the oxidation of these substrates leads to the establishment of a $\Delta\bar{\mu}_{H^+}$ across the membrane which can drive secondary transport of many solutes. The effect of some of these substrates on the rate of phosphorylation of αMGlc is presented in Figure 1. It shows that Asc-PMS, at concentrations sufficient to sustain a $\Delta\bar{\mu}_{H^+}$ across the mem-

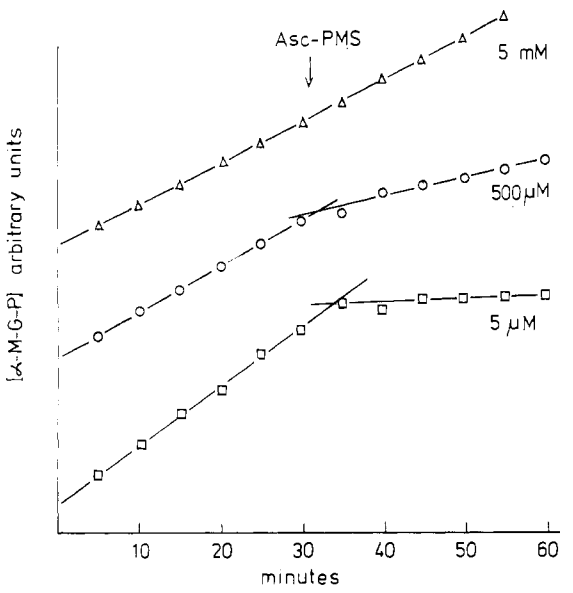

FIGURE 1: Rate of formation of αMGP by inverted membrane vesicles using the initial concentrations of αMGlc indicated at the right-hand side of each curve. At $T = 31$ min, ascorbate and PMS were added to final concentrations of 20 mM sodium ascorbate and 5 μM PMS. The specific activity of αMGlc ranged from 90 μCi/mmol to 184 mCi/mmol as required. The concentration of E_{II} was adjusted in each experiment to ensure that no more than 10% of the total sugar was converted to sugar phosphate during the course of the reaction. Since the concentration of E_{II} differs for each curve, comparison of rates between separate curves cannot be made from the graph itself. The rates for the first 30 min of each measurement are the following: 5 mM αMGlc, 8.2 nmol of αMG-P min^{-1} (mg of protein)$^{-1}$; 500 μM αMGlc, 2.4 nmol of αMG-P min^{-1} (mg of protein)$^{-1}$; 5 μM αMG, 0.28 nmol of αMG-P min^{-1} (mg of protein)$^{-1}$.

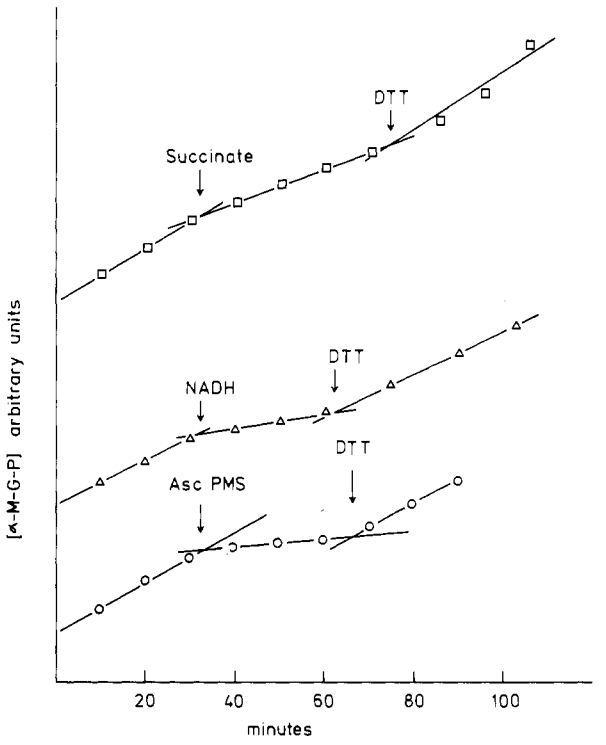

FIGURE 2: Influence of oxidizable substrates and DTT on the rate of formation of αMG-P by inverted membrane vesicles. NADH curve: 5 mM NADH, 2 mM DTT, and 5 μM αMGlc; specific activity 93 mCi/mmol. Succinate curve: 10 mM succinate, 10 mM DTT, and 3.7 μM αMGlc; specific activity 185 mCi/mmol. Asc-PMS curve: 2.5 mM Asc, 5 μM PMS, 3 mM DTT, and 5 μM αMGlc; specific activity 93 mCi/mmol.

brane, has little or no effect on the rate of αMGlc phosphorylation when this sugar is present at high concentrations. When the initial concentration of αMGlc is decreased, however, addition of Asc-PMS results in inhibition. The lower the initial sugar concentration, the stronger the inhibition. These data indicate that the oxidizable substrate influences predominantly the K_m for binding of PTS sugars to E_{II}. Quantitative data supporting this conclusion will be presented further on. Oxidation of NADH or succinate produces the same effect as Acs-PMS at low αMGlc concentrations (Figure 2). Parallel polarographic measurements confirmed that the substrates were being oxidized in the presence of the vesicles. Later in this paper, we will show that inhibition similar to that observed here for αMGlc can be measured with other PTS sugars such as D-fructose, 2-deoxy-D-glucose, and *N*-acetylmannosamine.

How Is Inhibition Achieved? (*1*) *Effect of Oxidizable Substrates.* The experiments in Figure 1 indicate that the oxidation of a respiratory chain substrate inhibits PTS activity by raising the K_m of E_{II} for αMGlc. However, we do not know what causes the change in K_m. It has been proposed that the change might be generated by a $\Delta\bar{\mu}_{H^+}$ established across the membrane as a result of substrate oxidation (Reider et al., 1979); thus the response of the Asc-PMS-induced inhibition to uncouplers and ionophores was monitored. The data are summarized in Table I. In these experiments, Asc-PMS was added to the complete PTS mixture lacking αMGlc. After preincubation, αMGlc was added. The resulting rate of phosphorylation was 22% that of the control. When uncouplers or ionophores were added after Asc-PMS but before αMGlc, stimulation back to between 50% and 65% of the control rate was observed. Complete reversal of the inhibition, however, was never achieved because higher concentrations of ionophores and uncouplers also inhibited αMGlc phosphorylation. A similar reversal of NADH-dependent inhibition was obtained in the presence of KCN, HOQNO, and CCCP together. From these data, we can conclude that a $\Delta\bar{\mu}_{H^+}$ resulting from substrate oxidation can inhibit αMGlc phosphorylation with inverted membrane vesicles.

Table I: Influence of Oxidizable Substrates and Ionophores on αMGlc Phosphorylation

additions	rate[a]	control rate (%)
no additions	0.27	100
2.5 mM Asc	0.26	96
2.5 mM Asc, 5 μM PMS	0.06	22
2.5 mM Asc, 5 μM PMS, 1 μM FCCP	0.14	52
2.5 mM Asc, 5 μM PMS, 1 μM nigericin	0.14	52
2.5 mM Asc, 5 μM PMS, 2 μM valinomycin	0.17	64
5 μM PMS	0.17	64

[a] Rates are given as nmol of αMG-P min^{-1} (mg of protein)$^{-1}$. Measurements were done at 25 °C in 50 mM potassium phosphate buffer, pH 7.5, 10 mM KF, 10 mM $MgSO_4$, and 5 mM PEP (potassium salt). The initial αMGlc concentration was 5 μM with a specific activity of 93 mCi/mmol. The HPr, E_I, and E_{II} concentrations were 5.2 μM, 55 nM, and 75 μg of protein/mL, respectively. The above components except for αMGlc were preincubated 30 min at 25 °C, after which ascorbate and/or PMS was added. After 5-min incubation, uncouplers and ionophores were added, and after a further 5-min incubation, sugar phosphorylation was initiated by the addition of αMGlc. After 30 min, the phosphorylation reaction was terminated by adding ice-cold water and immediately loading the diluted reaction mixture on Dowex columns as described under Materials and Methods.

(*2*) *Effects of Oxidants and Reductants.* The observation in Table I that low concentrations of PMS alone inhibited αMGlc phosphorylation was entirely unexpected. PMS is autooxidizable and, in the absence of excess ascorbate, will be maintained in the oxidized form by reaction with O_2. Since,

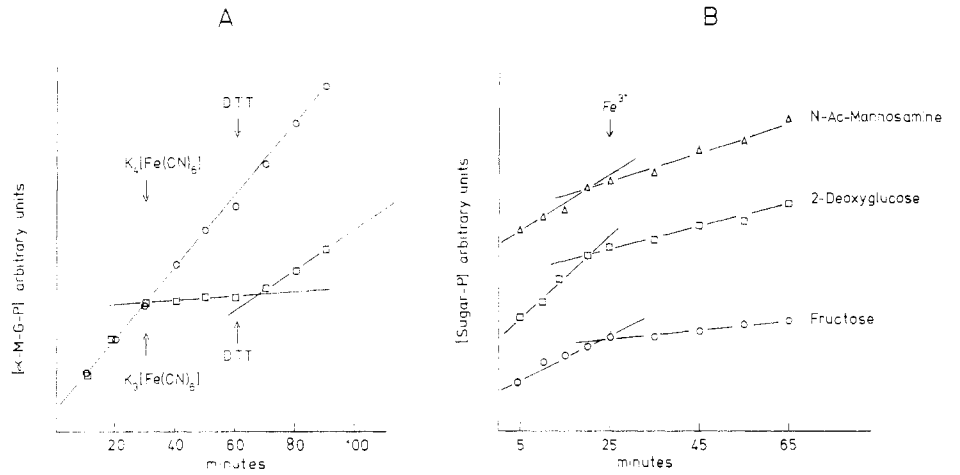

FIGURE 3: (A) Effect of the addition of 3 mM potassium ferrocyanide (○) or 3 mM potassium ferricyanide (□) on the rate of αMGlc phosphorylation by inverted membrane vesicles. The initial αMGlc concentration was 5 μM, and the specific activity was 69 mCi/mmol. At 61 min, the reaction mixtures were made 2 mM in DTT. (B) Effect of the addition of 1 mM potassium ferricyanide on the rate of phosphorylation of D-fructose, 2-deoxyglucose, and *N*-acetylmannosamine at pH 7 by inverted membrane vesicles. The specific activities, initial concentrations of the sugars, and the initial rates before the addition of ferricyanide were the following: D-fructose, 301 mCi/mmol, 1.65 μM, and 0.17 nmol min^{-1} (mg of protein)$^{-1}$; 2-deoxyglucose, 58.5 mCi/mmol, 9 μM, and 5.88 nmol min^{-1} (mg of protein)$^{-1}$; *N*-acetylmannosamine, 254 mCi/mmol, 1.9 μM, and 0.369 nmol min^{-1} (mg of protein)$^{-1}$.

in this form, it could not serve as an oxidizable substrate, the preliminary conclusion was that not only oxidizable substrates but also the oxidized product could inhibit the PTS activity. The inhibition could be prevented if DTT was added before PMS and reversed if it was added after PMS.

The concept of an oxidized substrate inhibiting the PTS was supported by experiments with solubilized membranes. When the closed structure of the vesicle was disrupted by 5% deoxycholate or 1% Triton X-100, inhibition of αMGlc phosphorylation in the presence of Asc-PMS could still be measured, and complete reversal could be achieved with DTT. In this case, there can be no question of a $\Delta\bar{\mu}_{H^+}$ arising from substrate oxidation, so one is forced to attribute the inhibition to the presence of dehydroascorbate on oxidized PMS.

The effect of DTT on the inhibition of PTS activity by oxidants is parallel to that measured when the inhibition was generated by oxidizable substrates. As shown in Figure 2, DTT was capable of completely reversing the Asc-PMS-induced inhibition as well as inhibition generated by NADH and succinate oxidation. Recovery of the initial activity could be achieved even with time spans as long as 1 or 2 h between the addition of the oxidizable substrates and the reducing agent. When DTT was added prior to the oxidizable substrate, no inhibition occurred.

Coupling between the Oxidation–Reduction Potential and the PTS Activity. The unexpected influence of oxidants and reductants on E_{II} activity and the ability of reducing agents to reverse the $\Delta\bar{\mu}_{H^+}$-generated PTS inhibition suggest that the $\Delta\bar{\mu}_{H^+}$ and oxidizing agents may be affecting the PTS by the same physical mechanism. For this reason, the coupling between E_{II} activity and the redox potential was examined in more detail. Figure 3A presents the results of directly altering the redox potential by addition of ferri- or ferrocyanide. In keeping with our hypothesis, addition of 3 mM ferricyanide results in complete inhibition of PTS-dependent αMGlc phosphorylation. Subsequent addition of 2 mM DTT restores most of the original activity. When added in excess of ferricyanide, DTT restores the activity completely. In contrast to these findings, addition of 3 mM ferrocyanide in a separate experiment has no effect on the PTS activity. Similar experiments have been carried out by monitoring other PTS substrates (see Figure 3B). The rates of phosphorylation listed in the figure legend are similar to those observed for similar concentrations of αMGlc. Inhibition was observed for all

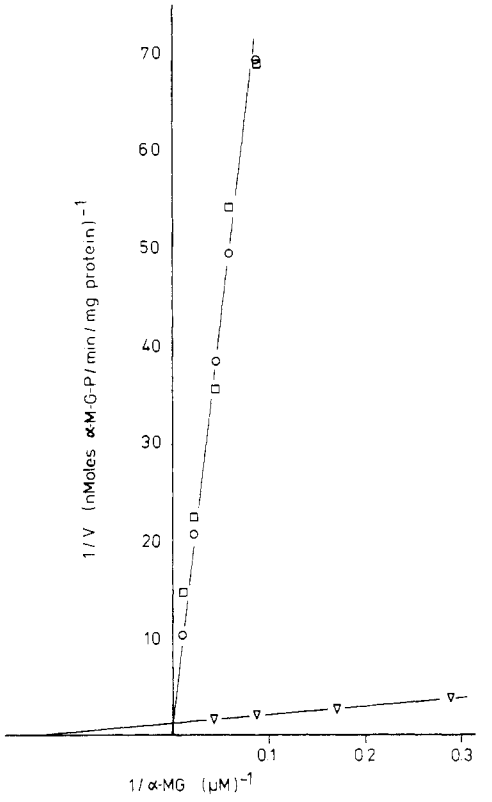

FIGURE 4: Double-reciprocal plot of the initial velocity of αMGP formation vs. the initial αMGlc concentration in the presence of 1.5 mM $K_3[Fe(CN)_6]$ (□) or 10 mM sodium ascorbate + 10 μM PMS (○). No oxidants were present in the control (▽). Measurements were performed at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM potassium phosphoenolpyruvate, 10 mM $MgSO_4$, 10 mM KF, 0.082 μM E_I, 3.3 μM HPr, and 57 μg of membrane protein/mL of assay volume. The reaction mixtures were incubated at 25 °C for 5 min in the presence of Fe^{3+} or Asc, PMS, and O_2 prior to measuring the phosphorylation rates. Phosphorylation was initiated by subsequently adding αMGlc.

substrates at initial concentrations of 10 μM or less. Thus, the inhibition is common to several PTS sugars, not only glucose analogues.

Quantitative information concerning the effect of adding oxidizable substrates or Fe^{3+} is presented in the double-reciprocal plot in Figure 4. The initial rates of αMGlc phos-

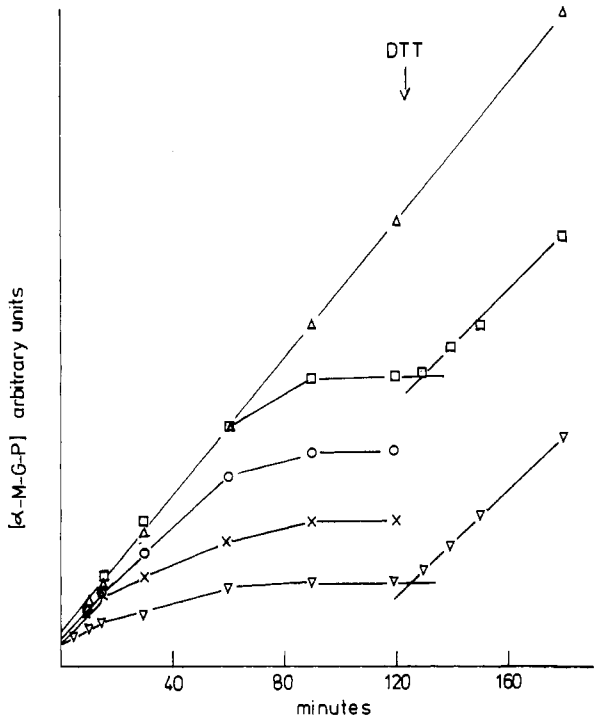

FIGURE 5: Influence of various concentrations of potassium ferricyanide on the rate of αMGP formation. (△) Zero K_3 [$Fe(CN)_6$]; (□) 66 μM; (○) 80 μM; (×) 106 μM; (▽) 120 μM. The reactions were carried out at pH 7, 37 °C, by using an initial αMGlc concentration of 7.5 μM with a specific activity of 185 mCi/mmol. All components except αMGlc and ferricyanide were preincubated 10 min at 37 °C before the start of the reaction. At $T = 0$, potassium ferricyanide was added, followed immediately by αMGlc. Where indicated, DTT was added to a final concentration of 2.5 mM. See Materials and Methods for further details.

phorylation in an E_{II} rate-limited system were measured over the αMGlc concentration range 5–90 μM. The apparent K_m of αMGlc for E_{II} in the absence of Fe^{3+} or a $\Delta\bar{\mu}_{H^+}$ is 7 μM. When the same measurements were carried out either in the presence of 1.5 mM ferricyanide or in the presence of 10 mM Asc plus 10 μM PMS, identical results were obtained, as shown by the superimposed curves for the two sets of measurements in Figure 4. The apparent K_m for αMGlc increased at least 100-fold, but no change was observed in the V_{max}.

Figure 5 presents the rate of αMGlc formation as a function of the ferricyanide concentration. The control, no ferricyanide, is linear over the entire span of the experiment. In the presence of ferricyanide, we observed decreased initial rates of phosphorylation and eventual plateauing of the curves when complete inhibition was achieved. The higher the ferricyanide concentration, the more rapid the inhibition. This response of the enzyme activity to the ferricyanide concentration is similar to the pattern of inhibition normally observed for inactivation of a protein via covalent chemical modification. It is different from the competitive inhibition which one finds for the noncovalent binding of an inhibitor in a substrate binding site. Figure 5 also shows that the inhibition at the lowest and highest ferricyanide concentration is completely reversed by the addition of 2.5 mM DTT.

The experiments presented in Figures 3–5 support the suggestion that the PTS is sensitive to the redox potential of the solution. Confirmation of this hypothesis can be found in the data presented in Table II. We have tested, in addition to the ferricyanide–ferrocyanide couple, 12 other biological and nonbiological redox reagents whose midpoint potentials range from −440 to +293 mV. Each oxidizing agent was tested at various concentrations in a manner analogous to that

Table II: Effect of Redox Reagents on E_{II}-Dependent αMGlc Phosphorylation

redox reagents	$E^{0'}$ (mV)[a]	inhibition
methylviologen	−440	−
benzylviologen	−360	−
NAD^+	−320	±
riboflavin	−208	+
plumbagin	−150	+
methylene blue	+11	+
PMS	+80	+
1,2-naphthoquinone	+143	+
DCPIP	+217	+
Fe^{3+} cytochrome *c*	+254	+
1,4-benzoquinone	+293	+
$CuCl_2$	+337 (E^0)	+
$Fe(CN)_6^{3-}$	+360	+

[a] $E^{0'}$ obtained from Sober (1968).

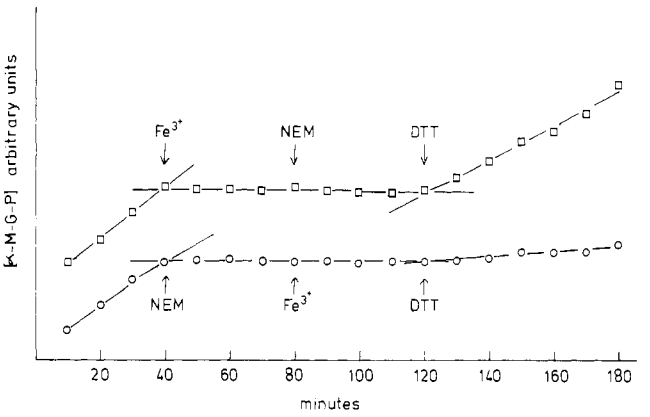

FIGURE 6: Influence of the order of addition of ferricyanide and *N*-ethylmaleimide on the E_{II}-catalyzed αMGlc phosphorylation. The reactions were carried out at pH 7, 25 °C. αMGlc was present at an initial concentration of 3.7 μM with a specific activity of 92 mCi/mmol. The reaction mixtures were preincubated 10 min at 25 °C before starting the experiment. The reactions were initiated by adding αMGlc. At the indicated times, ferricyanide, NEM, and DTT were added to final concentrations of 1, 1, and 5 mM, respectively.

used for the experiments reported in Figure 5. As shown in Table II, inhibition of PTS activity was observed at concentrations between 10 μM and 1 mM for all agents with redox potentials of −208 mV or higher. Subsequent addition of excess DTT completely reversed the inhibitions except in the case of the quinones where only partial reversal was observed. No inhibition was observed for benzylviologen or methylviologen while only slight inhibition was detected with 5 mM NAD^+. From these data we can tentatively conclude that the midpoint potential of the process modulating E_{II} activity is between −330 (the midpoint potential of DTT) and −200 mV.

Mechanism of Inhibition of PTS Activity. So far we have demonstrated that there is a component in the membrane associated with the PTS activity which is responsive to the oxidation–reduction potential of its surroundings. At low redox potentials, this component appears to be in the reduced form, and E_{II} exhibits a high affinity for PTS sugars. At high redox potentials, this component is oxidized, and the affinity for PTS sugars sharply decreases.

Since many membrane proteins including E_{II} possess sensitive sulfhydryl groups and since the midpoint potential of the inhibitory process would be consistent with an −SH to −SS− oxidation, we propose that inhibition occurs via an oxidation of two spatially adjacent SH groups which, at low redox potential, are in the reduced form. When the redox potential is elevated, the two groups are oxidized to a disulfide. The experiment reported in Figure 6 is a protection experiment

designed to test this hypothesis. The rationale is that if the oxidizable group is an SH it should be protected from reaction with *N*-ethylmaleimide when first oxidized to the –SS– form by Fe^{3+}. *N*-Ethylmaleimide is a known inhibitor of E_{II} (Haguenauer-Tsapis & Kepes, 1973, 1977; Gachelin, 1970). In preliminary experiments (data not shown), we determined the NEM concentration dependence for inhibition of αMGlc phosphorylation when E_{II} was the rate-limiting component. Approximately 1 mM NEM was necessary to provide rapid, complete inhibition. The protection experiments were then executed. After measuring the αMGlc phosphorylation for 40 min (lower curve, Figure 6), NEM was added to a final concentration of 1 mM. The PTS activity was immediately and completely inhibited. Forty minutes later, $K_3[Fe(CN)_6]$ was added to a final concentration of 1 mM. No change was observed. The activity remained inhibited. At 120 min, DTT was added to a final concentration of 5 mM, resulting in a return of only 15% of the initial phosphorylation rate. In the upper curve of Figure 6, the order of addition was reversed. $K_3[Fe(CN)_6]$, 1 mM final concentration, was added at 40 min before NEM. As expected from our Fe^{3+} concentration dependence studies (Figure 5), inhibition was immediate and complete. At $T = 80$ min, the reaction mixture was brought to 1 mM in NEM and, after incubating for an additional 40 min, it was made 5 mM in DTT. As can be seen in the upper curve of Figure 6, the DTT restored 70% of the initial rate of phosphorylation. These results demonstrate that raising the redox potential protects against inhibition by NEM, supporting the concept of an –SH to S–S conversion controlled by this potential.

Enzyme I is known to be sensitive to sulfhydryl reagents. Therefore, it could be argued that this redox conversion is taking place at the level to E_I, not E_{II}, and that the NEM inactivation is also occurring at the level of E_I. We have shown that addition of active E_I after DTT, in an experiment similar to that illustrated in the lower plot, does not result in the return of phosphorylating activity. Thus, the protection of the oxidizing agents against NEM inhibition is occurring at the level of E_{II}.

Relation to Other E_{II} SH Groups. Kepes and his associates have deomonstrated that there is an NEM-sensitive sulfhydryl group associated with E_{II} activity and that the accessibility of this SH group is modulated by the state of the PTS. The SH group is most accessible when a P-HPr generating system is lacking (i.e., PEP is absent) and αMGlc is present. It is most inaccessible when αMGlc is absent and a P-HPr generating system is present. The experiment presented in Figure 7 was performed to determine if the sulfhydryl group monitored by Kepes and co-workers was related to the oxidizable sulfhydryl group reported in this paper. In this experiment, we measured the inhibition of E_{II} activity resulting from preincubation with various concentrations of NEM in the presence of αMGlc, PEP, or αMGlc plus Fe^{3+}. Reaction mixtures containing E_I, HPr, and E_{II} buffers and the components listed at the right of each curve in Figure 7 were prepared. Then NEM was added to each mixture at the final concentration indicated on the abscissa. After incubating at 25 °C for 30 min, the NEM reaction was terminated with 5 mM DTT, and the rate of phosphorylation in each sample was measured after addition of the missing component, αMGlc or PEP. It is important to stress that each data point in Figure 7 is a rate calculated from a time curve which itself consists of at least 5 data points. Figure 7 confirms what has already been found by Haguenauer-Tsapis & Kepes (1973, 1977), namely, that PEP protects the PTS against inhibition by NEM

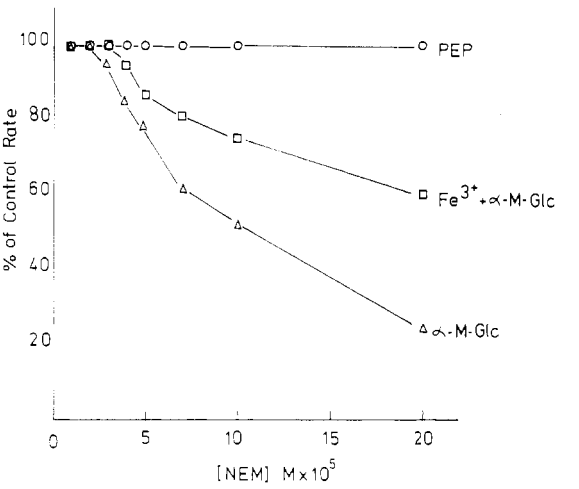

FIGURE 7: E_{II}-catalyzed αMGlc phosphorylation activity remaining after preincubating the PTS components with various concentrations of NEM in the presence of 50 μM αMGlc, 5 mM PEP, or 50 μM αMGlc plus 1 mM potassium ferricyanide. The activity remaining is plotted as percent of the control rate. A separate control was used for each curve, the control being a mixture of the specified components preincubated in the absence of NEM. See Material and Methods and text for experimental details.

whereas αMGlc sensitizes the PTS to inhibition by very low concentrations of NEM. However, when 1 mM Fe^{3+} is present with αMGlc during the NEM treatment, the αMGlc stimulation of inhibition is blocked. Only that degree of inhibition occurs which is normally found when 1 mM Fe^{3+} alone is present during the NEM treatment. These data suggest that the SH group protected by oxidation with Fe^{3+} and the SH group sensitized by the presence of αMGlc are most likely the same.

Discussion

The physical mechanism controlling E_{II} activity is the conversion between a high- and low-affinity form for binding substrate. The conversion between the two forms is a redox sensitive process involving an SH HS to S–S interconversion with a midpoint potential between –330 and –200 mV. The reduced (SH) form is the high-affinity form, and the oxidized (S–S) form is low affinity. We have shown that this redox conversion can be effected either by substrate oxidation or by direct addition of oxidizing agents. There is, most likely, more than one way to transmit the oxidizing effect to E_{II}. E_{II} activity is clearly responsive to the redox potential of the solution phase. Nevertheless, the redox potential appears to be transmitted through the membrane phase before affecting E_{II}. This can be concluded from the concentration dependence of the inhibition induced by the various oxidizing agents. The most effective oxidants listed in Table II are the most lipophilic ones. The quinones inhibit E_{II} activity immediately and completely at concentrations between 5 and 20 μM whereas the more polar agents such as riboflavin or ferricyanide generate similar effects only in the 0.2–1 mM concentration range. While we could envision that the low molecular weight oxidants, even the polar ones like ferricyanide, might gain direct access to E_{II} and thereby effect its oxidation, this would be an unlikely proposal for the mechanism of oxidation by ferricytochrome *c*. A more realistic picture would vizualize an interaction of ferricytochrome *c* on the surface of the membrane with a *b*-type cytochrome or cytochrome oxidase. The oxidation could be transmitted to mobile electron-transfer intermediates such as ubiquinone which, in turn, could oxidize E_{II}.

Data have been presented over the past years implicating similar redox processes in the control of transport activity for

other solutes. In 1971, Kaback & Barnes reported the effects of sulfhydryl blocking reagents on the D-lactate oxidation driven uptake of lactose and the efflux of lactose in response to uncouplers and respiratory inhibitors. They integrated these results into a general model in which the carrier protein itself was an electron-transfer intermediate which could undergo a reversible oxidation–reduction reaction involving an SH HS to S–S conversion. In their vision, the oxidized form of the carrier was a high-affinity form for binding substrate, and the reduced carrier was a low-affinity form. Since then, evidence has accrued which is in conflict with the concept of the carrier as an electron-transfer intermediate. There is, nevertheless, evidence that carriers do undergo changes in K_m or V_{max} in response to energization and that such changes are somehow related to SH group accessibility. Kaczorowski et al. (1979) demonstrated that the K_m for lactose uptake decreased approximately 100-fold upon energization of membrane vesicles. Earlier, Schachter et al. (1966) had provided evidence for an increase in the K_m for ONPG binding to the lactose carrier in *E. coli* which had been deenergized by treatment with KCN. The change was accompanied by a change in the availability of the SH group to NEM modification. More recently, Jacobs et al. (1978) presented data that the oxidation state regulates carrier activity in *Mycobacterium phlei*, in this case, at the level of the V_{max}. They showed that Cu^{2+} mediated the concentrative accumulation of certain amino acids in membrane vesicles. Cu^{+} was ineffective in the same process. Results from studies with sulfhydryl blocking reagents were consistent with the proposal that Cu^{2+} was responsible for the oxidation of an SH HS to an S–S form. Reducing agents prevented the Cu^{2+}-stimulated upttake and initiated the efflux of amino acids transported in the presence of Cu^{2+}. Since 50% of the DTNB-reactive SH groups in the membrane were protected by Cu^{2+}, Jacobs et al. (1978) proposed that the active, Cu^{2+}-generated conformation of the carrier was the oxidized, S–S, form.

Mechanism of the $\Delta\bar{\mu}_{H^+}$ Regulation of the PTS. The results presented in Table I are in agreement with the observations of Reider et al. (1979) linking the inhibition of transport by the PTS to a $\Delta\bar{\mu}_{H^+}$, and, moreover, they demonstrate that the influence of a $\Delta\bar{\mu}_{H^+}$ can be measured at the level of phosphorylation, not just transport. The limitations of the transport measurements (inability to regulate the levels of HPr and E_I) restricted Reider et al. (1979) to the conclusion that the $\Delta\bar{\mu}_{H^+}$ altered the K_m of the PTS carriers for their substrates without allowing them to measure the magnitude of the changes or examine the physical basis for them. The flexibility afforded by the inverted vesicle system, on the other hand, enabled us to quantitate the magnitude of the $\Delta\bar{\mu}_{H^+}$-induced changes and to compare these directly with changes induced by oxidants and reductants.

The changes in the PTS carriers brought about by substrate oxidation seem to be identical with those caused by oxidants (conversion of the PTS carriers from high-affinity to low-affinity states). It is reasonable, therefore, to assume that the physical basis for these changes is also the same (conversion from the reduced to the oxidized form). That being the case, we must find a mechanism for achieving a conversion between redox states with a $\Delta\bar{\mu}_{H^+}$. In 1970, Hinkle & Mitchell proposed that an electrical potential difference across a membrane ($\Delta\Psi$) could alter the redox potential in the membrane. This would be manifested as a shift in the midpoint potential for a membrane-bound redox couple [see Dutton & Wilson (1974) and Walz (1978) for detailed reviews]. The mechanism can be summarized for a two-phase system (membrane and solution) as follows. The electrochemical potential of an electron in a given phase (M) expressed in terms of a redox couple in that phase is

$$\tilde{\mu}_\epsilon^{M} = -FE^0 + \frac{RT}{n} \ln (a_R^{M}/a_0^{M}) - F\Psi^{M} \qquad (1)$$

Since the redox potential $E = E^0 + RT/n \ln (a_0/a_R)$, we can rewrite eq 1 in terms of the redox potential

$$\tilde{\mu}_\epsilon^{M} = -F(E^{M} + \Psi^{M}) \qquad (2)$$

A similar equation with the superscript S can be written for the solution phase. Due to the presence of electron carriers in the membrane (respiratory chain components), the electrons can be at equilibrium throughout the system ($\tilde{\mu}_\epsilon^{M} = \tilde{\mu}_\epsilon^{S}$), even when there is a difference in electrical potential between the two phases ($\Delta\Psi = \Psi^{S} - \Psi^{M} \neq 0$) because of the active transport of protons through the membrane (maintenance of a $\Delta\bar{\mu}_{H^+}$). This potential difference will be compensated by an equal but opposite shift in the redox potential

$$-F(E^{S} + \Psi^{S}) = \tilde{\mu}_\epsilon^{S} = \tilde{\mu}_\epsilon^{M} = -F(E^{M} + \Psi^{M}) \qquad (3)$$

$$\Psi^{S} - \Psi^{M} = E^{M} - E^{S} \qquad (4)$$

A decrease in Ψ^{M} relative to Ψ^{S} will be reflected in an increase in the redox potential of the membrane relative to that of the solution. The situation is analogous when considering the effect of the presence or absence of a $\Delta\bar{\mu}_{H^+}$ on the redox state of a membrane-bound redox couple. A negative $\Delta\Psi$ will generate a higher redox potential in the membrane (i.e., a more oxidized redox system) than would be found in the absence of a $\Delta\Psi$.

Hinkle & Mitchell (1970) monitored the changes in the oxidation state of mitochondrial cytochrome *a* in response to membrane potentials developed naturally by ATP hydrolysis or artificially by ion diffusion. The amount of oxidized cytochrome *a* increased 20%–30% in response to a transmembrane electrical potential of approximately 100 mV (interior negative). Similar studies have been done more recently which monitor the influence of surface potentials and membrane potentials on the midpoint potential of cytochrome *c*-555 in chromatophores of *Chromatium vinosum* (Itoh, 1980), of cytochrome c_2 in *R. sphaeroides* chromatophores (Matsuura et al., 1980a), and of the carotenoid spectrum of *R. sphaeroides* spheroplasts and chromatophores (Matsuura et al., 1980b). In all cases, shifts in the midpoint potentials of the redox components were measured in response to changes in the surface potential or transmembrane electrical potential differences.

We propose, therefore, that the activity of the PTS is regulated by the redox potential in the membrane which changes in response to a $\Delta\bar{\mu}_{H^+}$. A $\Delta\Psi$, interior negative for the intact bacteria, raises the redox potential in the membrane, and causes E_{II} to convert from the reduced, high-affinity, form to the oxidized, low-affinity, form. A type of fine control in the PTS can be exerted by the magnitude of the transmembrane electrical potential. This governs the extent of the shift in the redox potential and thus regulates the fraction of E_{II} in the oxidized and reduced form.

This mechanism of regulation by $\Delta\bar{\mu}_{H^+}$ may also be applicable to other types of "redox-sensitive" transport systems. The precise mode of expression (oxidized or reduced/high or low affinity or high or low V_{max}) would be dictated by the nature of the system.

Relationship of the Redox-Sensitive Site to E_{II}-Sensitive SH Groups. The data presented in Figure 7 show that oxidation of the redox-sensitive site with Fe^{3+} protects against

αMGlc-stimulated inactivation of E_{II} by NEM. The simplest interpretation of these data is that the redox-sensitive SH site is the same as the SH unmasked by the binding of αMGlc. In this model, Fe^{3+} and αMGlc have competing effects on the equilibrium

$$SX\ XS \xleftarrow[\alpha MGlc]{NEM} SH\ HS \xrightarrow{Fe^{3+}} -S-S-$$

Ferricyanide shifts the equilibrium strongly to the oxidized form and thereby protects from NEM inactivation whereas αMGlc binds more strongly to the reduced form, shifting the equilibrium back in that direction and simultaneously exposing the SH group to NEM inactivation. These results could, however, be fitted to a model in which the redox site was distinct from the αMGlc-sensitized site. In this model, the oxidation of the redox-sensitive site would raise the K_m for αMGlc, thereby weakening the binding and reducing the capability of αMGlc to unmask the second site to NEM inhibition. The present results cannot differentiate between the two models.

Acknowledgments

We acknowledge the expert technical assistance of R. ten Hoeve-Duurkens and helpful discussions with Drs. O. Misset, K. Hellingwerf, F. F. Roossien, and J. Lolkema.

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