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Evidence for the Existence of a Channel in the Glucose-Specific Carrier EII\textsuperscript{Glc} of the Salmonella typhimurium Phosphoenolpyruvate-Dependent Phosphotransferase System\textsuperscript{a}

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Abstract: The effect of membrane-impermeable sulfhydryl reagents on glucose-specific enzyme II (EII\textsuperscript{Glc}) activity has been studied in Salmonella typhimurium whole cells and in properly sealed inverted cytoplasmic membrane vesicles. Glutathione N-hexylmaleimide and N-polymethylenecarboxymaleimides inactivate methyl a-D-glucopyranoside (a-MeGlc) transport and phosphorylation in whole cell preparations at a dithiol that can be protected by nonpermeant mercurials, oxidants, and arsenicals as well as by phosphorylation of EIIG\textsuperscript{1}. Accessibility to this activity-linked site is restricted to small apolar reagents or to polar reagents with a hydrophobic spacer between the polar group and the reactive maleimide moiety. These same reagents inactivate a-MeGlc phosphorylation in inverted cytoplasmic membrane vesicles. Inhibition results from reaction at a dithiol that can be protected by nonpermeant mercurials, oxidants, and arsenicals as well as by phosphorylation of EIIG\textsuperscript{1}. The characteristics of this site are virtually identical with those of the activity-linked dithiol elucidated in intact cells. No evidence could be found for a second activity-linked site on the other side of the membrane when the permeable reagent N-ethylmaleimide was used. Since only one activity-linked dithiol can be detected with sealed inverted membrane vesicles or intact cells and it is accessible to membrane-impermeable sulfhydryl reagents from both sides of the cytoplasmic membrane, we suggest that it is located in a channel constructed by the carrier and that the channel spans the membrane. A second dithiol, not essential for activity, is located near the outer surface of the cytoplasmic membrane.

The phosphoenolpyruvate-dependent methyl a-D-glucopyranoside (a-MeGlc) transport activity has been studied in Escherichia coli and Salmonella typhimurium whole cells and in properly sealed inverted cytoplasmic membrane vesicles. Glutathione N-hexylmaleimide and N-polymethylenecarboxymaleimides inactivate methyl a-D-glucopyranoside (a-MeGlc) transport and phosphorylation in whole cell preparations at a dithiol that can be protected by oxidizing reagents, trivalent arsenicals, or phosphorylation of EII\textsuperscript{Glc}. Accessibility to this activity-linked site is restricted to small apolar reagents or to polar reagents with a hydrophobic spacer between the polar group and the reactive maleimide moiety. These same reagents inactivate a-MeGlc phosphorylation in inverted cytoplasmic membrane vesicles. Inhibition results from reaction at a dithiol that can be protected by nonpermeant mercurials, oxidants, and arsenicals as well as by phosphorylation of EIIG\textsuperscript{1}. The characteristics of this site are virtually identical with those of the activity-linked dithiol elucidated in intact cells. No evidence could be found for a second activity-linked site on the other side of the membrane when the permeable reagent N-ethylmaleimide was used. Since only one activity-linked dithiol can be detected with sealed inverted membrane vesicles or intact cells and it is accessible to membrane-impermeable sulfhydryl reagents from both sides of the cytoplasmic membrane, we suggest that it is located in a channel constructed by the carrier and that the channel spans the membrane. A second dithiol, not essential for activity, is located near the outer surface of the cytoplasmic membrane.

1 Abbreviations: PEP, phosphoenolpyruvate; EII\textsuperscript{Glc}, glucose-specific enzyme II of the PEP-dependent phosphotransferase system; EIIG\textsuperscript{1}, mannitol-specific enzyme II; GSH, glutathione N-hexylmaleimide; a-MeGlc, methyl a-D-glucopyranoside; DTT, dithiothreitol; AM2, -3, -5, or -10, N-polymethylenecarboxymaleimides containing 2, 3, 5, or 10 methylene moieties between the carboxylate group and the maleimide ring; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; PCMBS, p-(chloromercuri)benzenesulfonate; DNP, 2,4-dinitrophenol; thorin, 4-[2-arsenophenylazo]-3-hydroxy-2,7-naphthalenedisulfonic acid; BAL, British antilewesite (2,3-diemcapropropyl); Tris, tris(2-hydroxymethyl)aminomethane; NEM, N-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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(Hagunauer-Tsapis & Kepes, 1977a,b, 1980; Robillard & Konings, 1981) and EII\textsuperscript{Md} (Roossien & Robillard, 1984). A \( \Delta \mu_{H^+} \) can also influence the reactivity of various transport proteins, including EII\textsuperscript{Glc} toward sulfhydryl reagents (Robillard & Konings, 1981; Konings & Robillard, 1982; Poolman et al., 1983; Cohn et al., 1981; Le Quoc et al., 1979). The data suggested that dithiols occur in these proteins and that their oxidation states could be altered either by phosphorylation or by \( \Delta \psi \). As a result of these data we proposed that turnover of a carrier might be linked to a cycling of the carrier’s various redox centers at different positions across the membrane between the oxidized, disulfide, and the reduced, dithiol, state (Robillard & Konings, 1982). Our recent studies using membrane-impermeable sulfhydryl reagents have confirmed that the proline/H\textsuperscript{+} symport system in \textit{E. coli} possesses at least two dithiol redox centers, one accessible from the outer side of the cytoplasmic membrane and the other accessible from the inner side (Poolman et al., 1983). These sites alter their redox state in response to a \( \Delta \mu_{H^+} \) or \( \Delta \psi \) in a manner consistent with our earlier proposal.

The present investigations are intended to define the location, number, and oxidation state of the redox centers in membrane-bound EII\textsuperscript{Glc} as a function of the phosphorylation state of the enzyme and the energization state of the membrane.

We will examine those sites accessible from the exterior using whole cells and sites accessible from the interior using inverted cytoplasmic membranes vesicles. Both studies employ a variety of sulfhydryl-directed membrane reagents most of which are impermeable: irreversible alkylating agents, glutathione N-hexylmaleimide and N-polymethyleneoxycarboxy-maleimides; reversible mercurials, PCMBs and HgCl\textsubscript{2}; trivalent arsenicals, phenylarsine oxide and thorin; oxidants, CuCl\textsubscript{2}, potassium ferricyanide, hexaamineruthenium(II1) trichloride, and diamide. The differing size, charge, and polarity of these reagents offer the possibility of discriminating among various sites.

**Materials and Methods**

**Bacterial Strain and Culture Conditions.** \textit{S. typhimurium} PP1133 (ptsM416 trpB223) (Scholte et al., 1982) was grown on 0.1 M potassium phosphate, pH 7.2, 7.5 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and 0.4 mM MgSO\textsubscript{4} containing 20 mg/L tryptophan, 0.1% yeast extract, 0.2% glucose, and, for phosphoenolpyruvate-induced cells, 0.1% PEP (monopotassium salt).

**Preparation of Whole Cells.** A culture was grown overnight at 37 °C. In the morning it was diluted 10-fold with fresh medium. When the OD\textsubscript{560} reached 0.9–1.1, chloramphenicol was added to a final concentration of 50 \( \mu \)g/mL and the suspension was centrifuged. The cells were suspended in 0.1 M potassium phosphate, pH 7.3, or 0.1 M Tris-HCl, pH 8, recentrifuged, and resuspended in the same buffer. The cells were kept on ice and used within 12 h after harvesting.

**Transport Activity.** \( \alpha \)-MeGlc transport activity was measured by incubating cells with \( [\text{C}]\alpha \)-MeGlc at 30 °C. Aliquots were withdrawn, pipetted directly onto 0.45-\( \mu \)m cellulose acetate filters under vacuum. The filters were washed, under vacuum, within 30 s with 3 mL of 0.1 M potassium phosphate, pH 7.3. The vacuum was released immediately thereafter, and the filters were put into scintillation vials containing 10 mL of Packard Emulsifier Scintillator 299. The vials were counted at 10 °C in a Nuclear Chicago-Mark III liquid scintillation counter after the filters became fully transparent (30–60 min). All transport and phosphorylation activities are reported as nanomoles per milligram of total protein.

**Decryptification.** \( \alpha \)-MeGlc phosphorylation activity in permeabilized cells was measured by using the tolune per- meabilization procedure of Gachelin (1969).

**Preparation of Inverted Membrane Vesicles.** Inverted cytoplasmic membrane vesicles were prepared according to the procedure of Reenstra et al. (1980). The final vesicle preparation was suspended in 50 mM potassium phosphate buffer, pH 7.0, at a protein concentration of 7–10 mg/mL and stored in 50 \( \mu \)L volumes in liquid nitrogen. The vesicle suspension was rapidly thawed at 40–45 °C on the day of the experiment and kept on ice until use. As a routine precaution a vesicle suspension was used only during the day that it was thawed, and the remainder was discarded at the end of the day.

**Enzyme Purification.** HPp was purified from \textit{E. coli} P650 according to Dooljewaard et al. (1979). Enzyme I was purified from the same strain following the procedure of Robillard et al. (1979) as modified by Brouwer et al. (1982) and Misset and Robillard (1982).

**\( \alpha \)-MeGlc Phosphorylation Activity.** In general a 20–40-\( \mu \)L aliquot of a phosphorylating reaction mixture containing \( [\text{C}]\alpha \)-MeGlc as substrate was removed from a reaction vessel, diluted into 10 mL of water, and rapidly loaded on a Bio-Rad disposable polypropylene column containing 1 mL of Dowex AG 1-X2, 50–100 mesh, in the chloride form. The column was washed with 20 mL of distilled water to remove \( [\text{C}]\alpha \)-MeGlc. The \( [\text{C}]\alpha \)-MeGlc–P was then eluted into scintillation vials with 10 mL of 0.1 M HCl. Eight milliliters of Packard Emulsifier Scintillator 299 was added, and the samples were counted at 10 °C. The counting efficiency was 70%. Specific assay conditions are listed in the figure legends.

**Protein concentration** was determined according to Lowry et al. (1951).

**Reagents.** Glutathione N-hexylmaleimide (GSM) was prepared and isolated following the procedure of Abbot and Schachter (1976). The isolation method used by these authors involved chromatography on Sephadex G-15 in H\textsubscript{2}O. In order to determine whether the inhibition observed in our studies could be due to the copurification of a small amount of the apolar N,N'-hexane-1,6-diyldimaleimide starting material, a portion of the GSM eluted from the Sephadex column was purified by using an ion-exchange procedure developed for the purification of glutathione (Furano, 1981). No difference was observed in the pattern of inhibition by the GSM preparation subjected to this extra purification step. N-Polyethyleneoxycarboxy-maleimides (AM2, AM3, AM5, and AM10) were synthesized as indicated by Griffiths et al. (1981).

**Monobromotrimethylammonio)biamine** was purchased from Aldrich. Phosphoenolpyruvate (monopotassium salt), \( p \)-chloromercuribenzenesulfonate (PCMBs), diamide (azodicarboxylic acid bis(dimethylamide)), and phenylarsine oxide, were obtained from Sigma. Thorin [4-\( \text{N}_{2} \)-arsenophenyl]azo-3-hydroxy-2,7-naphthalenedisulfonic acid] was purchased from Ventron Corp., Alfa Division. Hexa-aminoruthenium(III) trichloride was purchased from Johnson Matthey Chemicals Ltd. \( [\text{C}]\)Methyl \( \alpha \)-D-gluco-pyranoside was obtained from Amersham Radiochemical Centre. All other chemicals were reagent grade.

**Results**

**GSM Inhibition in Cells Lacking a \( \Delta \mu_{H^+} \).** Earlier studies demonstrated that a \( \Delta \mu_{H^+} \) alters the redox state of dithiols and their reactivity toward sulfhydryl reagents. All experiments reported in this section have been carried out on cells preincubated with a respiratory chain inhibitor, KCN (10 mM), and an uncoupler dinitrophenol (DNP, 1 mM) that abolishes the \( \Delta \mu_{H^+} \) in the pH range 7–8 (data not shown). The following
protocol was employed for studying P-EII\textsuperscript{Glc} or the dephosphorylated enzyme in whole cells. The cells were grown in the presence of 0.2% glucose and 0.1% PEP. They were pretreated with fluoroide if we intended to examine the dephosphorylated enzyme’s response to sulfhydryl reagents (Haugenauer & Kepes, 1972). After treatment with sulfhydryl reagents, PEP was added, and the PEP-dependent \(\alpha\)-MeGlc transport activity was measured (Saier et al., 1975).

Cells grown on glucose plus PEP were used for the experiments reported in Figure 1. Treatment of cells with 50 mM KF eliminates virtually all \(\alpha\)-MeGlc transport activity [Figure 1A (\(\varpi\))]. Addition of PEP after the KF treatment (\(\Delta\)) restores the activity to the level of that found in non-KF-treated cells (\(\varangle\)). When the cells, depleted of PEP, are incubated with 5 mM GSM for 5 min followed by excess DTT and then loaded with PEP and assayed, \(\alpha\)-MeGlc accumulation is strongly inhibited [Figure 1A (\(\Omega\))]. However, if exogenous PEP is added after the KF preincubation but before the GSM treatment, no inhibition is observed (\(\Omega\)). These data establish that GSM inactivates \(\alpha\)-MeGlc transport in intact cells and that dephosphorylated EII\textsuperscript{Glc} is significantly more susceptible to inactivation than P-EII\textsuperscript{Glc}.

Oxidizing agents were employed in Figure 1B to determine whether the site of GSM reaction was a monothiol or dithiol. \(\alpha\)-MeGlc accumulation is strongly inhibited in the presence of 5 mM cupric chloride [Figure 1B (\(\bar{A}\))]. The inhibition can be reversed for 70% by excess DTT (\(\bar{A}\)). Preincubation with Cu\textsuperscript{2+} prior to treatment with GSM prevents irreversible inhibition by GSM (\(\varnothing\)). Diamide (5 mM), a sulfhydryl-specific oxidant, also inhibits \(\alpha\)-MeGlc accumulation reversibly (\(\varnothing\), \(\varnothing\)) and protects against GSM inhibition just as well as Cu\textsuperscript{2+} (\(\varnothing\)). These data are consistent with the existence of an EII\textsuperscript{Glc} di thiol accessible to the nonpermeant reagents GSM and Cu\textsuperscript{2+} from the outer surface of the cytoplasmic membrane.

**Influence of Other Sulfhydryl Reagents.** Haugenauer and Kepes (1980) reported that, in contrast to the GSM data presented above, PCMBs was incapable of inactivating glucose transport when added to whole cells. We have repeated this experiment and confirmed their observation. Since it is possible that access to the GSM site is restricted by factors such as size, charge density, and polarity of the reagents, we have examined various reagents for their ability to inhibit \(\alpha\)-MeGlc transport and to protect against GSM inhibition.

Ferricyanide, thorin, PCMBs, DTT, and hexammineruthenium at concentrations up to 5 mM provided no significant protection against GSM inhibition nor were they capable of inhibiting \(\alpha\)-MeGlc transport activity on their own. Phenylarsine oxide, Cu\textsuperscript{2+}, and diamide reversibly inhibited \(\alpha\)-MeGlc transport and protected against GSM inhibition. Slight inhibition was observed with the nonpermeant reagent monobromo(trimethylammonio)ribimane whereas the N-poly methylene carboxy maleimides possessing 2, 3, 5, or 10 methylene groups between the carboxylate and maleimide moieties inhibited strongly under conditions identical with those stated in the legend to Figure 1. The inhibition characteristics of AM2, the smallest and most polar of the N-poly methylene carboxy maleimides, are presented in Figure 1C. At 1 mM, AM2 strongly inhibits \(\alpha\)-MeGlc accumulation (\(\varnothing\)). The inhibition is prevented when the cells are preincubated with PEP (\(\varnothing\), 2.5 mM CuCl\(_2\) (\(\bar{O}\)), or 2.5 mM diamide (\(\bar{D}\)) prior to treatment with AM2. The patterns of inhibition and protection are identical with those observed for GSM, suggesting that both reagents are acting at the same di thiol.

**Inhibition of Transport and Phosphorylation Activity.** GSM and the AM reagents might inhibit the transport function but not the phosphorylating function of the enzyme. If so, permeabilizing the cells with tol uene would allow the sugar to reach the phosphorylation site. This possibility was examined by comparing the rates of transport in GSM-treated cells with the rates of phosphorylation after the same cells had been permeabilized with tol uene (Gachelin, 1969). An aliquot of the cell suspension exposed to 5 mM GSM for 10 min was permeabilized with tol uene and assayed for phosphorylation activity as described under Materials and Methods. After a 10-min exposure to 4 mM GSM, 92% inhibition of the
transport activity was observed and 90% inhibition of the phosphorylation activity occurred (data not shown). Both transport and phosphorylation of α-MeGlc are inhibited by the maleimides. These data also enable us to eliminate alkylation of the PEP carrier as the reason for inhibition of α-MeGlc transport. Even when PEP has free access to the PTS components, no α-MeGlc phosphorylation occurs. *Inhibition of P-ElIGlc*. Figure 1 demonstrates that phosphorylation protects virtually completely against GSM and AM2 inhibition at concentrations which, in the absence of phosphorylation, inhibit α-MeGlc transport for better than 90%. However, in the phosphorylated state, cells were not fully protected against inhibition by some of the other N-poly-methylene(carboxymaleimides (Figure 2A). When cells preincubated with KCN, DNP, and PEP are exposed to 3 mM AM2 for 5 min, there is a slight inhibition (O). More substantial inhibition occurs with 3 mM AM3 (O) and 3 mM AM5 (O), and virtually complete inhibition occurs with 3 mM AM 10 (O). The time dependence of AM3 and AM10 inhibition is presented in Figure 2B. Both reagents inactivate very rapidly at 1 mM if the cells are pretreated with 50 mM KF to eliminate endogenous PEP (O, ▲). When PEP is added in a parallel experiment, the pattern of inhibition is altered. Inhibition by AM3 appears to be a monophasic, but the τ1/2 for the inhibition is increased from 30 s (▲) to 7 min (∆). Inhibition by AM10 is biphasic. The initial phase is nearly as rapid as that in the nonphosphorylated state and accounts for approximately 80% of the inhibition. This is followed by a second slower phase with a τ1/2 similar to that observed with AM3. The slow inactivation is considerably more rapid than the rate at which the transport activity decreases in the absence of maleimides (O).

*Inhibition of PEIIGlc in the Presence of a ΔH+. A ΔH+ inhibits EIIGlc-catalyzed α-MeGlc phosphorylation by causing a dithiol to disulfide conversion (Robillard & Konings, 1981). The experiments presented in this section were intended to check whether the dithiol protected by oxidation in Figure 1 was also protected by a ΔH+.*

Parts A and B of Figures 3 show the effect of treating PEP containing cells with GSM and the AM series alone (open symbols) or in the presence (closed symbols) of an endogenous ΔH+. ΔH+ stimulates the inhibition by 3 mM AM2, AM3, and AM5 (Figure 3A), but it protects against inhibition by 3 mM AM10 and 10 mM GSM (Figure 3B). Examination of the time dependence of the inhibition process for AM3 and AM10 (Figure 3C) indicates the origin of these differences. AM10 inhibits 80% of the activity rapidly in deenergized cells pretreated with PEP (O). AM10 inhibition does not occur in energized cells (O). This is consistent with the results in Figure 3B where a 5-min preincubation with AM10 was employed. AM3 inhibits deenergized cells pretreated with PEP albeit more slowly than AM10 (▲). However, energization only offers protection for the first few minutes of AM3 treatment (▲). Thereafter the activity decreases more rapidly than in the case of deenergized cells. This behavior is consistent with AM3 reaction at two distinct sites. During the first few minutes AM3 apparently alkylates a site that does not directly affect the activity of EI1. Following reaction at the first site, the activity-linked site becomes alkylated. The higher the AM3 concentration, the shorter the period of protection and the more rapid the inactivation rate. This explains why the 5-min preincubation with 3 mM AM2, -3 and -5 resulted in more inactivation with energized than deenergized cells (Figure 3A).

The location of this new site was examined with PCMBs. Experiments reported above showed that PCMBs cannot reach the activity-linked dithiol. The data in Figure 3D shows that PCMBs is capable of protecting against reaction at the new site. When cells possessing an endogenous ΔH+ were preincubated with PEP and 5 mM PCMBs, the period of complete protection against AM3 was longer and the subsequent inactivation phase was slower [Figure 3D (▲)] than when PCMBs was omitted [Figure 3D (O)]. When 5 mM thorin or 5 mM Ru3+ was used exactly the same, protection was observed as found with PCMBs (data not shown). Since an impermeant dithiol-specific reagent and an oxidant can protect against reaction, this new site must be a dithiol near the periplasmic side of the membrane.

*Characteristics of α-MeGlc Phosphorylation by Inverted Cytoplasmic Membrane Vesicles of S. typhirnurium PP.1133*. α-MeGlc phosphorylation rates measured by using inverted cytoplasmic membrane vesicles and excess E1, HPr, and PEP yield nonlinear Lineweaver–Burk plots (Figure 4, open symbols). Detergents such as deoxycholate or octylpolyethylene glycol remove the nonlinearity largely or totally depending on the detergent concentration employed (Figure 4, filled sym-
with 0.1 M PEP, then for above for inhibitions in the presence of H⁺.

Cells in 0.1 M potassium phosphate-0.4 mM magnesium sulfate prepared as stated for (A) and (B). Aliquots were removed at the indicated times and added to the tubes containing DTT or, in the case indicated, the presence of a-MeGlc transport activity by 1 mM AM3 and AM10. The initial rates of a-MeGlc transport were measured as stated in the legend to Figure 1A. For inhibitions in the absence of a ΔH⁺, 10 mM KCN and 1 mM DNP were added along with 0.1 M PEP at the start of the incubation period. (A) ΔH⁺ stimulation of inhibition by 3 mM AM2, AM3, and AM5. Control, no inhibitor added (Δ); AM2 inhibition with (○) and without (■) ΔH⁺; AM3 inhibition with (●) and without (□) ΔH⁺; AM5 inhibition with (●) and without (□) ΔH⁺. (B) ΔH⁺ protection against inhibition by 10 mM GSM and 3 mM AM10. 10 mM GSM inhibition with (●) and without (□) ΔH⁺; 3 mM AM10 inhibition with (●) and without (□) ΔH⁺. (C) Rates of inhibition of a-MeGlc transport activity by 1 mM AM3 and AM10 in the presence and absence of a ΔH⁺. The reaction mixtures were prepared as stated for (A) and (B). Aliquots were removed at the indicated times and added to the tubes containing DTT or, in the case of energized cells, DTT, KCN, and DNP (final concentrations 10, 10, and 1 mM respectively). The initial rates of a-MeGlc transport were measured as stated in the legend to Figure 2B. Inhibition by AM3 (Δ) or AM10 (○) in the presence (closed symbol) or absence (open symbols) of a ΔH⁺. (D) Influence of PCMBs on the rate of inactivation of a-MeGlc transport activity by 1 mM AM3 in the presence of a ΔH⁺. The reaction mixtures were prepared as stated above for inhibitions in the presence of ΔH⁺. (Δ) Cells preincubated with 1 mM PCMBs for 5 min at 30°C prior to the addition of 3 mM AM3; (○) inactivation by 1 mM AM3 in the presence of PCMBs. Aliquots were removed at the indicated times and added to tubes containing 2 mM DTT, 10 mM KCN, and 1 mM DNP. The initial rates of a-MeGlc transport were measured as stated in the legend to Figure 2B.

FIGURE 4: Effect of detergent on a-MeGlc phosphorylation by inverted membrane vesicles. Membrane vesicles were suspended to a final protein concentration of 0.195 mg/mL in buffer containing 10 mM MgSO₄, 10 mM KF, 50 mM potassium phosphate buffer, pH 7.0, 10 mM PEP, 5 mM DTT, and 0.2% octylpoly(ethylene glycol)-300 (filled symbols) or no detergent (open symbols). Volumes of 110 μL were incubated 5 min at 30°C; 20 μL of each suspension was then added to 70 μM HPr, 1 μM EI, and 100 mM PEP was then added and the 30°C incubation continued for 5 min. Ten microliters of [α-32P]-α-MeGlc was added, and the rate of phosphorylation was determined by withdrawing four 30-μL aliquots over a period of between 1 and 15 min and processing for [α-32P]-α-MeGlc-P.

Rates of Inactivation of EI Activity by NEM and Nonpermeable Maleimides. The rates of inactivation of EI in inverted membrane vesicles have been examined by using equal concentrations of GSM, AM2, AM5, AM10, and NEM (see Figure 5). DTT (1 mM) has been routinely added to the vesicle stock solution in the maleimide inhibition studies because we have observed that the activity-linked dithiol converts to a disulfide in the presence of oxygen alone. In this state the thiols are protected from reaction with maleimides. All reagents show similar biphasic inhibition patterns. There is a rapid inactivation with a T1/2 of less than 15 s. There is also

fraction have their sugar binding site directly accessible to solution, but the major fraction have their binding sites facing the inner side of the cytoplasmic membrane. Sugar must first diffuse into the vesicle before it can be phosphorylated and exported into the external medium. This diffusion process is slow and only significant at sugar concentrations above 100 μM. The linearity at low sugar concentrations is attributable to that fraction of EI1 in leaky vesicles or membrane fragments whose binding sites are directly accessible to solution. Detergents cause all of the vesicles to become leaky so that the entire EI1 population is freely accessible to the sugar. The V₅₀ value² of the detergent-treated vesicles represents the total activity of the entire EI1 population. The V₅₀ extrapolated from the linear portion of the Lineweaver–Burk plot for untreated membranes can be used to estimate the fraction of EI1 in leaky vesicles or fragments. The measured V₅₀ for detergent-treated membranes is 14 nmol of a-Glc-P min⁻¹ (mg of protein)⁻¹. The extrapolated V₅₀ for untreated membranes is 3.6 nmol min⁻¹ mg⁻¹. Consequently 25% of the EI1 molecules are freely accessible to sugar in properly sealed inverted membrane vesicles. Experiments with these vesicles and nonpermeable sulfhydryl reagents can provide information concerning the accessibility of activity-linked thiols from the inner side of the cytoplasmic membrane.

Rates of Inhibition of EI Activity by NEM and Nonpermeable Maleimides. The rates of inactivation of EI in inverted membrane vesicles have been examined by using equal concentrations of GSM, AM2, AM5, AM10, and NEM (see Figure 5). DTT (1 mM) has been routinely added to the vesicle stock solution in the maleimide inhibition studies because we have observed that the activity-linked dithiol converts to a disulfide in the presence of oxygen alone. In this state the thiols are protected from reaction with maleimides. All reagents show similar biphasic inhibition patterns. There is a rapid inactivation with a T₁/₂ of less than 15 s. There is also

² EI1GC² is obligatory for the activity of this system. The vesicle preparation contains subsaturating amounts of EI1GC². No extra enzyme is added for activity measurements; consequently, the y-axis intercept is only an apparent V₅₀.
a slow inactivation process with a $T_{1/2}$ of approximately 2–5 min. Even though the patterns of inhibition are similar, the reagents seem to divide into two groups. The more hydrophobic AM10 and NEM inhibit more extensively in the first phase than do the less hydrophobic AM2, AM5, and GM. Since the inhibitions are carried out on inverted membrane vesicle suspensions prior to the addition of EI, inhibition must be attributed to the reaction of the maleimides with E1 and not with EI.

**Protection against Maleimide Inhibition.** Figure 6 examines the inhibition by 3 mM AM2 and the protection against AM2 inhibition. The pattern of inhibition by 3 mM AM2 and the extent of inhibition of the unprotected enzyme (●) were virtually identical with that observed in Figure 5. The substrate, α-MeGlc ($K_{m} = 7 \mu M$), had very little if any protective effect on either phase at concentrations up to 2 mM (●). When the inverted membrane vesicles were preincubated with 4 mM PCMBs (●) prior to the addition of 3 mM AM2, hardly any rapid or slow inhibition was observed. Since it protected, we can conclude that the rapid and slow inhibition resulted from reaction at sulfhydryl groups that were accessible from the cytoplasmic side of the membrane.

Preincubation with 2 mM thorin (●) prior to the addition of 3 mM AM2 protected against rapid inhibition, but the slow inhibition continued with essentially the same $T_{1/2}$ as in the unprotected sample (●). Consequently the rapid inhibition must have been at a dithiol that was accessible from the cytoplasmic side of the membrane.

Phenylarsine oxide (●), a membrane-permeable arsenical, also protected only against rapid inhibition, suggesting that the slow inhibition was not due to reaction at a buried dithiol. Phosphorylation protected against GMS and AM inactivation in whole cells (Figure 1). Phosphorylation (●) of the inverted membrane vesicles by preincubation with PEP, HPr, and EI prior to AM2 addition also prevented the rapid inhibition but not the slow inhibition. The oxidant CuCl2 inhibits α-MeGlc phosphorylation with inverted vesicles (data not shown), and it protected against AM2 inactivation [Figure 6 (●)]. CuCl2 clearly protected against both the rapid and slow inactivation; however, diamide (data not shown) and O2 [Figure 6 (×)] only
protected against the rapid inactivation. The lack of $\mathrm{O}_2$, diamide, and arsenical protection against the slow inhibition indicates that the site was incapable of being oxidized or converted to a cyclic dithiol arsenite. The observed $\mathrm{Cu}^{2+}$ protection is most likely a result of adduct formation with the sulfhydryl group. The biphasic inactivation patterns in Figures 5 and 6 are suggestive of an equilibrium between two conformations of the carrier that interconvert very slowly or not at all. The thiols react rapidly with maleimides and can be protected by oxidants, arsenicals, or phosphorylation only in one of the conformations. Similar biphasic kinetics have been found for maleimide inactivation of EIIG from $R$. sphaeroides. Treatment of those membranes with EDTA converted the slowly reacting form into the rapidly reacting form. Zinc specifically stabilized the slowly reacting conformation (Lolkema & Robillard, 1986). EDTA and $\mathrm{Zn}^{2+}$ do not affect the reactivity of EIIG in an analogous way. At the present time we do not know what factors stabilize the slowly reacting form of EIIG.

**Inactivation with N-Ethylmaleimide.** The patterns of inactivation in whole cells with impermeant maleimides suggest that there is an EIIG dithiol accessible from the periplasmic side of the membrane. The data just presented on inverted vesicles argue for a dithiol with similar inactivation and protection characteristics that is accessible from the cytoplasmic side of the membrane. The following experiment attempts to determine whether there are two distinct dithiols, one accessible from each side of the membrane or only one dithiol accessible from both sides. The dithiol accessible from the cytoplasmic side can be protected by PCMBs in the inverted vesicle experiments, but the dithiol accessible from the periplasmic side in the whole cell experiments cannot be protected by PCMBs. Therefore, we have preincubated the inverted vesicles with PCMBs and then exposed them to 10 mM N-ethylmaleimide. In the absence of PCMBs complete inhibition is achieved by 1 mM NEM in less than 15 s. In the presence of PCMBs the cytoplasmic dithiol will be protected. If there is a separate periplasmic dithiol, it will be alkylated by the membrane-permeable NEM and result in inactivation. Virtually no inactivation occurred in the presence of PCMBs even after a 30-min incubation with 10 mM NEM. Thus, we can conclude that the dithiol accessible from the periplasmic and cytoplasmic side of the membrane is one and the same dithiol.

**Discussion**

Phosphorylation, imposition of a $\Delta \mu_{\mathrm{H}^+}$, or exposure to oxidants protects carrier-associated dithiols from reaction with alkylating reagents (Robillard & Konings, 1981; Poolman et al., 1983; Konings & Robillard, 1982). These results plus theoretical considerations led us to propose that carrier turnover might be associated with cooperative dithiol-disulfide interchanges between different redox centers in a single carrier (Robillard & Konings, 1982). One feature of this proposal was that carriers functioning in this manner would contain at least two redox centers located at different depths in the membrane. We have provided data supporting this feature for the proline/H$^+$ symporter from $E$. coli (Poolman et al., 1983). The present investigation addresses the same issue for the phosphoenolpyruvate-dependent phosphotransferase carriers.

The results presented in this report indicate that there are at least two operationally distinct sulfhydryl sites on EIIG. One site is a dithiol essential for EIIG phosphorylation and transport activity. The second site is a dithiol not essential for EIIG activity but capable of influencing the reactivity or accessibility of the activity-linked dithiol. These sites are distinguishable because they react with sulfhydryl reagents under different conditions and are protected by different reagents.

**Location of the Activity-Linked Dithiol.** Combining the inactivation and protection data from all of the reagents employed in this study leads to the following view of the location of the activity-linked dithiol. It is situated in a channel or cleft of restricted dimensions no deeper than 18-20 Å from the outer surface of the cytoplasmic membrane. It is more readily accessible from the inside than from the outside of the cytoplasmic membrane. Alkylation from the outer side can only be prevented by $\mathrm{Cu}^{2+}$ or diamide but not by PCMBs, thorin, ferricyanide, or hexammineruthenium(III) trichloride. PCMBs and the smaller AM reagents are comparable in bulk and molecular weight. Since the site is accessible to the AM reagents but not to PCMBs, we suggest that access is restricted by the strongly ionized sulfate group. Both size and charge probably prevent access by thorin, ferricyanide, and hexammineruthenium(III) trichloride. All of the reagents, however, protect when added from the inner side (i.e., in the inverted membrane vesicle studies).

This view of the location of the activity linked dithiol assumes (i) that the distance of penetration of the maleimide group of GSM is limited by the impenetrability of the tripeptide moiety into the bulk of the membrane and also the length of the hexamethylene spacer chain and (ii) that the penetration of the acid maleimides is limited by the presence of the ionized carboxyl group and the length of the polymethylene chain. Various studies have shown that GSM and the acid maleimides are membrane impermeable at the pH, concentration, and reaction times used in the present investigations (Moore et al., 1984; Batt et al., 1976; Moore & Beechey, 1984; Poolman et al., 1983). None of the acid maleimides will inhibit 3-phosphoglycerate dehydrogenase activity in intact red blood cell ghosts (the enzyme is located on the inner face of this membrane); in contrast the corresponding $N$-alkylmaleimides have no difficulty in penetrating the membrane and inhibiting the enzyme activity. When red cell ghosts are made permeable by treatment with Triton X-100 to the AM and the substrate, there is a sharply graded ability of the acid maleimide to inhibit 3-phosphoglycerate dehydrogenase activity. The optimum chain length for inhibitory activity is found with AM2, one of the most polar of the AM series (Moore & Beechey, 1984). Consideration of the result with detergent-treated ghosts also suggests that the polymethylene chains do not fold back on themselves. If they did, the longer chain length AM would be expected to inhibit as well as AM2.

A number of reports indicate that the acid maleimides are also impermeable to mitochondrial membranes while NEM is permeable (Moore et al., 1984; Griffiths et al., 1983, 1981). Succinate dehydrogenase is accessible from the inner side of the mitochondrial membrane. When studied in submitochondrial particles, AM$\mathrm{I}^-$, -7, and -10 were found to inhibit 50-80% of the activity at reagent concentrations of 25 nmol/mg of protein. When studied in intact mitochondria, however, no inhibition was observed with these reagents even at 50 nmol/mg of protein, yet 25 nmol of NEM resulted in complete inhibition under the same conditions (Griffiths, 1983).

Batt et al. (1976) studied the topography of the glucose carrier in erythrocytes using dextranylmaleimide and glutathione maleimides. They reported that glutathione maleimide inhibits glucose efflux and that glucose protects against this inhibition. NEM also inhibits glucose efflux, but in this case,
glucose stimulates the inhibition. The authors concluded that NEM was able to reach sulfhydryl sites essential for glucose transport activity which were not accessible to GSM.

We have presented similar results indicating that GSM is incapable of penetrating the *E. coli* cytoplasmic membrane (Poolman et al., 1983). The *E. coli* proline/H⁺ symporter can be inhibited by GSM. The inhibition can be prevented completely by ferricyanide, thorin, and PCMBs, suggesting that the GSM site of inhibition is a dithiol near the outer side of the cytoplasmic membrane. NEM also inhibits transport activity, but in this case, PCMBs offers only slight protection. These data indicate that NEM, an apolar reagent, is able to gain access to sites not accessible to the impermeable PCMBs. Since GSM only reacted at the PCMBs site, we can conclude that GSM has permeability characteristics similar to those of PCMBs.

**Number of Activity-Linked Dithiols.** The GSM and AM inactivation data could be indicative of two activity-linked sites situated on opposite sides of the membrane or one activity-linked site accessible from either side of the membrane. We favor a single activity-linked site for the following reasons. (i) The protection characteristics are very similar; phosphorylation protects against inactivation in whole cells and in inverted vesicles. It is difficult to envision that phosphorylating a position near the inner surface of the cytoplasmic membrane provides equivalent protection to two sites located on opposite sides of the membrane irrespective of the mechanism of protection.

(ii) NEM does not inactivate by reacting at a second site in inverted vesicles (see Results). NEM inactivates transport in whole cells just as AM2 and GSM (data not shown); therefore, it does react at the site accessible from the exterior. NEM (0.1 mM) penetrates red blood cell ghosts and inhibits 95% of the glyceraldehyde-3-P dehydrogenase within 5 min. It is safe to conclude, therefore, that the conditions used in the present study (10 mM NEM, 30-min incubation) were sufficient to have allowed penetration of the inverted vesicles and reaction at the second site, if it existed.

**Nonactivity-Linked Dithiol.** A second dithiol appears to be located nearer to the outer surface of the cytoplasmic membrane. Reaction at this site does not lead directly to inactivation; however, it alters the reactivity or accessibility of the activity-linked dithiol. The biphasic nature of the inactivation time dependence with AM2, -3, and -5 shows that the activity-linked site is protected by a ΔΔH⁺ but that the protection is lost upon reaction at a second site. Since a ΔΔH⁺ protects against GSM and AM10 inactivation and since the AM10 inactivation under these circumstances is monophasic, both reagents probably penetrate too deeply into the membrane to be able to react at the outer site.

**Redox State of the Dithiols.** The activity-linked dithiol is in the reduced state in the absence of a ΔΔH⁺. The initial complete protection of the phosphorylated enzyme in the presence of a ΔΔH⁺ against all the maleimides tested suggests that this site might be oxidized when the membrane is energized. This would be in agreement with previous observations on this carrier (Robillard & Konings, 1981) and the proline/H⁺ symporter system in *E. coli* (Poolman et al., 1983). Possible mechanisms for altering the redox state with a ΔΔH⁺ have been explained (Robillard & Konings, 1982). It should be noted, however, that protection could also result from phosphorylation initiated changes in the reactivity of pK of the activity-linked dithiol.

No statement can be made concerning the redox state of the dithiol near the outer surface because reaction at this site can only be detected indirectly by its effect on the reactivity of the activity-linked site. Since the outer site does influence the activity-linked site, it is worthwhile considering that the two sites may respond to each others redox state.

**Comparison of Sulfhydryl Reactivities in EIIG⁺ vs. EIIM⁺**

EIIG⁺ and EIIM⁺ are both components of the phosphotransferase system, but the sequence of reactions in which they participate is not identical. The phosphoryl donor for EIIM⁺ is P–HP, while for EIIG⁺, it is P–EIIG⁺. Furthermore, the molecular mass of the two proteins differs significantly. EIIM⁺ is approximately 60 000 daltons and EIIG⁺ is approximately 45 000 daltons. The amino acid sequence of EIIM⁺ has been determined from the nucleotide sequence of the cloned gene (Lee & Saier, 1983). There are four cysteines in the protein. We have been able to label three of them in the purified protein with [¹⁴C]NEM (Roossien & Robillard, 1984a). Treatment of the active enzyme with NEM caused inactivation and incorporation of one label per peptide chain. This site was protected by oxygen, ferricyanide, phenylarsine oxide, and phosphorylation. Since only 1 mol of label was incorporated per peptide chain, the protection experiments suggested that an interpeptide disulfide bridge was involved. These data led to the proposal that EIIM⁺ existed as a dimer with an interpeptide activity-linked dithiol. We have confirmed this proposal by cross-linking the peptides using the short bifunctional sulfhydryl reagents 1,3-dibromoacetone and N,N'-1,2-phenylenedimaleimides and by oxidizing the dithiol to a disulfide with Cu²⁺ and with O₂ alone. In all cases EIIM⁺ dimer was observed on SDS–polyacrylamide gels (unpublished results). We have also obtained evidence for the existence of an EIIM⁺ dimer in the membrane. When gentle extraction procedures are employed, the dimer can be solubilized in an active state, labeled by [¹³P]HP, and visualized as a dimer on SDS–polyacrylamide gels. The dimer can be dephosphorylated by its substrate, mannitol (Roossien & Robillard, 1984b). Analytical ultracentrifugation on purified EIIG⁺ solubilized in octylpoly(oxyethylene) indicate that this enzyme also occurs as a dimer (Erni et al., 1982).

A different sulfhydryl group was labeled by [¹⁴C]NEM in P–EIIM⁺. Little, if any, labeling occurred at this site in the dephosphorylated enzyme. Phenylarsine oxide prevented labeling, suggesting that this sulfhydryl could also be capable of forming an interpeptide disulfide. Reaction at this site did not cause inactivation. This site may be equivalent to the nonactivity-linked dithiol on EIIG⁺.

**Transmembrane Channel Formed by EIIG⁺**

The inactivation data combined with the [¹⁴C]NEM labeling studies and the cross-linking results on purified EIIM⁺ lead to the following model of EI. An enzyme aggregate (possibly a dimer) forms a transmembrane channel. A dithiol essential for activity is located in the channel. Phosphorylation protects against alkylation presumably by effecting a conformational change that restricts access of reagents to the activity-linked site or by altering the reactivity of the dithiol. The dithiol can be oxidized by various nonpermeant oxidizing agents and, in intact cells, by a ΔΔH⁺. The oxidized enzyme is ineffective both in transport and in phosphorylation.

A nonactivity-linked dithiol is located near the outer surface of the cytoplasmic membrane. Although it effects the reactivity of the activity-linked site, we have no information concerning the proximity of the two sites.

Only analytical ultracentrifugation data have been supplied in support of an EIIG⁺ dimer. Until more evidence has been accumulated, the dimer hypothesis will remain speculative. Whether the EI dimers and the channel that allows access...
to the activity-linked dithiol play any role in the physical mechanism of sugar transport remain to be determined.

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