Vicinal Dithiol–Disulfide Distribution in the *Escherichia coli* Mannitol Specific Carrier Enzyme IImtl†

F. F. Roossien and G. T. Robillard

**Abstract:** *Escherichia coli* mannitol specific EII in membrane vesicles can be inhibited by the action of the oxidizable substrate-reduced phenazine methosulfate (PMS) in a manner similar to *E. coli* enzyme IIGlc [Robillard, G. T., & Konings, W. (1981) *Biochemistry* 20, 5025–5032]. The fact that reduced PMS and various oxidizing agents protect the enzyme from inactivation by the sulfhydryl reagents N-ethylmaleimide and bromopyruvate suggests that the active form possesses a dithiol which can be protected by conversion to a disulfide. The sulfhydryl–disulfide distribution has been examined in purified EIIImtl by labeling studies with N-[1-14C]ethylmaleimide ([14C]NEM). EIIImtl can be alkylated at three positions per peptide chain. When alkylation takes place in 8 M urea, only two positions are labeled. The third position becomes labeled in urea only after treatment with DTT, suggesting that the native enzyme is composed of two subunits linked by a disulfide bridge. The remaining two sulfhydryl groups per peptide chain appear to undergo changes in oxidation state as indicated by the following results. (1) Treatment of the active enzyme with NEM leads to complete inactivation and incorporation of 1 mol of [14C]NEM per peptide chain. Oxidizing agents protect the activity and prevent labeling presumably by forming a disulfide. (2) Phosphorylating the enzyme (one phosphoryl group per peptide chain) fully protects the activity, but 1 mol of NEM per peptide chain is still incorporated. Subsequent dephosphorylation by adding mannitol causes a second mole of [14C]NEM to be incorporated and results in complete inactivation. (3) The site that is labeled in the phosphorylated enzyme can also be protected by oxidizing agents. The possibility that dithiol–disulfide interchange occurs during the turnover of the carrier is discussed.

The activity of the glucose-specific carrier from *Escherichia coli* EIIBGlc is controlled by the redox potential. At low potentials EIIBGlc is in an active high-affinity form in which two vicinal thiol groups are reduced. At potentials greater than approximately −100 mV the dithiols convert to a disulfide (Robillard & Konings, 1981). Similar observations have been made for the lactose and proline carriers in *E. coli* which are totally different from the glucose carrier in their mode of energization (Konings & Robillard, 1982). On the basis of these data and similar observations for other transport and energy transducing systems, we suggested that dithiol–disulfide interchange could play a general role in energy-dependent processes (Robillard & Konings, 1982). Whether the redox changes serve a regulatory function or whether they are actually involved in the turnover of the enzymes has remained uncertain. The study presented in this report attempts to address this question for one transport system by examining the distribution of dithiols and disulfides in a purified EII and the various intermediates which occur during a turnover. The mannitol-specific EII has been used.

Recently a purification procedure for EIIImtl has been published (Jacobson et al., 1979). It is not known whether subunit interactions are involved in the catalytic activity of the enzyme. Kinetic studies have revealed that both EIIImtl and EIIGlc catalyze the phosphorylation of their respective substrates by ping-pong mechanisms involving P–EII intermediates as indicated in Scheme I (F. F. Rooslien, M. Blaauw, and G. T. Robillard, unpublished results; Misset et al., 1983; Rephaeli & Saier, 1980). The distribution of redox states has been determined in EIIImtl and P–EIIImtl before and after reaction with mannitol by monitoring the extent of incorporation of [14C]NEM in these intermediates. The data obtained indicate that the active enzyme possesses two sets of vicinal thiols whose reactivity toward NEM alternates with the phosphorylation state of the enzyme. One set is reactive in the dephosphorylated enzyme but not in the phosphorylated species. The other set has the opposite reactivity. Both sets can be protected by oxidizing agents or dithiol specific reagents.

**Scheme I**

\[
\begin{align*}
P-EII + EIIImtl & \rightarrow P-EIIImtl \\
P-EIIImtl & \rightarrow EIIImtl + mtl-1-P \\
P-EIIBGlc + EIIImtl & \rightarrow EIIImtl + Glc-6-P
\end{align*}
\]

**Experimental Procedures**

**Materials**

[1-14C]PEP (monocyclohexylammonium salt; specific activity 12 mCi/mmol) and [D-1-14C]mannitol (59 mCi/mmol) were obtained from Amersham. *N-[1-14C]Ethylmaleimide* (23.7 mCi/mmol) was from New England Nuclear. Lubrol PX, sodium deoxycholate, hexylagarose, and butylagarose were from Sigma. Sodium deoxycholate was recrystallized twice from acetone/H2O. All other chemicals were reagent grade from commercial sources.

EI and HPr were purified from *E. coli* P650 as described previously (Dooyewaard et al., 1979; Robillard et al., 1979).

**Methods**

**Growth Conditions.** *Escherichia coli* ML 308/225 was grown under aerobic conditions at 37 °C in medium 63 (Saier

† Abbreviations: PMS, phenazine methosulfate; PEP, phosphoenolpyruvate; EIIBGlc, glucose-specific enzyme II; EIIImtl, mannitol-specific enzyme II; DTT, dithiothreitol; NEM, N-ethylmaleimide; TDL, buffer containing 20 mM Tris-HCl, pH 8.4, 1 mM DTT, and 0.05% or 0.5% Lubrol PX (0.05% TDL and 0.5% TDL, respectively); Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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et al., 1976) containing 0.5% mannitol as the carbon source. Cells were harvested at OD550 = 1.2. Inside-out vesicles containing EIImtl were prepared as described by Reenstra et al. (1980). Membranes for EIImtl purification were prepared as described by Jacobson et al. (1979). Both membrane preparations were kept in liquid N2 until used.

Purification of EIImtl was performed according to a modified procedure of Jacobson et al. (1979). Membranes from 14 g of cells were extracted at 25 °C by stirring in 200 mL of extraction buffer (0.5% sodium deoxycholate, 20 mM Tris-HCl, pH 8.4, 0.2 M NaCl, and 1 mM DTT). After 30 min the solution was chilled on ice, and all further operations were performed at 4 °C. The extracted membrane suspension was centrifuged (14000g, 65 min), and the supernatant was loaded on a column of heylagarose (2 × 29 cm), preequilibrated in extraction buffer. The column was washed with 350 mL of extraction buffer. The EI activity was eluted with extraction buffer containing 0.5% Lubrol PX. The active fractions were pooled (46 mL) and diluted with 460 mL of extraction buffer. This solution was loaded on a second smaller heylagarose column (2 × 11 cm). The column was washed with 150 mL of extraction buffer followed by a 150-mL 0.0-1.5% Lubrol PX gradient in extraction buffer. The peak of EI activity eluted at 0.6% Lubrol PX. The peak fractions were pooled (18 mL) and dialyzed for 30 h against 2 volumes of 1 L of 20 mM Tris-HCl, pH 8.4, containing 0.5% Lubrol PX and 1 mM DTT. The dialyzed material was diluted with 150 mL of 20 mM Tris-HCl, pH 8.4, and 1 mM DTT and loaded on a butylagarose column (2 × 9 cm), preequilibrated with 20 mM Tris-HCl, pH 8.4, 0.05% Lubrol PX, and 1 mM DTT (0.05% TDL). The column was washed with 60 mL of 0.05% TDL, followed by a 100-mL 0–100 mM NaCl gradient in 0.05% TDL. The peak of the active fractions eluted at 20 mM NaCl. The pool (16 mL) was incubated for 10 min at 30 °C in the presence of 5 mM DTT (see Results). The DTT concentration was lowered to 0.05 mM by subsequent dialysis and concentration in an Amicon diaflow apparatus equipped with a YM-30 filter. This isolation procedure resulted in 2.5 mL of eluted E1 activity. SDS (10%)-polyacrylamide gel electrophoresis showed a single band with an apparent molecular weight of 58 000. Lubrol PX (0.025–0.05%) was present in all experiments performed with EIImtl.

Assays. EI activities were determined by an assay in which the rate of mannitol phosphorylation was linear with the EI concentration. EI was incubated at 30 °C in a buffer containing 25 mM Tris-HCl, pH 7.6, 10 mM NaF, 5 mM MgCl2, 1 mM DTT, 0.05% Lubrol PX, 10 mM PEP, 0.1–1.0 mM [1-14C]mannitol (4–0.4 mCi/mmol, respectively), and saturating amounts of E1 and HPr. Aliquots were withdrawn at various time intervals, and the phosphorylated sugar was separated from nonphosphorylated sugar by the Dowex AG 1-X2 ion-exchange column procedure (Misset et al., 1980) with 0.1 N HCl in place of LiCl as the eluent.

EI concentrations were determined in a procedure which measures the amount of phosphorylated EI from [14C]PEP (Misset et al., 1983). Various amounts of EI were incubated at 30 °C with 0.23 μM E1, 0.24 μM HPr, 50 mM sodium phosphate buffer, pH 7.0, 1 mM DTT, 0.5 mM MgCl2, and 5 μM [1-14C]PEP (12 mCi/mmol). The [14C]pyruvate formed was separated from [14C]PEP on an ion-exchange column (Brouwer et al., 1982). The pyruvate concentrations were corrected for the amounts arising from the phosphorylation and hydrolysis of phosphorylated EI and HPr, determined in the absence of EI. EI concentrations given in the text are always concentrations of available phosphoryl binding sites.

| Table I: Influence of Reduced PMS, Fe3+, and Phenylarsine Oxide on the EIImtl Activity in Inverted Membrane Vesicles and on the Inhibition by NEM |
|-----------------|-----------------|-----------------|
| EIImtl activity incubated in the presence of | EIImtl activity (%) |
|                | −NEM            | +NEM            |
| A               | no additions    | 100             | 18              |
| B               | reduced PMS     | 4               | 80              |
| C               | KCN/reduced PMS | 89              |                 |
| D               | Fe3+            | 15              | 85              |
| E               | phenylarsine oxide | 18        | 90              |

A EIImtl activity was measured as described under Experimental Procedures except that DTT was omitted from the assay mixture. The final protein concentration of the inverted vesicles in the assay mixture was 0.5–3 μg/mL. All experiments were performed at 30 °C. (A) Reference, no additions. (B) Vesicles were incubated for 15 min in the presence of 10 mM ascorbate and 10 μM PMS under a continuous flow of oxygen. (C) As in (B), except that prior to the addition of reduced PMS the vesicles were incubated for 5 min with 1 mM KCN. (D, E) Vesicles were preincubated for 10 min with 6.3 mM K3Fe(CN)6 (D) or 2 mM phenylarsine oxide (E). EIImtl activities were measured in the presence of 0.6 mM K3Fe(CN)6 and 0.2 mM phenylarsine oxide, respectively. NEM treatment: Vesicles were incubated for 5 min with 1 mM NEM in the presence of 1A, no additions. (B) 10 mM ascorbate and 10 mM PMS under a continuous flow of oxygen. (D) 6.3 mM K3Fe(CN)6, and (E) 2 mM phenylarsine oxide. After 5 min 25 mM DTT was added, and EIImtl activities were measured.

Reaction of EIImtl with [14C]NEM. Stock [14C]NEM was diluted 10-fold in pentane. Suitable aliquots were stored under nitrogen atmosphere in closed hydrolysis tubes. Prior to reaction with EIImtl a given amount of [14C]NEM in pentane was added to water. The pentane was removed from the water surface with a stream of dry N2 gas. After reaction of EIImtl with [14C]NEM (see table footnotes for specific details), nonreacted [14C]NEM was removed by extensive dialysis. The EIImtl-containing samples were diluted in 0.5% TDL buffer to a final volume of 2.5 mL and dialyzed against three 500-mL volumes of 0.5% TDL buffer. Sephadex G-15 gel filtration showed that no free label remained in the dialyzed samples. Control experiments showed that no EIImtl was absorbed on the dialysis membrane.

Radioactivity was counted in the presence of 8 mL of Packard emulsifier scintillator in a Nuclear Chicago Mark I liquid scintillation counter. The counting efficiency, determined by an internal standard, was 75%.

Results

Sulfhydryl Group Reactivity of Membrane-Bound EIImtl. Table I shows that reduced PMS, Fe3+, and phenylarsine oxide strongly inhibit EIImtl activity in inverted cytoplasmic membrane vesicles from E. coli (Table I). The results parallel those reported earlier (Robillard & Konings, 1981; Robillard et al., 1983) for the activity of E. coli EIImtl in inverted vesicles. Preincubation of EIImtl-containing membranes with KCN, which itself does not influence EIImtl activity, prevents the inhibition of EIImtl activity by reduced PMS. The inhibition of EIImtl activity by reduced PMS, Fe3+, and phenylarsine oxide was reversed by the addition of excess DTT (not shown). Incubation of EIImtl vesicles with the sulfhydryl alkylating reagents NEM or bromopyruvate resulted in an inactive EIImtl preparation. This inhibition was not reversed by DTT. When, prior to addition of NEM or bromopyruvate, the inside-out vesicles were preincubated with Fe3+, there was no irreversible loss of EIImtl activity. Similarly, preincubation with reduced PMS protected EIImtl against NEM inhibition. From the similarities between the results obtained with EIImtl and EIImtl containing inside-out vesicles, we conclude, analogous to the
conclusion of Robillard & Konings (1980) for EI\(\text{Glc}\), that
dithiol-disulfide interchanges play an important role in the
functioning of EI\(\text{m}^{\text{ntl}}\) in inside-out vesicles. The following
experiments with purified EI\(\text{m}^{\text{ntl}}\) were intended to answer two
questions: (1) whether the redox interchanges involve some
regulatory protein distinct from EI\(\text{m}^{\text{ntl}}\) or EI\(\text{m}^{\text{ntl}}\) itself, and (2)
whether the interchanges regulate the activity of the enzyme
or occur during the turnover of the enzyme.

**Sulfhydryl–Disulfide Distribution in Denatured EI\(\text{m}^{\text{ntl}}\).** The
number of reactive thiol groups in EI\(\text{m}^{\text{ntl}}\) was determined by
denaturing the enzyme in 7.8 M urea, followed by reaction with
\([^{14}\text{C}]\text{NEM}\). Table IIA shows that denatured EI\(\text{m}^{\text{ntl}}\)
contains two reactive sulfhydryls. The method of Zahler &
Cleland (1968) was used to determine whether EI\(\text{m}^{\text{ntl}}\) contained
any disulfide bridges. According to this method the available
disulfide bridges in the urea-treated protein are first reduced
by an excess of DTT. The excess DTT is removed by reaction
with arsenite, which complexes dithiols forming cyclic dithiol
arsenites. \([^{14}\text{C}]\text{NEM}\) is then reacted with the thiol groups
originating from the disulfides. When this method was applied
to denatured EI\(\text{m}^{\text{ntl}}\) in which the two sulfhydryls were already
reacted with \([^{14}\text{C}]\text{NEM}\), we found that only one extra thiol
could be labeled (Table IIB). Since this extra thiol must
originate from a disulfide bridge, the results suggest that the
denatured EI\(\text{m}^{\text{ntl}}\) preparation consists of two EI\(\text{m}^{\text{ntl}}\) monomers
connected by an intermolecular disulfide bridge, while the
monomer is defined as that unit which can be phosphorylated.
Direct reduction of denatured EI\(\text{m}^{\text{ntl}}\) before NEM treatment
leads to the same label incorporation as in the above experi-
ment (Table IIC). **Sulfhydryl Group Reactivity and Sulfhydryl–Disulfide
Distribution in Native EI\(\text{m}^{\text{ntl}}\).** The EI\(\text{m}^{\text{ntl}}\) purification procedure
results in a concentrated EI\(\text{m}^{\text{ntl}}\) preparation (2.5–4 \(\mu\)M) that is
very sensitive to inactivation by NEM. The enzyme is inac-
tivated after a 2-min incubation with 0.3 mM NEM. It is
important to note that if the incubation with 5 mM DTT after
the butyrylglucose column elution was omitted in the isolation
procedure, the concentrated EI\(\text{m}^{\text{ntl}}\) preparation could only
be inactivated by high NEM concentrations (>1 mM) in the
presence of substantial amounts of DTT (>0.4 mM). Further,
if DTT was omitted from the assay mixture, the apparent
activity of such a preparation was less than 40% of the activity
in the presence of 1 mM DTT. These results indicate that
the enzymatic properties of EI\(\text{m}^{\text{ntl}}\) strongly depend on its redox
state. For this reason all experiments described in this report
are performed with EI\(\text{m}^{\text{ntl}}\) preparations purified as described
under Experimental Procedures, including the DTT preincu-
bation before concentration.

When native EI\(\text{m}^{\text{ntl}}\) is incubated with 0.44 mM
\([^{14}\text{C}]\text{NEM}\), complete inactivation occurs, and 1.2 \([^{14}\text{C}]\text{NEM}\)
labels per EI are incorporated (Table IIA). Prolonging the
incubation for 15 min did not increase the label incorporation.
These data suggest that only one of the two thiols which occur
in denatured EI\(\text{m}^{\text{ntl}}\) is readily alkylated by \([^{14}\text{C}]\text{NEM}\) in native
EI. The rate of reaction of the second thiol is by comparison
much slower.

The data presented at the beginning of the Results show
that the oxidizing reagent K\(\text{Fe(CN)}_5\text{NO}\) and the dithiol blocking
reagent phenylarsine oxide inhibit the activity of EI\(\text{m}^{\text{ntl}}\)-
containing vesicles and protect the enzyme against NEM inac-
tivation. The same kind of inhibition and protection was
studied with purified EI\(\text{m}^{\text{ntl}}\) (Table IV). Fe\(^{3+}\) and phenylarsine
oxide strongly inhibit the activity of purified EI\(\text{m}^{\text{ntl}}\); if the
addition of DTT in the experiments in Table IVE.G was
omitted, the EI activity after preincubation was less than 10% of
the control value. The inhibition of EI\(\text{m}^{\text{ntl}}\) activity by Fe\(^{3+}\) is
not completely reversed by DTT. This is probably due to a
nonspecific salt effect because the same partial nonrevers-
sibility occurs after incubation with Fe\(^{2+}\). Preincubation of
EI\(\text{m}^{\text{ntl}}\) with Fe\(^{3+}\) protects the enzyme against NEM activation
for 85% (compare Table IVE.F). In contrast preincubation
of EI with Fe\(^{2+}\) did not result in any protection against NEM

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### Table II: Label Incorporation in Urea-Denatured EII\(\text{ntl}\)

<table>
<thead>
<tr>
<th>sequence of additions to denatured EII</th>
<th>([^{14}\text{C}]\text{NEM}/\text{EII} ) (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>([^{14}\text{C}]\text{NEM}/\text{DTT} )</td>
</tr>
<tr>
<td>B</td>
<td>([^{14}\text{C}]\text{NEM}/\text{DTT}/\text{AsO}_3^- )</td>
</tr>
<tr>
<td>C</td>
<td>(\text{DTT}/\text{AsO}_3^-/\text{[^{14}\text{C}]NEM}/\text{DTT} )</td>
</tr>
</tbody>
</table>

### Table III: Incorporation of \([^{14}\text{C}]\text{NEM}\) in Native EI\(\text{m}^{\text{ntl}}\)

<table>
<thead>
<tr>
<th>sequence of additions</th>
<th>EI activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>EII + ([^{14}\text{C}]\text{NEM} )</td>
</tr>
<tr>
<td>B</td>
<td>EII + phenylarsine oxide + ([^{14}\text{C}]\text{NEM} )</td>
</tr>
<tr>
<td>C</td>
<td>P-EII + ([^{14}\text{C}]\text{NEM} )</td>
</tr>
<tr>
<td>D</td>
<td>P-EII + (\text{[^{14}\text{C}]NEM} + \text{mannitol} )</td>
</tr>
<tr>
<td>E</td>
<td>P-EII + phenylarsine oxide + ([^{14}\text{C}]\text{NEM} )</td>
</tr>
<tr>
<td>F</td>
<td>P-EII + ([^{14}\text{C}]\text{NEM} + \text{P-HPr} )</td>
</tr>
</tbody>
</table>

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\( ^a \) EI was denatured by the addition of urea followed by a 60-
min incubation at 30°C. Final concentrations: EII, 1.9 \(\mu\)M;
urea, 7.8 M; sodium phosphate buffer, 35 mM, pH 7.0. \([^{14}\text{C}]\text{NEM}\) labeling of urea-denatured EII was performed at 30°C in
different ways: (A) Incubation with 0.31 mM \([^{14}\text{C}]\text{NEM} \),
1.6 mM DTT. (B) Incubation with subsequent addition of the following:
1.0 min, 0.31 mM \([^{14}\text{C}]\text{NEM} \); 0.1 min, 1.6 mM DTT; 0.6 mm
(14 mM) DTT. (C) Incubation with 0.31 mM \([^{14}\text{C}]\text{NEM} \),
1.6 mM DTT. (D) Incubation with subsequent addition of the following:
1.0 min, 0.31 mM \([^{14}\text{C}]\text{NEM} \); 0.1 min, 1.6 mM DTT; 0.6 mm
(14 mM) DTT. (E) Incubation with subsequent addition of the following:
1.0 min, 0.31 mM \([^{14}\text{C}]\text{NEM} \); 0.1 min, 1.6 mM DTT; 0.6 mm
(14 mM) DTT. (F) Incubation with subsequent addition of the following:
1.0 min, 0.31 mM \([^{14}\text{C}]\text{NEM} \); 0.1 min, 1.6 mM DTT; 0.6 mm
(14 mM) DTT.
and H after 5 min. E11 activities were assayed as described under Experimental Procedures and expressed as percentage of the control. The inhibition of E11 activity by phenylarsine oxide was almost completely reversed by addition of DTT. Preincubation of E11 with phenylarsine oxide protected the enzyme for more than 90% against NEM inactivation. When E11 was preincubated for 2 min with 2.5 mM phenylarsine oxide followed by a 10-min incubation with 0.4 mM [14C]NEM, 80% of the enzyme activity was retained, and only 0.3 mol of label was incorporated per E11 (Table IIIB). The protection afforded by Fe³⁺ and phenylarsine oxide indicates the presence of two adjacent thiols which can be converted to a disulfide and thereby be protected from reaction with NEM.

Sulfhydryl Group Reactivity and Sulfhydryl-Disulfide Distribution in P-E11. Addition of E11 to a mixture of [14C]PEP, E1, and HPr results in a burst of [14C]pyruvate due to the formation of P-E11 (see Figure 1). Kinetic studies have shown that this intermediate is a catalytically significant species in the reaction leading to mannitol-P (F. F. Roossien et al., unpublished results). Under conditions where the E11-catalyzed mannitol phosphorylation is inhibited by Fe³⁺ or NEM, 70% of the E11 phosphorylation sites can still be phosphorylated (Figure 1). Apparently the dephosphorylation reaction or the binding of the sugar is inhibited by oxidants or NEM. Phosphorylation of EIImtl protects the enzyme against NEM activation (Haguenauer-Tapis & Kepes, 1977; Robillard & Konings, 1981). Table IIIC shows that the same holds for purified EIImtl, virtually no activity loss is observed when phosphorylated E11 is incubated with 0.3 mM NEM. Even raising the NEM concentration to 1 mM did not result in considerable loss of EIImtl activity (not shown). Labeling experiments with [14C]NEM demonstrate that despite the retention of EIImtl activity, one label is still incorporated in P-E11. Incorporation of [14C]NEM in P-E11 can be prevented by preincubation with phenylarsine oxide (cf. Table IIIE) or with Fe³⁺ (data not shown). This protection again suggests that a second cysteine must be located in the vicinity of the reactive sulfhydryl such that formation of a disulfide or cyclic dithiol disulfide is possible. Reaction of P-E11 with NEM does not inactivate while reaction with dephosphorylated E11 does inactivate. We would expect, therefore, that mannitol-induced dephosphorylation of singly labeled P-E11 in the presence of [14C]NEM should result in incorporation of a second label and inactivation. The data in Table IIID show that inactivation is accompanied by an additional incorporation of 0.8 mol of label per E11. The analogous experiment, the phosphorylation of singly labeled E11 and mannitol-P (F. F. Roossien et al., unpublished results). The inhibition of EIImtl activity in inverted cytoplasmic membrane vesicles can be protected from inhibition from sulfhydryl reagents by prior exposure to oxidizable substrates, oxidizing agents, or the dithiol-specific reagent phenylarsine oxide. Since oxidizing agents and phenylarsine oxide exert the same protective effect on purified EIImtl as on the membrane-associated enzyme, we conclude that these agents and oxidizable substrates work directly at the level of EIImtl in the membrane instead of at the level of a regulatory protein which controls EIImtl activity.

The stoichiometry of label incorporation is based on an active site titration in which E11 is phosphorylated from [14C]PEP, producing a stoichiometric quantity of [14C]pyruvate. In a separate study, we have shown that essentially all of the phosphoryl groups on P-E11 can be converted to mannitol-P (F. F. Roossien et al., unpublished results). An EIImtl monomer is then defined as that unit which accepts one phosphoryl group. To test this, a total of three sites can be labeled per EIImtl monomer in the reduced denatured state. Since only two can be labeled without reduction, one is forced to suggest that the third site is a disulfide bridge between two monomers in the native enzyme. Each monomer in the native enzyme also contains two sulfhydryls, SH(A) and SH(B), capable of being alkylated. SH(A) is essential for enzymatic activity and is readily accessible only in the nonphosphorylated enzyme (see Figure 2). We have never observed alkylation of SH(A) in P-E11. SH(B) is most readily accessible in the phosphorylated enzyme. However, some label incorporation is observed at high NEM concentrations or long exposure times even in the nonphosphorylated state (Table III). The experiments with oxidants and phenylarsine oxide suggest that the SH groups occur in pairs capable of being oxidized to disulfides. The simplest construction consistent with the observed patterns of protection would be disulfides between the subunits involving...
present time there is no conclusive evidence that phosphorylation alters the redox state of the dithiols. Nevertheless the effects of phosphorylation/dephosphorylation are so similar to those of oxidation/reduction that such a mechanism must be seriously considered.

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Registry No. EII\textsuperscript{mt}, 37278-09-4; NEM, 128-53-0.

References


