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Published in:
Biochemistry

DOI:
10.1021/bi00297a006

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Document Version
Publisher’s PDF, also known as Version of record

Publication date:
1984

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Vicinal Dithiol–Disulfide Distribution in the *Escherichia coli* Mannitol Specific Carrier Enzyme II<sub>mtl</sub>†

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 II<sub>Glc</sub> [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 substrate-reduced phenazine methosulfate (PMS) in a manner similar to [14C]NEM. EIImtl 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* EII<sub>Glc</sub> is controlled by the redox potential. At low potentials EII<sub>Glc</sub> 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 EIImtl 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 EIImtl and EII<sub>Glc</sub> catalyze the phosphorylation of their respective substrates by ping-pong mechanisms involving P–EII intermediates as indicated in Scheme I (F. F. Roossien, M. Blaauw, and G. T. Robillard, unpublished results; Misset et al., 1983; Rephael & Saier, 1980). The distribution of redox states has been determined in EIImtl and P–EIImtl 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**

\[
P–\text{HPr} + \text{EIImtl} \rightarrow P–\text{EIImtl} \rightarrow \text{EIImtl} + \text{mtl–1–P} \]

\[
P–\text{EIImtl} + \text{Glc–6–P} \rightarrow \text{EIImtl} + \text{EIImtl} + \text{Glc–6–P} \]

**Experimental Procedures**

**Materials**

[1-14C]PEP (monocyclohexylammonium salt; specific activity 12 μCi/mmol) and [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/H<sub>2</sub>O. 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; EII<sub>Glc</sub>, glucose-specific enzyme II; EIImtl, mannitol-specific enzyme II; DTT, dithiothreitol; NEM, N-ethylmaleimide; TDL, buffer containing 20 mM Tris-Cl, 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 OD500 = 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 (140000g, 65 min), and the supernatant was loaded on a column of heparagrose (2 x 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 heparagrose column (2 x 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 x 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 pooled (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 dilution and concentration in an Amicon diaflow apparatus equipped with a YM-30 filter. This isolation procedure resulted in 2.5 mL of 20 mM Tris-HCl, pH 8.4, and 1 mM DTT containing EIImtl were prepared as described by Konings et al. (1983) for the activity of EIImtl in inverted membrane vesicles. The activity 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 reduced PMS, the inside-out vesicles were incubated 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 EIIGlc containing inside-out vesicles, we conclude, analogous to the

### Table 1: Influence of Reduced PMS, Fe3+, and Phenylarsine Oxide on the EIImtl Activity in Inverted Membrane Vesicles and on the Inhibition by NEM

<table>
<thead>
<tr>
<th></th>
<th>Inverted Vesicles</th>
<th>EIIImtl Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>no additions</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>reduced PMS</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>KCN/reduced PMS</td>
<td>89</td>
</tr>
<tr>
<td>D</td>
<td>Fe3+</td>
<td>15</td>
</tr>
<tr>
<td>E</td>
<td>phenylarsine oxide</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Inverted Vesicles</th>
<th>EIIImtl Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>NEMa</td>
<td>80</td>
</tr>
<tr>
<td>G</td>
<td>NEMb</td>
<td>90</td>
</tr>
</tbody>
</table>

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 1). The results parallel those reported earlier (Robillard & Konings, 1981; Robillard et al., 1983) for the activity of _E. coli_ EIIBGlc 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 EIIGlc containing inside-out vesicles, we conclude, analogous to the
conclusion of Robillard & Konings (1980) for EII\textsuperscript{Glc}, that dithiol–disulfide interchanges play an important role in the functioning of EII\textsuperscript{mt} in inside-out vesicles. The following experiments with purified EII\textsuperscript{mt} were intended to answer two questions: (1) whether the redox interchanges involve some regulatory protein distinct from EII\textsuperscript{mt} or EII\textsuperscript{mt} 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 EII\textsuperscript{mt}.** The number of reactive thiol groups in EII\textsuperscript{mt} was determined by denaturing the enzyme in 7.8 M urea, followed by reaction with \(^{14}\text{C}\)NEM. Table IIA shows that denatured EII\textsuperscript{mt} contains two reactive sulfhydryls. The method of Zahler & Cleland (1968) was used to determine whether EII\textsuperscript{mt} 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. \(^{14}\text{C}\)NEM is then reacted with the thiol groups originating from the disulfides. When this method was applied to denatured EII\textsuperscript{mt} in which the two sulfhydryls were already blocked with \(^{14}\text{C}\)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 EII\textsuperscript{mt} preparation consists of two EII\textsuperscript{mt} monomers connected by an intermolecular disulfide bridge, where the monomer is defined as that unit which can be phosphorylated. Direct reduction of denatured EII\textsuperscript{mt} before NEM treatment leads to the same label incorporation as in the above experiment (Table IIC).

**Sulfhydryl Group Reactivity and Sulfhydryl–Disulfide Distribution in Native EII\textsuperscript{mt}.** The EII\textsuperscript{mt} purification procedure results in a concentrated EII preparation (2.5–4 \(\mu\)M) that is very sensitive to inactivation by NEM. The enzyme is inactivated 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 butyrylase column elution was omitted in the isolation procedure, the concentrated EII 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 EII\textsuperscript{mt} strongly depend on its redox state. For this reason all experiments described in this report are performed with EII\textsuperscript{mt} preparations purified as described under Experimental Procedures, including the DTT preincubation before concentration.

When native EII\textsuperscript{mt} is incubated with 0.4 mM \(^{14}\text{C}\)NEM, the dithiol blocking reagent phenylarsine oxide inhibits the activity of EII\textsuperscript{mt}-containing vesicles and protect the enzyme against NEM inactivation. The same kind of inhibition and protection was studied with purified EII\textsuperscript{mt} (Table IV). \textsuperscript{15}Fe\textsuperscript{2+} and phenylarsine oxide strongly inhibit the activity of purified EII\textsuperscript{mt}; if the addition of DTT in the experiments in Table IV,E,G was omitted, the EII activity after preincubation was less than 10% of the control value. The inhibition of EII\textsuperscript{mt} activity by \textsuperscript{15}Fe\textsuperscript{2+} is not completely reversed by DTT. This is probably due to a nonspecific salt effect because the same partial nonreversibility occurs after incubation with \textsuperscript{15}Fe\textsuperscript{3+}. Preincubation of EII\textsuperscript{mt} with \textsuperscript{15}Fe\textsuperscript{2+} protects the enzyme against NEM activation for 85% (compare Table IV,E,F). In contrast preincubation of EII with \textsuperscript{15}Fe\textsuperscript{2+} did not result in any protection against NEM inhibition.
Table IV: Inactivation of EII\textsubscript{mt} by NEM in the Presence of Fe\textsuperscript{3+}, Fe\textsuperscript{2+}, and Phenylarsine Oxide\textsuperscript{a}

<table>
<thead>
<tr>
<th>EII preincubated with</th>
<th>NEM added</th>
<th>EII activity after DTT addition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>Fe\textsuperscript{3+}</td>
<td>73</td>
</tr>
<tr>
<td>D</td>
<td>Fe\textsuperscript{3+}</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>Fe\textsuperscript{3+}</td>
<td>73</td>
</tr>
<tr>
<td>F</td>
<td>Fe\textsuperscript{3+}</td>
<td>62</td>
</tr>
<tr>
<td>G</td>
<td>phenylarsine oxide</td>
<td>90</td>
</tr>
<tr>
<td>H</td>
<td>phenylarsine oxide</td>
<td>83</td>
</tr>
</tbody>
</table>

\textsuperscript{a} EII\textsubscript{mt} at 0.25 \mu M in 33 mM sodium phosphate buffer, containing 0.5 mM MgCl\textsubscript{2}, was incubated at 30 °C for 5 min with 1.6 mM K\textsubscript{3}FeCN\textsubscript{6} (C, D) or 1.6 mM K\textsubscript{3}FeCN\textsubscript{6} (E, F) and for 2 min with 2.5 mM phenylarsine oxide (G, H). After this preincubation 0.3 mM NEM was added to the indicated samples. DTT at 14 mM was added to samples A–F after 2.5 min and to samples G and H after 5 min. EII activities were assayed as described under Experimental Procedures and expressed as percentage of the control sample (A).

activation. The inhibition of EII\textsubscript{mt} by phenylarsine oxide was almost completely reversed by addition of DTT. Preincubation of EII with phenylarsine oxide protected the enzyme for more than 90% against NEM inactivation. When EII was preincubated for 2 min with 2.5 mM phenylarsine oxide followed by a 10-min incubation with 0.4 mM [\textsuperscript{14}C]NEM, 80% of the enzymatic activity was retained, and only 0.3 mol of label was incorporated per EII (Table IIIB). The protection afforded by Fe\textsuperscript{3+} 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–EII. Addition of EII to a mixture of [\textsuperscript{14}C]PEP, EI, and HPr results in a burst of [\textsuperscript{14}C]pyruvate due to the formation of P–EII (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 EII-catalyzed mannitol phosphorylation is inhibited by Fe\textsuperscript{3+} or NEM, 70% of the EII 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 EI\textsubscript{GDS} protects the enzyme against NEM activation (Haguenauer-Tapis & Kepes, 1977; Robillard & Konings, 1981). Table IIIC shows that the same holds for purified EII\textsubscript{mt}, virtually no activity loss is observed when phosphorylated EII is incubated with 0.3 mM NEM. Even raising the NEM concentration to 1 mM did not result in considerable loss of EII activity (not shown). Labeling experiments with [\textsuperscript{14}C]NEM demonstrate that despite the retention of EII activity, one label is still incorporated in P–EII. Incorporation of [\textsuperscript{14}C]NEM in P–EII can be prevented by preincubation with phenylarsine oxide (cf. Table IIIC) or with Fe\textsuperscript{2+} (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 arsinite is possible. Reaction of P–EII with NEM does not inactivate while reaction with dephosphorylated EII does inactivate. We would expect, therefore, that mannitol-induced dephosphorylation of singly labeled P–EII in the presence of [\textsuperscript{14}C]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 EII. The analogous experiment, the phosphorylation

![Figure 1: Pyruvate burst experiments performed as described under Experimental Procedures except that the [\textsuperscript{14}C]PEP concentration was 4.5 \mu M and EII was subjected to different preincubations. (C) No EII added, the formation of [\textsuperscript{14}C]Pyr originates from the phosphorylation of EI and HPr. (O) Control, EII not subjected to preincubation. (A) EII preincubated for 2 min with 0.33 mM NEM. The inactivation was stopped by addition of 14 mM DTT. (C) EII preincubated for 8 min with 5 mM K\textsubscript{3}FeCN\textsubscript{6}. The final Fe\textsuperscript{3+} concentration in the pyruvate burst assay is 1 mM. The formed [\textsuperscript{14}C]pyruvate is expressed as percentage of the original [\textsuperscript{14}C]PEP concentration.](image-url)
present 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.

Acknowledgments

The technical assistance of M. Blaauw and the stimulating discussions with J. Lolkema have attributed significantly to this work.

Registry No. EII$^{\text{mtl}}$, 37278-09-4; NEM, 128-53-0.

References


