Dimeric enzyme IImtl of the E. coli phosphoenolpyruvate-dependent phosphotransferase system. Cross-linking studies with bifunctional sulfhydryl reagents
Roossien, F.F.; Es-Spiekman, W. van; Robillard, G.T.

Published in:
FEBS Letters

DOI:
10.1016/0014-5793(86)80264-2

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

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

Publication date:
1986

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Dimeric enzyme II$_{\text{mtl}}$ of the E. coli phosphoenolpyruvate-dependent phosphotransferase system

Cross-linking studies with bifunctional sulfhydryl reagents

F.F. Roossien$^+$, W. van Es-Spiekman$^0$ and G.T. Robillard$^*$

Department of Physical Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

Received 20 September 1985; revised version received 25 November 1985

The occurrence of intermolecular dithiols on EIIm$^t$ has been studied with a number of thiol-specific cross-linking reagents. The reaction of EIIm$^t$ with bifunctional maleimide derivatives inactivates the enzyme. At the same time the enzyme is irreversibly cross-linked to a dimeric species. Under optimal conditions 50% of the protein is cross-linked upon reaction with the dimaleimides. The enzyme is also cross-linked under oxidizing conditions in the presence of CuCl₂, presumably by oxidizing an intermolecular dithiol to a disulfide. This oxidation can be reversed by the addition of the reducing agent dithiothreitol. The reaction of phosphorylated EIIm$^t$ with the same sulfhydryl-specific bifunctional reagents does not lead to any cross-linked product. The results are discussed in terms of the association state of the purified protein and the distribution of its thiol groups.

Dithiol Disulfide $\alpha$-Phenylenedimaleimide 1,3-Dibromoacetone 1,6-Hexanediimaleimide

1. INTRODUCTION

The phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) catalyze the concomitant transport and phosphorylation of a number of hexoses and hexitols across the cytoplasmic membrane. The mannitol specific PTS consists of 2 cytoplasmic proteins, EI and HPr, which are not sugar specific, and a sugar-specific integral membrane transport protein, EIIm$^t$. EIIm$^t$, originally purified by Jacobson et al. [1], consists of a single polypeptide chain with an $M_r$ of 58000 on SDS gels. Kinetic studies have shown that the EIIm$^t$ catalyzed mannitol phosphorylation proceeds via a phosphorylated EIIm species, P-EIIm$^t$ [2].

Several lines of evidence exist which indicate that the active forms of EIIm$^t$ and P-EIIm$^t$ are oligomeric (probably dimeric) species:

(i) The rate of the exchange reaction ([¹⁴C]mtl + mtl-P $\rightleftharpoons$ EIIm$_t$ [¹⁴C]mtl-P + mtl) shows a non-linear dependence on the EIIm$^t$ concentration [2,3].

(ii) EIIm$^t$ can be extracted as a dimer from the cytoplasmic membrane [4].

(iii) The reaction of EIIm$^t$ with [¹⁴C]MalNET results in the loss of enzymatic activity and incor-
poration of 1 mol label per peptide chain. The in-
activation and labeling can be prevented by prior
treatment of the enzyme with oxidizing or dithiol-
specific agents indicating a dimeric species with an
intermolecular dithiol (thiols (SHA) in fig.2 of [5]).
If P-EII \textsuperscript{ml} is reacted with \[^{14}\text{C}\]MalNET, en-
zymatic activity is retained, yet 1 mol label is still
incorporated per peptide chain. Preincubation of
P-EII \textsuperscript{ml} with dithiol-specific agents prevents the
incorporation of \[^{14}\text{C}\]MalNET, indicating a
dimeric P-EII \textsuperscript{ml} species with intermolecular dithiol
(thiols (SHB) in fig.2 of [5]). According to the
model presented, the 2 vicinal dithiols could altern-
ately undergo changes in accessibility or changes
in their redox states during turnover [5].

Although consistent with a dimeric enzyme
species, the labeling patterns and the influence of
oxidizing or dithiol-specific agents are not proof
for the existence of intermolecular dithiols. It is
 conceivable that there is asymmetry in the dimer so
that, in the presence of these reagents a dithiol on
1 subunit is labeled with 2 mol \[^{14}\text{C}\]MalNET while
a dithiol on the neighbouring subunit is not labeled
because it is oxidized to a disulfide or complexed
with the dithiol-specific agent. It is also con-
ceivable that only 1 thiol of a dithiol on each
subunit might be labeled because of steric restric-
tions. The issue of an inter- vs intramolecular
dithiol has been addressed in this study by using
sulfhydryl-specific cross-linking reagents.

2. EXPERIMENTAL

2.1. Materials

p-PDM and o-PDM were from Sigma;
1,6-HDM from ICN. 1,3-Dibromoacetone was ob-
tained as a dark coloured solution from ICN. This
material was crystallized from light petroleum
(b.p. 40–60°C) to give colourless needles. Stock
solutions of the dimaleimides and 1,3-dibromoace-
tone were made up daily in 100% acetone. The
final acetone concentration during cross-linking
experiments amounted to less than 2.5%.

2.2. Enzymes

Enzyme I and HPr were purified according to
[6,7], respectively. Enzyme II \textsuperscript{ml} was purified as
described in [5], except that the second hex-
ylagarose column was omitted.

2.3. Methods

EII \textsuperscript{ml} concentrations were determined by the
pyruvate-burst method as follows:

Four incubation mixtures were prepared each
containing 50 mM sodium phosphate buffer (pH
6.75) plus 1 mM MgCl\textsubscript{2}, 2 mM NaF, 2 mM DTT, 6–10 \textmu M phosphoenol\[^{14}\text{C}\]pyruvate and 0.1–0.3
\textmu M HPr and EI. Each of the mixtures was
incubated for 5 min at 30°C. Then 1, 2 or 3 vols
EII \textsuperscript{ml} were added to the incubation mixture. No
EII \textsuperscript{ml} was added to the fourth mixture which
served as the control for the \[^{14}\text{C}\]pyruvate burst
due to the phosphorylation of EI and HPr alone.
Samples (20–30 \mu l) were withdrawn from each
mixture at 3 min intervals, diluted into 1 ml H\textsubscript{2}O
and loaded directly onto columns containing 1 ml
BioRad AG 1-\times 2 resin. The flow rate of the col-
umns was \approx 0.5 ml/min. The columns were then
eluted with 4 ml of 125 mM NaCl. The load frac-
tion and the 125 mM NaCl fraction containing the
\[^{14}\text{C}\]pyruvate were collected in a scintillation vial.
The columns were then eluted with 5 ml of 1 M
NaCl to remove the phosphoenol\[^{14}\text{C}\]pyruvate.
This fraction was collected in separate scintillation
vials. Packard emulsifier scintillator was added
and the \(^{14}\text{C}\) levels were measured. The total
radioactivity per assay point was determined by
adding the counts from the phosphoenolpyruvate
and pyruvate fraction from each column. The frac-
tion of pyruvate at each assay point was then ob-
tained by dividing the counts in the pyruvate frac-
tion by the total counts.

Extrapolation of plots of \[^{14}\text{C}\]pyruvate to \(T = 0\)
yield the pyruvate burst for each incubation mix-
ture. The concentration of EII \textsuperscript{ml} was calculated
from the difference in the concentration of pyruvate produced in the burst with the 3 different
concentrations of EII \textsuperscript{ml} tested.

EII \textsuperscript{ml} enzymatic activity was determined by
measuring the rate of phosphoenolpyruvate-depen-
dent \[^{14}\text{C}\]mannitol phosphorylation in the
presence of saturating amounts of phosphoenol-
pyruvate, EI and HPr [5]. The activities of the
reacted EII \textsuperscript{ml} samples are expressed as percentages
of the rate of an untreated EII \textsuperscript{ml} sample, which
amounts to 210 \mu mol mannitol-1-P/min per \mu mol
EII \textsuperscript{ml}. The EII \textsuperscript{ml} catalyzed phosphoryl-group-
exchange reaction was measured at 30°C essen-
tially as described [8]. Samples contained 0.75 \mu M
EII \textsuperscript{ml} in 50 mM sodium phosphate buffer (pH
7.0), 5 mM MgCl₂, 0.05% Lubrol, 0.3 mM DTT and 3 μM ¹⁴C-labeled mannitol. At \( t = 0 \) min 0.8 mM mannitol-1-P was added, after which the isotope exchange rate was measured. In the absence of inhibitors this rate amounted about 80 pmol mannitol-1-P/min per nmol EIImtl.

2.4. Centricon method

The EIImtl purification procedure yields a preparation which contains about 1–2 μM EIImtl in 20 mM Tris-HCl (pH 8.4), 1 mM DTT and 0.1% Lubrol PX. Prior to cross-linking experiments DTT was removed from the EIImtl samples by diluting the sample in a degassed phosphate buffer of desired pH (containing 0.1% Lubrol, no DTT) and subsequent concentration in a Centricon microconcentrator (Amicon). This step was repeated as often as was necessary to bring the DTT to submicromolar concentrations. All steps were performed at 4°C. The yield of this procedure, on the basis of EIImtl enzymatic activity, was about 90%. An extra advantage of this method was that the final EIImtl concentration in the samples could be raised up to about 25 μM.

2.5. Solubilization and electrophoresis

Protein samples were diluted with solubilization buffer leading to a final concentration of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% mercaptoethanol (unless otherwise indicated). Solubilization was performed by incubating the samples for 30 min at 30°C. SDS-polyacrylamide gel electrophoresis was performed essentially as described in [9], except that the stacking gel was omitted. Usually a reference sample, containing the marker proteins myosin (200 kDa), β-galactosidase (116.5 kDa), phosphorylase (94 kDa), bovine serum albumin (68 kDa) and ovalbumin (46 kDa), was co-electrophoresed. The protein pattern of the gel was determined by staining with Coomassie brilliant blue or by silver staining [10].

3. RESULTS

3.1. Reaction of EIImtl with dimaleimide derivatives

The cross-linking reaction of EIImtl with dimaleimide derivatives cannot be properly studied in the presence of excess DTT. Therefore we developed a method (see section 2.4) which met with the following requirements: (i) DTT was almost completely removed; (ii) little loss of enzymatic activity occurred; (iii) the protein could be concentrated, if necessary. EIImtl samples obtained in this way were inactivated by MalNET under mild conditions ([MalNET] = 0.1 mM; pH 6.5; \( T = 30°C \)). This demonstrates that, although DTT is virtually completely removed by the Centricon method, the sulfhydryl groups of EIImtl do not

![Fig. 1. Cross-linking of enzyme IIml with different dimaleimides. The following additions were made to samples containing 21 μm EIImtl in 33 mM sodium phosphate buffer (pH 6.9) supplemented with 0.1% Lubrol: sample A, none; sample B, 62 μM 1,6-HDM; sample C, 62 μM α-PDM. After 10 min the reactions were terminated by the addition of 1.5 mM DTT. The reactions were performed at 30°C. A small aliquot of the samples was withdrawn to determine the phosphoenolpyruvate-dependent sugar phosphorylation activity: A, 100%; B, C, 10% (for details see section 2). Solubilization buffer was added to the remaining part of the samples after which electrophoresis on 7% acrylamide gels was performed. The protein pattern of the gel after staining with Coomassie brilliant blue is shown. A reference sample, containing marker proteins, was electrophoresed in the extreme left lane (for details see section 2).]
become oxidized in the course of this treatment; the oxidized enzyme cannot be inactivated by MalNET.

The reaction of EII\textsuperscript{mt} with 1,6-HDM or o-PDM leads to almost complete inactivation of the enzyme. Fig. 1 shows that, as a result of these reactions, an additional high-M\textsubscript{r} band is introduced in the protein pattern of the gel obtained after SDS-gel electrophoresis. The reaction of EII\textsuperscript{mt} with p-PDM leads to the same result as that obtained with o-PDM and 1,6-HDM. Husain and Lowe \cite{11} used the bifunctional reagent 1,3-dibromoacetone to locate a nucleophile close to a reactive thiol in the active site of papain. The reaction of EII\textsuperscript{mt} with 1,3-dibromoacetone, under essentially the same experimental conditions as those described in the legend to fig. 1, also resulted in loss of enzymatic activity and cross-linking of the protein (not shown). From fig. 1 we estimate that approx. 50% of the total protein is cross-linked upon reaction with the dimaleimides. A higher cross-linking percentage was never reached. As will be discussed later, this maximum yield of 50% is in agreement with the mechanism of the dimaleimide cross-linking reaction proposed by Knight and Offer \cite{12}. The activity-linked sulfhydryl groups of EII\textsuperscript{mt} are irreversibly blocked by reaction with MalNET. If the dimaleimides are added to EII\textsuperscript{mt}, preincubated with MalNET, no cross-linking is observed (not shown). On the basis of this result and the generally accepted specificity of maleimide derivatives for sulfhydryl groups, when the reaction is performed at neutral pH and submillimolar concentrations of maleimide (review \cite{13}), we conclude that the cross-link is introduced between the activity-linked sulfhydryl groups on different subunits of the EII\textsuperscript{mt} dimer.

3.2. \(M_r\) of the cross-linked EII\textsuperscript{mt} product

When the logarithm of the \(M_r\) of a number of reference proteins is plotted against their \(R_f\) values on SDS-polyacrylamide gels, a straight line is obtained. The \(M_r\) of an unknown protein can usually be deduced from such a plot. The \(M_r\) of EII\textsuperscript{mt}, determined in this way, was 58000 \cite{1}. When SDS gel electrophoresis was used to determine the \(M_r\) of the cross-linked EII\textsuperscript{mt} product, we observed a strong dependence of the apparent \(M_r\) on the polyacrylamide percentage of the gel (fig. 2). However, below 6% polyacrylamide the apparent

\[\text{Fig. 2. The dependence of the apparent } M_r \text{ of the cross-linked EII}^{\text{mt}} \text{ species on SDS-polyacrylamide gels as a function of the percentage of acrylamide. EII}^{\text{mt}} \text{ was reacted with o-PDM under essentially the same conditions as described in fig. 1. Samples were electrophoresed in the presence of SDS on gels with the indicated polyacrylamide percentages. A reference sample, containing the marker proteins ovalbumin (48 kDa), bovine serum albumin (68 kDa), phosphorylase (94 kDa), \(\beta\)-galactosidase (116.5 kDa) and myosin (200 kDa), was electrophoresed under the same conditions. The } M_r \text{ of the cross-linked EII}^{\text{mt}} \text{ species was determined as described in the text.}\]

\[M_r\] of the cross-linked species reaches a constant value of approx. 120000. This value corresponds with that of a cross-linked EII\textsuperscript{mt} dimer.

3.3. EII\textsuperscript{mt} cross-linking as a function of dimaleimide concentration

The specificity of the cross-linking process can be determined by examining the dependence of the amount of cross-linked product on the concentrations of protein and cross-linking reagent. This concentration dependence will be quite different when the cross-links are aspecifically created between protein monomers as opposed to when they are created between the 2 subunits of a dimeric protein. In the first case we expect that increase of the cross-linking reagent concentration beyond a 1:1 protein:cross-linker ratio will lead to a decrease in the amount of cross-linked product. However, in the second case we expect that the amount of cross-linked product will remain con-
stant if the concentration of cross-linking reagent is increased from stoichiometric to higher concentrations. In the experiment described in fig. 3, a fixed concentration of EII\textsuperscript{mtl} (3.6 \textmu M) was reacted with increasing amounts of o-PDM. A substantial amount of EII\textsuperscript{mtl} was cross-linked at a substoichiometric concentration of o-PDM (1 \textmu M). If the o-PDM concentration was increased to 2 \textmu M the amount of cross-linked product seemed to increase slightly. A further increase in o-PDM concentration had no influence on the cross-linking yield, consistent with the hypothesis that the association state of EII\textsuperscript{mtl} is a dimeric form. Reaction with o-PDM results in a covalent linkage between the 2 sulfhydryls on different subunits.

3.4. Cross-linking of EII\textsuperscript{mtl} under oxidizing conditions

Preincubation of EII\textsuperscript{mtl} with oxidizing agents such as K\textsubscript{3}Fe(CN)\textsubscript{6} protected the enzyme against MalN\textsubscript{ET} inhibition [5]. We assume that this protection results from the oxidation of the intermolecular activity-linked dithiol to a disulfide. According to this assumption, oxidation of EII\textsuperscript{mtl} leads to a cross-linked dimer in which the 2 subunits are attached by an intermolecular disulfide. In the experiment described in fig. 4, EII\textsuperscript{mtl} samples were incubated under different oxidizing conditions after which they were elec-

Fig. 3. The amount of cross-linked EII\textsuperscript{mtl} product upon reaction with increasing concentrations of o-PDM. EII\textsuperscript{mtl} containing samples (final concentration 3.6 \textmu M) in 46 mM sodium phosphate buffer (pH 6.5) supplemented with 0.1% Lubrol, were reacted for 18 min at 30°C with the following concentrations of o-PDM (\textmu M): A, 0; B, 1; C, 2; D, 3; E, 4; F, 6; G, 8. The reactions were terminated by the addition of 2.8 mM DTT. A portion of each sample was solubilized and electrophoresed on 7% SDS-polyacrylamide gels. The gel was stained by the silver-stain method. Small aliquots were withdrawn from the samples to determine the phosphoenolpyruvate-dependent sugar phosphorylation activity. The enzymatic activity of the samples, expressed as percentage of a non-treated sample, was: A, 100; B, 37; C, 18; D, 16; E–G, less than 12. A value of 100% corresponds with an activity of 200 \textmu mol mannitol-1-P/min per \textmu mol EII\textsuperscript{mtl}.

Fig. 4. Formation of EII\textsuperscript{mtl} dimers by oxidation (influence of CuCl\textsubscript{2}). The following additions were made to EII\textsuperscript{mtl} containing samples (final concentration 3.4 \textmu M) in 20 mM Tris-HCl (pH 8.4) supplemented with 0.1% Lubrol: A and B, none; C, 0.1 mM CuCl\textsubscript{2}; D, 0.5 mM CuCl\textsubscript{2}; E, 0.5 mM CuCl\textsubscript{2}. The samples were incubated for 30 min at 30°C. At $t = 30$ min, 7 mM DTT was added to samples A and E. At $t = 35$ min solubilization buffer, which did not contain mercaptoethanol, was added. Samples were electrophoresed on 7.5% SDS-polyacrylamide gels. The gel was silver stained.
trophoresed on SDS gels in the absence of the reducing agent, mercaptoethanol. EII\textsuperscript{ml} incubated in the presence of DTT migrates as usual, as a monomer (sample A). If DTT was omitted during incubation a relatively low intensity band at the EII\textsuperscript{ml} dimer position was detected. It is known that Cu\textsuperscript{2+} stimulates the oxidation of dithiols to disulfides \cite{13}. Incubation of EII\textsuperscript{ml} in the presence of CuCl\textsubscript{2} clearly enhanced the intensity of the EII\textsuperscript{ml} dimer band (samples C,D). The formation of the intermolecular disulfide could be completely reversed by the addition of an excess of the reducing agent, DTT (sample E).

Cd\textsuperscript{2+} is known to complex very strongly with vicinal dithiols \cite{14}. We measured the influence of Cd\textsuperscript{2+} on the EII\textsuperscript{ml} catalyzed phosphoryl-group-exchange reaction instead of on the phosphoenolpyruvate-dependent sugar phosphorylation to avoid the interference of a possible Cd\textsuperscript{2+}-induced inhibition of the EI catalyzed reaction. In the presence of 0.1 mM CdCl\textsubscript{2} the EII\textsuperscript{ml} catalyzed exchange reaction was completely inhibited (for details, see section 2). This inhibition could be reversed by the addition of 5 mM DTT. These results indicate the formation of a complex between Cd\textsuperscript{2+} and the exposed vicinal dithiol of the EII\textsuperscript{ml} dimer. However, if EII\textsuperscript{ml} in an identical experiment as shown in fig.4 was incubated with 0.6 mM CdCl\textsubscript{2} instead of CuCl\textsubscript{2}, no enhanced intensity of the dimer band could be observed on SDS gels (not shown). Apparently the complex between Cd\textsuperscript{2+} and the dimer dissociates in the presence of SDS.

3.5. Attempts to cross-link P-EII\textsuperscript{ml}

The \textsuperscript{[14C]}MalNET labeling studies on P-EII\textsuperscript{ml} were consistent with a dimeric species containing an exposed dithiol (thiols (SH\textsubscript{B}) in fig.2 of \cite{5}). Labeling of this dithiol with MalNET does not lead to a decrease in enzymatic activity. We attempted to cross-link the vicinal thiols by reacting P-EII\textsuperscript{ml} with o-PDM or p-PDM. The results obtained with o-PDM and p-PDM were quite different. Incubation of P-EII\textsuperscript{ml} with varying concentrations of o-PDM had no influence on the enzymatic activity and no cross-linked product could be observed after these incubations. On the other hand, incubation of P-EII\textsuperscript{ml} with increasing p-PDM concentrations resulted in an increasing loss of enzymatic activity. An increase in the amount of cross-linked product paralleled the loss of EII\textsuperscript{ml} enzymatic activity. In a series of kinetic experiments (not shown) we observed that preincubation of P-EII\textsuperscript{ml} with MalNET (which itself did not influence enzymatic activity) did not prevent the activity loss obtained upon subsequent incubation with p-PDM. From this we conclude that p-PDM does not react solely with SH\textsubscript{B} in P-EII\textsuperscript{ml}.

4. DISCUSSION

The data presented here show that EII\textsuperscript{ml} can be cross-linked through the activity-linked dithiol. This supports the view that the enzyme in the purified form is a dimer and that the activity-linked dithiol arises from 2 thiols contributed from separate subunits.

Knight and Offer \cite{12} postulated a mechanism for the p-PDM cross-linking reaction between the subunits of F-actin. In this reaction p-PDM initially reacts with a cysteine residue on one subunit, after which the cross-link is created by a relatively slow reaction with a lysine residue on a neighbouring subunit. Although, in the case of EII\textsuperscript{ml}, the cross-link is introduced between 2 cysteine residues, we assume that the reaction takes place via a mechanism analogous to that proposed by Knight and Offer \cite{12}. This involves hydrolysis of the maleimide ring after the first thiol is alkylated to instill sufficient flexibility into the cross-linker to enable the other maleimide moiety to react with the second thiol. If the unreacted maleimide moiety hydrolyzes first it cannot alkylate and cross-linking will not occur. Since the rates of hydrolysis of the 2 rings are the same a maximum of 50\% cross-linking is predicted. These predictions of the cross-linking yield agree very well with our observation of 50\% cross-linked EII\textsuperscript{ml} after reaction with the dimaleimides. There are other factors which might have a negative influence on the cross-linking yield. Incomplete removal of DTT by the Centricon method will decrease the cross-linking yield. Hydrolysis of one of the maleimide rings of the dimaleimide prior to the initial reaction with a cysteine residue decreases the yield as well.

A strong argument in favour of a functional dimeric EII\textsuperscript{ml} species comes from the observation that the membrane-bound protein can be extracted as a dimer if the extraction is performed in the presence of SDS and mercaptoethanol \cite{4}. The ex-
tracted EIIm dimer, in contrast to the cross-linked dimer described above, shows a normal behaviour on SDS gel electrophoresis. Its apparent $M_r$ does not depend on the acrylamide percentage of the gel. The interactions between the dimer subunits are markedly weakened by the non-ionic detergent Lubrol. When the membranes are treated with SDS and Lubrol the protein migrates as a monomer.

All cross-linking experiments described here were performed with purified EIIm in the presence of Lubrol. The $M_r$ of the dimaleimide cross-linked EIIm product shows a strong dependence on the acrylamide percentage of the gel (fig.2). If the enzyme is cross-linked by oxidation, in the presence of CuCl$_2$, the same dependence on the gel percentage is observed. Therefore, this behaviour is not caused by the nature of the cross-link created, a dimaleimide bridge or a disulfide, respectively. The different dependence on the acrylamide concentration may be due to the elongated structure of the cross-linked dimer in the presence of Lubrol and SDS. SDS alone leaves the subunit interactions of the non-cross-linked protein intact so that the protein migrates similar to a globular protein with $M_r$ of the dimer. Lubrol and SDS destroy the subunit interactions, however, when cross-linked, the subunits cannot dissociate. The acrylamide concentration dependence suggests that the cross-linked molecule is oblong and has a higher frictional coefficient than the dimer which has not been exposed to Lubrol.

On the basis of $[^{14}\text{C}]$MalNET labeling studies of urea denatured EIIm, we suggested that the enzyme, in addition to 2 intermolecular dithiol groups, might contain an intermolecular disulfide. This disulfide could not be demonstrated by electrophoresis of the enzyme in the absence of reducing agents. Under these conditions the protein almost completely migrated as a monomer (fig.4, sample B).

Labelling of P-EIIm with $[^{14}\text{C}]$MalNET resulted in an incorporation of 1 mol $^{14}$C per polypeptide chain. Since the labeling could be prevented by dithiol-specific reagents we suggested that there was a second intermolecular dithiol (thiols (SH$_A$) in fig.2 of [5]). The experiments presented here do not support that proposal. No cross-linked product could be obtained by reaction at the non-activity-linked dithiol in P-EIIm. The cross-linking which was observed using p-PDM resulted in the loss of enzymatic activity and could be attributed to reaction at the activity-linked-dithiol (SH$_A$). In light of the original labeling data, our inability to achieve intermolecular cross-linking of P-EIIm suggests that the non-activity-linked dithiol may be located within a single subunit where, because of steric hindrance, only one of the sulfhydryls could be labeled with $[^{14}\text{C}]$MalNET.

ACKNOWLEDGEMENT

This research has been supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES