

# Dimeric enzyme $\text{EII}^{\text{mtl}}$ of the *E. coli* phosphoenolpyruvate-dependent phosphotransferase system

## Cross-linking studies with bifunctional sulfhydryl reagents

F.F. Roossien<sup>+</sup>, W. van Es-Spiekman<sup>°</sup> 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  $\text{EII}^{\text{mtl}}$  has been studied with a number of thiol-specific cross-linking reagents. The reaction of  $\text{EII}^{\text{mtl}}$  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  $\text{CuCl}_2$ , 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  $\text{EII}^{\text{mtl}}$  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      *o*-Phenylenedimaleimide      1,3-Dibromoacetone      1,6-Hexanedimaleimide

## 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,  $\text{EII}^{\text{mtl}}$ .  $\text{EII}^{\text{mtl}}$ , 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  $\text{EII}^{\text{mtl}}$  catalyzed mannitol phosphorylation proceeds via a phosphorylated  $\text{EII}^{\text{mtl}}$  species,  $\text{P-EII}^{\text{mtl}}$  [2].

Several lines of evidence exist which indicate that the active forms of  $\text{EII}^{\text{mtl}}$  and  $\text{P-EII}^{\text{mtl}}$  are oligomeric (probably dimeric) species:

(i) The rate of the exchange reaction ( $[^{14}\text{C}]\text{mtl} + \text{mtl-P} \xrightleftharpoons{\text{EII}} [^{14}\text{C}]\text{mtl-P} + \text{mtl}$ ) shows a non-linear dependence on the  $\text{EII}^{\text{mtl}}$  concentration [2,3].

(ii)  $\text{EII}^{\text{mtl}}$  can be extracted as a dimer from the cytoplasmic membrane [4].

(iii) The reaction of  $\text{EII}^{\text{mtl}}$  with  $[^{14}\text{C}]\text{MalNET}$  results in the loss of enzymatic activity and incor-

\* To whom correspondence should be addressed

<sup>+</sup> Present address: Antoni van Leeuwenhoekhuis, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX Amsterdam, The Netherlands

<sup>°</sup> Present address: Philips Research Laboratories (Natuurkundig Laboratorium) Eindhoven, The Netherlands

**Abbreviations:**  $\text{EII}^{\text{mtl}}$ , mannitol-specific enzyme II;  $\text{P-EII}^{\text{mtl}}$ , phosphorylated  $\text{EII}^{\text{mtl}}$ ; DTT, dithiothreitol; PTS, phosphotransferase system; *o*-PDM, *N,N'*-*o*-phenylenedimaleimide; *p*-PDM, *N,N'*-*p*-phenylenedimaleimide; 1,6-HDM, 1,6-hexanedimaleimide; MalNET, *N*-ethylmaleimide

poration of 1 mol label per peptide chain. The inactivation 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 (SH<sub>A</sub>) in fig.2 of [5]). If P-EII<sup>mtl</sup> is reacted with [<sup>14</sup>C]MalNET, enzymatic activity is retained, yet 1 mol label is still incorporated per peptide chain. Preincubation of P-EII<sup>mtl</sup> with dithiol-specific agents prevents the incorporation of [<sup>14</sup>C]MalNET, indicating a dimeric P-EII<sup>mtl</sup> species with intermolecular dithiol (thiols (SH<sub>B</sub>) in fig.2 of [5]). According to the model presented, the 2 vicinal dithiols could alternately 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 [<sup>14</sup>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 conceivable that only 1 thiol of a dithiol on each subunit might be labeled because of steric restrictions. 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 obtained 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-dibromoacetone 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 EII<sup>mtl</sup> was purified as described in [5], except that the second hexylagarose column was omitted.

### 2.3. Methods

EII<sup>mtl</sup> 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<sub>2</sub>, 2 mM NaF, 2 mM DTT, 6–10 μM phosphoenol[<sup>14</sup>C]pyruvate and 0.1–0.3 μM HPr and EI. Each of the mixtures was incubated for 5 min at 30°C. Then 1, 2 or 3 vols EII<sup>mtl</sup> were added to the incubation mixture. No EII<sup>mtl</sup> was added to the fourth mixture which served as the control for the [<sup>14</sup>C]pyruvate burst due to the phosphorylation of EI and HPr alone. Samples (20–30 μl) were withdrawn from each mixture at 3 min intervals, diluted into 1 ml H<sub>2</sub>O and loaded directly onto columns containing 1 ml BioRad AG 1-×2 resin. The flow rate of the columns was ≈0.5 ml/min. The columns were then eluted with 4 ml of 125 mM NaCl. The load fraction and the 125 mM NaCl fraction containing the [<sup>14</sup>C]pyruvate were collected in a scintillation vial. The columns were then eluted with 5 ml of 1 M NaCl to remove the phosphoenol[<sup>14</sup>C]pyruvate. This fraction was collected in separate scintillation vials. Packard emulsifier scintillator was added and the <sup>14</sup>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 fraction of pyruvate at each assay point was then obtained by dividing the counts in the pyruvate fraction by the total counts.

Extrapolation of plots of [<sup>14</sup>C]pyruvate to *T* = 0 yield the pyruvate burst for each incubation mixture. The concentration of EII<sup>mtl</sup> was calculated from the difference in the concentration of pyruvate produced in the burst with the 3 different concentrations of EII<sup>mtl</sup> tested.

EII<sup>mtl</sup> enzymatic activity was determined by measuring the rate of phosphoenolpyruvate-dependent [<sup>14</sup>C]mannitol phosphorylation in the presence of saturating amounts of phosphoenolpyruvate, EI and HPr [5]. The activities of the reacted EII<sup>mtl</sup> samples are expressed as percentages of the rate of an untreated EII<sup>mtl</sup> sample, which amounts to 210 μmol mannitol-1-P/min per μmol EII<sup>mtl</sup>. The EII<sup>mtl</sup> catalyzed phosphoryl-group-exchange reaction was measured at 30°C essentially as described [8]. Samples contained 0.75 μM EII<sup>mtl</sup> in 50 mM sodium phosphate buffer (pH

7.0), 5 mM  $\text{MgCl}_2$ , 0.05% Lubrol, 0.3 mM DTT and  $3 \mu\text{M}$   $^{14}\text{C}$ -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  $\text{EII}^{\text{mtl}}$ .

#### 2.4. Centricon method

The  $\text{EII}^{\text{mtl}}$  purification procedure yields a preparation which contains about 1–2  $\mu\text{M}$   $\text{EII}^{\text{mtl}}$  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  $\text{EII}^{\text{mtl}}$  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  $4^\circ\text{C}$ . The yield of this procedure, on the basis of  $\text{EII}^{\text{mtl}}$  enzymatic activity, was about 90%. An extra advantage of this method was that the final  $\text{EII}^{\text{mtl}}$  concentration in the samples could be raised up to about 25  $\mu\text{M}$ .

#### 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  $30^\circ\text{C}$ . 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),  $\beta$ -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 $\text{EII}^{\text{mtl}}$ with dimaleimide derivatives

The cross-linking reaction of  $\text{EII}^{\text{mtl}}$  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.  $\text{EII}^{\text{mtl}}$  samples obtained in this way were inactivated by MalNET under mild conditions ( $[\text{MalNET}] = 0.1$  mM; pH 6.5;  $T = 30^\circ\text{C}$ ). This demonstrates that, although DTT is virtually completely removed by the Centricon method, the sulfhydryl groups of  $\text{EII}^{\text{mtl}}$  do not

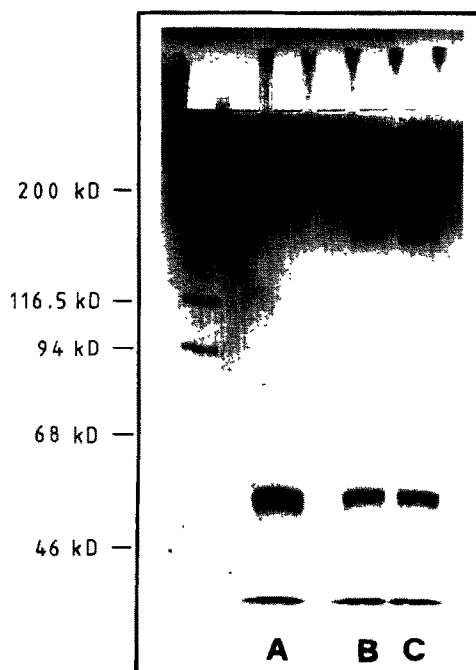


Fig.1. Cross-linking of enzyme  $\text{EII}^{\text{mtl}}$  with different dimaleimides. The following additions were made to samples containing 21  $\mu\text{M}$   $\text{EII}^{\text{mtl}}$  in 33 mM sodium phosphate buffer (pH 6.9) supplemented with 0.1% Lubrol: sample A, none; sample B, 62  $\mu\text{M}$  1,6-HDM; sample C, 62  $\mu\text{M}$  o-PDM. After 10 min the reactions were terminated by the addition of 1.5 mM DTT. The reactions were performed at  $30^\circ\text{C}$ . 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  $EII^{mtl}$  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- $M_r$  band is introduced in the protein pattern of the gel obtained after SDS-gel electrophoresis. The reaction of  $EII^{mtl}$  with p-PDM leads to the same result as that obtained with o-PDM and 1,6-HDM. Husain and Lowe [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  $EII^{mtl}$  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 [12]. The activity-linked sulfhydryl groups of  $EII^{mtl}$  are irreversibly blocked by reaction with MalNET. If the dimaleimides are added to  $EII^{mtl}$ , 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 [13]), we conclude that the cross-link is introduced between the activity-linked sulfhydryl groups on different subunits of the  $EII^{mtl}$  dimer.

### 3.2. $M_r$ of the cross-linked $EII^{mtl}$ 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  $EII^{mtl}$ , determined in this way, was 58000 [1]. When SDS gel electrophoresis was used to determine the  $M_r$  of the cross-linked  $EII^{mtl}$  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

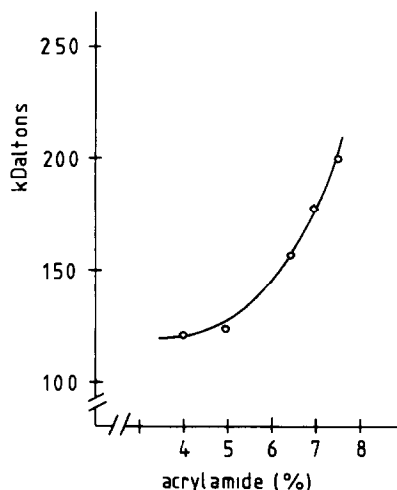


Fig.2. The dependence of the apparent  $M_r$  of the cross-linked  $EII^{mtl}$  species on SDS-polyacrylamide gels as a function of the percentage of acrylamide.  $EII^{mtl}$  was reacted with o-PDM under essentially the same conditions as described in fig.1. Samples were electrophoresed in the presence 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  $M_r$  of the cross-linked  $EII^{mtl}$  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  $EII^{mtl}$  dimer.

### 3.3. $EII^{mtl}$ 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  $EII^{mtl}$  ( $3.6 \mu M$ ) was reacted with increasing amounts of o-PDM. A substantial amount of  $EII^{mtl}$  was cross-linked at a sub-stoichiometric concentration of o-PDM ( $1 \mu M$ ). If the o-PDM concentration was increased to  $2 \mu M$  the amount of cross-linked product seemed to increase slightly. A further increase in o-PDM concentration had no influence on the cross-linking

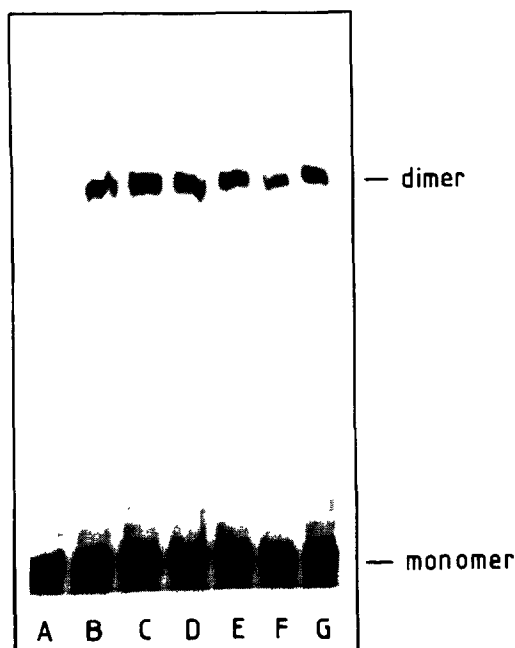


Fig.3. The amount of cross-linked  $EII^{mtl}$  product upon reaction with increasing concentrations of o-PDM.  $EII^{mtl}$  containing samples (final concentration  $3.6 \mu M$ ) in 46 mM sodium phosphate buffer (pH 6.5) supplemented with 0.1% Lubrol, were reacted for 18 min at  $30^\circ C$  with the following concentrations of o-PDM ( $\mu M$ ): 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  $200 \mu mol$  manitol-1-P/min per  $\mu mol$   $EII^{mtl}$ .

yield, consistent with the hypothesis that the association state of  $EII^{mtl}$  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 $EII^{mtl}$ under oxidizing conditions

Preincubation of  $EII^{mtl}$  with oxidizing agents such as  $K_3FeCN_6$  protected the enzyme against MalNET 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  $EII^{mtl}$  leads to a cross-linked dimer in which the 2 subunits are attached by an intermolecular disulfide. In the experiment described in fig.4,  $EII^{mtl}$  samples were incubated under different oxidizing conditions after which they were elec-

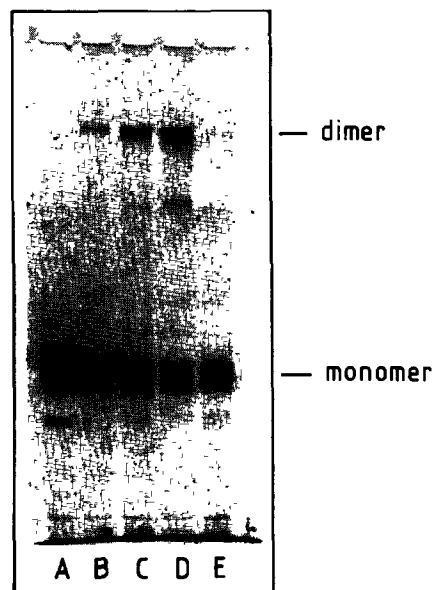


Fig.4. Formation of  $EII^{mtl}$  dimers by oxidation (influence of  $CuCl_2$ ). The following additions were made to  $EII^{mtl}$  containing samples (final concentration  $3.4 \mu M$ ) in 20 mM Tris-HCl (pH 8.4) supplemented with 0.1% Lubrol: A and B, none; C, 0.1 mM  $CuCl_2$ ; D, 0.5 mM  $CuCl_2$ ; E, 0.5 mM  $CuCl_2$ . The samples were incubated for 30 min at  $30^\circ C$ . 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<sup>mtl</sup> 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<sup>mtl</sup> dimer position was detected. It is known that Cu<sup>2+</sup> stimulates the oxidation of dithiols to disulfides [13]. Incubation of EII<sup>mtl</sup> in the presence of CuCl<sub>2</sub> clearly enhanced the intensity of the EII<sup>mtl</sup> 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<sup>2+</sup> is known to complex very strongly with vicinal dithiols [14]. We measured the influence of Cd<sup>2+</sup> on the EII<sup>mtl</sup> catalyzed phosphoryl-group-exchange reaction instead of on the phosphoenolpyruvate-dependent sugar phosphorylation to avoid the interference of a possible Cd<sup>2+</sup>-induced inhibition of the EI catalyzed reaction. In the presence of 0.1 mM CdCl<sub>2</sub> the EII<sup>mtl</sup> 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<sup>2+</sup> and the exposed vicinal dithiol of the EII<sup>mtl</sup> dimer. However, if EII<sup>mtl</sup> in an identical experiment as shown in fig.4 was incubated with 0.6 mM CdCl<sub>2</sub> instead of CuCl<sub>2</sub>, no enhanced intensity of the dimer band could be observed on SDS gels (not shown). Apparently the complex between Cd<sup>2+</sup> and the dimer dissociates in the presence of SDS.

### 3.5. Attempts to cross-link P-EII<sup>mtl</sup>

The [<sup>14</sup>C]MalNET labeling studies on P-EII<sup>mtl</sup> were consistent with a dimeric species containing an exposed dithiol (thiols (SH)<sub>2</sub>) in fig.2 of [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<sup>mtl</sup> with o-PDM or p-PDM. The results obtained with o-PDM and p-PDM were quite different. Incubation of P-EII<sup>mtl</sup> 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<sup>mtl</sup> 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<sup>mtl</sup> enzymatic activity. In a series of kinetic experiments (not shown) we observed that preincubation of P-EII<sup>mtl</sup> 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<sub>2</sub> in P-EII<sup>mtl</sup>.

## 4. DISCUSSION

The data presented here show that EII<sup>mtl</sup> 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 [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<sup>mtl</sup>, 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 [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<sup>mtl</sup> 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<sup>mtl</sup> 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 [4]. The ex-

tracted EII<sup>mtl</sup> 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 EII<sup>mtl</sup> in the presence of Lubrol. The  $M_r$  of the dimaleimide cross-linked EII<sup>mtl</sup> 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<sub>2</sub>, 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 [<sup>14</sup>C]MalNET labeling studies of urea denatured EII<sup>mtl</sup>, 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-EII<sup>mtl</sup> with [<sup>14</sup>C]MalNET resulted in an incorporation of 1 mol <sup>14</sup>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<sub>B</sub>) 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-EII<sup>mtl</sup>. 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<sub>A</sub>). In light of the original labeling data, our inability to achieve intermolecular cross-linking of P-EII 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 [<sup>14</sup>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

- [1] Jacobson, G.R., Lee, C.A. and Saier, M.H. jr (1979) *J. Biol. Chem.* 254, 249–252.
- [2] Roossien, F.F., Blaauw, M. and Robillard, G.T. (1984) *Biochemistry* 23, 4934–4939.
- [3] Saier, M.H. jr (1980) *J. Supramol. Struct.* 14, 281–294.
- [4] Roossien, F.F. and Robillard, G.T. (1984b) *Biochemistry* 23, 5682–5685.
- [5] Roossien, F.F. and Robillard, G.T. (1984a) *Biochemistry* 23, 211–215.
- [6] Robillard, G.T., Dooijewaard, G. and Lolkema, J. (1979) *Biochemistry* 18, 2990–2996.
- [7] Dooijewaard, G., Roossien, F.F. and Robillard, G.T. (1979) *Biochemistry* 18, 2980–2989.
- [8] Brouwer, M., Elferink, M. and Robillard, G.T. (1980) *Biochemistry* 21, 82–88.
- [9] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [10] Wray, W., Bouliskas, T., Wray, V.P. and Hancock, R. (1981) *Anal. Biochem.* 118, 197–203.
- [11] Husain, S.S. and Lowe, G. (1968) *Biochem. J.* 110, 53–57.
- [12] Knight, P. and Offer, G. (1978) *Biochem. J.* 175, 1023–1032.
- [13] Torchinsky, Yu.M. (1981) *Sulfur in Proteins*, Pergamon Press, Oxford.
- [14] Gaber, B.P. and Fluharty, A.L. (1972) *Bioinorg. Chem.* 2, 135–148.