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Enzyme II^{Mtl} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Identification of the Activity-Linked Cysteine on the Mannitol Carrier†

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ABSTRACT: The cysteines of the membrane-bound mannitol-specific enzyme II (EII^{Mtl}) of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system have been labeled with 4-vinylpyridine. After proteolytic breakdown and reversed-phase HPLC, the peptides containing cysteines 110, 384, and 571 could be identified. *N*-Ethylmaleimide (NEM) treatment of the native unphosphorylated enzyme results in incorporation of one NEM label per molecule and loss of enzymatic activity [Roossien, F. F., & Robillard, G. T. (1984) *Biochemistry 23*, 211–215]. NEM treatment and inactivation prevented 4-vinylpyridine incorporation into the Cys-384-containing peptide, identifying this residue as the activity-linked cysteine. Both oxidation and phosphorylation of the native enzyme protected the enzyme against NEM labeling of Cys-384. Positive identification of the activity-linked cysteine was accomplished by inactivation with [^{14}C]iodoacetamide, proteolytic fragmentation, isolation of the peptide, and amino acid sequencing.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] is responsible for uptake of a number of hexoses and hexitols in both Gram-negative and Gram-positive organisms [for a review see Postma and Lengeler (1985)]. The driving force for uptake is phosphoenolpyruvate. During transport over the membrane the substrate becomes phosphorylated. The PTS consists of two general proteins, EI and HPr, which are cytoplasmic, and a sugar-specific EII, which is membrane bound. Some PTS systems, for instance the glucose PTS of *Escherichia coli*, use a fourth protein, $EIII^{Glc}$, which acts between HPr and EII^{Glc}.

Cysteines often play important roles in transport processes in both procaryotic and eucaryotic systems. However, their exact role is uncertain. Localization of the cysteines is a prerequisite for understanding their mechanistic importance. Recently, Menich et al. (1987) showed by site-directed mutagenesis that, of the eight cysteines of the *E. coli* lac permease, Cys-154 is obligatory for lactose/H^+ symport. Robillard and Konings (1981) showed that the activity of the PTS glucose-specific carrier EII^{Glc} was sensitive to oxidants and the redox potential. The activity decreased at potentials greater than −100 mV. Furthermore, the reduced enzyme could be irreversibly inactivated by the thiol reagent *N*-ethylmaleimide (NEM), while the oxidized form was protected. On the basis of these results it was suggested that dithiol–disulfide interchange could play an important role in catalytic activity. Studies on EII^{Fru} from *Rhodopseudomonas sphaeroides* expanded on this concept by demonstrating that, at intermediate redox potentials, turnover of the carrier is accompanied by cycling through the oxidized and reduced states (Lolkema & Robillard, 1986). Roossien and Robillard (1984) quantitated the NEM labeling of the purified mannitol permease EII^{Mtl}. The reduced unphosphorylated enzyme was inhibited by incorporation of NEM at one site per peptide chain. The labeled thiol was named SH_A. Oxidation prevented labeling at this site. One label per peptide chain was also incorporated in the

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; EII^{Mtl}, mannitol-specific enzyme II; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TFA, trifluoroacetic acid; decyl-PEG, decylpoly(ethylene glycol); PEC, *S*-(β-pyrid-4-ylethyl)cysteine; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); HPLC, high-performance liquid chromatography.

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phosphorylated enzyme but without loss of activity. The cysteine labeled this way was named SH_B. EII^{Mtl} contains four cysteines (Lee & Saier, 1983), of which two are present in the membrane-bound hydrophobic domain and two in the cytoplasmic hydrophilic domain. The aim of the present study was to identify which of the four thiols play the roles of SH_A and SH_B. The data presented below show that Cys-384 is SH_A. The peptide containing SH_B could not be isolated. Labeling of Cys-384 occurs in the nonphosphorylated enzyme and is responsible for activity loss; it does not become labeled in the phosphorylated enzyme.

Materials and Methods

N-Ethylmaleimide and 4-vinylpyridine were from Jansen. Trypsin-TPCK and α-chymotrypsin were from Worthington. Acetonitrile (HPLC grade) and trifluoroacetic acid were obtained from Rathburn. 2-Propanol (p.a.) was from Merck. Urea was recrystallized 3 times from 30% acetone in H_2O. Nucleosil C_{18} was from Macherey Nagel & Co. EI and HPr were purified as described previously (Dooijewaard et al., 1979; Robillard et al., 1979). EII^{Mtl} was purified by using the nonionic detergent decyl-PEG as described by Robillard and Blaauw (1987).

EII^{Mtl} Activity Assays. The phosphorylation activities were measured by following the amount of [^{14}C]Mtl-P formed from [^{14}C]Mtl in the presence of PEP, EI, and HPr. The sugar phosphate was separated from nonphosphorylated sugar by the Dowex AG-X2 ion-exchange column procedure with 0.2 N HCl in place of LiCl as the eluent (Robillard & Blaauw, 1987).

NEM Labeling Procedure. Purified EII^{Mtl} was concentrated 10-fold by use of Amicon Centricon microconcentrators to a concentration of 40 μM. Prior to NEM labeling of the reduced unphosphorylated enzyme, 5 μL of 100 mM DTT was added to 100 μL of enzyme and incubated at 30 °C for 15 min to achieve complete reduction of the enzyme. Next, 900 μL of buffer containing 36 mM sodium phosphate, pH 7.0, 0.7 mM $MgCl_2$, and 0.18% decyl-PEG was added. The labeling was started by adding 150 μL of 10 mM NEM and terminated after 2 min by adding 143 μL of 100 mM DTT. The reaction was followed by taking samples and monitoring them for PEP-dependent phosphorylation of mannitol in the presence of EI and HPr. EII^{Mtl} labeled this way was inactivated to 5–10% of the initial activity.

The same procedure was followed to label oxidized enzyme except that, before labeling, 5 μL of 100 mM diamide instead of DTT was added to the concentrated enzyme. NEM treatment of oxidized enzyme did not result in any activity loss.

To label the phosphorylated enzyme, 100 μL of concentrated EII^{Mtl} was incubated with DTT as above. Then 900 μL of the above buffer was added, but now containing 0.4 μM EI, 8.7 μM HPr, and 2.9 mM PEP. After incubation of this mix for 15 min at 30 °C, 150 μL of 10 mM NEM was added. The reaction was again terminated after 10 min with 143 μL of 100 mM DTT. Under these conditions the enzyme was completely protected from NEM inactivation. After the reaction was stopped, EII^{Mtl} was dephosphorylated by adding excess mannitol, and the preparation was dialyzed against 20 mM Tris-HCl, pH 8.4, containing 0.35% decyl-PEG and 1 mM DTT.

4-Vinylpyridine Labeling. To label unmodified EII^{Mtl}, purified enzyme was concentrated as described before with Amicon microconcentrators. The DTT concentration was raised to 3 mM, and solid urea was added to give a final concentration of 8 M. The mixture was incubated overnight at 30 °C on a rotator. The next morning DTT was added to 2 mM followed, after 15 min, by 20% 4-vinylpyridine in ethanol until the solution was 25 mM in vinylpyridine.[2] After 3 h of rotating at 30 °C, the reaction mixture was extensively dialyzed against 100 mM Tris-HCl, pH 8.0, containing 2 M urea and 0.35% decyl-PEG. The NEM-labeled enzyme preparations were concentrated 10-fold and subjected to this same 4-vinylpyridine labeling procedure.

[^{14}C]Iodoacetamide Labeling. Inactivated enzyme was prepared as described for NEM except that the reaction was 5 min with 5 mM iodoacetamide.

Proteolysis of 4-Vinylpyridine-Labeled Enzyme. Both trypsin and α-chymotrypsin were added to the dialyzed enzyme preparations, each at a concentration of 3% compared to EII, followed by an overnight incubation on a rotator at 30 °C. The incubation was continued the next day after addition of fresh trypsin/chymotrypsin (3% w/w) and the following night after a third addition (3% w/w). The digested preparations were stored at −20 °C until used.

Proteolysis of the [^{14}C]Iodoacetamide-Labeled Enzyme. The labeled enzyme was digested as described above but for only 4 h at 37 °C with 10% (w/w) of both trypsin and chymotrypsin. After HPLC purification, sequences were determined on an Applied Biosystems Model 470A protein sequencer.

HPLC Procedures and Amino Acid Composition of 4-Vinylpyridine-Containing Peptides. Reversed-phase HPLC chromatography was carried out using a 250 × 4.6 mm column filled with Nucleosil C_{18}. The peptides were eluted with a gradient of 0.1% TFA in H_2O to 0.067% TFA in 25% 2-propanol/75% acetonitrile. The gradient ran from 0 to 100% in 100 min with a flow rate of 2 mL/min. The collected peptides were subjected to a second chromatography on the same column with the same gradients but now using 0.1% NH_4Ac, pH 6.0, instead of the stated concentrations of TFA. The purified peptides were freeze-dried and hydrolyzed for 20 h at 110 °C in 6 N HCl containing 0.5% phenol. Amino acid analysis was performed on a Kontron Liquimat III analyzer. PEC concentrations were calculated by using an extinction coefficient determined from amino acid analysis of 4-vinylpyridine-labeled glutathione hydrolyzed under the same conditions as those used for labeled EII.

Results

Localization of Cysteine-Containing Peptides. Unmodified EII^{Mtl} was labeled with 4-vinylpyridine in 8 M urea, and the labeled enzyme was enzymatically digested with trypsin and chymotrypsin (for details see Materials and Methods). The resulting peptides were then separated by reversed-phase HPLC on a Nucleosil C_{18} column. For detection of the vinylpyridine-labeled peptides, the effluent was simultaneously monitored at 214 and 254 nm (Figure 1). Peptide peaks having high A_{254}/A_{214} ratios were further purified by an additional reversed-phase HPLC on Nucleosil C_{18}, but now at pH 6 instead of pH 2. The 4-vinylpyridine-labeled cysteine *S*-(β-pyrid-4-ylethyl)cysteine is stable to acid hydrolysis (Friedman et al., 1970), allowing the peptides to be prepared for automated amino acid analysis by hydrolysis in 6 N HCl for 24 h at 110 °C. Under our conditions PEC appeared to oxidize, especially with the low amount of peptide used in our experiments (1–3 nmol). The yield of PEC was raised con-

[2] Care should be taken not to use too high a concentration of 4-vinylpyridine since, in combination with DTT or mercaptoethanol, it gives rise to nondialyzable complexes with high absorption coefficients at 254 nm that obscure the HPLC reversed-phase patterns.

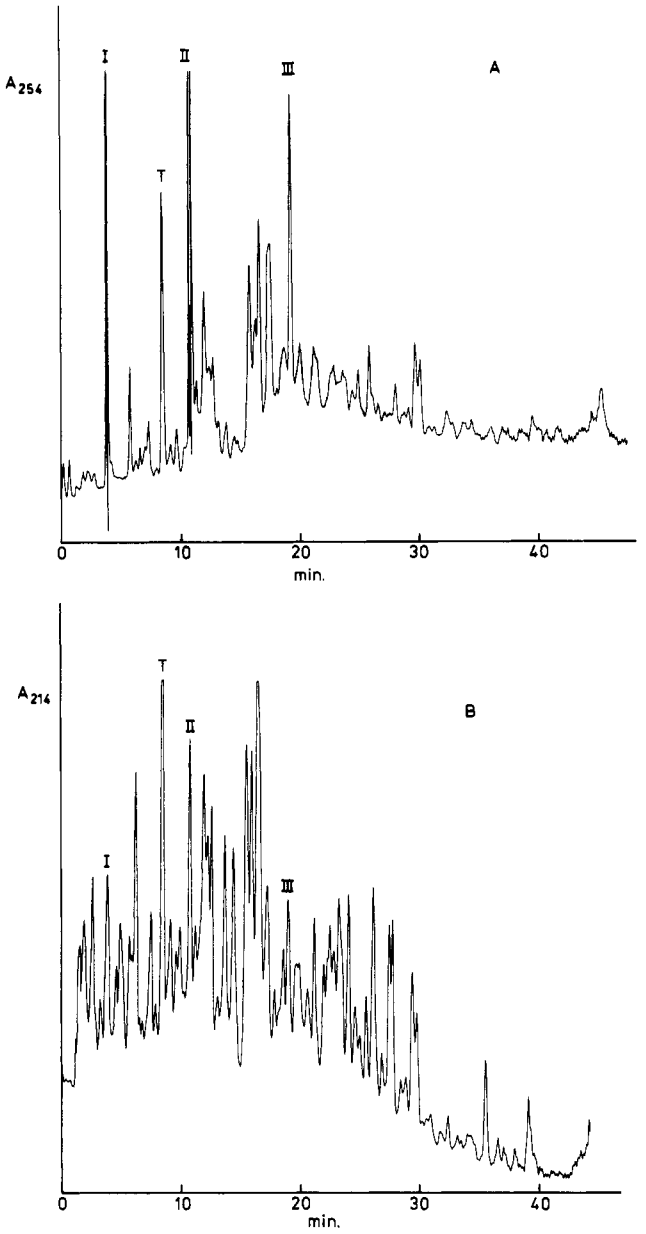

FIGURE 1: HPLC elution profile of digested 4-vinylpyridine-labeled EII^{Mtl}. The enzyme (750 μg) was labeled and digested as described in the text. The peptides were separated by HPLC using the TFA-containing gradient as described under Materials and Methods. The effluent was monitored at 254 nm (A) and at 214 nm (B). PEC-containing peptides are indicated as I–III. The peak indicated as T is the tryptophan-containing peptide Trp-117–Lys-121, illustrating the difference in A_{254}/A_{214} ratio for peptides containing PEC or tryptophan.

siderably by hydrolysis in the presence of 0.5% phenol, an oxygen scavenger. Three peaks having high A_{254}/A_{214} ratios (Figure 1, peaks I–III) were identified as cysteine-containing peptides. Table I shows the amino acid compositions of these peptides. Since the primary structure of EII^{Mtl} is known from the gene sequence (Lee & Saier, 1983), theoretical compositions can be calculated for the expected peptides (Table I). The values obtained correlated very well with the expected values. Peak I was identified as the peptide Cys-110–Lys-112, peak II as peptide Cys-571–Arg-578, and peak III as Lys-379–Leu-398. Under the conditions used trypsin appears to hydrolyze on the carboxyl side of Arg-378 instead of Lys-379. The gene sequence predicts a fourth cysteine (Cys-320). This peptide has not been isolated because it does not elute as an identifiable peak (see discussion).

Table I: Amino Acid Analysis of PEC-Containing Peptides[a]

	I		II		III	
peptide	analysis	gene seq	analysis	gene seq	analysis	gene seq
Asx					1.9	1
Glx			5.1	2		
Arg			1.6	1		
Lys	1.5	1			1.0	1
His						
Ser					2.3	2
Thr					0.6	
Tyr			1.9	1		
Met					2.0	2
Pro	nd		nd	1	nd	
Gly			2.1	1	3.2	4
Phe						
Trp	nd		nd		nd	
Ala					3.9	4
Val			2.0	1	1.7	2
Ile	1.2	1			1.2	2
Leu					0.8	1
PEC	1.0	1	1.9	1	1.4	1

[a] Amino acid analysis was performed as described under Materials and Methods. The values obtained (nanomoles) are compared with the expected ratios from the gene sequence (Lee & Saier, 1983). nd, not determined.

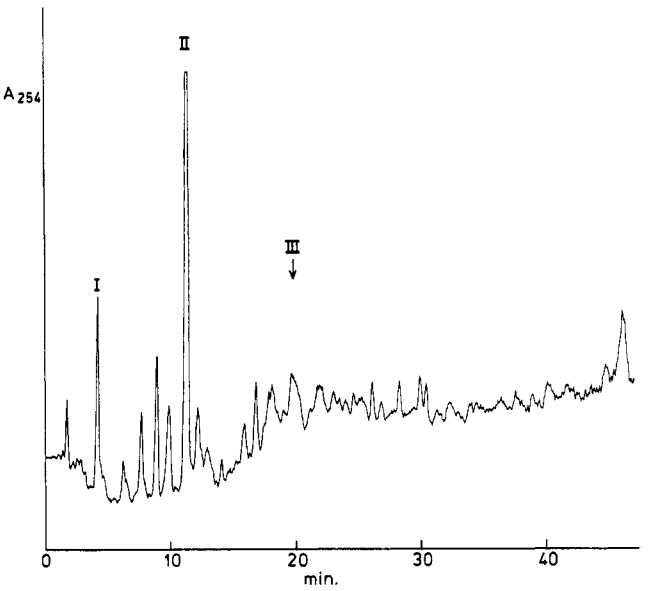

FIGURE 2: Elution profile of digested, SH_A-modified, labeled enzyme. EII^{Mtl} (250 μg) inactivated with NEM, was labeled with 4-vinylpyridine and digested as described in the text. HPLC chromatography was identical with Figure 1. The effluent was monitored at 254 nm.

Identification of Essential Thiol. After reduction with DTT, the native unphosphorylated enzyme was reacted with the thiol reagent *N*-ethylmaleimide. As has been shown by Roossien and Robillard (1984), this results in incorporation of one NEM label per monomer and in loss of catalytic activity.

After NEM labeling, the urea-denatured enzyme was subjected to 4-vinylpyridine labeling as before and digested. HPLC analysis (Figure 2) shows no change in peak heights of the peptides containing the cysteines 110 and 571. The peak containing Cys-384, however, is now totally absent. Since only one NEM label is incorporated per monomer, this means that Cys-384, in the unphosphorylated enzyme, reacts with NEM and that modification of this thiol is responsible for loss of its functional activity.

Protection of Cys-384 from NEM Labeling by Oxidation. Oxidation of EII^{Mtl} resulted in inactivation of the enzyme (Roossien & Robillard, 1984; Grenier et al., 1985). Fur-

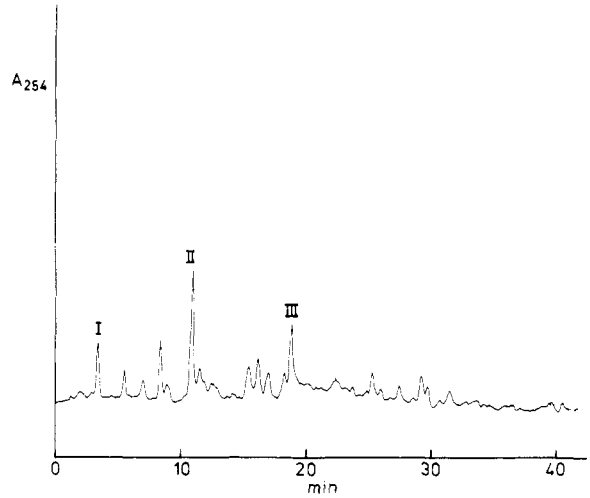

FIGURE 3: Influence of oxidation on NEM modification. The oxidized enzyme (250 μg) was treated with NEM, labeled with 4-vinylpyridine, and digested as described in the text. HPLC chromatography was identical with Figure 2 except that the peptides were monitored at 254 nm on another vertical scale.

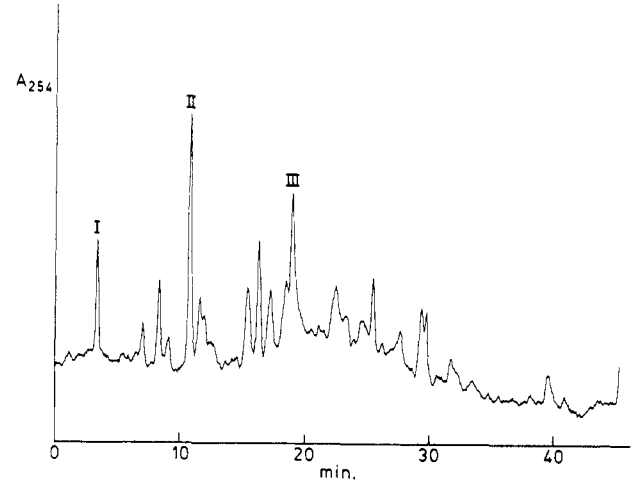

FIGURE 4: Influence of phosphorylation on NEM modification. The phosphorylated enzyme (250 μg) was treated with NEM, labeled with 4-vinylpyridine, and digested as described in the text. HPLC chromatography was identical with Figure 2.

thermore, under this condition, the enzyme was protected against NEM labeling. Figure 3 shows that, when the enzyme was reversibly oxidized with 5 mM diamide before treatment with NEM, reduced with DTT, urea denatured, and labeled with 4-vinylpyridine, no significant decrease in labeling of any of the cysteine peptides was observed. This indicates that Cys-384, peak III, is protected by oxidation from NEM labeling.

Protection of Cys-384 from NEM Labeling by Phosphorylation. Roossien and Robillard (1984) also showed that when EII^{Mtl} was phosphorylated from PEP by EI and HPr it was protected against inactivation by NEM. However, under these conditions, 1 mol of label was still incorporated into EII^{Mtl}. Apparently, a second cysteine (SH_B), not important for catalytic activity, was modified.

The HPLC pattern in Figure 4 shows that Cys-384 was still available to react with 4-vinylpyridine when NEM labeling occurred on SH_B. Therefore, phosphorylation protected Cys-384 in the native enzyme. Figure 4 also shows that neither the Cys-110 nor the Cys-571 peptide was affected by NEM labeling. Since the three cysteine-containing peptides were not influenced by NEM labeling of SH_B and control experiments with fluorescent thiol reagents confirmed that labeling of our enzyme preparation under phosphorylated condition had occurred, SH_B is probably Cys-320. Isolation of a labeled peptide will be necessary before this assignment can be confirmed.

Positive Identification of Cys-384 as the Activity-Linked Cysteine. The absence of vinylpyridine labeling of peptide III in the NEM-inactivated enzyme is negative proof for the assignment of the activity-linked cysteine. Positive proof was obtained with EII^{Mtl} inactivated by exposure to iodoacetamide. Proteolytic digestion, as described under Materials and Methods, followed directly by reversed-phase HPLC, resulted in a labeled peak. The peak was collected and rechromatographed at pH 6, and the resulting peaks were subjected to gas-phase sequencing. These data confirmed that the activity-linked cysteine is Cys-384.

DISCUSSION

In 1984, Roossien and Robillard reported on the different susceptibility of enzyme II^{Mtl} to sulfhydryl reagents under various conditions. One thiol (SH_A) was exposed in the reduced unphosphorylated state, and one (SH_B) was exposed under phosphorylated conditions.

In this paper we describe the localization of the activity-linked cysteine on the EII^{Mtl} monomer. We used specific cysteine alkylation followed by proteolytic digestion and HPLC separation. As specific cysteine label we chose to use the sulfhydryl reagent 4-vinylpyridine instead of [^{14}C]NEM since the latter is known to be relatively unstable. If ring opening would occur during processing, up to three different reaction products could be expected, leading to several peptide peaks per cysteine during HPLC separation or at least to broad overlapping peaks. On the other hand, *S*-(β-pyrid-4-yl-ethyl)cysteine, the reaction product of 4-vinylpyridine and cysteine, is both stable and has a high A_{254}/A_{214} ratio, making it easy to detect with HPLC. Furthermore, it can be detected with automated amino acid analysis, since it is stable to acid hydrolysis at elevated temperatures (Friedman et al., 1970). We observed, however, that PEC is sensitive to oxygen during hydrolysis at 110 °C in 6 N HCl. This effect is more extreme with low amounts of peptide (1–2 nmol). Addition of 0.5% phenol solved this problem. High concentrations of 4-vinylpyridine during labeling also had to be avoided. Labeling with 100–250 mM 4-vinylpyridine, in combination with DTT or mercaptoethanol, gave rise to formation of nondialyzable reaction products with very high A_{254}/A_{214} ratios, which during HPLC elution result in "false" peaks. Low 4-vinylpyridine concentrations (~25 mM) solved this problem. When purified denatured protein was labeled with 4-vinylpyridine, three PEC-peptide peaks could be identified after proteolytic processing and HPLC analysis. Amino acid analysis confirmed the presence of PEC in these peaks and, on the basis of the amino acid composition, the peptides were identified as those containing Cys-110, Cys-384, and Cys-571. Not all peptides had the same high A_{254} absorption. Apparently, not all cysteines react to the same extent with 4-vinylpyridine. Increases in the 4-vinylpyridine concentration did result in higher degrees of labeling, but byproducts interfered with the chromatography as discussed above. Partial labeling of cysteines has been reported before for the γ subunit of coupling factor 1 (Moroney et al., 1984). The reason for this partial labeling is not clear. Cys-320 could not be detected at all. Either it did not react with 4-vinylpyridine or the peptide containing Cys-320 did not elute from the reversed-phase column as a defined peak (see the discussion below).

When SH_A-NEM-modified enzyme was subjected to 4-vinylpyridine labeling, the peptide containing Cys-384 was not

labeled. Thus, modification of Cys-384 is most likely responsible for loss of catalytic activity. Oxidation and phosphorylation protected the enzyme to NEM inactivation, and under these conditions, Cys-384 is prevented from NEM labeling, confirming it as the activity-linked cysteine. Positive identification of the activity-linked cysteine was accomplished by labeling with [^{14}C]iodoacetamide and isolation and sequencing of the labeled peptide. Cys-110 and Cys-571 did not become labeled with NEM in either the phosphorylated or dephosphorylated enzyme. One label should, however, be incorporated in the phosphorylated enzyme on SH_B. We have shown that SH_B was labeled by using both fluorescent and radioactive sulfhydryl reagents. Since one label was incorporated but did not show up on Cys-110, 384, or 571, it most probably was located on Cys-320. When the enzyme was labeled on SH_B with [^{14}C]iodoacetamide and degraded by using, in succession, cyanogen bromide, trypsin, and chymotrypsin, the HPLC pattern only showed a smear of radioactivity. The reason for this behavior is most likely the hydrophobic nature of the domain containing Cys-320, which either does not unfold properly in 8 M urea or refolds upon reducing the urea concentration to 2 M before adding the proteolytic enzymes, thereby hampering the digestion and giving rise to a number of peptides of different lengths. Phosphorylation of EII results in incorporation of NEM at SH_B and leaves SH_A unmodified because, after dephosphorylation, SH_A could still be labeled with 4-vinylpyridine as shown in Figure 4. The HPLC pattern shown could only be obtained however if, after removal of excess NEM with DTT, the enzyme was dephosphorylated with mannitol followed by dialysis. Otherwise only a small fraction of 4-vinylpyridine-modified Cys-384 peptide could be detected (not shown). The same effect was observed if phosphorylated enzyme not treated with NEM was used. Apparently, phosphorylation protects against labeling of Cys-384 in the urea-denatured enzyme as well as in the native enzyme. This could indicate that the phosphorylation site is close to Cys-384 in the primary structure or that phosphorylation stabilizes the enzyme against urea denaturation.

The results presented above show very clearly that Cys-384 is the activity-linked cysteine called SH_A in our previous publication. The mechanistic role of this cysteine is not yet known. It could play a part in the catalytic turnover of the enzyme. On the other hand, it is possible that the enzyme can only function when Cys-384 is in the reduced, unmodified state and that an alteration in this state inhibits an essential step in turnover. We recently obtained evidence that modification of Cys-384 blocks the movement of a phosphoryl group from the first to the second phosphorylation site on EII^{Mtl} (Pas et al., 1988). Site-directed mutagenesis on Cys-384 should provide additional insight into the actual functioning of this thiol in the transport process.

ACKNOWLEDGMENTS

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ADDED IN PROOF

Cys-384 has been identified as one of the phosphorylation sites on EII^{Mtl} (Pas & Robillard, 1988).

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