Accelerated Publications

S-Phosphocysteine and Phosphohistidine Are Intermediates in the Phosphoenolpyruvate-Dependent Mannitol Transport Catalyzed by *Escherichia coli* EII^{Mtl} †

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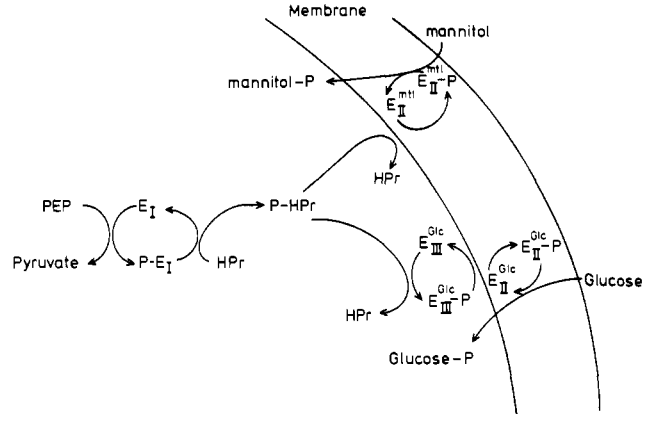
ABSTRACT: During a cycle of mannitol transport and phosphorylation, the phosphoryl group originating on P-enolpyruvate is transferred, consecutively, to two sites on the *Escherichia coli* mannitol-specific carrier (EII^{Mtl}) before being placed on mannitol [Pas et al. (1988) *Biochemistry* (in press)]. The peptides constituting the two EII^{Mtl} phosphorylation sites have been isolated and identified after labeling with [^{32}P]-P-enolpyruvate. The first site is localized in peptide Leu 541–Lys 560. The hydrolysis characteristics of the phosphorylated peptide indicate that a histidine residue is phosphorylated. The second site is located in peptide Ile 380–Met 393, which contains the activity-linked cysteine (384) [Pas & Robillard (1988) *Biochemistry* (in press)]. The hydrolysis characteristics of the phosphopeptide indicate that Cys 384 is the site of phosphorylation.

The mannitol-specific enzyme II (EII^{Mtl})[1] of *Escherichia coli* is a 67 893-dalton polypeptide consisting of a hydrophobic N-terminal domain, which contains seven hydrophobic stretches, and a hydrophilic C-terminal half, which is cytoplasmic (Lee & Saier, 1983; Stephan & Jacobson, 1986). The enzyme catalyzes the transport and phosphorylation of mannitol and can catalyze two partial reactions, phosphoryl group exchange between mannitol phosphate and mannitol (Saier et al., 1977) and that between P-HPr and HPr (Sutrina et al., 1987). A sulfhydryl group is essential for EII^{Mtl} activity. The enzyme is only active in the reduced form. NEM alkylation or reaction with phenylarsine oxide abolishes the mannitol phosphorylation and exchange activities. Cysteine 384 has been shown to be the redox-sensitive, activity-linked residue (Pas & Robillard, 1988).

Some PTS systems use in addition to EI, HPr, and EII a cytoplasmic protein, EIII, which transfers the phosphoryl group from HPr to EII (see Scheme I). The EIII's are specific for their respective EII's. The occurrence of these two types of systems led Saier and co-workers (1985) to suggest that the larger EII's, which function without an EIII, share a common evolutionary origin with the EII–EIII couples. According to this theory the cytoplasmic C-terminal part of EII^{Mtl} should function as a covalently attached EIII. This theory implies that EII^{Mtl} should contain two phosphorylation sites. The first phosphorylation site, the "EIII-like" site, should be phosphorylated from HPr, and this phosphoryl group should then be transferred to the second site, the "real EII" site. We have recently shown that that EII^{Mtl} does contain two phosphorylation sites per monomer and that only the reduced enzyme can be phosphorylated at both sites. If cysteine 384 is oxidized or alkylated, only one phosphoryl group is incorporated per monomer (Pas et al., 1988).

Scheme I



All phosphoenzyme intermediates of the PTS analyzed to date, EI, HPr, $EIII^{Glc}$, and $EIII^{Lac}$, carry their phosphoryl group on a histidine residue (Alpert et al., 1985; Weigel et al., 1982; Dörschug et al., 1984; Kalbitzer et al., 1981). The general expectation was that EII^{Mtl} was also phosphorylated on two histidine residues. On the basis of the sequence homologies between different EII's, histidines 554 and 195 were suggested as phosphorylation sites 1 and 2, respectively (Saier et al., 1988; Bramley & Kornberg, 1987a). Histidine 256 has also been suggested as phosphorylation site 2 (Manayan et al., 1988). Pas and Robillard (1988) showed that phosphorylation

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[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; P-enolpyruvate, phosphoenolpyruvate; Pyr, pyruvate; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; EIII, enzyme III; EII^{Mtl}, mannitol-specific enzyme II; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; NaP_i, inorganic sodium phosphate; decyl-PEG, decylpoly(ethylene glycol) 300; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; Gut, glucitol; Man, mannose; Glc, glucose; Bgl, β-glucoside; Nag, *N*-acetylglucosamine.

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blocked alkylation of cysteine 384 both in the native enzyme and in the 8 M urea denatured enzyme. Consequently, it was suggested that phosphorylation could take place close to or at this site.

The purpose of the present study was to determine the phosphorylation sites on EII^{Mtl}.

Materials and Methods

The following reagents were used: *N*-ethylmaleimide from Jansen, γ-^{32}P-labeled adenosine triphosphate from Amersham, trypsin–TCPK and α-chymotrypsin from Worthington, calf intestine alkaline phosphatase (special quality for molecular biology) from Boehringer, "HPLC grade" acetonitrile and trifluoroacetic acid from Rathburn, 2-propanol (p.a.) from Merck, and Nucleosil C_{18} from Macherey-Nagel & Co. EI and HPr were purified as described previously (Dooijewaard et al., 1979; Robillard et al., 1979). The procedures for the purification of EII^{Mtl} and the determination of the PTS enzyme concentrations by the pyruvate burst method have been described by Robillard and Blaauw (1987). NEM-labeled EII^{Mtl} was prepared as described previously (Pas & Robillard, 1988).

Synthesis of ^{32}P-Labeled Phosphoenolpyruvate. The synthesis was performed as described by Roossien et al. (1983), using triethanolamine in the incubation buffer instead of triethylamine.

^{32}P Labeling and Proteolysis of Labeled Enzymes. The labeling reaction mixture contained 35 mM Tris-HCl, 25 mM NaP_i, pH 8.5, 5 mM $MgCl_2$, 2.5 mM NaF, 0.6 mM DTT, 0.2% decyl-PEG, 0.2 μM EI, 1.5 μM HPr, and, depending on the experiment, no EII^{Mtl}, 2.3 μM NEM-alkylated EII^{Mtl}, or 2.3 μM native EII^{Mtl} in a total volume of 3.5 mL. The labeling was achieved by a 5-min preincubation at 37 °C, followed by addition of 12 μM [^{32}P]-P-enolpyruvate and incubation for 15 min. Next 0.15 mg each of trypsin and chymotrypsin was added, and the incubation was prolonged for 2 h. The mixture was then immediately loaded on the HPLC column or stored in liquid N_2 until use.

HPLC Procedures for Peptide Separation. Reversed-phase HPLC chromatography was carried out with a 250 × 4.6 mm column filled with Nucleosil C_{18}. The peptides were eluted with a gradient of 0.1% NH_4OAc, pH 6.0, in H_2O to 0.1% NH_4OAc 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 peaks were collected and counted by Cherenkov radiation, and the labeled fractions were subjected to a second chromatography on the same column using a gradient of 0.1% TFA in H_2O to 0.067% TFA in 25% 2-propanol/75% acetonitrile. The running conditions were identical with those of the first column. The third and last purification step was a repeat of the second step.

Amino Acid Sequence Determination of ^{32}P-Labeled Peptides. The purified peptides were lyophilized and subjected to gas-phase sequence determination on an Applied Biosystems Model 470A protein sequencer equipped with a Model 120A PTH analyzer.

Alkaline Phosphatase Treatment. ^{32}P-Labeled peptide in HPLC elution buffer, pH 2 (20% organic phase), was mixed with an equal volume 0.5 M Tris-HCl, pH 11, containing 2 mM $ZnCl_2$. Alkaline phosphatase (2 units) was added and the mixture was incubated overnight at 15 °C. HPLC analysis showed that no label remained on the peptide under these conditions. Parallel experiments without phosphatase showed that more than 95% of the label was still present on the peptide at the end of the incubation.

Determination of pH-Dependent Hydrolysis Rate of the ^{32}P-Labeled Peptide. A ^{32}P-peptide batch prepared by one reversed-phase chromatography step at pH 6 was used for this study. Three hundred microliter volumes were mixed with equal volumes of the desired buffer, and the pH was determined after mixing. EDTA was added to a concentration of 1 mM. The mixtures were incubated at 37 °C, and at the indicated times, samples were withdrawn and the ratio peptide-bound phosphorus/free phosphate was determined by a fast HPLC reversed-phase separation at pH 2.

Results

Identification of Phosphorylation Sites on EII^{Mtl}. Pas et al. (1988) have demonstrated that only one phosphoryl group becomes phosphorylated in NEM-inactivated EII^{Mtl} whereas two sites become phosphorylated in active EII^{Mtl}. We have used the difference in the labeling patterns of native versus NEM-inactivated EII^{Mtl} to determine which peptides constitute sites 1 and 2.

Figure 1A shows the HPLC pattern obtained after proteolytic digestion of a mixture containing only ^{32}P-EI and ^{32}P-HPr. The first peak results from EI and the latter peak from HPr. Figure 1B shows a chromatogram of a similar reaction mixture in which NEM-labeled EII was also present. This resulted in one extra peak (I). Labeling of native EII resulted in two more peaks (Figure 1C, peaks II and III). The peaks were further purified by two additional reversed-phase HPLC separations at pH 2, and the amino acid sequence was determined by gas-phase sequencing. Peptide I, which became labeled in the NEM-reacted enzyme, had the sequence Leu-Thr-Pro-Thr-Tyr-Leu-Gly-Glu-Ser-Ile-Ala-Val-Pro-His-Gly-Thr-Val-Glu-Ala-Lys, which corresponded with the peptide Leu 541–Lys 560 of enzyme II^{Mtl}. Peptide II, which became labeled only in active EII^{Mtl}, had the sequence Lys-Ile-Ile-Val-Ala-Dha-Asp-Ala-Gly-Met-Gly-Ser-Ser-Ala-Met, corresponding to the peptide Lys 379–Met 393. Dha (dehydroalanine) arises from cysteine during the sequencing procedure (see Discussion). The sequence of peptide III was identical with that of peptide II except that the N-terminal Lys was not present.

pH-Dependent Hydrolysis Rate of the ^{32}P-Labeled Peptides. The hydrolysis characteristics of the peptide Leu 541–Lys 560 were examined according to the procedure stated under Materials and Methods. The phosphorylated peptide was acid labile ($k = 1.3 \times 10^{-3}$ min^{-1}) at pH 4 and it was stable ($k < 10^{-5}$ min^{-1}) at pH 12.

The isolated site 2 peptide contains four residues that are possible phosphorylation sites: Cys 384, Asp 385, Ser 390, and Ser 391. A pH-dependent hydrolysis pattern of the isolated peptide was determined in order to characterize the second phosphorylation site (see Materials and Methods). Figure 2 clearly shows that the phosphoryl group on the peptide (□) hydrolyzes below pH 9 and is rapidly lost at low pH. It is very stable in the pH range 10–12. The dashed line is the published pH dependence of hydrolysis rate constants for phosphoaspartate in the tripeptide Pro-(P)Asp-Lys under similar conditions of temperature and buffer (Post & Kume, 1973). In contrast to the P_2 peptide phosphoaspartate is destabilized by high pH and is stable down to pH 2. Moreover, the rate constants for the P_2 peptide at basic pH are 100–1000 times lower than those for phosphoaspartate.

Discussion

Site 1. EII^{Mtl} contains two phosphorylation sites per monomer. Oxidation or alkylation of cysteine 384 results in a reduction of phosphoryl group incorporation to one per monomer (Pas et al., 1988). The present results confirm these observations. ^{32}P labeling and proteolytic processing of al-

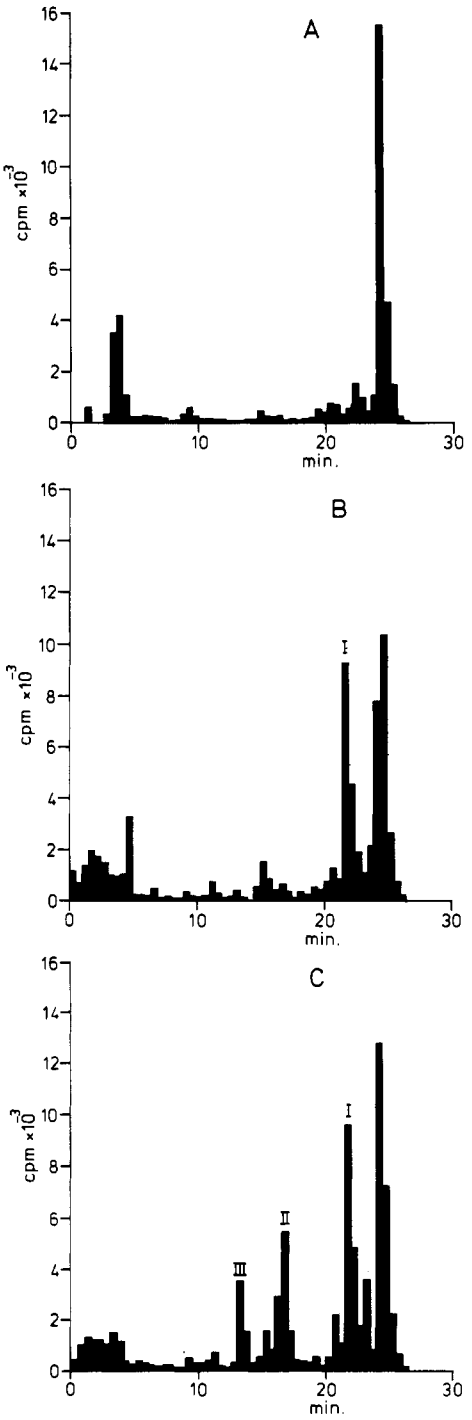

FIGURE 1: HPLC elution profile after ^{32}P labeling and proteolytic processing of EII^{Mtl}. The elution time is plotted versus the radioactivity. (A) EI and HPr; (B) EI, HPr, and NEM-alkylated EII; (C) EI, HPr, and native EII. See Materials and Methods for concentrations and chromatographic conditions.

kylated P-EII resulted in one labeled peptide, Leu 541–Lys 560. The same peptide was also phosphorylated in the native, reduced enzyme. This peptide represents the site that becomes phosphorylated from P-HPr (P_1). The site is present in the C-terminal part of the protein. Saier et al. (1985) proposed that this part of the protein functions as a covalently coupled EIII molecule. Homology studies suggested that histidine 554 might represent the EIII-like phosphorylation site on EII^{Mtl} (Saier et al., 1988). Recently, the homology between *E. coli* EII^{Mtl} and the cytoplasmic $EIII^{Mtl}$'s from both *Staphylococcus aureus* and *Staphylococcus carnosus* has been investigated (Reiche et al., 1988). $EIII^{Mtl}$ peptides showed good homology

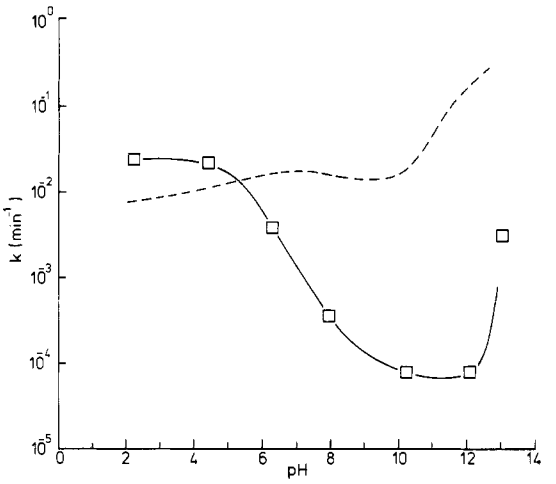

FIGURE 2: Influence of pH on the hydrolysis rate of the site 2 peptide. One volume of peptide in HPLC elution buffer containing 20% organic phase, pH 6, was mixed with 1 volume of the following buffers (the pH was measured after mixing): 0.86 M formic acid (pH 2.2); 0.1 M citric acid (pH 4.4 and 6.3); 0.1 M Tris-HCl (pH 8.0); 0.1 M $KHCO_3$ and 0.1 M K_2CO_3 (pH 10.2); 0.5 M triethylamine (pH 12.1); 0.15 M KOH (pH 13.1). The rate constants were determined as indicated under Materials and Methods and are plotted versus the pH (□). The dashed line gives the rate constants measured under similar conditions and with the same buffer systems for the tripeptide Pro-(P)Asp-Lys as given by Post and Kume (1973).

with the C-terminal sequence from EII^{Mtl}, starting at residue 493. The phosphorylation site on *S. aureus* $EIII^{Mtl}$ was identified by ^{32}P labeling, proteolytic fragmentation, and purification procedures similar to those employed above. A 19-residue peptide containing 7 residues identical with our site 1 peptide sequence was isolated. Both peptides contain the sequence Pro-His-Gly-Thr. One histidine and two threonines are the only homologous phosphorylatable residues found in the entire 19-residue stretch of both peptides. Our site 1 phosphopeptide is unstable at acid pH. Phosphothreonine and phosphoserine are stable to acid hydrolysis in 4 N HCl at 105 °C for several hours (Hunt, 1985). These data and comparisons indicate that the phosphorylated residue in the site 1 peptide of EII^{Mtl} is a histidine. This is in agreement with the results of Sutrina et al. (1987), who showed that treatment of EII^{Mtl} with the histidine reagent diethyl pyrocarbonate inhibited EII-catalyzed phosphoryl group exchange between HPr and P-HPr.

Site 2. Two extra labeled peptides were obtained from the labeled reduced enzyme. The sequence of both peptides appeared to be similar except that one peptide contained an extra N-terminal lysine. The sequences were equivalent to the EII peptides Lys 379–Met 393 and Ile 380–Met 393. Under the conditions employed trypsin apparently splits at either Arg 378 or Lys 379. These results fix the second phosphorylation site to be somewhere on the peptide Ile 380–Met 393. This peptide contains four possible sites of phosphorylation, Cys 384, Asp 385, Ser 390, and Ser 391. The serines can be eliminated since the phosphorylated peptide hydrolyzes below neutral pH. Moreover, in gas-phase sequencing, serine phosphate can be detected by a relative increase in dehydroalanine. This results from a β-elimination reaction catalyzed by the anhydrous TFA used in the Edman degradation. Both serines of the phosphorylated peptide eluted as normal serines. These two lines of evidence therefore eliminate serine as the phosphorylation site. The two remaining phosphorylation candidates are Cys 384 and Asp 385. Surprisingly, dehydroalanine was detected at the position of the cysteine residue. The sequencing was done under reducing conditions; therefore,

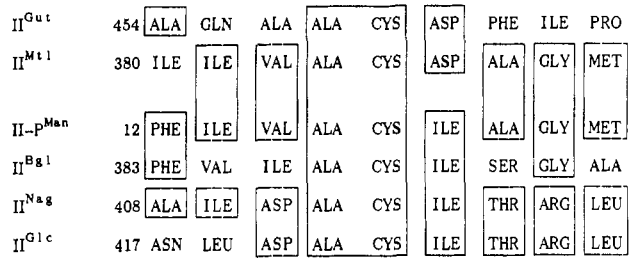

FIGURE 3: Possible phosphorylation sites for *E. coli* EII^{Gut} (Yamada & Saier, 1987), EII^{Man} (Erni et al., 1987), EII^{Glc} (Erni & Zanolari, 1986), EII^{Bgl} (Bramley & Kornberg, 1987b), and EII^{Nag} (Rogers et al., 1988).

the dehydroalanine did not result from cystine, which has been reported to produce dehydroalanine upon gas-phase sequencing (Marti et al., 1987). The dehydroalanine could not be attributed to phosphocysteine since it was observed upon sequencing the alkaline phosphatase dephosphorylated peptide as well. The dehydroalanine peak was not observed if the unphosphorylated peptide was isolated and sequenced after reaction with iodoacetamide. The most probable cause for dehydroalanine formation is the adjacent aspartic acid. A cyclization reaction can occur between the thiol and carboxylate side chains during the acid step of the degradation sequence to yield a thiol ester, which then decomposes during the base step to give dehydroalanine at the cysteine position.

Evidence in favor of phosphocysteine was obtained by characterization of the pH-dependent hydrolysis of the phosphoryl group on the isolated peptide. Like other thiophosphates, the compound is unstable at low pH. Sikkema and O'Leary (1988) measured the hydrolysis rate of the S–P linkage in phosphoenolthiopyruvate at different pH's. At 25 °C they reported the following half-lives: pH 4, 30 min ($k = 23 \times 10^{-3}$ min^{-1}); pH 6, ≤2 h ($k \geq 5.8 \times 10^{-3}$ min^{-1}); pH 7.5, 30 h ($k = 3.9 \times 10^{-4}$ min^{-1}). At pH 13 and 5 °C they measured a k of 4.8×10^{-5} min^{-1}. These values are very similar to the values obtained for the P_2 peptide. *S*-Phosphocysteine has also been observed in thioredoxin. The pH-dependent hydrolysis showed maximal instability between pH 2 and 3. The absolute value of the rate is the same as observed for the P_2 peptide at this pH, and the maximum stability is observed above pH 7 (Pigiet & Conley, 1978). The hydrolysis pattern of phosphoaspartate, on the other hand, is very different. Phosphoaspartate is most stable between pH 2 and 10. At high pH it rapidly decomposes with a half-life of a few minutes (Post & Kume, 1973). The difference in the hydrolysis patterns for the site 2 peptide and the tripeptide containing phosphoaspartate eliminates Asp 385 as the residue that is phosphorylated.

Sequence comparisons of different EII species support cysteine as the phosphorylation site (Figure 3). Identities are indicated by the boxed residues. Both hexitol-specific systems, EII^{Gut} and EII^{Mtl}, contain an Ala-Cys-Asp sequence. EII^{Mtl} and EII^{Man} possess a 10-residue stretch, which is identical except that Asp is replaced by Ile. All other hexose-specific systems possess an Ala-Cys-Ile. Recently Erni and colleagues have replaced Cys 421 in EII^{Glc} by Ser. The mutated enzyme was inactive and incapable of being phosphorylated (B. Erni, personal communication). These results are further evidence for the essential role of cysteine.

Observations from EII^{Mtl} itself also support the cysteine as the phosphorylation site. Native EII^{Mtl} cannot be alkylated or oxidized if site 2 is phosphorylated (Roossien & Robillard, 1984; Grenier et al., 1985). It is also impossible to alkylate Cys 384 when the phosphorylated enzyme is denatured in 8 M urea (Pas & Robillard, 1988). Conversely, EII^{Mtl}, oxidized or alkylated at Cys 384, cannot be phosphorylated at site 2 (Pas et al., 1988). Similar observations have been reported for *E. coli* EII^{Glc}. Phosphorylation protects against inactivation by alkylating and oxidizing agents (Robillard & Konings, 1981; Robillard & Beechey, 1986).

Enzyme II^{Mtl} is thought to function as an integrated EII–EIII couple in which EIII is phosphorylated from HPr and transfers its phosphoryl group to EII. As discussed above, the site 1 peptide that we have isolated is similar to the phosphorylation site peptide isolated from *S. aureus* $EIII^{Mtl}$ (Reiche et al., 1988). These investigators also showed that the C-terminal part of *E. coli* EII^{Mtl} is homologous to this $EIII^{Mtl}$. Consequently, the site 1 phosphorylation site must be considered as the EIII-like site, which accepts its phosphoryl group from P-HPr. Since EII is phosphorylated from P-EIII, the phosphorylation of site 2 in EII^{Mtl} must reflect the actual EII site. The phosphoryl group on histidine 554 in EII^{Mtl} has to be translocated to cysteine 384 via an internal transfer step. This second phosphorylation site then participates in sugar phosphorylation as has been shown for EII^{Glc} (Erni, 1986).

The present findings drastically alter the prevailing ideas on the catalytic mechanism of EII^{Mtl}. First, they show that the second phosphorylation site is not histidine (Saier et al., 1988; Bramley & Kornberg, 1987a; Manayan et al., 1988). We cannot, however, rule out the possibility that other histidines are involved in phosphoryl group transfer as transient intermediates which are too unstable to be isolated. Second, this site is not present, as was generally assumed, in the hydrophobic, integral membrane-bound domain of the enzyme but in the cytoplasmic domain. The present results imply that the cytoplasmic domain of the protein not only serves as the phosphoryl group acceptor but also functions as the phosphoryl group donor with respect to sugar phosphorylation. This restricts the integral membrane domain to function merely as a permease, supposedly by forming a channel as shown for glucose-specific EII (Robillard & Beechey, 1986). Furthermore, this domain might participate in sugar phosphorylation by supplying the sugar binding site. The unphosphorylated enzyme does bind sugar (Pas et al., 1988), but no efficient transport without phosphorylation has been observed. Obviously transport by the permease part of the enzyme remains coupled to phosphorylation of the cytoplasmic domain.

In summary, the results reported here show that EII^{Mtl} is phosphorylated successively on two residues, histidine 554 and cysteine 384. The latter residue is most probably involved in phosphorylation of substrate.

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Helically Organized Macroaggregates of Pigment–Protein Complexes in Chloroplasts: Evidence from Circular Intensity Differential Scattering†

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ABSTRACT: Angle dependence of circular intensity differential scattering (CIDS) and of nonpolarized scattering was determined in isolated spinach chloroplasts at 514.5 nm. CIDS between 0° and 170° was independent of the nonpolarized scattering and showed intense lobes of alternating signs, exhibiting the negative and positive maxima around 15° and 70°, respectively. These results provide experimental evidence for the existence of large helically organized macroaggregates of pigment–protein complexes in thylakoid membranes. Modeling of the CIDS data by a simple helical array of uniaxial polarizable groups suggests that the chiral structure is left-handed with pitch and radius of the order of 385 nm.

In all photosynthetic organisms, the light-harvesting pigment–protein complexes and the reaction centers form a cooperative highly organized and regulated energy transfer and trapping system. The primary structure, the orientation of pigment molecules with respect to molecular axes, embedding of complexes in the membrane, and the three-dimensional model are known for many pigment–protein and reaction center complexes [for recent reviews, see Michel and Deisenhofer (1986), Zuber et al. (1987), Breton and Nabedryk (1987) and Garab et al. (1987)]. Our knowledge about the supramolecular organization of complexes is derived mainly from freeze–fracture (–etch) microscopy (Staehelin, 1986). The micrographs, however, do not carry information about possible interactions between different complexes or particles.

Circular intensity differential scattering (CIDS)[1] is a newly emerging structure-analysis technique (Tinoco et al., 1983).

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[1] Abbreviations: CD, circular dichroism; CIDS, circular intensity differential scattering $[(I_L - I_R)/(I_L + I_R)$, where I_L and I_R are the intensities scattered upon illumination with left and right circularly polarized light, respectively]; LHC II, chlorophyll *a*/*b* light harvesting pigment–protein complex of photosystem II.

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