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Communication

³¹Phospho-NMR Demonstration of Phosphocysteine as a Catalytic Intermediate on the *Escherichia coli* Phosphotransferase System *EII*^Mtl*

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The mannitol-specific phosphotransferase system transport protein, Enzyme II^Mtl, contains two catalytically important phosphorylated amino acid residues, both present on the cytoplasmic part of the enzyme. Recently, this portion has been subcloned, purified, and shown to be an enzymatically active domain. The N-terminal half has also been subcloned and shown to be the mannitol-binding domain. When combined the two domains catalyze mannitol phosphorylation at the expense of phospho-HPr (van Weeghel, R. P., Meyer, G. H., Pas, H. H., Keck, W. H., and Robillard, G. T., *Biochemistry* in press). The phospho-NMR spectrum of the purified phosphorylated cytoplasmic domain, taken at pH 8.0, shows two signals, one at −6.9 ppm compared with inorganic phosphate resulting from phosphohistidine and one at +11.9 ppm originating from phosphocysteine. Addition of mannitol plus membranes containing the N-terminal mannitol-binding domain results in the formation of mannitol 1-phosphate and the disappearance of the two signals at −6.9 and +11.9 ppm.

The bacterial phosphoenolpyruvate-dependent phosphotransferase system is responsible for uptake of a number of hexoses and hexitols by bacteria. It is a group translocation system in which the substrate becomes modified, in this case phosphorylated, during turnover. As shown in Fig. 1, the phosphoryl group originates from phosphoenolpyruvate and is transferred by two intermediate cytoplasmic proteins, EI^1 and HPr, to the membrane-bound protein, Enzyme II, which is the actual transport protein. We have shown that the mannitol-specific Enzyme II (EII^{Mtl}) contains two sites of covalent phosphorylation (1), which we refer to as P_1 and P_2, both being catalytic intermediates (2). The route of phosphorylation is as follows; P_1 accepts a phosphoryl group from phospho-HPr and transfers it to P_2 after which it is coupled

to mannitol. In order to identify the amino acids which become phosphorylated as P_1 and P_2, the enzyme was labeled with [³²P]phosphoenolpyruvate, proteolyzed, and the labeled peptides were isolated (3). Sequencing gave His-554 as the P_1 site and Cys-384 as the P_2 site. The presence of phosphocysteine as a catalytic intermediate is unique. All preceding phosphoenolpyruvate-dependent phosphotransferase system phosphoenzyme intermediates (EI, HPr, and $EIII$) were shown to contain either N1-histidine or N3-histidine as the phosphoryl acceptor.

All $EIIs$ are proposed to originate from a primordial ancestor gene (4). Sugar specificity developed during evolution, but the mechanism of sugar transport is assumed to be the same. Today we can discriminate between three types of EII. The first types are $EIIs$ such as EII^{Mtl} containing both phosphorylation sites on the protein. Both of these sites are present on the hydrophilic part of the protein which protudes into the cytoplasm. The second types are $EIIs$ containing only the P_2 site. In order to function they need a cytoplasmic partner, called $EIII$, which contains the P_1 site. An example is the glucose-specific EII. The third types are $EIIs$ containing no phosphorylation site. Both sites are now present on the matching $EIII$ (Fig. 1). The mannose-specific system and the recently discovered sorbose-specific system are, until now, the only examples of this class (5, 6). EII^{Mtl} has been divided into two portions, the cytoplasmic portion containing two phosphorylation sites and an integral membrane-bound domain (7). Several studies have shown that the membrane-bound domain contains the sugar-translocating unit (7–11). Since all $EIIs$ and $EII/EIII$ pairs are believed to be similar, one would expect all systems to contain a histidine as P_1 and a cysteine as P_2. Data gathered on the mannose and glucose system indeed confirm histidine as P_1. However, the labeled P_2 peptide of the mannose system was characterized as containing a N1-phosphorylated histidine (5). No data are yet available for the other systems, although it was claimed that, after a complete amino acid digestion of labeled glucose-specific EII, a compound with the same R_F on paper chromatography as N1-phosphohistidine was obtained (5).

Considering the novelty of the EII^{Mtl} P-cysteine observation, its contrast with the EII^{Glc} and EII^{Man} observations and the fact that phosphoryl groups on enzymes are known to migrate, particularly during degradation, we have chosen to reinvestigate the nature of the EII^{Mtl} P_1 and P_2 site using ³¹P-NMR. This technique can distinguish between a phosphoryl group on histidine which resonates in the region of −7 ppm and on cysteine which resonates around +12 ppm. Since the measurements can be done *in situ* possible phosphoryl group migration during chemical degradation can be avoided.

MATERIALS AND METHODS

Proteins—The cytoplasmic domain of EII^{Mtl}, CIII, was obtained from *Escherichia coli* strain JM101 containing the plasmid pMcCIII. CIII was isolated from the cytoplasmic cell fraction as described by van Weeghel *et al.* (7). The preparation obtained showed one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After dialysis against 50 mM Tris-Cl, pH 8.0, containing 1 mM DTT, the enzyme was concentrated by Centricon-P10 centrifugation and stored in liquid N_2 until use. The CIII concentration was determined by the method of Bradford (12). *N*-Ethylmaleimide (NEM)-inactivated CIII was prepared as described for EII^{Mtl} (1, 21). Membrane vesicles containing NIII were prepared and stored as stated (7). The EI and

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HPr used were preparations routinely purified in our laboratory (13, 14) and additionally dialyzed against 10 mM Tris-Cl, pH 8.0.

Phosphocysteamine—Phosphocysteamine was synthesized according to Åkerfeldt (15).

Spectra—NMR spectra were recorded on a 300-MHz Varian VXR spectrometer operating at 121 Mhz (^{31}P frequency) using a 10-μs (90°) pulse and a repetition time of 3.2 s, which satisfies complete relaxation of the protein-bound phosphorous signals (peaks 4 and 5 in Fig. 3). Protons were decoupled by broad band decoupling. A line broadening of 3 Hz was used, the spectral width was 6700 Hz, and the number of data points was 16,000. All spectra were recorded at 30 °C.

The chemical shifts reported in this article are relative to the inorganic phosphate signal in the spectra which is set at 0.0 ppm. The pH of the sample was 8.0, at which pH inorganic phosphate resonates at −2.5 ppm relative to 85% phosphoric acid. To avoid confusion, all chemical shifts discussed hereafter and originally reported relative to 85% phosphoric acid have, in this article, also been converted to shifts relative to inorganic phosphate.

RESULTS AND DISCUSSION

^{31}P-NMR measurements require concentrations of protein in the 0.5–1 mM range which for EII^{Mtl} is 34–68 mg/ml. The hydrophobic nature of the protein makes such concentrations difficult to achieve. As stated in the introduction, EII^{Mtl} is constructed of three domains, two cytoplasmic domains in the C-terminal half (CIII) and a membrane-bound domain in the N-terminal half (NIII). The C-terminal half, consisting of the domains carrying the P_1 and P_2 sites, has been subcloned, overexpressed, and purified (7) (see Fig. 1). The phosphorylation of mannitol is restored when the C-terminal half is combined with the subcloned N-terminal half. Since the C-terminal domain is water-soluble and available in large quantities, it was used in place of intact EII^{Mtl} in these NMR investigations.

Fig. 2 shows that both sites in CIII can be phosphorylated and that hydrolysis is not increased compared with EII^{Mtl} (1). The combination of CIII with [^{14}C]PEP, EI, and HPr shows the stoichiometric production of 2 eq of pyruvate by phosphorylation of CIII (Fig. 2). A total of 12.5 μM [^{14}C]PEP was used. Nineteen percent or 2.4 μM [^{14}C]pyruvate was produced in the control (■) in which a total of 2.5 μM HPr plus EI only were present. An additional 26% or 3.0 μM [^{14}C]pyruvate was produced when 1.5 μM CIII was added (●). Thus 2 phosphoryl equivalents are incorporated per CIII molecule. Previous studies have shown that *N*-ethylmaleimide inactivates EII^{Mtl} by alkylating only one site, Cys-384. Alkylation of this site blocks phosphorylation of the second phosphorylation site (1, 3). NEM inactivation of CIII halved the [^{14}C]pyruvate burst in the present experiment. Thirteen percent or 1.5 μM [^{14}C]
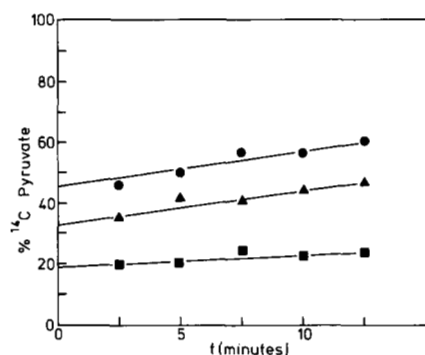

FIG. 2. **Determination of the number of phosphorylation sites on CIII by the pyruvate burst method (1, 16).** The assay mixtures contained 50 mM Tris-Cl, pH 7.5, 5 mM MgCl$_2$, 5 mM DTT, 0.2 μM EI, 2.3 μM HPr, and either no CIII (■), 1.5 μM *N*-ethylmaleimide-inactivated CIII (▲), or 1.5 μM native CIII (●). At $t = 0$ 12.5 μM [^{14}C]PEP was added, and at 2.5-min intervals samples were taken and monitored for [^{14}C]pyruvate.

pyruvate was produced when *N*-ethylmaleimide-inactivated CIII was added to the phosphorylation mix (▲). This is similar to intact EII^{Mtl} where NEM alkylation leaves only His-554 available for phosphorylation. These data are in keeping with the observations of Stephan *et al.* (10) that NEM alkylation of the C-terminal domain prepared by mild proteolysis reduced the phosphoryl group incorporation by 50%.

Fig. 3A shows the ^{31}P-NMR spectrum of phosphorylated CIII. Five resonances can be observed. Two of them, numbered 1 and 2, could also be observed in the control spectrum without CIII (not shown). Peak 1 results from phosphoenolpyruvate and peak 2 from inorganic phosphate formed through hydrolysis. Peak 3 results from a compound formed by a minor protein contaminant in the CIII preparation; it does not arise from CIII itself as will be discussed below. Peaks 4 and 5 are the phosphorylated residues present on CIII. Both peaks have widths at half-height approximately a factor of 3 larger than the remaining signals suggesting shorter T_2 as expected for protein resonances. Under turnover conditions both signals disappear. Addition of mannitol to the reaction mixture consisting of purified CIII- and NIII-containing vesicles results in a gradual increase of the mannitol 1-phosphate signal (peak 6) and in a loss of peaks 4 and 5 (Fig. 3B).

The chemical shift of peak 4 is −6.9 ppm relative to inorganic phosphate. This is a typical value expected for phosphohistidine (17, 18) and is consistent with the phosphorylation of histidine 554 on the $EIII$ domain of EII^{Mtl}. Model ^{31}P-NMR studies on 3-P-histidine and 1-P-histidine showed chemical shifts at pH 8 of −7.3 and −8.2 ppm, respectively, relative to inorganic phosphate (17). Our value of −6.9 ppm is closest to 3-P-histidine. Early work on $EIII$ by the group of Roseman revealed the phosphoryl accepting sites of *Staphylococcus aureus* $EIII^{Lac}$ and *E. coli* $EIII^{Glc}$ (20) as N3-histidines. In 1985 Saier *et al.* (4) proposed that all $EIIIs$ and $EIII$ domains of independent $EIIs$ be phosphorylated at the N3 position of a histidine. The ^{31}P spectrum presented here is consistent with this postulate.

The chemical shift of peak 5, +11.9 ppm, does not fit in with the phosphorylation of a second histidine residue. In fact, all phosphorylated amino acid residues, the phosphomonoesters phosphoserine, phosphothreonine, and phosphotyrosine, the acylphosphate phosphoaspartate, and the phosphoamidates phospholysine, phosphoarginine, and phosphohistidine all resonate much further downfield (21). To ascertain that this signal results from phosphorylated cysteine we synthesized phosphocysteamine. After adding this com-
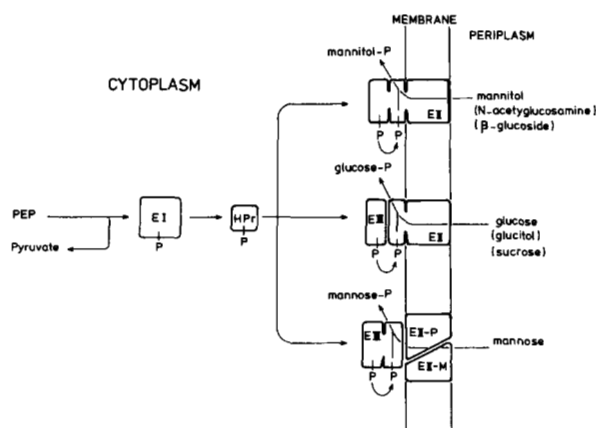

FIG. 1. **E. coli EII types.** *Brackets* indicate other sugar-specific Enzymes II of the same type.

FIG. 3. ^{31}P-NMR spectrum of phosphorylated CIII. *A*, phosphorylated CIII. The sample contained 30 mM Tris-Cl, pH 8.0, 1.3 mM MgCl$_2$, 5 mM DTT, 19% D$_2$O, 0.5 μM Enzyme I, 23 μM HPr, 0.47 mM CIII, 0.8 μM membrane-bound NIII, and 20.3 mM PEP. *B*, dephosphorylated CIII. Mannitol (60 mM) was added to sample A. The chemical shifts are reported relative to the inorganic phosphate signal at 0 ppm.
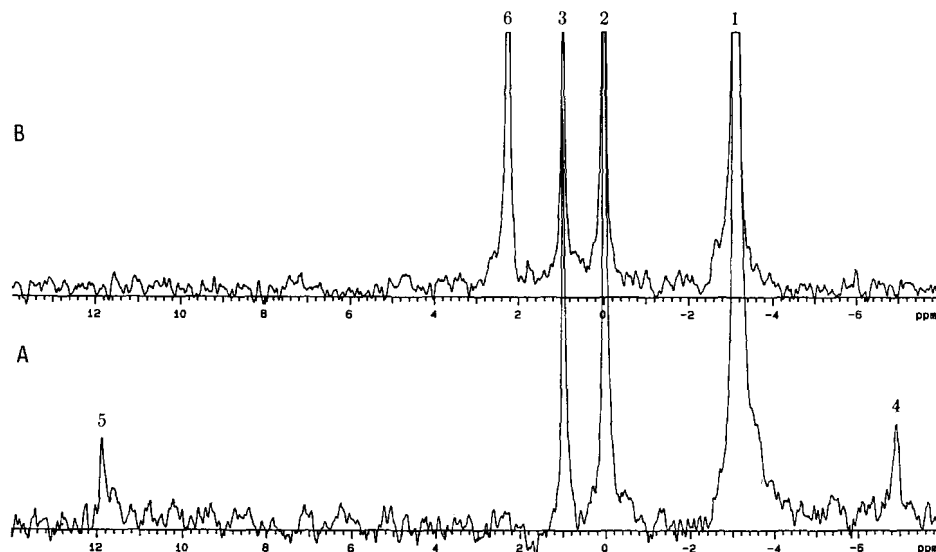
pound to the phosphorylated CIII mixture an extra signal was obtained close to peak 5 at +13.3 ppm (not shown). The slight difference in chemical shift could be due to local effects around the phosphoryl group in the intact protein or arise from the amino group of the model compound.

Peak 3 cannot result from a protein-bound phosphoryl group. When in a separate experiment (not shown) spectra were measured repeatedly overnight, the signal rose to a maximum of about 4 mM, far exceeding the protein concentration. In contrast signals 4 and 5 remained almost constant. Peak heights indicated 0.8–0.9 mM Cys(P) and 0.9–1.0 mM His(P) which is in good agreement with the CIII concentration of 1.1 mM. Since peak 3 originates from phosphoenolpyruvate it most probably reflects 2-phosphoglycerate. Adding this compound in pure form to the reaction mixture indeed raised the signal intensity of peak 3. Spore contamination of enolase in the CIII preparation far below the sodium dodecyl sulfate-polyacrylamide gel electrophoresis detection limit would be sufficient to account for the observed signal intensity. We checked for contamination by comparing CIII samples from different phases in the isolation procedure. The purification procedure consists of three column chromatography steps, Q-Sepharose chromatography followed by hydroxyapatite chromatography, and finally high pressure liquid chromatography Mono-P chromatofocusing. NMR spectra of a Q-Sepharose CIII pool were compared with fully purified CIII. The CIII mannitol phosphorylation activities measured by complementation assays (7) were comparable, but the initial rate of formation of the 2-phosphoglycerate peak dropped to 17% in the more purified CIII preparation.

In summary, the above data show that two phosphorylated residues can be detected in the native intact protein. The first is a histidine, and the second is a cysteine. Together with the data obtained in a previous study (3) we can now state that, in the P$_2$ domain of the mannitol-specific Enzyme II, the phosphoryl group is *covalently* attached to a cysteine. For the mannose system good evidence has been presented that the second phosphorylation site is a histidine. Apart from the isolation of a phosphorylated histidine, this *E*III does not contain a cysteine. Interestingly enough, one of the membrane-bound subunits, II-P^{Man}, contains a decapeptide at its N terminus which is identical for 9 of the 10 amino acids, including the cysteine, with the phosphocysteine-containing

peptide of *E*II^{Mtl} (22). The fact that the cysteine in this subunit does not become phosphorylated is in agreement with the absence of sensitivity of *E*II^{Man} to sulfhydryl reagents (23). *E*II^{Mtl} sulfhydryl reagent sensitivity is caused by a direct reaction on cysteine 384 (24). All other *E*IIs investigated until now react comparably with *E*II^{Mtl} with a loss of catalytic activity upon alkylation. Site-directed replacement of the homologous cysteines in *E*II^{Glc}, *E*II^{Mtl}, and *E*II^{Bgl} also resulted in inactive mutants (25–27). This is in favor of phosphocysteine as a catalytic intermediate in these enzymes. More data concerning the nature of the phosphorylated residues will have to be provided to determine whether the mannose system is the only exception.

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