The Transport/Phosphorylation of \(N, N'\)-Diacetylchitobiose in Escherichia coli

CHARACTERIZATION OF PHOSPHO-IIB\(^{\text{Chb}}\) AND OF A POTENTIAL TRANSITION STATE ANALogue IN THE PHOSPHOTRANSFER REACTION BETWEEN THE PROTEINS IIA\(^{\text{Chb}}\) AND IIB\(^{\text{Chb}}\)

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Enzyme II permeases of the phosphoenolpyruvate:glycose phosphotransferase system comprise one to five separately encoded polypeptides, but most contain similar domains (IIA, IIB, and IIC). The phosphoryl group is transferred from one domain to another, with histidine as the phosphoryl acceptor in II and cysteine as the acceptor in certain IIB domains. IIB\(^{\text{Chb}}\) is a phosphocarrier in the uptake/phosphorylation of the chitin disaccharide (GlcNAc\(_2\)) by Escherichia coli and is unusual because it is separately encoded and soluble. Both the crystal and solution structures of a IIB\(^{\text{Chb}}\) mutant (C10S) have been reported. In the present studies, homogeneous phospho-IIB\(^{\text{Chb}}\) was isolated, and the phosphoryl-Cys linkage was established by \(^{31}\)P NMR spectroscopy. Rate constants for the hydrolysis of phospho-IIB\(^{\text{Chb}}\) plotted \textit{versus} pH gave the same shape peak reported for the model compound, butyl thiophosphate, but was shifted about 4 pH units. Evidence is presented for a stable complex between homogeneous Cys10SerIIB\(^{\text{Chb}}\) (which cannot be phosphorylated) and phospho-IIA\(^{\text{Chb}}\), but not with IIA\(^{\text{Chb}}\). The complex (a tetramer (3)) contains equimolar quantities of the two proteins and has been chemically cross-linked. It appears to be an analogue of the transition state complex in the reaction: phospho-IIA\(^{\text{Chb}}\)+IIB\(^{\text{Chb}}\)\(\rightarrow\)IIA\(^{\text{Chb}}\)+phospho-IIB\(^{\text{Chb}}\). This is apparently the first report of the isolation of a transition state analogue in a protein-protein phosphotransfer reaction.

The accompanying papers\(^1\) present evidence that the chitin disaccharide (GlcNAc\(_2\))\(^2\) is taken up in Escherichia coli by the phosphoenolpyruvate:glycose phosphotransferase system (PTS). The three genes involved in this process were previously characterized as part of a cryptic cellobiose operon (6), and the three proteins were designated IIA\(^{\text{Cel}}\), IIB\(^{\text{Cel}}\), and IIC\(^{\text{Cel}}\), respectively. We suggested (7) that the appropriate nomenclature is IIA\(^{\text{Chb}}\), IIB\(^{\text{Chb}}\), and IIC\(^{\text{Chb}}\) (Chb for \(N\)-acetylchitobiose). We report here the isolation and characterization of phospho-IIB\(^{\text{Chb}}\). Our concept of how (GlcNAc\(_2\))\(^2\) is taken up by \(E.\) \(c\)oli is summarized schematically in Fig. 1.

In most phospho-PTS proteins, the phosphoryl group is linked to a His residue. However, the active site amino acid in the IIB domain of the \(E.\) \(c\)oli mannitol Enzyme II complex was shown to be cysteine (8), and shortly thereafter the same result was found with the IIB domain of the glucose-specific Enzyme II complex of \(E.\) \(c\)oli (9). The definitive method for characterizing this novel linkage was by \(^{31}\)P NMR spectroscopy (see “Discussion”). Sequence similarity of the amino acids around the active site in other IIB domains (10), including IIB\(^{\text{Chb}}\), suggests that the phosphoryl group may be linked to Cys in these proteins as well, although they have not been definitively characterized. In the present studies, homogeneous phospho-IIB\(^{\text{Chb}}\) was isolated and the phosphoryl linkage to Cys\(^10\) (the only Cys in the protein) was established by \(^{31}\)P NMR.

A C10S mutant of IIB\(^{\text{Chb}}\) (or IIB\(^{\text{Cel}}\)) has been crystallized, and both its crystal and solution structures have been determined (11, 12). Apparently the Ser replacement was used because the Cys caused technical difficulties. As shown here, the mutant protein cannot be phosphorylated. But perhaps the most significant result reported in the present studies is that Cys10SerIIB\(^{\text{Chb}}\) forms a stable complex with phospho-IIA\(^{\text{Chb}}\) (but not with IIA\(^{\text{Chb}}\)). Further, the complex can be chemically cross-linked. It seems likely that the complex is a transition state analogue for the phosphotransfer reaction between the two native proteins. To our knowledge, there are no reports of the isolation of a transition state complex, or of an analogue of such a complex, involving a protein-protein phosphotransfer reaction.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

The materials and biochemical and molecular biological methods are the same as described in the accompanying paper on IIA\(^{\text{Chb}}\) (2).

**Construction of IIB\(^{\text{Chb}}\) Overexpression Vector**

The open reading frame corresponding to the \(chbB\) gene was cloned into the pET21a (Novagen, Madison, WI) overexpression vector using polymerase chain reaction and primers specific to the ends of the gene.

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1. The subject matter of the accompanying manuscripts is as follows: (GlcNAc\(_2\))\(^2\) is a PTS sugar in \(E.\) \(c\)oli (1); characterization of IIA\(^{\text{Chb}}\) from \(E.\) \(c\)oli (2); analytical sedimentation studies on IIA\(^{\text{Chb}}\), IIB\(^{\text{Chb}}\), the phosphoproteins and a model transition state analogue (3); identification and molecular cloning of a chitinoporin from \(Vibrio\) \(f\)urnissii (4); and cloning and characterization of a (GlcNAc\(_2\))\(^2\) phosphorylase from \(V.\) \(f\)urnissii (5).

2. The abbreviations used are: (GlcNAc\(_2\)), \(\beta\)-1,4-linked oligomers of GlcNAc where \(n = 2\)–6; PTS, phosphoenolpyruvate:glycose phosphotransferase system; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; TAPS, 3-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-amino)-1-propanesulfonic acid; PEP, phosphoenolpyruvate; BS\(_3\), \(\text{bis(sulfosuccinimidyl)suberate; DTSSP, 3',3''-dithiobis(sulfosuccinimidyl propionate).}

33102 This paper is available on line at http://www.jbc.org
The primers were designed with unique restriction sites at each end to facilitate the cloning procedure. Polymerase chain reaction generated fragments were agarose gel purified and cloned into the pNOTa shuttle vector (5 Prime —→ 3 Prime, Inc., Boulder, CO) and then subsequently subcloned into pET21a using standard procedures. The nucleotide sequences of the primers are given below. The engineered restriction sites are underlined, and the start site of the gene is in bold type. A mutant version of the protein was also constructed in which the active site Cys (amino acid 10) was converted to a Ser. This was achieved by designing a primer with a mismatch in the sequence converting the codon from Cys to Ser, with the mutation given below in underlined italics.

The primers used were: for chbB, 5’-GTCATATGGAGAAGAAAAACATTATCTCTG-3’ (NdeI) and 5’-GATGAATTCTTGAACACCATCTTTTGACGG-3’ (EcoRI); for Cys10SerchbB, 5’-GTCATATGGAGAAGAAAAACATTATCTCTG-3’ (NdeI) and 5’-GAACACCATCTTTTGACGG-3’ (EcoRI).

**Phosphorylation Assay**

The IIB<sub>Chb</sub> proteins were purified essentially as described (12). Two liters each of LB media supplemented with 100 μg/ml ampicillin in three-liter flasks were inoculated with 40 ml (each) of an overnight culture of *E. coli* strain BL21 (DE3) (containing a deletion in Enzyme I of the PTS) harboring the plasmid pET:chbB (or pET: Cys10SerchbB). The culture was shaken vigorously at 37 °C until a<sub>opt</sub> = 0.8–1.0 (2–3 h) before being induced by the addition of 1 mM (final concentration) isopropyl-1-thio-β-D-galactopyranoside. Cells were allowed to grow for an additional 2–3 h and harvested by centrifugation at 4000 × g for 10 min at 4 °C. The following steps were conducted at 0–4 °C unless otherwise stated. The cell pellet was washed twice with TG buffer (10 mM Tris, acetate buffer, pH 6.5, containing 1% glycerol, 1 mM NaN<sub>3</sub>, and 1 mM DTT) and resuspended in the same buffer using 4.0 mg/l (wt weight) of cells. After passage twice through a French Press, cell debris was removed by centrifugation at 12,000 × g for 15 min. Membranes were removed by high speed centrifugation at 180,000 × g for 1 h.

**Step 1: Mono-S- Sepharose Chromatography** — The high speed supernatant (40–50 ml, 5–10 mg protein/ml) was applied to a 2.6 × 15 cm (75 ml) Mono S-Sepharose column equilibrated in TG buffer, at a flow rate of 1 ml/min, after which the column was washed overnight with TG buffer before being eluted with a 1-liter gradient of 0–300 mM NaCl in TG buffer. Fractions were analyzed by SDS-PAGE. The protein eluted between 100 and 150 mM NaCl. Pooled, concentrated fractions from Step 1 were transferred to the column and eluted with the same buffer. Protein fractions were pooled based on purity as determined by SDS-PAGE. The purified protein was concentrated as described above (to 5–10 mg/ml) and dialyzed against 25 mM sodium phosphate buffer, pH 8.0. Wild type protein was dialyzed against the same buffer containing 0.2 mM DTT. Purified protein aliquots were stored at ~70 °C until used.

**Phosphorylation Assay**

The assay was performed as described (2) for the phosphorylation of IIA<sub>Chb</sub>. For measurement of IIB<sub>Chb</sub> phosphorylation, the assay reaction mixture contained (20 μl) 50 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 5 mM NaF, 2–5 pmol of purified Enzyme I, 5–10 pmol of purified HPr, 2–10 pmol of purified IIA<sub>Chb</sub>, and 100–1000 pmol of purified IIB<sub>Chb</sub>. Reactions were initiated by the addition of 0.2–2 nmol of [γ<sup>32</sup>P]ATP (10–20 cpm/pmol). Aliquots were taken over the time course, and the reaction was stopped by dilution with 1.0 ml of ice-cold buffer (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl) and filtered through polyvinylidene difluoride filters (Sartosorb). The filters were washed twice with 1 ml of the same buffer, immersed in 4 ml of Packard Ultima-Gold XR liquid scintillation counter mixture, and counted in a Packard Liquid Scintillation Spectrometer. Control incubation mixtures lacked either Enzyme I, HPr, or IIA<sub>Chb</sub>.

The assay was performed as described (2) for the phosphorylation of IIB<sub>Chb</sub>. The high speed supernatant (40–50 ml, 5–10 mg protein/ml) was applied to a 2.6 × 15 cm (75 ml) MonoS-Sepharose column equilibrated in TG buffer (10 mM Tris, acetate buffer, pH 6.5, containing 1% glycerol, 1 mM NaN<sub>3</sub>, and 1 mM DTT) and resuspended in the same buffer using 4.0 mg/l (wt weight) of cells. After passage twice through a French Press, cell debris was removed by centrifugation at 12,000 × g for 15 min. Membranes were removed by high speed centrifugation at 180,000 × g for 1 h.

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**Phosphorylation Assay**

The assay was performed as described (2) for the phosphorylation of IIA<sub>Chb</sub>. For measurement of IIB<sub>Chb</sub> phosphorylation, the assay reaction mixture contained (20 μl) 50 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 5 mM NaF, 2–5 pmol of purified Enzyme I, 5–10 pmol of purified HPr, 2–10 pmol of purified IIA<sub>Chb</sub>, and 100–1000 pmol of purified IIB<sub>Chb</sub>. Reactions were initiated by the addition of 0.2–2 nmol of [γ<sup>32</sup>P]ATP (10–20 cpm/pmol). Aliquots were taken over the time course, and the reaction was stopped by dilution with 1.0 ml of ice-cold buffer (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl) and filtered through polyvinylidene difluoride filters (Sartosorb). The filters were washed twice with 1 ml of the same buffer, immersed in 4 ml of Packard Ultima-Gold XR liquid scintillation counter mixture, and counted in a Packard Liquid Scintillation Spectrometer. Control incubation mixtures lacked either Enzyme I, HPr, or IIA<sub>Chb</sub>.

**Kinetics of Hydrolysis of Phosphoprotein as a Function of pH**

The rate of hydrolysis of [γ<sup>32</sup>P]phospho-IIB<sub>Chb</sub> was determined as a function of pH at 25 °C. The following buffers were used: McIlvaine’s sodium phosphate-citric acid broad range buffer from pH 2.0 to 8.8, Bates and Bowers boric acid-KCl buffer (pH 8.0–10), sodium phosphate, sodium acetate, sodium borate, Tris-HCl, MOPS-HCl, and TAPS-HCl. At each pH, the kinetics of hydrolysis were determined using the DEAE paper method for separating [γ<sup>32</sup>P]PIP, from [γ<sup>32</sup>P]phospho-IIB<sub>Chb</sub> (13), and initial rates were determined to calculate the respective rate constants.

**Detection, Isolation, and Analysis of a Complex between Phospho-IIA<sub>Chb</sub> and Cys10SerIIB<sub>Chb</sub>**

The mutant IIB<sub>Chb</sub> protein could not be phosphorylated, as expected. However, when stoichiometric amounts of phospho-IIA<sub>Chb</sub> were added to the mutant protein, a complex was detected using native gel electrophoresis (see “Results”). Two methods were developed to purify the complex.

**Electroelution from Native Gel—Equimolar amounts of IIA<sub>Chb</sub> and IIB<sub>Chb</sub>** were incubated with Enzyme I and HPr at 1:250 the molar concentrations of IIA<sub>Chb</sub> and IIB<sub>Chb</sub>. Reactions were initiated by adding a 40-fold excess of PEP and allowed to incubate for 1 h at 37 °C. Samples were electrophoresed in a 16% polyacrylamide gels under native conditions, and the protein band corresponding to the complex was electroeluted from the gel.

**Superdex-75 Gel Filtration** — A two-step procedure was employed to purify the complex. First, IIA<sub>Chb</sub> was phosphorylated and purified by gel filtration chromatography using a Superdex-75 (10 × 300 mm; Amersham Pharmacia Biotech) fast protein liquid chromatography column and system. The column was equilibrated and eluted with 25 mM sodium phosphate pH 8.0 buffer. The protein concentration in the pooled fractions containing the purified phospho-IIA<sub>Chb</sub> was estimated by A<sub>280</sub> and a 1.1-fold excess Cys10SerIIB<sub>Chb</sub> was added to the phosphoprotein. The mixture was maintained at room temperature for 15–20 min before being transferred to a Superdex-75 gel filtration column. The three proteins (IIB<sub>Chb</sub>, IIA<sub>Chb</sub>, and the complex) could easily be separated on the column (see “Results”).
Isolation of Phospho-IIB\textsubscript{Chb} and a Transition State Analogue

Cross-linking Experiments

Phosphorylation reactions were performed in 25 mM sodium phosphate, pH 8.0, buffer containing 5 mM MgCl\textsubscript{2} and 0.2 mM DTT. Typical reaction mixtures (10 \mu l) contained equimolar amounts (0.7–1.4 nmol) of IIA\textsubscript{Chb} and Cys10SerIIB\textsubscript{Chb}, 5–10 pmol of Enzyme I, 5–10 pmol of HPr, and 20–50 nmol of PEP. Reaction mixtures were incubated at 37 °C for 30 min prior to the addition of the cross-linking reagent (20–30-fold excess) and then allowed to stand at room temperature for 30–60 min. Unless otherwise indicated, reactions were quenched by adding 1 \mu l of 0.5 M Tris-HCl buffer, pH 7.5, and incubated for an additional 5 min at room temperature. SDS loading buffer (5 \mu l) was then added, and samples were analyzed by SDS-PAGE. The following cross-linking reagents (Pierce) were tested: bis(sulfosuccinimidyl)suberate (BS3), 1-ethyl-3-(dimethylaminopropyl)carbodiimide, dimethylaminopropyl imidate, dimethylsuberimidate, and DTSSP were prepared in 25 mM sodium phosphate buffer, pH 8.0, and solutions of disuccinimidyl tartrate, dithiobis(succinimidyl propionate) were prepared in Me\textsubscript{2}SO. For reactions containing the cross-linkers dithiobis(succinimidyl propionate) and DTSSP, DTT was omitted from all buffers and solutions. 1-Ethyl-3-(dimethylaminopropyl)carbodiimide reaction mixtures were quenched using 2-mercaptoethanol (final concentration, 10 mM) instead of Tris-HCl buffer.

Analysis of DTSSP Cross-linked Product

The NMR experiments were kindly performed by Dr. Charles Long (Department of Chemistry, Johns Hopkins University). A large scale sample (3.0 pmol) of IIB\textsubscript{Chb} was phosphorylated and purified as described above. The final reaction mixture (100 \mu l) was incubation mixtures where: (a) Enzyme I, HPr, and 20–50 nmol of PEP; (b) Enzyme I, 50 pmol of HPr, and 50 pmol of PEP in buffer (25 mM sodium phosphate buffer, pH 8.0, containing 5 mM MgCl\textsubscript{2}) and DTSSP were prepared in 25 mM sodium phosphate buffer, pH 8.0, and solutions of disuccinimidyl tartrate and dithiobis(succinimidyl propionate) were prepared in Me\textsubscript{2}SO. Each reaction mixture (10 \mu l) was treated with DTT (10 mM) for 30 min and then dialyzed against (100 ml) 25 mM sodium phosphate buffer, pH 8.0, prior to SDS-PAGE analysis.

\textsuperscript{31}P NMR Spectroscopy

Purification of IIB\textsubscript{Chb}—IIB\textsubscript{Chb} was overexpressed and purified from E. coli BL21:ΔEI harboring pET:IIB\textsubscript{Chb}. As in the case of IIA\textsubscript{Chb} (2), IIB\textsubscript{Chb} was purified from a deletion of Enzyme I to ensure that it was isolated in its unphosphorylated form. SDS-PAGE of the purified protein is shown in Fig. 2A. The protein migrates with an apparent molecular mass of 11–12 kDa, which agrees with a predicted molecular mass of 11,400 Da from the gene sequence (see Ref. 7). The protein is not processed during expression because the N-terminal amino netics and requirements for phosphorylation of IIB\textsubscript{Chb}. Phosphorylation was measured by DEAE-paper chromatography as described under “Experimental Procedures.” Aliquots (50 \mu l) were taken over the indicated time course from 0.5-mL reaction mixtures incubated at 37 °C. Each reaction mixture contained 25 mM sodium phosphate buffer, pH 8.0, 5 mM MgCl\textsubscript{2}, 5 mM [\textsuperscript{32}P]PEP and the following proteins: (a) IIB\textsubscript{Chb} (450 pmol), Enzyme I (4.5 pmol), HPr (4.5 pmol); (b) IIB\textsubscript{Chb} (450 pmol), Enzyme I (2.25 pmol), HPr (2.25 pmol), IIB\textsubscript{Chb} (2.25 pmol). Controls (○) were incubation mixtures where: (a) Enzyme I, HPr, IIB\textsubscript{Chb}, or PEP were omitted; (b) IIA\textsubscript{Chb} was substituted for IIB\textsubscript{Chb}; and (c) Cys10SerIIB\textsubscript{Chb} was substituted for IIB\textsubscript{Chb}.

RESULTS

The NMR experiments were kindly performed by Dr. Charles Long (Department of Chemistry, Johns Hopkins University). A large scale sample (3.0 pmol) of IIB\textsubscript{Chb} was phosphorylated and purified as described above. The final reaction mixture (100 \mu l) contained 12 nmol of IIA\textsubscript{Chb} and Cys10SerIIB\textsubscript{Chb}, 50 pmol of Enzyme I, 50 pmol of HPr, and 50 pmol of PEP in buffer (25 mM sodium phosphate buffer, pH 8.0, containing 5 mM MgCl\textsubscript{2}). The sample was analyzed by SDS-PAGE, and the band corresponding to a 26-kDa molecular mass protein was electroeluted from the gel. After electrosolution a portion of the sample (100 \mu l) was treated with DTT (10 mM) for 30 min and then dialyzed against (100 ml) 25 mM sodium phosphate buffer, pH 8.0, prior to SDS-PAGE analysis.

Purification of IIB\textsubscript{Chb}—IIB\textsubscript{Chb} was overexpressed and purified from E. coli BL21:ΔEI harboring pET:IIB\textsubscript{Chb}. As in the case of IIA\textsubscript{Chb} (2), IIB\textsubscript{Chb} was purified from a deletion of Enzyme I to ensure that it was isolated in its unphosphorylated form.
acids. No phospho-compound, synthetic phosphocysteamine gave a signal at 85% H$_3$PO$_4$. Preparations of phospho-IIB$_{Chb}$ were used only when there was no detectable unphosphorylated protein (less than 5%). The kinetics of phosphorylation of IIB$_{Chb}$ shown in Fig. 2C. IIB$_{Chb}$ was incubated with PEP, Mg$^{2+}$, and catalytic quantities of homogeneous Enzyme I, HPr, and IIA$_{Chb}$. No phospho-IIB$_{Chb}$ was detected when any of the four proteins were omitted from the incubation. Thus, there is no detectable transfer from phospho-HPr to IIB$_{Chb}$. Direct transfer of the phosphoryl group from phospho-IIA$_{Chb}$ to IIB$_{Chb}$ was also demonstrated (data not shown), and the kinetics of this reaction will be presented elsewhere.

The phosphoprotein was isolated in mg quantities, and after gel chromatography for final purification and analysis by native gel electrophoresis, it was used for the following studies. Preparations of phospho-IIB$_{Chb}$ were used only when there was no detectable unphosphorylated protein (less than 5%).

$^3$P NMR spectra of purified phospho-IIB$_{Chb}$ and a transition state analogue were recorded as described under “Experimental Procedures.” A, spectral accumulations at 81 min, 3 Hz line broadening, 1536 transients collected, 9728 data points. B, 17 h, 10 Hz line broadening, 19,290 transients collected, 9728 data points.

**Isolation of Phospho-IIB$_{Chb}$ and a Transition State Analogue**

$^3$P NMR of phospho-IIB$_{Chb}$—Although SDS-PAGE cannot separate IIB$_{Chb}$ and phospho-IIB$_{Chb}$, the proteins are separable by native gel electrophoresis (Fig. 2B). Densitometric scans of the stained gels permitted quantitation of the two proteins and were used to determine the extent of phosphorylation of IIB$_{Chb}$.

The kinetics of phosphorylation of IIB$_{Chb}$ are shown in Fig. 2C. IIB$_{Chb}$ was incubated with PEP, Mg$^{2+}$, and catalytic quantities of homogeneous Enzyme I, HPr, and IIA$_{Chb}$. No phospho-IIB$_{Chb}$ was detected when any of the four proteins were omitted from the incubation. Thus, there is no detectable transfer from phospho-HPr to IIB$_{Chb}$. Direct transfer of the phosphoryl group from phospho-IIA$_{Chb}$ to IIB$_{Chb}$ was also demonstrated (data not shown), and the kinetics of this reaction will be presented elsewhere.

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$^3$P NMR Spectrum of Phospho-IIB$_{Chb}$—$^3$P NMR spectroscopy is a powerful tool for determining the structures of phosphorylated compounds. For example, there is a significant and specific chemical shift in for virtually each phosphoryl linkage found in proteins (for reviews see Refs. 14 and 15).

The $^3$P NMR spectrum of homogenous phospho-IIB$_{Chb}$ is shown in Fig. 3 relative to 85% phosphoric acid. A single signal was observed downfield, at 16.3 ppm at 10 °C, pH 8.6. This 16.3 ppm signal can be compared with those reported for phosphocysteine in the following: (a) The IIB domain of the *E. coli* Enzyme II$^{Mtl}$ (16), +11.9 ppm at pH 8.0 relative to P$_1$, or approximately +14–15 relative to 85% H$_3$PO$_4$. The model compound, synthetic phosphocysteamine gave a signal at +13.3 ppm and, when corrected relative to 85% H$_3$PO$_4$, was at +15.3–16.3 ppm. (b) The IIB domain of the *E. coli* Enzyme II$^{Mtl}$ (17), +11.7 ppm at pH 6.8 relative to P$_1$ or −13.7–15 relative to 85% H$_3$PO$_4$. (c) The IIB domain of the *Staphylococcus carnosus* Enzyme II$^{Mtl}$ (18) was cloned linked to a His tag (for purification), +11.8 ppm at 10 °C, pH 7.5 relative to 85% H$_3$PO$_4$. (d) Both a synthetic phosphocysteinylpeptide and a leukocyte tyrosine phosphatase phosphoprotein intermediate (19) in the hydrolytic reaction showed a chemical shift of +16.1 ppm in the rapid quench fluid (0.2 N NaOH) relative to 85% H$_3$PO$_4$, where the P$_1$ signal was +5.5 ppm. (e) A similar human dual specific phosphatase (20) showed a chemical shift at +13.7 ppm relative to 85% H$_3$PO$_4$ at pH 7.0, 22 °C.

The chemical shifts observed with thiophosphoryl derivatives are far downfield from all other known $^3$P chemical shifts for both low molecular mass phosphoryl derivatives and phosphoamino acids (14, 15). These include O-phosphoserine and threonine, N-phospholysine, N-phosphoarginine, O-phosphotyrosine, acylphosphate (e.g. to aspartate), phospho-N$^2$- and phospho-N$^3$-histidine, and the pyrophosphate linkage. At pH 8.0, the chemical shifts for these substances lie in the range +4 to −11 relative to 85% H$_3$PO$_4$ set at 0 ppm. Our $^3$P NMR results therefore lead to the conclusion that phosphoryl group in phospho-IIB$_{Chb}$ is linked to Cys$^{10}$. This linkage had previously been surmised based on amino acid sequence similarity but had not been experimentally demonstrated.

The first spectrum shown in Fig. 3 was collected over a period of 81 min at pH 8.6, 10 °C. On repeated scans of the sample, over a period of 17 h, another peak was observed at +3.1 ppm, which increased with time, whereas the peak at +16.3 ppm decreased. The new peak was identified as inorganic phosphate, corresponding in its chemical shift to a standard in the same buffer (data not shown). Phospho-IIB$_{Chb}$ therefore slowly hydrolyzes under these conditions.

**Stability of Phospho-IIB$_{Chb}$—**Aside from the early studies on the properties of a thioester phosphate ester, butyl thiophosphate (21), there are only a few reports on the properties of the thioester group in proteins or peptides derived by proteolysis of the phosphoprotein.

The rate constants for the hydrolysis of [32P]phospho-IIB$_{Chb}$ as a function of pH at 25 °C are shown in Fig. 4. The maximum instability is pH −8. At this pH, phospho-IIB$_{Chb}$ is hydrolyzed at about seven times the rate of phospho-IIA$_{Chb}$ at the concentration used for the latter (2).

The bell-shaped curve in Fig. 4 is similar in shape to that observed for the spontaneous hydrolysis of the model compound, butyl thiophosphate (21), except that the peak of the curve for the latter has a pH of −3–4. The shift to the right of about 4 pH units for the phosphoprotein must reflect the effects of the local environment surrounding the thioester group in the protein. Different results were obtained with other thiophosphates: (a) *E. coli* phospho-Enzyme II$^{Mtl}$ was subjected to trypsin and chymotrypsin digestion (8), a phosphopeptide (14 amino acids) was isolated, and the phosphonyl group was found to be linked to Cys (the first such report). The stability of the phosphopeptide was studied in the range pH 2–13 and gave an...
inverted bell-shaped curve, with maximum instability at pH 2–4 and 13 and maximum stability at pH 10–12. (b) Similar results were obtained with a phosphododecapeptide isolated from the E. coli IIIBCt translocator (22). The curve, resembling an hyperbola, was virtually superimposable over that obtained with the IIICt phosphopeptide in the range pH 2–12. (c) Finally, the intact phosphoprotein-tyrosine phosphatase showed a bell-shaped profile, similar to the model compound butyl thiophosphate, with maximum instability at pH 2–3 and maximum stability at pH 8–10 (23). Thus, phospho-IIIB behaves differently from other known thiophosphates with respect to its stability as a function of pH. This observation is discussed below.

Formation of Complex between Phospho-IIAChb and Cys10SerIIIBChb—When [32P]phospho-IIAChb and the mutant protein Cys10SerIIIBChb were mixed in equimolar quantities and subjected to native gel electrophoresis, a new labeled band was observed in the gel (Fig. 5A), intermediate in its position between the two proteins. The band was cut from the gel, eluted, and analyzed by SDS-PAGE, and [32P]phospho-IIAChb and Cys10SerIIIBChb were found (Fig. 5B). The two proteins were present in equimolar quantities in the complex, as indicated by quantitative densitometry of the bands (data not shown).

The complex was also isolated by gel filtration chromatography (Fig. 6). In this experiment, [32P]phospho-IIAChb was added to a 10% excess of Cys10SerIIIBChb, and the mixture was fractionated by gel filtration chromatography using a Superdex-75 column. The first and largest protein peak contained the [32P] followed by a small peak of Cys10SerIIIBChb. Fig. 6 shows that the higher molecular mass or major peak (labeled Complex) is eluted before the standards used to calibrate the column, including IIAChb or phospho-IIAChb, plus (IIIBChb or phospho-IIIBChb); IIAChb and Cys10SerIIIBChb. The higher molecular mass radiolabeled peak from the column in Fig. 6 was analyzed by SDS-PAGE and, as shown in Fig. 5B, contained both of the starting proteins, [32P]phospho-IIAChb and Cys10SerIIIBChb, in equimolar quantities.

From these results we conclude that phospho-IIAChb and Cys10SerIIIBChb form a stable complex containing equimolar quantities of the proteins and that IIAChb does not substitute for phospho-IIAChb. The binding constant holding the proteins in the complex is sufficiently high so that they do not separate on a gel filtration column. The same results were obtained by subjecting the complex eluted from the column to analytical ultracentrifugation (3). A schematic version of these and the following results is shown in Fig. 7.
The stability of the phosphoryl linkage in the $^{32}$P-labeled complex was studied as a function of pH, over the range 6–11 (Fig. 4). The complex precipitates at lower pH values. Unexpectedly, we found that the complex was slightly less stable than phospho-IIAChb, suggesting that the phosphoryl linkage is not shielded from attack by the solvent in the complex.

Cross-linking of Phospho-IIAChb and Cys10SerIIBChb—When efforts were made to crystallize the complex, it partially dissociated. To eliminate this problem, attempts were made to covalently cross-link the two proteins in the complex. A number of reagents were tested, and the most satisfactory results were obtained with BS$_3$, a noncleavable cross-linking reagent that reacts with amines. When the reaction products were subjected to SDS-PAGE, they gave the results shown in Fig. 8. A major band was detected in the gel that migrated at a molecular mass of 24–25 kDa. The calculated molecular masses (not corrected for the cross-linkers) of potential products of a reaction mixture containing phospho-IIAChb and Cys10SerIIBChb are: the homodimer (phospho-IIA)$_2$, 25 kDa; the homodimer (IIB)$_2$, 22.8 kDa; the heterodimer phospho-IIA/IIB, 24.3; and the heterotetramer (phospho-IIA/IIB)$_2$, 48.3. The observed band could be either of the two homodimers or the desired product, a heterodimer of the two proteins, which may have been derived from the tetramer upon SDS treatment (Fig. 7).

Control experiments offered strong evidence that the 24–25-kDa band consisted of the desired product. Fig. 8 shows that the new protein band was formed when the reaction mixture...
under these conditions, the covalently cross-linked complex could be the heterodimer phospho-IIA/IIB or the heterotetramer (phospho-IIA/IIB)₂ (Fig. 7). Both the gel filtration and analytical ultracentrifugation experiments suggest that the native cross-linked complex is, in fact, the tetramer, schematically illustrated in Fig. 7.

DISCUSSION

Although the solution and crystal structures of the mutant protein Cys₁₀SerIIBChb have been established (11, 12) and IIBChb has been isolated, there are no reports on the properties of phospho-IIBChb. As demonstrated by analytical sedimentation (3), IIBChb and phospho-IIBChb are monomeric, unlike both IIAChb and phospho-IIAChb, which form stable dimers. In addition, we show that phosphate transfer proceeds as depicted in Fig. 1, from PEP through Enzyme I, HPr to IIAChb to IIBChb, and finally to (GlcNAc)₂, the last step mediated by the membrane receptor, IICChb. Kinetic and thermodynamic studies on the reversible transfer of the phosphoryl group between IIAChb and IIBChb are now in progress.

Early work on phospho-PTS proteins established that the phosphoryl group was generally linked to a histidine. However, in 1988 Pas and Robillard (8, 24) reported a unique linkage, a thiophosphate, in a phosphoryl-polypeptide isolated from the IIB domain of II₄₅. The thiophosphate linkage was subsequently reported in the IIB domain of IICBChb (22) and in the IIB domain of the Staphylococcus carnosus Enzyme II₄₅ (18). Amino acid sequence similarities also suggested that Cys is the active site amino acid in a number of IIB domains. The thiophosphate linkage has also been found in a different family of enzymes, protein-tyrosine phosphatases (23), where they act as catalytic intermediates in the overall hydrolysis reaction (19, 20).

The phosphoryl group in phospho-IIBChb was presumed to be linked to Cys¹⁰ (25) based on sequence similarity. In the present studies, phospho-IIBChb was isolated in homogeneous form, and the linkage was shown to be a thiophosphate by ³²P NMR; IIBChb contains only one Cys. IIBChb offers a unique advantage for these experiments because it is a separately encoded protein. Thus, NMR can be directly applied without resorting to protease digestion and the isolation of a phosphopeptide and therefore without the danger of an artificial result because of phosphoryl migration.

For structural and other experiments on phospho-IIBChb, it was necessary to determine its stability. Like butyl thiophosphate (21), the curve of hydrolysis rate versus pH is bell-shaped, but whereas butyl thiophosphate shows maximum instability at pH ~3–4, phospho-IIBChb shows maximum instability at pH ~8. This behavior is different from thiophosphate-binding peptides isolated from other IIB domains and from that of a protein-tyrosine phosphatase, all of which more closely resemble butyl thiophosphate. The most likely explanation for this large difference in behavior of the thiophosphate group in phospho-IIBChb are neighboring group effects. It should be noted that IIBChb is a very basic protein, with a calculated pI of 8.0. Furthermore, two crystal structures have been solved, one a protein-tyrosine phosphate phosphatase mutant with the thiophosphate, in a phosphoryl-polypeptide isolated from the IIB domain of II₄₅ (26). In both cases, the structures show that a conformationally flexible loop closes over the phosphate (or tautomeric phosphate) (27). In both cases, the structures show that a conformationally flexible loop closes over the phosphate (or tautomeric phosphate), thereby increasing the number of amino acid side chains surrounding the phosphoryl moiety. The ³²P NMR results obtained here with phospho-IIBChb are also consistent with the idea of strong neighboring groups interactions, because the 16.3 ppm downfield shift at pH 8.6 was significantly greater than in the other thiophosphoryl proteins and was
similar to that of the leukocyte tyrosine phosphatase phospho-
protein, where the chemical shift was +16.1 in 0.2 N NaOH
(19). In other words, the phosphoryl group in phospho-IIA\textsuperscript{Chb}
may be completely ionized at pH 8.6. In this connection, it
should be noted that the pK\textsubscript{a} of the thiophosphoryl group in the
IIB domain of \textit{S. carnosus} could not be measured in the pH
range 3.9–8.4 (18), and von Strandmann \textit{et al.} concluded that
the pK\textsubscript{a} was likely to be <2.5 and that the phosphoryl group is
doubly charged over a very broad pH range. There were no pH
stability studies reported on this thiophosphoryl domain, and
additionally, the IIB domain was subcloned with a His\textsubscript{6} tag to
aid in the purification. Conceivably, the latter could influence
the pK\textsubscript{a} of the phosphoryl group in this protein.

The most unexpected result of the present studies, was to
find that phospho-IIA\textsuperscript{Chb} and the mutant protein
Cys\textsubscript{10}SerIIB\textsuperscript{Chb} form a stable complex, stable, that is, to gel
filtration column chromatography, native gel electrophoresis,
and analytical ultracentrifugation (some dissociation was not-
ed). The complex was dissociated to its components by SDS-
filtration column chromatography, native gel electrophoresis,
and analytical ultracentrifugation (some dissociation was not-
ed). The complex was dissociated to its components by SDS-
PAGE, and they were present in equimolar quantities in the
PAGE, and they were present in equimolar quantities in the
gels.

The complex was cross-linked with BS\textsuperscript{3}, shown schematically in
Fig. 7. The complex and the cross-linked complex are, we
believe, analogues of a transition state complex that occur as
intermediate in the phosphotransfer reaction between II-
A\textsuperscript{Chb} and IIB\textsuperscript{Chb}. Insofar as we are aware, no such transition
state analogues involving protein-protein phosphotransfer re-
actions have been reported. Attempts are now in progress to
 crystallize the cross-linked analogue.

REFERENCES

1. Keyhani, N. O., Wang, L., Lee, Y. C., and Roseman, S. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33084–33090
2. Keyhani, N. O., Boudker, O., and Roseman, S. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33091–33101
3. Keyhani, N. O., Rodgers, M., Demeler, B., Hansen, J., and Roseman, S. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33110–33115
4. Keyhani, N. O., Li, X., and Roseman, S. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33068–33076
5. Park, J. K., Keyhani, N. O., and Roseman, S. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33077–33083
6. Parker, L. L., and Hall, B. G. (1990) \textit{Genetics} \textbf{124}, 455–471
7. Keyhani, N. O., and Roseman, S. (1997) \textit{Proc. Natl. Acad. Sci., U. S. A.} \textbf{94}, 14367–14371
8. Pas, H. H., and Robillard, G. T. (1988) \textit{Biochemistry} \textbf{27}, 5835–5839
9. Erni, B. (1989) \textit{FEBS Microbiol. Rev.} \textbf{63}, 15–25
10. Postma, P. W., Lengeler, J. W., and Jacobson, G. R. (1993) \textit{Microbiol. Rev.} \textbf{57}, 543–594
11. van Montford, R. L. M., Pijning, T., Kalk, K. H., Reizer, J., Saier, M. H., Thunnissen, M. M. G. M., Robillard, G. T., and Dijkstra, B. W. (1997) \textit{Structure} \textbf{5}, 217–225
12. Ab, E., Schuurman-Wolters, G. K., Saier, M. H., Reizer, J., Jacuinod, M., Roepstorff, P., Dijkstra, K., Scheek, R. M., and Robillard, G. T. (1994) \textit{Protein Sci.} \textbf{3}, 282–290
13. Weigel, N., Kakuruzinska, M. A., Nakazawa, A., Waygood, E. B., and Rose-
man, S. (1982) \textit{J. Biol. Chem.} \textbf{257}, 14477–14491
14. Matheis, G., and Whitaker, J. R. (1984) \textit{Int. J. Biochem.} \textbf{16}, 867–873
15. Vogel, H. J. (1989) \textit{Methods Enzymol.} \textbf{177}, 283–283
16. Pas, H. H., Meyer, G. H., Kruizinga, W. H., Tamminga, K. S., van Weeghel,
R. P., and Robillard, G. T. (1991) \textit{J. Biol. Chem.} \textbf{266}, 6690–6692
17. Gemmecker, G., Eberstadt, M., Buhr, A., Lanz, R., Grudanolnik, S. G., kessler,
H., and Erni, R. (1997) \textit{Biochemistry} \textbf{36}, 7408–7417
18. Pogge von Strandmann, R., Weigt, C., Fischer, R., Karschki, H. R., and Hengstenberg,
W. (1995) \textit{Eur. J. Biochem.} \textbf{233}, 116–122
19. Cho, H., Krishnaraj, R., Kitas, E., Bannwarth, W., Walsh, C. T., and Andersson,
K. S. (1992) \textit{J. Am. Chem. Soc.} \textbf{114}, 7296–7298
20. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E.
(1996) \textit{Proc. Natl. Acad. Sci., U. S. A.} \textbf{93}, 2493–2498
21. Herr, E. B., Jr., and Koshland, D. E. J. (1957) \textit{Biochem. Biophys. Acta} \textbf{25}, 219–220
22. Meins, M., Jani, P., Muller, D., Richter, W. J., Rosenbusch, J. P., and Erni, B.
(1993) \textit{J. Biol. Chem.} \textbf{268}, 11604–11609
23. Guan, K. L., and Dixon, J. E. (1991) \textit{J. Biol. Chem.} \textbf{266}, 17026–17030
24. Pas, H. H., and Robillard, G. T. (1988) \textit{Biochemistry} \textbf{27}, 5515–5519
25. van Montford, R. L. M., Pijning, T., Kalk, K. H., Schuurman-Wolters, G. K.,
Reizer, J., Saier, M. H., Robillard, G., and Dijkstra, B. W. (1994) \textit{J. Mol.
Biol.} \textbf{239}, 588
26. Pannifer, A. D., Flint, A. J., Tenks, N. K., and Barford, D. (1998) \textit{J. Biol. Chem.} \textbf{273}, 10454–10462
27. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z. Y., Dixon, J. E., and
Saper, M. A. (1994) \textit{Nature} \textbf{370}, 571–575