Isolation and Characterization of IIA\textsubscript{Chb}, a Soluble Protein of the Enzyme II Complex Required for the Transport/Phosphorylation of \(N,N'\)-Diacetylchitobiose in \textit{Escherichia coli}\textsuperscript{*}

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\(N,N'\)-Diacetylchitobiose is transported/phosphorylated in \textit{Escherichia coli} by the (GlcNAc)\(_2\)-specific Enzyme II permease of the phosphoenolpyruvate:glycose phosphotransferase system. IIA\textsubscript{Chb}, one protein of the Enzyme II complex, was cloned and purified to homogeneity. IIA\textsubscript{Chb} and phospho-IIA\textsubscript{Chb} form stable homodimers (3). Phospho-IIA\textsubscript{Chb} behaves as a typical e2-N (i.e. N-2) phospho-IIs protein. However, the rate constants for hydrolysis of phospho-IIA\textsubscript{Chb} at pH 8.0 unexpectedly increased 7-fold between 25 and 37 °C and increased ~4-fold with decreasing protein concentration at 37 °C (but not 25 °C). The data were explained by thermal denaturation studies using CD spectroscopy. IIA\textsubscript{Chb} and phospho-IIA\textsubscript{Chb} exhibit virtually identical spectra at 25 °C (~80% α-helix), but phospho-IIA\textsubscript{Chb} loses about 30% of its helicity at 37 °C, whereas IIA\textsubscript{Chb} shows only a slight change. Furthermore, the \(T_m\) for thermal denaturation of IIA\textsubscript{Chb} was 54 °C, only slightly affected by concentration, whereas the \(T_m\) for phospho-IIA\textsubscript{Chb} was much lower, ranging from 40 to 46 °C, depending on concentration. In addition, divalent cations (Mg\(^{2+}\), Cu\(^{2+}\), and Ni\(^{2+}\)) have a dramatic and differential effect on the structure, depending on the state of phosphorylation of the protein. Thus, phosphorylation destabilizes IIA\textsubscript{Chb} at 37 °C, potentially affecting the monomer/dimer transition, which correlates with its chemical instability at this temperature. The physiological consequences of this phenomenon are briefly considered.

In \textit{Escherichia coli}, the operon that encodes the proteins required for catabolism of the chitin disaccharide \(N,N'\)-diacetylchitobiose, (GlcNAc)\(_2\),\textsuperscript{1} was previously thought to be a “cryptic” cellulobiose operon (6, 7). The cryptic cellulobiose operon is, in fact, a normally inducible catabolic operon required for the utilization of (GlcNAc)\(_2\). We have proposed that the nomenclature for the proteins encoded by the operon be changed, from Cel (cellulobiose) to Chb (di-N-acetylchitobiose).

We report (1, 5)\textsuperscript{2} that (GlcNAc)\(_2\) uptake is mediated by the phosphoenolpyruvate:glycose phosphotransferase system (PTS). Transport requires the PTS general proteins, Enzyme I and HPr, as well as two soluble sugar-specific proteins, IIA\textsubscript{Chb} and IIB\textsubscript{Chb}, and a membrane protein IIC\textsubscript{Chb}. The overall reaction sequence leading to cytoplasmic sugar phosphate is summarized in Fig. 1 of one of the accompanying papers (2).

The structures of the Enzyme II complexes, i.e. the sugarspecific components or permeases of the PTS, are currently under intense investigation. One suggested nomenclature of the complexes derives from amino acid sequence and functional homologies (8, 9). They can be divided into distinct domains, designated IIA, IIB, and IIC. For some sugars the IIA, IIB, and IIC domains are encoded by a single (membrane bound) polypeptide, for others IBC forms a single membrane protein component with a separate soluble IIA protein. The (GlcNAc)\(_2\) permease offers many advantages for studying the structure/function of an Enzyme II complex because the IABC domains are encoded by three separate genes, one each for the IIA, IIB, and IIC. But more importantly, IIB\textsubscript{Chb} is cytoplasmic, which offers a rare opportunity to conveniently study its interactions with its partners, IIA\textsubscript{Chb} and IIC\textsubscript{Chb}.

Based on sequence homologies IIA\textsubscript{Chb} has been assigned to the lactose family of PTS transporters (8, 10), but there is virtually no published information on IIA\textsubscript{Chb} nor, of course, on phospho-IIA\textsubscript{Chb}. Although both the crystal and solution structures of an active site mutant protein of IIB\textsubscript{Chb} have been reported (11), there is little published information on IIB\textsubscript{Chb} itself and nothing on phospho-IIB\textsubscript{Chb}, the subjects of the accompanying papers.

In the present report, homogeneous IIA\textsubscript{Chb} and phospho-IIA\textsubscript{Chb} are described along with some important properties of the proteins. Analytical sedimentation studies of the proteins are presented in an accompanying paper (3).

**Experimental Procedures**

**Materials**

Buffers and reagents were of the highest purity commercially available. \(^{32}\)P-PEP was a kind gift from Dr. N. Meadow (Johns Hopkins University). Cellulose nitrate filters (diameter, 25 mm; pore size, 0.45

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\† The abbreviations used are: (GlcNAc)\(_n\), \(\beta,1,4\)-linked oligomers of GlcNAc where \(n = 2–6\); PTS, phosphoenolpyruvate:glycose phosphotransferase system; PEP, phosphoenolpyruvate; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

\textsuperscript{1} The subject matter of the accompanying manuscripts is as follows: (GlcNAc)\(_2\) is a PTS sugar in \textit{E. coli} (1); characterization of phospho-IIB\textsubscript{Chb} and of a potential transition state analogue in the phosphotransfer reaction between IIA\textsubscript{Chb} and IIB\textsubscript{Chb} in \textit{E. coli} (2); analytical sedimentation studies on IIA\textsubscript{Chb}, IIB\textsubscript{Chb}, the phosphoproteins and a model transition state analogue (3); identification and molecular cloning of a chitoporin from \textit{Vibrio furnissii} (4); and cloning and characterization of a (GlcNAc)\(_2\) phosphorylase from \textit{V. furnissii} (5).
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µm) were obtained from Sartorius. *E. coli* strain BL21(DE3)::ΔEI, containing a kanamycin cartridge in Enzyme I was a kind gift from Dr. F. Chaavin.

**Molecular Analysis and Sequencing of DNA**

Preparations, analyses, restriction enzyme digests, ligations, and transformations were performed using standard techniques (12, 13). Double-stranded DNA was prepared from recombinant clones and sequenced by the dideoxy method using a U.S. Biochemical Corp. Sequenase version 2.0 sequencing kit or alternately by the Genetics Core Facility (Johns Hopkins Medical School) using an ABI-373 automated sequencer.

**Construction of IIA<sub>Chb</sub> Overexpression Vector**

The open reading frame corresponding to the chbA gene was cloned into the pET21a (Novagen, Madison, WI) overexpression vector using polymerase chain reaction and primers specific to the ends of the gene. The primers were designed with unique restriction sites at each end to facilitate the cloning procedure. The polymerase chain reaction generated fragments were first cloned into the pNoTA/T7 shuttle vector (Prime PCR Cloner Cloning System; 5 Prime → 3 Prime, Inc., Boulder, CO) and then subcloned into pET21a (according to the manufacturer's recommendations and procedures). Primers were designed (at the 5’ end of the gene) such that the start site (ATG) codon would be ligated directly to the NdeI site of pET21a. 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. The primers used were as follows. 5'-AGGAAAGCCATAGTAGGGATCTC-3' (NdeI site) and 5'-TACCTGCTGATGATCCTTGCC-3' (BamHI). The isolated subclones in pET21a were confirmed by sequencing the entire insert.

**Purification of IIA<sub>Chb</sub>**

When the protein was overexpressed from pET21a and crude extracts were subjected to SDS-PAGE, a new major band was detected that migrated at the expected molecular mass (12.75 kDa). SDS-PAGE was therefore used to detect the protein throughout the purification procedure.

**Step 1: Crude Extract—**Two liters each of LB media supplemented with 100 µg/ml ampicillin in two 6-liter flasks were inoculated with 80 ml of an overnight culture of *E. coli* strain BL21(DE3)::ΔEI harboring the plasmid pET-chbA. The culture was shaken vigorously at 37 °C until A<sub>600</sub> was about 1.0 (2–3 h) before being induced by the addition of 1 mM IPTG. The expression was induced for 45–60 min at 37 °C. The cells were then allowed to grow for an additional 2 h and harvested by centrifugation at 20,000 g for 30 min. The pellet was resuspended in 100 mM dibasic sodium phosphate buffer, pH 7.5, containing 100 mM NaF, 2–5 pmol of purified Enzyme I, 5–10 pmol of purified HPr, and 100–1000 pmol of purified IIA<sub>Chb</sub>. The reaction stopped by dilution with 1.0 ml of ice-cold buffer (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl) and rapidly filtered through cellulose nitrate filters (Sartorius). The filters were washed twice with 1 ml each of ice-cold buffer, immersed in 4 ml of Packard Ultima-Gold XR liquid scintillation counter mixture, and counted in a Packard Liquid Scintillation Spectrometer.

**Filter Binding Assay**

The assay was performed essentially as described (14). For measurement of IIA<sub>Chb</sub> phosphorylation the assay reaction mixture (20 µl) contained 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, and 100–1000 pmol of purified IIA<sub>Chb</sub>. The reaction was started by the addition of 2 pmol of [γ<sup>32</sup>P]PEP (10–20 cpm/pmol). Aliquots were taken over the time course, the reaction stopped by dilution with 1.0 ml of ice-cold buffer (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl) and rapidly filtered through cellulose nitrate filters (Sartorius). The filters were washed twice with 1 ml each of ice-cold buffer, immersed in 4 ml of Packard Ultima-Gold XR liquid scintillation counter mixture, and counted in a Packard Liquid Scintillation Spectrometer.

**Phosphorylation of IIA<sub>Chb</sub>**

Phosphorylation reactions for preparative isolation of phospho-IAA<sub>Chb</sub> were performed on a large scale as described above. Typically, 1.0-m1 reaction mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1–0.3 nmol of purified Enzyme I, 0.25–0.5 nmol of purified HPr, and 35–100 nmol of purified IIA<sub>Chb</sub>. The mixture was incubated for 45–60 min at 37 °C. The phosphorylated protein was purified by native gel electrophoresis using 15% acrylamide gels. Stacking and resolving gels and buffer were identical to the SDS-PAGE procedure except that SDS was omitted from all buffers. Protein samples were maintained at 4 °C prior to loading onto the gel, and the sample loading buffer was modified to contain the following (final concentrations): 0.1 mM Tris-HCl buffer, pH 9.3, 5% glycerol, 0.1% (w/v) bromphenol blue, 2 mM DTT. To identify the position of the phosphorylated protein in the gel after electrophoresis, lanes on both sides of the gel were cut and either blotted with Coomassie
Blue or (where proteins were labeled with $^{32}\text{P}$) further cut into slices, immersed in 3.0 ml of scintillation fluid, and counted as described above. The remaining portion of the gel was then sliced and the desired (phospho)-protein product recovered by electrophoresion at 250 V for 2 h at 4 °C in an Amicon Centrifull. Electrophoresion buffer was either 12.5 mM Tris hydrochloride, pH 6.8 for 15 or 15 mM Tris-Caps, pH 9.3. The eluate was concentrated to 0.5 ml by ultrafiltration in a Centricon-3 and stored in aliquots at −80 °C until used.

**Alternate Procedure for Phosphorylation and Purification of Phosphoproteins**

The phosphorylation reaction was performed as described above or using HPr coupled to Affi-Gel-15 agarose beads (see below). The reaction was terminated by sedimenting the HPr beads (10,000 × g, 5 min), and the supernatant was applied to a Superdex 75 HR 10/30 column (10 ml × 300 mm; Amersham Pharmacia Biotech) equilibrated and eluted with 25 mM sodium phosphate buffer, pH 8.0, using an Amersham Pharmacia Biotech FPLC system. Protein in the eluate was monitored by absorbance at 280 nm. Fractions corresponding to phospho-IIAChb were either used immediately or stored at −70 °C; the phosphoprotein was stable for at least 3 days under these conditions.

**Coupling of HPr to Affi-Gel 15 Beads**

Purified HPr (kind gift of Dr. R. Matteo) was coupled to Affi-Gel 15 (Bio-Rad) according to the manufacturer's recommendations. Briefly, 3.6 mg of purified HPr in 2 ml of 10 mM MOPS buffer, pH 6.5, were added to 1.5 ml of washed Affi-Gel 15 resin and reacted at 4 °C for 4 h on a rotating shaker. About 50% of the protein remained in the solution and was removed. Unreacted groups on the resin were blocked with ethanolamine as recommended by the manufacturer.

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

The rate of hydrolysis of $^{14}\text{P}$-phospho-IIAChb was determined as a function of pH. The following buffers were used: McIlvaine's sodium phosphate-citric acid broad range buffer from pH 2.0 to 8.8, Bates' and phosphate-citric acid broad range buffer from pH 2.0 to 8.8, Bates' and borate, pH 8.7, or 15 mM Tris-CAPS, pH 9.3. The eluate was subjected to electrophoresion at 50 V/cm for 30–45 min. After drying the paper was immersed in 3.0 ml of scintillation fluid, and counted as described above.

**Paper Electrophoresis—Reaction mixtures containing $^{32}\text{P}$-labeled proteins were subjected to electrophoresion on a PhastGel system loaded with Whatman 3MM paper saturated with 1% tetraborate, pH 9.3, and subjected to electrophoresion at 50 V/cm for 30–45 min. After drying the paper was cut into segments, immersed in 3 ml of HOMIC fluor (Packard), and counted.

**Filter Binding Assay—**The filter binding assay was as described above.

**Protein Determination**

Protein concentrations and extinction coefficients were calculated for the purified proteins by quantitation of nitrogen content by the Kjeldahl method, modified by Jannickie (18) using the routine protein measurements, the Bio-Rad dye binding assay was employed using bovine serum albumin as the standard.

**CD Spectroscopy**

CD experiments were conducted using a Jasco-J710 spectropolarimeter with a Peltier type cell holder (Jasco model PTC-345W). Each wavelength spectrum scan was obtained by averaging 4–5 spectra using a scan speed of 50 nm/min and a resolution of 0.3 nm rectangular cell. Buffer scans (25 mM sodium phosphate, pH 8.0) were accumulated and subtracted from the sample scans, and the mean residue ellipticity was calculated. CD temperature scans were performed by varying the temperature over the indicated range at a rate of 1 °C/min, and the mean ellipticity was measured at 222 nm. Experiments measuring the reversibility of thermal denaturation were conducted at a rate of 2 °C/min.

**Effect of Divalent Cations on CD Spectra and Thermal Denaturation of IIAChb and Phospho-IIAChb**

Because IIAChb binds to a Ca$^{2+}$ chelate column, it was of interest to study the effects of divalent cations on the CD spectra and on thermal denaturation. Three metals were tested, Ca$^{2+}$, Mg$^{2+}$, and Ni$^{2+}$. Final cation concentrations (as their chlorides) were 1 mM, whereas the proteins were used at 20–200 μM concentrations.

**RESULTS**

**Purification of IIAChb—**The gene encoding IIAChb was cloned into the pET21a overexpression vector using a polymerase chain reaction approach as described under “Experimental Procedures.” The protein was purified from *E. coli* BL21(DE3):AEI harboring pET:IIAChb. A deletion of Enzyme I was used to ensure that IIAChb was isolated in its unphosphorylated form. During purification of IIAChb, it eluted as a blue solution, i.e. complexed to Cu$^{2+}$, from the Cu$^{2+}$ chelating column. The Cu$^{2+}$ ion was removed by dialysis against buffer (typically, 20 mM sodium phosphate, pH 8.0) containing 1 mM EDTA and 0.1 mM DTT.

SDS-PAGE of the purified protein is shown in Fig. 1A. The protein migrates with an apparent molecular mass of 12–14 kDa, which agrees with a predicted molecular mass of 12.75 kDa from the gene sequence (7, 19). As we report elsewhere (3), the native protein is a homodimer.

The N-terminal amino acid sequence of the purified protein was obtained as described under “Experimental Procedures” and agreed with that predicted from the coding sequence, indicating that the protein is not processed during its expression (data not shown).

**Phosphorylation of IIAChb—**In the accompanying report (1) phosphorylation of (GlcNac)$_2$ and its analogue Me-TCB was reconstituted in *vitro* using a mixture of (purified) Enzyme I, HPr, IIAChb (for which IIAChb could not substitute), and membranes containing IICChb.

Direct measurement of the phosphorylation of IIAChb (and IIBChb) could be monitored using $^{32}\text{P}$-labeled proteins and autoradiography after SDS-PAGE (phospho-IIAChb migrates with IIAChb under these conditions). Native, nondenaturing gel electrophoresis was also used. These gels offered several advantages over SDS-PAGE. Fig. 1B shows that IIAChb is phosphorylated by HPr in the presence of Enzyme I and PEP. The Coomassie stain shows a light band corresponding to Enzyme I in each lane. Furthermore, Fig. 1B also shows that a shift in the gel mobility of IIAChb occurs when it is phosphorylated and that the phosphoproteins were stable in the native gels and were easily separated from the unphosphorylated proteins. Use of $^{32}\text{P}$-labeled HPr and autoradiography confirmed the identities of the phosphoproteins (data not shown). Omission of Enzyme I, HPr, or PEP yielded no phosphorylation of IIAChb.

The kinetics of phosphorylation of IIAChb are shown in Fig. 1C. The Chb proteins can be phosphorylated using either catalytic or stoichiometric quantities of Enzyme I and HPr. The degree of phosphorylation was assayed by determining the relative ratios of the differentially migrating forms of the stained proteins in the native gels (scanning densitometry).

**Stability of Phospho-IIAChb—**To systematically study the stability of the phosphorylated protein, it was purified using Superdex-75 gel filtration chromatography as described under “Experimental Procedures.” Gel filtration chromatography is capable of separating Enzyme I and HPr from IIAChb but cannot separate IIAChb from P-IIAChb. The phosphoprotein preparations were analyzed for the degree of phosphorylation using native gel electrophoresis. For the studies described below, preparations of phospho-IIAChb were used only where there was no detectable unphosphorylated protein (i.e., <5%). As reported in the accompanying paper, both IIAChb and phospho-
IIA\textsubscript{Chb} and the IIA\textsubscript{Lac} proteins (discussed below), phospho-Enzyme I or HPr or PEP were omitted. IIA\textsubscript{Chb} should be a phosphohistidinyl protein, with the phospho-IIA\textsubscript{Chb} was purified as described under "Experimental Procedures." Aliquots (50 μg) were measured by the filter binding assay described under "Experimental Procedures." The protein (5 and 10 μg, lanes 2 and 3, respectively) was analyzed by SDS-PAGE (16% polyacrylamide gel) and stained with Coomassie Brilliant Blue. B, phosphorylation of IIA\textsubscript{Chb}. Phosphorylation was assayed by native gel electrophoresis (no SDS, 16% polyacrylamide gel). Reaction mixtures (20 μl) were incubated at 37 °C for 1 h, and contained 25 mM sodium phosphate buffer, pH 8.0, 5 mM MgCl\textsubscript{2}, 0.1 μg of Enzyme I and 0.1 μg of HPr. Lane 1, IIA\textsubscript{Chb} (4 μg) without PEP; lane 2, IIA\textsubscript{Chb} (4 μg) + 10 mM PEP. The gel was stained using Coomassie Brilliant Blue. Phospho-IIA\textsubscript{Chb} could also be visualized in reaction mixtures containing \[^{32}P\]PEP by autoradiography (not shown). C, kinetics and requirements for phosphorylation of IIA\textsubscript{Chb}. Phosphorylation was measured by the filter binding assay described under "Experimental Procedures." Aliquots (50 μl) were taken over the indicated time course from reaction mixtures incubated at 37 °C containing 25 mM sodium phosphate buffer, pH 8.0, 5 mM MgCl\textsubscript{2}, 5 mM \[^{32}P\]PEP and the following proteins: •, IIA\textsubscript{Chb} (425 pmol), Enzyme I (4.5 pmol), HPr (4.5 pmol); ■, IIA\textsubscript{Chb} (425 pmol), Enzyme I (1.5 pmol), HPr (1.5 pmol); ○, Enzyme I or HPr or PEP were omitted.

IIA\textsubscript{Chb} are dimers (3). The corresponding monomers were not detected in the sedimention experiments. Phospho-IIA\textsubscript{Chb} can therefore contain (on the average) from 1 to 2 mol of phosphate/dimer. When it is phosphorylated under the conditions described above, 1.5–2.0 mols of phosphate were incorporated per dimer based on protein concentration determined by a micro-Kjeldahl procedure and the specific activity of the \[^{32}P\]PEP. From the amino acid sequence similarity/identity of IIA\textsubscript{Chb} and the IIA\textsubscript{Lac} proteins (discussed below), phospho-IIA\textsubscript{Chb} should be a phosphohistidinyl protein, with the phospho-IIA\textsubscript{Chb} are dimers (3). The corresponding monomers were not detected in the sedimentation experiments. Phospho-IIA\textsubscript{Chb} can therefore contain (on the average) from 1 to 2 mol of phosphate/dimer. When it is phosphorylated under the conditions described above, 1.5–2.0 mols of phosphate were incorporated per dimer based on protein concentration determined by a micro-Kjeldahl procedure and the specific activity of the \[^{32}P\]PEP. From the amino acid sequence similarity/identity of IIA\textsubscript{Chb} and the IIA\textsubscript{Lac} proteins (discussed below), phospho-IIA\textsubscript{Chb} should be a phosphohistidinyl protein, with the phospho-IIA\textsubscript{Chb} therefore compared but showed no detectable dif-

**Fig. 1.** Purity and phosphorylation of recombinant IIA\textsubscript{Chb}. A, SDS-PAGE. IIA\textsubscript{Chb} was purified as described under "Experimental Procedures," The protein (5 and 10 μg, lanes 2 and 3, respectively) was analyzed by SDS-PAGE (16% polyacrylamide gel) and stained with Coomassie Brilliant Blue. B, phosphorylation of IIA\textsubscript{Chb}. Phosphorylation was assayed by native gel electrophoresis (no SDS, 16% polyacrylamide gel). Reaction mixtures (20 μl) were incubated at 37 °C for 1 h, and contained 25 mM sodium phosphate buffer, pH 8.0, 5 mM MgCl\textsubscript{2}, 0.1 μg of Enzyme I and 0.1 μg of HPr. Lane 1, IIA\textsubscript{Chb} (4 μg) without PEP; lane 2, IIA\textsubscript{Chb} (4 μg) + 10 mM PEP. The gel was stained using Coomassie Brilliant Blue. Phospho-IIA\textsubscript{Chb} could also be visualized in reaction mixtures containing \[^{32}P\]PEP by autoradiography (not shown). C, kinetics and requirements for phosphorylation of IIA\textsubscript{Chb}. Phosphorylation was measured by the filter binding assay described under "Experimental Procedures." Aliquots (50 μl) were taken over the indicated time course from reaction mixtures incubated at 37 °C containing 25 mM sodium phosphate buffer, pH 8.0, 5 mM MgCl\textsubscript{2}, 5 mM \[^{32}P\]PEP and the following proteins: •, IIA\textsubscript{Chb} (425 pmol), Enzyme I (4.5 pmol), HPr (4.5 pmol); ■, IIA\textsubscript{Chb} (425 pmol), Enzyme I (1.5 pmol), HPr (1.5 pmol); ○, Enzyme I or HPr or PEP were omitted.

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**Fig. 2.** Effect of pH on rate of hydrolysis of phospho-IIA\textsubscript{Chb}. \[^{32}P\]-Labeled phospho-protein was purified by electroelution from a native gel or by gel filtration on a Superdex-75 FPLC column as described under "Experimental Procedures." Assays for hydrolysis were conducted at 25 °C by the DEAE-paper method. Rate constants were determined from logarithmic plots (phosphoprotein remaining versus time) as a function of pH in the following buffers: •, sodium acetate; ■, sodium chloride; □, Tris-HCl buffer; ○, McIlvaine's and Bates/Bowers'.

A characteristic feature of the phosphohistidinyl linkage in phosphorylated PTS proteins is the sensitivity of this bond to acid, hydroxylamine, and pyridine and its stability to alkali (20). Hydrolysis of \[^{32}P\]phospho-IIA\textsubscript{Chb} was therefore followed at 25 °C, and Fig. 2 gives the rate constants for hydrolysis (as \(t_{1/2}\) values) as a function of pH. The protein precipitated below pH 5, prohibiting accurate assessment at lower pH values. Fig. 2 shows that the phosphoryl linkage is stable at high pH. The rate was also determined at 37 °C at pH 8.0 in the presence and absence of hydroxylamine and pyridine (Fig. 3). The phosphoryl linkage is sensitive to hydroxylamine and pyridine, characteristic of a phosphoramide linkage.

**Effect of Temperature and Concentration on Hydrolysis Rates of Phospho-IIA\textsubscript{Chb} at pH 8.0**—For physico-chemical studies, it was important to determine the conditions of maximum stability of the phosphoprotein at or near physiological pH. Gel electrophoresis was therefore used to analyze the hydrolysis process for 24 h at different temperatures and at pH 8.0 and gave the following results (as a percentage of phosphoprotein remaining): 37 °C, 30–40%; 25 °C, 75%; 16 °C, >90%; 10 °C, stable. Unexpected results were obtained, however, when the experiments were conducted kinetically to obtain rate constants.

First order hydrolysis rate constants were determined at two temperatures, 25 and 37 °C, and the results are given in Table I. The rates, expressed as \(t_{1/2}\) were 1800 and 250 min, respectively. This 7-fold change seemed inordinately large for a 12 °C change in temperature.

The second unexpected finding was obtained when the hydrolysis rates were determined at different concentrations of phospho-IIA\textsubscript{Chb}. The results are also presented in Table I. As can be seen, at pH 8.0 and 37 °C, the rates of hydrolysis increase 4-fold as the phosphoprotein is diluted. The effects of temperature and concentration did not readily fit a pseudo first order reaction (the activity of water remains constant), and an explanation was sought.

**Fluorescence Studies**—The protein IIA\textsubscript{Chb} contains no Trp and only one tyrosine per monomer. The fluorescence excitation and emission spectra of the Tyr in phospho-IIA\textsubscript{Chb} and IIA\textsubscript{Chb} were therefore compared but showed no detectable dif-
of these experiments were conducted at temperatures of phospho-IIA Chb at 37 °C.

The rate of hydrolysis of purified 32P-labeled phospho-protein was determined by the DEAE-paper method. Samples (5–10 μM phospho-IIA-Chb), in 5 mM sodium carbonate buffer pH 8.0, were diluted 1:2 with concentrated buffer solutions adjusted to pH 8.0 with HCl, giving the following final concentrations: □, 0.4 M Tris-HCl buffer; □, 0.05 M pyridine; ▲, 0.15 M hydroxylamine. The samples were maintained at 37 °C, and at the indicated time, aliquots (20–50 μl) were removed and analyzed.

![Graph](image.png)

**Fig. 3.** Effect of pyridine and hydroxylamine on the hydrolysis of phospho-IIA-Chb at 37 °C. The rate of hydrolysis of purified 32P-labeled phospho-IIA-Chb, determined by the DEAE-paper method, was plotted as a function of time. Samples (5–10 μM phospho-IIA-Chb) were diluted 1:2 with concentrated buffer solutions adjusted to pH 8.0 with HCl, giving the following final concentrations: □, 0.4 M Tris-HCl buffer; □, 0.05 M pyridine; ▲, 0.15 M hydroxylamine. The samples were maintained at 37 °C, and at the indicated time, aliquots (20–50 μl) were removed and analyzed.

| Phosphoprotein | t_{1/2} | Reference |
|----------------|--------|-----------|
| E. coli phospho-HPr | ~13 | Refs. 29 and 30 |
| E. coli phospho-Enzyme 1 | 1400 | Refs. 29 and 30 |
| E. coli phospho-IIA-Chb | 116 | Refs. 29 and 30 |
| S. aureus phospho-IIA-Lac | 58 | Ref. 20 |
| E. coli phospho-IIA-Chb | These studies |

| Concentration (μM) | Time (min) |
|-------------------|------------|
| 10 μM, 25 °C | 1800 |
| 10 μM | 250 |
| 69 μM | 480 |
| 274 μM | 720 |
| 345 μM | 1080 |

* Rate constants were obtained from the progress curves for the pseudo first order hydrolysis reactions, and are presented as t_{1/2} values in minutes.

* Phospho-HPr is the only phosphoprotein shown where the phospho-ri boyl group is linked to the 61-N position of the His residue.

* The molar concentrations of the phosphoprotein were calculated based on the monomer molecular mass, 12,850 g/mol. Monomeric species were not detected in the equilibrium sedimentation experiments (3) with IIA-Chb and phospho-IIA-Chb. However, it should be stressed that all of these experiments were conducted at temperatures <25 °C. Temperatures such as 37 °C could not be used because of hydrolysis of the phosphoprotein over the prolonged times required for equilibrium sedimentation experiments.

* One experiment was conducted at 25 °C with IIA-Chb.

CD spectroscopy was then used to study thermal denaturation of the two proteins. Here, in contrast to the data discussed above, a dramatic difference was observed. The phosphoprotein was much more sensitive to thermal denaturation. Two types of experiments were conducted. In the first, the CD spectra are shown from 190 to 250 nm for IIA-Chb and phospho-IIA-Chb respectively. In the second, discussed below, thermal denaturation was followed at 220 nm.

**Fig. 4.** shows that the helical content of both proteins decreased as they unfolded, but phospho-IIA-Chb is much less stable than IIA-Chb. For example, at 45 °C there is only a slight decrease in the signal for IIA-Chb (Fig. 4B), whereas more than 50% of the helicity is lost from phospho-IIA-Chb (Fig. 4C). More pertinent to the hydrolysis results reported above, at 37 °C phospho-IIA-Chb exhibits about 35% less helicity than at 25 °C, whereas IIA-Chb was essentially unaffected.

**Fig. 5.** shows the effect of temperature on ellipticity at 220 nm. The temperature of a solution containing either IIA-Chb or phospho-IIA-Chb was slowly raised over the range 10–90 °C at pH 8.0, 25 mM phosphate buffer. Both IIA-Chb and phospho-IIA-Chb showed at least two transitions (Fig. 5A). The thermal denaturation of IIA-Chb was reversible. Likewise, a second temperature scan of phospho-IIA-Chb was identical to the first if the phosphoprotein was heated to the end point of the first transition (65 °C) but not higher. Less than 10% of the protein was dephosphorylated during the thermal denaturation (data not shown). Continued repeat scans of phospho-IIA-Chb resulted in ellipticity curves intermediate between the original phosphoprotein and the native protein, eventually resulting in a curve superimposable on the nonphosphorylated protein (data not shown). Native gel electrophoretic analysis revealed a gradual increase in the fraction of dephosphoprotein until eventually the protein was completely dephosphorylated. These results indicate that the thermal denaturation resulting over the course of the first transition is reversible, both for IIA-Chb and phospho-IIA-Chb.

**TABLE 1**

| Phosphoprotein | t_{1/2} | Reference |
|----------------|--------|-----------|
| E. coli phospho-HPr | ~13 | Refs. 29 and 30 |
| E. coli phospho-Enzyme 1 | 1400 | Refs. 29 and 30 |
| E. coli phospho-IIA-Chb | 116 | Refs. 29 and 30 |
| S. aureus phospho-IIA-Lac | 58 | Ref. 20 |
| E. coli phospho-IIA-Chb | These studies |

**Effect of Divalent Cations on Thermal Denaturation of IIA-Chb and Phospho-IIA-Chb**—The results described above showed that the t_{1/2} of hydrolysis of the phosphoprotein changed with protein concentration. As a consequence, the effects of protein concentration on the thermal denaturation of IIA-Chb and phospho-IIA-Chb were examined, and the results are presented in Fig. 6. As the concentration decreases from 150 to 10 μM the T_{m} of IIA-Chb changes slightly, from about 56 to 54 °C. By contrast, there is a large change in the T_{m} of phospho-IIA-Chb from about 45 to 34 °C. These results are discussed below.
Fig. 4. Circular dichroic spectra of IIA$^{Chb}$ and phospho-IIA$^{Chb}$. A, CD spectra at 25 °C. The spectra of IIA$^{Chb}$ (50 μM, solid line) and (purified) phospho-IIA$^{Chb}$ (40 μM, dashed line) in 25 mM phosphate buffer, pH 8.0, were determined at 25 °C. The data are presented in terms of the mean residue ellipticity. B, effect of temperature on CD spectrum of IIA$^{Chb}$. The temperature was increased from 15 to 90 °C at a rate of 1 °C/min, and the spectra of IIA$^{Chb}$ (150 μM) were recorded at the indicated temperatures, and also at 90 °C in the presence of 4 M guanidinium-HCl. C, effect of temperature on CD spectrum of phospho-IIA$^{Chb}$. The spectra of purified phospho-IIA$^{Chb}$ (160 μM) were recorded at the indicated temperatures.
column with an imidazole gradient, but the solution was blue, indicating that IIA\(\text{Chb}\) binds Cu\(^{2+}\) with high affinity; the copper was removed by dialysis against EDTA. The effects of the divalent cations Mg\(^{2+}\), Cu\(^{2+}\), and Ni\(^{2+}\) on the thermal denaturation of IIA\(\text{Chb}\) and phospho-IIA\(\text{Chb}\) was studied because of the affinity of the protein for Cu\(^{2+}\) and because Mg\(^{2+}\) has been found in the crystal structure of a similar protein, IIA\(\text{Lac}\). The Mg\(^{2+}\) was bound to Asp81 in the core of the protein between its subunits (10). Control experiments (data not shown) established that 2 mM EDTA had no effect on the thermal denaturation curves of IIA\(\text{Chb}\) and phospho-IIA\(\text{Chb}\) and that EDTA completely reversed the divalent cation effects discussed below including the marked effects on thermal denaturation of the proteins.

The results are presented in Figs. 7 (IIA\(\text{Chb}\)) and 8 (phospho-IIA\(\text{Chb}\)), containing three panels in each. Panel A is the wavelength scan from 190 to 250 nm. Panel B is the thermal denaturation experiment, followed at 220 nm. Panel C consists of the data in Panel B normalized for the first transition.

Fig. 7 and Table II show that there is a noticeable increase in stability of 20 \(\mu\)M IIA\(\text{Chb}\) in the presence of 1 mM Mg\(^{2+}\), but the effects of 1 mM Cu\(^{2+}\) and Ni\(^{2+}\) were entirely unexpected. The \(T_\text{m}\) values increased as follows (1 mM chloride salts): Mg\(^{2+}\), 2.75 °C; Cu\(^{2+}\), 15.75 °C; Ni\(^{2+}\), 29.25 °C. Mg\(^{2+}\) is considered in detail below. Cu\(^{2+}\) and Ni\(^{2+}\) were also tested over the concentration range 20 \(\mu\)M to 1 mM using 20 \(\mu\)M IIA\(\text{Chb}\). An effect was observed on the CD spectrum at the lowest concentration, and the full effect was found at 100 \(\mu\)M concentrations of the ions (data not shown).

Thermal unfolding experiments (Fig. 8, B and C) were also conducted with phospho-IIA\(\text{Chb}\) in the presence of the ions. In view of the effects of protein concentration on stability of the phosphoprotein, the ion studies were conducted at 22 and 150 \(\mu\)M phospho-IIA\(\text{Chb}\). Mg\(^{2+}\) (Fig. 8 and Table II) showed a greater effect with phospho-IIA\(\text{Chb}\) than with IIA\(\text{Chb}\). With II-A\(\text{Chb}\), the \(T_\text{m}\) increased about 2 °C in the presence of Mg\(^{2+}\). By contrast, at each phospho-IIA\(\text{Chb}\) concentration tested, Mg\(^{2+}\) increased the \(T_\text{m}\) by 9 °C. Interestingly, at the high phospho-
IIA^{Chb} concentration, Mg^{2+} brought the $T_m$ to 55 °C, about the same value as obtained with IIA^{Chb} ± Mg^{2+}. Cu^{2+} and Ni^{2+} gave complex results: (a) ions appeared to decrease the helicity of the protein at room temperature; (b) the thermal unfolding process appears to proceed via several intermediates with two or three temperature transitions observed.

**DISCUSSION**

(GlcNAc)$_2$ is a PTS sugar in *E. coli* (1). The chb or chitobiase operon contains three genes originally thought to be required for the uptake and phosphorylation of cellobiose but now known to encode the three (GlcNAc)$_2$-specific PTS transport proteins, IIA^{Chb}, IIB^{Chb}, and IIC^{Chb}, respectively (7). There appear to be no published reports on the properties of IIA^{Chb} nor on the respective phosphoprotein.

The phosphoryl group in phospho-IIA^{Chb} exhibits properties of a typical e2-N (i.e. N-3) phospho-His derivative with respect to akali stability, acibility, and sensitivity to hydroxylamine and pyridine (Figs. 2 and 3). A plot of the first order rate constants as a function of pH also indicate that the phosphoryl group is linked to e2-N (i.e. N-3) of the imidazole His ring rather than to the δ1-N (i.e. N-1), as in phospho-HPr. Table I compares the rate constants for the hydrolysis of some phospho-PTS proteins with phospho-IIA^{Chb} at pH 8.0, 37 °C. The most interesting comparison is between *E. coli* phospho-IIA^{Chb} and *Staphylococcus aureus* phospho-IIA$^{lac}$ because of their sequence and structural similarities. The $t_{1/2}$ for phospho-IIA$^{lac}$ (called III$^{lac}$ in Ref. 20) is 58 min, whereas the corresponding values for phospho-IIA$^{Chb}$ varied from 250 to 1080 min, depending upon protein concentration. It was this concentration effect that led to the experiments discussed below.

IIA$^{Chb}$ (previously IIA$^{Cel}$) is a member of the lactose/cellobiose PTS Enzyme II permeases. There is considerable amino acid sequence identity/similarity within this group of proteins (8, 10). For example, IIA$^{Chb}$ shows 33% identity in its sequence to the *S. aureus* IIA$^{lac}$ protein, and 35% to the homologous protein from *Lactococcus lactis*. The latter has been crystallized, and its structure has been determined (10). The structures of the protein monomers also show similarity. *L. lactis* IIA$^{lac}$ is 83% α helix, whereas the CD spectrum indicates that IIA$^{Chb}$ is from 75–85% helix.

There are also marked differences between *E. coli* IIA$^{Chb}$ and the IIA$^{lac}$ proteins of *S. aureus* and *L. lactis*. For one, both IIA$^{lac}$ proteins form stable trimers (10, 20), whereas IIA$^{Chb}$ forms a very stable dimer. Second, phosphorylation of *S. aureus* IIA$^{lac}$ trimer is thought to result in dissociation to phospho-IIA$^{lac}$ monomers (21), but no monomer of either IIA$^{Chb}$ or phospho-IIA$^{Chb}$ was detected in the analytical sedimentation experiments. Third, phosphorylation has only a small effect on the CD spectrum of IIA$^{Chb}$ at pH 8.0 and 25 °C (Fig. 4A), whereas there was a major change in the structure of phospho-IIA$^{lac}$ as judged by relative reactivity to antibodies.

Nevertheless, phosphorylation of IIA$^{Chb}$ has a profound effect on the structure of the protein. The most obvious change was observed on thermal denaturation, where phospho-IIA$^{Chb}$ was found to have a $T_m$ 15 °C lower than the unphosphorylated protein. Thus, phosphorylation significantly destabilizes II-A$^{Chb}$. Below 25 °C, at pH 8.0, the CD spectra of IIA$^{Chb}$ and phospho-IIA$^{Chb}$ are very similar. At 37 °C, there is a minimal change in the spectrum of IIA$^{Chb}$, but there is a striking change in the spectrum of phospho-IIA$^{Chb}$. At this temperature, there is a loss of close to 35% of the helicity of the phosphoprotein. At the same time and under the same conditions, there is a large change in the rate constant for the hydrolysis of phospho-IIA$^{Chb}$ (Table I). The $t_{1/2}$ is 1800 min at 25 °C and 250 min at 37 °C, a substantially greater effect than expected for a 12 °C change in temperature.

A second set of data required explanation. The rate constants for the hydrolysis of phospho-IIA$^{Chb}$ varied with protein concentration at 37 °C (Table I). The same result was obtained in the thermal unfolding experiments, where the $T_m$ of phospho-IIA$^{Chb}$ changed significantly with concentration, whereas there was only a slight change with IIA$^{Chb}$ (Fig. 6 and Table II).

We interpret these results as follows: (a) At pH 8.0, in the temperature interval 25–37 °C, there is little change in the structure of IIA$^{Chb}$ but a profound change in the structure of phospho-IIA$^{Chb}$, leading to a loss of stability of the phosphoryl linkage in the phosphoprotein. (b) The effects of protein concentration on both the $T_m$ and the $t_{1/2}$ of hydrolysis of phospho-IIA$^{Chb}$ reflect a change in the monomer/dimer transition of the phosphoprotein relative to IIA$^{Chb}$. In monomer/dimer transitions of this type, the fraction of monomer/dimer increases significantly as the absolute concentration is decreased, and the data are explained if the phosphomonomer is less stable than the phosphodimer. The monomer/dimer transition has a $K_{dissoc} \leq 10^{-7}$ M at 25 °C, as deduced from the sedimentation experiments (3). Using this $K_{dissoc}$ value, one can calculate that
FIG. 7. Effect of divalent metal ions and temperature on the ellipticity and spectrum of IIAChb. A, the mean residue ellipticity of IIAChb (20 μM) was determined at 220 nm. The protein was dissolved in 25 mM sodium phosphate buffer, pH 8.0, and the solution was supplemented with 1 mM MgCl₂, CuCl₂, or NiCl₂. The ellipticity of each sample was determined by changing the temperature over the range 10–90 °C at a rate of 1 °C/min. B, the data in A are normalized for the first transition. The mid-point temperatures (T_m) derived from the figure are presented in Table II. C, the spectra of IIAChb at 25 °C in the presence of divalent metal ions.
FIG. 8. Effect of divalent metal ions and temperature on the ellipticity of phospho-IIA\textsuperscript{Chb}. The procedures described in Fig. 7 for measuring the ellipticity of IIA\textsuperscript{Chb} were applied to the phosphoprotein. A, mean residue ellipticity at 220 nm of purified phospho-IIA\textsuperscript{Chb} (22 μM). B, data in A normalized for the first transition. The mid-point temperatures (T\textsubscript{m}) derived from the figure are presented in Table II. C, The spectra of phospho-IIA\textsuperscript{Chb} at 25 °C in the presence of divalent metal ions.
Table II

| Additions (1 mM) | IIAChb (20 μM) | Phospho-IIAChb (22 μM) | Phospho-IIAChb (150 μM) |
|-----------------|----------------|------------------------|-------------------------|
| None            | 54.5°C         | 40.5°C                  | 46°C                    |
| EDTA            | 54.5°C         | 40.5°C                  | 55°C                    |
| MgCl₂           | 7.0°C          | 32°C                    | 36°C                    |
| CuCl₂           | 83.5°C         | 31°C                    | 56°C                    |
| NiCl₂           | 83.5°C         | 31°C                    | 56°C                    |

Mid-point temperature (Tm) of the first transition during thermal denaturation of IIAChb and phospho-IIAChb.

Data were collected as described in Figs. 4–8.

at 1 mM total protein, the monomer would constitute 1% of the total, whereas at 1 μM protein, the monomer would comprise 27% of the mixture.

Finally, the structure of IIAChb was surprisingly affected by certain divalent cations (Figs. 7 and Table II). In the presence of 1 mM divalent cation, the Tm increased 3°C in Mg²⁺, about 16°C in Cu²⁺, and 29°C in Ni²⁺. Mg²⁺ increased the Tm of phospho-IIAChb by 9°C. We do not know whether these cations have a stabilizing effect on the monomers or on the dimers or both nor the mechanism for these large effects. But it is important to note that Mg²⁺, for example, increases the stability of phospho-IIAChb to accelerate its interaction with or insertion into the membrane sugar receptor, IIBChb. It is conceivable, for instance, that phosphoryltransfer from phospho-IIAChb to IIBChb to the (GlcNAc)₂/IICChb complex proceeds as a quaterary complex of the three proteins and the sugar and not as usually visualized as a separate sequence of bimolecular reactions. If this is true, then it may be important to destabilize phospho-IIAChb to stabilize its interaction with or insertion into the membrane. Appropriate kinetic studies are in progress to test these ideas. The effect of Mg²⁺ on the kinetics will also be measured because Mg²⁺ appears to stabilize phospho-IIAChb.

Is destabilization of the active site domain a prerequisite for rapid phosphoryltransfer between proteins? Both the crystal and NMR structures of an analogous PTS protein, IIAGlc (also called IIIGlc), are well established, as is the NMR structure of phospho-IIA-Glc (22–28). NMR studies were conducted at 36.5°C, and some chemical shifts were found in phospho-IIA-Glc, but only in four dipetide segments around the active site His. There were no significant structural changes in large segments of the protein as is reported here for phospho-IIAChb or as has been reported for phospho-IIALac. It remains to be seen whether the structural effects observed upon phosphorylation of the monomeric IIA-Glc or the dimeric IIAChb (and trimetric IIA-Lac) best exemplifies the IIA proteins as a group.

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