The phosphoenolpyruvate-glycose transferase system (PTS) is a prototypic signaling system responsible for the vectorial uptake and phosphorylation of carbohydrate substrates. The accompanying papers describe the proteins and product of the Escherichia coli N,N-diacytelchitobiose ((GlcNAc)₂) PTS-mediated permease. Unlike most PTS transporters, the Chb system is composed of two soluble proteins, IIA\textsubscript{Chb} and IIB\textsubscript{Chb}, and one transmembrane receptor (IIC\textsubscript{Chb}). The oligomeric states of PTS permease proteins and phosphoproteins have been difficult to determine. Using analytical ultracentrifugation, both dephospho and phosphorylated IIA\textsubscript{Chb} are shown to exist as stable dimers, whereas IIB\textsubscript{Chb}, phospho-IIB\textsubscript{Chb} and the mutant Cys10SerIIB\textsubscript{Chb} are monomers. The mutant protein Cys10SerIIB\textsubscript{Chb} is unable to accept phosphate from phospho-IIB\textsubscript{Chb} but forms a stable higher order complex with phospho-IIA\textsubscript{Chb} (but not with dephospho-IIA\textsubscript{Chb}). The stoichiometry of proteins in the purified complex was determined to be 1:1, indicating that two molecules of Cys10SerIIB\textsubscript{Chb} are associated with one phospho-IIA\textsubscript{Chb} dimer in the complex. The complex appears to be a transition state analogue in the phosphotransfer reaction between the proteins. A model is presented that describes the concerted assembly and disassembly of IIA\textsubscript{Chb}-IIB\textsubscript{Chb} complexes contingent on phosphorylation-dependent conformational changes, especially of IIA\textsubscript{Chb}.

The phosphoenolpyruvate-glycose phosphotransferase system (PTS) consists of two soluble general proteins, Enzyme I and HPr, required for the uptake of all PTS sugars. These proteins are coupled to substrate-specific permeases or transporters that are ultimately responsible for the concomitant uptake and phosphorylation of carbohydrate substrates. Phosphorylation proceeds sequentially, beginning with the auto-phosphorylation of Enzyme I by phosphoenolpyruvate. A frequent sequence is as follows: phospho-Enzyme I donates its phosphate to HPr, phospho-HPr to sugar-specific IIA proteins, phospho-IIA to IIB, and phospho-IIB in conjunction with the membrane receptor, IIC, phosphorylates and transports the substrate. The structure and oligomeric state(s) of both Enzyme I and HPr have been extensively studied (for reviews see Refs. 6–8). Several sugar-specific PTS transporters have also been well characterized, including the Escherichia coli glucose and mannitol transporters. To date, however, there have been few reports on the oligomeric states of the sugar-specific proteins, particularly in their phosphorylated states. Furthermore, although phosphate is transferred from one protein to another as summarized above, there have been no reports describing the isolation of a transition state intermediate or stable complex between two reacting PTS proteins.

In the accompanying papers (1–3), we characterize the transport kinetics, product, and two of the sugar-specific transport proteins of the (GlcNAc)₂ (N,N'-diacytelchitobiose) or chb catabolic operon of E. coli. The phosphoryl transfer reaction sequence resulting in the uptake of (GlcNAc)₂ was studied, and the soluble proteins IIA\textsubscript{Chb}, IIB\textsubscript{Chb}, and an active site mutant (C10S), of IIB\textsubscript{Chb} were purified to apparent homogeneity. A phosphoryl group is transferred from phospho-HPr to IIA\textsubscript{Chb} and from phospho-IIA\textsubscript{Chb} to IIB\textsubscript{Chb}. Phospho-IIA\textsubscript{Chb} was found to be 5–10-fold more stable than homologous phosphate-IIA proteins. This stability, as well as that of phospho-IIB\textsubscript{Chb}, enabled us to use analytical ultracentrifugation to determine the oligomeric states of IIA\textsubscript{Chb} and IIB\textsubscript{Chb} in both their unphosphorylated and phosphorylated forms and of an analogue of a potential transition state intermediate in the phosphotransfer reaction between the proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Buffers and reagents were of the highest purity available. The solvent used in all analytical ultracentrifuge experiments was 25 mM sodium phosphate buffer, pH 8, unless otherwise specified. The values of solvent density and viscosity were calculated from composition as described in Laue et al. (9) using either the program SEDNTERP or Ultrasan.

**Protein Samples**—Purified proteins IIA\textsubscript{Chb}, IIB\textsubscript{Chb}, and Cys10SerIIA\textsubscript{Chb}, their respective phosphorylated forms, and phospho-IIA\textsubscript{Chb}-Cys10SerIIB\textsubscript{Chb} complex were prepared as described in the accompanying reports (2, 3). Native gel and SDS-denaturing polyacryl

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amide gel electrophoresis were performed using standard protocols. Protein bands were visualized using Coomassie Brilliant Blue and where indicated quantitated by densitometric scanning using the Eagle Eye II Still Video System and software (Stratagene). The partial specific volume (\(\gamma\)) of each sample was estimated from the amino acid sequence using the method of Cohn and Edsall (10) as implemented in the program SEDNTERP (9).

Data Acquisition and Analysis—Ultracentrifugation experiments were performed in a Beckman XL-I analytical ultracentrifuge. All samples prepared for analytical ultracentrifugation experiments were in 25 mM sodium phosphate buffer, pH 8.0, unless otherwise noted. Samples were either dialyzed against the buffer prior to experiments or equilibrated with the buffer using gel filtration chromatography.

Velocity runs were conducted at either 4 or 20 °C at 45,000 rpm in an An-60 rotor. Data scans were recorded continuously throughout the experiment using the absorption optical system at either 280 or 230 nm. Protein concentrations were adjusted to about 0.5 absorbance at the detection wavelength. Sample cells with 12-mm double sector charcoal filled epon centerpieces and quartz windows were used for all velocity runs. All data were analyzed using the method of van Holde and Weischet (11, 12) as implemented in the program Ultrascan. Following preliminary analysis, data were further fitted using the finite element method of Demeler and Saber (13) also as implemented in Ultrascan.

Equilibrium runs were conducted at 20 °C using either standard cells or short column eight channel cells equipped with sapphire windows. Several loading concentrations and several speeds were used for each sample. Data were collected using the absorption optical system at either 280 or 230 nm for runs in standard cells and with the interference optical system for short column runs. All sedimentation equilibrium data were analyzed using the program NONLIN (14).

RESULTS

Velocity Sedimentation Analyses of IIA\(^{Chb}\), Phospho-IIA\(^{Chb}\), IIB\(^{Chb}\), Phospho-IIB\(^{Chb}\), and Cys10SerIIB\(^{Chb}\)—Samples for velocity sedimentation were prepared as described under "Experimental Procedures." Phosphorylated forms of the indicated proteins were used immediately after preparation. Phosphorylation was monitored both before and after centrifuge runs using the gel shift mobility assay (2). Total phosphorylation was at least 95% in each sample, and unless otherwise specified, less than 10% dephosphorylation occurred during the time of the run.

Sedimentation velocity data on IIA\(^{Chb}\), phospho-IIA\(^{Chb}\), IIB\(^{Chb}\), phospho-IIB\(^{Chb}\), and the active site mutant Cys10SerIIB\(^{Chb}\) were collected at 20 °C, and analyzed by the method of van Holde and Weischet (11, 12). For each protein, an extrapolation plot of \(S_{apparent} \times V \times \sqrt{I} \times \gamma\) across the boundary converged to a single point indicating homogeneity of the samples. Integral distribution plots, \(G(s)\), of the diffusion-corrected sedimentation coefficient versus boundary fraction are shown in Fig. 1 for all five samples. IIB\(^{Chb}\) and Cys10SerIIB\(^{Chb}\) show essentially identical sedimentation behavior, indicating that there is no significant hydrodynamic change introduced by the mutation. Phosphorylation of IIB\(^{Chb}\) leads to a 20% increase in \(s_{20,w}\) for IIA\(^{Chb}\) has a much higher \(s_{20,w}\) than IIB\(^{Chb}\), and in contrast to IIB\(^{Chb}\), phosphorylation of IIA\(^{Chb}\) results in a 15% decrease in the sedimentation coefficient.

To derive the hydrodynamic parameters for each protein and better understand the implications of these changes in \(s_{20,w}\), all data were fitted directly to the Lamm equation using the finite element fitting method as implemented in the program Ultrascan. A summary of the fitted sedimentation and diffusion coefficients, calculated molecular weights, hydrodynamic radii, and frictional coefficients are given in Table I. Given that the known molecular weight of the IIA\(^{Chb}\) dimer is about 25,496, the results indicate that IIA\(^{Chb}\) is a stable dimer. Because no monomeric species were detected, the upper limit of the \(K\) is 10\(^{-7}\) M (15). Additionally, no monomeric species were detected after phosphorylation of IIA\(^{Chb}\), even though the \(s_{20,w}\) value for phospho-IIA\(^{Chb}\) was 10–15% lower than for the unphosphorylated protein. This observation was consistent and reproducible and suggests that phosphorylation of IIA\(^{Chb}\) induces a more elongated conformation. Furthermore, samples that were cycled between the phosphorylated and dephosphorylated states showed a reproducible shift between the corresponding low and high \(s\) values when analyzed by sedimentation velocity centrifugation (data not shown).

Possible nonideality effects because of the low ionic strength of the solvent (25 mM NaPO\(_4\), pH 8.0) were investigated by repeating the experiments in the same buffer containing 0.1 M NaCl. Similar results as those summarized in Table I were obtained. Although there was a slight change in the calculated \(s\) value for each species, the \(s_{20,w}\) value for IIA\(^{Chb}\) dropped 10–15% upon phosphorylation, and finite element fitting indicated that both species are stable dimers (data not shown). The estimated \(s_{20,w}\) values were also found to be dependent on the temperature of the experiment. For runs carried out at 4 °C, \(s_{20,w}\) for IIA\(^{Chb}\) and phospho-IIA\(^{Chb}\) were found to be nearly identical (Table I), whereas they were different at 20 °C. Here again, fitting of the data showed both species to be dimeric.

In contrast to the results obtained with IIA\(^{Chb}\) and its phospho derivative, a comparison of the known molecular masses with those obtained by finite element fitting of the velocity sedimentation data indicated that IIB\(^{Chb}\), phospho-IIB\(^{Chb}\), and the mutant Cys10SerIIB\(^{Chb}\) sediment as monomers under these conditions.

Equilibrium Sedimentation Analysis of IIA\(^{Chb}\), IIB\(^{Chb}\), and Cys10SerIIB\(^{Chb}\)—Equilibrium sedimentation data were collected for IIA\(^{Chb}\), IIB\(^{Chb}\), and Cys10SerIIB\(^{Chb}\) in standard double sector cells with a 3-mm column (Fig. 2). Samples of these proteins were brought to equilibrium at three initial loading concentrations and three speeds (for IIA\(^{Chb}\) or five speeds (for IIB\(^{Chb}\) and Cys10SerIIB\(^{Chb}\)). The data were analyzed by global
fitting to a single ideal species model, and the results are summarized in Table II. In close agreement with the velocity sedimentation results, sedimentation equilibrium data indicate that IIAChb exists as a dimer, whereas both IIBChb and Cys10SerIIBChb are monomers at the concentrations used in these experiments.

Short Column Equilibrium Sedimentation Analysis of IIAChb and Phospho-IIAChb—Using a standard column height, equilibrium sedimentation data could not be gathered for the phosphorylated form of IIAChb because of the lability of the phosphate group during the several days required for a typical equilibrium run. To circumvent this limitation, we conducted short column equilibrium studies with both IIAChb and phospho-IIAChb. This permitted equilibrium data to be obtained at three speeds, using four loading concentrations each, in less than 5 h. The data were globally fitted to a single ideal species model using the program NONLIN, and results are summarized in Table II. Results with IIAChb are fully consistent with the standard sedimentation equilibrium results described above. Similarly, short column data with phospho-IIAChb are well described by a single ideal species model with a molecular weight indicative of a dimer.

Velocity Sedimentation Analysis of Mixtures of IIAChb and IIBChb—Attempts were made to determine whether complexes could be detected in mixtures of IIAChb and IIBChb and/or their phospho derivatives. Equimolar quantities of the protein pairs were mixed and maintained at room temperature until loaded into the centrifuge (30–60 min). Under these conditions, phospho-transfer can occur between the proteins, until presumably an equilibrium is reached.

The following mixtures were tested: IIAChb and IIBChb, phospho-IIAChb and IIBChb, IIAChb and phospho-IIBChb, and Cys10SerIIBChb. Extrapolation plots indicated that each data set was composed of a mixture of the two components with s values similar to those of the individual protein species. Diffusion corrected integral distribution plots for various mixtures are presented in Fig. 3. Finite element fitting of the data to a noninteracting two species model in each case gave sedimentation coefficients consistent with those of the individual proteins. Thus, we concluded that none of the protein pairs tested formed significant concentrations of stable higher order complexes, i.e. complexes detectable by analytical sedimentation.

In sharp contrast to the results obtained above, when a sample containing the mutant Cys10SerIIBChb, which cannot accept the phosphoryl group, was incubated with phospho-IIAChb, a higher order complex of approximately 4.2 s was observed (Fig. 3). As indicated above, this species was not detected in the mixture of Cys10SerIIBChb and (unphosphorylated) IIAChb. These results indicate that a complex between phospho-IIAChb and IIBChb is stabilized under conditions where phosphotransfer cannot occur.

Short Column Equilibrium Sedimentation Analysis of Phospho-IIAChb and Cys10SerIIBChb—To verify the sedimentation velocity results above and to determine the molecular weight of the complex, it was desirable to obtain equilibrium data on the mixture of phospho-IIAChb and Cys10SerIIBChb. The instability of the phosphate group in the phospho-IIAChb, even at lowered temperatures, precluded standard equilibrium experiments because of the time required to make the measurements. It is, however, possible to gather data on the complex using a short solution column. Short column data on a stoichiometric mixture of phospho-IIAChb and Cys10SerIIBChb were obtained at four initial loading concentrations and two speeds in about 5 h (Fig. 4). These data were fitted to a single ideal species model and yielded an estimate on the molecular weight of the complex of 55,600. The best fit lines and combined residuals for the fit versus both the dependent and independent variable are also presented. Attempts to fit these data to more complex models yielded no significant improvement in the fit.

Stoichiometry of Phospho-IIAChb and Cys10SerIIBChb in the Complex—Although the velocity centrifugation data clearly showed the formation of a complex between phospho-IIAChb and Cys10SerIIBChb, the estimated molecular weights did not allow discrimination between a ternary and a quaternary complex. That is, it could be either phospho-IIAChb dimer and 1 mol of Cys10SerIIBChb (molar mass, 36 kDa) or phospho-IIAChb dimer and 2 mol of Cys10SerIIBChb (molar mass, 48 kDa). The short column equilibrium data gave a molecular weight 55,631 (± 1870) consistent with a complex of one phospho-IIAChb dimer and either two IIBChb monomers (48,000) or possibly three IIBChb monomers (60,000).

To independently determine the stoichiometry of the proteins in the complex, the purified complex was subjected to denaturing SDS-polyacrylamide gel electrophoresis, and protein bands were quantitated and compared with known amounts of each individual protein as standards (3). The results showed that the complex was composed of equimolar quantities of phospho-IIAChb monomer and the mutant protein Cys10SerIIBChb. We therefore concluded that the complex is a tetramer, composed of 1 mol of a dimer of phospho-IIAChb and 2 mol of Cys10SerIIBChb.

**DISCUSSION**

There are only a few studies on the hydrodynamic properties of the sugar-specific PTS proteins, and insofar as we know, none on the corresponding phosphoproteins (6, 7). In the present experiments, analytical sedimentation, both velocity and

| Sample           | \(x_{20, w}\) | \(D_{20, w}\) | \(M_w\, app\) | \(R_{h, obs}\) | \(f/f_0\) | Temperature |
|------------------|--------------|--------------|---------------|---------------|----------|------------|
| IIAChb           | 2.00         | 8.04         | 23270         | 19.0          | 1.40     | 4.0        |
| IIBChb           | 2.31         | 7.82         | 27655         | 20.1          | 1.36     | 20.1       |
| phospho-IIAChb   | 2.03         | 7.85         | 24258         | 19.2          | 1.42     | 4.0        |
| phospho-IIBChb   | 1.99         | 7.54         | 24770         | 19.4          | 1.47     | 20.1       |
| IIBChb           | 1.39         | 12.67        | 10983         | 14.9          | 1.14     | 4.0        |
| IIBChb           | 1.33         | 12.69        | 10477         | 16.8          | 1.15     | 20.0       |
| phospho-IIBChb   | 1.41         | 10.06        | 10453         | 16.2          | 1.32     | 4.0        |
| Cys10SerIIBChb   | 1.30         | 11.96        | 10924         | 17.8          | 1.20     | 20.0       |
equilibrium, were used to characterize the following proteins of the \(N_N\)-diacetylchitobiose transport system: IIA\(^{\text{Chb}}\), phospho-IIA\(^{\text{Chb}}\), IIB\(^{\text{Chb}}\), phospho-IIB\(^{\text{Chb}}\), a mutant Cys10SerIIB\(^{\text{Chb}}\), and an apparent complex between phospho-IIA\(^{\text{Chb}}\) and the mutant protein. The latter is a likely candidate as a transition state analogue in the phosphotransfer reaction between IIA\(^{\text{Chb}}\) and IIB\(^{\text{Chb}}\).

IIA\(^{\text{Chb}}\) has considerable amino acid sequence similarity to IIA\(^{\text{Lac}}\), part of the lactose PTS permease in Gram-positive organisms; IIA\(^{\text{Chb}}\) is 33% identical to IIA\(^{\text{Lac}}\) of \textit{Staphylococcus aureus} and 35% identical to the same protein from \textit{Lactococcus lactis} (7, 16). The crystal structure of the latter protein reveals that it is 83% \(\alpha\)-helix (16), whereas in the accompanying paper (2) we report that IIA\(^{\text{Chb}}\) is 75–85% helix. Nevertheless, IIA\(^{\text{Chb}}\) is very different from IIA\(^{\text{Lac}}\). Sedimentation equilibrium experiments (17) clearly established that IIA\(^{\text{Lac}}\) forms a stable trimer, whereas the results reported here show that IIA\(^{\text{Chb}}\) forms a very stable dimer. Because no monomer was detected, the dissociation constant for the dimer would have to be less than \(10^{-7}\) M (15).

Further, there are other major differences between phospho-IIA\(^{\text{Lac}}\) and phospho-IIA\(^{\text{Chb}}\). IIA\(^{\text{Lac}}\) is thought to dissociate when it is phosphorylated (18). No monomeric phospho-IIA\(^{\text{Chb}}\) was detected in the sedimentation experiments reported here. Although they have the same molecular weight, the sedimentation coefficient of phospho-IIA\(^{\text{Chb}}\) was 10–15% less than that of IIA\(^{\text{Chb}}\), suggesting that phosphorylation yielded a significantly less compact protein. These results agree with those obtained from the CD spectra, where it appeared that at 37 °C, phospho-IIA\(^{\text{Chb}}\) loses \(\sim 35\%\) of the helicity of IIA\(^{\text{Chb}}\) (2) and was much more sensitive to thermal denaturation. The effects of protein concentration both on the stability to hydrolysis of the phosphoprotein and on its thermal denaturation suggested that the phosphodimer dissociates to phosphomonomer, but this effect was only apparent at 37 °C and above. The sedimentation experiments were conducted at temperatures \(\sim 20^\circ\text{C}\) because significant hydrolysis of the phosphoprotein does occur over prolonged periods at the higher temperatures. The sedimentation velocity data indicate a change in \(s_{20,\text{w}}\) for IIA\(^{\text{Chb}}\) but not for phospho-IIA\(^{\text{Chb}}\) as a function of temperature (Table I). Because no change in extent of dimerization is observed, this result suggests an important change in shape and/or hydration with temperature. We are currently investigating this observation in greater detail.

The solution and crystal structures of the mutant protein Cys10SerIIB\(^{\text{Chb}}\) have been described (19, 20), but there are no reports on the properties of IIB\(^{\text{Chb}}\) nor phospho-IIB\(^{\text{Chb}}\). The sedimentation results presented here show that: (a) IIB\(^{\text{Chb}}\), phospho-IIB\(^{\text{Chb}}\), and Cys10SerIIB\(^{\text{Chb}}\) each behaves as a single monomeric species. (b) IIB\(^{\text{Chb}}\) and the mutant Cys10SerIIB\(^{\text{Chb}}\) exhibited identical sedimentation behavior, therefore suggesting that this mutation causes no significant change in structure of each of the unphosphorylated proteins. Results are presented for IIA\(^{\text{Chb}}\) (A), IIB\(^{\text{Chb}}\) (B), and Cys10SerIIB\(^{\text{Chb}}\) (C). Three cell loading concentrations were used in A and B (0.2, 0.25, and 0.3 optical density units at 230 nm), and two cell loading concentrations were used in C (0.2 and 0.25 optical density units at 230 nm). Samples were brought to equilibrium at either three speeds (A, 36,000, 20,000, and 42,000 rpm) or five speeds (B and C, 20,000, 40,200, 45,000, 49,000, 57,000, and 60,000 rpm). All data were globally fit to a single ideal species model using the program NONLIN. The fitted curves are superimposed on the data in the main chart, and the residuals of the fit are plotted above the main chart (\textit{versus} the independent variable). A summary of the fitted molecular weights is presented in Table II. This run was performed using cells with 12-mm double-sector charcoal filled epon centerpieces and quartz windows. Equilibrium was established when data obtained from scans taken 2 h apart were indistinguishable. The solvent comprised 25 mM sodium phosphate buffer, pH 8, and the run temperature was 20 °C.
Molecular Sedimentation of IIA\textsuperscript{Chb} and IIB\textsuperscript{Chb}

Table II

| Sample          | Sequence molecular weight | \(M_w\text{,app}^{b}\) | Variance of fit | Type of run |
|-----------------|----------------------------|-------------------------|-----------------|-------------|
| IIA\textsuperscript{Chb} | 12,748 (25,496)          | 25,204 (± 500)         | 2.79e-05        | Standard    |
| IIB\textsuperscript{Chb} | 12,826 (25,652)          | 23,010 (± 839)         | 3.76e-04        | Short column|
| Phospho-IIA\textsuperscript{Chb} | 11,431                   | 11,119 (± 172)         | 1.88e-04        | Standard    |
| Cys10SerIIB\textsuperscript{Chb} | 11,415                   | 10,891 (± 340)         | 1.21e-04        | Short column|
| Phospho-IIA\textsuperscript{Chb} + Cys10SerIIB\textsuperscript{Chb} | 24,241 (48,482)\textsuperscript{c} | 55,450 (± 1720)        | 5.00e-04        | Short column|

\(a\) Molecular weights were calculated from the known amino acid sequence. Values in parentheses are calculated for a dimer species.

\(b\) Best fit value of molecular weight assuming a single ideal species. Values in parentheses represent the 95\% joint confidence interval.

\(c\) The calculated molecular weight is for a complex with 1:1 (2:2) stoichiometry.

(a) As reflected in the hydrodynamic properties. In contrast to IIA\textsuperscript{Chb}, where phosphorylation resulted in a lower sedimentation coefficient, phospho-IIA\textsuperscript{Chb} exhibited a significantly higher coefficient than IIB\textsuperscript{Chb}, implying that the phosphoprotein may be more compact than unphosphorylated IIB\textsuperscript{Chb}. (Similar effects can result from changes in hydration and/or shape.) As discussed elsewhere in these papers (3), phospho-IIA\textsuperscript{Chb} is a thiophosphoryl protein, analogous to protein-tyrosine phosphatases. In the latter, phosphorylation at the active site Cys causes a conformationally flexible loop in the peptide chain to close over the phosphoryl group, which conceivably would yield a more compact protein with a higher sedimentation coefficient. Finally, it should also be noted that IIB\textsuperscript{Chb} is a basic protein (pI about 8.0) and that the phosphoryl group may interact with basic groups in the protein, thereby resulting in a more compact structure than IIB\textsuperscript{Chb}.

When phospho-IIA\textsuperscript{Chb} was mixed with an equimolar quantity of Cys10SerIIIB\textsuperscript{Chb}, a new molecular species was observed that was stable to both native gel electrophoresis and to gel filtration chromatography (3). No other combination of proteins gave this result. Fig. 3 presents sedimentation velocity data using the following mixtures of proteins: IIA\textsuperscript{Chb} and IIB\textsuperscript{Chb}, phospho-IIA\textsuperscript{Chb} and IIB\textsuperscript{Chb}, IIA\textsuperscript{Chb} and phospho-IIB\textsuperscript{Chb}, and IIA\textsuperscript{Chb} and Cys10SerIIIB\textsuperscript{Chb}. Sedimentation equilibrium data were collected on an equimolar mixture of phospho-IIA\textsuperscript{Chb} and Cys10SerIIIB\textsuperscript{Chb}. The chart shows eight concentration distributions collected from samples at four loading concentrations (2.5, 1.25, 0.625, and 0.31 mg/ml) and two speeds (20,000 and 30,000 rpm). All data were globally fit to a single species model using the program NONLIN. The fitted curves are superimposed on the data in the main chart, and the residuals of the fit are plotted above the main chart (versus the independent variable) and to the right of the main chart (versus the dependent variable). This run was performed using a short column 8-channel centerpiece to minimize the time required to reach equilibrium. The cell was equipped with sapphire windows, and all data were collected using the interference optics of the XL-I. Scans were taken every 15 min until no change in the fringe pattern was detectable, about 90 min at each speed. The solvent comprised 25 mM sodium phosphate buffer, pH 8, and the run temperature was 20 °C.
formed a stable complex. (Because phosphoryl transfer does occur between two of the pairs of proteins, a transient complex, at least, must be formed, but its concentration was either too low to be detected or the transfer was complete prior to sedimentation.) By sharp contrast to these results, an equimolar mixture of phospho-IIAChb and Cys10SerIIBChb yielded a new species of much higher sedimentation coefficient than each of the individual proteins. A short column sedimentation equilibrium experiment revealed that the complex had about the expected molecular weight for a tetramer, which agreed with the analyses (3). The composition of the complex is therefore: 1 mol of phospho-IIAChb dimer and 2 mol of Cys10SerIIBChb. The dissociation constant of the complex must be less than $10^{-7}$ M (15). Thus, we have been able to characterize a complex formed between phospho-IIAChb and the mutant, Cys10SerIIBChb. As far as we have been able to determine, there are no previous reports on transition state analogue intermediates in a phosphotransfer reaction between two proteins.

A model depicting our conclusions of these and related CD spectral studies is schematically depicted in Fig. 5. Phosphorylation of the IIAChb dimer results in a conformational change that permits binding of two mols of IIBChb to form one or more transient transition state complexes. Phosphate transfer to IIBChb and dissociation to the products completes the reaction, with concomitant change of IIAChb back to its original conformation.

**FIG. 5. Model of interaction between phospho-IIAChb and IIBChb.** The IIAChb dimer is phosphorylated by phospho-HPr. The model depicts a change in conformation of the dimer when it is phosphorylated, consistent with the sedimentation and CD spectral data. The phosphoprotein binds two molecules of IIBChb to form a transient complex, which dissociates when the phosphoryl group is transferred to IIBChb, and the IIAChb dimer returns to its original conformation.

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