The solution structure of the IIA-IIB complex of the \( N,N' \)-diacetylchitobiose (Chb) transporter of the Escherichia coli phosphotransferase system has been solved by NMR. The active site His-89 of IIA\(^{Chb}\) was mutated to Glu to mimic the phosphorylated state and the active site Cys-10 of IIB\(^{Chb}\) was substituted by serine to prevent intermolecular disulfide bond formation. Binding is weak with a \( K_D \) of \( \sim 1.3 \) mM. The two complementary interaction surfaces are largely hydrophobic, with the protruding active site loop (residues 9 – 16) of IIB\(^{Chb}\) buried deep within the active site cleft formed at the interface of two adjacent subunits of the IIA\(^{Chb}\) trimer. The central hydrophobic portion of the interface is surrounded by a ring of polar and charged residues that provide a relatively small number of electrostatic intermolecular interactions that serve to correctly align the two proteins. The conformation of the active site loop in unphosphorylated IIB\(^{Chb}\) is inconsistent with the formation of a phosphoryl transition state intermediate because of steric hindrance, especially from the methyl group of Ala-12 of IIB\(^{Chb}\). Phosphorylation of IIB\(^{Chb}\) is accompanied by a conformational change within the active site loop such that its path from residues 11 – 13 follows a mirror-like image relative to that in the unphosphorylated state. This involves a transition of the \( \phi/\psi \) angles of Gly-13 from the right to left \( \alpha \)-helical region, as well as smaller changes in the backbone torsion angles of Ala-12 and Met-14. The resulting active site conformation is fully compatible with the formation of the His-89-P-Cys-10 phosphoryl transition state without necessitating any change in relative translation or orientation of the two proteins within the complex.

The solution structure of the IIA-IIB complex of the \( N,N' \)-diacetylchitobiose transporter IIChb of the Escherichia coli phosphotransferase system; EI, enzyme I; HPr, histidine-containing phosphocarrier protein; Chb, \( N,N' \)-diacetylchitobiose; II\(^{Chb}\), II\(^{Chb}\), and IIC\(^{Chb}\), A, B, and C domains, respectively, of the \( N,N' \)-diacetylchitobiose transporter II\(^{Chb}\);
IIAChb is a small 11-kDa protein that has been studied by both x-ray crystallography (9) and NMR (10, 11) and is structurally similar to IIBMtl (7, 8), despite the absence of any significant sequence similarity (<10%). The active site residue, Cys-10, of IIBChb is located within an 8-residue protruding loop (residues 9–16) whose conformation is very similar to that of the low molecular weight protein-tyrosine phosphatases (42), including hydrogen-bonding interactions in the phosphorylated state between the phosphoryl group and backbone amide protons (11). (Note, throughout the text residues of IIBChb are shown in italics.) Wild-type IIAChb is highly prone to nonspecific aggregation promoted by a disordered 13-residue N-terminal tail and by metal ions that coordinate three buried aspartic acid residues (Asp-92), each from one subunit, at the center of the trimer interface (26). It has previously been shown that aggregation can be completely eliminated by removing the N-terminal tail and mutating Asp-92 to Leu to generate a mutant, which we refer to hereafter as IIAChb (26). In the present work, we made use of an active site H89E mutation introduced into IIAChb* to mimic the phosphorylated state. For IIBChb we introduced a C10S mutation to prevent intermolecular disulfide bridge-mediated dimer formation (9, 10). The IIAChb*-IIBChb complex is transient and weak with an equilibrium dissociation constant (KD) in the millimolar range. The affinity of IIAChb*(H89E) for IIBChb(C10S) is a factor of ~1.5 higher than that of IIAChb* (Kd ~1.3 versus ~2 mM) making the phosphomimetic mutant more suitable for NMR structural studies by increasing the population of the complex at the concentrations used in the NMR experiments. The structure of the IIAChb*(H89E)-IIBChb(C10S) complex reveals the structural basis of specific recognition and the interactions involved in phosphoryl transfer.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Mutagenesis—**Genes encoding IIAChb* (corresponding to a NA13/D92L mutant of wild-type IIAChb) and IIBChb (kindly provided by Dr. Saul Roseman, Johns Hopkins University, Baltimore) were cloned into the pET-11 vector. Additional H89E and C10S mutations of the active site residues of IIAChb* and IIBChb*, respectively were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The H89E mutation in IIAChb* was designed to mimic the charge effects of phosphorylation of His-89, and the C10S mutation of IIBChb was introduced to prevent any potential complications arising from possible intermolecular disulfide bridge formation.

The IIAChb*, IIAChb*(H89E), IIBChb*, and IIBChb(C10S) plasmids were introduced into *E. coli* BL21(DE3) (Novagen) cells for protein expression and induced at an A600 ~0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C. Cells were grown in either Luria-Bertani medium or minimum medium (in either H2O or D2O) with 15NH4Cl or 14NH4Cl as the main nitrogen source, and U-[13C3,3-2H2] glucose as the main carbon source. Because Leu, Val, Ile, Met, Gly, Tyr, and Ser residues are involved in the IIAChb*/IIBChb interface, selective labeling was also employed in the preparation of NMR samples. For 1H/13C/15N-(Ile/Leu/Val)-methyl-protonated (but otherwise fully deuterated) protein samples, 100 mg of α-[13C3,3-2H2]ketosiovalerate and 50 mg of α-[13C3,3-2H2]ketoisovalerate (Cambridge Isotopes) were added to 1 liter of D2O medium 1 h prior to induction (43). 1H/12C/14N-(Ile/Met/Thr-protonated)-IIAChb*(H89E) and 1H/12C/14N-(Gly/Met/Pro/Tyr-protonated)-IIBChb(C10S) samples were prepared by supplementing 1 liter of D2O medium with 300 mg of Gly/Ile/Met/Pro/Tyr (Sigma-Aldrich) at natural isotopic abundance 1 h prior to induction. After induction (4 and 7 h for growths in H2O and D2O, respectively), cells expressing IIAChb*(H89E) or IIBChb(C10S) were harvested by centrifugation at 15,900 × g for 25 min. IIAChb* and IIBChb*(H89E) Purification—The cell pellet was resuspended in 50 ml of buffer A (20 mM Tris, pH 8.0, 1 mM EDTA, 0.2 mM sodium azide) with 1 mM phenylmethylsulfonyl fluoride. The cell suspension was lysed by two passages through a microfluidizer at 15,000–23,000 psi and centrifuged at 75,600 × g for 30 min. The supernatant was loaded onto a 5-ml HiTrap SP FF column (Amersham Biosciences), and IIAChb* was eluted with a gradient of buffer B. After induction (4 and 7 h for growths in H2O and D2O, respectively), cells expressing IIAChb*(H89E) or IIBChb(C10S) were harvested by centrifugation at 15,900 × g for 25 min. IIAChb* and IIBChb*(H89E) Purification—The cell pellet was resuspended in 50 ml of buffer A (20 mM Tris, pH 8.0, 1 mM EDTA, 0.2 mM sodium azide) with 1 mM phenylmethylsulfonyl fluoride. The cell suspension was lysed by two passages through a microfluidizer at 15,000–23,000 psi and centrifuged at 75,600 × g for 30 min. The supernatant was loaded onto a 5-ml HiTrap QFF column (Amersham Biosciences), and IIAChb* was eluted with a gradient of buffer B (20 mM Tris, pH 8.0, 1 mM EDTA, 0.2 mM sodium azide, 1 mM NaCl). The eluted protein was subsequently denatured with 4 mM guanidine-HCl for 15 min. The protein solution was then dialyzed against 2 liters of buffer A overnight. After centrifugation of the dialyzed solution to remove precipitated proteins, the supernatant was purified by size exclusion chromatography on a Superdex-75 column (Amersham Biosciences) in buffer C (20 mM Tris, pH 8.0, 1 mM EDTA, 0.2 mM sodium azide, 0.5 mM NaCl). The fractions containing IIAChb* were exchanged into buffer A using an Amicon Ultra-15 (Millipore) filter, loaded onto a mono Q (10/100GL column (Amersham Biosciences), and eluted with a gradient of buffer B.

IIAChb* and IIBChb*(H89E) Purification—The cell pellet was resuspended in 50 ml of buffer D (20 mM sodium phosphate, pH 4.5, 0.2 mM sodium azide) with 1 mM phenylmethylsulfonyl fluoride. The cell suspension was lysed by two passages through a microfluidizer at 15,000–23,000 psi and centrifuged at 75,600 × g for 30 min. The supernatant was loaded onto a 5-ml HiTrap SP FF column (Amersham Biosciences), and IIBChb*(C10S) was eluted with a gradient of buffer E (20 mM sodium phosphate, pH 4.5, 0.2 mM sodium, 1 mM NaCl). The eluted protein was further purified by size exclusion chromatography in buffer F (20 mM sodium phosphate, pH 6.5, 0.2 mM...
sodium azide) on a Superdex-75 column (Amersham Biosciences).

Phosphorylation of $IIB^{Chb}$ — $^{15}$N-labeled $IIB^{Chb}$ was phosphorylated by the addition of 5 $\mu$M enzyme, 1 $\mu$M HPr, 5 $\mu$M $IIA^{Chb}$, 5 mM MgCl$_2$, and 20 mM phosphoenolpyruvate in 20 mM sodium phosphate, pH 6.5, 100 mM NaCl, 0.2 mM sodium azide, and 90% H$_2$O/10% D$_2$O. Phosphorylation was confirmed by two-dimensional $^1$H-$^1$H correlation spectroscopy, which showed large chemical shift perturbations of residues 10–16 comprising the active site loop (11), relative to unphosphorylated $IIB^{Chb}$. Full-length Enzyme I and HPr were expressed and purified as described previously (44).

NMR Data Collection and Analysis — All NMR samples were prepared in a buffer of 20 mM sodium phosphate, pH 6.5, 100 mM NaCl, 0.2 mM sodium azide, and either 90% H$_2$O/10% D$_2$O or 99.99% D$_2$O. $IIA^{Chb}$ is a symmetric trimer with three equivalent binding sites for $IIB^{Chb}$ (26). To achieve optimal linewidths for NMR spectroscopy, a 1:1 mixture of $IIB^{Chb}$($H76E)$ trimer to $IIB^{Chb}(C105)$ monomer was employed. NMR spectra were recorded at 20 and 35 °C on Bruker DMX500, DMX600, DRX600, DRX800, and DRX900 spectrometers equipped with either $x$-, $y$-, and $z$-shielded gradient triple resonance probes or $z$-shielded gradient triple resonance cryoprobe. Spectra were processed with the NMRPipe package (45) and analyzed using the program PIPP (46).

Sequential and side chain assignments of $IIB^{Chb}$($H89E)$ and $IIB^{Chb}(C105)$ were derived from the following three-dimensional double and triple resonance through-bond correlation experiments (47–49): HNCA, HN(CO)CA, HN(CO)CB, CBCA(CO)NH, HNCACB, HN(CO)CA-TROSY, HN(CO)CB-TROSY, C(CCO)NH, H(CC)CNH, HCCCH-TOCSY. Three-dimensional $^{15}$N-separated, $^{13}$C-separated, and $^{13}$C/$^{13}$C-separated nuclear Overhauser enhancement (NOE) experiments were used to facilitate side chain assignments (47, 48).

Backbone $^1$D$_{NH}$ residual dipolar couplings (RDC) were obtained from the difference in $^1$H$_{NN}$ scalar couplings measured in dilute liquid crystalline medium (phage pf1 (50, 51)) and isotropic (water) medium, measured using two-dimensional in-

![Figure 1. Binding of $IIA^{Chb}(H89E)$ and $IIB^{Chb}(C105)$. Backbone amide chemical shift perturbations upon titrating unlabeled $IIA^{Chb}(H89E)$ into a solution of $^{15}$N-labeled $IIB^{Chb}(C105)$ at 20 °C. The chemical shifts were monitored using $^1$H-$^1$N HSQC spectroscopy at a spectrometer $^1$H frequency of 600 MHz. $\Delta\delta_{HN} = [(\Delta\delta_{HN})/25 + (\Delta\delta_{HN})/21/2]$ (78). The $IIA^{Chb}(H89E)$: $IIB^{Chb}(C105)$ molar ratios, expressed in terms of subunit concentration of $IIA^{Chb}(H89E)$, are 0.4, 0.5, 0.6, 0.7, and 0.8, respectively, and concentrations of $IIB^{Chb}(C105)$ of 0.5, 0.49, 0.47, 0.45, 0.44 mM, respectively, and concentrations of $IIB^{Chb}(C105)$ of 0.5, 0.49, 0.47, 0.45, 0.44 mM, respectively, and concentrations of $IIB^{Chb}(C105)$ of 0.5, 0.49, 0.47, 0.45, 0.44 mM, respectively.

**TABLE 2**

| RDC R-factor for $IIA^{Chb}$ | X-ray | NMR | NMR | NMR |
|-------------------------------|------|-----|-----|-----|
| $^1$D$_{NH}$ | $IIB^{Chb}(C105)$ | $IIB^{Chb}(C105)$ | phospho-$IIB^{Chb}$ |
| 100 mM NaCl and 10 mg/ml pf1 | 12.1/8.0 | 44.7/43.8 | 18.8/14.4 | 88.2 |
| Active site loop (residues 9–16) (6)** | 70.1 | 79.5 | |
| 400 mM NaCl and 17 mg/ml pf1 | 14.7/8.4 | 40.2/34.8 | 16.2/11.5 | 50.2 |
| Active site loop (residues 9–16) (6)** | 50.9 | 88.4 | 50.2 | |
| $^1$D$_{NH}$ | $IIB^{Chb}(C105)$ | $IIB^{Chb}(C105)$ | phospho-$IIB^{Chb}$ |
| 100 mM NaCl and 10 mg/ml pf1 | 38.8/16.8 | 55.4/49.3 | 19.9/20.8 | 15.5 |
| Active site loop (residues 9–16) (7)** | 73.2 | 70.0 | 16.6/16.8 | |
| 200 mM NaCl and 15 mg/ml pf1 | 34.1/16.0 | 52.2/46.6 | 70.6 | 15.3 |
| Active site loop (residues 9–16) (7)** | 70.6 | 79.0 | |

**The RDC R-factor is defined as $100 \times (1 - R_{D}) = (R_{D}^\text{obs} - R_{D}^\text{calc})^2 < 2\cdot(R_{D}^\text{obs} + R_{D}^\text{calc})^{1/2}$ (71), where $R_{D}^\text{obs}$ are the observed RDCs, and $R_{D}^\text{calc}$ are the calculated RDCs obtained by singular value decomposition against the coordinates of the indicated protein (72). The values for the magnitude of the principal component of the alignment tensor ($D_{\varphi}^\text{calc}$) and the rhombicity ($\eta$) are as follows. For the RDCs measured on free $IIB^{Chb}(C105)$, the values are 25.6 Hz and 0.4, respectively, in 100 mM NaCl and 10 mg/ml pf1, and 8.9 Hz and 0.3, respectively, in 400 mM NaCl and 17 mg/ml pf1; the normalized scalar product between the alignment tensors (80) at the two salt concentrations is 0.96 between the two alignment tensors.**

*The PDB accession codes are 1IIB and 1E2B for the x-ray (9) and NMR (10) structures of $IIB^{Chb}(C105)$, respectively, and 1H9C for the NMR structure of phospho-$IIB^{Chb}$ (11). The active site region is located at position 10, and the active site loop comprises residues 9–16. The number of experimentally measured $^1$D$_{NH}$ RDCs is shown in parentheses.

$^a$ RDCs were measured for residues 9, 10, 11, 12, 13, and 16. The cross-peaks for the backbone amide groups of Met-14 and Ser-15 were too broad to permit measurement of the $^1$J$_{HN}$ splitting in the alignment medium.

$^b$ RDCs were measured for residues 9, 10, 11, 12, 13, 14, and 16. The cross-peak for the backbone amide group of Ser-15 was too broad to permit measurement of the $^1$J$_{HN}$ splitting in the alignment medium.
Solution Structure of the IIA<sup>Chb</sup>-IIB<sup>Chb</sup> Complex

Intermolecular NOEs in the IIA<sup>Chb</sup>(H89E)-IIB<sup>Chb</sup>(C10S) complex. NOEs in a three-dimensional <sup>12</sup>C-filtered/<sup>13</sup>C-separated NOE experiment recorded in D<sub>2</sub>O are specifically observed from protons attached to <sup>12</sup>C<sup>NOEs</sup> in a three-dimensional <sup>12</sup>C-filtered/<sup>13</sup>C-separated NOE experiment (53, 54). Nine different phase/anti-phase (IPAP) <sup>1</sup>H-<sup>15</sup>N heteronuclear single quantum coherence (HSQC) spectra (52).

Intermolecular NOEs were observed on the IIA<sup>Chb</sup>(H89E)-IIB<sup>Chb</sup>(C10S) complex in D<sub>2</sub>O buffer using three-dimensional <sup>12</sup>C-filtered(F<sub>3</sub>)/<sup>13</sup>C-separated(F<sub>3</sub>) or <sup>13</sup>C-separated(F<sub>3</sub>)/<sup>12</sup>C-filtered(F<sub>3</sub>) NOE experiments, and in H<sub>2</sub>O buffer using two-dimensional <sup>15</sup>N-separated/<sup>13</sup>C-edited and <sup>13</sup>C-separated/<sup>15</sup>N-edited NOE experiments (53, 54). Nine different combinations of isotope-labeled complexes were used for analysis of intermolecular NOEs (Table 1).

Structure Calculations—NOE-derived interproton distance restraints were classified into loose approximate distance ranges of medium, weak, and very weak NOE cross-peak intensities, respectively (55); an empirical correction of 0.5 Å was added to the upper distance bounds of distance restraints involving methyl groups to account for the higher apparent intensity of methyl resonances (56). NOEs involving non-stereospecifically assigned methyl, methylene, and aromatic protons were represented by a (Σ r<sup>−6</sup>)<sup>−1/6</sup> sum (57). Backbone torsion angle restraints for the active site loop of IIB<sup>Chb</sup>(C10S) and phospho-IIB<sup>Chb</sup>, and for the

![Table 3](image-url)
mobile loop (residues 75−84) of IIAChb(H89E) were derived from chemical shifts using the program TALOS+ (58). The minimized target function comprises NOE-derived interproton distance restraints, torsion angle restraints, RDC restraints (62), 13Ca/13Cβ chemical shift restraints (63), a quartic van der Waals repulsion term for the non-bonded con-
tacts (64), a multidimensional torsion angle data base potential of mean force (65), and a gyration volume potential to ensure optimal packing (66). Structure figures were generated using the programs VMD-XPLOR (67) and GRASP (68). Reweighted atomic probability density maps were calculated as described previously (69).

The atomic coordinates and NMR experimental restraints (access code 2WVV for the unphosphorylated complex and 2WY2 for the phosphoryl transition state complex) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ.

RESULTS AND DISCUSSION

Equilibrium Binding of IIAChb−(H89E) and IIBChb(C10S)—The binding of IIAChb and the active site IIAChb*(H89E) phosphomimetic mutant (at natural isotopic abundance) to U-15N-labeled IIBChb(C10S) was monitored by 1H-15N correlation spectroscopy. Exchange between the complex and free proteins is fast on the chemical shift time scale. The pattern of 1H/15N chemical shift perturbations observed for the binding of IIAChb and IIAChb*(H89E) to IIBChb(C10S) is very similar but the magnitude of the perturbations is smaller for IIAChb than IIAChb*(H89E). This is caused by the fact that the binding of IIAChb to IIBChb(C10S) is weaker (Kd ~ 2.1 ± 0.5 mM) than that of IIAChb*(H89E) (Kd ~ 1.3 ± 0.3 mM; see Fig. 1), and hence the fraction of complex formed under the same experimental conditions (protein concentrations ~ 1 mM) is considerably reduced. For this reason, all structural studies were conducted with the IIAChb*(H89E) phosphomimetic mutant.

Structural studies were carried out on samples comprising 1 mM IIAChb*(H89E) trimer and 1 mM IIBChb(C10S). Under these conditions, 51.6% of IIAChb*(H89E) and 64.4% of IIBChb(C10S) are in the bound state. Because IIAChb is a symmetric trimer with three equivalent binding sites for IIBChb, one can calculate that the percentage of IIAChb*(H89E) with one, two, and three IIBChb(C10S) molecules bound is 39.7, 10.9, and 1.0%, respectively. Hence, the fraction of IIAChb*(H89E) that is bound to IIBChb(C10S) is 64.4%.
Solution Structure of the IIA\(^{\text{Chb}}\)-IIB\(^{\text{Chb}}\) Complex

tively, the corresponding percentages, expressed in terms of IIB\(^{\text{Chb}}\)(C10S) are 39.7, 21.7, and 3.0%, respectively. Given molecular masses of 33.6 kDa and 11.4 kDa for free IIA\(^{\text{Chb}}\)(H89E) and IIB\(^{\text{Chb}}\)(C10S), respectively, and the fact that all species are in fast exchange with one another, the linewidths of IIA\(^{\text{Chb}}\)(H89E) and IIB\(^{\text{Chb}}\)(C10S) in the NMR sample are determined by population weighted-average molecular masses of 41.0 and 36.2 kDa, respectively.

Structure Determination—Because the chemical shift perturbations observed upon complex formation are small, one can conclude that there are no significant backbone structural changes (within the limits of the NMR method) induced within either IIA\(^{\text{Chb}}\)(H89E) or IIB\(^{\text{Chb}}\)(C10S). We, therefore, proceeded to solve the structure of the complex using conjoined rigid body/torsion angle dynamics simulated annealing (59, 70), largely on the basis of intermolecular NOE data. In this approach, the backbone (except for certain selected regions, see below) and non-interfacial side chain coordinates are treated as rigid bodies with rotational and translational degrees of freedom, whereas the interfacial sidechains are given full torsional degrees of freedom.

The chemical shift differences between IIA\(^{\text{Chb}}\) and IIA\(^{\text{Chb}}\)(H89E) are limited to the immediate vicinity of the mutation, and \(^{1}\)\(D_{\text{NH}}\) RDC measurements on free IIA\(^{\text{Chb}}\)(H89E) indicate excellent agreement between observed RDCs for helical residues and those calculated from the NMR structure (restrained regularized mean coordinates) of IIA\(^{\text{Chb}}\)(26). The RDC R-factor is 8.7% (defined as \(\langle(D_{\text{obs}} - D_{\text{calc}})^2\rangle^{1/2}\)) (71), where \(D_{\text{obs}}\) are the observed RDCs, and \(D_{\text{calc}}\) are the calculated RDCs obtained by singular value decomposition against the coordinates of the protein (72)). The alignment tensor of IIA\(^{\text{Chb}}\)(H89E) is axially symmetric, as expected for a symmetric trimer, with a magnitude of ~11.3 Hz for the axial component (\(D_{a,\text{NH}}\)). We, therefore, used the NMR structure of IIA\(^{\text{Chb}}\) in the conjoined rigid body/torsion angle dynamics simulated annealing calculations. However, because the loop connecting helices 2 and 3 is partially disordered (i.e. highly mobile) in solution and contributes to the interface with IIB\(^{\text{Chb}}\), the backbone of residues 75–84 was also given torsional degrees of freedom.

IIB\(^{\text{Chb}}\)(C10S) strongly interacts with the alignment medium (phage pf1) in the absence of salt; RDC measurements were therefore carried out in 100 and 400 mM NaCl using 10 and 17 mg/ml phage pf1, yielding values of 25.5 and ~8.5 Hz for \(D_{a,\text{NH}}\), respectively, and a rhombicity \(\eta\) of ~0.4. The RDC R-factors are summarized in Table 2 and allow one to conclude the following: (a) both the 1.8-Å resolution x-ray structure of IIB\(^{\text{Chb}}\)(C10S) (9) and the NMR structure of phospho-IIB\(^{\text{Chb}}\)(C10S) (11) provide a much better representation of the actual solution structure of IIB\(^{\text{Chb}}\)(C10S) than does the NMR structure of IIB\(^{\text{Chb}}\)(C10S) (10), reflecting the lower coordinate accuracy of the latter; (b) there are very large discrepancies between observed and calculated RDCs within the active site loop (residues 9–16) for both the x-ray structure of IIB\(^{\text{Chb}}\)(C10S) and the NMR structure of phospho-IIB\(^{\text{Chb}}\), reflected in very high RDC R-factors for this region; (c) removing the RDCs for the active site residues results in excellent agreement between observed and calculated RDCs with lower RDC R-factors for the x-ray structure of IIB\(^{\text{Chb}}\)(C10S). Therefore, the coordinates of the x-ray structure of IIB\(^{\text{Chb}}\)(C10S) (9) were used in the calculations, with the backbone of the active site loop (residues 9–16), given torsion degrees of freedom. The observed RDC R-factors of 8–9% observed for the x-ray structure of IIB\(^{\text{Chb}}\)(C10S), excluding the active site loop, are as expected for a crystal structure solved at 1.5 to 2 Å resolution (73, 74). It is worth noting that phosphorylation of IIB\(^{\text{Chb}}\) is accompanied by a large conformational change within the active site loop as manifested both by RDCs (Table 2) and by significant differences in backbone (\(N, C_{\alpha}, H_{\alpha}\), and \(C_{\beta}\)) chemical shifts (11). Excluding the active site loop, the crystal structure of IIB\(^{\text{Chb}}\)(C10S) still displays lower RDC R-factors than the NMR structure of phospho-IIB\(^{\text{Chb}}\) (Table 2). However, the RDCs within the active site loop are now in excellent agreement with the NMR structure of phospho-IIB\(^{\text{Chb}}\) but exhibit very large discrepancies with respect to the x-ray structure of IIB\(^{\text{Chb}}\)(C10S) (Table 2).

The intermolecular NOE data were derived from a large series of isotope-filtered/isotope-separated intermolecular NOE experiments (53). Because of the relatively large size of the complex and extensive chemical shift overlap, nine different labeling combinations (Table 1), including amino acid specific labeling, were used to eliminate any ambiguities in intermolecular NOE assignments. Examples of the quality of the intermolecular NOE data are shown in Fig. 2.

In protein–protein complexes of the PTS that we have solved previously (30, 31, 33–37), it is usually possible to derive sidechain torsion angle restraints for interfacial side chains based on heteronuclear \(\text{j}^\prime\) scalar couplings and short mixing time NOE data (53). In this instance, the complex is not fully saturated because of weak binding (\(K_{d} \sim 1.3 \text{mM}\)), and there is a significant proportion of each component in the free state. We therefore employed a heuristic approach in which the interfacial side chains were given torsional degrees of freedom but restrained within the \(X_{i}\) and, where appropriate, \(\gamma_{i}\) rotamers occupied in the free structures, unless these were inconsistent with the intermolecular NOE data.

Unfortunately we were not able to use RDC data to provide information on the relative orientation of the two components

| TABLE 4 RDC R-factors for IIB\(^{\text{Chb}}\) in the IIA\(^{\text{Chb}}\)(H89E)-IIB\(^{\text{Chb}}\)(C10S) complex and corresponding phosphoryl transition state |
|-----------------|-----------------|-----------------|-----------------|
|                 | Complex         | Transition state |
| \(1^{\text{D}_{\text{obs}}}\) RDCs measured on free IIB\(^{\text{Chb}}\)(C10S) \(^a\) | \(100 \text{ mM NaCl and 10 mg/ml pf1}\) | \(8.1\) | 16.7 |
| All data (73)   | Active site loop (residues 9–16) (6) | 7.8 | 105.4 |
| \(400 \text{ mM NaCl and 17 mg/ml pf1}\) All data (80) | Active site loop (residues 9–16) (6) | 9.8 | 13.1 |
| \(1^{\text{D}_{\text{calc}}}\) RDCs measured on free phospho-IIB\(^{\text{Chb}}\) | \(100 \text{ mM NaCl and 10 mg/ml pf1}\) All data (66) | 44.1 | 15.1 |
| 200 mM NaCl and 15 mg/ml pf1 All data (76) | Active site loop (residues 9–16) (7) | 86.9 | 2.8 |

\(\text{d}^\text{a}\) The number of experimentally measured \(1^{\text{D}_{\text{NH}}}\) RDCs is shown in parentheses.
\(\text{d}^\text{b}\) RDCs were measured for residues 9, 10, 11, 12, 13, and 16 (see Table 2, Footnote d).
\(\text{d}^\text{c}\) RDCs were measured for residues 9, 10, 11, 12, 13, and 14 (see Table 2, Footnote c).
\(\text{d}^\text{e}\) See Table 2, Footnote a for definition.
within the complex. In a simple case of a weak binding binary complex in fast exchange on the chemical shift scale, the observed RDCs are weighted averages of the RDCs in the free and bound state, so that it is possible in principle to back-calculate the RDCs for the pure complex providing one knows exactly the fraction of the bound species (35, 75). However, for the IIA\textsuperscript{Chb}(H89E)-IIB\textsuperscript{Chb}(C10S) complex, the bound species comprises a mixture of three states with one, two and three IIB\textsuperscript{Chb}(C10S) molecules bound to the IIA\textsuperscript{Chb}(H89E) trimer, each with its own alignment tensor. Deconvolution of the alignment tensors for the individual bound states is not feasible, thereby precluding the use of RDCs in this system. Moreover, the alignment media that we explored (pf1, strained gels and PEG/hexanol (72)) all displayed differential interaction with one of the partners, making any extrapolation of average RDCs for the bound states unreliable.

A summary of the structural statistics is given in Table 3, and a best-fit superposition of the final 90 simulated annealing structures is shown in Fig. 3A. The NOE-derived interproton distance restraints comprised 40 intermolecular NOEs (per bound IIB\textsuperscript{Chb} molecule), as well as intramolecular NOEs related to those portions of the IIB\textsuperscript{Chb}(C10S) backbone that were given torsional degrees of freedom. The agreement of the RDCs within the active site loop of IIB\textsuperscript{Chb}(C10S) is comparable to that of the rest of the protein (Table 4). The relative orientation of IIB\textsuperscript{Chb}(C10S) to IIA\textsuperscript{Chb}(H89E) is well defined with a precision of 0.3 ± 0.1 Å for the backbone of the complete complex (IIA Chb* and IIB\textsuperscript{Chb} best-fitted overall), 0.9 ± 0.3 Å for the backbone of IIB\textsuperscript{Chb} with best-fitting to IIA\textsuperscript{Chb*}, and 1.2 ± 0.4 Å for IIA\textsuperscript{Chb*} with best-fitting to IIB\textsuperscript{Chb}. The coordinate precision for the interfacial side chains is 0.9 ± 0.1 Å.

Overall Structure of the IIA\textsuperscript{Chb*}(H89E)-IIB\textsuperscript{Chb}(C10S) Complex—The overall structure of the complex is shown in Fig. 3 with one, two, and three molecules of IIB\textsuperscript{Chb}(C10S) bound. IIA\textsuperscript{Chb*} is a symmetric trimer (26). Each subunit comprises a three-helix bundle (α1, residues 14–44; α2, residues 46–74, α3, residues 84–114) in an up-down topology with α2 antiparallel to α1 and α3. The trimer interface consists of a parallel coiled-coil formed by helix α3 (H89E) colored in orange because its backbone carbonyl accepts a hydrogen bond from Ser-17 of IIB\textsuperscript{Chb}(C10S). The active site residues, H89E of IIA\textsuperscript{Chb}(H89E), and C10S of IIB\textsuperscript{Chb}(C10S) are colored purple.
Solution Structure of the IIA\textsuperscript{Chb}-IIB\textsuperscript{Chb} Complex

FIGURE 5. Interaction surfaces for the IIA\textsuperscript{Chb}(H89E)-IIB\textsuperscript{Chb}(C10S) complex. The left panel display the interaction surface (formed by the A and C subunits) on IIA\textsuperscript{Chb}(H89E) for IIB\textsuperscript{Chb}(C10S); the right panel shows the interaction surface on IIB\textsuperscript{Chb}(C10S) for IIA\textsuperscript{Chb}(H89E). The surfaces are color coded as follows: hydrophobic residues, green; uncharged residues bearing a polar functional group, cyan; negatively charged residues, red; positively charged residues, blue. Relevant portions of the backbone and active site residue of the interacting partner are displayed as tubes and bonds, respectively. Residues of IIB\textsuperscript{Chb}(C10S) are labeled in italics.

IIB\textsuperscript{Chb} is a mixed $\alpha/\beta$ protein comprising 5 helices (residues 12–30, 43–49, 63–71, 80–86, and 88–105), and 4 $\beta$-strands (residues 4–9, 34–39, 53–56 and 76–78) arranged in a $\alpha_1x_12$ topology (9–11). The active site residue, Cys 10, is located in an exposed active site loop (residues 9–16) that forms a protrusion on the surface of the protein. The overall topology of IIB\textsuperscript{Chb} is similar to that of IIA\textsuperscript{Mtl} (7) although the percentage sequence identity is only 8%. In the NMR structure of phospho-IIB\textsuperscript{Chb}(C10S) (9), the active site loop has a conformation that is similar to that of IIB\textsuperscript{Mtl} (both unphosphorylated (7) and phosphorylated (8)) and the low molecular weight protein-tyrosine phosphatases (42), except that the residues located at $i+1$ and $i+2$ of the active site cysteine are replaced by only a single residue in IIB\textsuperscript{Chb}.

Because the three IIB\textsuperscript{Chb} binding sites on IIA\textsuperscript{Chb}$^r$ are identical, we will simply consider the interaction of IIB\textsuperscript{Chb}(C10S) with the A and C subunits of IIA\textsuperscript{Chb}$^r$(H89E), where the contributing active site histidine (His 89) originates from the A subunit (Fig. 4). A total of 1836 $\AA^2$ is buried upon complexation, with 927 $\AA^2$ originating from IIA\textsuperscript{Chb}(H89E) and 909 from IIB\textsuperscript{Chb}(C10S). The interface accessible surface area contributed by the A and C subunits of IIA\textsuperscript{Chb}$^r$(H89E) is approximately equal. The interface comprises 15 residues each from the A and C subunits of IIA\textsuperscript{Chb}$^r$(H89E) and 29 residues from IIB\textsuperscript{Chb}(C10S); 4 residues of the latter interact simultaneously with the A and C subunits of IIA\textsuperscript{Chb}$^r$(H89E) (Fig. 4C). The interfacial gap volume index (ratio of gap volume to interface accessible surface area) is 3.3 Å, and the r.m.s. deviation of the interface atoms from a least-squares plane through these atoms is 3.7 Å (concave; Fig. 5, left panel) for IIA\textsuperscript{Chb}$^r$(H89E) and 3.3 Å (convex; Fig. 5, right panel) for IIB\textsuperscript{Chb}(C10S). These values are typical of transient complexes (76, 77).

Stereoviews depicting details of the interface are shown in Fig. 4, A and B, and a summary of the intermolecular contacts is provided in Fig. 4C. The active site loop, a 3$\alpha$ helix (residues 58–62) and helix $\alpha_4$ of IIB\textsuperscript{Chb}(C10S) are in contact with helices $\alpha_1$ and $\alpha_3$ of the A subunit of IIA\textsuperscript{Chb}(H89E) (Fig. 4A). Helices $\alpha_1$ and $\alpha_2$, the C-terminal end of strand $\beta_2$ and the subsequent 3$\alpha$ helix (residues 40–42) of IIB\textsuperscript{Chb}(C10S) contact helices $\alpha_2$ and $\alpha_3$ of the C subunit of IIA\textsuperscript{Chb}$^r$(H89E), as well as some residues in the disordered loop connecting these two helices in

IIA\textsuperscript{Chb}$^r$(H89E) (Fig. 4B). The intermolecular interactions are largely hydrophobic with 50–60% of the interfacial residues being nonpolar. Met-14 of the active site loop of IIB\textsuperscript{Chb}(C10S) is involved in a very large number of intermolecular hydrophobic interactions with methyl group clusters of Val and Leu residues, specifically Val-21A and Val-86C of the A subunit and Val-83C, Leu-87C, and Val-88C of the C subunit of IIA\textsuperscript{Chb}$^r$(H89E). Two other methionine residues, Met-22A and Met-81C of IIA\textsuperscript{Chb}(H89E) are also involved in an array of intermolecular hydrophobic interactions, with C10S, Pro-58, Gln-59, and Tyr-84, and with Leu-18, Lys-22, and Val-87, respectively, of IIB\textsuperscript{Chb}(C10S). There are only two intermolecular salt bridge/hydrogen bonding interactions: between Arg-24 and Glu-72C, and between Ser-17 and the backbone carbonyl of Ile-72C (Fig. 4B). These are supplemented by 7 longer range electrostatic interactions, 5 involving the A chain and 2 the B chain of IIA\textsuperscript{Chb}$^r$(H89E) (Fig. 4C). Of these, two involve interactions between charged side chains (Glu-15A and Lys-86, and Lys-70C and Glu-73C). The remainder involve interactions either between polar groups or between polar and charged groups. An example of the former would include the interaction between the sulfur atom of Met-22A and the hydroxyl group of Tyr-84 (Fig. 4A). An example of the latter is the interaction between the carboxylate of Glu-19A and the hydroxyl group of Ser-81 and the amide group of Gln-59 (Fig. 4A).

As in previously solved complexes of the PTS (30, 31, 33–37), the center of each interface is largely hydrophobic, surrounded
by a ring of polar and charged residues (Fig. 5). Of note is that the interaction surface of IIBChb contains both positively and negatively charged residues, similar to IIBGlc (31), IIBMtl (36) and IIBMan (35), but in contrast to HPr (33, 34, 37) where the charged residues on the interaction surface are entirely positive. This ensures that the IIB domains interact with their corresponding IIA domains, but not with enzyme I, despite the fact that the binding site on all the sugar-specific enzymes IIA is common to both HPr and IIB, and HPr uses the same binding surface to interact with both enzymes IIA and EI.

The Active Site and the Phosphoryl Transition State—The conformation of the active site loop (residues 9–16) of IIBChb(C10S) in the complex is not compatible with the formation of a pentacoordinate phosphoryl transition state in which the phosphorus atom lies in the plane of the imidazole ring of His-89A and the P-Sy-Cβ bond angle of Cys-10 lies between 90 and 130°. This is because the location of Ala-12 sterically occludes the formation of a phosphoryl transition state (Fig. 6A). The same is true of the x-ray structure of IIBChb(C10S) (9). Although there is a small displacement (1.3 Å) of the backbone of the active site loop of IIBChb(C10S) between the current structure (based on RDCs within the active site loop) and the crystal structure, the conformation of the loop in the two structures is actually very similar and the differences in backbone torsion angles are relatively small. In the conformation of the active site loop of phospho-IIBChb (11), however, the path of the polypeptide chain from residues 11–13 follows a mirror-like image relative to the unphosphorylated active site loop.

To model the transition state, therefore, we kept the coordinates of IIAChb* and IIBChb within the complex fixed, with the exception of the backbone of the active site loop of IIBChb(C10S) between the current structure (based on RDCs within the active site loop) and the crystal structure, the conformation of the loop in the two structures is actually very similar and the differences in backbone torsion angles are relatively small. In the conformation of the active site loop of phospho-IIBChb (11), however, the path of the polypeptide chain from residues 11–13 follows a mirror-like image relative to the unphosphorylated active site loop.

To model the transition state, we kept the coordinates of IIAChb* and IIBChb within the complex fixed, with the exception of the backbone of the active site loop (residues 9–16) of IIBChb(C10S), the backbone immediately adjacent to the active site histidine (residues 87–91 of subunit A) of IIAChb*, and the interfacial side chains in close proximity to Cys-10 and His-89A, which were given torsional degrees of freedom and subjected to simulated annealing refinement on the basis of the experimental intermolecular NOE restraints and the RDCs collected on free phospho-IIBChb. In addition, restraints were included to define the geometry of the phosphoryl group relative to Cys-10 and
Solution Structure of the IIA\textsubscript{Chb}-IIB\textsubscript{Chb} Complex

Although the structure of phospho-IIA\textsubscript{Chb} has not been solved, the phosphorylated state is easily modeled from the structure of unphosphorylated IIA\textsubscript{Chb} (26): minimal changes in the $x_1$ and $x_2$ angles of His-89\textsubscript{A} are all that are required to permit the formation of hydrogen bonds from the side chain amide of Gln-91\textsubscript{C} and the Ne2-H proton of His-93\textsubscript{A} to the phosphoryl group. In the transition state, these distances are lengthened but bridging hydrogen bonds involving water molecules could clearly be formed (Fig. 6C). The phosphoryl group in the transition state is stabilized by an array of hydrogen bonds from the IIB\textsubscript{Chb} active site loop, including the backbone amides of Ala-12, Met-14, and Ser-15, and the hydroxyl groups of Ser-11 and Ser-15; in addition, hydrogen bonds from the backbone amides of Ser-11 and Gly-13 to the sulfur atom to Cys-10 further stabilize the conformation (Fig. 6, B and C). Many of these interactions are maintained upon shortening of the S-P bond to form phospho-IIB\textsubscript{Chb} (11). The interactions stabilizing the phosphoryl group in both the IIA\textsubscript{Chb}*-P-IIB\textsubscript{Chb} transition state and phospho-IIB\textsubscript{Chb} (11) are very similar to those observed in phospho-IIB\textsubscript{Mtl} (8) and the phosphorylated state of the low molecular weight protein-tyrosine phosphatases (42). Presumably, the reason that the active site loop of IIB\textsubscript{Chb} undergoes a conformational change upon phosphorylation, whereas that of IIB\textsubscript{Mtl} does not, resides in the fact that the single residue deletion within the active site loop of IIB\textsubscript{Chb} relative to IIB\textsubscript{Mtl}, results in stereochemical strain that can only be overcome by numerous interactions between the protein backbone and the phosphoryl group.

Further examination of the IIA\textsubscript{Chb}*-P-IIB\textsubscript{Chb} transition state reveals that the phosphoryl group is buried within a largely hydrophobic environment provided by Ala-12 and Met-14 of IIB\textsubscript{Chb} and Val-21\textsubscript{A}, Met-22\textsubscript{A}, Ile-25\textsubscript{A}, Ile-72\textsubscript{C}, and Leu-87\textsubscript{C} of IIA\textsubscript{Chb}. This configuration is common to all protein-protein complexes of the PTS solved to date (30, 31, 33–37), including the EIN-HPr complex that is common to all branches of the pathway, as well as the complexes involving sugar-specific components.

Concluding Remarks—We have determined the solution structure of the IIA\textsubscript{Chb}*(H89E)-IIB\textsubscript{Chb}(C10S) complex, and shown that this structure is fully compatible with the formation of a phosphoryl transition state when the active site loop of IIB\textsubscript{Chb} adopts the conformation found in phosphorylated...
IIBChb. As previously noted, the overall topology of IIBChb and IIIMtl are remarkably similar, despite 8% sequence identity, and the Ca atoms of 71 out of 106 residues can be superimposed with an atomic r.m.s. difference of 2 Å. Fig. 7 shows a comparison of the phosphoryl transition states of the two complexes with the coordinates of IIBChb and IIIMtl superimposed. The structures of the IIAChb* trimer and IIIMtl monomer bear no resemblance to one another, and with the exception of a single turn of helix that fortuitously overlaps, the structural elements making up the binding site are entirely different. Nevertheless, the position of the His-P-Cys phosphoryl transition state is remarkably similar. In addition, the distribution of hydrophobic, polar, and charged residues within the binding sites of IIAChb* and IIIMtl, and likewise IIBChb and IIIBMtl, is broadly similar (compare Fig. 5 of this report with Fig. 6 of Ref. 36), although the interaction of IIAChb* with IIBChb involves a somewhat larger preponderance of hydrophobic residues than that between IIAChb and IIIMtl, and the cleft in which the active site histidine of IIAChb* is located is both deeper and narrower than that for IIIMtl. Thus, one might argue that these two PTS complexes from distinct sugar branches of the pathway illustrate an example of convergent evolution of the surfaces of active sites (in terms of shape and distribution of residue type) generated by completely different underlying backbone structural elements.

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