Solution Structure of a Post-transition State Analog of the Phosphotransfer Reaction between the A and B Cytoplasmic Domains of the Mannitol Transporter II\textsuperscript{Mannitol}\ of the \textit{Escherichia coli} Phosphotransferase System

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The solution structure of the post-transition state complex between the isolated cytoplasmic A (IIA\textsuperscript{Mtl}) and phosphorylated B (phospho-IIB\textsuperscript{Mtl}) domains of the mannitol transporter of the \textit{Escherichia coli} phosphotransferase system has been solved by NMR. The active site His-554 of IIA\textsuperscript{Mtl} was mutated to glutamine to block phosphoryl transfer activity, and the active site Cys-384 of IIB\textsuperscript{Mtl} (residues of IIBMtl are denoted in italic type) was substituted by serine to permit the formation of a stable phosphorylated form of IIB\textsuperscript{Mtl}. The two complementary interaction surfaces are predominantly hydrophobic, and two methionines on IIBMtl, Met-388 and Met-393, serve as anchors by interacting with two deep pockets on the surface of IIA\textsuperscript{Mtl}. With the exception of a salt bridge between the conserved Arg-538 of IIA\textsuperscript{Mtl} and the phosphoryl group of phospho-IIBMtl, electrostatic interactions between the two proteins are limited to the outer edges of the interface, are few in number, and appear to be weak. This accounts for the low affinity of the complex ($K_d \sim 3.7$ mM), which is optimally tuned to the intact biological system in which the A and B domains are expressed as a single polypeptide connected by a flexible 21-residue linker. The phosphoryl transition state can readily be modeled with no change in protein-protein orientation and minimal perturbations in both the backbone immediately adjacent to His-554 and Cys-384 and the side chains in close proximity to the phosphoryl group. Comparison with the previously solved structure of the IIA\textsuperscript{Mtl}-HPr complex reveals how IIA\textsuperscript{Mtl} uses the same interaction surface to recognize two structurally unrelated proteins and explains the much higher affinity of IIA\textsuperscript{Mtl} for HPr than II\textsuperscript{Mtl}.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)\cite{1} comprises a fundamental signal transduction pathway whereby phosphotransfer via a series of biomolecular steps is coupled to sugar transport across the membrane (1–4). The phosphoryl group originates on phosphoenolpyruvate, and the initial transfer steps, involving first enzyme I and subsequently HPr, are common to all components of the pathway. Thereafter, the phosphoryl group is transferred to sugar-specific enzymes II, of which there are four major classes, glucose (Glc), mannitol (Mtl), mannoside (Man), and chitobiase (Chb), which share no sequence similarity with one another (2, 3) and, with one exception (the B domains of enzymes II\textsuperscript{Mtl} and II\textsuperscript{Chb}), no structural similarity with one another either (4–15). Enzymes II generally comprise three domains, two cytoplasmic, A and B, and one transmembrane, C, which may or may not be covalently linked to one another (2–4). The A domain accepts the phosphoryl group from HPr and donates it to the B domain. Subsequently, the phosphoryl group is transferred from the B domain to the incoming sugar bound to the C domain. The complexes of the PTS are of considerable interest from the perspective of protein-protein interactions, since similar binding surfaces can recognize multiple, structurally different, targets. We have embarked on a long term structural study of the complexes of the PTS, and to date we have determined the solution NMR structures of the enzyme I-HPr complex (16), complexes of HPr with IIAGlc (17), IIA\textsuperscript{Mtl} (18), and IIA\textsuperscript{Man} (19), and the complex of IIA\textsuperscript{Glc} with IIB\textsuperscript{Glc} (19). In this paper, we present the solution structure of a post-transition state analog of the IIA\textsuperscript{Mtl}, IIB\textsuperscript{Mtl} complex. Specifically, we make use of the active site C384S mutant of IIB\textsuperscript{Mtl} to generate a stably phosphorylated form of IIB\textsuperscript{Mtl} (20, 21) and the active site H554Q mutant of IIAMtl to prevent phosphoryl transfer between IIA\textsuperscript{Mtl} and IIB\textsuperscript{Mtl}. (Throughout this work, residues of IIBMtl are denoted in italic type.) The complex reveals the structural basis of specific recognition between IIA\textsuperscript{Mtl} and IIB\textsuperscript{Mtl} and the interactions involved in phosphoryl transfer.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of II\textsuperscript{Mtl}(HS54Q) and Phospho-II\textsuperscript{Bmtl}(C384S)—** The region corresponding to the A domain (IIA\textsuperscript{Mtl}) of the mannitol transporter, residues 490–637, was cloned as described previously (18). From this original construct, the active site histidine residue (His-554) was mutated to a glutamine to disable its phosphoryl transfer activity. The new construct was verified by DNA sequencing and then subcloned into a modified pET-32a vector (14) to form a thioredoxin fusion protein with a His\textsuperscript{tag}. After transformation with an expression vector, \textit{Escherichia coli} strain BL21(DE3) (Novagen) was grown in either Luria Bertini or minimal media (with $^{15}$NH$_4$Cl and/or $^{13}$C$_6$-glucose as the sole nitrogen or carbon source, respectively), induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an $A_{600}$ of ~0.8, and harvested by centrifugation after 4 h of induction. After harvesting, the cell pellet was resuspended in 50 ml (per liter of culture) of 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride. The suspension was lysed by three passages through a microfluidizer and centrifuged at 10,000 × g for 20 min. The supernatant fraction was loaded...
Solution Structure of the IIA\textsuperscript{Mtl}-Phospho-IIB\textsuperscript{Mtl} Complex

onto a nickel-Sepharose column (~20 ml, Amersham Biosciences), and the fusion protein was eluted with a 100-ml gradient of imidazole (25–500 mM). The fusion protein was then dialyzed against 20 mM Tris, pH 8.0, 200 mM NaCl, and 2 mM β-mercaptoethanol, and digested with thrombin (10 NIH units/ml of protein). Thrombin was removed by passage over a benzamidine-Sepharose column (1 ml; Amersham Biosciences), followed by the addition of 1 mM phenylmethylsulfonyl fluoride. The cleaved His\textsubscript{6}-thioredoxin was removed by loading the digested proteins over a nickel-Sepharose column. IIA\textsuperscript{Mtl}(H554Q) was further purified by Sephadex-75 gel filtration column (Amersham Biosciences) equilibrated with 20 mM Tris, pH 7.4, 1 mM EDTA, and 0.01% (w/v) sodium azide.

The active site mutant of the B domain, IIB\textsuperscript{Mtl}(C384S), of the mannitol transporter (residues 375–476) was cloned, expressed, phosphorylated, and purified as described previously (21).

With the exception of the titration experiments, NMR samples contained 3 mM IIA\textsuperscript{Mtl}(H554Q) and 3 mM phospho-IIB\textsuperscript{Mtl}(C384S) in 20 mM Tris-d\textsubscript{4}, 0.01% (w/v) sodium azide, and either 90% H\textsubscript{2}O, 10% D\textsubscript{2}O or 99.996% D\textsubscript{2}O. Each sample contained complexes of one uniformly \textsuperscript{15}N/\textsuperscript{13}C-labeled protein with its unlabeled partner. For titration experiments, a 0.6 mM concentration of either protein was titrated with up to 1:12 molar ratios in 20 mM Tris, pH 7.4, and 0.01% (w/v) sodium azide.

NMR Spectroscopy—NMR spectra were collected at 30 °C on Bruker DRX800, DMX750, DMX600, DRX600, and DMX500 spectrometers equipped with either an x,y,z-shielded gradient triple resonance probe or a z-shielded gradient triple resonance cryoprobe. Spectra were processed with the NMRPipe package (22) and analyzed using the program PIPP/CAPP/STAPP (23).

Sequential and side-chain assignments of IIA\textsuperscript{Mtl}(H554Q) and phospho-IIB\textsuperscript{Mtl}(C384S) in a 1:1 mixture were performed using three-dimensional triple resonance through-bond scalar correlation experiments (three-dimensional HNCACB, CBCA(CO)NH, HBHA(CBCACO)NH, C(CO)NH, and H(CCO)NH) in conjunction with three-dimensional triple resonance through-bond scalar correlation experiments (NOE-derived interproton distances and torsion angles), a quartic van der Waals repulsion term for the nonbonded contacts (32), a multidimensional torsion angle data base potential of mean force (33), and a radius of gyration potential to ensure optimal packing (34).

Structure figures were generated using the programs VMD-XPLOR (35), RIBBONS (36), and GRASP (37). Reweighted atomic probability density maps were calculated as described previously (38).

RESULTS AND DISCUSSION

Equilibrium Binding of IIA\textsuperscript{Mtl}(H554Q) and Phospho-IIB\textsuperscript{Mtl}(C384S)—The mannitol transporter consists of a single polypeptide comprising three independent domains (C, B, and A from N to C terminus), connected by long flexible linkers (39, 40). The linker between the B and A domains is 21 residues and extends from residues 472–492 of the full-length protein (10, 14). We studied the interaction of constructs comprising the isolated B (residues 371–474) and A domains (490–637) by \textsuperscript{1}H–\textsuperscript{15}N correlation spectroscopy. The combined results from two titration experiments, in which unlabeled phospho-IIB\textsuperscript{Mtl}(C384S) was titrated into \textsuperscript{15}N-labeled IIA\textsuperscript{Mtl}(H554Q) and vice versa, are displayed in Fig. 1. Exchange between the free proteins and the complex is fast on the chemical shift scale, and binding is readily monitored by following the chemical shift perturbation of the \textsuperscript{15}N-labeled partner upon the addition of the unlabeled second protein. The binding of the two proteins is weak, and nonlinear least squares optimization of all of the titration data simultaneously yields an equilibrium dissociation constant, K\textsubscript{d} of 3.7 ± 0.2 mM. Essentially identical results were obtained using the wild type proteins.
FIGURE 2. Intermolecular NOEs in the IIAMtl(H554Q)-phospho-IIBMtl(C384S) complex. NOEs in a three-dimensional $^{13}$C-separated ($F_2$)/$^{12}$C-filtered ($F_3$) NOE experiment recorded in D$_2$O are specifically observed from protons attached to $^{13}$C (in the $F_1$ dimension) to protons attached to $^{12}$C (in the $F_3$ dimension). In A, IIAMtl(H554Q) is $^{15}$N/$^{13}$C-labeled, and phospho-IIBMtl(C384S) is unlabeled; in B, phospho-IIAMtl(H554Q) is $^{15}$N/$^{13}$C-labeled, and IIAMtl(C384S) is unlabeled. The asterisks denote residual diagonal (autocorrelation) peaks arising from the labeled partner. (Note that $^{13}$C-decoupling is not employed in the $F_3$ acquisition dimension, and hence residual autocorrelation peaks are split into two components separated by $\sim$130 Hz, corresponding to the $J_{CH}$ coupling; similarly, any incompletely suppressed cross-peaks arising from very high intensity intramolecular NOEs within the labeled partner will also be split by $\sim$130 Hz and are therefore easily distinguishable from intermolecular NOEs (50).) Residues of phospho-IIBMtl(C384S) are labeled in italic type.

TABLE 1

Structural statistics

The notation of the NMR structures is as follows: (SA) are the final 270 simulated annealing structures, (SA), is the restrained regularized mean structure.

|                          | (SA) | (SA) |
|--------------------------|------|------|
| Number of experimental NMR restraints |      |      |
| Intermolecular interproton distance restraints | 84   |      |
| IIAMtl intramolecular interproton distance restraints | 37   |      |
| IIAMtl intramolecular interproton distance restraints | 46   |      |
| IIAMtl interfacial side-chain torsion angle restraints | 36   |      |
| IIAMtl interfacial side-chain torsion angle restraints | 19   |      |
| Root mean square deviation from interproton distance restraints ($\AA$)$^b$ | 0.012 ± 0.031 | 0.009 |
| Root mean square deviation from side-chain torsion angle restraints (degrees)$^b$ | 0.007 ± 0.001 | 0.31  |
| Measures of structural quality$^c$ |      |      |
| Intermolecular repulsion energy (kcal/mol$^{-1}$) | 3.8 ± 0.9 | 6.5   |
| Intermolecular Lennard-Jones energy (kcal/mol$^{-1}$) | −31.2 ± 3.1 | −36.7 |
| Coordinate precision of the complex ($\AA$)$^d$ |      |      |
| Complete backbone (N, Ca, C, O) atoms | 0.10  |      |
| Interfacial side chain heavy atoms | 0.65  |      |

$^a$ The intramolecular NOE-derived interproton distance restraints relate only to interfacial side chains.

$^b$ None of the structures exhibit interproton distance violations $>0.3 \text{ \AA}$ or torsion angle violations $>5^\circ$.

$^c$ The intermolecular repulsion energy is given by the value of the quartic van der Waals repulsion term calculated with a force constant of 4 kcal/mol$^{-1}$Å$^{-4}$ and a van der Waals radius scale factor of 0.78. The intermolecular Lennard-Jones term van der Waals interaction energy is calculated using the CHARMM19/20 parameters and is not included in the target function used to calculate the structures.

$^d$ Defined as the average root mean square difference between the final 270 simulated annealing structures and the mean coordinates. The value quoted for the complete backbone provides only a measure of the precision with which the orientation and translation of the two proteins in the complex have been determined and does not take into account the backbone accuracy of the x-ray/NMR coordinates of IIAMtl and the NMR coordinates of phospho-IIBMtl used for conjoined rigid body/torsion angle dynamics docking. The accuracy of the x-ray coordinates of IIAMtl (including NMR refinement of the four variable regions comprising residues 540–543, 555–567, 580–585, and 593–599 on the basis of backbone RDCs) is likely to be around 0.3 Å, judging from the crystallographic resolution and R-factor (10) as well as the dipolar coupling R-factors (this paper). The accuracy of the restrained regularized mean coordinates of phospho-IIBMtl can be estimated from the coordinate precision (0.3 Å) and the values of 14–16% for cross-validated N-H dipolar coupling R-factors in two alignment media (phage pf1 and neutral polyacrylamide gel (21)), which suggest that the coordinate accuracy is comparable with a 1.5–2 Å resolution crystal structure (19, 45). The percentage of residues in the most favored region of the Ramachandran map (51) is 92% for IIAMtl and 91% for phospho-IIBMtl.
Such weak yet specific binding may at first appear surprising but is in fact optimally tuned to the intact biological system. The average end-to-end distance of an unstructured 21-residue linker (given by $\sqrt{C_n n l}$, where $n$ is the number of residues, $C_n$ is the characteristic ratio, which has a value of 11 for $n = 21$, and $l$ is the average C–C distance, which has a value of 3.8 Å) (41) would be expected to be ~46 Å. If one considers one of the domains to be fixed in space, then the effect of the linker is to constrain the second domain to a sphere of average volume $4.1 \times 10^{-22}$ liters, which corresponds to an effective concentration of ~4 mM.

Thus, in the intact protein one would expect the A domain to be bound to the B domain ~50% of the time, which is perfectly reasonable, given that the B domain must use approximately the same interaction surface, centered around the active site cysteine at position 384, to accept the phosphoryl group from the A domain and subsequently transfer the phosphoryl group onto the incoming sugar on the C domain.

It is also worth noting that the intact mannitol transporter is a dimer, with dimerization occurring solely through the transmembrane C domain (42). No homodimeric interactions between A domains or B domains are likely to occur, since the isolated A and B domains do not self-associate in free solution, even at high concentrations (in the millimolar range) (14, 18). Clearly, phosphoryl transfer between the A and B domains can occur via either intra- or intersubunit interactions between the A and B domains. Mutagenesis and complementation experiments indicate that whereas phosphoryl transfer can occur via both routes, intrasubunit phosphoryl transfer is dominant (43). The above argument is consistent with the latter experimental observation, since the volume in which the A domain would be constrained relative to the B domain would be larger in the context of the intersubunit than the intrasubunit interaction, and hence the occupancy of the intersubunit IIAMtl-IIBMtl complex would be expected to be less than that of the intrasubunit complex.

**Structure Determination**—The solution NMR structures of IIBMtl (14) and phospho-IIBMtl(C384S) (21) have been determined previously, and the only structural differences between the phosphorylated and unphosphorylated forms are confined to the active site loop (resides 383–393) and involve a backbone atomic root mean square shift of only ~0.7 Å. Extensive use of residual dipolar couplings (RDC) was made in these structure determinations (14, 21), and cross-validation using RDCs in multiple alignment media (21) indicate that the accuracy of the coordinates is high, with cross-validated dipolar coupling R-factors (44) of 14–16% (in phage pf1 and neutral polyacrylamide gel), which is equivalent to values expected for 1.5–2 Å resolution crystal structures.
Solution Structure of the IIA(Mtl)-Phospho-IIBMtl Complex

The crystal structure of IIA(Mtl) has been determined at 2.0 Å resolution (10). The unit cell in the crystal comprises a dimer of dimers (note that IIA(Mtl) does not self-associate in solution even at the high concentrations employed by NMR) (18). The structure of the four molecules of IIA(Mtl) in the unit cell are very similar, with an atomic root mean square difference of 0.3 Å, but four regions (residues 540–543, 555–567, 580–585, and 593–599), comprising loops, exhibit differences between the two pairs of molecules in the unit cell with maximal backbone atomic displacements in the range 1.5–3 Å. The structure of these loops in solution, both free and bound to HPr, coincides with that found in molecule D of the crystal structure as judged from RDC analysis, and these four regions were refined independently using RDCs in two alignment media in the solution structure determination of the IIA(Mtl)-HPr complex (18). RDCs measured in neutral polyacrylamide gel on free IIA(Mtl)(H554Q) are in excellent agreement with the coordinates of IIA(Mtl) in the IIA(Mtl)-HPr complex (18) with a dipolar coupling R-factor of 17%, a correlation coefficient of 0.98, and no deviations above average for the RDCs in the region of the mutation, indicating that the structure remains unaltered within coordinate errors (45, 46). Since the chemical shift perturbations for both proteins upon formation of the IIA(Mtl)(H554Q)-phospho-IIBMtl(C384S) complex are very small, the largest being less than 0.3 ppm in 1H (for Asp-454 of IIB(Mtl)) and 1 ppm in 13N (for Leu-611 of IIA(Mtl)), we conclude that the backbone of both IIA(Mtl)(H554Q) and phospho-IIBMtl(C384S) remains essentially unchanged upon complex formation. Consequently, the structure determination was carried out using conjoined rigid body/torsion angle dynamics (28) based on the coordinates of free phospho-IIBMtl(C384S) (21) (Protein Data Bank accession code 1VRV) and the coordinates of IIA(Mtl) in the IIA(Mtl)-HPr complex (18) (Protein Data Bank accession code 1J6T). The coordinates of the backbone and interfacial side chains were held fixed, and rigid body docking with full torsional degrees of freedom for the interfacial side chains was driven by interproton distance restraints derived from intermolecular NOE data coupled with torsion angle restraints derived from both heteronuclear coupling constant and short mixing time NOE data. Examples of the quality of the intermolecular NOE data obtained from three-dimensional 13C-separated/12C-filtered NOE experiments that provide exclusively intermolecular NOEs from protons attached to 13C on the 13C-labeled protein to protons attached to 12C on the unlabeled protein, are shown in Fig. 2. A summary of the structural statistics is given in Table 1, a superposition of the backbone of the final 270 simulated annealing structures is shown in Fig. 3A, and an atomic density probability map representation of some interfacial side chains is depicted in Fig. 3B.

The NMR experiments were carried out on samples comprising a 1:1 mixture of the proteins, each at a concentration of 3 mM. Under these conditions, only 30% of each protein is in the complex (i.e. the concentration of the complex in the sample is ~1 mM). This does not affect the observation of intermolecular NOEs. However, it does impede the use of RDCs to provide accurate and reliable information on the relative orientation of the two proteins in the complex. Since the observed RDCs in a fast exchanging system are a weighted average of the RDCs for the free and complexed protein, one could in principal back-calculate the RDCs for the pure complex on the basis of experimental RDCs measured on the free proteins and the mixture of the two proteins (19, 47). However, this requires very accurate RDC measurements under nearly identical alignment conditions as well as accurate knowledge of the fraction of bound protein. Moreover, in a mixture of labeled and unlabeled partners, the fraction of the labeled partner (on which the RDCs are measured in the mixture) should typically exceed ~30% to ensure that the complex makes a significant contribution to the observed RDCs. Because, in this instance, the two proteins bind so weakly to one another, relatively high concentrations of the unlabeled partner (>2 mM) are required. Despite attempts using a variety of alignment media, we were not able to find experimental conditions that permitted reliable back-calculation of the RDCs due to either differential interaction of one of the partners with the alignment medium or differential perturbation of the alignment tensor by the unlabeled partner arising from molecular crowding at these protein concentrations.

Overall Structure of the Complex—Ribbon diagrams providing an overall view of the complex are shown in Fig. 4. The Ca–Ca separation between the last ordered residue at the C terminus of phospho-IIBMtl (residue 471) and the first ordered residue of IIA(Mtl) (residue 493) is 38 Å, a distance that can readily be accommodated by the flexible 21-residue linker. The interaction surface on IIA(Mtl) comprises 27 residues involving three segments of polypeptide chain: residues 538–557 include the C-terminal end of helix α2, a small antiparallel β-sheet formed by strands β2 and β3 connected by a hairpin turn, and the active site residue at position 554; residues 480–585 comprise a loop connecting strands β4 and β5; and residues 598–612 encompass helix α3 (residues 600–610). The interaction surface on IIBMtl is made up of 18 residues located in four segments of polypeptide chain: residues 384–400 include the active site loop (residues 384–389) and most of helix α1 (residues 390–404); residue 414 is the C-terminal residue of strand β2; residues 430–433 make up the first turn of helix α2; and residues 451–453 are located in the loop connecting strand β4 and helix α3. The residues comprising the interaction surface of IIBMtl are highly conserved throughout Gram-positive and Gram-negative bacteria (48): 14 of 18 residues are conserved absolutely; two are subject to highly conservative
changes and preserve the functional group (Arg-399 to lysine, His-430 to glutamine); and one residue, Gly-396, can be substituted conservatively by a serine. Only Lys-400 is subject to nonconservative substitutions from lysine in Gram-negative bacteria to asparagine or aspartate in Gram-positive bacteria. It is also worth noting that residues 382–394 comprising the complete active site loop and the first turn of helix H9251 are conserved throughout.

The interaction surfaces of IIAMtl and IIBMtl are approximately circular in shape and comparable in size (28 × 27 Å for IIAMtl and 22 × 27 Å for IIBMtl) and are composed of predominantly hydrophobic residues, with 60–65% of the atoms being nonpolar. The interaction surface on IIAMtl is concave, whereas that on IIBMtl is largely convex, thereby providing overall complementarity of fit. The total accessible surface buried at the interface is 1575 Å², of which 735 Å² originates from IIAMtl and 840 Å² from IIBMtl. The gap volume index (defined as the ratio of gap volume to interface accessible surface area) is 2.7, a value typical of optional complexes (i.e., heterocomplexes where the individual components of the complex can also exist as monomers) (49). The predominant intermolecular contacts between secondary structure elements involve a helix-helix interaction between helix α3 of IIAMtl and helix α1 of IIBMtl, oriented at an angle of ~60°.

The IIAMtl-IIBMtl Interface—A stereoview of the interface is shown in Fig. 5A, together with selected close-ups in Fig. 5B and C; surface representations of the interfaces are given in Fig. 6A; and a schematic summary of the intermolecular contacts is provided in Fig. 7. A virtually complete, horseshoe-shaped ring of hydrophobic residues surrounds the active site phospho-Ser-384 of IIBMtl (Fig. 6A, bottom panel). Several intermolecular interactions are noteworthy. The two methionines on IIBMtl, Met-393 and Met-388, make extensive hydrophobic contacts to residues in two deep pockets located at the top and bottom halves, respectively, of the IIAMtl interface (in the views shown in Figs. 5 and 6) that serve to anchor IIBMtl onto IIAMtl. The top pocket is formed by
Leu-546, Gly-547, Glu-548, Ile-550, Ile-604, and Thr-608. The bottom pocket comprises Arg-538, the active site residue Gln-554, Val-557, His-600, and Ile-601. The residues in both pockets are either conserved or substituted conservatively in Gram-negative and positive bacteria (48). The close contacts between Met-338 of IIIBMtl and Arg-538 and Gln-554 of IIAMtl serve to position the latter two residues such that the carboxyamide of Gln-554 is directed toward phospho-Ser-384, and the guanidino group of Arg-538 makes a direct salt bridge with the phosphate group of phospho-Ser-384. The critical role of Arg-538 is supported by its conservation throughout Gram-negative and Gram-positive bacteria, (48). The negative charge on Asp-385, located adjacent to phospho-Ser-384, is partially offset by a hydrogen bonding interaction with the hydroxyl group of Thr-542. There are also a number of weak complementary electrostatic interactions, asymmetrically situated along the outer edges of the interaction surfaces that facilitate the correct orientation of the two proteins. These include interactions between Lys-400 and Asp-612 and Asn-609, between Arg-399 and Thr-605 and Asn-609, between Asn-451 and Glu-582 and Asp-585, and between His-430 and Glu-581. These electrostatic interactions, however, are likely to be relatively weak, since the functional groups are separated by \( \approx 4 \) Å.

The Phosphoryl Transition State—The phosphoryl transition state can be readily modeled by substituting Gln-554 for His and Ser-384 for Cys and minimizing the restrained regularized mean coordinates of the IIAMtl- phospho-IIIBMtl complex subject to geometrical restraints for the His-phosphoryl-Cys transition state in conjunction with the experimental NMR restraints, only allowing the backbone and active site residue of the interacting partner are displayed as tubes and bonds, respectively. Also shown in the top panel of A are the side chains of Met-388 and Met-383 in gold, illustrating how their methyl group points directly into deep hydrophobic pockets on the surface of IIAMtl. Residues of phospho-IIIBMtl(C384S) and HPr are labeled in italic type. The coordinates of the IIAMtl-HPr complex are taken from Ref. 18.
553–555 of IIAMtl) and the side chains of the interfacial residues to move (15). The geometric restraints include N–P and S–P bond lengths, a planarity term to ensure that the phosphorus atom lies in the plane of the imidazole ring of His-554 and the sulfur atom of Cys-384, and bond angle terms to enforce trigonal bipyramidal geometry for the phosphoryl group (15). A dissociative transition state complex (N–P and S–P bond lengths given by the sum of the van der Waals radii of the atoms, 3.4 and 3.7 Å, respectively) can be formed with minimal atomic root mean square shifts of the backbone in the vicinity of the active site residues (0.2 Å) and compensatory minor displacements of the side chains of Cys-384, Met-388, Arg-438, and His-554 to accommodate the change in the position of the phosphoryl group (Fig. 8). An associative transition state complex (N–P and S–P bond lengths of 2.4 and 2.8 Å) can also be formed but involves slightly larger backbone displacements (0.33 Å for residues 383–385 and 0.22 Å for residues 553–555). The transition state thus preserves all of the intermolecular interactions seen in the post-transition state analogue, including the neutralization of the phosphate group by the guanidino group of Arg-438. However, the hydrogen bonds to the phosphoryl group from the hydroxyl groups of Ser-390 and Ser-391 and from the backbone amide of Ser-390 seen in phospho-IIAMtl (C384S) are no longer present in the transition state due to the shift in the position of the phosphoryl group toward His-554 of IIAMtl.

Mechanism of Phosphoryl Transfer—In vivo phosphoryl transfer proceeds from IIAMtl to IIAMtl. A proposed mechanism for phosphoryl transfer is shown in Fig. 9. Modeling phospho-IIAMtl on the basis of the crystal structure of IIAMtl (10) suggests that the phosphoryl group, bonded to the N9280 atom of His-554, accepts three hydrogen bonds: two from the guanidino group of Arg-538 and a potential third from the imidazole ring of His-600 (N9280 atom) if the 1/H110021/1/H110022/1/H110011/1/H110022 conformation of the latter is changed from g/H11002/g/H11002 (seen in the IIAMtl-phospho-IIB Mtl complex) to g/H11001/g/H11002. (Note in the crystal structure of IIAMtl both rotamers are observed (10).) The N92541-H atom of His-554 accepts a hydrogen bond from the backbone of Val-452, thereby stabilizing the N92541-H tautomeric state. The thiolate state of Cys-384 of IIBMtl is stabilized by numerous hydrogen bonding interactions within the active site loop (14). Upon formation of the pretransition state phospho-IIAMtl-IIIB Mtl encounter complex, the hydrogen bond...
between the imidazole ring of His-600 and the phosphoryl group is broken due to steric clash with IIIBMtl, which precludes the conformation. Nucleophilic attack at the phosphoryl group by the thiolate of Cys-384 results in the formation of a transition state in which the phosphoryl group accepts two hydrogen bonds from the backbone amide of Met-388 and Gly-389 of IIAMtl and one hydrogen bond from the guanidino group of Arg-538 of IIAMtl (Fig. 8). Resolution of the transition state to the post-transition state complex results in further electrostatic interactions in which the phosphoryl group accepts five hydrogen bonds from the active site loop of IIIBMtl (the backbone amides of Met-388, Gly-389, and Ser-391 and the hydroxyl groups of Ser-390 and Ser-391) and one from the guanidino group of Arg-538 of IIAMtl and is partially neutralized by the positive helix dipole at the N terminus of helix H9251 of IIBMtl. Thus, the number of hydrogen bonding/electrostatic interactions involving the phosphoryl group in the post-transition state IIA Mtl-phospho-IIBMtl complex is larger than in the pretransition state phospho-IIA Mtl-IIBMtl complex. In addition, in the transition state, more hydrogen bonds to the phosphoryl group originate from IIBMtl than IIAMtl. Thus, whereas phosphoryl transfer between IIAMtl and IIBMtl is fully reversible in vitro, one would expect the flow of the phosphoryl group from IIAMtl to IIBMtl to be favored, in accord with the biological function of the PTS pathway.

Comparison of the Interactions of IIBMtl and HPr with IIA Mtl—A comparison of the IIA Mtl(H554Q)-phospho-IIBMtl(C384S) and IIA Mtl-HPr (18) complexes is provided in Figs. 6 and 7. Of the 27 residues of IIA Mtl that interact with IIBMtl and the 25 that interact with HPr, 23 are shared by the two interfaces (Fig. 7). Thus, IIA Mtl uses essentially the same interaction surface to recognize two structurally different proteins, HPr and IIBMtl. The shapes of the IIBMtl and HPr interaction surfaces are similar but bear no similarity to one another in terms of the underlying backbone topology or the orientation of secondary structure elements relative to the IIA Mtl surface (Fig. 6). Indeed, helix α2 of IIA Mtl lies approximately orthogonal to helices 1 and 2 of HPr (cf. top panels of Fig. 6, A and B). There are, however, a number of key differences between the interaction surfaces of IIBMtl and HPr that are noteworthy. First, the number of positively charged residues on the interaction surface of IIBMtl is half that for HPr (2 compared with 4). Three of the positively charged residues on the HPr interaction surface are involved in electrostatic interactions with negatively charged residues on IIA Mtl, whereas for IIBMtl, only Lys-400 interacts with a negatively charged residue (Asp-612) on IIA Mtl. This probably accounts for the much lower affinity (about 2 orders of magnitude) of the IIA Mtl-IIBMtl complex relative to the IIA Mtl-HPr complex (18). Second, the interaction surface on IIBMtl is significantly more hydrophobic than that on HPr (Fig. 6, A and B, bottom panels). Finally, the interaction surface on IIBMtl includes a nega-
tively charged residue (Asp-385), whereas there are no negative charges in the case of HPr. The latter is probably important in ensuring that direct interaction and phosphoryl transfer between IIBMtl and enzyme I, bypassing HPr and IIAmtl, does not occur; the presence of a negatively charged residue in close proximity to the active site residue in IIBMtl would lead to unfavorable electrostatic interactions with the interaction surface on enzyme I that contacts HPr (16).

Comparison of the IIAmtl(H554Q)-phospho-IIBMtl(C384S) and IIAmtl-IBBmtl Complexes—Although the A and B components of enzymes IIAmtl and IIBMtl bear no sequence, secondary structure or topological similarity to one another (2, 3, 5, 6, 14, 15), the two complexes share a number of features in common as well as some significant differences. In both cases, phosphoryl transfer occurs from a histidine on the A domain to a cysteine on the B domain (2, 3). Unlike IIBMtl, the A and B domains of IIAmtl are expressed as separate polypeptides (2, 3). The general surface features of the binding interfaces on IIAmtl and IIBMtl for their target proteins (HPr and the corresponding B domains) are broadly similar in terms of size and shape, each comprising a central hydrophobic region surrounding the active site residue and an outer ring of charged residues (this work) (15). However, the IIAmtl interface has a much larger preponderance of negatively charged residues (8 versus 4 for IIBMtl; cf. Ref. 15). The active site loops of IBBmtl and IIBMtl display similarities in so far that the thiolate state of the active site cysteine is stabilized by hydrogen bonding interactions with backbone amide protons located in the active site loop, reminiscent of the active site loop of eukaryotic protein tyrosine kinases (14, 15). However, the structural correspondence with the latter is far more extensive for IIBMtl (14, 21) than IIBmtl (15).

The affinity of IIAmtl for IIBMtl (15) is about 2 orders of magnitude higher than that of IIBMtl for IIBMtl. From a functional perspective, this and downstream, IIBMtl (this paper), interaction partners, although surface (72), thus, a cluster of two positive charges at one edge of the HPr interaction surface, may play a key role in the initial docking event. For the IIAmtl(H554Q)-phospho-IIBMtl(C384S) complex, on the other hand, the electrostatic interactions are clearly very weak, and interactions between complementary, localized hydrophobic features of the interaction surfaces may be more important, with the two conserved methionines of IIBMtl serving as hooks that latch on to the two deep hydrophobic pockets on the surface of IIAmtl (Figs. 5, B and C, and 6A). Methionine is ideally suited to such a role, since its unbranched hydrophobic side chain can sample extensive configurational space, thereby permitting optimization of its interactions with a target surface. Indeed, extensive use of methionines is employed by calmodulin to recognize many different interaction partners (50). Thus, like other complexes of the PTS, the IIAmtl(H554Q)-phospho-IIBMtl(C384S) complex illustrates the versatility of a protein interaction network in which each protein recognizes its upstream and downstream partner using the same interaction surface. Key features of these interactions are complementarity of shape and residue type that can be achieved using a wide array of underlying structural elements; surface side-chain conformational plasticity, particularly involving long side chains (both hydrophobic and charged), to optimize intermolecular interactions; asymmetric distribution of complementary, intermolecular electrostatic interactions to aid in guiding correct docking; and finally extensive redundancy, thereby ensuring that the overall interaction energy is not dominated by any single interaction but by a multitude of interactions, each contributing only a small proportion of the total interaction energy. The last is important, since, as clearly illustrated by the structures of various protein-protein complexes of this PTS (this paper) (15–19), not every charged residue located in the binding surface of a particular PTS protein need be involved in electrostatic interactions with complementary residues of its diverse interaction partners. Whereas the presence of inherent redundancy does not impact specificity, it will generally result in relatively weak protein-protein interactions. In the case of the PTS, such weak interactions, which are tuned to the microto millimolar range, are critical to function that requires rapid dissociation of transient protein-protein complexes to permit efficient phosphoryl transfer down the reaction cascade.

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