The solution of the complex between the cytoplasmic A domain (IIAMtl) of the mannitol transporter II(Mannitol) and the histidine-containing phosphocarrier protein (HPr) of the *Escherichia coli* phosphotransferase system has been solved by NMR, including the use of conjoined rigid body/torsion angle dynamics, to permit accurate orientation of the two proteins. A convex surface on HPr, formed by helices 1 and 2, interacts with a complementary concave depression on the surface of IIAMtl formed by helices 3, portions of helices 2 and 4, and β-strands 2 and 3. The majority of intermolecular contacts are hydrophobic, with a small number of electrostatic interactions at the periphery of the surface. The active site histidines, His-15 of HPr and His-65 of IIAMtl, are in close spatial proximity, and a pentacoordinate phosphoryl transition state can be readily accommodated with no change in protein-protein orientation and only minimal perturbations of the backbone immediately adjacent to the histidines. Comparison with two previously solved structures of complexes of HPr with partner proteins of the phosphotransferase system, the N-terminal domain of enzyme I (EIN) and enzyme IIAGlucose (IIAGlc), reveals a number of common features despite the fact that EIN, IIAGlc, and IIAMtl bear no structural resemblance to one another. Thus, entirely different underlying structural elements can form binding surfaces for HPr that are similar in terms of both shape and residue composition. These structural comparisons illustrate the roles of surface and residue complementarity, redundancy, incremental build-up of specificity and conformational side chain plasticity in the formation of transient specific protein-protein complexes in signal transduction pathways.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (1) is a classical example of a signal transduction pathway whereby the transfer of a phosphoryl group through a series of biomolecular protein-protein complexes is coupled with the transport of sugars across the membrane (2–4). The initial events of the cascade involve a common pathway: enzyme I, which is autophosphorylated by phosphoenolpyruvate at His-189 (in *Escherichia coli*), transfers the phosphoryl group to His-15 (in *E. coli*) of the histidine-containing phosphocarrier protein (HPr). Subsequently, the phosphoryl group on HPr is transferred to a variety of sugar-specific carbohydrate transporters, known as enzymes II (5). The enzymes II are organized into several domains, some of which are covalently linked (5). There are two cytoplasmic domains, IIA and IIB; IIA recognizes the phosphoryl group from HPr and then transfers it to IIB. The transmembrane domain IIC (and in some cases IID as well) catalyzes the translocation and phosphoryl transfer from IIB to the incoming sugar. There are five classes of enzymes II as follows: glucose-sucrose, mannitol-fructose, mannose-sorbitose, lactose-cellobiose, and glucitol (3, 5). The membrane-bound IIC domains comprise a variable number of transmembrane helices (5). The IIA and IIB domains for the different sugar classes bear no sequence or structural similarity to one another (6–15).

The bacterial phosphoenolpyruvate:sugar phosphotransferase system provides a paradigm for understanding protein-protein interactions and the factors governing their specificity. Thus, for example, HPr recognizes enzyme I and the various sugar-specific IIA domains, although all these target proteins are structurally dissimilar. We have recently solved the structures of the complexes between the N-terminal phosphoryl transfer domain of EI (EIN) and HPr (16) and between IIAGlucose (IIAGlc) and HPr (17). In the present paper, we extend these studies to the solution structure determination of the complex between IIAMtl (IIAMtl) and HPr.

**EXPERIMENTAL PROCEDURES**

*Expression Vector for IIAMtl*—E. coli chromosomal DNA was used as a template to amplify by PCR the region corresponding to the A domain of the mannitol permease. The forward primer 5′-GACAGCCTTT-GACGATCATATGGCTAACAAGTGTTCAAG-3′ contained an engineered *NdeI* restriction site (underlined), and the reverse primer 5′-TTAAC-CCCCACCTTCATCATCGAGGAGGATGGGATTGAGG-3′ contained an engineered *SacII* site (underlined). The *NdeI* and *SacII*-cut PCR product was purified and cloned into the corresponding sites of the vector pREI**.

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1 The abbreviations used are: HPr, histidine-containing phosphocarrier protein; EI, enzyme I; EIN, N-terminal domain of enzyme I, IIAMtl, cytoplasmic A domain of the mannitol-specific transporter II(Mannitol); IIAGlc, glucose-specific enzyme II; NOE, nuclear Overhauser effect; r.m.s., root mean square.
The expressed product from the recombinant plasmid (pLP1572) was identical in sequence to that from the clone described by Van Weeghel et al. (19).

**Protein Expression**—The recombinant plasmids, pSP100 for HP expression (20) and pLP1572 for IIAmt expression, were introduced into E. coli GIB96 for protein expression induced by tryptophan (21). Four-liter cultures were grown in minimal labeling medium (MLM) (22) supplemented with ampicillin (100 μg/ml). For labeling with 15C2glucose and/or 15NH4Cl, the media contained 2.5 g of 15C2glucose/liter and/or 1 g of 15NH4Cl, respectively. Induction was continued overnight.

**Protein Purification**—E. coli HPr, unlabeled and/or isotopically labeled with 15N (>95%) and 13C (>95%), was purified as described elsewhere (17, 23).

The 148 residue IIAmt domain, containing a methionine residue in front of alanine 491, was purified as follows. Washed cells from a 4-liter culture were suspended in 40 ml of 10 mM Tris-Cl (pH 7.5), 1 mM EDTA (Buffer A). The cell suspension was passed twice through a French press at 10,000 pounds/square inch. The ruptured cell fraction was centrifuged at 100,000 g to yield the supernatant fraction which was applied to a DE52 anion-exchange column (1.5 x 40 cm) equilibrated with 20 mM Tris-Cl (pH 8.5), 1 mM dithiotreitol (Buffer E). The column was washed with 100 ml of Buffer E supplemented with 100 mM NaCl (Buffer EE). Then a linear gradient (from 500 ml of Buffer EE to 500 ml of Buffer E + 400 mM NaCl (Buffer F)) was run. Column fractions were screened by SDS-PAGE. Fractions enriched in IIAmt were pooled and concentrated by ultrafiltration through Filtron 3K membranes. Further purification was achieved by gel filtration chromatography on a AcA-44 Urtregel column (2.6 x 100 cm) run with 10 mM Tris-Cl (pH 7.5) + 100 mM NaCl. Fractions enriched in IIAmt were pooled and concentrated as before. All protein samples used for the NMR studies were greater than 95% pure, as judged by both SDS-PAGE and two-dimensional 1H-15N correlation spectra.

The majority of NMR samples contained 1% 15N- and 1% 13C-labeled IIAmt-HPr complex in 10 mM phosphate buffer, pH 7.0. The following samples were employed (only the presence of 15N and 13C isotopes are indicated; if no C or N isotope is mentioned, then the sample contained 12C or 14N at natural isotopic abundance): IIAmt(15N)-HPr, IIAmt(13C)-HPr(15N), IIAmt(15N)-HPr(13C), IIAmt(15N/13C)-HPr, IIAmt(15N)-HPr(13C), and IIAmt(15N/13C)-HPr(13C).

**NMR Spectroscopy**—All spectra were recorded at 35 °C on Bruker DMX500, DMX600, DMX750, and DRX800 spectrometers equipped with x,y,z-shielded gradient triple resonance probes. Spectra were processed with the NMRPipe package (24) and analyzed using the programs NMRDraw and NMRShine (24), and PIPP, CAPP, and STAPP (25). 1H, 15N, and 13C sequential assignments were obtained using three-dimensional double- and triple-resonance through bond correlation experiments (26–28). Three-dimensional experiments employed for sequential assignments included HINCACB, CBCA(CO)NH, HBHA(CB-CACO)NH, H(CCO)NH, C(CCO)NH, and HCCH-TOCSY experiments. 1JHC−1JC−1JC and 1JCC−1JC couplings were measured using quantitative 1JHC−1JC−1JC−1JC-NOE (29–31). Residual dipolar couplings were derived from multidimensional NOE spectra recorded with mixing times ranging from 75 to 120 ms. NOE experiments included three-dimensional 15N-separated, 13C-separated, and 15N-separated/13C-filtered NOE spectra, and two-dimensional 15N-separated/13C-filtered, 15N-filtered/13C-separated, and 13C-filtered/15N-filtered NOE spectra (26).

Residual 1DHN, 1DCO, and 1DCN dipolar couplings were obtained by taking the difference in the corresponding J splittings measured in magnetically oriented and isotropic (in water) media. The orienting liquid crystalline medium employed consisted of a 4–5% C12E5 polyethylene glycol (PEG)hexanol mixture with a surfactant to alcohol ratio of 1:35. Dipolar couplings were measured on IIAmt-HPr(15N/13C)-HPr complexes using two-dimensional in-phase/anti-phase (15N,H) heteronuclear single quantum coherence experiments (33). 1DCO and 1DCN couplings were measured on IIAmt-HPr(15N/13C) and IIAmt(15N/13C)-HPr(15N/13C) complexes using three-dimensional (HCACO)NH and (TCOSY)HNOE experiments (34), respectively. In all cases the coupled magnetically aligned proteins ranged in size from 0.6 to 1.0 ms.

The isolated IIAmt domain is catalytically active and can accept a phosphoryl group from HPr and transfer it to both the isolated IIBmt domain as well as IICmt (19, 55). We have solved the structure of the IIAmt-HPr complex by multidimensional NMR. For consistency with previous structural work (11, 57, 58), residues 490–637 of IIBmt are numbered 1–148 in the cloned, expressed IIAmt domain; hence, in this numbering scheme, the active site histidine (residue 554 in the full-length IIBmt sequence (59, 60)) is located at position 65.

IIAmt and HPr are in fast exchange on the chemical shift scale. The equilibrium association constant derived from NMR titration studies by monitoring 1H,15N cross-peaks of 15N-labeled IIAmt upon addition of unlabeled HPr is ~2 x 104 M−1. The lower limit for the dissociation rate constant, derived from the maximal observed 1HShift between free and bound states, is ~4500 s−1.

A combination of isotopically (15N and/or 15C) labeled proteins was used to simplify the spectra for assignment purposes and to specifically observe intermolecular nuclear Overhauser effects (NOEs) (26). An example of the quality of the data is illustrated in Fig. 1 which shows a series of strips from three-dimensional 13C-separated/13C-filtered NOE spectra in which NOEs are observed specifically from protons attached to 13C on one protein to protons attached to 14C on the other.

High resolution crystal structures of both HPr (37) and IIAmt (11) become available and therefore permit the structure of the complex to be solved by conjoined

RESULTS AND DISCUSSION

**Structure Determination**—The physiological form of enzyme IIBmt consists of the three domains, A–C, joined together in a single polypeptide chain by flexible linkers of variable length (51, 52). The membrane-bound IICmt domain is at the N terminus (residues 1–547), followed by the cytoplasmic IIBmt domains 348–489 with the IIAmt (residues 490–637) domain at the C terminus (19, 53–56). The isolated IIAmt domain is catalytically active and can accept a phosphoryl group from HPr and transfer it to both the isolated IIBmt domain as well as IICmt (19, 55). We have solved the structure of the IIAmt-HPr complex by multidimensional NMR. For consistency with previous structural work (11, 57, 58), residues 490–637 of IIBmt are numbered 1–148 in the cloned, expressed IIAmt domain; hence, in this numbering scheme, the active site histidine (residue 554 in the full-length IIBmt sequence (59, 60)) is located at position 65.

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High resolution crystal structures of both HPr (37) and IIAmt (11) become available and therefore permit the structure of the complex to be solved by conjoined
rigid body/torsion angle dynamics (40, 41), in a manner analo-
gous to that employed for the HPrt-IIAMtl-Glc complex (17). In this
approach, it is necessary to either demonstrate that no signif-
icient conformational changes occur upon complex formation or
to localize regions where such changes may occur. This can be
rapidly assessed by the measurement of residual dipolar cou-
plings, which provide highly sensitive long range orientational
information (61–65).

For HPrt, agreement between observed \( ^1 \text{D}_{\text{NH}} \) dipolar cou-
plings measured on the complex dissolved in a dilute liquid
crystalline medium of polyethylene glycol/hexanol (33) and
those calculated from the x-ray structure of free HPrt is excel-
icient with a \( ^1 \text{D}_{\text{NH}} \) dipolar coupling R-factor, \( R_{\text{dip}}^{\text{NH}} \), of 19.1% and
a correlation coefficient of 0.96, consistent with the expec-
ted accuracy of a 2.5 Å resolution crystal structure.

The situation for IIAMtl is more complex, because the crystal
structure has four molecules (A–D) in the unit cell (11); mole-
cules A and B, and molecules C and D are very similar with an
overall backbone root mean square difference of \( -0.3 \) Å, con-
sistent with the expected coordinate errors for a 1.8 Å reso-
lation structure. However, the backbone of one region (I, residues
51–54) is displaced by up to \( -2 \) Å in molecule A relative to the
other three molecules, and three regions (II, 66–78; III, 91–96;
and IV, 104–110) exhibit maximal backbone displacements of
up to \( 1.5–3 \) Å between the two pairs (A/B and C/D) of struc-
tures. Excluding these regions, the backbone root mean square
difference between the four structures remains at \( -0.3 \) Å. For
the invariant core residues (i.e. all residues with the exception
of those in the four variable regions), the average values of
\( R_{\text{dip}}^{\text{NH}} \) and the correlation coefficients are 20.5 \pm 1.2% and
0.96 \pm 0.05, respectively, for the four molecules. For the four
variable regions, on the other hand, \( R_{\text{dip}}^{\text{NH}} \) and the correlation
coefficient have values of 32% and 0.92 for molecule A, 29% and
0.93 for molecule B, 28% and 0.94 for molecule C, and 22% and
0.96 for molecule D. Thus, the overall agreement between the
measured and calculated \( ^1 \text{D}_{\text{NH}} \) dipolar couplings for the four
variable regions in molecule D is essentially the same as that of
the core residues, whereas the discrepancies for molecules A–C
are significantly (~50%) higher. The improvement for molecule
D relative to the other three molecules is statistically signif-
icient, because it is larger than the intrinsic error, and implies
that the structure of molecule D of IIAMtl is closest to that in
the IIAMtl–HPrt complex observed in solution.

Because the variable regions I–IV of IIAMtl compose several
residues (specifically residues 52–54, 68, 92, 93, 96, and 109) at
the periphery of the interface with HPrt, the following strategy
was employed. The starting coordinates comprised the free
x-ray structures of \( E. \) coli HPrt (37) and IIAMtl (molecule D (11)),
placed 30–50 Å apart in random orientations. Structures of the
complex were calculated from the experimental restraints by
rigid body minimization (66), followed by conjoined rigid body/
torsion angle dynamics (40, 41). The backbone coordinates and
non-interfacial side chains (excluding the four variable regions
of IIAMtl) were treated as rigid bodies throughout, with IIAMtl
held fixed, HPrt allowed to rotate and translate, and the axis of
the dipolar coupling alignment tensor free to rotate. The inter-
facial side chains of both IIAMtl and HPrt were given their full
torsional degrees of freedom. The backbone and side chains of
the four variable regions of IIAMtl were also given torsional
degrees of freedom; the \( \phi/\psi \) angles were restrained by square-
well potentials to the ranges covered by all four molecules in
the crystal structure of free IIAMtl, consistent with chemical
shift data (39), and the side chain torsion angles of the non-
interfacial residues were similarly restrained to the rotamers
observed in the crystal structure, consistent with hetero-
nuclear \( ^3J \) coupling measurements. In the case of region II,
however, residues 64–67 and residues 77–78 were treated as a rigid body because the \( \phi/\psi \) angles for this region differed by less than 5° between all four molecules of the crystal
structure. (Note residues 64 and 65 are included in region II to
allow appropriate hinge movements to occur.) Within the con-
finse of these torsion angle restraints, the conformations of the
four variable regions of IIAMtl in the complex are determined by
the dipolar coupling restraints and intramolecular NOE-de-
duced interproton distance restraints relating to these regions.

The IIAMtl–HPrt complex was solved on the basis of 872 ex-
perimental restraints, including 107 intermolecular NOE-de-
duced interproton distance restraints and 528 residual dipolar
couplings. A summary of the structural statistics is provided in
Tables I and II. A best fit superposition of the backbone for the
final ensemble of 200 simulated annealing structures is shown in
Fig. 2A, and an atomic density map, calculated from the
complete ensemble, is shown in Fig. 2B to illustrate the distri-
bution of interfacial side chain conformations.

The backbone atomic r.m.s. displacements for variable re-
Figure 1. Intermolecular NOEs in the IIAMtl–HPrt complex. Strips
from three-dimensional \( ^{13} \text{C} \)-separated/\( ^{15} \text{N} \)-filtered NOE spectra
recorded on a 1:1 IIAMtl(\( ^{13} \text{C} \)/\( ^{15} \text{N} \))-HPrt(\( ^{12} \text{C} \)/\( ^{14} \text{N} \)) complex (A) and
IIAMtl(\( ^{13} \text{C} \)/\( ^{15} \text{N} \))-HPrt(\( ^{12} \text{C} \)/\( ^{14} \text{N} \)) complex (B), illustrating specifically inter-
molecular NOE contacts from protons attached to \( ^{13} \text{C} \) on one protein to
protons attached to \( ^{12} \text{C} \) on the other. Residues from HPrt are denoted in
italics.

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Figure 1. Intermolecular NOEs in the IIAMtl–HPrt complex. Strips
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molecular NOE contacts from protons attached to \( ^{13} \text{C} \) on one protein to
protons attached to \( ^{12} \text{C} \) on the other. Residues from HPrt are denoted in
italics.
Solution Structure of the IIA^Mtl-HPr Complex

The notation of the NMR structures are as follows: (SA) are the final 200 simulated annealing structures; (SA)r is the restrained regularized mean structure. The number of terms for the various restraints is given in parentheses.

| TABLE I STRUCTURAL STATISTICS |
|--------------------------------|
| (SA)                          |
| Number of experimental restraints | 107 |
| Intermolecular interproton distance restraints | 105 |
| Intramolecular interproton distance restraints\(\alpha\) | 70 |
| Interfacial side chain torsion angle restraints\(\alpha\) | 62 |
| Residual dipolar couplings | 528 |
| R.m.s deviation from interproton distance restraints (Å)\(\alpha\) | 0.006 ± 0.001 |
| R.m.s deviation from sidechain torsion angle restraints (°)\(\alpha\) | 0.25 ± 0.16 |
| Overall dipolar coupling R-factors (%) | 1.2 ± 1.18 |
| Dipolar coupling R-factor (%) | |
| \(\text{D}_{\text{NH}}\) HPr (71) | 19.4 ± 0.02 |
| \(\text{D}_{\text{NH}}\) IIA^Mtl (114) | 20.0 ± 0.02 |
| \(\text{D}_{\text{Ca}}\) HPr (63) | 26.0 ± 0.05 |
| \(\text{D}_{\text{Ca}}\) IIA^Mtl (121) | 20.8 ± 0.08 |
| \(\text{D}_{\text{NC}}\) HPr (56) | 34.3 ± 0.03 |
| \(\text{D}_{\text{NC}}\) IIA^Mtl (103) | 32.8 ± 0.08 |
| Measures of structure quality\(\alpha\) | |
| Intermolecular repulsion energy (kcal · mol\(^{-1}\)) | 8.8 ± 1.5 |
| Intermolecular Lennard-Jones Energy (kcal · mol\(^{-1}\)) | -28.8 ± 1.5 |
| Coordinate precision (Å) | 0.09 |
| Backbone (N, C, O) | 0.56 |
| Interfacial side chains | 0.11 |
| Backbone of variable regions of IIA^Mtl | 0.14 |
| Precision of protein-protein orientation (°) | 1.4 |

The intramolecular interproton distance restraints relate to the interface side chains of IIA^Mtl and HPr and to the four variable regions of IIA^Mtl; there are 24 intraside residues and 40 sequential (\(|j-j'|=1\), 21 medium range (1 < \(|j-j'|<5\)), and 20 long range (\(|j-j'|>5\)) interresidue restraints. In addition to the side chain torsion angle restraints related to the interface side chains, 23 \(\phi\), 23 \(\psi\), and 16 side chain torsion angle restraints related to the variable regions of IIA^Mtl were also included in the calculations (see text).

None of the structures exhibited interproton distance violations \(\geq 0.2\) Å or torsion angle violations \(>5°\).

The dipolar coupling R-factor is defined as the ratio of the r.m.s. deviation between observed and calculated values to the expected r.m.s. deviation if the vectors were randomly oriented. The latter is given by \(2D_i (\pi / 2)_{5/2}\), where \(D_i\) is the magnitude of the axial component of the alignment tensor and \(\pi\) the rhombicity (67). \(\text{D}_{\text{NH}}\) and \(\gamma\) have values of \(-13.3\) Hz and 0.43, respectively, for the \(\text{D}_{\text{NH}}\) set of dipolar couplings, and \(-9.4\) Hz and 0.58, respectively, for the \(\text{D}_{\text{Ca}}\) and \(\text{D}_{\text{NC}}\) set of dipolar couplings. For reference, the \(\text{D}_{\text{NH}}\), \(\text{D}_{\text{Ca}}\), and \(\text{D}_{\text{NC}}\) dipolar coupling R-factors are 19.1, 25.2, and 34.1%, respectively, for the free x-ray structure of HPr (1POH (37)); 21.3, 21.1, and 33.6%, respectively, for the free x-ray structure of IIA^Mtl (molecule D of 1A3A (11)); and 19.2, 18.0, and 32.0% for the restrained regularized mean structure of IIA^Mtl in the complex fitted with an individual alignment tensor for IIA^Mtl alone.

The intermolecular repulsion energy is given by the value of the intermolecular quartic van der Waals repulsion term (38) calculated with a force constant of 4 kcal · mol\(^{-1}\) · Å\(^{-2}\) and a van der Waals radius scale factor of 0.8. The intermolecular Lennard-Jones van der Waals energy is calculated using the CHARMM19/20 parameters and is not included in the target function employed in the structure calculations. The percentages of residues present in the most favorable region of the Ramachandran plot (78) are 93 and 92% for the x-ray structures of HPr (1POH) and IIA^Mtl (molecule D of 1A3A), respectively, and 92% for the restrained regularized mean structure of IIA^Mtl in the complex.

The numbers in parentheses refer to the total number of each particular type of dipolar coupling used in cross-validation. In the case of IIA^Mtl, the dipolar couplings from the four variable regions (22 \(\text{D}_{\text{NH}}\), 20 \(\text{D}_{\text{Ca}}\), and 22 \(\text{D}_{\text{NC}}\)) are excluded from cross-validation.


### TABLE II DIPOLAR COUPLING CROSS-VALIDATION

The dipolar couplings, excluding those of the four variable regions of IIA^Mtl, were randomly divided into two equal groups, A and B, each comprising 50% of the dipolar couplings. In the Set 1 calculations, group A was used as the work set and group B as the free set; in the Set 2 calculations, group B was used as the work set and group A as the free set. Thus, \(R_{\text{dip}}\)(work) from Set 1 and \(R_{\text{dip}}\)(free) from Set 2 comprise the same dipolar couplings; likewise for \(R_{\text{dip}}\)(free) from Set 1 and \(R_{\text{dip}}\)(work) from Set 2.

| Dipolar couplings\(\alpha\) | \(R_{\text{dip}}\)(work) | \(R_{\text{dip}}\)(free) | \(R_{\text{dip}}\)(work) | \(R_{\text{dip}}\)(free) |
|-----------------------------|----------------------|----------------------|----------------------|----------------------|
| \(\text{D}_{\text{NH}}\) HPr (71) | 17.6 ± 0.02 | 21.8 ± 0.09 | 20.6 ± 0.04 | 17.7 ± 0.02 |
| \(\text{D}_{\text{NH}}\) IIA^Mtl (92) | 20.9 ± 0.02 | 21.7 ± 0.03 | 21.6 ± 0.01 | 21.2 ± 0.03 |
| \(\text{D}_{\text{Ca}}\) HPr (63) | 27.3 ± 0.10 | 27.3 ± 0.08 | 27.1 ± 0.07 | 28.1 ± 0.09 |
| \(\text{D}_{\text{Ca}}\) IIA^Mtl (101) | 18.4 ± 0.05 | 22.4 ± 0.13 | 22.0 ± 0.09 | 19.1 ± 0.11 |
| \(\text{D}_{\text{NC}}\) HPr (56) | 35.1 ± 0.02 | 33.4 ± 0.09 | 33.5 ± 0.08 | 35.3 ± 0.03 |
| \(\text{D}_{\text{NC}}\) IIA^Mtl (81) | 35.8 ± 0.02 | 30.1 ± 0.07 | 29.9 ± 0.05 | 35.9 ± 0.03 |

The numbers in parentheses refer to the total number of each particular type of dipolar coupling used in cross-validation. In the case of IIA^Mtl, the dipolar couplings from the four variable regions (22 \(\text{D}_{\text{NH}}\), 20 \(\text{D}_{\text{Ca}}\), and 22 \(\text{D}_{\text{NC}}\)) are excluded from cross-validation.
The quality of the fit to the experimental dipolar coupling data is good, and the dipolar coupling data have not been overfitted (67). Comparison of the relative orientations of the two proteins in these two sets of calculations with that obtained using all the dipolar couplings indicates that the relative rotation of the two proteins is determined with a precision of \( \pm 1.5° \), which corresponds approximately to the expected uncertainty in the orientation of the dipolar alignment tensor (68). In this regard it is worth noting that there is only a 1.9° difference in orientation between the alignment tensor used for the \( ^1\text{D}_{\text{NH}} \) couplings obtained from one stock of PEG/hexanol and that used for the \( ^1\text{D}_{\text{CA}} \) and \( ^1\text{D}_{\text{NC}} \) couplings obtained from a different PEG/hexanol stock. The location of the four variable regions (I–IV) of IIAMtl are indicated (residues 51–54, 66–78, 91–96, and 104–110). B, isosurface of the reweighted atomic density map drawn at a value of 25% of maximum, calculated from the final 200 simulated annealing structures, for selected interfacial side chains of IIAMtl (red) and HPr (purple); the backbones of IIAMtl (blue) and HPr (green) are displayed as tubes. The side chain coordinates displayed within the atomic density map are those of the restrained regularized mean structure; note that the side chain of His-111 of IIAMtl displayed within the atomic density map are those of the restrained regularized mean structure. Residues from HPr are denoted in italics.

Overall Description of the IIAMtl-HPr Interface—Two views of the overall IIAMtl-HPr complex, displayed as ribbon diagrams, are shown in Fig. 3 (throughout the text and figures, residues of HPr are denoted in italics). The IIAMtl binding surface on HPr is convex and comprises two segments of the polypeptide chain (residues 12–27 and 46–56) encompassing helices a1 (residues 16–28) and a2 (residues 47–52), consistent with previous chemical shift mapping studies (69). HPr docks into a concave cleft on the surface of IIAMtl formed by three distinct segments of polypeptide chain as follows: residues 49–68 that include the C-terminal end of helix a2 (residues 41–52), an antiparallel b-sheet formed by b-strands 2 (residues 56–58) and 3 (residues 61–62), and the active site histidine at position 65; residues 92, 93, and 96 located in the loop connecting b-strands 4 and 5; and residues 109–128 that includes helix a3 (residue 111–121) and the N-terminal portion of helix a4 (residues 125–133). The interface is \( \sim 27 \) Å long and \( \sim 22 \) Å wide and comprises a total of 42 residues, 25 from IIAMtl and 17 from HPr. The interfacial residues are distributed between \( \sim 60% \) non-polar residues and \( \sim 40% \) polar ones. The total accessible surface area buried upon complexation is \( \sim 1450 \) Å\(^2\) of which 685 Å\(^2\) originate from IIAMtl and \( \sim 765 \) Å\(^2\) from HPr.

A detailed view of the interface is shown in Fig. 4A together with a diagrammatic summary of the intermolecular contacts.
The majority of interactions are hydrophobic in nature. The most extensive of these involves the aromatic ring of Phe48 of HPr which is surrounded by a hydrophobic cluster of residues on IIA^{Mtl} including Leu-57, Gly-58, Ile-61, Thr-119, Leu-122, and Ile-128. In addition, there are several potential electrostatic and/or hydrogen bonding interactions (either direct or water-mediated) between the side chains of Asn-12 and Asn-109, Thr-16 and Arg-49, Lys-24 and Glu-92, Lys-27 and Glu-93/Asp-96, Ser-46 and Glu-59, Lys-49 and Asp-123, and Gln-51 and Thr-116.

The conformations of two side chains, His-111 and Glu-59 of IIA^{Mtl}, cluster into two distinct conformations. For His-111, the g' and g'' rotamers (labeled a and b, respectively, in both Figs. 2B and 4A) are approximately equally populated. This is consistent both with previous NMR measurements on free IIA^{Mtl} which indicated the presence of two conformations for His-111 in slow exchange (57), as well as with the observation of two alternate conformations in different molecules of the crystal structure of free IIA^{Mtl} (11). In the case of Glu-59, the x_2 angle is ~70% in the g' rotamer (conformer a) and ~30% in the
The transition state of the IIA\textsuperscript{Mt}-HPr complex. A, detailed view around the active site histidines, illustrating the backbone and side chain positions in the unphosphorylated complex, the dissociative transition state, and the associative transition state. The backbones of IIA\textsuperscript{Mt} and HPr are shown in dark blue and dark green, respectively, for the unphosphorylated complex, and in light blue and light green, respectively, for the putative dissociative and associative transition states; the active site histidines and pentacoordinate phosphoryl group (in the case of the transition state) are shown in purple for the unphosphorylated complex, in red for the dissociative transition state (Ne\textsuperscript{2-N}\textsubscript{M} distance of ~6 Å between His-65 and His-15), and in orange for the associative transition state (Ne\textsuperscript{2-N}\textsubscript{M} distance of ~4 Å between His-65 and His-15). Small changes in the backbone of residues 64–66 of IIA\textsuperscript{Mt} and residues 14–16 of HPr are required to accommodate the transition states. B, detailed view of the active site in the putative transition state illustrating the interactions that stabilize the phosphoryl group. The color coding is as follows: the backbone and side chains of IIA\textsuperscript{Mt} are shown in blue and red, respectively; the backbone and side chains of HPr are shown in green and gray, respectively; the active site histidines are in purple, and the pentacoordinate phosphoryl group is in yellow. Residues from HPr are labeled in italics.

Some degree of assessment of the relative importance of the various intermolecular interactions can be gauged by sequence comparisons of the interfacial residues of IIA\textsuperscript{Mt} and HPr among a variety of bacterial species. The species chosen comprise examples of Gram-negative (Yersinia pestis and Vibrio cholera) and Gram-positive (Bacillus anthracis, Bacillus subtilis, and Staphylococcus carnosus) bacteria, and the sequence alignments are shown in Fig. 4C. The overall sequence identities for the complete protein sequences range from 28 to 97% relative to the E. coli sequences (50). In the case of the Gram-negative bacteria, E. coli, Y. pestis, and V. cholera, sequence conservation among the interfacial residues is higher than that for the overall proteins. In addition, the few substitutions are highly conservative in terms of the intermolecular interactions in which they participate. Thus, for example, substitution of Thr-56 of HPr by Val in V. cholera still permits hydrophobic interactions with Ile-112 of IIA\textsuperscript{Mt}; likewise, substitution of Thr-53 in IIA\textsuperscript{Mt} by Ser in Y. pestis and Val in V. cholera maintains hydrophobic interactions with the aliphatic portion of the side chain of Arg-17 and the methyl group of Ala-20 of HPr. However, it seems likely that the potential salt bridges involving Lys-24 and Lys-27 of HPr and Glu-92, Glu-93, and Asp-96 of IIA\textsuperscript{Mt} seen in the E. coli complex may not be that critical, because Glu-92 and Glu-93 are substituted by a shorter Asp side chain in Y. pestis and V. cholera IIA\textsuperscript{Mt}, respectively. Nevertheless, these substitutions at positions 92 and 93 in Y. pestis and V. cholera, respectively, would still permit water-bridged electrostatic interactions at the periphery of the interface.

Sequence conservation between the E. coli proteins and those of the Gram-positive bacteria, B. anthracis, B. subtilis, and S. carnosus, is lower, ranging from 28 to 42% overall and 21 to 35% for the interfacial residues (Fig. 4C). These sequence comparisons highlight the critical importance of the intermolecular hydrophobic interactions involving the side chains of residues (Thr or Ala), 17 (Arg), 47 (Leu or Ile), 48 (Phe or Met), and 51 (Gln or Met) of HPr and the side chains of residues 52 (Leu, Thr, or Val), 57 (Leu, Met, or Val), 112 (Ile or Leu), 115 (Ile or Leu), 119 (Thr, Ala, or Leu) and 122 (Leu, Phe, Ile, or Cys) of IIA\textsuperscript{Mt}. The intermolecular electrostatic interactions observed in the E. coli complex, however, are much less conserved, suggesting that these are not critical determinants of specificity. Thus, the salt bridge between Lys-49 of HPr and Asp-123 of IIA\textsuperscript{Mt} in the E. coli complex is abrogated by substitution of Lys-49 to an aliphatic side chain (Val or Gly) and Asp-123 to a neutral Ser. Salt bridges involving Lys-24/Lys-27 and Glu-92/Glu-93/Asp-96 in the E. coli complex can no longer occur because of substitution of the two lysines to neutral residues (Gln or Asn) at position 24 and Ser at position 27, although water-bridged hydrogen bonding interactions are still possible. The probable water-mediated hydrogen bonding interaction between residue 12 (Asn, Thr or Ser) of HPr and 109 (Asn or Asp) of IIA\textsuperscript{Mt} does appear to be preserved except in the case of S. carnosus where Asn-109 is replaced by a Gly.

Although many features of the HPr and IIA\textsuperscript{Mt} interfaces are preserved between the Gram-negative and -positive bacteria, there is still considerable species specificity in terms of the interaction of HPr and IIA\textsuperscript{Mt}. Thus, phosphoryl transfer between E. coli (Gram-negative) HPr and S. carnosus (Gram-positive) IIA\textsuperscript{Mt} is ~10-fold lower than that involving S. carnosus HPr (70). Because the disposition of intermolecular hydrophobic interactions is preserved, the reduction in the rate of the heterologous phosphoryl transfer is presumably due to a reduction in the surface complementarity for the inter-species interactions. Thus, for example, Phe-48 of HPr in the Gram-negative bacteria is substituted by Met in the Gram-positive bacteria (Fig. 4C) which will necessarily change the details of...
the surface contour. As a consequence, there are compensatory changes in the hydrophobic residues of IIA^{Mt} (at positions 57, 61, 119, 122, and 128; cf. Fig. 4C) that interact with Met-48 that serve to maintain surface complementarity.

The Phosphoryl Transition State Intermediate—The phosphoryl group, originating on the Nδ1 atom of His-15 of HPr (71, 72), is transferred to the Nε2 atom of His-65 of IIA^{Mt} (59) with inversion of the configuration at phosphorus (73). This indicates a transition state involving a pentacoordinate phosphoryl group, originating on the N2 atom of His-65 of IIA^{Mt} and EIN, and EIN are shown in blue, red, and green, respectively, in B. The location of the phosphor group in the transition state is depicted in yellow. Residues of HPr are labeled in italics. The coordinates of the EIN-HPr and IIA^{Mt}-HPr complexes are taken from Garrett et al. (16) (code 1GGR), respectively.

We modeled the transition state using essentially the same approach as that described previously (17) in the case of the IIA^{Glc}-HPr complex; in particular, structures were recalculated introducing appropriate geometric restraints for the phospho-

Fig. 6. Surface representations illustrating the binding surfaces involved in the IIA^{Mt}-HPr (left panel), IIA^{Glc}-HPr (middle panel), and EIN-HPr (right panel) complexes. The binding surfaces on IIA^{Mt}, IIA^{Glc}, and EIN are shown in A, and the binding surfaces on HPr are shown in B. The binding surfaces are color-coded with hydrophobic residues in green, polar residues in light blue, the active site histidines in purple, positively charged residues in dark blue, and negatively charged residues in red. The relevant portions of the backbone of HPr are shown in gold in A; the relevant portions of the backbone of IIA^{Mt}, IIA^{Glc}, and EIN are shown in blue, red, and green, respectively, in B. The location of the phosphor group in the transition state complexes. The phosphor group is depicted in purple, positively charged residues in dark blue, and negatively charged residues in red.

To accommodate the phosphoryl transition state, only small displacements (0.2 and 0.4 Å for the dissociative and associative transition states, respectively) in the backbone coordinates of the residues immediately adjacent to the active site histidines are required (Fig. 5A). This is mirrored by equally minor changes in backbone ϕ/ψ angles. The only noteworthy difference appears to be in the ϕ/ψ angles of Thr-16 at the beginning of the first helix of HPr; these change from −42°/−67° in the unphosphorylated complex to the more helical values of −65°/−54° in the transition state complexes. The χ1 and χ2 rotamers of His-65 ε(1/ε(2)) of IIA^{Mt} and His-15 ε(1/ε(2)) of HPr remain unaltered. The χ1 and χ2 angles of His-65 change by less than 5 and −30°, respectively; the χ3 and χ4 angles of His-15 change by 20−30 and −1°, respectively. In addition, the side chain of Arg-49 is slightly displaced (~0.7−0.9 Å) by the presence of the phosphoryl group; this is achieved by very small changes (5−20°) in χ1/χ2/χ3/χ4 side chain torsion angles that remain in the tig’/lilt rotamers.

A detailed view of the active site illustrating interactions that stabilize the transition state is shown in Fig. 5B. The phosphoryl group sits in a hydrophobic cleft comprising Leu-52, Ile-112, and Ile-115 of IIA^{Mt}; there are no negatively charged carboxylate groups in close proximity. The phosphoryl group is within hydrogen bonding distance of the guanidino group of Arg-49 of IIA^{Mt} and the hydroxyl group of Thr-16 and the backbone amides of Thr-16 and Arg-17 of HPr. The b conformer of His-111 does not interact with the phosphoryl group, but its Nδ1 atom may be hydrogen-bonded to the carboxamide of Asn-109; in the a conformer, the latter interaction is preserved but the Nε2 atom is now in close proximity to both the phosphoryl group and the side chain carboxamide group of Asn-12 of HPr. Although Arg-17 of HPr does not participate in any electrostatic interactions with IIA^{Mt}, it contributes to the po-
Comparison with the IIA\textsuperscript{Glc}-HPr and EIN-HPr Complexes—

The interaction surfaces employed by HPr to recognize IIA\textsuperscript{Mtl} and IIA\textsuperscript{Glc} (17) and EIN (16) are very similar, overlap extensively, and consist of 17, 18, and 23 residues, respectively (Fig. 6B, left, middle, and right panels). Of the 17 HPr residues that participate in the IIA\textsuperscript{Mtl}-HPr complex, 15 are involved in the IIA\textsuperscript{Glc}-HPr complex and 16 in the EIN-HPr complex (Fig. 4B). In the case of the IIA\textsuperscript{Mtl}-HPr and IIA\textsuperscript{Glc}-HPr (17) complexes, the overall accessible surface areas buried at the interface are essentially the same (1350–1450 Å\textsuperscript{2}) and distributed similarly between HPr (~45%) and the partner protein (~55%). The surface area buried at the interface of the EIN-HPr complex (16) is about 40% larger and approximately equally divided between HPr and EIN.

The backbone scaffolds comprising the interaction surfaces on IIA\textsuperscript{Mtl}, IIA\textsuperscript{Glc}, and EIN are topologically different both in terms of structure and connectivity: IIA\textsuperscript{Mtl} employs a mixture of helix and sheet (Fig. 7A); IIA\textsuperscript{Glc} predominantly makes use of sheet (Fig. 7B); and EIN uses only helices (Fig. 7C). Despite these differences, some elements of commonality can be observed (Fig. 7D). Thus, strands β3 of IIA\textsuperscript{Mtl} and β7 of IIA\textsuperscript{Glc} which bear the active site histidine, are aligned; helix α3 of IIA\textsuperscript{Mtl} coincides with strands β5 and β6 of IIA\textsuperscript{Glc}; and helix α2 of IIA\textsuperscript{Mtl}, helix α1 of IIA\textsuperscript{Glc} and helix α2 of EIN partially overlap (Fig. 7D).

Surface representations of the interaction interfaces for the IIA\textsuperscript{Mtl}-HPr, IIA\textsuperscript{Glc}-HPr, and EIN-HPr complex are shown in Fig. 6 (left, middle, and right panels, respectively). The target surfaces that interact with HPr are broadly similar in all three cases comprising a central hydrophobic core located within a concave cleft surrounded by negatively charged residues (Fig. 6A). A number of electrostatic interactions are preserved in the three complexes. Thus, for example, Glu51 of HPr is buried in all three complexes (Fig. 6B) and adopts its conformation to form potential hydrogen bonds with Thr-116 of IIA\textsuperscript{Mtl}, Ser-78 of IIA\textsuperscript{Mtl}, and Arg-126 of EIN (Fig. 6A). Likewise, Lys-49 of HPr forms potential salt bridges with Asp-123 of IIA\textsuperscript{Mtl}, Glu-80 and Glu-86 of IIA\textsuperscript{Glc}, and Glu-84 of EIN; Lys-27 of HPr form potential salt bridges with Glu-92, Glu-93, and Asp-96 of IIA\textsuperscript{Mtl} and Asp-144 of IIA\textsuperscript{Glc}, and Glu-74 and Asp-82 of EIN. Ser-46 of HPr interacts with a negatively charged residue in both the complexes with IIA\textsuperscript{Mtl} (Glu-59) and EIN (Asp-82), consistent with the finding that mutation of Ser-46 to Asp significantly reduces activity for phosphoryl transfer to these two enzymes (75).

In addition to the similarities noted above, there are also a number of noteworthy differences between the three complexes. Although the negatively charged residues are almost uniformly distributed around the periphery of the interaction surfaces on IIA\textsuperscript{Glc} and EIN, they are clustered in essentially one region of IIA\textsuperscript{Mtl} (along the top rim of the interaction site in the view shown in Fig. 6A). From a structural perspective, this has consequences with regard to the role of the conserved Arg-17 of HPr. Arg-17 is critical for phosphoryl transfer between EIN and HPr and between HPr and IIA\textsuperscript{Glc} (76, 77), and its function is to neutralize a pair of negatively charged residues that are located in close spatial proximity to the active site histidines: Glu-67 and Glu-68 of EIN (16), and Asp-38 and Asp-94 of IIA\textsuperscript{Glc} (17) (Fig. 6, A and B). In the case of IIA\textsuperscript{Mtl}, these negatively charged residues are replaced by one positively charged residue, Arg-49, and two hydrophobic residues, Leu-52 and Val-68 (Fig. 6A, left panel). Thus, in the IIA\textsuperscript{Mtl}-HPr complex, the neutralizing role of the guanidino group of Arg-17 is no longer required, and the aliphatic portion of the Arg-17 side chain simply packs against the methyl groups of Leu-52 and Thr-53 (Fig. 4A). The guanidino group of Arg-49 of IIA\textsuperscript{Mtl}, on the other hand, forms a potential hydrogen bond with Thr-16 in the unphosphorylated complex (Fig. 4A) and interacts with the phosphoryl group in the transition state (Fig. 5). This suggests that mutation of Arg-17 to a neutral residue would have little effect on phosphoryl transfer between HPr and IIA\textsuperscript{Mtl}. This is borne out experimentally where mutation of Arg-17 to His, Ser, Cys, or Gly has no effect on V\textsubscript{max} and only increases K\textsubscript{m} by a factor of ~2 (76). Mutation of Arg-17 to a negatively charged residue (Glu), on the other hand, increases the value of K\textsubscript{m} 100-fold, while leaving V\textsubscript{max} unaltered (76); this is presumably due to either the introduction of a negative charge in close proximity to the phosphoryl group or possibly to a potential electrostatic contact with Arg-49, thereby hindering the interaction of Arg-49 with the phosphoryl group. Mutation of Arg-49 of IIA\textsuperscript{Mtl}, however, would be predicted to abrogate phosphoryl transfer between HPr and IIA\textsuperscript{Mtl}. This prediction is supported by the observation that Arg-49 appears to be absolutely conserved among IIA\textsuperscript{Mtl}'s from different bacterial species (Fig. 4C).

The location of the active site histidines of IIA\textsuperscript{Mtl}, IIA\textsuperscript{Glc}, and EIN in the three complexes is also of interest. In the case of EIN (16), the active site His-189 is located at the very edge of the interaction surface, and adjacent residues in the sequence of EIN are not in contact with HPr (Fig. 6A, right panel). In contrast, in the case of both IIA\textsuperscript{Mtl} and IIA\textsuperscript{Glc}, the active site histidines, His-65 and His-90, respectively, are located just off-center of the interaction surface (Fig. 6A, left and middle panels, respectively). In all three complexes, the phosphoryl group in the transition state intermediate is located between the N\textsubscript{δ1} of His-15 of HPr and the N\textsubscript{ε2} atom of the active site histidine of the partner protein, and the χ\textsubscript{1}/χ\textsubscript{2} angles of His-15...
are in the same rotamere states (g<sup>-</sup>/l<sup>+</sup>). Consequently, the side chain conformation of the active site histidine of the partner protein must accommodate its different location relative to His-15 of HPp in the three complexes. This is achieved by different combinations of rotameric states for χ<sub>1</sub> and χ<sub>2</sub>/g<sup>-</sup>/l<sup>+</sup> for His-65 of IIA<sup>M1</sup>, tlg<sup>-</sup> for His-90 of IIA<sup>M2</sup>, and tlg<sup>-</sup> for His-189 of EIN.

An example of conformational side chain plasticity is afforded by Phe-48 of HPp. Phe-48 participates in crucial intermolecular hydrogen bonding interactions in all three complexes (Fig. 6) and adapts its side chain rotamer conformation to optimize these; in the IIA<sup>M1</sup> and IIA<sup>G</sup> complex, the χ<sub>1</sub> angle of Phe-48 is in the t rotamer, whereas in the EIN complex it is in the g<sup>-</sup> rotamer.

Concluding Remarks—The structure of the E. coli IIA<sup>M1</sup>-HPp complex reveals that specificity is achieved through a large set of hydrophobic interactions, supplemented by a few electrostatic/hydrogen bonding interactions around the phosphoryl interaction. The combination of the IIA<sup>M1</sup>-HPp (this paper), IIA<sup>G</sup>-HPp (17), and EIN-HPp (16) complexes show how HPp can use essentially the same interaction surface to recognize a plethora of structurally unrelated proteins. Three key factors are involved as follows: shape and residue complementarity of the interaction surfaces, even though the underlying backbone structural elements of the target surfaces are dissimilar; large interaction surfaces that can support redundancy of interactions and that minimize the effect of any single interaction (that is specificity is built up by a large series of interactions, each of which makes only a small contribution to the overall interaction energy); and side chain conformational plasticity to optimize intermolecular contacts and surface complementarity. These features are likely to be characteristic of many protein-protein interactions in signal transduction pathways that involve the rapid formation and dissociation of temporally transient, yet specific protein-protein complexes.

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