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Notes:
Intramolecular domain–domain association/dissociation and phosphoryl transfer in the mannitol transporter of *Escherichia coli* are not coupled

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The *Escherichia coli* mannitol transporter (II*Mtl*) comprises three domains connected by flexible linkers: a transmembrane domain (C) and two cytoplasmic domains (A and B). II*Mtl* catalyzes three successive phosphoryl-transfer reactions: one intermolecular (from histidine phosphocarrier protein to the A domain) and two intramolecular (from the A to the B domain and from the B domain to the incoming sugar bound to the C domain). A key functional requirement of II*Mtl* is that the A and B cytoplasmic domains be able to rapidly associate and dissociate while maintaining reasonably high occupancy of an active stereospecific AB complex to ensure effective phosphoryl transfer along the pathway. We have investigated the rate of intramolecular domain–domain association and dissociation in II*B*Mtl* by using 1H relaxation dispersion spectroscopy in the rotating frame. The open, dissociated state (comprising an ensemble of states) and the closed, associated state (comprising the stereospecific complex) are approximately equally populated. The first-order rate constants for intramolecular association and dissociation are 1.7 (±0.3) × 106 and 1.8 (±0.4) × 105 s<sup>−1</sup>, respectively. These values compare to rate constants of ~500 s<sup>−1</sup> for A → B and B → A phosphoryl transfer, derived from qualitative line-shape analysis of 1H-15N correlation spectra taken during the course of active catalysis. Thus, on average, ~80 association/dissociation events are required to effect a single phosphoryl-transfer reaction. We conclude that intramolecular phosphoryl transfer between the A and B domains of II*Mtl* is rate-limited by chemistry and not by the rate of formation or dissociation of a stereospecific complex in which the active sites are optimally apposed.

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The bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) is a signaling pathway whereby sequential, reversible phosphoryl transfer via a series of transient bimolecular complexes is coupled to sugar uptake across the membrane (1). The transporter (enzyme II*C*) of the mannitol branch of the PTS consists of a single polypeptide chain organized into three domains: IIC*Mtl*, II*B*Mtl*, and IIA*Mtl* (from the N to C terminus), connected by flexible linkers (1). The phosphoryl group is transferred from the histidine phosphocarrier protein (HPr) to II*A*Mtl* via a bimolecular event and subsequently from IIA*Mtl* to II*B*Mtl*, and finally onto the incoming sugar bound to the cytoplasmic side of IIC*Mtl* via unimolecular reactions. A key functional feature of II*C* is that intramolecular association and dissociation between the A and B domains must be fast to allow for effective phosphoryl transfer along the pathway.

Relaxation measurements, including relaxation dispersion, have been used to study dynamics of enzyme function and protein folding on milli- to microsecond time scales (2–10). Recent work has suggested that the dynamics of atomic motions observed by relaxation dispersion are directly linked to catalysis (5, 7, 8). For adenylyl kinase, the rate of lid opening, which involves large-amplitude correlated motions, seems to represent the rate-limiting step in catalysis (5). This observation led to the suggestion that many enzymes have evolved such that the catalytic reaction itself is so fast that catalytic power is limited not by chemistry but by the rate at which conformational rearrangements can take place to optimally align the reactive atoms (5). However, this generalization has been called into question, because the correlation between conformational changes and kinetics observed for adenylyl kinase does not necessarily imply a direct link between dynamics, correlated motions, and catalysis (11).

Previously, we determined the solution structure of an analog of the isolated IIA*Mtl* domain and a stably phosphorylated IIB*Mtl* domain from *Escherichia coli* (12). IIB*Mtl* was stably phosphorylated by mutating the active-site cysteine (Cys-384) to serine (13), whereas the active-site histidine (His-554) of IIA*Mtl* was mutated to glutamine to prevent transfer of the phosphoryl group back to IIA*Mtl* in the complex. The interaction between the isolated IIA*Mtl* and phosphoIIB*Mtl* domains is weak, with an equilibrium dissociation constant (K<sub>d</sub>) of ~3.7 mM. In the native enzyme, however, the two domains are connected by an ~21-residue flexible linker (1). The linker renders the equilibrium between associated (closed) and dissociated (open) forms of intact IIA*Mtl* concentration independent and is expected to stabilize the associated state, because the reduction in configurational space that accompanies tethering is equivalent to raising the effective concentration of the domains (12, 14). In this article we investigate the dynamics of intramolecular domain–domain association and dissociation between the A and B domains of single-chain II*B*Mtl* of *E. coli* by using 1H relaxation dispersion spectroscopy in the rotating frame and compare these rates with estimates of the reversible phosphoryl-transfer rates between the A and B domains derived from qualitative line-shape analysis of cross-peaks in 1H-15N correlation spectra during the course of active catalysis. We show that the intramolecular domain–domain association and dissociation rates are not rate-limiting and that phosphoryl transfer occurs, on average, once for every ~80 association and dissociation events.

Results

**Chemical-Shift Perturbation.** For simplicity, phosphorylated IIB*Mtl*(C384S/H554Q/C571A) is denoted as phosphoIIB*Mtl*, phosphorylated IIB*Mtl*(C384S) as phosphoII*B*Mtl*, IIA*Mtl*(H554Q/C571A) as IIA*Mtl*, and the wild-type construct as IIB*Mtl*.

The profile of 1HN/15N chemical-shift perturbations relative to the free phosphoII*B*Mtl* and free IIA*Mtl* is essentially the same for...
the mixture of isolated IA^\text{Mli} and phosphoIB^\text{Mli} (comprising ~30% complex) and the intact phosphoIB^\text{Mli}, indicating that the interaction of the two domains is the same in both systems (Fig. 1). The magnitudes of the perturbations, however, are larger for phosphoIB^\text{Mli} than for the mixture, indicating that the fraction of closed (associated) form in the single-chain phosphoIB^\text{Mli} is >30%. The \textbf{H^1} chemical shifts of phosphoIB^\text{Mli} are not affected by concentration (over a range of 50–500 μM), indicating that the chemical-shift perturbation relative to the isolated IA^\text{Mli} and phosphoIB^\text{Mli} domains is solely caused by intramolecular association of the two domains.

Large-Scale Interdomain Motion. The values of the \textbf{H^1} heteronuclear NOEs for the linker (residues 472–492) are all <0.6, indicating that the linker region is flexible. D^\text{H11002} residual dipolar couplings (RDCs) measured for phosphoIB^\text{Mli} weakly aligned in a polyacrylamide gel reveal that the magnitude of the principal component of the alignment tensor for the B domain (−8.2 Hz) is approximately twice as large as that for the A domain (−4.2 Hz) (Fig. 2). Alignment in the neutral gel is dominated by steric effects approximately twice as large as that for the A domain (Fig. 2).

Association of the A and B domains of II Mtl in the context of phosphoIB^\text{Mli} and the isolated phosphoIB^\text{Mli} and IIAM^\text{Mli} domains. Portion of the 2D \textbf{H^1}-15N HSQC spectra of 15N-labeled phosphoIB^\text{Mli} (blue) (a) and 15N-labeled IIAM^\text{Mli} (red) (b); a 1:1 mixture of 3 mM 15N-labeled IA^\text{Mli} and 15N-labeled phosphoIB^\text{Mli} (green) comprising ~30% complex (c) superimposed on the spectra of the individual domains shown in a and b; and \textbf{H^1},15N-labeled phosphoIB^\text{Mli} (cyan) (d) superimposed on the spectra of the individual domains shown in a and b. Cross-peaks with large chemical-shift changes after association of the A and B domains are highlighted in c and d.

Fig. 1. Association of the A and B domains of II^\text{Mli} in the context of phosphoIB^\text{Mli} and the isolated phosphoIB^\text{Mli} and IIAM^\text{Mli} domains. Portion of the 2D \textbf{H^1}-15N HSQC spectra of 15N-labeled phosphoIB^\text{Mli} (blue) (a); 15N-labeled IIAM^\text{Mli} (red) (b); a 1:1 mixture of 3 mM 15N-labeled IA^\text{Mli} and 15N-labeled phosphoIB^\text{Mli} (green) comprising ~30% complex (c) superimposed on the spectra of the individual domains shown in a and b; and \textbf{H^1},15N-labeled phosphoIB^\text{Mli} (cyan) (d) superimposed on the spectra of the individual domains shown in a and b. Cross-peaks with large chemical-shift changes after association of the A and B domains are highlighted in c and d.

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Kinetics of Intramolecular Association and Dissociation of the A and B Domains of IB^\text{Mli}. The intramolecular association/dissociation studied can be described by the scheme:

\[
\begin{align*}
\text{IIA}^\text{Mli} \cdot \text{P-IIB}^\text{Mli} & \quad k_{\text{OC}} \quad \text{IIA}^\text{Mli} \cdot \text{P-IIB}^\text{Mli} \\
\text{open} & \quad \equiv \quad \text{closed}
\end{align*}
\]

where II^\text{Mli} \cdot P-IIBM^\text{Mli} is the closed state in which the A and B domains are associated to form a stereospecific complex. II^\text{Mli} \cdot P-IIBM^\text{Mli} is the open, dissociated state (which, in reality, is an ensemble of states) in which there is no interaction between the A and B domains, and \(k_{\text{OC}}\) and \(k_{\text{CO}}\) are the unimolecular rate constants for the open-to-closed and closed-to-open transitions, respectively. For this two-state system, the overall relaxation rate in the rotating frame, \(R_{1p}\), is given by the sum of the intrinsic relaxation rate, \(R_{1p}^0\), and the exchange of chemical-shifts contribution, \(R_{\text{ex}}(9)\):

\[
R_{1p} = R_{1p}^0 + R_{\text{ex}}.
\]

Because exchange is fast on the relaxation time scale, \(|R_{1p}^\text{closed} - R_{1p}^\text{open}| \ll k_{\text{ex}}\), \(R_{1p}^0\) is given by

\[
R_{1p}^0 = R_{\text{ex}}(9)
\]

where \(R_{1p}^\text{open}\) and \(R_{1p}^\text{closed}\) are the intrinsic relaxation rates for the open and closed forms, respectively, and \(R_{\text{ex}}\) is the exchange of populations of the open and closed states, respectively, with \(R_{\text{ex}}(9)\). Note that the values of \(R_{1p}\) for the open and closed states in the absence of chemical exchange are not accessible experimentally because this is a unimolecular system in equilibrium.

In the fast-exchange limit, \((\Delta k_{\text{ex}})^2 \ll 1\), on the chemical-shift time scale, the \(R_{\text{ex}}\) contribution to \(R_{1p}\) is given by \((10, 19, 20)\).
the values of the chemical shifts of the two states; IIBMtl, orange), and the active-site residues, phosphorylated Ser-384 of IIBMtl that although Glu-530 lies outside the interface, the perturbation in its 1HN chemical shift is caused by a secondary effect arising from close proximity to 

where both $\Delta\omega_0$ and $\omega_{\text{spec}}$ are known, because they are the same as those for the two isolated domains ($\omega_{\text{free}}$). The fraction of the closed state, therefore, is given by

$$p_{\text{closed}} = (\omega_{\text{obs}} - \omega_{\text{spec}})/(\omega_{\text{closed}} - \omega_{\text{free}}),$$

where $\omega_{\text{obs}}$ is the observed shift measured for phosphoIIBAMtl. Thus, $R_{\text{ex}}$ can be expressed as

$$R_{\text{ex}} = p_{\text{closed}}(1 - p_{\text{closed}})/(\omega_{\text{obs}} - \omega_{\text{free}})^2 k_{\text{ex}}/(k_{\text{ex}} + \omega_{\text{SL}}^2),$$

thereby enabling $p_{\text{closed}}$ to be determined independently, because both $\omega_{\text{obs}}$ and $\omega_{\text{free}}$ are directly accessible experimentally.

Mapping of the residues used for analysis of the relaxation dispersion data onto the structure of the previously determined IIAMtl-phosphoIIBAMtl complex (12) is shown in Fig. 3. The titration spectra obtained with the isolated IIAMtl and IIBMtl domains were used to estimate the lower limit of $k_{\text{ex}}$ and the range of $\Delta\omega$. Because the values of $\Delta\omega$ were larger in the $^1$H than $^{15}$N dimension and stronger spin-lock field strengths can be applied for $^1$H than $^{15}$N to suppress the exchange contribution to $R_{\text{ex}}$, we made use of backbone amide proton (1HN) relaxation dispersion measurements in the rotating frame (20) by using perdeuterated phosphoIIBAMtl. Among the titrating residues of IIBA10, 10 residues with both well resolved cross-peaks in the 2D $^1$H-15N correlation spectrum and large $^1$HN chemical-shift differences ($\Delta\omega_{\text{IIBA}}/2\pi > 60$ Hz at a $^1$H frequency of 750 MHz) relative to the free protein were selected for further analysis. Note that simulations of Eq. 4, varying the $\Delta\omega_0$, $k_{\text{ex}}$ (for values larger than the estimated lower limit of $k_{\text{ex}}$), and $\omega_{\text{SL}}$, indicate that a 60-Hz cutoff is required to ensure that $R_{\text{ex}}$ values larger than the 3–4% experimental errors in $R_{\text{ex}}$ can be detected.

The relaxation dispersion data for the 10 selected residues were fit simultaneously by nonlinear least-squares minimization by using Eqs. 2 and 5, optimizing two global parameters, $p_{\text{closed}}$ and $k_{\text{ex}}$, and one residue-specific parameter, $R_{\text{ex}}^{\text{calc}}$, by using the error function

$$\chi^2 = \sum_j \sum_i [R_{\text{obs}}^{\text{calc}}(i, j) - R_{\text{obs}}^{\text{calc}}(i, j)]^2/\sigma_{ij}^2,$$

where $R_{\text{obs}}^{\text{calc}}(i, j)$ and $R_{\text{obs}}^{\text{calc}}(i, j)$ are the calculated and observed $R_{\text{ex}}$ values for the $i$th spin-lock field and $j$th residue, respectively, and $\sigma_{ij}$ is the error obtained from Monte Carlo simulation for the single exponential fitting of $R_{\text{ex}}$. Fig. 4a shows typical $R_{\text{ex}}$ data measured for the backbone amide proton of Asp-454 at three different field strengths, illustrating that the data follow single exponential decays. Selected relaxation dispersion profiles for residues with (Asp-454 and Gly-394) and the data follow single exponential decays. Selected relaxation dispersion profiles for residues with (Asp-454 and Gly-394) and $R_{\text{ex}}$ can be expressed as

$$R_{\text{ex}} = p_{\text{closed}}(1 - p_{\text{closed}})/(\omega_{\text{obs}} - \omega_{\text{free}})^2 k_{\text{ex}}/(k_{\text{ex}} + \omega_{\text{SL}}^2),$$

Fig. 4b shows typical $R_{\text{ex}}$ data measured for the backbone amide proton of Asp-454 at three different field strengths, illustrating that the data follow single exponential decays. Selected relaxation dispersion profiles for residues with (Asp-454 and Gly-394) and without (Ala-513 and Gly-592) a chemical-exchange contribution to $R_{\text{ex}}$ are displayed in Fig. 4b. Global fitting of the data for the 10 selected residues yielded values of $k_{\text{ex}} = 3.5(\pm 0.5) \times 10^4$ s$^{-1}$ and $p_{\text{closed}} = 0.48 \pm 0.06$ with $\chi^2/N = 0.8$ (where $N$ is the number of degrees of freedom). The value of $p_{\text{closed}}$ obtained from the relaxation dispersion data are in agreement with the approximate estimate derived from the observed chemical shifts of phosphoIIBAMtl, the chemical shifts of the isolated IIAMtl and phosphoIIBAMtl domains free in solution, and the extrapolated chemical shifts of the fully saturated IIAMtl-phosphoIIBAMtl complex derived from titration data with the isolated domains (note >90–95% saturation is difficult to achieve given the $K_d$ of 3.7 mM). Because $k_{\text{ex}} = k_{\text{OC}} + k_{\text{CO}}$ and $p_{\text{closed}}k_{\text{CO}} = p_{\text{open}}k_{\text{OC}}$ at equilibrium, the unimolecular association ($k_{\text{OC}}$) and dissociation ($k_{\text{CO}}$) rate constants in Eq. 1 are given by $p_{\text{closed}}k_{\text{ex}}$ and $p_{\text{open}}k_{\text{ex}}$, respectively, yielding values of $k_{\text{OC}} = 1.7(\pm 0.3) \times 10^4$ s$^{-1}$ and $K_{\text{OC}} = 1.8(\pm 0.4) \times 10^4$ s$^{-1}$.

The pathway from the open to closed states probably involves the formation of an ensemble of very low populated, nonspecific encounter complexes, as has been demonstrated recently for several weak protein–protein complexes by using paramagnetic relaxation enhancement measurements (21). Because the relaxation dispersion data obtained for the 10 sites can be fit simultaneously within experimental error by using a simple two-site exchange model, the
population of nonspecific encounter complexes must be very low (<5%). Consequently, the presence of such intermediates will not impact to any significant extent the values of the $k_{OC}$ and $k_{CO}$ rate constants obtained from the two-state fit. In addition, the average chemical shifts for the ensemble of nonspecific encounter complexes are anticipated to be virtually identical to those of the open state, thereby further reducing the impact of their presence on the estimated values of the rate constants to negligible levels. We note, however, that $k_{OC}$ and $k_{CO}$ can be expressed as $k_{OC} = k_{IC}(k_{IO} + k_{IC})$ and $k_{IO} \cdot k_{CI}(k_{IO} + k_{IC})$, respectively, where $k_{OI}$ and $k_{IO}$ are the forward and backward rate constants, respectively, for the formation of nonspecific encounter complexes from the open state, and $k_{IC}$ and $k_{CI}$ are the forward and backward rate constants, respectively, for the formation of the closed state from nonspecific encounter complexes.

**Kinetics of Reversible Intramolecular Phosphoryl Transfer Between the A and B Domains of IIA^Mtl.** Under conditions of catalytic amounts of enzyme I and HPr and excess PEP, both the A and B domains of wild-type IIA^Mtl are fully phosphorylated. Preliminary experiments indicated that the isolated phosphorylated IIA^Mtl domain is relatively unstable at neutral pH, and irreversible dephosphorylation by hydrolysis occurs with a half-life of $\approx 20$ min. In contrast, the isolated phosphorylated IIB^Mtl domain is rather stable, and the phosphorylated form has a half-life of $\approx 17$ h. Thus, PEP is continually consumed and inorganic phosphorus generated during the time course of the reaction. The $^{31}P$-NMR spectrum of fully phosphorylated IIA^Mtl displays two resonances of equal intensity: one at 122.2 ppm corresponding to phospho-Cys-384 (B domain) and the other at $-6.7$ ppm corresponding to phospho-His-554 (A domain) (referenced relative to inorganic phosphate at 0 ppm), in perfect agreement with previous reports on full-length IIA^Mtl [ref. 22; see supporting information (SI) Fig. 6]. Once PEP is fully consumed, hydrolysis of phospho-His-554 occurs. The intensity of the resonances of phospho-His-554 and phospho-Cys-384 remain equal and decrease in concert to finally disappear after $\approx 4$ h (SI Fig. 6). The two phosphorus resonances remain in slow exchange on the chemical-shift time scale throughout the reaction. Therefore, one can conclude that the overall phosphoryl-transfer reaction rate, $k_{ex}$ (given by the sum of the $A \rightarrow B$ and $B \rightarrow A$ phosphoryl-transfer reaction rates), is at least 10-fold slower than $\Delta \omega_{PP} = 2.9 \times 10^4$ s$^{-1}$ (calculated from the chemical-shift difference between the two signals, 18.9 ppm, and the $^{31}P$ frequency of 242.9 MHz).

To further probe the rate of reversible phosphoryl transfer between the A and B domains we recorded a series of $^1H$-$^{15}N$ heteronuclear single quantum coherence (HSQC) spectra (at a $^1H$ frequency of 800 MHz) during active phosphoryl transfer between the two domains subsequent to the depletion of PEP. The results are shown in Fig. 5. At time 0 h (the point at which PEP is fully depleted), the $^1H$-$^{15}N$ HSQC spectrum reflects fully phosphorylated IIA^Mtl, that is, the species in which both the B and A domains are phosphorylated. At 4 h subsequent to PEP depletion, the $^1H$-$^{15}N$ HSQC spectrum corresponds to the fully unphosphorylated IIB^Mtl species. The intermediate time points reflect a mixture of biphosphorylated, monophosphorylated, and unphosphorylated species, with the monophosphorylated species reaching a maximum between 1 and 2 h subsequent to PEP depletion. In some instances, the chemical shifts of the cross-peaks progressively shift during the course of the reaction from the fully phosphorylated to the unphosphorylated positions without any significant line broadening.
This is the fast-exchange regime on the chemical-shift time scale and is exemplified by the cross-peak for AlA-383 (Fig. 5r). $\Delta_{\text{obs}}$ for AlA-383 is 151 s$^{-1}$, indicating that the overall phosphoryl-transfer reaction rate $k_{\text{PT}}$ is in excess of $\approx 750$ s$^{-1}$ ($k_{\text{PT}} > 3\Delta_{\text{obs}}$). In the case of Asp-432 (Fig. 5t), a continual shift is also observed, but extensive line broadening is seen in the spectra taken at 1 and 2 h, characteristic of an exchange rate on the fast side of intermediate exchange: $\Delta_{\text{obs}}$ for Asp-432 is 437 s$^{-1}$, suggesting that $k_{\text{PT}} > 1,000$ s$^{-1}$ ($2\Delta_{\text{obs}} < k_{\text{PT}} < 3\Delta_{\text{obs}}$). This estimate is fully confirmed by the behavior of the cross-peaks of Gly-555 ($\Delta_{\text{obs}} = 905$ s$^{-1}$; Fig. 5c) and Thr-556 ($\Delta_{\text{obs}} = 1,116$ s$^{-1}$; Fig. 5d) where the cross-peaks completely disappear in the $^{1}H,^{15}N$ HSQC spectra taken at 1 and 2 h, which is indicative of the intermediate exchange regime where $k_{\text{PT}} > \Delta_{\text{obs}}$. Because the intensities of the $^{31}P$ resonances of phospho-Cys-384 and phospho-His-554 decrease in concert and remain approximately equal to each other throughout the reaction, one can conclude that the forward and backward rate constants for phosphoryl transfer are approximately equal with a value of $\approx 500$ s$^{-1}$.

Discussion

Impact of Flexible Linker Between the A and B Domains of IIBAM$_{\text{M}}$. The $K_d$ for the interaction of the isolated IA$_{\text{M}}$ and phosphoIB$_{\text{M}}$ domains is $3.7$ mM (12). Because the occupancy of the closed (associated) state of phosphoIB$_{\text{M}}$ determined from the relaxation dispersion measurements is $\approx 45\%$, one can conclude that the 21-residue linker connecting the A and B domains in IIBA$_{\text{M}}$ results in an effective local concentration of $\approx 4$ mM for each domain, a value that is in good accord with that predicted from polymer chain theory (12, 23) and configurational entropy calculations (14). Moreover, if the unimolecular association rate constant, $k_{\text{OC}} (1.7 \times 10^6$ M$^{-1}$s$^{-1})$, is divided by the effective local concentration, the value of $\approx 5 \times 10^6$ M$^{-1}$s$^{-1}$ for the apparent bimolecular rate constant is reasonably close to what one might expect for a diffusion-limited reaction between two proteins, particularly when factors such as the chain-diffusion coefficient of the linker are taken into account. Thus, intradomain association in the IIBA$_{\text{M}}$ system is tuned to function, which requires reasonably high occupancy coupled with rapid association and dissociation ($\approx 2 \times 10^6$ s$^{-1}$ in both directions) to efficiently carry out three sequential phosphoryl-transfer reactions, namely from HPr to IA$_{\text{M}}$, from IB$_{\text{M}}$ to IIAM$_{\text{M}}$, and finally from IIBAM$_{\text{M}}$ onto the incoming sugar bound to the cytosolic side of IIC$_{\text{M}}$.

Relationship Between Phosphoryl Transfer and Domain Dynamics. The NMR data presented in this article clearly indicate that the rate constants for the forward (A $\rightarrow$ B) and backward (B $\rightarrow$ A) phosphoryl-transfer reactions are approximately equal with values of $\approx 500$ s$^{-1}$, which is $\approx 40$-fold lower than the rate constants for intramolecular domain–domain association and dissociation. Thus, on average, $\approx 80$ association/dissociation events take place for every phosphoryl-transfer reaction. One can conclude, therefore, that the rate-limiting step for intramolecular phosphoryl transfer in IIBA$_{\text{M}}$ is governed by the chemistry of the phosphoryl-transfer reaction rather than the time it takes for the two domains to form a stereospecific complex in which the active sites of the A and B domains are optimally positioned for phosphoryl transfer. In this regard, it is worth noting that in the case of all of the bimolecular complexes of the PTS solved to date, including the N-terminal domains of enzyme I (EIN)–HPr (24), IA$_{\text{G}}$–HPr (25), IA$_{\text{M}}$–HPr (26), IA$_{\text{M}}$–HPr (27), IA$_{\text{G}}$–IB$_{\text{G}}$ (28), and IA$_{\text{M}}$–IB$_{\text{M}}$ (12) complexes, either minimal or no backbone changes are required to form an optimal pentacoordinate phosphoryl-transfer transition state.

Phosphoryl-Transfer Rates in the PTS. There have been a number of kinetic studies on the glucose-branch PTS using rapid-quench techniques (29, 30). (Note that the kinetics of the intramolecular phosphoryl-transfer reaction studied here is not accessible to classical biochemical methodology.) The reported apparent second-order rate constants for reversible phosphoryl transfer along the enzyme I, HPr, IA$_{\text{G}}$, and IB$_{\text{G}}$ pathway range from $\approx 10^6$ to $2 \times 10^7$ M$^{-1}$s$^{-1}$ (29). By using estimated intracellular protein concentrations of $\approx 5$, 20–100, 20–60, and 10 $\mu$M for enzyme I (monomer), HPr, IA$_{\text{G}}$, and IB$_{\text{G}}$, respectively (29), one can deduce that the upper limits of the pseudo-first-order rate constants for the forward and backward phosphoryl-transfer reactions in vivo are $\approx 1,000$ and 160–800 s$^{-1}$, respectively, between enzyme I and HPr; 1,200–6,000 and 1,000–3,000 s$^{-1}$, respectively, between HPr and IA$_{\text{G}}$, and 200–600 and 40 s$^{-1}$ between IA$_{\text{G}}$ and IIC$_{\text{G}}$, respectively. Thus, the intramolecular phosphoryl-transfer rates of $\approx 500$ s$^{-1}$ observed here for IIBA$_{\text{M}}$ are quite comparable to those in the glucose branch of the PTS.

Comparison with Adenylate Kinase. Adenylate kinase catalyzes the reversible transfer of phosphorus from two molecules of ADP to form ATP and AMP. Its structure comprises a core and two mobile domains (the lid and the AMP-binding domains) that close over the nucleotide-binding sites (31). The two mobile domains exist in either open or closed forms, and the transition between these two states involves large-scale correlated motions within the hinge regions connecting the two domains to the core (31). The opening rates for both a thermophilic and mesophilic adenylate kinase ($\approx 40$ and 290 s$^{-1}$, respectively) are essentially identical to the respective catalytic rates, whereas the closing rate (1,400–1,600 s$^{-1}$) is much faster (5). These data suggest that the conformational transition from closed to open states required for product release is rate-limiting in catalysis (5). In contrast, in the IIBA$_{\text{M}}$ system, the intramolecular domain–domain opening (dissociation/product release) and closing (association/substrate binding) rates are comparable ($\approx 2 \times 10^6$ s$^{-1}$), and it is the phosphoryl-transfer reaction that seems to be rate-limiting ($\approx 500$ s$^{-1}$).

The different behaviors observed for IIBA$_{\text{M}}$ and adenylate kinase may be rationalized in structural terms. In the case of IIBA$_{\text{M}}$, the two domains are connected by a long 21-residue linker that behaves essentially as a random-coil polymer. Thus, concerted backbone motions within the linker are not required to bring the two domains together or move them apart; rather the function of the linker is to restrict diffusion of one domain relative to another to a volume limited by the average rms end-to-end distance of the linker. For adenylate kinase, however, motion of the lid and AMP-binding domains relative to the core domain is determined by concerted backbone conformational changes of a few residues at the hinge points between the domains. The concerted nature of these changes is presumably more complex and, therefore, energetically more costly and slower relative to the essentially random, largely uncorrelated nature of the motions within the IIBA$_{\text{M}}$ linker.

Biological Implications for the PTS. In the absence of external sugar, the PTS is in the resting state with all signal transducers phosphorylated (32). When the bacterium encounters external sugar in the medium, the first event involves the transfer of the phosphoryl group from IIB to the incoming sugar located on IIC, thereby switching on the PTS signaling pathway. Dephosphorylated IIB and subsequently dephosphorylated upstream mediators (e.g., IIA and enzyme I) interact with several transcription factors to turn on or off a variety of proteins involved in sugar uptake. The fast intramolecular domain–domain association between the A and B domains of IIBA$_{\text{M}}$ and subsequent phosphoryl transfer represents an efficient means for rapid initiation and amplification of sugar-uptake signaling in bacteria.

Materials and Methods

Cloning, Expression, and Purification. IIBA$_{\text{M}}$ was cloned to encompass the entire cytosolic AB component (residues 375–637) of the E. coli mannitol transporter (II$_{\text{M}}$). The B domain extends from...
residues 375 to 471, the A domain from residues 472 to 492, the linker from residues 493 to 637, and Cys-571 to Ala to improve protein stability (12). The construct was verified by DNA sequencing and then subeloned into a modified pET-32a vector to form a thiorodoxin fusion protein with a 6-His tag. After transformation with an expression vector, E. coli strain BL21 Star (DE3) (Novagen) was grown in minimal medium (either supplemented by 15NH4Cl and 13C-glucose in H2O or 15NH4Cl and 13C2H2O in H2O) as the sole nitrogen or carbon sources, respectively), induced with 1 mM isopropyl-15-β-D-thiogalactopyranoside at an A600 of 0.8 and harvested by centrifugation after 4 h of induction. Wild-type IIBAMtl and phosphoIIBAMtl in 20 mM Tris-d11 (pH 7.4), 0.01% sodium azide, and 10%2H2O (vol/vol). 13C,15N-labeled phosphoIIBAMtl was used to assign the linker region and determine RDC data to the coordinates of each domain (12) individually.

Wild-type IIBAMtl and phosphoIIBAMtl were expressed and purified as described (12). RDCs were obtained by taking the difference in the corresponding amide gel, prepared as described (13), and1JNH couplings were measured in aligned and isotropic (water) medium (15). The alignment medium comprised a neutral 5% polyacrylamide gel. After transformation with an expression vector, the A and B domains of wild-type IIBAMtl in unphosphorylated and phosphorylated by addition of 5 mM MgCl2 and 2 mM ATP was used to assign the linker region and determine RDCs were measured by taking the difference in the corresponding amide gel, prepared as described (13), and1JNH couplings were measured in a 2D 1H-15N HSQC (1H frequency 242.94 MHz) spectroscopy.15N-labeled IIBAMtl (0.5 mM) was prepared in 20 mM Tris-d11 buffer (pH 7.4) and phosphorylated by addition of 5 μM enzyme I/5 μM HPr/5 mM MgCl2/1.5 mM PEP. The PEP stock solution was prepared in Trizma base, and the Trizma base buffer maintained the pH of the solution at 7.4 throughout the course of the reaction.

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