Conformational Selection and Substrate Binding Regulate the Monomer/Dimer Equilibrium of the C-terminal domain of Escherichia coli Enzyme I

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Conformational Selection and Substrate Binding Regulate the Monomer/Dimer Equilibrium of the C-terminal domain of *Escherichia coli* Enzyme I*1*,

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**Background:** Conformational changes in the EIC domain of enzyme I upon ligand binding are thought to regulate the phosphotransfer system by modulating the monomer/dimer equilibrium.

**Results:** Binding of phosphoenolpyruvate shifts a preexisting conformational equilibrium in EIC.

**Conclusion:** Conformational selection provides a direct structural link between ligand binding and dimer affinity.

**Significance:** Isolated EIC is an optimal system for investigating dynamic processes regulating EI.

The bacterial phosphotransferase system (PTS) is a signal transduction pathway that couples phosphoryl transfer to active sugar transport across the cell membrane. The PTS is initiated by the binding of phosphoenolpyruvate (PEP) to the C-terminal domain (EIC) of enzyme I (EI), a highly conserved protein that is common to all sugar branches of the PTS. EIC exists in a dynamic monomer/dimer equilibrium that is modulated by ligand binding and is thought to regulate the overall PTS. Isolation of EIC has proven challenging, and conformational dynamics within the EIC domain during the catalytic cycle are still largely unknown. Here, we present a robust protocol for expression and purification of recombinant EIC from *Escherichia coli* and show that isolated EIC is capable of hydrolyzing PEP. NMR analysis and residual dipolar coupling measurements indicate that the isolated EIC domain in solution adopts a stable tertiary fold and quaternary structure that is consistent with previously reported crystallographic data. NMR relaxation dispersion measurements indicate that residues around the PEP binding site and in the \( \beta_3 \alpha_3 \) turn (residues 333–366), which is located at the dimer interface, undergo a rapid transition on the sub-millisecond time scale (with an exchange rate constant of \(~1500 \text{s}^{-1}\)) between major open (\(~97\%) and minor closed (\(~3\%) conformations. Upon PEP binding, the \( \beta_3 \alpha_3 \) turn is effectively locked in the closed state by the formation of salt bridges between the phosphate group of PEP and the side chains of Lys\(^{340}\) and Arg\(^{358}\), thereby stabilizing the dimer.

Enzyme I (EI)\(^2\) is the first protein in the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS), a signal transduction pathway that couples phosphoryl transfer through a series of bimolecular protein–protein complexes to sugar transport across the membrane (1, 2). The PTS is also involved in the regulation of diverse cellular processes, including transcription, chemotaxis, and glycolysis (2). There are four sugar-specific branches of the PTS, but all require EI to initiate the phosphoryl transfer cascade. EI is autophosphorylated by phosphoenolpyruvate (PEP) and subsequently donates the phosphoryl group to the histidine phosphocarrier protein HPr (3, 4). EI and HPr are common to all branches of the PTS. Thereafter, the phosphoryl group is transferred from HPr to the sugar-specific enzymes II and ultimately onto the incoming sugar. It has been shown recently that, under conditions of nitrogen limitation, EI is inhibited by \( \alpha \)-ketoglutarate (5), the carbon substrate for ammonia assimilation, thereby providing a regulatory link between central carbon and nitrogen metabolism in bacteria. EI is ubiquitous in bacteria and does not have any eukaryotic counterparts. Thus, given the central role of EI in the control and regulation of bacterial metabolism, it is no surprise that EI has been described as an ideal pharmaceutical target for the identification of novel and highly specific antimicrobials (6).

The functional form of EI is a \(~128\)-kDa dimer of identical subunits (7) comprising two structurally and functionally distinct domains (8, 9). The N-terminal phosphoryl-transfer domain (EIN, residues 1–249) contains the site of phosphorylation (His\(^{189}\)) and the binding site for HPr (8, 10, 11). The C-terminal domain (EIC, residues 261–575) is responsible for dimerization and contains the binding site for PEP (12–14). The EIN and EIC domains are connected to one another (15–18) by a long helical linker.

The isolated EIN domain can transfer a phosphoryl group to HPr but only intact dimeric EI can be autophosphorylated by PEP (18) by a long helical linker.

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1. This work was supported by the Intramural Program of the National Institutes of Health, NIDDK, and the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health (to G. M. C.).
2. To whom correspondence should be addressed: Lab. of Chemical Physics, Bldg. 5, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0520. Tel.: 301-496-0782; Fax: 301-496-0825; E-mail: mariusc@mail.nih.gov.
3. The abbreviations used are: EI, enzyme I (residues 1–575); EIN, N-terminal domain of EI (residues 1–249); EIC, C-terminal domain of EI (residues 261–575); PTS, phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine phosphocarrier protein; TROSY, transverse relaxation optimized spectroscopy; RDC, residual dipolar coupling; SVD, singular value decomposition; CPMG, Carr-Purcell-Meiboom-Gill; \( ^{1}D_{\text{HH}} \), one-bond backbone amide RDC; MESG, 2-amino-6-mercaptop-7-methylpurine riboside.

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PEP (8, 9, 13, 19–21). It has therefore been suggested that the monomer/dimer equilibrium for the EIC domain plays a central role in the regulation of the overall PTS (22). Indeed, the interaction of EI with the physiological ligands for the EIC domain, Mg$^{2+}$ and PEP, decreases the equilibrium dissociation constant for dimerization ($K_D$) by ~30-fold (from 4.8 to 0.15 $\mu$M (14, 22). Given the intracellular EI concentration of ~10 $\mu$M subunits (23), binding of Mg$^{2+}$ and PEP to EI results in a significant shift in the monomer/dimer equilibrium and a concomitant substantial increase in the cellular population of dimeric EI (from ~60 to 90%).

There is now a wealth of structural studies on EI. The isolated EIN domain from *Escherichia coli* has been solved in its free form by crystallography (10) and NMR (11) as well as in its phosphorylated state (24) and bound to HPr (25) by NMR. In all three cases, the structure of the EIN domain and the relative orientation of the $\alpha$ and $\alpha/\beta$ subdomains remain unchanged. There are three crystal structures of intact EI, two of free EI from *Staphylococcus carnosus* (26) and *Staphylococcus aureus* (16), and one of a trapped phosphorylated intermediate of *E. coli* EI bound to the inhibitor oxalate (15). In addition, there are two solution structures of *E. coli* EI, free and bound to HPr, determined by the combined use of NMR and x-ray scattering (17, 18). The EI structures reveal large rigid body conformational transitions involving domain reorientation of EIN relative to EIC, as well as reorientation of the two subdomains of EIN relative to each other. These large-scale conformational changes permit transfer of the phosphoryl group from PEP bound to the EIC domain to His$^{189}$ located on EIN in the conformation found in the trapped phosphorylated intermediate (15), and subsequent transfer of the phosphoryl group on His$^{189}$ to HPr in the conformation found in the structures of free EI and the EI-HPr complex where the structure of the EIN domain is identical to that of the isolated EIN domain (17). In addition, crystal structures of the isolated EIC domain from *Thermoanaerobacter tengcongensis* in the free form (27) and in complexes with PEP and pyruvate (28) have been obtained. Although the structure of the EIC domain is the same in intact EI and the isolated EIC domain, spectroscopic and kinetic investigations have suggested that in solution the EIC domain may be present as an ensemble of different conformations that are not apparent in the crystal structures (14).

The recombinant *E. coli* EIC domain has been reported to be proteolytically unstable and difficult to isolate (14, 29). Here, we present a robust protocol for expression and purification of recombinant *E. coli* EIC. We show that the purified protein adopts a single, stable fold in solution and is able to hydrolyze PEP into pyruvate and inorganic phosphate. Using relaxation dispersion NMR spectroscopy (30, 31), we demonstrate the existence of a rapid local conformational transition on the millisecond time scale between two states, a major open state and a minor closed state, involving residues in the vicinity of the active site. Chemical shift analysis indicates that the conformation of the minor species is similar to that of the PEP-bound state, indicating that PEP binding likely proceeds via conformational selection. Furthermore, in combination with the available crystal structures, the data provide a rationale for understanding dimer stabilization by PEP. The data presented here suggest that EIC represents a good model system for studying the combined effects of substrate binding and conformational dynamics on enzymatic regulation.

**EXPERIMENTAL PROCEDURES**

Protein Expression and Purification—Intact EI (residues 1–575) and the EIN domain (residues 1–249) were expressed and purified as described previously (17, 24). The EIC domain (residues 261–575) was cloned into a pET11a vector (Novagen) without tags. The plasmid was introduced into *E. coli* strain BL21star(DE3) (Invitrogen), and the transformed bacteria were plated onto an LB-agar plate containing ampicillin (100 $\mu$g/ml) for selection. Cells were grown at 37 °C in either Luria Bertani (LB) or minimal medium (with $^{13}$NH$_4$Cl and $^{13}$C$_6$-glucose as the sole nitrogen and carbon sources, respectively) in H$_2$O or D$_2$O, respectively. At $A_{600}$ ~ 0.4 (for LB cultures) or $A_{600}$ ~ 0.8 (for cultures in minimal medium), the temperature was reduced to 20 °C, and expression was induced with 1 mM isopropyl-$d$-thiogalactopyranoside. Cells were harvested by centrifugation after 16 h of induction, and the pellet was resuspended in 20 ml of 20 mM Tris, pH 8.0, 0.2 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was lysed using a microfluidizer and centrifuged at 70,000 × g for 40 min. The supernatant was filtered and loaded onto a DEAE anion exchange column (20 ml; GE Healthcare), and the protein was eluted with a 400 ml gradient of 1 mM NaCl. The fractions containing the protein were confirmed by SDS-PAGE and purified by gel filtration on a Superdex-75 column (GE Healthcare) equilibrated with 20 mM Tris, pH 7.4, 200 mM NaCl, 2 mM DTT, and 1 mM EDTA. Relevant fractions were loaded on a monoQ anion exchange column (GE Healthcare), and the protein was eluted with a 400-ml gradient from 150 mM to 400 mM NaCl.

NMR Spectroscopy—All of the NMR samples were prepared in 20 mM Tris buffer, pH 7.4, 100 mM NaCl, 4 mM MgCl$_2$, 1 mM EDTA, 2 mM DTT, and 90% H$_2$O/10% D$_2$O (v/v). The protein concentration (in subunits) was 300–400 $\mu$M unless stated otherwise.

NMR spectra were recorded at 37 °C on Bruker 900 and 600 MHz spectrometers equipped with either a z-shielded gradient triple resonance cryoprobe or, for $^{13}$C NMR, an x/y/z-shielded gradient quadruple resonance probe. Spectra were processed using NMRPipe (32) and analyzed using the program SPARKY. Sequential $^1$H/$^{15}$N/$^{13}$C backbone assignment of the EIC domain was carried out using transverse relaxation optimized (TROSY) versions (33, 34) of conventional three-dimensional triple resonance correlation experiments (HNCO, HNCA, HNCA/CB, HNCACB, HN(CO)CA, and HN(CO)CACB) (35). The $^1$H/$^{15}$N/$^{13}$C backbone chemical shifts have been deposited in the BioMagResBank (accession no. 18392) (36). Assignment of the $^1$H–$^{15}$N correlations for the EIC-PEP complex was performed by titration experiments, following the change in $^1$H–$^{15}$N cross-peak positions as a function of added PEP in $^1$H–$^{15}$N TROSY spectra. Weighted combined $^1$H/$^{15}$N chemical shift perturbations ($\Delta_{HN}$) resulting from the addition of 10 mM PEP were calculated using the following equation (37): $\Delta_{HN} = ((\Delta\delta_{H} W_{H})^2 + (\Delta\delta_{N} W_{N})^2)^{1/2}$, where $W_{H}$ and $W_{N}$ are weighing fac-

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M. For the activity assay, to the unnatural amino acid 2-amino-6-mercapto-7-methylpurine. Enzymatic activity of EI and EIC for the enzyme, 362.1-x-y l aliquots of the reaction mixture were assayed spectrophotometrically at 37 °C using the EnzChek 70-kDa dimer exhibiting an 8-barrel fold with the active site Cys502 located in the β7α7 turn (15). Comparison of the x-ray structures of T. tengcongensis EIC free and bound to PEP indicates that substrate binding causes rearrangements of a few side chains (Arg296, Phe354, Gln458, Arg465, Met466, and Glu504) in the active site but leaves the overall tertiary fold and quaternary structure unperturbed (28). By way of contrast, sedimentation velocity and thermal denaturation exper-
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FIGURE 1. Isolation and purification of E. coli EIC. A, size exclusion chromatography elution profile. B and C, chromatograms of the final anion exchange step obtained for species 1 and 2, respectively. The salt gradient is shown in red, D and E, 600 MHz 1H-15N TROSY correlation spectra obtained for EIC from species 1 and 2, respectively. Buffer conditions are as described under “Experimental Procedures.” F, selected regions of the 1H-15N TROSY spectrum showing the overlap of E (blue) and EIC (red) cross-peaks. The cross-peaks for the isolated EIN domain are displayed in green for completeness. NMR spectra for the isolated EIN and EIC domains were acquired at 600 MHz. The full-length EI spectrum was acquired at 900 MHz. The full-length EI spectrum was acquired at 900 MHz.

Relaxation Dispersion on Free EIC—To characterize the dynamics in the region of the PEP binding site, we carried out CPMG 15N-relaxation dispersion experiments (31) at 600 and 900 MHz. These experiments probe exchange dynamics between species with distinct chemical shifts on a time scale ranging from ~50 μs to 10 ms. Significant relaxation dispersion was observed for the backbone amides of a number of residues in the vicinity of the PEP binding site: Ile326 and Gly327 at the N-terminal end of strand β5; Arg465 and Asn467 located in the turn/loop (residues 453–477) connecting strand β6 to helix α6; and Gly337, Lys340, Glu341, Leu355, Trp357, Arg358, and Ala359 located in the turn/loop (residues 333–366) connecting strand β3 to helix α3 (Fig. 2B). Three of the residues, Leu355, Trp357, and Asn467, are also located at the dimer interface (Fig. 2C). The 15N-relaxation dispersion curves for all the above residues at both fields (600 and 900 MHz) were fit simultaneously to a two-state model (Equation 1 in “Experimental Procedures”) describing the interconversion of two conformational states, optimizing the values of the exchange rate (kex), the fractional population of the minor state (pM), and the residue-specific 15N chemical shift differences between the two conformational states (ΔωN). An example of the fits is provided in Fig. 2E, and a summary of the results is provided in Table 1. The population of the minor species is ~3%, and the overall exchange rate (sum of forward and backward rate constants) is ~1500 s⁻¹. These

ments have shown that the addition of PEP stabilizes the protein fold and decreases the equilibrium dissociation constant for dimerization (14). PEP binding also results in marked changes in the near UV CD and fluorescence spectra, suggesting that EIC undergoes conformational rearrangements upon PEP binding (14). These spectroscopic techniques, however, only provide averaged information on aromatic side chains, so that one cannot ascertain whether the observed spectral changes reflect global effects or local conformational transitions.

Here, we investigated the effect of PEP on the solution structure E. coli EIC by NMR. 1H, 15N, and 13C backbone resonances of free EIC were assigned for 278 of 316 amino acids. The backbone ψ/φ torsion angles derived from the backbone chemical shifts using the program TALOS+ (49) are in excellent agreement with the x-ray structure of the trapped phosphorylated intermediate of E. coli Ei (Fig. 2A) (15). Thus, one can conclude that the tertiary structure of the isolated EIC domain in solution is essentially the same as that in the crystal structure of full-length EI. 1H-15N cross-peaks for several residues located in the PEP binding site (Val326–Thr332, Gly452–Tyr509, Cys502–Glu504) are not visible in the 1H-15N TROSY spectrum of EIC, indicating that this region of the protein is undergoing a conformational transition on the submillisecond to millisecond time scale (i.e. intermediate exchange on the chemical shift time scale resulting in line-broadening beyond the level of detection).
parameters yield values of the rate constants for the conversion from the major to the minor species and from the minor to the major species of ~50 and ~1500 s⁻¹, respectively.

Consistent with the relaxation dispersion results, an overlay of the crystal structures of the EIC domain (15, 16, 26–28) obtained to date (Fig. 2D) shows that the backbone of the Gly337–Tyr344 segment of the β3α3 turn can adopt one of two conformations: an open state observed in the crystal structures of phosphorylated EI from S. aureus (15). The data for the β3α3 turn is in the open conformation and colored in red; in all other cases, the β3α3 turn is in the open conformation and colored in cyan. PEP is displayed as a green bond. The amide groups (blue for N, white for HN) of Gly337 and Ala359, as well as the carbonyl groups (red for O) of Ile336 and Lys340 (red), are shown as spheres on the phosphorylated EI structure. E. coli examples of typical relaxation dispersion data at 600 MHz (left panel) and 900 MHz (right panel). Data are shown for Glu341 (red) and Ala359 (blue) with the experimental data represented by filled circles and the best-fit curves for a two-site exchange model as solid lines. The optimized values of the kinetic, population, and chemical shift parameters derived from the relaxation dispersion data are provided in Table 1.

FIGURE 2. Structure and dynamics of the free EIC domain. A. Correlation between the backbone ϕ/ψ torsion angles derived from backbone 1H/15N/13C chemical shifts using the program TALOS +49) and those in the crystal structure of the trapped phosphorylated intermediate of intact E. coli EI (15). The data for loop regions (where conformational differences between solution and crystal are to be expected) are depicted as open circles, whereas the data for secondary structure elements are reported as filled circles. The overall correlation coefficient is 0.90, and the correlation coefficient computed using only the data from secondary structure elements is 0.95. B, structural model for the E. coli EIC-PEP complex. The model was obtained by rigid-body least-square fitting of the backbone atoms of the x-ray structure of the EIC-PEP complex from T. tengcongensis (28) onto the EIC coordinates of the x-ray structure of phosphorylated EI from E. coli (15). The fitting was carried out using residues 261–333 and 367–570, and the Cα rms difference is 1.1 Å. Ribbons of the two identical subunits are depicted in yellow and white. The PEP molecule is displayed as green bonds, and the side chains of Lys340, Arg358, and Arg465 are shown as red bonds. The nitrogen atoms of amide groups exhibiting significant 15N relaxation dispersion, characteristic of dynamics on the submillisecond to millisecond time scale, are shown as blue spheres. The inset provides a close-up of the β3α3 turn. The turn is highlighted in cyan, the carbonyl groups of Ile336 and Lys340 are shown as spheres (gray for carbon, red for oxygen), and the amide groups of residues in this region displaying relaxation dispersion are shown as spheres, blue for nitrogen and white for the associated amide proton. C, close-up view of the EIC dimer interface. The two subunits are colored in white and yellow. The atoms of Leu355, Trp357, and Asn467 are represented as red spheres. D, superposition of the x-ray structures of EIC (15, 16, 26–28), illustrating the conformational variability in the β3α3 turn. The β3α3 turn in the closed conformation seen in the x-ray structure of phosphorylated EI is highlighted in red; in all other cases, the β3α3 turn is in the open conformation and colored in cyan. PEP is displayed as a green bond. The amide groups (blue for N, white for HN) of Gly337 and Ala359, as well as the carbonyl groups (red for O) of Ile336 and Lys340, are shown as spheres on the phosphorylated EI structure. E. coli examples of typical relaxation dispersion data at 600 MHz (left panel) and 900 MHz (right panel). Data are shown for Glu341 (red) and Ala359 (blue) with the experimental data represented by filled circles and the best-fit curves for a two-site exchange model as solid lines. The optimized values of the kinetic, population, and chemical shift parameters derived from the relaxation dispersion data are provided in Table 1.

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### TABLE 1

Kinetic, population, and chemical shift parameters for conformational exchange between open and closed states of EIC derived from relaxation dispersion experiments

All of the relaxation dispersion curves at two fields (600 and 900 MHz) were fit simultaneously to a two-site exchange model, optimizing the values of two global parameters, the overall exchange rate ($k_ex$) and the population of the minor state ($p_B$), and the residue-specific $|\Delta\omega_{ex}|$ values, which represent the absolute $^{15}$N chemical shift difference between the major and minor states.

<table>
<thead>
<tr>
<th>Gly$^{327}$</th>
<th>Lys$^{400}$</th>
<th>Glu$^{141}$</th>
<th>Leu$^{555}$</th>
<th>Trp$^{357}$</th>
<th>Arg$^{468}$</th>
<th>Ala$^{329}$</th>
<th>Ile$^{306}$</th>
<th>Gly$^{327}$</th>
<th>Arg$^{465}$</th>
<th>Asn$^{467}$</th>
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<tr>
<td>$k_{ex}$  (s$^{-1}$)</td>
<td>1520 ± 350</td>
<td>3 ± 1</td>
<td>46 ± 26</td>
<td>1474 ± 385</td>
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<td>$p_B$ (%)</td>
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<td>$k_{BA}$  (s$^{-1}$)$^a$</td>
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<td>$k_{AB}$  (s$^{-1}$)$^a$</td>
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<tr>
<td>$</td>
<td>\Delta\omega_{ex}</td>
<td>$ (ppm)$^b$</td>
<td>2.0</td>
<td>0.6</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
<td>0.7</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>15N chemical shift perturbation from PEP titration experiment</td>
<td>0.9$^d$</td>
<td>1.3</td>
<td>2.3</td>
<td>2.2</td>
<td>1.3</td>
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</table>

$^a$ The open (major) and closed (minor) states are referred to as A and B. $k_{AB}$ and $k_{BA}$ are the rate constants for the transition from A to B and from B to A, respectively, and are calculated from the values of the optimized parameters $k_{ex}$, ($k_{BA}$ + $k_{AB}$), and $p_B$.

$^b$ The error in the values of $|\Delta\omega_{ex}|$ is ± 0.1 ppm with the exception of those for Leu$^{555}$ and Arg$^{465}$, which are ± 0.2 ppm.

$^c$ 15N chemical shift perturbation upon addition of 10 mM PEP are provided for comparison with the values of $|\Delta\omega_{ex}|$ obtained from the relaxation dispersion experiments on free EIC.

$^d$ The significant difference between $|\Delta\omega_{ex}|$ and $\Delta\delta$ observed for Gly$^{327}$ can be ascribed to the different conformations adopted by the side chain of Arg$^{358}$ in free EIC and the EIC-PEP complex. In the crystal structures of free EIC, the guanidino group of Arg$^{358}$ is located 4–5 Å away from the backbone amide of Gly$^{337}$. However, in the structural model of the EIC-PEP complex displayed in Fig. 2, the Arg$^{358}$ side chain directly interacts with PEP and the positively charged guanidino group is located > 6 Å away from the backbone amide of Gly$^{337}$. Because the relaxation dispersion experiments were acquired in the absence of substrate, the effect of this conformational change on the 15N chemical shifts of EIC are observable in the $|\Delta\delta|$ values only.

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**FIGURE 3. Structure and dynamics of the EIC-PEP complex.** A, 600 MHz $^1$H-$^15$N TROSY correlation spectrum of EIC in the absence (red) and presence (blue) of 10 mM PEP. B, structural model for the EIC-PEP complex (see legend to Fig. 2) showing the extent of $^1$H-$^15$N chemical shift perturbation ($|\Delta\omega_{ex}|$) see “Experimental Procedures” for details) upon addition of 10 mM PEP to the protein sample. Assigned backbone amides are depicted as spheres and colored according to their $|\Delta\omega_{ex}|$ values (color scale ranges from blue to red). Amide groups assigned only for free EIC are displayed as gray spheres. The inset shows a close-up of the dimer interface. The amide groups exhibiting $|\Delta\omega_{ex}|$ values > 0.2 ppm are displayed as spheres. C, selected regions of the $^1$H-$^15$N TROSY spectrum of EIC showing the effect of increasing concentrations of PEP on cross-peaks originating from residues in the $\beta3\alpha3$ turn. The color code is as follows: red, 0 mM PEP; green, 0.2 mM PEP; yellow, 0.4 mM PEP; purple, 1 mM PEP; blue, 10 mM PEP. It should be noted that because PEP is hydrolyzed by EIC (see main text), the listed PEP concentrations are approximate values. D, $|\Delta\omega_{ex}|$ profile.

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**Effect of PEP on Spectrum of EIC**—Addition of 10 mM PEP to EIC results in substantial changes in the $^1$H-$^15$N TROSY spectrum (Fig. 3A). However, as we discuss in depth in a subsequent section, we noticed that formation of the EIC-PEP complex also results in degradation of PEP into pyruvate and inorganic phos-
phosphate, making the lifetime of the EIC-PEP complex too short (<4 h at 37 °C with 400 μM EIC and 10 mM PEP) to allow acquisition of the three-dimensional triple resonance experiments necessary for sequential backbone resonance assignment. Thus, only those \(^1H-^{15}N\) cross-peaks that could be tracked unambiguously in a PEP titration experiment were assigned in the EIC-PEP complex and used for further analysis.

The assigned backbone amides for the EIC-PEP complex and the corresponding \(^1H-^{15}N\) chemical shift perturbations (\(\Delta_{\text{HUN}}\)) arising from PEP binding are depicted on the EIC structure in Fig. 3B. Although the \(^1H-^{15}N\) cross-peaks of residues directly facing PEP were completely broadened out after addition of ligand, large \(\Delta_{\text{HUN}}\) values were still observed for the flanking residues (Ile\(^{277}\), Gly\(^{276}\), Ile\(^{426}\), and Gly\(^{427}\)) suggesting that structural rearrangements are occurring at the active site (Fig. 3, B and D). Moreover, the \(^1H-^{15}N\) cross-peaks of residues in the β3α3 turn experience extensive line broadening upon addition of PEP (Fig. 3C), indicating that the binding of the substrate affects the conformational dynamics detected in this area.

The perturbations on the \(^{15}N\) chemical shifts (\(\Delta_{\text{N}}\)) of EIC observed upon PEP binding are in excellent agreement with the \(\Delta_{\text{N}}\) values obtained from the relaxation dispersion measurements on free EIC (Table 1), strongly suggesting that binding of PEP shifts the conformational equilibrium detected in free EIC from the open to the closed state. This is consistent with the model of the \(E.\ coli\) EIC-PEP complex (Fig. 2B), derived from the structures of \(E.\ coli\) phosphorylated El (15) and the \(T.\ tengcongensis\) EIC-PEP complex (28), which shows that salt bridges between the phosphate group of PEP and the side chains of Lys\(^{340}\) and Arg\(^{358}\) lock the β3α3 turn in the closed conformation, providing further stabilization to the Gly\(^{337}\) → Lys\(^{340}\) and Ala\(^{359}\) → Ile\(^{366}\) backbone hydrogen bonds. These key interactions, together with an additional salt bridge between the phosphoryl group of PEP and the guanidino group of Arg\(^{465}\) (Fig. 2B), also serve to stabilize the backbone conformation of Leu\(^{355}\), Trp\(^{357}\), and Asn\(^{476}\) in the closed state observed in the \(\beta\)-turn structure of phosphorylated El. Because Leu\(^{355}\), Trp\(^{357}\), and Asn\(^{476}\) are involved in several intersubunit contacts (Fig. 2C), the above observations explain the lower dimerization \(K_D\) measured for EI in the presence of PEP (22), as well as the large \(\Delta_{\text{HUN}}\) perturbations induced by PEP for Glu\(^{351}\), Asn\(^{352}\), Gly\(^{356}\), and Trp\(^{1}\) in the β3α3 turn, and for Arg\(^{465}\), Asp\(^{468}\), Ile\(^{470}\), and Asn\(^{475}\) at the dimer interface (Fig. 3, B and C). In addition, the β3α3 turn is rich in aromatic residues (Tyr\(^{344}\), Phe\(^{347}\), Phe\(^{354}\), and Trp\(^{357}\)), so that conformational rearrangements in this region are fully consistent with the changes observed in the CD and fluorescence spectra of EIC upon PEP binding (14).

**Effect of PEP on Quaternary Structure of EIC**—To assess whether the local conformational changes in the β3α3 turn observed in EIC upon PEP binding affect the subunit orientation in the EIC dimer, backbone amide (\(^1\text{DNH}\)) RDCs for well resolved \(^1\text{H}-^{15}N\) cross-peaks were measured on samples of weakly aligned EIC and the EIC-PEP complex in a dilute liquid crystalline medium of phase \(pff1\) (39). To avoid structural noise from flexible regions, only the backbone amides from secondary-structure elements were included in the analysis. This necessary precaution, together with the fact that a low protein concentration was used in the EIC-PEP sample to limit the rate of PEP hydrolysis, reduced the number the experimental RDCs available for the EIC-PEP complex to 37 (Table 2). However, because \(^1\text{DNH}\) RDCs provide orientational information on NH bond vectors relative to an external alignment tensor (38, 50), the subunit structure of EIC is known, and one of the principal components of the alignment tensor must lie along the \(C_s\) symmetry axis of the dimer (17), this small number of RDCs is sufficient to fully describe the relative orientation of the two symmetry-related subunits (51).

**TABLE 2**

SVD analysis of \(^1\text{DNH}\) RDCs for the monomeric and dimeric forms of EIC and EIC-PEP

<table>
<thead>
<tr>
<th>Monomer/dimer</th>
<th>Number of RDCs</th>
<th>Euler angles (°)</th>
<th>(D_o) (Hz)</th>
<th>(\eta) (%)</th>
<th>R-factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIC</td>
<td>86/172</td>
<td>99/93</td>
<td>21/20</td>
<td>167/172</td>
<td>18.8/18.8</td>
</tr>
<tr>
<td>EIC-PEP</td>
<td>37/74</td>
<td>107/95</td>
<td>17/17</td>
<td>178/170</td>
<td>13.1/13.2</td>
</tr>
</tbody>
</table>

\(^a\) The first and second numbers listed give the results of the SVD fits to a single subunit and to the dimer.

\(^b\) The alignment tensor is described by five parameters: three Euler angles (φ, θ, and ψ), the magnitude of the alignment tensor \(D_o\), and the rhombicity \(\eta\). For a symmetric dimer, one of the axes of the principal components of the alignment tensor coincides with the \(C_s\) symmetry axis of the dimer. The fact that the values of the alignment tensor and RDC R-factor are the same for the SVD fits to a monomer and a dimer indicates that the orientation of the two subunits in the dimer in solution is the same as that in the crystal structure.

\(^c\) The RDC R-factor is given by \([1/(D_{obs} - D_{calc})^2] \times (2/D_{obs}^2)\), where \(D_{obs}\) and \(D_{calc}\) are the observed and calculated RDCs, respectively (52).

**FIGURE 4.** RDC analysis of free EIC and the EIC-PEP complex. A, free EIC. B, EIC-PEP complex. The two panels show a comparison of the observed and calculated RDCs obtained by SVD to the coordinates of an individual subunit of EIC (blue open circles) and the EIC dimer (red filled circles). The coordinates of the x-ray structure of phosphorylated El from \(E.\ coli\) were used (15).

**Conformational Dynamics of \(E.\ coli\) EIC**

The coordinates used for SVD analysis are those of EIC in the crystal structure of phosphorylated El (15). The RDCs for the EIC-PEP complex were measured on samples containing 100 μM EIC and 50 mM PEP. The stability of the NMR samples used for measuring the RDCs was confirmed by acquiring \(^1\text{H}-^{15}N\) TROSY spectra immediately before and after the acquisition of the ARTSY experiment.
Conformational Dynamics of E. coli EIC

**A**

![Image of Enzymatic activity of EIC](http://www.jbc.org/)

**B**

![Image of 1H-15N TROSY correlation spectrum](http://www.jbc.org/)

**C**

![Image of Michaelis-Menten parameters](http://www.jbc.org/)

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**Enzymatic Activity of E. coli EIC**—During the course of our NMR studies on the EIC-PEP complex, we noticed that the $^{1}$H-$^{15}$N TROSY correlation spectrum for the EIC-PEP complex changes over time and slowly reverts to that of the free protein, indicating that EIC degrades PEP and that the degradation products are no longer able to interact with the protein. This is confirmed by $^{31}$P NMR, which shows that a 100 μM EIC sample is able to fully hydrolyze 10 mM PEP into phosphate and pyruvate in <16 h (Fig. 5A). Similar results are obtained with full-length EI (Fig. 5A).

An enzymatic assay that detects the amount of inorganic phosphate released as a consequence of PEP degradation (see “Experimental Procedures”), reveals that the hydrolysis of PEP by both EI and EIC follows Michaelis-Menten kinetics with $K_m$ values of 327 and 374 μM, respectively (Fig. 5B and Table 3). These values agree well with the previously reported $K_m$ (~400 μM) for the EI-PEP interaction (20) and indicate that the isolated EIC domain binds PEP with essentially the same affinity as the full-length protein.

Differences, however, are observed for the E1 and EIC kinetics, with full-length EI hydrolyzing PEP with ~1.5 times higher efficiency (reported as $k_{cat}/K_m$ in Table 3) than the isolated EIC domain. A schematic diagram for the PEP degradation pathways catalyzed by EI and EIC is shown in Fig. 5C. The autophosphorylation reaction (equilibrium $d$ in Fig. 5C), which initiates the PTS and occurs in only the full-length protein, is ~6 orders of magnitude faster than the direct hydrolysis of PEP into pyruvate and inorganic phosphate catalyzed by EIC (equilibria $a$ and $c$ in Fig. 5C). Phosphorylated EI, which is rapidly formed after addition of PEP to the full-length protein, can generate inorganic phosphate through two independent pathways that occur with similar velocities (equilibria $e$ and $f$ in Fig. 5C), explaining the higher efficiency of PEP degradation exhibited by the full-length protein relative to EI. It is also worth noting that no

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**TABLE 3**

<table>
<thead>
<tr>
<th>Michaelis-Menten parameters for the hydrolysis of PEP into inorganic phosphate and pyruvate by EI and EIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (μM)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>EI</td>
</tr>
<tr>
<td>EIC (species 2)*</td>
</tr>
<tr>
<td>EIC (species 1)**</td>
</tr>
</tbody>
</table>

* EIC purified from species 2 exhibits a well dispersed $^{1}$H-$^{15}$N TROSY correlation spectrum typical of a folded protein (Fig. 1F).

** Exponentially over 3 orders of magnitude faster than the direct hydrolysis of PEP into pyruvate and inorganic phosphate catalyzed by EIC (equilibria $a$ and $c$ in Fig. 5C).

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**FIGURE 5.** Enzymatic activity of EIC. A, $^{31}$P NMR spectra showing hydrolysis of PEP catalyzed by EI and EIC. The blue spectra were acquired on 100 μM samples of EI (left panel) and EIC (right panel) immediately after addition of 10 mM PEP. The red spectra were acquired after 16 h of incubation at 37°C at which time all the PEP has been hydrolyzed to inorganic phosphate and pyruvate. B, Michaelis-Menten kinetics for EI (blue) and EIC (red) with the substrate PEP. C, schematic diagram showing the possible PEP degradation pathways by EI (top) and EIC (bottom). For each equilibrium, the velocity of the forward reaction is provided. Velocities for the equilibria $a$, $b$, $c$, and $f$ were calculated based on the $k_{cat}/K_m$ values reported in Table 3 for EI (b) and EIC (a, c, and f). The velocity for the equilibrium $d$ was calculated based on the second-order rate constant obtained for the EI autophosphorylation reaction at 25°C (21). The velocity for the equilibrium $e$ was calculated based on the half-life ($t_1/2$) for the decay of phosphorylated EI to unphosphorylated EI reported by Suh *et al.* (24). For all conversions, $K_m$ was set to 350 μM, and the EI concentration was set to 5 μM.
Conformational Dynamics of E. coli EIC

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The bacterial phosphotransferase system is a ubiquitous enzyme complex that transfers phosphate from phosphoenolpyruvate to a wide range of acceptor molecules. The structural and functional properties of this enzyme are regulated by the conformational dynamics of the enzyme complex. The phosphotransferase system consists of several components, including enzyme I (EI), which is responsible for the transfer of phosphate from phosphoenolpyruvate (PEP) to acceptor molecules. The conformational dynamics of EI have been studied extensively, and it has been shown that this enzyme can adopt multiple conformational states, including an open and a closed state.

The conformational dynamics of EI are important for the regulation of the phosphotransferase system. For example, the open state of EI is associated with the active transfer of phosphate, while the closed state is associated with the inactivation of the enzyme. The conformational dynamics of EI are also important for the regulation of EI by other enzymes, such as EI-III, which is responsible for the activation of EI.

The conformational dynamics of EI are controlled by several factors, including the concentration of PEP, the presence of acceptor molecules, and the presence of regulatory proteins. The conformational dynamics of EI are also important for the regulation of other aspects of EI, such as its ability to interact with other components of the phosphotransferase system.

The conformational dynamics of EI are important for the regulation of the phosphotransferase system. The regulation of EI by the conformational dynamics of the enzyme complex is important for the regulation of the phosphotransferase system as a whole, and for the regulation of other enzymes that are regulated by the phosphotransferase system. The understanding of the conformational dynamics of EI is important for the development of new strategies for the regulation of the phosphotransferase system, and for the development of new strategies for the treatment of diseases that are associated with the dysregulation of the phosphotransferase system.

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Protein Structure and Folding:
Conformational Selection and Substrate Binding Regulate the Monomer/Dimer Equilibrium of the C-terminal domain of *Escherichia coli* Enzyme I

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