Large interdomain rearrangement triggered by suppression of micro- to millisecond dynamics in bacterial Enzyme I

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Enzyme I (EI), the first component of the bacterial phosphotransfer signal transduction system, undergoes one of the largest substrate-induced interdomain rearrangements documented to date. Here we characterize the perturbations generated by two small molecules, the natural substrate phosphoenolpyruvate and the inhibitor α-ketoglutarate, on the structure and dynamics of EI using NMR, small-angle X-ray scattering and biochemical techniques. The results indicate unambiguously that the open-to-closed conformational switch of EI is triggered by complete suppression of micro- to millisecond dynamics within the C-terminal domain of EI. Indeed, we show that a ligand-induced transition from a dynamic to a more rigid conformational state of the C-terminal domain stabilizes the interface between the N- and C-terminal domains observed in the structure of the closed state, thereby promoting the resulting conformational switch and autophosphorylation of EI. The mechanisms described here may be common to several other multidomain proteins and allosteric systems.
Large rigid body reorientations of one structural domain relative to another induced by small-molecule binding is a key feature of many biological systems, governing diverse processes such as catalysis, the formation of protein assemblies, cellular locomotion and motor translocation. Although the structural changes underlying domain reorientations have long been recognized, the fundamental forces that trigger and drive large conformational rearrangements remain unknown, with the exception of trivial cases in which ligands bind directly to the interface or hinge point between two domains.

Here we investigate the effect of two small-molecule ligands, the substrate phosphoenolpyruvate (PEP) and the competitive inhibitor α-ketoglutarate (αKG), on the structure and dynamics of E. coli Enzyme I (EI). Autophosphorylation of EI by PEP activates the bacterial phosphotransfer signal transduction system, a phosphorylation cascade involving multiple bimolecular phosphoryl transfer steps that results in eventual active sugar transport across the cell membrane. EI is also competitively inhibited by αKG, the carbon substrate for ammonia assimilation. If bacteria are cultured under conditions of nitrogen limitation, EI inhibition by αKG is strong enough to abolish active sugar uptake by the phosphotransfer signal transduction system, thereby providing a regulatory link between central carbon and nitrogen metabolisms.

The functional form of EI is a 128-kDa dimer of identical subunits comprising two structurally and functionally distinct domains (Fig. 1a). The N-terminal phosphoryl-transfer domain (EIN, residues 1–249) contains the phosphorylation site (H189) and the binding site for PEP. The C-terminal domain (EIC, residues 261–575) is responsible for dimerization and contains the binding site for PEP. The EIN and EIC domains are connected to one another by a long helical linker. Isolated EIN can transfer a phosphoryl group to HPr but cannot be autophosphorylated by PEP. The solution structure of EI in its apo form has been solved by a combination of NMR residual dipolar coupling and X-ray scattering data. In the absence of ligands, EI adopts an open conformation (state A) in which the EIN domains of the two monomeric subunits are more than 60 Å apart. The holo structure of the enzyme is more elusive and has currently not yet been determined. Addition of PEP to EI results in autophosphorylation of the enzyme at the H189 position and formation of pyruvate as a by-product of the enzymatic reaction. The X-ray structure, however, of a phosphorylated EI intermediate captured immediately after autophosphorylation by PEP has been solved by crystallizing the protein in the presence of PEP and Mg2+ and quenching the autophosphorylation reaction by addition of the inhibitor oxalate. In the phosphorylated intermediate, the enzyme adopts a closed conformation (state B) in which the EIN domains of the two subunits are in direct contact, and the active site H189 is inserted in the PEP-binding pocket of the EIC domain. The open-to-closed transition involves two coupled, rigid body rotations of the two subdomains of EI: namely, a ~90° reorientation of the α/β subdomain relative to EIC (Fig. 1a) and a ~70° reorientation of the α subdomain relative to the β/α subdomain (Fig. 1b). Modelling the position of PEP by replacing oxalate and the phosphoryl group indicates that the closed conformation is fully consistent with in-line phosphoryl transfer from PEP bound to the EIC domain to H189 on the α/β subdomain of EIN (Fig. 1c). However, it is not clear whether the closed conformation corresponds to the holo form of the enzyme or is only transiently sampled to permit the phosphoryl transfer reaction. Once H189 in the α/β subdomain is phosphorylated, conformational rearrangement rapidly occurs to a structure that resembles the apo form, thereby exposing the phosphoryl group and making it available for in-line phosphoryl transfer to HPr bound to the α subdomain (Fig. 1a).

Here using solution NMR and small-angle X-ray scattering (SAXS) we show that binding of PEP to the EIC domain induces a

**Figure 1 | EI adopts several conformations during its catalytic cycle.** (a) Experimental structures of full-length E. coli EI. The EIC domain is coloured pink. The α and α/β subdomains of EIN are coloured blue and light blue, respectively. HPr is shown in green. The active site histidines, H189 of EI and H15 of HPr, are shown as red and orange spheres, respectively. (b) Conformations adopted by EIN in the open (state A) and closed (state B) structures. (c) Structural model of PEP bound to the active site of closed EI. PEP is shown as solid sticks. The phosphorylated H189 and the oxalate molecule in the X-ray structure of the closed phosphorylated intermediate are shown as transparent sticks. The magnesium ion present in the X-ray structure is displayed as a yellow sphere.
dynamic equilibrium in which both the open and closed forms of EI are populated. Interestingly, we observe that no contacts between PEP and the EIN domain are needed to trigger the open-to-closed transition of EI. Although this finding cannot be explained in terms of the available static structures of EI, we show that a transition from a dynamic to a more rigid EIC structure, induced by PEP-binding, is responsible for the large conformational rearrangement. Our results provide direct evidence for large-scale interdomain rearrangements induced by a change in dynamics within one of the protein domains, further supporting the recently emerging idea of protein flexibility as a general mechanism for transmitting signals between distant sites in macromolecules or macromolecular assemblies.

Results

Effect of PEP and αKG on the open-to-closed conformational equilibrium of EI. The effect of PEP and αKG on the global structure of EI was assessed by means of backbone (1H N/15N) and side chain (1H/13C methyl) chemical shift perturbation mapping and SAXS. Experiments were acquired on the wild-type protein (EI WT) and on mutants of EI in which the active site H189 was replaced by Gln (EIQ) or Ala (EIA). The EIQ and EIA mutants allow the PEP-induced structural perturbations to be probed without interference caused by phosphorylation of H189, and have been extensively employed in structural and biochemical studies of EI.

Addition of 5 mM PEP to EI WT, EIQ and EIA results in substantial changes in the 1H N/15N and 1H/13C methyl transverse relaxation optimized (TROSY) correlation spectra (Fig. 2a and Supplementary Fig. 1). As expected, larger chemical shift perturbations (ΔH/N,C) are observed in the EIC domain and involve residues located in the PEP-binding site and at the dimer interface (Fig. 2a). These perturbations closely resemble the PEP-induced chemical shift perturbations observed previously for isolated EIC (Supplementary Fig. 2). In addition, chemical shift perturbations are also observed in the EIN domain at sites located more than 20 Å away from the PEP-binding site in the open EI structure (Fig. 2a). In particular, the largest perturbations on the EIN domain are observed in the linkers connecting the α and γ/β subdomains, in the vicinity of the helical linker connecting EIN to EIC, at the interface between the α and γ/β subdomains, and at the interfaces between EIN and EIC and between the two EIN subunits in the closed EI structure (Fig. 2a). All these regions are expected to experience chemical shift perturbations upon transition from the open to the closed form of EI. In contrast, addition of 10 mM αKG to EI WT, EIQ or EIA does not result in any significant chemical shift perturbation within the EIN domain (Fig. 2b and Supplementary Fig. 1); the only chemical-shift changes observed are located within the EIC domain, and closely resemble the chemical shift perturbations induced by αKG on isolated EIC (Supplementary Fig. 2).

Overall, the chemical shift perturbation data suggest that only PEP binding to EIC is able to trigger the transition from the open to the closed form of EI, whereas binding of αKG leaves the overall structure of EI unperturbed in the open state. To test this hypothesis, SAXS data were acquired for EI WT, EIQ and EIA in the presence and absence of ligands. SAXS curves are linear time-averaged and ensemble weighted experimental measurements that are highly sensitive to the overall size and shape of a molecule. Thus, SAXS is a good reporter of conformational equilibria involving large interdomain rearrangements. The SAXS data were fit (up to q = 0.16 Å⁻¹) with a helper programme distributed with Xplor-NIH (see Supplementary Methods) using a grid search (step size = 1%) in which increasing populations of the closed EI conformer were added to the starting system comprising EI in the open configuration. A plot of χ² versus population of closed state is shown in Fig. 3a. In general, raising the population of the closed EI state results in larger χ² values for the majority of the scattering curves, indicating that the...
SAXS data acquired for most of the EI samples are best fit by the open structure of the enzyme. However, exceptions to this trend are observed for the scattering curves obtained for EI\textsuperscript{WT} and EI\textsuperscript{A} in the presence of PEP where the $\chi^2$ minimum is reached at closed-state populations of $\sim 11\%$ and $\sim 62\%$ for the EI\textsuperscript{Q}-PEP and the EI\textsuperscript{A}-PEP complexes, respectively (Fig. 3a,b), confirming that only the interaction of PEP with EI\textsuperscript{C} is able to trigger the open-to-closed conformational transition of EI.

We note that although the SAXS curves in the absence of ligands are fit very well by the open structure of EI ($\chi^2 \leq 1$), small, systematic deviations between the experimental and calculated SAXS curves are observed for the EI\textsuperscript{A}-PEP complex (Fig. 3b), for which the largest population of closed EI was obtained from the fits (Fig. 3a). This suggests that the crystal structure of the phosphorylated intermediate does not fully describe the solution structure of holo EI. Assessment of the small discrepancy between the structures of closed EI in the crystal and solution states is beyond the scope of the present work and will be the subject of future investigations.

The SAXS data in Fig. 3a,b indicate that PEP binding does not fully stabilize the closed state of EI, resulting in a dynamic equilibrium in which both open and closed forms are populated. This equilibrium is modulated by the nature of the amino-acid side chain at position 189 in a manner that can be readily rationalized by simple steric and electrostatic considerations.

In particular, addition of PEP to samples of EI\textsuperscript{WT} results in rapid phosphorylation of the active site H189. Steric and electrostatic repulsion between the phosphoryl group of PEP and phosphorylated H189 destabilizes the closed form of EI (Fig. 4a), thereby keeping the enzyme in the open state (Fig. 3a) with the phosphoryl group of H189 exposed and available for in-line phosphoryl transfer to HPr. For the EI\textsuperscript{Q}-PEP complex, Q189 cannot be phosphorylated by PEP, and only minor contacts between PEP and the side chain of Q189 are formed (Fig. 4b). The weaker repulsion between Q189 and PEP results in partial stabilization of the closed state of EI, consistent with the larger population ($\sim 11\%$) of closed state obtained from analysis of the SAXS data for the EI\textsuperscript{Q}-PEP complex (Fig. 3a). When the active site H189 is mutated to Ala, no contacts are formed between PEP and A189 (Fig. 4c), resulting in the largest population of closed state ($\sim 62\%$) derived from analysis of the SAXS data (Fig. 3a). Thus, we conclude that no direct contacts between PEP and the EIN domain are required to trigger the open-to-closed transition of EI.

The conclusions drawn from the SAXS analysis correlate well with the affinities of EI\textsuperscript{WT}, EI\textsuperscript{A} and EI\textsuperscript{Q} for PEP and $\alpha$KG. Enzyme kinetic assays indicate that the affinity of PEP for EI\textsuperscript{WT} ($K_m \sim 330 \pm 20 \mu M$) is two times lower than for EI\textsuperscript{Q} ($K_m \sim 130 \pm 10 \mu M$) and about an order of magnitude lower than for EI\textsuperscript{A} ($K_m < 50 \mu M$; Fig. 5a), reflecting the fact that only the...
open form of EI can release the substrate/product from the binding pocket on the EIC domain. In contrast, NMR titration experiments show that mutations of H189 have no effect on the affinity of EI WT for PEP (K<sub>D</sub>~2.0 ± 0.2 mM, Fig. 5b), confirming that this small-molecule inhibitor does not perturb the overall structure of EI. It is also worth noting that the affinities of isolated EIC for PEP (K<sub>D</sub>~370 ± 30 μM)<sup>17</sup> and zKG (K<sub>D</sub>~2.2 ± 0.2 mM)<sup>8</sup>, are the same (within experimental error) as the affinities of full-length EI WT for PEP and of EI WT, EIQ and EIA for zKG, in agreement with the conclusion from SAXS analysis that the EI WT-PEP and all the investigated EI complexes with zKG adopt a fully open conformation (Fig. 3a).

**Effect of PEP and zKG on μs-ms dynamics of EI.** The results presented in the previous section clearly indicate that the open-to-closed transition of EI is induced by PEP binding to the EIC domain with no requirement for direct interactions between the substrate and EIN domain. On the other hand, zKG, which shares most of the same interactions as PEP with the EIC domain (Fig. 5c,d), does not affect the relative positioning of the EIN domain. Inspection of the various EI X-ray structures shows very little variability in the coordinates of the EIC domain (backbone r.m.s.d. to the mean coordinate positions of 2 Å for residues 261–572, Supplementary Fig. 3), irrespective of the presence of ligands bound to EI. It is therefore not evident how PEP-binding triggers the massive conformational rearrangement from the open to closed state.

Using <sup>15</sup>N NMR relaxation dispersion experiments, we recently showed that the PEP-binding pocket of isolated EIC undergoes a dynamic conformational equilibrium on the submillisecond timescale (κ<sub>ex</sub>~1,500 s<sup>−1</sup>) between a major (‘expanded’) state (population ~97%) and a minor state (population ~3%) that is more ‘compact’ (in terms of several loops) and closely resembles the PEP-bound state<sup>17</sup>. To investigate whether PEP and zKG affect this conformational equilibrium in full-length EI, we analysed the μs-ms dynamics of EI WT, EIQ and EIA, in the presence and absence of ligands, using <sup>1</sup>H–<sup>13</sup>C Multiple-Quantum (MQ)<sup>21</sup> and <sup>13</sup>C Single-Quantum (SQ)<sup>22</sup> Carr–Purcell–Meiboom–Gill (CPMG)<sup>23</sup> relaxation dispersion spectroscopy. Experiments were acquired at 600, 800 and 900 MHz on the methyl groups of U-[<sup>3</sup>H, <sup>15</sup>N]<Ile(δ1)</Ile, <sup>13</sup>C<sub>1</sub>-labelled and U-[<sup>3</sup>H, <sup>15</sup>N]<Val,Leu-(<sup>13</sup>C<sub>1</sub><sup>2</sup>C<sub>2</sub>H<sub>3</sub>)]-labelled EI. Like <sup>15</sup>N-CPMG relaxation dispersion, the <sup>13</sup>C<sub>me</sub> relaxation dispersion experiments probe exchange dynamics between species with distinct chemical shifts on a time scale ranging from ~50 μs to ~10 ms. However, owing to favourable relaxation properties, the absence of exchange with water and the threefold degeneracy of methyl proton chemical shifts, the <sup>13</sup>C<sub>me</sub> relaxation dispersion experiments yield data of superior quality for large macromolecular systems<sup>24</sup> such as the full-length 128 kDa EI.

In the absence of ligands, large exchange contributions to the transverse MQ-relaxation rates (M–R<sub>ex</sub>) were detected for several Ile, Val and Leu side chains in the EIC domain (Fig. 6a and Supplementary Fig. 4). The majority of methyl groups experiencing conformational exchange are located within or in the vicinity of the β3α3 (residues 333–366) and β6α6 (residues 453–477) loops that were shown to be dynamic in isolated EIC (Fig. 6b). In addition, large MQ-R<sub>ex</sub> values were observed for

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**Figure 5** | Effect of mutations at position 189 on the affinity of EI for PEP and zKG. (a) Michaelis–Menten kinetics for EI WT (black), EIQ (blue) and EIA (red) with the substrate PEP. Experimental velocities were normalized to the maximum velocity obtained by the individual fits (solid lines). (b) Δ<sub>H/N</sub> values for EI WT (black), EIQ (blue) and EIA (red) as a function of zKG concentration. Data for all residues showing Δ<sub>H/N</sub> > 0.1 ppm at 10 mM zKG were fit simultaneously using a one-site binding model per subunit (see Methods). The three data sets can be fit with the same parameters, irrespective of the presence of ligands, using 1H–13C Multiple-Quantum (MQ) and 13C Single-Quantum (SQ) Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments. Experiments were acquired at 600, 800 and 900 MHz on the methyl groups of U-[<sup>3</sup>H, <sup>15</sup>N]<Ile(δ1)</Ile, <sup>13</sup>C<sub>1</sub>-labelled and U-[<sup>3</sup>H, <sup>15</sup>N]<Val,Leu-(<sup>13</sup>C<sub>1</sub><sup>2</sup>C<sub>2</sub>H<sub>3</sub>)]-labelled EI. Like <sup>15</sup>N-CPMG relaxation dispersion, the <sup>13</sup>C<sub>me</sub> relaxation dispersion experiments probe exchange dynamics between species with distinct chemical shifts on a time scale ranging from ~50 μs to ~10 ms. However, owing to favourable relaxation properties, the absence of exchange with water and the threefold degeneracy of methyl proton chemical shifts, the <sup>13</sup>C<sub>me</sub> relaxation dispersion experiments yield data of superior quality for large macromolecular systems such as the full-length 128 kDa EI.

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Figure 6 | μs-ms dynamics in full-length EI. (a) Exchange contribution to the transverse MQ-relaxation rates (MQ-Rex) at 800 MHz measured for samples of EIWT, EIα, EIα-αKG (in the presence of 50 mM αKG) and EIα-PEP (in the presence of 50 mM PEP). Boundaries of the EIN and EIC domains in the full-length EI sequence are highlighted by red horizontal lines. Similar plots are provided for all the analysed samples in Supplementary Fig. 4 (error bars: 1 s.d.). (b) The 19 methyl groups showing MQ-Rex values >3 s⁻¹ in free EI are plotted as red spheres on the structure of EI in the open state. The EIN and EIC domains are shown as light blue and pink cartoons, respectively. The second subunit of the EI dimer is shown as a transparent surface. Backbone amides showing 15N relaxation dispersions in the isolated EIC domain are shown as blue spheres. The β3α3 and β6α6 loops are coloured cyan and purple, respectively. PEP is shown as green sticks and the magnesium ion as a yellow sphere. (c) Examples of typical MQ and SQ relaxation dispersion data acquired on EI in the presence of 50 mM αKG at 800 MHz (blue), 600 MHz (red), 500 MHz (green), 400 MHz (magenta) and 300 MHz (cyan) for two representative residues. The experimental data are represented by filled-in circles, and the best-fit curves for a two-site exchange model are shown as solid lines. (d) Summary of relaxation dispersion data plotted on the structure of the closed state of EI. Colour coding is the same as in (b).

L264, V269 and I533 located in the vicinity of the long helical linker connecting the EIC and EIN domains (Fig. 6a,b). All the MQ and SQ relaxation dispersion curves of the 19 methyl groups exhibiting MQ-Rex values >3 s⁻¹ at 800 MHz were fit simultaneously to a model describing the interconversion of two conformational states (see Supplementary Methods). In the global fitting procedure, the exchange rate (kex) and the fractional population of the minor state (pβ) were optimized as global parameters, whereas the 13C chemical shift differences between the two conformational states (ΔωC) were treated as methyl-specific parameters. MQ relaxation dispersion curves also depend upon the 1H chemical shift differences between the two conformational states (ΔωH, see Supplementary Methods). However, an initial residue-based fitting (in which kex, pβ, ΔωC and ΔωH were all optimized as methyl-specific parameters) indicated that the contribution of ΔωH to the observed dispersions is negligible for all analysed methyl groups (Supplementary Fig. 4). Therefore, to improve convergence of the calculation, the methyl-specific ΔωH values were set to zero in the subsequent global fit of the relaxation dispersion curves. An example of the global fit is provided in Fig. 4c. Curves for all the 19 methyl groups as well as the optimized ΔωC values are provided in Supplementary Fig. 6 and Supplementary Table 1, respectively. The overall exchange rate (sum of forward and backward rate constants) is 1,200 ± 50 s⁻¹, and agrees with experimental error with the kex value obtained for the isolated EIC domain (1,500 ± 350 s⁻¹)17. The population of the minor species, however, is 13 ± 5%, which is considerably larger than the value of 3 ± 1% obtained from fitting the 15N-CPMG data for isolated EIC17. Consistent with this latter finding, an overlay of the NMR spectra acquired for full-length EI and the isolated EIC domain shows that, in the absence of ligands, the NMR signals of the full-length EI protein than in the isolated EIC domain (Fig. 7), indicating that the presence of the EIN domain promotes sampling by the EIC domain of the more compact conformational state that resembles the substrate-bound form.

Thus, the 13Cmethyl-CPMG relaxation dispersion data presented above for free EI describe the same conformational equilibrium previously detected in the PEP-binding site of the isolated EIC domain by 15N backbone CPMG relaxation dispersion experiments17. The 1H-13Cmethyl MQ CPMG relaxation dispersion experiments acquired on EI in the presence of ligands indicate that PEP and αKG affect the conformational equilibrium differently. In particular, analysis of the MQ-Rex data obtained at 800 MHz for the EIα-αKG and EIα-PEP complexes shows that PEP binding completely suppresses μs-ms dynamics within the EIC domain (Fig. 6a and Supplementary Fig. 4). In this regard, the closed form of EI is stabilized by contacts between the α/β subdomain of EIN and the
β323 and β626 loops of EIC, which have been observed to undergo dynamics in the free enzyme (Fig. 6d and Supplementary Fig. 7). Structural stabilization of the β323 and β626 loops in the compact PEP-bound form allows optimal docking of the EIN domain onto the EIC domain, and is therefore responsible for triggering the open-to-closed transition of EI.

We also noticed that, although the chemical shift perturbations induced by PEP on the backbone 15N resonances of isolated EIC closely match the residue-specific ΔΔνN parameters obtained from the fit of the 15N relaxation dispersion data acquired on the C-terminal domain of the enzyme17, and a moderate correlation is observed between the methyl-specific ΔΔνC parameters derived from relaxation dispersion data on wild-type EI versus the chemical shift perturbations induced by PEP on the 13C methyl resonances of full-length EI16 and EIA (ΔΔνC, Supplementary Fig. 8), the measured ΔΔνC values for several methyl groups located within or in the proximity of the β323 loop are considerably larger than the corresponding ΔΔνC values (Supplementary Fig. 8). Taken together, these observations suggest that the compact state transiently sampled by the EIC domain in the absence of PEP, while overall is similar to the PEP-bound state, differs from the PEP-bound structure in terms of side-chain packing within and in the proximity of the β323 loop (Supplementary Fig. 8). Interestingly, contacts between the α/β subdomain of EIN and the β323 loop of EIC account for ~20% of the interdomain interface in closed EI (Supplementary Fig. 7). The different conformations adopted by the β323 loop in the compact states sampled by EIC in the absence (compactI) and presence (compactII) of PEP are consistent with our SAXS analysis indicating that the closed state of EI is significantly populated only in the presence of PEP (Fig. 3a).

zKG, on the other hand, is not able to provide full stabilization of the EIC domain, as evidenced by the fact that several methyl groups with MQ-Rex values > 3 s⁻¹ are still observed for the zKG complexes with EIWT, EIA and EIQ (Fig. 6c and Supplementary Fig. 4). Specifically, large MQ-Rex values for the zKG complexes are observed for residues 331, 336, 342, 355 and 378 within or in the proximity of the β323 loop (Fig. 6b and Supplementary Fig. 9), which does not make contacts with zKG but does interact with PEP (Fig. 5c,d and Supplementary Fig. 9); likewise large MQ-Rex values are also observed for residues 482, 488 and 505 that are located close to the active site Cys502, which forms hydrophobic contacts with PEP and is involved in one hydrogen bond with the C2 carbonyl group of zKG (Fig. 5c,d and Supplementary Fig. 9). Other MQ-Rex values > 3 s⁻¹ are detected for residues 264 and 269 that are spatially close to the linker helix. Thus, in the case of the complexes with zKG, the more dynamic state of the long β323 loop hampers effective

Figure 7 | Comparison of the NMR spectra of EI and EIC in the free and bound forms. Overlay of the 1H,15N TROSY correlation spectra for isolated EIC in the absence (red) and presence (blue) of 50 mM PEP, and for full-length EI in the absence (pink) and presence (cyan) of 50 mM PEP. Close-up views of the cross peaks for residues I426 and G427 are provided. The amide groups of I426 and G427 show very large 15N relaxation dispersion in isolated EIC, and experience large PEP-induced chemical shift perturbations17. Interestingly, I426 and G427 are located more than 15 Å away from the PEP-binding site in the structure of the EI-PEP complex, and the changes in chemical shift for these two residues are exclusively dependent upon the change in the populations of the expanded and compact conformational states of EIC induced by PEP-binding17. Considering that (i) for the isolated EIC in the absence of PEP the population of compact conformer is ~3% (ref. 17), (ii) at saturating concentrations of PEP the population of compact EIC is ~100% (ref. 17) and (iii) the observed chemical shift is a population weighted average of the chemical shifts of the expanded and compact states, the theoretical position of the cross-peaks for I426 and G427 in the presence of 13% compact state can easily be calculated from the position of the peaks in the spectra of isolated EIC (~3% compact state) and of the isolated EIC-PEP complex (~100% compact state). The positions of the calculated cross-peaks are reported as black crosses, and agree very well with the position of the cross peaks in the spectra of the full-length enzyme in the absence of ligand. These results provide an independent validation of the accuracy of the global fit of the MQ and SQ relaxation dispersion curves.

Figure 8 | Coupling between open-to-closed and expanded-to-compact conformational equilibria of EI. The EIN and EIC domains are coloured blue and red, respectively. The PEP molecule is coloured green. kex is the exchange rate constant for the conformational equilibrium, and Km the Michaelis constant for the EI-PEP complex. The compact state of EIC exists in two different conformations (compactI and compactII) that differ in side chain packing within and in the proximity of the β323 loop (see main text). Populations of the different conformational states are indicated. Populations on the left side of the free/bound equilibrium correspond to the populations obtained from the fit of the relaxation dispersion data acquired on EIWT; populations on the right side correspond to the populations obtained from the fit of the SAXS curves acquired for the EI(WT)-PEP complex.
docking of EIN over EIC, thereby destabilizing the closed EI configuration. As a final note, although the EIN domain of EI<sup>Δ</sup> experiences significant PEP-induced chemical shift perturbations (Fig. 2a), and the SAXS data reveal that the closed EI form is highly populated in the presence of PEP (population > 50% for the EI<sup>Δ</sup>–PEP complex, Fig. 3), no relaxation dispersions are observed in the EIN domain (Fig. 6a,d), and a single set of cross-peaks is observed in the NMR spectra of all the investigated EI–PEP complexes. These observations indicate that the interdomain rearrangement in EI is too fast (＞10,000 s<sup>−1</sup>) to be detected by CPMG-type relaxation dispersion measurements. The intricate coupling between the open-to-closed dynamics for EI and the expanded-to-compact conformational exchange within the EIC domain revealed by the present work is summarized in Fig. 8.

Discussion
Addressing the mechanisms whereby small-molecule ligands modulate the interactions between protein domains is of paramount importance for understanding fundamental phenomena in biology such as protein switches and allostery. In this communication, we have investigated the effect of two small molecules on the interaction between the EIN and EIC domains of EI, the first component of the bacterial phosphotransferase system. The open-to-closed conformational change induced by substrate binding to EI (Fig. 1a) is one of the largest ligand-induced interdomain rearrangements reported in the literature so far. Although there is now a wealth of structural studies on EI and its complexes<sup>30–33</sup>, the fundamental forces driving this conformational rearrangement were not evident. Here, we have shown that binding of the substrate PEP to the EIC domain of EI results in structural stabilization of the otherwise dynamic loop D2 HWG in EIC that forms a large portion of the interface between the EIC and EIN domains in the structure of the closed state of EI. This finding provides a direct link between ligand binding and the open-to-closed conformational transition, and is a clear example of how conformational dynamics and small-molecule binding have co-evolved to ensure the proper biological function upon this enzyme. Ligand-induced stabilization (or destabilization) of the interface between protein domains may be a common mechanism by which small-molecule binding propagates into large interdomain rearrangements, and may serve as the basis for interdomain communication in a recently discovered class of allosteric proteins where disorder-to-order transitions induced by ligand binding within one domain were shown to be associated with changes in the spectral and thermodynamic properties of adjacent structural motifs<sup>30–33</sup>.

Methods

Protein expression and purification. Mutants of <i>E. coli</i> EI (H189Q and H189A) were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). EI (wild type and mutants) was expressed and purified as described previously<sup>17</sup>. U-[<sup>15</sup>N]<sup>2</sup>ValLeu-[<sup>13</sup>C<sub>5</sub>;<sup>13</sup>C<sub>5</sub>;<sup>13</sup>C<sub>5</sub>]<sup>2</sup>His<sub>2</sub> and U-[<sup>15</sup>N]<sup>2</sup>ValLeu-[<sup>13</sup>C<sub>5</sub>;<sup>13</sup>C<sub>5</sub>;<sup>13</sup>C<sub>5</sub>]<sup>2</sup>His<sub>2</sub> labelled EI samples were prepared following standard protocols for specific isotope labelling of the methyl groups of Ile, Leu and Val side chains<sup>34</sup>.

Small-angle X-ray scattering. SAXS data were collected on samples of EI (6 mg ml<sup>−1</sup> corresponding to ＞48 μM dimer) in 20 mM Tris buffer, pH 7.4, 100 mM NaCl, 10 mM DTT, 4 mM MgCl<sub>2</sub>, 1 mM EDTA and one tablet of complete, EDTA-free protease inhibitor cocktail (Roche). Ligands were added to a final concentration of 20 mM to immediately before data acquisition. SAXS data were acquired at Beamline 12-IDC at the Advanced Photon Source (Argonne National Laboratory) at protein concentrations ranging from 1.5 to 6 mg ml<sup>−1</sup>. Data collection was done using a Gold CCD detector positioned at 2 m from the sample capillary. Incident radiation with an energy of 18 keV was used, resulting in observable q-ranges of 0.014–0.24 Å<sup>−1</sup>. Q-axis mapping was done using scattering from a silver behenate standard sample. A total of 20 sequential data frames with exposure times of 1.5 s were recorded with the samples kept at 25 °C throughout the measurement. To prevent radiation damage, volumes of 100 μl of samples and buffers were oscillated during data collection using a flow-through setup. Individual data frames were masked, corrected for the detector sensitivity, corrected for background and normalized against corresponding transmitted beam intensities. The final 1D scattering profiles and their uncertainties were calculated as means and standard deviations over the 20 individual frames.

Subtraction of the buffer data and analysis of the scattering profiles was carried out using the Xplor-NIH calsXAS-Subsift helper programme (see Supplementary Information). The SAXS data were used to build a linear combination of the experimental 3D structures of <i>E. coli</i> EI in the open (PDB code: 2KX9<sup>19</sup>) and closed (PDB code: 2HWG<sup>10</sup>) conformational states. Fits were performed for χ values in the range 0.02–0.16 Å<sup>−1</sup>. In this range, SAXS data are characterized by very high signal-to-noise ratio and are mainly dependent on the overall shape of the molecule (that is, contributions from small rearrangements within the domains are negligible)<sup>17</sup>.

NMR spectroscopy. NMR samples were prepared in 20 mM Tris buffer, pH 7.4, 100 mM NaCl, 4 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM DTT and either 90% H<sub>2</sub>O/10% D<sub>2</sub>O (for NMR experiments acquired on the backbone amides) or 100% D<sub>2</sub>O (for experiments acquired on methyl groups). The protein concentration (in subunits) was 0.3 and 1.0 mM for experiments run in the presence and in the absence of PEP, respectively. The concentrations of PEP and xkG used in the relaxation dispersion experiments were 50 mM.

NMR spectra were recorded at 37 °C on Bruker 800, 800 and 600 MHz spectrometers equipped with z-gradient nuclear triple resonance cryoprobes. Spectra were processed using NMRPipe<sup>35</sup> and analysed using the programme SPARKY<sup>36</sup>. The 1H–15N TROSY spectra of free EI were acquired using previously described pulse schemes. Resonance assignments of the 1H–15N and 1H–13C methyl TROSY spectra of free EI were achieved by transferring the assignments obtained for the isolated EIC<sup>17</sup> and EIC<sup>17</sup> domains on the SAXS data reveal that the closed EI form is highly observable q-values and are mainly dependent on the overall shape of the molecule (that is, contributions from small rearrangements within the domains are negligible)<sup>34</sup>.

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