The oligomerization state of bacterial enzyme I (EI) determines EI’s allosteric stimulation or competitive inhibition by α-ketoglutarate

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ABSTRACT

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 phosphorylation of enzyme I (EI) by phosphoenolpyruvate (PEP). The EI phosphorylation state determines the phosphorylation states of all other PTS components and is thought to play a central role in the regulation of several metabolic pathways and to control the biology of bacterial cells at multiple levels, for example, affecting virulence and biofilm formation. Given EI’s pivotal role in bacterial metabolism, an improved understanding of the mechanisms controlling its activity could inform future strategies for bioengineering and antimicrobial design. Here, we report an enzymatic assay, based on SOFAST NMR experiments, to investigate the effect of the small-molecule metabolite α-ketoglutarate (αKG) on the kinetics of the EI-catalyzed phosphoryl transfer reaction. We show that, at experimental conditions favoring the monomeric form of EI, αKG promotes dimerization and acts as an allosteric stimulator of the enzyme. However, when EI’s oligomerization state is shifted toward the dimeric species, αKG functions as a competitive inhibitor of EI. We developed a kinetic model that fully accounted for the experimental data and indicated that bacterial cells might use the observed interplay between allosteric stimulation and competitive inhibition of EI by αKG to respond to physiological fluctuations in the intracellular environment. We expect that the mechanism for regulating EI activity revealed here is common to several other oligomeric enzymes.

Enzyme I (EI) is the first protein of the bacterial phosphotransferase system (PTS), a signal transduction pathway that results in active sugar transport across the cell membrane (1-3). The PTS is initiated by phosphorylation of EI by the small molecule phosphoenolpyruvate (PEP). Phosphorylated EI transfers the phosphoryl group to the phosphocarrier protein HPr. Thereafter, the phosphoryl group is transferred to a sugar-specific enzyme II (EII), and finally to the incoming sugar (Figure 1a). Recently, the small-molecule metabolite α-ketoglutarate (αKG) was shown to act as a competitive inhibitor of EI (inhibition constant, $K_i \sim 2.2$ mM) (4,5). The intracellular concentration of αKG varies considerably in response to a change in the availability of nitrogen source in the culturing medium (from 0.5 mM, in the presence of 10 mM NH$_4$Cl, to 10 mM, in the absence of nitrogen source) (4). Thus, inhibition of EI by αKG has been proposed as a biochemical mechanism that links the uptake of sugars to the availability of nitrogen source (4,5). In addition to playing a primary role in coupling carbon and nitrogen metabolism in bacteria, the phosphorylation state of EI strictly controls the phosphorylation state of all other PTS components (6), which, in turn, regulates a large number of bacterial functions, including catabolic gene expression, virulence, biofilm formation,
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chemotaxis, potassium transport, and inducer exclusion, via phosphorylation-dependent protein-protein interactions (2). Therefore, EI is a central regulator of bacterial metabolism, and obtaining a comprehensive understanding of the mechanisms tuning its biological activity may suggest new strategies in bioengineering and antimicrobial design, and might help elucidating the coupling between metabolic networks that controls the biology of all living cells.

EI is a multidomain protein comprising a N-terminal domain (EIN, residues 1-249) that contains the phosphorylation site (His189) and the binding site for HPr, and a C-terminal domain (EIC, residues 261-575) that is responsible for protein dimerization and contains the binding site for PEP and the competitive inhibitor αKG. The EIN and EIC domains are connected by a short helical linker (residues 250-260) (1,7). EI undergoes a series of large scale conformational rearrangements during its catalytic cycle (Figure 1b), including: (i) a monomer-dimer transition (8), (ii) an expanded-to-compact conformational change within EIC (9), and (iii) an open-to-close transition describing a reorientation of EIN relative to EIC (10-12). PEP binding to EIC shifts the conformational equilibria toward the catalytically competent dimer/compact/close form and activates the enzyme for catalysis (Figure 1b) (11). The monomer-dimer equilibrium of EI has been often suggested as a major regulatory element for PTS since (i) only dimeric EI can be phosphorylated by PEP (13), (ii) the interaction of the enzyme with its physiological ligands Mg2+ and PEP (Michaelis constant, KM, measured in the presence of 4 mM Mg2+ ~ 300 μM) decreases the equilibrium dissociation constant for dimerization (KD) by more than 10-fold (from ~5 to <0.1 μM) (5,8), and (iii) the intracellular concentrations of EI and PEP were reported to vary substantially depending on the experimental conditions (from ~30 to ~300 μM for PEP and from ~1 to ~10 μM for EI) (14-16).

Here, we develop a flexible enzymatic assay to investigate the effect of perturbations of the monomer-dimer equilibrium of Escherichia coli EI on the activity of αKG against the enzyme. We show that, at physiological concentrations of EI and PEP that promote dimerization of EI ([EI] > KD, [PEP] > KM), αKG acts as a competitive inhibitor of EI. In contrast, at physiological conditions favoring the monomeric form of the enzyme ([EI] < KD, [PEP] < KM), αKG allosterically stimulates EI autophosphorylation. To our knowledge, this is one of the few examples of a small molecule metabolite being reported to both inhibit and stimulate the activity of the same enzyme depending on the experimental conditions (the other known case is ATP that can be a substrate or an allosteric inhibitor of phosphofructokinase) (17). The fact that the intracellular concentrations of EI, PEP and αKG are modulated by the composition of the culturing medium (4,14-16) suggests that this interplay between allosteric stimulation and competitive inhibition of EI might be used by bacterial cells to regulate the phosphorylation state of PTS in response to a change in the extracellular environment.

Results

Effect of PEP and αKG on the monomer-dimer equilibrium of EI – The effect of the EI ligands, PEP and αKG, on the monomer-dimer equilibrium of the enzyme was investigated by Analytical Ultracentrifugation (AUC). The sedimentation velocity data indicate that the monomer-dimer equilibrium of EI is shifted toward the monomeric species at concentrations of the enzyme < 1 μM (Figure 2a), and that addition of PEP or αKG results in a substantial stabilization of the dimeric state (Figure 2b,c). Our results are consistent with the more than 10-fold decrease in dimerization KD reported previously for EI upon addition of PEP or αKG (5,8).

Kinetics of the phosphoryl transfer reaction – Addition of 10 mM PEP to a NMR sample containing 1 mM 15N-labeled E. coli HPr and ~0.05 μM E. coli EI (unlabeled) results in substantial chemical shift perturbations for the 1H-15N TROSY (Transverse Relaxation Optimized SpectroscopY) (18) peaks originating from HPr residues located in the vicinity of the phosphorylation site (His15, Figure 3a and 3c). As previously noted, HPr does not interact directly with PEP, nor it can be phosphorylated in the...
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absence of EI (19). Therefore, the observed spectral changes are attributed to HPr phosphorylation via EI. After 24 h incubation at 37°C, the HPr spectrum relaxes back to the unphosphorylated form (Figure 3a), which is consistent with the low thermodynamic stability of phosphorylated histidine residues (20).

Here, we use 1H-15N SOFAST-TROSY spectra (21) to monitor the time evolution of the phosphoryl transfer reaction from PEP to HPr via EI. SOFAST NMR experiments are ideally suited for real-time investigations on reaction kinetics, because they allow acquisition of 2D NMR spectra within seconds (21). For this particular case, ~0.05 μM unlabeled EI and 1 mM 15N-labeled HPr are mixed in 500 μl of reaction buffer (see Experimental procedures) and incubated at 37°C for 30 minutes in a conventional 5 mm NMR tube. Thereafter, the reaction is started by addition of the desired amount of PEP (note that the PEP stock solution is preincubated at 37°C). The sample is mixed in the NMR tube and equilibrated at 37°C for 1 minute in the NMR magnet. The reaction is then monitored for 20 minutes by running a series of 2D 1H-15N SOFAST-TROSY spectra (1 minute each). The phosphoryl transfer reaction is slow on the chemical-shift timescale, and distinct NMR peaks are observed for the phosphorylated and unphosphorylated species (Figure 3b). To monitor the evolution of the phosphoryl transfer reaction, we have used the NMR peak intensities of residues Ala¹⁰, Gly¹³ and Gly⁵⁴ because they are characterized by high signal-to-noise ratio (S/N) and are well resolved throughout the experiment (Figure 3b). Since the early time points are more important in determining the initial rate of the reaction, we limited our analysis to the disappearance of the unphosphorylated species, for which NMR peaks with high S/N are obtained at the beginning of the phosphoryl transfer reaction (note the phosphorylated HPr peaks are not present at time zero, Figure 3b). Signal intensities are plotted versus time, and the linear portion of the decay is fit to obtain the initial rate of change (Figure 3d). In order to convert the reaction rate from change in signal intensity over time to change in concentration of unphosphorylated HPr over time, the NMR signal intensities at time zero for Ala¹⁰, Gly¹³ and Gly⁵⁴ were obtained by extrapolation (Figure 3d) and considered to correspond to the expected signal intensity for a 1 mM HPr sample. Unphosphorylated HPr concentration at any time-point is reported as the average over the three analyzed peaks (Figure 3e).

To evaluate the effect of an increased concentration of dimeric EI on the activity of the enzyme, enzyme kinetic data were collected at a fixed concentration of EI (~0.05 μM), PEP (1 mM) and HPr (1 mM), and with increasing concentration of the inactive EI mutant His¹⁸⁹Gln (EIQ). His¹⁸⁹ is located within the N-terminal domain of the enzyme and does not participate in the dimer interface or in PEP/αKG-binding to EIC. Indeed, EIQ cannot receive the phosphoryl group from PEP, but can still interact with the wild-type protein (EIWT) to form an identical EIC dimer as the wild-type EI (11,22). Therefore, EIQ cannot be considered to correspond to the expected signal intensity for a 1 mM HPr sample. It is worth noticing that EIQ is inactive in the absence of EIWT (Figure 4a). Therefore, the increased enzymatic activity observed by adding EIQ to a sample with a low concentration of EIWT (~0.05 μM) is due to an increased population of dimeric EI (which goes from 8% in the absence of EIQ, to 80% in the presence of 10 μM EIWT) and not to the eventual presence of EIWT contaminations in purified EIQ. The dependence of the HPr phosphorylation rate on the total concentration of EI ([EI_TOT] = [EIWT] + [EIQ]) can be fit considering that (i) only dimeric EI can catalyze the phosphoryl transfer reaction (13), (ii) binding of PEP to both monomeric subunits results in stabilization of the EI dimer, and (iii) binding of PEP to one monomeric subunit affects the KD for EI dimerization to a minor extent. To reduce the number of fitted parameters we have assumed that the dimer KD is not affected by binding of a single molecule of PEP to EI (see equations 1-12 in Experimental procedures). The fit was performed in DynaFit 4.0 (23) by keeping KM and KD for the free enzyme (KD,free) to their measured values (300
and 1 μM, respectively – note that the 5 μM value for $K_{D,\text{free}}$ reported in Figure 1b was measured in the absence of Mg$^{2+}$) (5), and by optimizing the dissociation constant for the EI dimer saturated with PEP ($K_{D,\text{bound}}$) and the catalytic rate constant for phosphoryl transfer ($k_{\text{phosph}}$). Results of the fitting are shown in Figure 4a and are consistent with the pronounced stabilization of the EI dimer induced by PEP binding observed by AUC experiments (fitted $K_{D,\text{bound}} < 10^{-7}$ M). A similar kinetic model (equations 13-20 in Experimental procedures) and the same equilibrium constants were used to fit the dependence of the rate of HPr phosphorylation on the concentration of PEP at a fixed concentration of enzyme (~0.05 μM, Figure 4b). It is worth noticing that increasing the concentration of PEP beyond 1.3 mM makes the phosphoryl transfer reaction too fast to be monitored by our method at our experimental conditions (37°C and ~0.05 μM enzyme). Therefore, $k_{\text{phosph}}$ cannot be accurately determined by the available data. However, our fitted results ($k_{\text{phosph}} > 10,000$ s$^{-1}$) are in good agreement with the fast conversion rates previously reported for the EI autophosphorylation reaction (24).

**Effect of αKG on the activity of EI**  – Data reported in the previous sections indicate that dimerization stimulates the phosphoryl transfer activity of EI (Figure 4a), and that increasing the concentration of αKG from 0 to 20 mM shifts the monomer-dimer equilibrium toward the enzymatically active EI dimer (Figure 2). In this section, we evaluate the effect of αKG on the phosphoryl transfer activity of EI at experimental conditions that promote the monomeric or dimeric form of the enzyme.

At low concentration of enzyme ($< K_{D,\text{free}}$) and substrate ($< K_M$), we expect EI to exist predominantly as a monomer. In this case, addition of small concentrations of αKG ($< K_I$) will act synergistically with PEP in saturating the binding sites on EI (Figure 5a). The increased population of EI-ligand adducts will result in stabilization of the enzymatically active EI dimer and allosteric stimulation of the phosphoryl transfer reaction (Figure 5a). In contrast, increasing the concentration of αKG to values larger than $K_I$ will result in oversaturation of the binding sites on EI, and consequential competitive inhibition of enzymatic activity (Figure 5a). Indeed, enzyme kinetic data collected at ~0.05 μM EI, 200 μM PEP and increasing concentrations of αKG (0-10 mM) show an initial stimulation of enzymatic activity followed by a decrease in the rate of phosphoryl transfer at high concentration of αKG (> 2 mM, Figure 6a). At concentrations of EI > $K_{D,\text{free}}$ and/or concentrations of PEP > $K_M$, we expect EI to exists predominantly as a dimer, and αKG to act exclusively as an inhibitor of the enzyme (Figure 5b-d). Experimental data collected at ~0.05 μM EI and 1000 μM PEP (Figure 6b), 10 μM EI and 200 μM PEP (Figure 6c), and 10 μM EI and 1000 μM PEP (Figure 6d) confirm the expected behavior. Interestingly all kinetic data reported in Figure 6 can be fit considering that (i) only dimeric EI can catalyze the phosphoryl transfer reaction (13), (ii) saturation of the EI dimer binding sites with PEP and/or αKG (dissociation constants $K_M$ and $K_I$, respectively) decreases the $K_D$ for EI dimerization, and (iii) binding of PEP or αKG to one monomeric subunit affects the $K_D$ for EI dimerization to a minor extent. As done in the previous section when fitting the dependence of the phosphoryl transfer reaction on the concentration of enzyme, the model has been simplified by setting the dissociation constant of the EI dimer occupied by a single ligand molecule to $K_{D,\text{free}}$ (see equations 21-37 in Experimental procedures). Fits were performed by keeping $K_M$, $K_I$ and $K_{D,\text{free}}$ to their measured values (300, 2200 and 1 μM, respectively) (5), and optimizing values for $K_{D,\text{bound}}$ and $k_{\text{phosph}}$. In all cases a $K_{D,\text{bound}} < 10^{-7}$ M was obtained.

The kinetic model summarized by equations 21-37 was used to simulate the effect of physiological fluctuations in the intracellular environment on the activity of αKG against EI (Figure 7). In this simulation, $K_M$ and $K_I$ were set to the literature values for the EI-PEP and EI-αKG interactions (5), respectively. $K_{D,\text{bound}}$ was set to $10^{-7}$ M, the upper bound value obtained by fitting the enzyme kinetic data in Figures 4 and 6 (this work). The intracellular concentrations of EI, PEP and αKG were considered to vary in the 0.5-10 μM (16), 30-300 μM (14,15) and 0-10 mM (4) range, respectively. $K_{D,\text{free}}$ is strongly affected by

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the presence of divalent cations in the buffer (8). Therefore, $K_{D,free}$ was set to 5 or 1 µM (8) to simulate low (0.1 mM) or high (4 mM) intracellular concentration of free Mg$^{2+}$, respectively. Our simulation (Figure 7) suggests that αKG binding can provide up to 1.5 times stimulation of EI activity at physiological conditions that promote the monomeric form of the enzyme (low concentrations of EI, PEP and Mg$^{2+}$), but results in strong inhibition of enzymatic activity at physiological concentrations of EI, PEP and Mg$^{2+}$ that stabilize the EI dimer.

**Discussion**

In this work, we describe a novel method based on fast NMR techniques to assay the activity of EI under a wide range of experimental conditions. Previously reported methods to assay the activity of EI required quantification by mass spectrometry of pyruvate (formed as a by-product of the phosphoryl transfer reaction) (4), or quantification of phospho-histidine containing proteins (either EI or some other PTS component) by radioactive-labeling (24,25) or by using a recently developed antibody (26). Compared to these methods, our protocol allows for observation of the phosphoryl transfer reaction in real-time, therefore reducing the number of reagents and experimental steps required by the assay. On the other hand, our approach does not allow to monitor multiple reactions (i.e. multiple substrate concentrations) simultaneously, and can only be applied if a 10% (or larger) reduction in NMR signal intensity is obtained for unphosphorylated HPr upon phosphorylation. This latter condition implies that phosphoryl transfer kinetics at concentrations of PEP lower than 100 µM cannot be characterized accurately by our approach.

Using our NMR-based assay we show that the small molecule metabolite αKG can act either as an allosteric stimulator or as a competitive inhibitor of EI depending on the oligomeric state of the enzyme (Figures 5 and 6). Indeed, at experimental conditions favoring the dimeric form of EI, αKG inhibits the phosphoryl transfer activity of the enzyme (Figure 6b-d). In contrast, at experimental conditions favoring monomeric EI, addition of αKG results in a shift of the monomer-dimer equilibrium toward the enzymatically active dimeric form and a consequential stimulation of enzymatic activity (Figure 6a). Interestingly, the intracellular concentration of EI was measured to be close to the equilibrium dissociation constant for protein dimerization (16), and the dimer $K_D$ of the free enzyme was shown to be affected substantially by varying the concentration of Mg$^{2+}$ in the experimental buffer (from 5 to 1 µM moving from 0 to 4 mM Mg$^{2+}$) (8). In addition, the intracellular amount of PEP and αKG are close to the dissociation constants for PEP and αKG binding to the enzyme, respectively (4,14,15). In this scenario, small fluctuations in the intracellular concentrations of EI, Mg$^{2+}$, PEP and αKG induced by a change in the extracellular environment would drastically affect the activity of αKG on the PTS (Figure 7). The PTS plays multiple regulatory functions in bacterial metabolism (including sugar uptake, virulence, biofilm formation and chemotaxis) (1-3). These PTS-mediated regulatory mechanisms are based either on direct phosphorylation of the target protein by one of the PTS components, or on phosphorylation-dependent interactions (2). Therefore, the interplay between allosteric stimulation and competitive inhibition of EI by αKG revealed here may be required to tune the phosphorylation state of PTS in response to a change in the extracellular environment. While the inhibitory activity of αKG on EI has been already proven to regulate the uptake of PTS-sugars by bacterial cells in response to the availability of nitrogen source (4), understanding the effect of the weak stimulatory activity of αKG at low concentration of PEP on the biology of bacterial cells will require further investigations.

Finally, this work shows how the activity of small molecule metabolites against their biological targets can change significantly in response to small changes in experimental conditions, and illustrates that the dependence of the oligomeric state of the enzyme on the experimental conditions must be considered with great care when interpreting enzyme kinetic data.

**Experimental procedures**
Protein expression and purification – Uniformly
15N-labeled E. coli HPr was expressed and purified as previously described (27). The
His189Gln (EIQ) mutant of E. coli EI was created using the QuikChange Site-Directed Mutagenesis
Kit (Stratagene). Genes for EI and EIQ were cloned into a pET-15b vector (Novagen)
incorporating a N-terminal His-tag. 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 µg/ml) for selection. Cell were grown at 37°C in Luria Bertani (LB) medium. At A600 ~ 0.4
the temperature was reduced to 20°C and expression was induced with 1 mM isopropyl-D-
thiogalactopyranoside (IPTG). Cells were harvested by centrifugation (4,000g for 30 min)
after 16 h of induction and the pellet was resuspended in 20 ml of 20 mM Tris, pH 8.0
(buffer A). The suspension was lysed using a microfluidizer and centrifuged at 40,000g for 40
min. The supernatant was filtrated through a 0.45
µm filter membrane to remove cell debris and
applied to a His affinity column (GE Healthcare).
After the sample was loaded, the column was
washed with buffer B (bufferA containing 20 mM
imidazole), and the target protein was eluted with
buffer C (buffer A containing 300 mM imidazole).
The fractions containing the protein were
confirmed by SDS-polyacrylamide gel
electrophoresis and further purified by gel
filtration on a Superdex-200 column (GE
Healthcare) equilibrated with 20 mM Tris, pH 7.4,
200 mM NaCl, 2 mM dithiothreitol (DTT), and 1
mM ethylenediaminetetraacetic acid (EDTA).
Relevant fractions were loaded on an EnrichQ
anion exchange column (Biorad), and the protein
was eluted with a 400 ml gradient from 150 mM
to 400 mM NaCl.

Analytical ultracentrifugation – Sedimentation
velocity experiments were carried out on a Beckman Coulter ProteomeLab XL-I analytical
ultracentrifuge at 50 krpm and 20°C following
standard protocols (28). A 2.0 mM stock solution
of EI was diluted 50-fold in 100 mM NaCl, 20
mM Tris buffer, pH 7.4, 2 mM DTT and 1 mM
EDTA (buffer A), and used to prepare a series of
solutions ranging from approximately 1 to 40 µM
by serial dilution. Samples were loaded into 2
channel epon centerpiece cells (12 or 3 mm
pathlength depending on the concentration). Absorbance (280 nm) and Rayleigh interference
(655 nm) scans were collected, time-corrected
(29), and analyzed in SEDFIT 15.01c (30) in
terms a continuous c(s) distribution covering an s
range of 0.0 – 10.0 S with a resolution of 200 and
a maximum entropy regularization confidence
level of 0.68. Good fits were obtained with
typically r.m.s.d. values corresponding to typical
instrumental noise values. Identical experiments
were carried out in buffer A containing 20 mM
PEP (buffer B) or 20 mM αKG (buffer C).

Enzyme kinetic assay – The ability of EI to
transfer the phosphoryl group from PEP to HPr
was assayed at 37°C using fast NMR methods
as described in Results. NMR spectra were
recorded on a Bruker 700 MHz spectrometer
equipped with a z-shielded gradient triple
resonance cryoprobe. Spectra were processed
using NMRPipe (33) and analyzed using the
program SPARKY (http://www.cgl.ucsf.edu/
home/sparky). Spectra were assigned according
to previously reported chemical shift tables (34).
enzymatic assays were run in a reaction volume of 500 µl and at fixed concentrations of wild-type EI (~0.05 µM) and HPr (1 mM). Assays were run in triplicate. Initial velocities for the phosphoryl transfer reaction in the presence of different amount of EI (see Results and Discussion) were fit in DynaFit 4.0 (23) using the following kinetic model:

\[
\begin{align*}
E + S & \leftrightarrow ES \quad K_M \quad (1) \\
Q + S & \leftrightarrow QS \quad K_M \quad (2) \\
E + Q & \leftrightarrow EQ \quad K_{D,\text{free}} \quad (3) \\
EQ + S & \leftrightarrow EQS \quad K_M \quad (4) \\
EQS + S & \leftrightarrow ESQS \quad K_M \quad (5) \\
ESQ + S & \leftrightarrow ESQS \quad K_M \quad (6) \\
E + QS & \leftrightarrow EQS \quad K_{D,\text{free}} \quad (8) \\
ES + Q & \leftrightarrow ESQ \quad K_{D,\text{free}} \quad (9) \\
ES + QS & \leftrightarrow ESQS \quad K_{D,\text{free}} \quad (10) \\
ESQS \Rightarrow EQS + P & \quad k_{\text{phosph}} \quad (11) \\
ESQ \Rightarrow EQ + P & \quad k_{\text{phosph}} \quad (12)
\end{align*}
\]

where \( E \) is the wild-type enzyme (EI\(^{WT} \)), \( Q \) is the concentration of EI\(^Q \), \( S \) is the substrate (PEP), \( ES \) is the EI\(^{WT}\)-PEP complex, \( QS \) is the EI\(^Q\)-PEP complex, \( EQ \) is the mixed EI\(^{WT}E^Q \) dimer, \( EQS \) is the mixed dimer with PEP bound to the EI\(^Q\) subunit, \( ESQ \) is the mixed dimer with PEP bound to the EI\(^{WT}\) subunit, \( ESQS \) is the mixed dimer with two PEP molecules, \( P \) is the product, \( K_{D,\text{free}} \) (1 µM) is the dimer dissociation constant for free EI, \( K_{D,\text{bound}} \) (fitted) is the dimer dissociation constant for EI when saturated with ligands, \( K_M \) (300 µM) is the Michaelis constant for the EI-PEP interaction, \( k_{\text{phosph}} \) (fitted) is the rate constant for the phosphoryl transfer interaction, \( \Leftrightarrow \) indicates a thermodynamic equilibrium, and \( \Rightarrow \) indicates the unidirectional chemical step. Note that given the small amount of EI\(^{WT}\) compared to EI\(^Q\), the amount of EI\(^{WT}EI^{WT}\) dimer is considered to be negligible in this model.

Initial velocities for the phosphoryl transfer reaction in the presence of different amount of PEP (see Results and Discussion) were fit in DynaFit 4.0 (23) using the following kinetic model:

\[
\begin{align*}
E + S & \leftrightarrow ES \quad K_M \quad (13) \\
E + E & \leftrightarrow E_2 \quad K_{D,\text{free}} \quad (14) \\
E_2 + S & \leftrightarrow E_2S \quad K_M \quad (15) \\
E_2S + S & \leftrightarrow E_2S_2 \quad K_M \quad (16) \\
E + ES & \leftrightarrow E_2S \quad K_{D,\text{free}} \quad (17) \\
ES + ES & \leftrightarrow E_2S_2 \quad K_{D,\text{bound}} \quad (18) \\
E_2S_2 & \Rightarrow E_2S + P \quad k_{\text{phosph}} \quad (19) \\
E_2S & \Rightarrow E_2 + P \quad k_{\text{phosph}} \quad (20)
\end{align*}
\]

where \( E_2 \) is the EI\(^{WT}EI^{WT}\) dimer, \( E_2S \) is the EI dimer complexed to one molecule of PEP and \( E_2S_2 \) is the dimer complexed with two molecules of PEP.

Enzyme kinetic data measured at different concentration of αKG were fit in DynaFit 4.0 (23) using the following kinetic model:

\[
\begin{align*}
E + S & \leftrightarrow ES \quad K_M \quad (21) \\
E + I & \leftrightarrow EI \quad K_I \quad (22) \\
E + E & \leftrightarrow E_2 \quad K_{D,\text{free}} \quad (23) \\
E_2 + S & \leftrightarrow E_2S \quad K_M \quad (24) \\
E_2S + S & \leftrightarrow E_2S_2 \quad K_M \quad (25) \\
E_2 + I & \leftrightarrow E_2I \quad K_I \quad (26) \\
E_2I + I & \leftrightarrow E_2I_2 \quad K_I \quad (27)
\end{align*}
\]
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$$E_2I + S \Leftrightarrow E_2SI \quad K_M \quad (28)$$

$$E_2S + I \Leftrightarrow E_2SI \quad K_I \quad (29)$$

$$E + ES \Leftrightarrow E_2S \quad K_{D,\text{free}} \quad (30)$$

$$ES + ES \Leftrightarrow E_2S_2 \quad K_{D,\text{bound}} \quad (31)$$

$$E + EI \Leftrightarrow E_2I \quad K_{D,\text{free}} \quad (32)$$

$$EI + EI \Leftrightarrow E_2I_2 \quad K_{D,\text{bound}} \quad (33)$$

$$ES + EI \Leftrightarrow E_2SI \quad K_{D,\text{bound}} \quad (34)$$

$$E_2S_2 \Rightarrow E_2S + P \quad k_{\text{phosp}} \quad (35)$$

$$E_2S \Rightarrow E_2 + P \quad k_{\text{phosp}} \quad (36)$$

$$E_2SI \Rightarrow E_2I + P \quad k_{\text{phosp}} \quad (37)$$

where $I$ is the inhibitor ($\alpha$KG), $EI$ is the EI-$\alpha$KG complex, $E_2I$ is the EI dimer complexed with one $\alpha$KG molecule, $E_2I_2$ is the EI dimer complexed with two $\alpha$KG molecules, $E_2SI$ is the EI dimer complexed with one $\alpha$KG molecule and one PEP molecule, and $K_I$ (2.2 mM) is the dissociation constant for free EI-$\alpha$KG interaction. In the fits, the concentration of EI is considered to be the sum of the active (EI$^{\text{WT}}$) and inactive (EI$^{\text{Q}}$) species.

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
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Figure 1. The bacterial PTS. (a) Diagram of the E. coli PTS. The first two steps are common to all branches of the pathway. Thereafter, the pathway splits into four sugar-specific classes: glucose, mannitol, mannose, and lactose/chitobiose. Color code for the enzymes in the pathway is as follow: EI, blue; HPr, pink; EIIA, red; EIIB, orange; EIIC/EIID, yellow. EIIC/EIID enzymes are shown in a lipid bilayer. The phosphorylated site is indicated by –P. (b) Schematic summary of the conformational equilibria of EI during its catalytic cycle. The EIN domain is colored blue, the EIC domain is colored red and PEP is colored green. Equilibrium constants reported in previous research articles (5,8) are shown. $K_D^{\text{Free}}$ and $K_D^{\text{Bound}}$ are the equilibrium dissociation constants for dimerization in the absence and in the presence of saturating concentrations of Mg$^{2+}$ and PEP, respectively.
Figure 2. PEP and αKG shift the monomer-dimer equilibrium of EI. c(s) distributions for EI obtained at different loading concentrations (ranging from ~5 to ~1 μM) based on sedimentation velocity absorbance data collected at 50 krpm and 20.0°C (see Experimental procedures). Data acquired for the free EI (a) revealed concentration dependent c(s) absorbance profiles typical of a monomer-dimer equilibrium. In the presence of 20 mM αKG (b) and 20 mM PEP (c) the sedimentation experiments indicate that EI is dimeric within the tested concentration range. Peaks at $s_{20,w} < 4$ S that do not show concentration dependent c(s) absorbance profiles (i.e. they do not report on the monomer-dimer equilibrium) are attributed to small amounts of contaminants in the AUC sample.
**Figure 3. Activity assay for the phosphoryl transfer reaction.** (a) $^1$H-$^{15}$N TROSY spectrum of $^{15}$N-labeled HPr in the presence of 0.05 µM unlabeled EI in the absence (red) and in the presence (blue) of 10 mM PEP. Spectra in the presence of PEP were measured after incubation for 10 minutes (upper panel) or 24 hours (lower panel) at 37°C. Cross-peaks showing chemical shift perturbation upon addition of PEP are labeled. The question mark indicates a peak of unknown assignment. (b) Close-up views of a $^1$H-$^{15}$N SOFAST-TROSY spectrum of 1 mM HPr in the presence of 0.05 µM EI and 1 mM PEP showing the cross-peaks for residues Ala$^{10}$, Gly$^{13}$ and Gly$^{54}$ at three different time points during the activity assay: 1 minute, red; 5 minutes, yellow; 10 minutes, blue. For each residue, distinct peaks are observed for the unphosphorylated and phosphorylated HPr species (labeled HPr and HPr-P in the figure, respectively). (c) 3D structure of HPr. Backbone amide groups experiencing chemical shift perturbation upon addition of PEP to a sample containing HPr and EI are shown as spheres. Amide groups for Ala$^{10}$, Gly$^{13}$ and Gly$^{54}$ are colored green. The phosphorylation site (His$^{15}$) is shown as red spheres. (d) Intensities of the $^1$H-$^{15}$N SOFAST-TROSY cross-peaks of Ala$^{10}$ (red), Gly$^{13}$ (black) and Gly$^{54}$ (blue) are plotted versus time. Intensities at time 0 were obtained by extrapolation. The displayed data were measured on a 1 mM sample of HPr containing 0.05 µM EI$^{WT}$ and 1 mM PEP. The extrapolated intensities at time 0 (corresponding to 1 mM HPr) were used to calculate the time-dependence of the unphosphorylated HPr concentration. (d) The concentration of unphosphorylated HPr is plotted versus time. The displayed data were measured on a 1 mM sample of HPr containing 0.05 µM EI$^{WT}$ and 1 mM PEP. Concentrations of EI$^Q$ were 0 (blue), 1 (red) and 10 µM (black).
Figure 4. Dependence of the phosphoryl transfer reaction on the concentration of substrate and enzyme. (a) The phosphoryl transfer activity of EI (measured in the presence of 1 mM PEP) is plotted versus the concentration of an inactive mutant of the enzyme (EI<sup>Q</sup>) in the presence of 0 (red) or 0.05 µM wild-type EI (EI<sup>WT</sup>). Data were fit using the kinetic model summarized by equations 1-12 (Experimental procedures). Results of the fits are shown as solid lines. (b) The phosphoryl transfer activity of EI is plotted versus the concentration of PEP. Data were fit using the kinetic model summarized by equations 13-20 (Experimental procedures). Results of the fits are shown as solid lines.
\[ \alpha \text{KG-binding regulates EI of the bacterial PTS} \]

**Figure 5. The monomer-dimer equilibrium of EI regulates the activity of \( \alpha \text{KG} \) on the enzyme.** (a) At low concentration of enzyme (< \( K_{D,\text{free}} \)) and PEP (< \( K_M \)) addition of \( \alpha \text{KG} \) stabilizes the catalytically active EI dimer and stimulates the activity of the enzyme. Increasing the concentration of \( \alpha \text{KG} \) to values higher than \( K_I \) results in displacement of PEP from the active site and inhibition of EI. (b) At high concentration of PEP (> \( K_M \)) addition of \( \alpha \text{KG} \) does not affect the population of dimeric EI (which is already stabilized by PEP binding to both subunits) and results in inhibition of the enzyme. At EI concentration larger than \( K_{D,\text{free}} \) the monomer dimer equilibrium is already shifted toward the dimer form, and no stimulatory effect of \( \alpha \text{KG} \) is detected at low (c) or high (d) concentration of PEP. The total concentration of enzyme (EI<sub>TOT</sub>) is the sum of the concentrations of the wild-type EI (EI<sub>WT</sub>) and of an inactive EI mutant (EI<sub>Q</sub>, see Results). PEP and \( \alpha \text{KG} \) are colored yellow and green, respectively. EI<sub>WT</sub> and EI<sub>Q</sub> are colored white and red, respectively.
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Figure 6. Dependence of the phosphoryl transfer reaction on the concentration of αKG. Enzyme kinetic data were measured at a fixed concentration of EI\textsuperscript{WT} (~0.05 µM). EI\textsuperscript{Q} and PEP concentrations were as follows: (a) 0 µM EI\textsuperscript{Q} and 200 µM PEP; (b) 0 µM EI\textsuperscript{Q} and 1000 µM PEP; (c) 10 µM EI\textsuperscript{Q} and 200 µM PEP; (d) 10 µM EI\textsuperscript{Q} and 1000 µM PEP. Data in (a)-(d) were fit using the kinetic model summarized by equations 11-37 (Experimental procedures). Results of the fits are shown as solid lines.
Figure 7. Effect of physiological fluctuations of the intracellular environment on the activity of αKG against EI. The rate of the phosphoryl transfer reaction is simulated in DynaFit 4.0 (23) using the kinetic model summarized by equations 18-32. $K_M$, $K_i$ and $K_{D,\text{bound}}$ were set to 300 µM (5), 2200 µM (5) and $10^{-7}$ M (this work), respectively. The concentration of EI was set to 0.5 µM (dashed line) or 10 µM (solid line) (16). PEP concentration was 30 µM (dashed line) or 300 µM (solid line) (14,15). The value of $K_{D,\text{free}}$ depends on the concentration of Mg²⁺. Here, a $K_{D,\text{free}}$ of 5 µM (dashed line) or 1 µM (solid line) was used to simulate an intracellular environment poor (~0.1 mM) or rich (~4 mM) of Mg²⁺, respectively (8).
The oligomerization state of bacterial enzyme I (EI) determines EI's allosteric stimulation or competitive inhibition by α-ketoglutarate
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