

Biochemical Characterization of a Nitrogen-Type Phosphotransferase System Reveals that Enzyme EI^{Ntr} Integrates Carbon and Nitrogen Signaling in *Sinorhizobium meliloti*

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In *Sinorhizobium meliloti*, catabolite repression is influenced by a noncanonical nitrogen-type phosphotransferase system (PTS^{Ntr}). In this PTS^{Ntr}, the protein HPr is phosphorylated on histidine-22 by the enzyme EI^{Ntr} and the flux of phosphate through this residue onto downstream proteins leads to an increase in succinate-mediated catabolite repression (SMCR). In order to explore the molecular determinants of HPr phosphorylation by EI^{Ntr}, both proteins were purified and the activity of EI^{Ntr} was measured. Experimentally determined kinetic parameters of EI^{Ntr} activity were significantly slower than those determined for the carbohydrate-type EI in *Escherichia coli*. Enzymatic assays showed that glutamine, a signal of nitrogen availability in many Gram-negative bacteria, strongly inhibits EI^{Ntr}. Binding experiments using the isolated GAF domain of EI^{Ntr} (EI_{GAF}^{Ntr}) showed that it is the domain responsible for detection of glutamine. EI^{Ntr} activity was not affected by α -ketoglutarate, and no binding between the EI_{GAF}^{Ntr} and α -ketoglutarate could be detected. These data suggest that in *S. meliloti*, EI^{Ntr} phosphorylation of HPr is regulated by signals from both carbon metabolism (phosphoenolpyruvate) and nitrogen metabolism (glutamine).

The alphaproteobacterium *Sinorhizobium meliloti* is a free-living soil bacterium capable of forming symbioses with certain leguminous plants, such as *Medicago sativa* (alfalfa) and *Medicago truncatula* (barrel medic) (1). *S. meliloti* is a metabolically diverse organism, capable of catabolizing a wide range of carbon compounds (2, 3). Of these compounds, C₄-dicarboxylic acids, such as succinate, play an important role for *S. meliloti*. During symbiosis, dicarboxylates provided by the plant are required for nitrogen fixation (4). While dicarboxylates are also available in the rhizosphere, they are present in a heterogeneous mixture containing many other carbon sources (5). A likely adaptation to this environment is a form of carbon catabolite repression (CCR) termed succinate-mediated catabolite repression (SMCR), which differs from the well-studied *Escherichia coli* and *Bacillus subtilis* CCR. SMCR is a global physiological response to nutrient availability that culminates in the preferential use of succinate over other carbon sources during growth. SMCR can impose inducer exclusion and repression of genes needed for the transport and catabolism of secondary carbon sources, such as lactose and raffinose (6, 7).

CCR in bacteria is often controlled by a group of proteins collectively termed the phosphotransferase system (PTS). The canonical Gram-negative model of the PTS (based on the PTS of enteric bacteria) includes two general proteins: enzyme I (EI) and HPr, as well as proteins with sugar-specific EIIA, EIIB, and EIIC domains (Fig. 1A). During growth on glucose, EI will autophosphorylate, using phosphoenolpyruvate (PEP) as the phosphate donor (8). EI transfers the phosphate to HPr histidine-15 and then onto EIIA^{Glc}. EIIA^{Glc} will pass the phosphate to the EIICB^{Glc} transport protein, which phosphorylates the incoming sugar (9). When EIIA^{Glc} is unphosphorylated, it is capable of binding secondary solute transporters (e.g., the lactose permease), preventing uptake and ensuring the genes required for secondary catabolism are not induced (inducer exclusion) (10–12). Once the PTS substrate is exhausted, phosphorylated EIIA^{Glc} begins to accumulate. The phosphorylated form of EIIA^{Glc} has a low affinity for secondary

substrate transporters and stimulates adenylate cyclase activity, which synthesizes cyclic AMP (cAMP; a global inducer of catabolic operons) (13).

The traditional Gram-positive PTS model (based on that of *B. subtilis*) is similar structurally to the Gram-negative model of PTS, but it differs mechanistically. There is an additional enzyme, HPrK, that phosphorylates HPr at serine-46 by using ATP as a substrate (14). HPr-Ser46-P interacts with the regulatory protein CcpA to repress the genes required for utilization of secondary carbon substrates (15). In certain Gram-positive organisms, HPr-Ser46-P is also responsible for inducer exclusion (16).

Transport operons (largely the ABC type) account for 12% of the *S. meliloti* genome, allowing the organism to import a wide variety of carbon sources present in soil (17). Despite the abundance of transporters, *S. meliloti* lacks PTS-type membrane transport proteins. The genome encodes a set of PTS proteins homologous to the *E. coli* PTS^{Ntr}, as well as the enzyme HPrK (initially thought to be present only in Gram-positive organisms) and a mannose-type EIIA (EIIA^{Man} or ManX) (Fig. 1B). N-terminal GAF domains are a hallmark of EI^{Ntr}, including the enzyme produced by *S. meliloti* (18). These domains are widely distributed and often responsible for detection of small-molecule signals, such as cGMP and α -ketoglutarate (19, 20). This abbreviated PTS organization that includes a HPrK is found in many of the alphaproteobacteria (21).

Previous work in our lab demonstrated that the PTS proteins expressed by *S. meliloti* regulate SMCR. HPr of *S. meliloti* can be phosphorylated on either histidine-22 by EI^{Ntr} or serine-53 by

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binding buffer (50 mM NaCl, 1 mM DTT, 50 mM Tris-Cl [pH 7.5], 10% glycerol). The column was washed with 50 ml of Q binding buffer and 5 ml of the same buffer with 150 mM NaCl and then eluted in 20 ml of Q binding buffer with 0.5 M NaCl. The purified EI^{Ntr} was dialyzed overnight at 4°C against 50 mM NaCl, 5 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 25 mM HEPES (pH 7.5), 10% glycerol, passed through a 0.22- μ m syringe filter, and stored in aliquots at -80°C. This resulted in approximately a 10 mg liter⁻¹ culture of >90% pure EI^{Ntr}, based on Coomassie staining (24). The concentration of EI^{Ntr} was measured spectrophotometrically at 280 nm by using a molar extinction coefficient (ϵ_{280}) of 37,360 M⁻¹ cm⁻¹ with a Biotek Synergy HT apparatus and UV-transparent 96-well plates (Falcon).

Purification of recombinant HPr and EI_{GAF}. For purification of HPr, cells were lysed as described above with DTT omitted from all buffers and with NaHPO₄ replacing Tris-Cl at the same concentration and pH. All purification steps were performed at 4°C. After elution from the HisTrap column, HPr was dialyzed overnight against 0.25 M NaCl, 5 mM Na₂SO₄, 1 mM EDTA, 50 mM NaHPO₄ (pH 7.4), 10% glycerol at 4°C. For native electrophoresis assays, the His₆ tag was removed during dialysis with 20 U of bovine thrombin, and uncut protein was removed as described above. The protein was passed through a 0.2- μ m syringe filter and concentrated to 1 ml with an Amicon 3-kDa centrifugal filter (Millipore), then loaded onto a 65-ml S75 column equilibrated in 150 mM NaOAc, 5 mM Na₂SO₄, 1 mM EDTA, 25 mM HEPES (pH 7.4), 10% glycerol run at 1 ml min⁻¹. Purified protein was filtered and concentrated as above, then stored in aliquots at -80°C. This resulted in an approximately 5-mg liter⁻¹ culture of homogenous HPr, as determined by Coomassie staining. EI_{GAF} was purified using the same method as for HPr, but the dialysis buffer consisted of 150 mM NaCl, 20 mM NaHPO₄ (pH 7.5) and the size exclusion buffer was 150 mM NaOAc, 25 mM HEPES (pH 7.5), 10% glycerol, with a final yield of approximately 25 mg liter⁻¹. The concentrations of HPr and EI_{GAF} were determined by measuring the A₂₀₅, with extinction coefficients (ϵ_{205}) of 27 mg⁻¹ cm⁻¹ (HPr) and 27.6 mg⁻¹ cm⁻¹ (EI_{GAF}) (25).

Native PAGE HPr phosphorylation assay. Reaction mixtures consisting of 0.1 M Tris-OAc (pH 8.0), 10 mM Mg(OAc)₂, 3 μ g HPr (14 μ M final concentration), and the indicated amount of EI^{Ntr} were set up in 20- μ l volumes, which were incubated at room temperature for 15 min to ensure EI^{Ntr} dimerization (26). PEP (5 mM) was added to initiate the reactions, which were allowed to proceed for 15 min at room temperature and then halted by the addition of ice-cold sample buffer (final concentrations, 180 mM Tris, 120 mM CAPS [N-cyclohexyl-3-aminopropanesulfonic acid], 20 mM EDTA, 12% sucrose). A 12.5- μ l aliquot of each reaction mixture was loaded onto a 7.5% acrylamide gel buffered in 180 mM Tris, 120 mM CAPS (pH 9.6) (27). Gels were run for 65 min at 75 V and then Coomassie stained.

EI^{Ntr} LDH assay. EI^{Ntr} lactate dehydrogenase (LDH) activity was assayed as described in reference 28, with modifications. Assays were performed in 96-well UV-transparent microtiter plates (Falcon) at 30°C in a Synergy HT automated plate reader (BioTek). Prior to the assay, 1.6 μ M EI^{Ntr} was preincubated in 0.1 M NaOAc, 10 mM Mg(OAc)₂, 2 mM DTT, and 0.1 M HEPES (pH 8.0) at 30°C for 15 min to 1 h (no change in activity was detected during this period). Reactions were performed in the same buffer with 150 μ M NADH, 10 U LDH (MP Bio), 10 mM PEP, and the specified amount of HPr in a 200- μ l final volume. All solutions were prewarmed to 30°C, and EI^{Ntr} was added to a final concentration of 40 nM. Phosphorylation was measured by the change in absorbance at 340 nm based on an ϵ_{340} for NADH of 6,220 M⁻¹ cm⁻¹ and path length of 0.45 cm for a 200- μ l volume. Reaction mixtures were corrected for NADH autooxidation by subtracting the decrease in absorbance of samples lacking EI^{Ntr}. Reactions were performed in triplicate, and values for k_{cat} and K_m were determined by using nonlinear regression curve fitting to the Michaelis-Menten equation using Kaleidagraph 3.1 (Synergy Software). The data were obtained from a single preparation of EI^{Ntr} and three

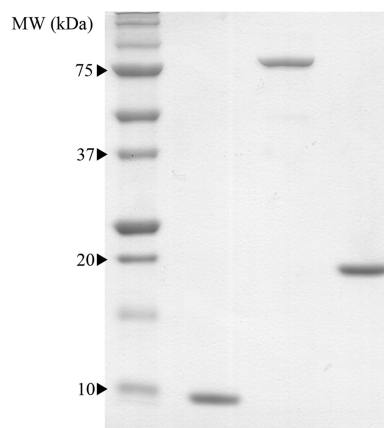


FIG 2 One microgram of purified HPr (left), EI^{Ntr} (center), and EI_{GAF} (right) were run on a 12% Tris-OAc SDS-polyacrylamide gel (43). All three proteins ran to their expected molecular masses of 10 kDa, 83 kDa, and 20 kDa, respectively.

preparations of HPr. There was no detectable variation between the HPr preparations.

Isothermal titration calorimetry (ITC). EI_{GAF} was dialyzed overnight at 4°C against 150 mM NaOAc, 50 mM HEPES (pH 7.5), 1% glycerol. After dialysis, the protein was filtered and the final concentration was measured. The ligand for each experiment was dissolved in the dialysis buffer. Binding experiments were performed in a VP-ITC apparatus (MicroCal) at 25°C with 300 rpm stirring. A 2- μ l test injection of ligand was performed, followed by 28 injections of 10 μ l each. The data were corrected for background by titrating ligand into buffer alone. The data for the test injection were discarded, and the data were analyzed using the Origin software package (MicroCal).

RESULTS

Purification of EI^{Ntr}, EI_{GAF}, and HPr. The genes encoding HPr and EI^{Ntr} were successfully cloned into pET-28a(+), which allowed for overexpression of the His₆-tagged proteins. Both EI^{Ntr} and HPr were largely insoluble when expressed under standard conditions (data not shown), and we subsequently searched for a method that would result in sufficient soluble protein for biochemical characterization. When the induction temperature and IPTG concentration were reduced to 18°C and 0.5 mM, respectively, approximately 10 mg liter⁻¹ of soluble EI^{Ntr} could be purified from Tuner cells. HPr solubility was not significantly improved by altering the IPTG concentration, by reducing the growth temperature, or by induction in 1 M sorbitol and 2.5 mM betaine (29). Soluble expression of HPr was achieved with an optimized autoinduction protocol (23) that ultimately resulted in approximately 5 mg of purified HPr per liter of culture medium. Autoinduction improved the yield of HPr to the point where we could study its phosphorylation by EI^{Ntr}. The isolated GAF domain of EI^{Ntr} could be expressed at levels significantly greater than full-length EI^{Ntr}, resulting in 25 mg of purified protein per liter of culture medium. The purification procedures described here resulted in highly pure proteins that migrated at their expected molecular masses of 10 kDa, 83 kDa, and 20 kDa for HPr, EI^{Ntr}, and EI_{GAF}, respectively (Fig. 2). All three proteins remained stable, with no loss of activity, after storage at -80°C for at least 8 months.

Phosphorylation of HPr. Using an *in vitro* phosphorylation assay that included native electrophoresis, we found that *S. meliloti* EI^{Ntr} could use PEP to phosphorylate HPr (Fig. 3). To measure

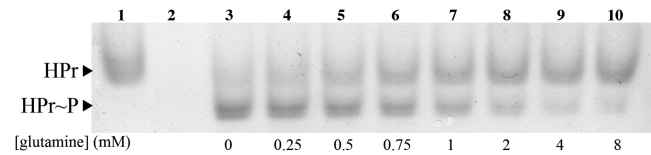


FIG 3 Native gel analysis of HPr phosphorylation by 100 ng of EI^{Ntr}. Lane 1, reaction mixture contained EI^{Ntr} and HPr without PEP; lane 2, reaction mixture lacked HPr; lanes 3 to 10, full phosphorylation reaction mixtures containing the indicated concentrations of glutamine.

the kinetic parameters of this reaction, an LDH coupled assay was adapted for a microplate reader. The Michaelis-Menten constants of EI^{Ntr} for HPr were determined: K_m of $12.8 \pm 1.5 \mu\text{M}$, k_{cat} of $1.11 \pm 0.4 \text{ s}^{-1}$, and k_{cat}/K_m ratio of $8.67 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Values are the result of a single fit to all of the data, with the standard error of the fit calculated by the software. The determined k_{cat}/K_m ratio is approximately 3×10^3 -fold lower than that of the *E. coli* carbohydrate EI (30), although the two enzymes were purified and assayed under different conditions. The kinetic data were obtained with a single preparation of EI^{Ntr}. After these experiments were performed, we developed an autoinduction protocol that allowed for soluble expression of EI^{Ntr} at 37°C. This enzyme was purified using the same protocol and while the yield was improved, its specific activity was unchanged (data not shown).

Inhibition of EI^{Ntr} by glutamine. In addition to kinetic characterization, we searched for effector molecules that would alter EI^{Ntr} activity at physiologically relevant concentrations. Previously, we isolated transposon insertions in *gln* genes during screens for mutants displaying altered SMCR phenotypes (unpublished results), suggesting that glutamine might modulate SMCR and provide a connection between nitrogen and carbon regulation within the cell. Glutamine was added to phosphorylation reaction mixtures at concentrations similar to those found within *E. coli* (31), and the reactions were analyzed by native-PAGE. This qualitative assay showed significant inhibition of EI^{Ntr} when glutamine was present (Fig. 3). For a more precise measure of EI^{Ntr} inhibition, the enzyme velocity was measured by using the LDH assay across multiple glutamine concentrations. The kinetic assays confirmed the results of the native electrophoresis experiments, showing that EI^{Ntr} only retained 20% of its activity in 1 mM glutamine (Fig. 4).

The GAF domain of EI^{Ntr} binds glutamine. The N-terminal GAF domain is a defining feature of EI^{Ntr} and was a likely candidate for glutamine binding. In order to detect binding of glutamine by EI_{GAF} and to measure the affinity, we used ITC. Titration of glutamine into isolated EI_{GAF} produced an exothermic binding curve that was fit with a one-site model (Fig. 5). From the fit, we determined that the K_D was 35 μM with an n value of 1.1. These values are similar to those reported for the GAF domain of *Azotobacter vinelandii* NifA binding with α -ketoglutarate (20).

EI^{Ntr} is insensitive to α -ketoglutarate. We screened a number of other metabolites for inhibition of EI^{Ntr}. These included compounds present in the same metabolic pathways as glutamine, such as glutamate, as well as chemically similar amino acids, including lysine, arginine, and asparagine. Other than glutamine, none of the other amino acids caused a measurable decrease in EI^{Ntr} activity (data not shown). It was recently shown that α -ketoglutarate stimulates the activity of EI^{Ntr} in *E. coli* (32). To determine if the *S. meliloti* enzyme responds the same way to the

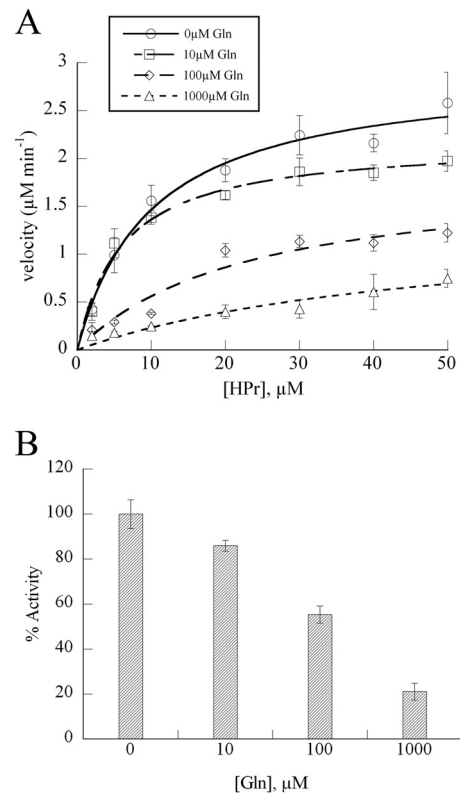


FIG 4 Kinetics of EI^{Ntr} inhibition by glutamine. (A) Reaction rates were measured with 40 nM EI^{Ntr} and the indicated HPr concentrations in the coupled LDH assay. Data points represent the means of three independent experiments (bars indicate standard errors) and were fit to the Michaelis-Menten equation. (B) Percent activity of EI^{Ntr} at 20 μM HPr and the indicated concentration of glutamine. Bars indicate standard errors.

presence of α -ketoglutarate, we adapted the native electrophoresis assay to detect enzyme activation. In the modified assay, the amount of EI^{Ntr} was reduced such that it would phosphorylate half of the HPr in 10 min. When the reaction mixtures were incubated in up to 10 mM α -ketoglutarate, the fraction of phosphorylated HPr did not increase (Fig. 6A). To show that all of the HPr could be phosphorylated, a reaction mix was included that contained 10-fold more EI^{Ntr}. Increasing the enzyme concentration resulted in complete phosphorylation of HPr. Addition of glutamine prevented phosphorylation of HPr, indicating that EI^{Ntr} was still sensitive to this ligand. Additional experiments were performed to detect an indirect activation of EI^{Ntr} by α -ketoglutarate via competition with glutamine for the binding site. Inhibition of EI^{Ntr} by 5 mM glutamine was not relieved by α -ketoglutarate (Fig. 6B). Finally, we could not detect direct binding of α -ketoglutarate by EI_{GAF} based on ITC (Fig. 5).

DISCUSSION

The nitrogen-type phosphotransferase system is a variant of the carbohydrate PTS found in many Gram-negative bacteria. The systems controlled by the PTS^{Ntr} vary greatly among bacteria, from nitrogen fixation and polyhydroxybutyrate accumulation in *Azotobacter vinelandii*, to oligopeptide transport in *Bradyrhizobium japonicum*, pathogenesis in *Brucella melitensis*, biofilm formation in *Vibrio cholerae*, and potassium homeostasis and tricar-

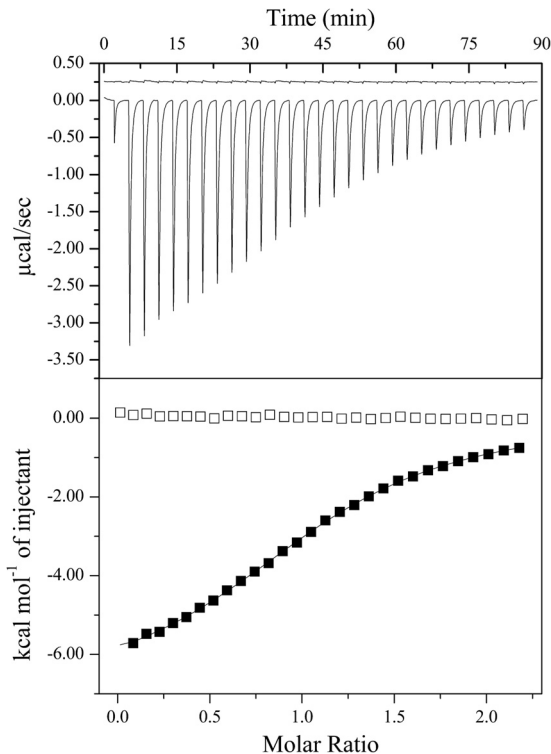


FIG 5 Titration of $143 \mu\text{M EI}_{\text{GAF}}$ with glutamine and α -ketoglutarate. The upper panel shows the raw heat of titration for α -ketoglutarate (top) and glutamine (bottom). The data for α -ketoglutarate have been shifted upwards by $0.5 \mu\text{cal/s}$ for clarity. The lower panel shows the binding isotherms for glutamine (filled boxes) and α -ketoglutarate (empty boxes). The isotherm for glutamine was fit with a one-site binding model.

boxylic acid (TCA) cycle flux in *E. coli* (33–39). In *S. meliloti*, the PTS^{Ntr} lacks membrane-bound transporters, making it strictly regulatory, and leaving the uptake of catabolites primarily to ABC and TRAP transporters (17). Understanding how the *S. meliloti* PTS^{Ntr} controls central metabolism and other aspects of its physiology is a crucial step for better understanding how *S. meliloti* senses and interacts with its environment both in the soil and during symbiosis.

The molecular mechanisms governing the PTS^{Ntr} have not been well studied outside of the major model bacteria, and in order to rectify this we have begun biochemical characterization of the phosphotransfer from EI^{Ntr} to HPr in *S. meliloti*. In measuring the kinetics of *S. meliloti* EI^{Ntr} activity, we found that it phosphorylates HPr at a much lower rate than the carbohydrate EI of *E. coli* (30). We believe that such a large difference in rates reflects a true difference in enzymatic activities, though we cannot rule out the possibility that the discrepancy was due to purification or assay artifacts. This difference in activities may reflect the function of the PTS in each of these organisms: in a carbohydrate PTS, the activity of the PTS proteins will determine the transport rate of sugar and by extension cell growth, favoring high enzymatic activity; conversely, in the case of a regulatory PTS with no known phosphate sink, a highly active EI would rapidly saturate the system. An oversaturated PTS would subsequently lose sensitivity and become unable to respond to changes in the state of the cell.

In a transposon screen to find mutations that relieved strong SMCR in certain PTS mutants, our lab isolated insertions in the

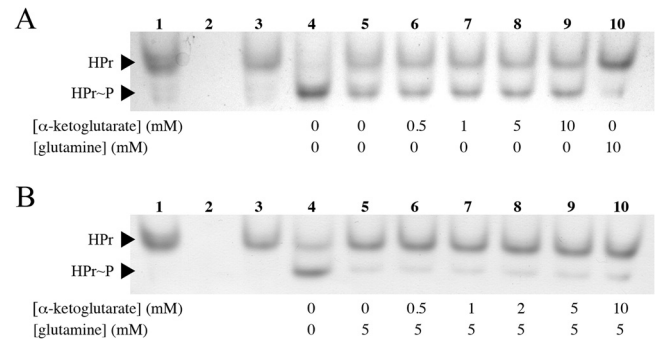


FIG 6 Phosphorylation of HPr by $50 \text{ ng of EI}^{\text{Ntr}}$ in the presence of α -ketoglutarate. (A) α -Ketoglutarate does not stimulate EI^{Ntr} activity. Lane 1, no EI^{Ntr} ; lane 2, no HPr; lane 3, no PEP; lane 4, full reaction mixture with $500 \text{ ng of EI}^{\text{Ntr}}$; lanes 5 to 10, reaction mixtures contained the indicated amounts of α -ketoglutarate or glutamine (in mM). (B) α -Ketoglutarate does not compete with glutamine to relieve EI^{Ntr} inhibition. Lanes 1 to 4, same reaction mixtures as in panel A; lanes 5 to 10, reaction mixtures contained the indicated concentrations of α -ketoglutarate and glutamine (in mM).

genes *glnD* and *glnE*. Since these genes encode proteins that regulate glutamine levels in the cell (40), we assayed glutamine for its ability to alter EI^{Ntr} activity. These assays showed that glutamine was a potent inhibitor of EI^{Ntr} , likely coupling the activity of the PTS^{Ntr} to a critical signal of nitrogen levels in both free-living and symbiotic *S. meliloti* (40). Direct binding measurements showed that the GAF domain of EI^{Ntr} is responsible for binding glutamine. When α -ketoglutarate was tested as an activator of EI^{Ntr} , we could not find any increase in activity, nor could we detect direct binding of α -ketoglutarate by EI^{Ntr} .

The data presented here can be used to further refine the model developed for the *S. meliloti* PTS^{Ntr} . Reduction of EI^{Ntr} velocity relative to carbohydrate EI likely prevents the PTS^{Ntr} from becoming saturated with phosphate, which would result in excess SMCR and other regulatory consequences. In Gram-positive bacteria, phosphorylation of HPr by HPrK renders it either a poor substrate for EI or unphosphorylatable (41, 42). If this holds true in *S. meliloti*, then the presence of HPrK would further reduce the rate of HPr phosphorylation by EI^{Ntr} *in vivo*. Currently, our attempts to purify active HPrK have failed, preventing us from exploring this hypothesis.

EI^{Ntr} senses the cellular carbon/energy state via the [PEP]:[pyruvate] ratio (11). EI^{Ntr} balances this activation against the glutamine availability in the cell, resulting in an intimate link between the availability of carbon and nitrogen. The GAF domain, which is a defining attribute of EI^{Ntr} in proteobacteria, is likely responsible for transmitting the signal from glutamine to the catalytic domain in order to coordinate EI^{Ntr} activity in response to carbon and nitrogen levels in the cell. Unlike the *E. coli* PTS^{Ntr} , *S. meliloti* does not use α -ketoglutarate to regulate EI^{Ntr} , possibly because succinate, another TCA cycle intermediate, is a primary carbon source for *S. meliloti*. Further studies using a combination of genetic and physiological techniques are needed to provide concrete evidence for the role of glutamine inhibition of EI^{Ntr} *in vivo*.

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REFERENCES

- Beringer JE, Brewin N, Johnston AW, Schulman HM, Hopwood DA. 1979. The *Rhizobium*-legume symbiosis. *Proc. R. Soc. Lond. B Biol. Sci.* 204:219–233.
- Mauchline TH, Fowler JE, East AK, Sartor AL, Zaheer R, Hosie AH, Poole PS, Finan TM. 2006. Mapping the *Sinorhizobium meliloti* 1021 solute-binding protein-dependent transportome. *Proc. Natl. Acad. Sci. U. S. A.* 103:17933–17938. <http://dx.doi.org/10.1073/pnas.0606673103>.
- Stowers MD. 1985. Carbon metabolism in *Rhizobium* species. *Annu. Rev. Microbiol.* 38:89–108.
- Finan TM, Wood JM, Jordan DC. 1983. Symbiotic properties of C4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J. Bacteriol.* 154:1403–1413.
- Hinsinger P, Gobran GR, Gregory PJ, Wenzel WW. 2005. Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. *New Phytol.* 168:293–303. <http://dx.doi.org/10.1111/j.1469-8137.2005.01512.x>.
- Pinedo CA, Bringham RM, Gage DJ. 2008. *Sinorhizobium meliloti* mutants lacking phosphotransferase system enzyme HPr or EIIA are altered in diverse processes, including carbon metabolism, cobalt requirements, and succinoglycan production. *J. Bacteriol.* 190:2947–2956. <http://dx.doi.org/10.1128/JB.01917-07>.
- Bringham RM, Gage DJ. 2002. Control of inducer accumulation plays a key role in succinate-mediated catabolite repression in *Sinorhizobium meliloti*. *J. Bacteriol.* 184:5385–5392. <http://dx.doi.org/10.1128/JB.184.19.5385-5392.2002>.
- Weigel N, Waygood EB, Kukuruzinska MA, Nakazawa A, Roseman S. 1982. Sugar transport by the bacterial phosphotransferase system. Isolation and characterization of enzyme I from *Salmonella typhimurium*. *J. Biol. Chem.* 257:14461–14469.
- Stolz B, Huber M, Markovic-Housley Z, Erni B. 1993. The mannose transporter of *Escherichia coli*. Structure and function of the IIABMan subunit. *J. Biol. Chem.* 268:27094–27099.
- Dills SS, Schmidt MR, Saier MH, Jr. 1982. Regulation of lactose transport by the phosphoenolpyruvate-sugar phosphotransferase system in membrane vesicles of *Escherichia coli*. *J. Cell. Biochem.* 18:239–244.
- Hogema BM, Arents JC, Bader R, Eijkemans K, Yoshida H, Takahashi H, Aiba H, Postma PW. 1998. Inducer exclusion in *Escherichia coli* by non-PTS substrates: the role of the PEP to pyruvate ratio in determining the phosphorylation state of enzyme IIA^{Glc}. *Mol. Microbiol.* 30:487–498.
- Chen S, Oldham ML, Davidson AL, Chen J. 2013. Carbon catabolite repression of the maltose transporter revealed by X-ray crystallography. *Nature* 499:364–368. <http://dx.doi.org/10.1038/nature12232>.
- Saier MH, Jr, Feucht BU, Hofstadter LJ. 1976. Regulation of carbohydrate uptake and adenylate cyclase activity mediated by the enzymes II of the phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*. *J. Biol. Chem.* 251:883–892.
- Saier MH, Jr. 1996. Cyclic AMP-independent catabolite repression in bacteria. *FEMS Microbiol. Lett.* 138:97–103.
- Deutscher J, Kuster E, Bergstedt U, Charrier V, Hillen W. 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. *Mol. Microbiol.* 15:1049–1053.
- Dossonnet V, Monedero V, Zagorec M, Galinier A, Perez-Martinez G, Deutscher J. 2000. Phosphorylation of HPr by the bifunctional HPr kinase/P-Ser-HPr phosphatase from *Lactobacillus casei* controls catabolite repression and inducer exclusion but not inducer expulsion. *J. Bacteriol.* 182:2582–2590. <http://dx.doi.org/10.1128/JB.182.9.2582-2590.2000>.
- Galibert F, Finan TM, Long SR, Puhler A, Abola P, Ampe F, Barloy-Hubler F, Barnett MJ, Becker A, Boistard P, Bothe G, Boutry M, Bowser L, Buhrmester J, Cadieu E, Capela D, Chain P, Cowie A, Davis RW, Dreano S, Federspiel NA, Fisher RF, Gloux S, Godrie T, Goffeau A, Golding B, Gouzy J, Gurjal M, Hernandez-Lucas I, Hong A, Huizar L, Hyman RW, Jones T, Kahn D, Kahn ML, Kalman S, Keating DH, Kiss E, Komp C, Lelaure V, Masuy D, Palm C, Peck MC, Pohl TM, Portetelle D, Purnelle B, Ramsperger U, Surzycki R, Thebault P, Vandenbol M, Vorholter FJ, Weidner S, Wells DH, Wong K, Yeh KC, Batut J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293:668–672. <http://dx.doi.org/10.1126/science.1060966>.
- Hu KY, Saier MH, Jr. 2002. Phylogeny of phosphoryl transfer proteins of the phosphoenolpyruvate-dependent sugar-transporting phosphotransferase system. *Res. Microbiol.* 153:405–415. [http://dx.doi.org/10.1016/S0923-2508\(02\)01339-6](http://dx.doi.org/10.1016/S0923-2508(02)01339-6).
- Martinez SE, Beavo JA, Hol WG. 2002. GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol. Interv.* 2:317–323. <http://dx.doi.org/10.1124/mi.2.5.317>.
- Martinez-Argudo I, Little R, Dixon R. 2004. Role of the amino-terminal GAF domain of the NifA activator in controlling the response to the anti-activator protein NifL. *Mol. Microbiol.* 52:1731–1744. <http://dx.doi.org/10.1111/j.1365-2958.2004.04089.x>.
- Cases I, Velazquez F, de Lorenzo V. 2007. The ancestral role of the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) as exposed by comparative genomics. *Res. Microbiol.* 158:666–670. <http://dx.doi.org/10.1016/j.resmic.2007.08.002>.
- Pinedo CA, Gage DJ. 2009. HPrK regulates succinate-mediated catabolite repression in the Gram negative symbiont *Sinorhizobium meliloti*. *J. Bacteriol.* 191:298–309. <http://dx.doi.org/10.1128/JB.01115-08>.
- Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* 41:207–234. <http://dx.doi.org/10.1016/j.pep.2005.01.016>.
- Kang DH, Gho YS, Suh MK, Kang CH. 2002. Highly sensitive and fast protein detection with Coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bull. Korean Chem. Soc.* 23:1511–1512. <http://dx.doi.org/10.5012/bkcs.2002.23.11.1511>.
- Scopes RK. 1974. Measurement of protein by spectrophotometry at 205 nm. *Anal. Biochem.* 59:277–282.
- Rabus R, Reizer J, Paulsen I, Saier MH. 1999. Enzyme I-Ntr from *Escherichia coli*: a novel enzyme of the phosphoenolpyruvate-dependent phosphotransferase system exhibiting strict specificity for its phosphoryl acceptor, NPr. *J. Biol. Chem.* 274:26185–26191.
- McLellan T. 1982. Electrophoresis buffers for polyacrylamide gels at various pH. *Anal. Biochem.* 126:94–99.
- Waygood EB, Meadow ND, Roseman S. 1979. Modified assay procedures for the phosphotransferase system in enteric bacteria. *Anal. Biochem.* 95:293–304.
- Ackerley DF, Caradoc-Davies TT, Lamont IL. 2003. Substrate specificity of the nonribosomal peptide synthetase PvdD from *Pseudomonas aeruginosa*. *J. Bacteriol.* 185:2848–2855. <http://dx.doi.org/10.1128/JB.185.9.2848-2855.2003>.
- Meadow ND, Mattoo RL, Savtchenko RS, Roseman S. 2005. Transient state kinetics of enzyme I of the phosphoenolpyruvate:glycose phosphotransferase system of *Escherichia coli*: equilibrium and second-order rate constants for the phosphotransfer reactions with phosphoenolpyruvate and HPr. *Biochemistry* 44:12790–12796. <http://dx.doi.org/10.1021/bi0502846>.
- Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. 2009. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* 5:593–599. <http://dx.doi.org/10.1038/nchembio.186>.
- Lee CR, Park YH, Kim M, Kim YR, Park S, Peterkofsky A, Seok YJ. 2013. Reciprocal regulation of the autophosphorylation of enzyme INtr by glutamine and alpha-ketoglutarate in *Escherichia coli*. *Mol. Microbiol.* 88:473–485. <http://dx.doi.org/10.1111/mmi.12196>.
- Segura D, Espin G. 1998. Mutational inactivation of a gene homologous to *Escherichia coli* ptsP affects poly-beta-hydroxybutyrate accumulation and nitrogen fixation in *Azotobacter vinelandii*. *J. Bacteriol.* 180:4790–4798.
- King ND, O'Brian MR. 2001. Evidence for direct interaction between enzyme I(Ntr) and aspartokinase to regulate bacterial oligopeptide transport. *J. Biol. Chem.* 276:21311–21316. <http://dx.doi.org/10.1074/jbc.M1101982200>.
- Lee CR, Cho SH, Yoon MJ, Peterkofsky A, Seok YJ. 2007. *Escherichia coli* enzyme IIA(Ntr) regulates the K⁺ transporter TrkA. *Proc. Natl. Acad. Sci. U. S. A.* 104:4124–4129. <http://dx.doi.org/10.1073/pnas.0609897104>.
- Houot L, Chang S, Pickering BS, Absalon C, Watnick PI. 2010. The phosphoenolpyruvate phosphotransferase system regulates *Vibrio cholerae* biofilm formation through multiple independent pathways. *J. Bacteriol.* 192:3055–3067. <http://dx.doi.org/10.1128/JB.00213-10>.
- Jahn S, Haverkorn van Rijsewijk BR, Sauer U, Bettenbrock K. 2013. A role for EIIA(Ntr) in controlling fluxes in the central metabolism of *E. coli* K12. *Biochim. Biophys. Acta* 1833:2879–2889. <http://dx.doi.org/10.1016/j.bbamcr.2013.07.011>.
- Luttmann D, Heermann R, Zimmer B, Hillmann A, Rampp IS, Jung K, Gorke B. 2009. Stimulation of the potassium sensor KdpD kinase activity

- by interaction with the phosphotransferase protein IIA(Ntr) in *Escherichia coli*. *Mol. Microbiol.* 72:978–994. <http://dx.doi.org/10.1111/j.1365-2958.2009.06704.x>.
39. Dozot M, Poncet S, Nicolas C, Copin R, Bouraoui H, Maze A, Deutscher J, De Bolle X, Letesson JJ. 2010. Functional characterization of the incomplete phosphotransferase system (PTS) of the intracellular pathogen *Brucella melitensis*. *PLoS One* 5(9):e12679. <http://dx.doi.org/10.1371/journal.pone.0012679>.
 40. Yurgel SN, Kahn ML. 2008. A mutant GlnD nitrogen sensor protein leads to a nitrogen-fixing but ineffective *Sinorhizobium meliloti* symbiosis with alfalfa. *Proc. Natl. Acad. Sci. U. S. A.* 105:18958–18963. <http://dx.doi.org/10.1073/pnas.0808048105>.
 41. Casabon I, Couture M, Vaillancourt K, Vadeboncoeur C. 2006. Synthesis of HPr(Ser-P)(His~P) by enzyme I of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus salivarius*. *Biochemistry* 45:6692–6702. <http://dx.doi.org/10.1021/bi060278p>.
 42. Reizer J, Sutrina SL, Wu LF, Deutscher J, Reddy P, Saier MH, Jr. 1992. Functional interactions between proteins of the phosphoenolpyruvate:sugar phosphotransferase systems of *Bacillus subtilis* and *Escherichia coli*. *J. Biol. Chem.* 267:9158–9169.
 43. Cubillos-Rojas M, Amair-Pinedo F, Tato I, Bartrons R, Ventura F, Rosa JL. 2010. Simultaneous electrophoretic analysis of proteins of very high and low molecular mass using Tris-acetate polyacrylamide gels. *Electrophoresis* 31:1318–1321. <http://dx.doi.org/10.1002/elps.200900657>.