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

Reed A. Goodwin, Daniel J. Gage

University of Connecticut, Department of Molecular and Cell Biology, Storrs, Connecticut, USA

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}) 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} and α-ketoglutarate could be detected. These data suggest that in *S. melilloti*, 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, C_4-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 carbons 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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Address correspondence to Daniel J. Gage, daniel.gage@uconn.edu.

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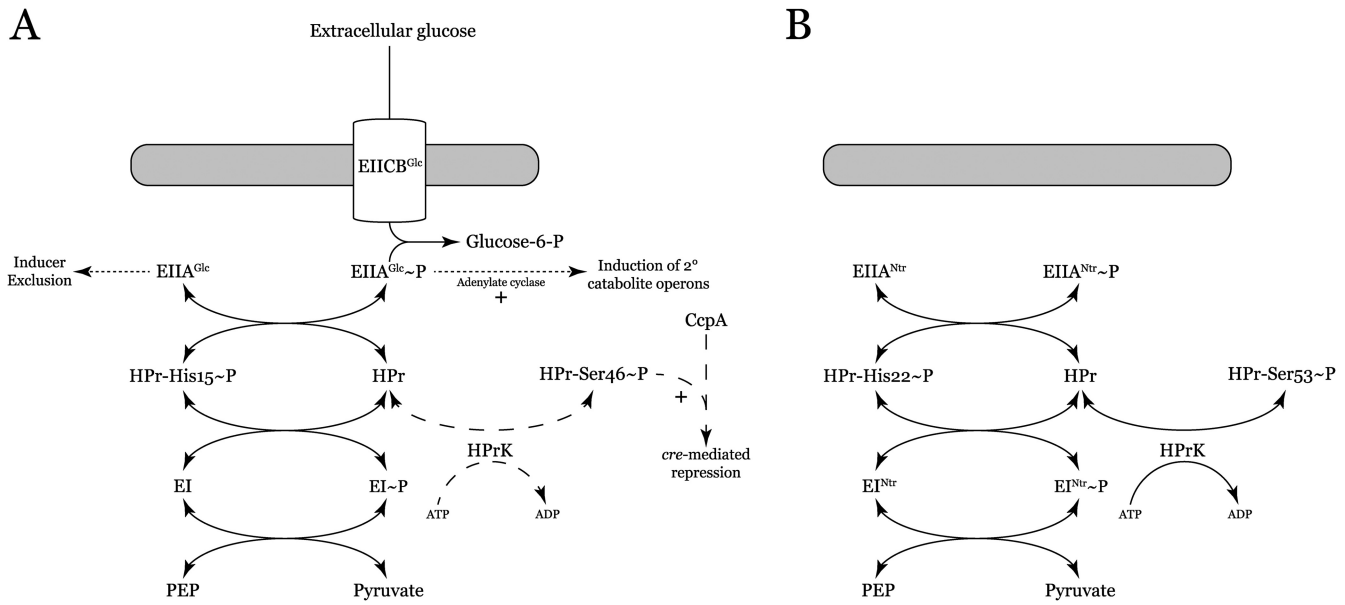

FIG 1 (A) Model of the canonical PTS. Dotted lines represent pathways specific to *E. coli*, and dashed lines are those specific to *B. subtilis*. (B) Proposed model of the *S. meliloti* PTS. Note the lack of membrane permeases and the presence of an HPrK in a Gram-negative bacterium. Not shown is phosphorylation of $EIIA^{man}$ (ManX) by HPr-His22-P.

HPrK (Fig. 1B). An increase in the phosphate flow through HPr histidine-22 results in a concomitant increase in SMCR. In addition to SMCR, we have shown that the PTS^{Ntr} regulates symbiosis, succinoglycan production, and trace element requirements (6, 22). To elucidate the mechanism of the PTS^{Ntr}, we have characterized the transfer of phosphate from PEP to HPr via EI^{Ntr} by using biochemical techniques.

MATERIALS AND METHODS

Media, strains, and plasmids. *E. coli* XL1-Blue (Stratagene) was used for all cloning steps and was grown in LB supplemented with either ampicillin (100 μg/ml) or kanamycin (25 μg/ml). EI^{Ntr} was expressed in Tuner (DE3)pLysS (Novagen), and HPr and EI_{GAF} were expressed in BL21(DE3) (Novagen). Media recipes and the nomenclature used for protein expression are those of reference 23, with tryptone used in place of N-Z-Amine, and all cultures contained 100 μg/ml kanamycin.

Construction of expression plasmids. The genes for HPr and EI^{Ntr}, *hpr* (*smc02754*) and *ptsP* (*smc02437*), respectively, were amplified using high-fidelity Phusion polymerase (New England BioLabs) and primers that allowed for cloning into the His-tagging vector pET28a(+) (Novagen). The primers for amplifying *hpr* were hpr_UP (5′-atgatgGCTAGCcgccccgacacggctct-3′) and hpr_DOWN (5′-atgtttaCTCGAGtcacatctcttcg-3′. The portions of the sequences in capital letters indicate NheI and XhoI sites in the upstream and downstream primers used for cloning the amplified fragment into pET28a(+). The primers for amplifying full-length *ptsP* were ptsP_UP (5′-cacgcgatgGCTAGcctttccgcaggtccgcg-3′) and ptsP_DOWN (5′-ctcttgGAATTCctaaaccggtatgccgt-3′). The capitalized portions of the sequences indicate NheI and EcoRI sites in the upstream and downstream primers used for cloning the amplified fragment into pET28a(+). The N-terminal GAF domain of EI^{Ntr} (encoding residues 2 to 162) was amplified using primers ptsP_UP and GAF_DOWN (5′-GGATCCtcaggtcgcgaccatctcgg-3′). The capitalized portion of the sequence indicates the BamHI site in the downstream primer used for cloning the amplified fragment into pET28a(+). Following amplification, the products were A-tailed and cloned into pGEM T-Easy (Promega). Next, the inserts were excised from the plasmid by cutting at the sites indicated in the primers above and cloned into pET28a(+), giving pDG142H, pDG142P, and pRG07, which expressed His_6-tagged versions of HPr, EI^{Ntr}, and EI_{GAF}, respectively. All three plasmids were sequenced to confirm that errors had not been introduced during amplification or cloning.

Expression of recombinant proteins. All three strains were grown overnight in MDG (medium phosphate plus aspartate and glucose) prior to inoculation of expression media. To express EI^{Ntr}, Tuner(DE3)pLysS/pDG142P was diluted into ZYM 505 and grown at 37°C for 5 h. The culture was cooled to 18°C and induced overnight with 0.5 mM isopropyl-β-D-galactopyranoside (IPTG). BL21(DE3)/pDG142H was diluted into ZYM 522, grown at 37°C for 2 h, and then incubated for an additional 24 h at 18°C. BL21(DE3)/pRG07 was diluted into ZYM 5052 and grown overnight at 37°C. Following induction, the cultures were harvested by centrifugation and stored as pellets at −20°C.

Purification of recombinant EI^{Ntr}. All purification steps were performed at 4°C. Cell pellets were resuspended in 50 mM NaCl, 5 mM $MgSO_4$, 50 mM Tris-Cl (pH 8.0) supplemented with 2.5 U/ml benzonase and 0.1 mg/ml lysozyme and with Halt protease inhibitors (Thermo) and incubated for 2 h. Cells were lysed by sonication for 6 min in 30-s bursts. The extract was clarified by centrifugation at 3,500 × *g* for 15 min and then 30,000 × *g* for 30 min. The crude extract was passed through a 0.45-μm syringe filter (Corning), and imidazole was added to 20 mM. The protein was loaded onto a 1-ml HisTrapFF Crude Ni^{2+} column (GE Healthcare) equilibrated in binding buffer (0.5 M NaCl, 25 mM imidazole, 50 mM Tris-Cl [pH 7.5], 10% glycerol). The column was washed with 20 ml of binding buffer, and then EI^{Ntr} was eluted in 20 ml of binding buffer containing 0.5 M imidazole. The protein was immediately diluted 2-fold in 50 mM NaCl, 0.1 M imidazole, 1 mM dithiothreitol (DTT), 1 mM EDTA, 5 mM magnesium acetate [$Mg(OAc)_2$], 50 mM Tris-Cl (pH 7.5), and 10% glycerol, and 20 U of bovine thrombin (GE Healthcare) was added to remove the His tag. The protein was dialyzed overnight against the dilution buffer, followed by a 2-hour dialysis against the same buffer with imidazole omitted. The dialyzed protein was centrifuged at 15,000 × *g* for 30 min, and uncut protein was removed with the HisTrap column after equilibration in dialysis buffer. The untagged protein was loaded onto a 5-ml Q Sepharose FF column (GE Healthcare) equilibrated in Q

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-μm 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 $NaHPO_4$ 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 Na_2SO_4, 1 mM EDTA, 50 mM $NaHPO_4$ (pH 7.4), 10% glycerol at 4°C. For native electrophoresis assays, the His_6 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-μm 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 Na_2SO_4, 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 $NaHPO_4$ (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 A_{205}, 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 μg HPr (14 μM final concentration), and the indicated amount of EI^{Ntr} were set up in 20-μl 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-μl 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 μM 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 μM NADH, 10 U LDH (MP Bio), 10 mM PEP, and the specified amount of HPr in a 200-μl 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-μl 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 preparations of HPr. There was no detectable variation between the HPr preparations.

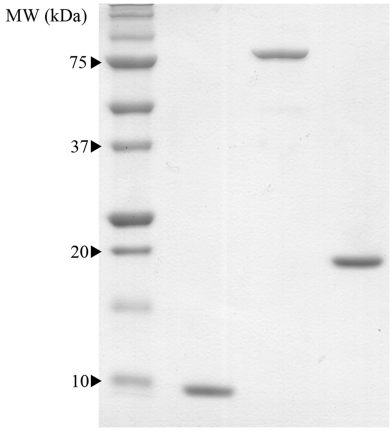

FIG 2 One microgram of purified HPr (left), EI^{Ntr} (center), and EI_{GAF} (right) were run on a 12% Tris-OAc SDS-polyacrylamide gel (43). All three proteins ran to their expected molecular masses of 10 kDa, 83 kDa, and 20 kDa, respectively.

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-μl test injection of ligand was performed, followed by 28 injections of 10 μl 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 His_6-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

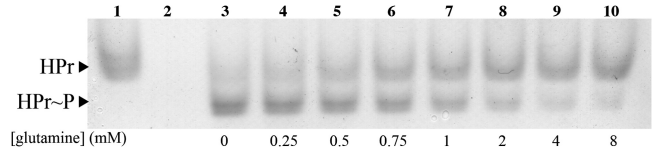

FIG 3 Native gel analysis of HPr phosphorylation by 100 ng of EI^{Ntr}. Lane 1, reaction mixture contained EI^{Ntr} and HPr without PEP; lane 2, reaction mixture lacked HPr; lanes 3 to 10, full phosphorylation reaction mixtures containing the indicated concentrations of glutamine.

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 ± 1.5 μM, k_{cat} of 1.11 ± 0.4 s^{-1}, and k_{cat}/K_m ratio of 8.67 × 10^4 M^{-1} 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

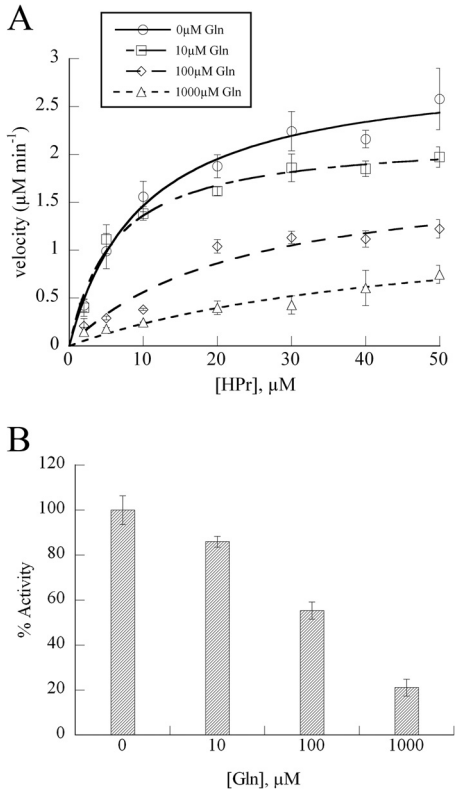

FIG 4 Kinetics of EI^{Ntr} inhibition by glutamine. (A) Reaction rates were measured with 40 nM EI^{Ntr} and the indicated HPr concentrations in the coupled LDH assay. Data points represent the means of three independent experiments (bars indicate standard errors) and were fit to the Michaelis-Menten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

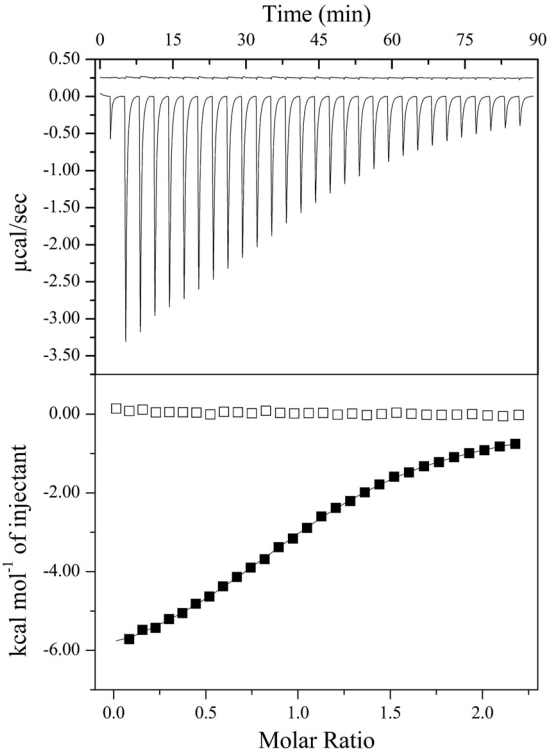

FIG 5 Titration of 143 μM EI_{GAF} with glutamine and α-ketoglutarate. The upper panel shows the raw heat of titration for α-ketoglutarate (top) and glutamine (bottom). The data for α-ketoglutarate have been shifted upwards by 0.5 μcal/s for clarity. The lower panel shows the binding isotherms for glutamine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

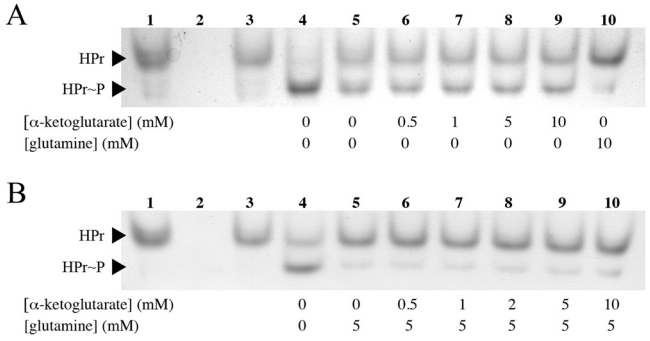

FIG 6 Phosphorylation of HPr by 50 ng of EI^{Ntr} in the presence of α-ketoglutarate. (A) α-Ketoglutarate does not stimulate EI^{Ntr} activity. Lane 1, no EI^{Ntr}; lane 2, no HPr; lane 3, no PEP; lane 4, full reaction mixture with 500 ng of EI^{Ntr}; lanes 5 to 10, reaction mixtures contained the indicated 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).

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 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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