Evidence for Direct Interaction between Enzyme I*Ntr and Aspartokinase to Regulate Bacterial Oligopeptide Transport

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‡ The abbreviations used are: Dpp, dipeptide permease; Opp, oligopeptide transport (Opp) permease systems; Dpp, dipeptide permease; Opp, oligopeptide permease; ALA, δ-aminolevulinic acid; EI Ntr, Enzyme I Ntr; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; kb, kilobase(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

Bacteria utilize small peptides as nutrients, chemotacticants, and quorum sensing signals, and their metabolism is a target for antibiotics (1–3). Escherichia coli and Salmonella typhimurium contain distinct dipeptide (Dpp) and oligopeptide permease (Opp) systems with some overlap in substrate specificity. The two permease systems are structurally homologous, each one containing five proteins, including a periplasmic peptide-binding protein (4, 5). The Opp system binds peptides two to five peptides in length, with the highest affinity for tripeptides (6, 7). For both permease systems, the amino acid side chain appears not to be important for specificity, and therefore these systems transport peptides independent of sequence.

Our interest in oligopeptide transport in the bacterium Bradyrhizobium japonicum is founded on studies of heme biosynthesis, where it has been demonstrated that the heme precursor δ-aminolevulinic acid (ALA) is taken up by a system that also transports oligopeptides (8). ALA is structurally similar to glycolylic acid. It is taken up by the Dpp system in E. coli (9) and S. typhimurium (10), but ALA is taken up in a dpp mutant if the opp system is activated (8). B. japonicum lives as a free-living soil bacterium or as an endosymbiont of soybeans within root nodules. In symbiosis, B. japonicum may utilize ALA synthesized by the plant host for heme formation (11, 12).

The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) couples the transfer of a high energy phosphoryl group from PEP to a sugar with the concomitant transport into the cell (13–15). Paralogues of the PTS enzymes EI, Hpr and IIA, called EI*Ntr, Npr, and IIA*Ntr, respectively, have been identified in several organisms (16–21), and evidence for a parallel phosphoryl transfer chain has been presented (22). Phenotypes of mutants in the parallel PTS system (herein called the PTS*Ntr system), gene organization and the structure of EI*Ntr, suggest a role for this system coordinating nitrogen and carbon metabolism. However, the physiological functions of the PTS*Ntr system is unclear. Although the conventional PTS system transports sugars, no solute transport activity has been linked to the PTS*Ntr proteins. Herein, we demonstrate a role for EI*Ntr, encoded by the ptsP gene, in oligopeptide transport in B. japonicum. Furthermore, a novel function for the amino acid biosynthesis enzyme aspartokinase was also implicated, and evidence for interaction between EI*Ntr and aspartokinase is presented.

MATERIALS AND METHODS

Chemicals and Reagents—All chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, MO, Fisher Scientific, Fair Lawn, NJ, or from J. T. Baker Inc., Phillipsburg, NJ. Granulated agar and yeast extract were obtained from Difco Laboratories, Detroit, MI. δ-Aminolevulinic acid was purchased from Porphyrin Products, Logan, UT. [14C]ALA (47.6 mCi/mmol), [32P]ATP (3000 Ci/mmOL), and [α-32P]dCTP (3000 Ci/mmOL) were purchased from PerkinElmer Life Sciences, Boston, MA. [α-32P]UTP (800 Ci/mmOL) was purchased from ICN Biomedicals, Irvine, CA. Peptides were obtained from Bachem, Torrance, CA.

Bacterial Strains, Plasmids, Media, and Growth—B. japonicum strain N110proC is a proC mutant derivative of strain N110 and is a proline auxotroph (23). All B. japonicum strains were grown at 29 °C in GSY media or minimal media as described previously (24). Tetracycline (75 μg/ml) was added to the media for growth of strains bearing the
broad host range plasmid pVK102 (25) and its derivatives. Strains 120, Q5, 111020, and 1110Q5 were grown in the presence of kanamycin (50 μg/ml) and streptomycin (50 μg/ml). Strains 1110proC, 120, and Q5 were grown in the presence of spectinomycin (50 μg/ml) and streptomycin. Strains 1110proC, 120, and Q5 required the addition of proline (500 μg/ml) for growth. E. coli strains DH5α and HB101 were used for propagation of plasmids and was grown at 37 °C on Luria-Bertani (LB) medium with appropriate antibiotics. E. coli strains HB101 (pDS4101) and HB101(pRK2013) were grown in media containing ampicillin (200 μg/ml) and kanamycin (50 μg/ml), respectively, for tri-parental matings.

Plasmid pSUP101 is a pACYC184 derivative carrying the transposon Tn5. Tn5 Mutagenesis and Construction of B. japonicum Mutant Strains 111020 and 1110Q5—A random transposon Tn5 mutagenesis of B. japonicum strain 1110proC was carried out as described previously (26).

Approximately 3500 mutant colonies were streaked onto media containing either proline or prolyl-glycyl-glycine (50 μg/ml) to screen for mutants that are unable to use the latter as a proline source. Two mutants displaying this phenotype were identified and designated strains 120 and Q5. Southern blot analysis revealed that Tn5 from each mutant were contained on EcoRI fragments ~12 kb in length, which were then isolated as described previously (26). These fragments, cloned into pBR322, were used to generate strains for the corresponding mutants in parent strain 1110 by homologous recombination as described previously (26). The nucleotide sequence of both strands of the coding region bearing the lysC region of B. japonicum lysC was determined.

B. japonicum lysC and ptsP genes were removed by introducing a pUH3 expression vector (Invitrogen). PCR reactions were performed using primers 5'-CTCGGATCCATGCGGAGCGCGTCGG-3' and 5'-GAGCCCCGGCGTAACCGTCTAGCCG-3', respectively. Expression was induced by adding 1 mM isopropyl-β-D-thiogalactosidase to the media, and cultures were grown overnight at 15 °C. Cells were lysed and cleared as described above, and protein was purified with prewashed glutathione-Sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

GST Pull-down Assay—Crude lysates of E. coli BL21(DE3)pLysS containing the overexpressed proteins GST, GST-AspK, or GST-EINtr were prepared as described. 1 ml of each cleared lysate preparation was added to a separate Eppendorf tube containing 50 μl of prewashed glutathione-Sepharose beads (Amersham Pharmacia Biotech) and rotatated on an Orbitor for 30 min at 4 °C. Beads were washed five times with PBS + 0.1% Triton X-100 by centrifugation at 2000 × g for 5 min at 4 °C. Strains I110 and I20 were grown at 37 °C in 250 ml of LB medium with 200 μg of 0.1% Triton X-100, and the amount of protein bound to the beads was determined using the Bradford protein assay (Bio-Rad).

The results of this assay were normalized against a control containing unbound glutathione-Sepharose beads. 5 μg of purified B. japonicum EINtr-c-myc/His6 fusion protein was added to a volume of beads that contained 20 μg of GST or GST-AspK, and 5 μg of purified B. japonicum AspK-c-myc/His6 fusion protein was added to a volume of beads that contained 20 μg of GST or GST-EINtr, in a total volume of 0.5 ml. Reactions were rotated on an Orbitor for 60 min at 4 °C. Beads were washed five times with PBS plus 0.1% Triton X-100 by centrifugation at 2000 × g for 5 min at 4 °C followed by aspiration of any remaining supernatant. Beads were resuspended in 25 μl of 2X SDS sample buffer, boiled for 5 min, and resolved by SDS-PAGE. After transferring to Immobilon-P (0.45 μm polyvinylidene difluoride, Millipore), proteins were detected with anti-c-myc/peroxidase conjugate (Roche Molecular Biochemicals) by chemiluminescence (Renaissance, PerkinElmer Life Sciences).

Assay for Aspartokinase Activity—Aspartokinase activity was determined by measuring aspartohydrolase produced when aspartate is introduced into the presence of ATP and hydroxyamine (30). The assay mixture contained 10.4 mM Mg-ATP, 94 mM Tris, HCl buffer (pH 8.0), 1.6 mM MgSO4, 10 mM β-mercaptoethanol, 10 mM L-α-aspartate, 800 mM NH4OH, 800 mM KCl, and purified B. japonicum AspK-c-myc/His6 fusion protein in a total volume of 0.5 ml. After incubation at room temperature for 15 min, the reaction was stopped by the addition of 0.5 ml of a 1.7% solution of FeCl3, in 1X HCl. After centrifugation, the absorbance at 540 nm of the supernatant was measured using a Beckman DU spectrophotometer. Enzyme activity is expressed as the optical density units × 1000. A blank reaction mixture that contained all components except for enzyme served as a control. Each reaction was performed in triplicate in the presence or absence of Mg-ATP.

Pep-dependent Phosphorylation of EINtr—B. japonicum EINtr-c-myc/His6 fusion protein was overexpressed and purified as described above. [32P]PEP was synthesized in a 0.1 ml reaction mixture containing 0.1 μM triethylamine (pH 7.6), 3 mM MgCl2, 15 mM KC1, 1 mM pyruvate, 0.1 mM phosphoenolpyruvate (cyclohexammonium salt), 10 μM γ-[32P]ATP (2× 106 Ci/mmol), and 4 units of pyruvate kinase (Sigma Chemical Co.) (31). The mixture was incubated for 90 min at 30 °C. Because this is an exchange reaction, the concentrations of pyruvate and PEP do not change; therefore, the specific activity of the [32P]PEP can be calculated from the known specific activity of the γ-[32P]ATP and the initial ATP and PEP concentrations to be ~2× 106 Ci/mmol. The reaction mixture was used as a [32P]PEP source for further purification. The EINtr phosphorylation assay contained, in a 20-μl volume, 94 mM Tris, HCl buffer (pH 8.0), 1.6 mM MgCl2, 10 μM β-mercaptoethanol, 4 μM of purified B. japonicum AspK-c-myc/His6 fusion protein for EINtr-c-myc/His6 and 125 μl [32P]PEP (220 Ci/mol) or 10 μM [γ-32P]ATP (50 Ci/mol). The reactions were incubated for 30 min at 37 °C. Reactions were stopped by adding an equal volume of 2× SDS-sample buffer and incubated at room temperature before resolving on a 7.5% SDS-PAGE. Gels were stained to visualize protein standards, and labeled proteins were detected by autoradiography.
Eco mutants were constructed by isolation of the Tn5-containing type background, and ALA uptake activity was measured. The mutations in strains I20 and Q5 were reconstructed in a wild or solid media supplemented with 50 mM Gly, to satisfy its auxotrophy as discerned by growth in liquid could use the proline-containing peptides Pro-Gly or Pro-Gly-proline for growth (23). We established that strain I110proC 10 mM

components, where added, were as follows: 95 mM Tris, pH 8, 1.6 mM MgSO4, and 18 μg of cell extracts prepared from lysC strain I110Q5 were added. The final concentrations of other components, where added, were as follows: 95 mM Tris, pH 8, 1.6 mM MgSO4, 10 mM β-mercaptoethanol, 0.1 mM [γ-32P]ATP (9 μCi/mM). Reactions were carried out for 10 min at 37 °C. Afterward, glutathione-agarose beads were added, centrifuged, washed, and then analyzed as autoradiograms of SDS-PAGE gels.

Results

Isolation of Oligopeptide Uptake Mutants of B. japonicum—B. japonicum strain I110proC is disrupted in the proline biosynthesis gene proC and requires an exogenous source of proline for growth (23). We established that strain I110proC could use the proline-containing peptides Pro-Gly or Pro-Gly-Gly, to satisfy its auxotrophy as discerned by growth in liquid or solid media supplemented with 50 μg/ml of either compound (Table I). Thus, the strategy for obtaining oligopeptide transport mutants was to screen for mutants of strain I110proC that could not use prolyl-glycyl-glycine (Pro-Gly-Gly) as a proline source. Strain I110proC was mutagenized with Tn5, and kanamycin-resistant colonies were screened for those that could no longer use Pro-Gly-Gly as a proline source, but still grew on proline. Two mutants, strains I20 and Q5, exhibited this phenotype on plates and in liquid cultures and were also unable to use Pro-Gly as a proline source (Table I). Both mutant strains retained the ability to grow on proline as well as strain I110proC.

Mutations in Strains I20 and Q5 AffectALA Uptake—Radio-labeled triptides were not commercially available, but [14C]ALA was available to carry out uptake experiments. The mutations in strains I20 and Q5 were reconstructed in a wild type background, and ALA uptake activity was measured. The mutants were constructed by isolation of the Tn5-containing EcoRI fragment from strains I20 and Q5 followed by introduction into the genome of strain I110 by homologous recombination to generate strains I110I20 and I110Q5 (see “Materials and Methods”). The uptake of [14C]ALA by the mutant and wild type strains was assessed using cells cultured in minimal media. Both mutants had severely reduced ALA uptake activities compared with the parent strain I110 (Fig. 1). These data show that the mutations in strain I20 or Q5, which affect their ability to use proline-containing peptides, also severely inhibit ALA uptake activity. The defect in uptake of ALA, a dipeptide analogue, indicates that the inability of Pro-Gly or Pro-Gly-Gly to satisfy the proline auxotrophy in strains I20 and Q5 is the result of a defect in transport rather than another step of oligopeptide metabolism.

Identification and Characterization of the lysC Gene and Its Product Aspartokinase—Initial subcloning and analysis of Tn5-containing genomic fragments from strains I20 and Q5 revealed that the transposon from each strain was inserted into one of two different regions of the same EcoRI fragment. Consequently, the EcoRI fragment isolated from strain I20 contained the wild type copy of the DNA mutated in strain Q5, and vice versa. Therefore, from these EcoRI fragments, the wild type genes were cloned and sequenced, and DNA fragments containing one or the other gene were constructed (see “Materials and Methods”). These two open reading frames have the same orientation, and are separated by a 288-bp intergenic region (Fig. 2A).

The upstream gene corresponding to that mutated in strain Q5 is 1257 bp in length and encodes a 418-amino acid polypeptide with extensive similarity to aspartokinase from numerous organisms, with the greatest identity (44.6%) to that from Corynebacterium glutamicum (32) (Fig. 3). Aspartokinase, encoded by the lysC gene, catalyzes the ATP-dependent phosphorylation of aspartate yielding aspartyl β-phosphate. The putative lysC gene was analyzed further by overexpression of the gene in E. coli (Fig. 2B). The purified recombinant had aspartokinase activity as determined by as the production asparthydroxamate from aspartate, ATP, and hydroxylamine (30). Thus, a bona fide lysC gene was identified.

Aspartyl β-phosphate formed by aspartokinase is the first intermediate in the lysine, threonine, and methionine biosynthetic pathways (33). Although only one gene was detected using the lysC open reading frame as a probe against B. japonicum wild type DNA in a Southern blot (data not shown), the lysC mutant strain I110Q5 is not an amino acid auxotroph. Thus, it is likely that B. japonicum contains an additional aspartokinase gene. Indeed, E. coli contains three aspartokinase isozymes, all of which must be mutated to obtain an amino acid auxotrophic phenotype (34, 35). We note, however, that the phenotypes of the B. japonicum lysC mutants indicate that a

Table I

| Strain (genotype) and gene on plasmid | Growth on medium with |
|--------------------------------------|----------------------|
|                                      | Proline | Pro-Gly-Gly | Pro-Gly |
| I110proC (proC lysC+ ptsP+ )         | None     | +           | +       |
|                                      | llysC+   | +           | +       |
|                                      | ptsP+    | +           | +       |
| I20 (proC lysC+ ptsP+ )              | None     | +           | -       |
|                                      | llysC+   | +           | +       |
|                                      | ptsP+    | +           | +       |
| Q5 (proC lysC ptsP+ )                | None     | +           | -       |
|                                      | llysC+   | +           | +       |
|                                      | ptsP+    | +           | +       |
putative second aspartokinase gene cannot compensate for the mutated gene described in this study. Finally, addition of lysine, threonine, or methionine, or combinations of them, to growth media did not complement the lysC strain Q5 for oligopeptide-dependent growth in the absence of proline. We suggest that lysC has a role other than, or in addition to, amino acid biosynthesis in B. japonicum.

Identification and Characterization of the ptsP Gene and Its Product Enzyme I_{Ntr}—The downstream open reading frame corresponding to the gene mutated in strain I20 is 2268 bp in length and encodes a 755-amino acid polypeptide that is homologous to an unusual protein called Enzyme I_{Ntr} (EINtr), which has been identified in several organisms. B. japonicum EINtr shows greatest identity (36.4%) to EINtr from Azotobacter vinelandii (21) (Fig. 4). EINtr is a paralogue of Enzyme I (EI) of the PTS system except that it contains an additional domain at the N terminus that is homologous to the N-terminal sensory domain of NifA from A. vinelandii (36). NifA is a regulatory protein of the s^{54}-dependent family of transcriptional activators responsible for the activation of genes related to nitrogen metabolism.

FIG. 3. Amino acid sequence comparison of aspartokinase from B. japonicum and C. glutamicum. Solid lines denote amino acid identity, and dotted lines represent similar residues.

FIG. 4. Amino acid sequence comparison of EIN_{Ntr} from B. japonicum and Azotobacter vinelandii. Solid lines denote amino acid identity, and dotted lines represent similar residues.
fixation in a wide variety of diazotrophs (37), including *B. japonicum* (38).

We overexpressed the *ptsP* gene in *E. coli*, and found that the recombinant *EI^Ntr* can be phosphorylated by PEP, but not by ATP (Fig. 2C), similar to what is observed for other EI proteins, including the *EI^Ntr* from *E. coli* (22). The current work indicates that like EI, *EI^Ntr* is involved in solute transport into cells. However, *EI^Ntr* is involved in transport of oligopeptides, which is not a known PTS substrate. Furthermore, the *ptsP* strain, as well as the *lysC* mutant, grew as well as the parent strain on glycerol, succinate, or glucose as sole carbon sources.

Therefore, it is unlikely that the phenotypes of those mutants is an indirect consequence of a defect in the ability to metabolize a carbon source. Data base searches reveal that the gene organization of *lysC* and *ptsP* in *B. japonicum* is not found in other bacteria where either gene, along with flanking sequence, has been identified.

**Evidence that lysC and ptsP Are Expressed as Separate Transcriptional Units and Are Both Required for Oligopeptide Transport**—The genetic organization of *lysC* and *ptsP* led us to ask whether the phenotype of the mutants was due to disruption of the respective gene, or whether it was due to a polar effect of the Tn5 on a downstream gene. To evaluate the necessity for each gene in the utilization of oligopeptides, we tested for complementation of *lysC* strain Q5 and *ptsP* strain 120 in trans using wild type copies of *lysC* or *ptsP*, respectively, harbored in the broad host range vector pVK102. The results show that each mutant strain can be complemented in trans for growth on Pro-Gly or Pro-Gly-Gly by a wild type copy of the respective gene (Table I) and indicates that the phenotypes exhibited by strains Q5 and 120 are due to the loss-of-function of *lysC* and *ptsP*, respectively.

Expression of *lysC* and *ptsP* was examined at the RNA level to determine the effect of the Tn5 on the transcription of both genes in the mutant strains. RNA protection analyses of total RNA isolated from strains Q5 and 120 and the parent strain revealed that *lysC* and *ptsP* mRNA accumulated in the parent strain, confirming that they are expressed genes (Fig. 5). *lysC* mRNA was not detected in the *lysC* mutant, but that strain did accumulate normal levels of *ptsP* transcript. Therefore, the Tn5 inserted in *lysC* did not have a polar effect on the downstream *ptsP* gene, which is consistent with the complementation data. Finally, *lysC* mRNA was found in the *ptsP* mutant; the levels were somewhat higher than found in the parent strain, for which we offer no explanation. These results support the conclusion that *lysC* and *ptsP* each have an effect on oligopeptide and Ala uptake in *B. japonicum*, and disruption of either gene results in a loss of those activities.

**Overexpression of ptsP in Trans Compensates for the lysC Mutation—** *lysC* and *ptsP* are each involved in the same cellular process, but their relationship to each other does not appear to be at the level of gene activation. Nevertheless, we found that *ptsP* complemented the *lysC* mutant in trans for growth on oligopeptides, suggesting that *ptsP* can compensate for the *lysC* mutation when expressed on a low copy plasmid (Table I). This is an interesting result given that transcription of the endoge-

**Fig. 5.** RNase protection analysis of *ptsP* and *lysC* expression in *B. japonicum* strains 110proC, 120, and Q5. Cells were grown in a yeast extract-based media. 8 μg of total RNA was analyzed per reaction.

**Fig. 6.** Protein-protein interactions between *B. japonicum* *EI^Ntr* and AspK. A, purified, recombinant *EI^Ntr* (His-myc-tagged) was incubated with either GST or GST-AspK, and protein bound to glutathione-Sepharose beads was analyzed by Western blots using anti-myc antibodies. B, purified, recombinant aspartokinase (His-myc-tagged) was incubated with either GST or GST- *EI^Ntr* and protein bound to glutathione-Sepharose beads was analyzed by Western blots using anti-myc antibodies.

**Evidence for Direct Interaction between Aspartokinase and Enzyme I^Ntr—** Complementation analysis suggests that aspartokinase affects *EI^Ntr* activity. Therefore, we examined whether the two proteins interact with each other by “pull-down” experiments using purified, recombinant proteins. In the first experiment, we tested for the ability of a GST-aspartokinase fusion, immobilized on glutathione-Sepharose, to interact with an *myc*-tagged *EI^Ntr* protein. The results show that the *myc*-tagged *EI^Ntr* was pulled down by the GST-aspartokinase fusion protein but not when GST alone was used as bait. The reverse experiment was carried out using a GST-EI^Ntr* fusion and a *myc*-tagged aspartokinase protein, and the results show an interaction between these two proteins as well (Fig. 6). The findings suggest that the mechanism by which aspartokinase affects *EI^Ntr* activity involves direct protein-protein interaction.

**Recombinant EI^Ntr* Is Phosphorylated by ATP in the Presence of *B. japonicum* Cell Extract, Which Is Negatively Affected in the Presence of Aspartokinase—** Aspartokinase catalyzes the transfer of phosphate from ATP to aspartate, whereas *EI^Ntr* is autophosphorylated by PEP. Initial experiments were carried out to determine whether PEP and ATP were interchangeable in the respective reactions, either alone or in combination with the other protein. Using purified recombinant proteins, we did not find evidence supporting phosphoryl transfer between aspartokinase and *EI^Ntr*, nor did one protein affect the activity of the other in vitro. It seemed plausible that a cellular factor was required for a functional interaction that was not present in these preliminary experiments. Therefore, we carried out a series of experiments where *B. japonicum* cell extract was included in the reactions. Purified GST-EI^Ntr* fusion protein was used so that it could be separated from the other components after the reaction was completed using glutathione-Sepharose beads, and subsequently analyzed by autoradiography of SDS-PAGE gels. GST-EI^Ntr* was not phosphorylated when incubated with [γ-32P]ATP alone, but it was strongly radio-labeled when cell extracts from the *lysC* strain 1110Q5 were included in the reaction (Fig. 7). Extracts from the *lysC* strain were used so that the only aspartokinase present was that added as purified protein. GST alone was not phosphorylated under those conditions. These observations indicate a factor in
The intriguing primary structure of Enzyme INtr suggested a physiological role for it or for the other PTSINtr. In this study, we show that EINtr is involved in oligopeptide transport, and therefore it, and perhaps the PTSINtr system as a whole, has a role in transport of a non-sugar solute. The N-terminal domain of EINtr is similar to the N-terminal sensory domain of NifA, and therefore it is probably significant that this protein is involved in transport of a nitrogen-containing compound. The present study identified a novel role for aspartokinase, the lysC gene product, in oligopeptide transport. The B. japonicum lysC strain is not an amino acid auxotroph, and thus its role in transport differs from its amino acid biosynthetic function. Collectively, the data indicate that aspartokinase interacts directly with EINtr to control its activity.

EINtr (GST-EINtr) was phosphorylated by ATP in the presence of cell extract in vitro, indicating a cellular factor that acts as an EINtr kinase or that allows EINtr to phosphorylate itself. Roseman’s group identified two ATP-dependent EI kinase activities in E. coli (39, 40), and therefore the input signal to EI and EINtr is not limited to PEP. ATP-dependent phosphorylation of EINtr was severely inhibited in the presence of aspartokinase, which provides a plausible regulatory function that the complementation data suggested. These findings, along with the fact that both aspartokinase and EINtr are required for oligopeptide uptake, strongly suggests that EINtr positively affects transport activity in the unphosphorylated state. Thus, it is likely that the lysC mutant was complemented in trans by the ptsP gene from a multicopy plasmid, because the overexpressed protein was primarily in the upper phosphorylated state, thereby obviating the need for aspartokinase. An activity for unphosphorylated EINtr to promote oligopeptide transport contrasts sharply with conventional EI enzymes, which transfer a phosphoryl group to the next protein in the cascade, ultimately resulting in phosphorylation and concomitant uptake of a sugar. In that case, the high energy phosphate drives the transport process. However, oligopeptide transport complexes bind ATP (4), and therefore EINtr need not directly couple transport with energy. The ability of EINtr to function differently depending on the phosphorylation status may allow it to serve as a branch point in different signal transduction systems. Recent studies involving a ptsN mutant of Pseudomonas putida indicate that the PTSINtr protein IIAINtr is a general regulator in that organism (17). Bacterial proteome and transcriptome analyses should help reveal the extent of control that these PTS paralogues exert on cellular activities.

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FIG. 7. ATP-dependent phosphorylation of EINtr in the presence of cell extracts and inhibition by aspartokinase. GST-EINtr or GST could be incubated with [γ-32P]ATP either alone or with one of the components labeled in the figure. When the reaction was complete, glutathione-agarose beads were added, centrifuged, washed, and proteins that bound were analyzed by autoradiography of SDS-PAGE gels.

B. japonicum extracts that allow GST-EINtr to be phosphorylated by ATP. However, when recombinant aspartokinase was included in the reaction, GST-EINtr was substantially underphosphorylated. Addition of aspartate or dialysis of cell extracts did not affect the phosphorylation of GST-EINtr, nor did it affect the inhibition by aspartokinase (data not shown). Thus, the underphosphorylated GST-EINtr in the presence of aspartokinase could not be explained by consumption of ATP from the enzyme activity of aspartokinase. We suggest that aspartokinase controls EINtr function by regulating the phosphorylation state of EINtr. The complementation data (Table I), indicating that aspartokinase exerts an affect on EINtr, are consistent with the in vitro phosphorylation experiments.
Evidence for Direct Interaction between Enzyme I^Ntr and Aspartokinase to Regulate Bacterial Oligopeptide Transport
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