The phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) phosphorylates sugars and regulates cellular metabolic processes using a phosphotransfer chain including the general energy coupling proteins, Enzyme I (EI) and HPr as well as the sugar-specific Enzyme II complexes. Analysis of the Escherichia coli genome has revealed the presence of 5 paralogues of EI and 5 paralogues of HPr, most of unknown function. The ptsP gene encodes an EI paralogue designated Enzyme I ntr (EI ntr), and two genes located in the rpoN operon encode PTS protein paralogues, NPr and IIA ntr, both implicated in the regulation of $\sigma^{54}$ activity. The EI ntr gene was polymerase chain reaction amplified from the E. coli chromosome and cloned into an overexpression vector allowing the overproduction and purification of EI ntr. EI ntr was shown to phosphorylate NPr in vitro using either a [32P]PEP-dependent protein phosphorylation assay or a quantitative sugar phosphorylation assay. EI ntr phosphorylated NPr but not HPr, whereas Enzyme I exhibited a strong preference for HPr. These two pairs of proteins (EI ntr/NPr and EI/HPr) thus exhibit little cross-reactivity. Phosphorylation transfer from PEP to NPr catalyzed by EI ntr has a pH optimum of 8.0, is dependent on Mg$^{2+}$, is stimulated by high ionic strength, and exhibits two $K_m$ values for NPr (2 and 10 $\mu$M) possibly because of negative cooperativity. The results suggest that E. coli possesses at least two distinct PTS phosphoryl transfer chains, EI ntr $\rightarrow$ NPr $\rightarrow$ IIA ntr and EI $\rightarrow$ HPr $\rightarrow$ IIA sugar. Sequence comparisons allow prediction of residues likely to be important for specificity. This is the first report demonstrating specificity at the level of the energy coupling proteins of the PTS.

The phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS) is a complex protein system that mediates uptake and concomitant phosphorylation of carbohydrates (1–3). Characterized PTS proteins include the cytoplasmic Enzyme I and HPr, which lack sugar specificity, and membrane Enzyme II complexes, each specific for one or a few sugars. The latter complexes usually consist of three proteins or protein domains that are designated IIA, IIB, and IIC (4). The phosphoryl relay proceeds sequentially from PEP to Enzyme I, HPr, IIA, IIB, and finally to the incoming sugar that is transported across the membrane and concomitantly phosphorylated by IIC. The PTS is present in a wide variety of Gram-positive and Gram-negative bacteria, but PTS protein homologues have not been found in archaea or eukaryotes. In addition to its primary functions in sugar transport, sugar phosphorylation, and chemoreception, the PTS is involved in regulatory processes such as catabolite repression and inducer exclusion (5, 6).

Novel PTS proteins, NPr and IIA ntr (paralogues of HPr and IIA ntr, respectively), are encoded by the npr and ptsN genes, respectively, localized to the rpoN operon of Escherichia coli, which also encodes the nitrogen-related $\sigma$ factor, $\sigma^{54}$ (7–10). ptsN deletion mutants lacking IIA ntr exhibit a growth defect in the presence of an organic nitrogen source and a sugar or tricarboxylic acid cycle intermediate. Protein phosphorylation involving NPr and IIA ntr was suggested to function in linking carbon and nitrogen metabolism, and IIA ntr has also been implicated in the regulation of the essential GTPase, Era, which appears to function in cell cycle progression and the initiation of cell division (10, 11). IIA ntr homologues have been identified in numerous Gram-negative bacteria (10), and a link between the ptsN gene and nitrogen regulation has been suggested for Rhizobium etli (12), Pseudomonas aeruginosa (13), and Klebsiella pneumoniae (14). The crystal structure of Enzyme IIA ntr has recently been determined (15).

Analysis of the E. coli genome revealed a gene, ptsP, encoding Enzyme I ntr (EI ntr), consisting of 2 domains, an N-terminal domain of 127 amino acids homologous to the N-terminal “sensory” domain of the NifA protein of Azotobacter vinelandii (16) and a C-terminal domain of 578 amino acids homologous to all currently sequenced enzymes I. EI ntr was suggested to serve a sensory function linking carbon and nitrogen metabolism (17). A mutation in the orthologous EI ntr-encoding ptsP gene of A. vinelandii resulted in impaired metabolism of poly-$\beta$-hydroxybutyrate as well as diminished respiratory protection of nitrogenase under carbon-limiting conditions (18).

In the present study we report the cloning and overexpression of the ptsP gene from E. coli, the purification of EI ntr, and the characterization of its biochemical activities. The results of these studies establish for the first time the existence of parallel but independent PTS phosphoryl transfer chains in which distinct Enzyme I paralogues exhibit specificity for their cognate HPr paralogues. Residues are identified that may account for this specificity.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Chemicals Used—Salmonella typhimurium strain SB2950ΔcysKptsHrrΔtrpB223 has been described** (19). The cloning vector pCR2.1TM was from Invitrogen (San Diego, CA).
Plasmid pJRENtr used for overexpression of EINtr is described below. Purified ppGpp was kindly provided by Mike Cashel (National Institutes of Health, Bethesda, MD). [\(\text{U-14C}\)]\(\text{D-Mannitol}\) (specific activity 32 mCi/mmol) and \([\gamma-32P]\)ATP (specific activity >4000 Ci/mmol) were obtained from ICN (Costa Mesa, CA). Other chemicals were of analytical grade.

Cloning of ptsP and Purification of EINtr—A DNA fragment containing the \(\text{ptsP}\) gene encoding EI from \(E.\ coli\) was amplified by polymerase chain reaction using \(E.\ coli\) genomic DNA. The top strand primer (NtrI) contained a Ndel site within the initiation codon (underlined) of \(\text{ptsP}\), 5′-\(\text{ACACGAAATCTCATATGCTCACTCGCCTGCGAAATAG}\)′. The bottom strand (Ntr2) contained a SalI site (underlined) 5′-\(\text{GACATGATCCGCGCTATAACCCTCCGCGAA}\)′. Amplification was performed with a Hybaid thermal reactor (Hybaid Ltd., Teddington, Middlesex, United Kingdom) in a reaction mixture containing Taq DNA polymerase, 100 mM Tris/HCl, pH 8.8, 15 mM MgCl\(_2\), and 250 mM KCl (Stratagene, La Jolla, CA) in a total volume of 100 μl. The amplification mixture was overlaid with 50 μl of mineral oil and subjected to 30 cycles of amplification as follows: 1-min denaturation at 94 °C, 1-min annealing at 55 °C, and 2-min extension at 72 °C. The polymerase chain reaction-amplified DNA was ligated to pCR2.1TM (Invitrogen), and the overexpression reaction-amplified DNA was ligated to pCR2.1TM (Invitrogen), and the overexpression plasmid pROEXTM-1 (Life Technologies, Inc.) to create the plasmid pJRENtr. Cloning of \(\text{ptsP}\) was confirmed by nucleotide sequencing using polymerase chain reaction dye terminator sequencing on an ABI 373 sequencer (Applied Biosystems) with a series of oligo-sequencing using polymerase chain reaction dye terminator sequencing encoding EINtr. DH10B cells bearing the overexpression plasmid were prepared from strain SB2950, which lacks EI, HPr, and NPr, were overproduced and purified as described previously (10, 21, 22). \([\text{32P}]\text{PEP}\) was separated from \([\gamma-32P]\)ATP using phosphoenolpyruvate carboxykinase from \(E.\ coli\) (23). \([\text{32P}]\text{PEP}\) was separated from \([\gamma-32P]\)ATP and \([\text{32P}]\text{Pi}\), by ion-exchange chromatography on AG-1-X8 bicarbonate resin (analytical grade anion exchange resin, 20–50 mesh, chloride form (Bio-Rad)). Protein phosphorylation reactions were modified after Powell et al. (10). The EI-specific phosphorylation reaction (at 37 °C for 15 min; 20 μl final volume) contained 250 mM HEPES, pH 8.0, 2.3 mM dithiothreitol, 5 mM MgCl\(_2\), 0.125 mM \([\text{32P}]\text{PEP}\) (1.2 × 10\(^6\) counts/min/μmol), 0.6 μg of EI, and 10 μg of NPr. The EI-specific phosphorylation reaction (at 37 °C for 15 min; 20 μl final volume) contained 50 mM Tris/HCl, pH 7.2, 2.0 mM dithiothreitol, 5 mM MgCl\(_2\), 0.125 mM \([\text{32P}]\text{PEP}\) (1.2 × 10\(^6\) counts/min/μmol), 0.6 μg of EI, and 1.25 μg of HPr. Proteins were separated by SDS-polyacrylamide gel electrophoresis as described previously (20, 24). Proteins labeled with \([\text{32P}]\text{PEP}\) were detected by autoradiography as described (24).

Concentrations of soluble PTS proteins, EINtr, EI, NPr, and HPr, were determined according to the method described by Bradford (25). Protein concentrations in butanol-urea-extracted membranes were measured using the DC Protein Assay as described by the manufacturer (Bio-Rad). Bovine plasma \(\gamma\)-globulin was used as a standard. Membrane samples and standards were boiled for 5 min in the presence of 1% SDS or N-octyl-3-b-glucopyranoside prior to the addition of the reagents.

Preparation of Butanol-Urea-Extracted Membranes—Membranes containing high levels of Enzyme IICBA\(_{\text{II}}\), used for assaying EI and EINtr, were prepared from strain SB2950, which lacks EI, HPr, and IIA\(_{\text{II}}\) (26). Batch cultures (5 liters) of strain SB2950 were grown overnight in nutrient broth (Difco). Cultures were centrifuged at 16,000 × \(g\) (GSA rotor, RC-5 centrifuge) for 5–10 min at 4 °C, washed in mineral medium (50 mM potassium phosphate buffer, pH 7.5 containing 15 mM \(\text{NaH}_{2}\text{PO}_{4}\) and 1.7 mM MgSO\(_4\), and resuspended in 25 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT, 1 mM EDTA, and 10 μg/ml DNase I. The cells were resuspended at 10,000 p.s.i. in a French pressure cell, and intact cells and cell debris were removed by centrifugation at 14,500 × \(g\) (SS34 rotor, RC-6 centrifuge) for 10 min at 4 °C. Membranes were recovered from the supernatant by centrifugation at 100,000 × \(g\) (Ti rotor, L-7–65 Ultracentrifuge, Beckman Instruments) and resuspended in a few ml of 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM DTT and 1 mM EDTA. These membranes were extracted with urea and 1-butanol (27). Urea (480 mg/ml) was added and dissolved by stirring on ice. Then 1-butanol (40 μl/ml) was added, and this solution was gently stirred for 2 h on ice. Membrane...
branes were recovered by centrifugation at 100,000 \( \times g \) (Ti rotor) for 4 h at 4 °C. Extracted membranes from 5 liters of stationary phase cells were resuspended in 10 ml of 25 mM Tris/HCl, pH 7.5, containing 1 mM DTT and 1 mM EDTA to a final protein concentration of 11–32 mg/ml and transferred to boiled dialysis tubes (MWCO: 12–14,000, Spectrum Medical Industries Inc., Houston, TX). Membranes were dialyzed 3 times for 8 h against the same buffer and were then aliquoted in small plastic vials for storage at \( 2 \pm 20 \) °C. Extraction of peripheral proteins from the membranes eliminated background sugar phosphorylation activity.

Sugar Phosphorylation Assay—The \([^{32}P]\)PEP-dependent protein phosphorylation assay described above does not provide a rate but instead represents an equilibrium situation. To allow estimation of relative rates of phosphoryl transfer via various PTS proteins, a quantitative assay was required. We found that phosphorylation of \([^{14}C]\)mannitol (or another PTS sugar) could be used for this purpose although the rate of phosphoryl transfer involving EI\({ \rm Ntr} \) and NPr was very low relative to that involving EI and HPr. \([^{14}C]\)Mannitol was selected as the phosphoryl acceptor because this sugar is PTS-specific.

**FIG. 3.** Dependence of EI\(^{\rm Ntr}\) activity on the concentration of PEP. A, activity of EI\(^{\rm Ntr}\) was determined using the sugar phosphorylation assay as described under "Materials and Methods" with potassium phosphate as the buffer. Three experiments were carried out with different amounts of NPr added to the reaction mixture: ●, 0.5 \( \mu \)g; ■, 1 \( \mu \)g; and ▲, 5 \( \mu \)g. B, in these double-reciprocal plots (1/\( V \) versus 1/S), two straight lines can be drawn through the data for each concentration of NPr used, one corresponding to a low \( K_m \) low \( V_{\max} \) value and one corresponding to a high \( K_m \) high \( V_{\max} \) value. C, the intercepts on the \( y \) axis from B were plotted versus 1/NPr to give the absolute \( K_m \) and \( V_{\max} \) values of 2 \( \mu \)M and 0.3 \( \mu \)mol \([^{14}C]\)mannitol-phosphate/mg EI\(^{\rm Ntr}\) for the high affinity, low velocity curve (●) and values of 10 \( \mu \)M and 1 \( \mu \)mol \([^{14}C]\)mannitol-phosphate/mg EI\(^{\rm Ntr}\) for the low affinity, high velocity curve (▲).

**FIG. 4.** Time courses of EI\(^{\rm Ntr}\) activity revealing that preincubation of EI\(^{\rm Ntr}\) with NPr eliminates the lag phase. Activity of EI\(^{\rm Ntr}\) was determined using the sugar phosphorylation assay as described under "Materials and Methods." HEPES (250 mM) was used as the buffer. Reaction mixtures were preincubated for 1 h at 37 °C in the absence of one or more selected component(s), and then at zero time the missing component(s) were added. The components that were missing during the preincubation are indicated to the right of the curves. In the controls, all components were present, but there was no preincubation period.

**FIG. 5.** Phosphoryl transfer from EI\(^{\rm Ntr}\) or EI to NPr or HPr. The figure shows autoradiographs from protein phosphorylation experiments as described under "Materials and Methods." Experiments in A show that EI only marginally phosphorylates NPr (lane 2) as compared with HPr (lane 1), whereas EI\(^{\rm Ntr}\) does not phosphorylate HPr (lane 3) although it readily phosphorylates NPr (lane 4). B shows that EI\(^{\rm Ntr}\)-dependent phosphorylation of NPr (control in lane 1) is inhibited by GDP (10 \( \mu \)M, lane 2).
and no phosphatase cleaving the product ([14C]mannitol-1-phosphate) is known. The standard assay for PTS sugar phosphorylation employs membranes isolated from disrupted E. coli cells that overproduce several Enzyme II complexes because of a pts operon deletion (28). Because the inverted membrane preparations are “leaky,” protein components and sugar phosphate products are not compartmentalized. Thus, this assay provides a useful measure of relative rates of overall phosphoryl transfer employing EI and HPr, even though the former reaction is not physiologically significant.

The sugar phosphorylation assay used was modified from the method described by Kundig and Roseman (27) as required by the exceptionally low activity of EI in this assay. The reaction mixture (50 ml) generally consisted of 0.1 M g of EINtr, 5 M g of NPr, 5–10 M l of extracted membranes, 10 mM PEP, 10 mM MgCl2, 5 M KF, 2.5 M DTT, 250 M FIG. 6. Multiple alignments of EINtrs versus EIs and NPrs versus HPrs. A, two regions of the multiple alignment of all recognized EINtrs (top) and selected recognized EIs (bottom). B, multiple alignment of the entire sequences of all recognized NPrs (top) and selected HPrs (bottom). Residues that are fully conserved among all proteins are shown in bold and are presented below the alignment (identities). Residues that are fully conserved only in one group (e.g., EI, but not NPr) are shaded (below the alignment) represents the phosphorylation site. Numbers in parentheses indicate the residue numbers. Abbreviations used and accession numbers (O and P for Swiss-Prot; AF, L and Y for Genbank) are as follows: EINtr of E. coli (Eco, P37177), A. vinelandii (Avi, Y14681), and P. aeruginosa (Pae, Contig 84); EI of Streptococcus mutans (Smu, L15191), Lactobacillus sakei (Lsa, P08839), and B. subtilis (Bsu, P07006); Staphylococcus aureus (Sau, P02907), Listeria monocytogenes (Lmo, AF030824), Mycoplasma genitalium (Mge, P45596), and Hemophilus influenzae (Hin, P43921); NPr of E. coli (Eco, P33996), K. pneumoniae (Kpn, P51185), and Pae (Contig 95); and HPr of Smu (P45596), Lsa (O07125), Bsu (P08877), Sau (P02907), Lmo (O31148), Mge (P47287), Eco (P07006), and Hin (P43921).

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The sugar phosphorylation assay used was modified from the method described by Kundig and Roseman (27) as required by the exceptionally low activity of EI in this assay. The reaction mixture (50 Ml) generally consisted of 0.1 M of EI, 5 M of NPr, 5–10 M of extracted membranes, 10 M PEP, 10 M MgCl2, 25 M RF, 2.5 M DTT, 250 M
Results

pH and Divalent Cation Dependence of the EI<sup>Ntr</sup>-catalyzed Reaction—To study the properties of EI<sup>Ntr</sup>, we developed a sugar phosphorylation assay coupling phosphoryl transfer from PEP via purified EI<sup>Ntr</sup>, NPr, and Enzyme IICBA Mtl to [14C]mannitol (see "Materials and Methods"). Under the conditions described by Kundig and Roseman (27) (20 mM potassium phosphate buffer, pH 7.5), EI<sup>Ntr</sup> showed only marginal activity, with three different concentrations of NPr (Fig. 3). The y intercepts (1/V<sub>max</sub> apparent) shown in Fig. 3B were plotted versus 1/NPr for the two parts of the curves (Fig. 3C). Straight lines were observed in each case, and these lines extrapolated to give V<sub>max</sub> and K<sub>m</sub> values, respectively, of 1 μmol of [14C]mannitol phosphate/mg of EI<sup>Ntr</sup>/min and 10 μM for the high velocity, low affinity curve, and 0.3 μmol of [14C]mannitol phosphate/mg of EI<sup>Ntr</sup>/min and 2 μM for the low velocity, high affinity curve.

Time-dependent, NPr-dependent activation of EI<sup>Ntr</sup>—Analysis of time courses for EI<sup>Ntr</sup> activity showed that the enzyme exhibited a lag phase of 30–40 min under our standard assay conditions (Fig. 4). When the otherwise complete reaction mixtures were preincubated for 1 h without either [14C]mannitol or the extracted membranes, no lag for sugar phosphorylation was observed (Fig. 4). However, the absence of extracted membranes during preincubation yielded lower activity than when mannitol was omitted, suggesting that the membranes might facilitate EI<sup>Ntr</sup>-NPr association. In contrast, when either EI<sup>Ntr</sup> or NPr was absent during the preincubation, a lag was still observed. Preincubation of EI<sup>Ntr</sup> with NPr was therefore required for optimal activity, suggesting that a slow association of these two proteins accounts for the lag phase observed in the control sample.

Specificity of EI<sup>Ntr</sup> for NPr and of EI for HPr—Selective phosphoryl transfer by EI and EI<sup>Ntr</sup> could be demonstrated using the [32P]-protein phosphorylation assay. NPr was found to be a specific protein substrate of EI<sup>Ntr</sup> as the latter could not phosphorylate HPr (Fig. 5A, lanes 3 and 4). In contrast, Enzyme I was found to be specific for HPr and could barely phosphorylate NPr (Fig. 5A, lanes 1 and 2). These observations were confirmed using the [14C]mannitol phosphorylation assay.

Search for Specific Inhibitors of EI<sup>Ntr</sup>—In a previous report, a regulatory function was suggested for the N-terminal domain of EI<sup>Ntr</sup> (17). We employed the sugar phosphorylation assay to screen various compounds for effects on the NPr-dependent phosphoryl transfer activity of EI<sup>Ntr</sup>. The following salts increased the activity of EI<sup>Ntr</sup> when added to the potassium phosphate (50 mM, pH 8.0) -buffered reaction mixture: (NH₄)₂SO₄, NH₄Cl, Na₂SO₄, NaCl, K₂SO₄, KCl, and LiCl (tested in a concentration range of 10–400 mM). This demonstrated that EI<sup>Ntr</sup> activity requires high ionic strength, but no evidence for a specific effect by any one ionic species was obtained. Other compounds (concentrations between 0.1 and 10 mM) tested in the presence of 20 mM Mg<sup>2+</sup> (to avoid Mg<sup>2+</sup> limitation because of chelation) were l-glutamine, l-glutamate, α-ketoglutarate, ATP, ADP, AMP, GTP, GDP, GMP, UDP, ppGpp, and adenine 3'-phosphate 5'-phosphosulfate. Only GDP and ppGpp at concentrations of 5 mM or higher had effects on the activity of EI<sup>Ntr</sup> (50–60% inhibition at 10 μM). The inhibitory effect of GDP was confirmed using the [32P]P-dependent protein phosphorylation assay. EI<sup>Ntr</sup>-dependent phosphorylation of NPr was significantly reduced although the amount of phosphorylated Enzyme I<sup>Ntr</sup> remained similar (Fig. 5B, lanes 1 and 2). Although phosphorylation of EI was not affected, the inhibition of NPr phosphorylation is not likely to be of physiological
Enzyme \(I^{Ntr}\) from E. coli

significance. Cyclic AMP and cyclic GMP were without effect in the concentration range 0.01–1 mM.

A number of redox cofactors were examined with respect to potential regulatory effects. The following compounds were tested in the concentration range of 1–10 mM: NAD, NADH, NADP, NADPH, and FAD. Of these compounds, only FAD was inhibitory (50% with 1 mM FAD). EI was similarly inhibited.

**DISCUSSION**

\(E^{Ntr}\) resembles the classical EI except for the N-terminal NifA-like putative sensory domain of \(E^{Ntr}\). Both enzymes catalyze PEP-dependent phosphoryl transfer to an HPr-like protein in a pH-, salt-, and Mg\(^{2+}\)-or Mn\(^{2+}\)-dependent process (30, 33, 34). The effects of ionic strength may be because of stabilization of the dimeric forms of these enzymes, and Mg\(^{2+}\) may similarly promote association (35–37). However, \(E^{Ntr}\) exhibits biphasic kinetics with respect to PEP concentration, whereas EI exhibits monophasic kinetics; \(E^{Ntr}\) exhibits essentially absolute specificity for NPr, whereas EI could phosphorylate NPr at a rate that was only a small fraction of that at which it phosphorylates HPr. The turnover number (38) for \(E^{Ntr}\) using the sugar phosphorylation assay proved to be 0.8 pmol/pmole \(E^{Ntr}\)/min, whereas that for EI is 1.34 nmol/pmol EI/min (39). This 1000-fold difference explains, in part, why EI is absolutely required for sugar phosphorylation under \textit{in vivo} conditions. \textit{pts} deletion mutants can be mutated so as to express an EI paralogue that can phosphorylate HPr (40), but preliminary evidence suggests that this enzyme is not \(E^{Ntr}\).

The nearly absolute specificity of \(E^{Ntr}\) for NPr and of EI for HPr provides the first evidence that two different EIs in a single organism exhibit specificity at the level of their phosphoryl acceptor PTS proteins. This finding clearly leads to both functional and mechanistic predictions. \(E^{Ntr}\) does not appear to function in sugar phosphorylation and may function exclusively in regulation, possibly controlling the activities of NPr and IIA\(^{Ntr}\). These latter proteins are encoded within the \(rpoD\) operon of \(E.\ \textit{coli}\) and have been implicated in the regulation of \(\sigma^{54}\)-dependent transcriptional initiation of genes concerned with organic nitrogen utilization (10). Moreover, in \textit{A. vinelandroidii}, the \(E^{Ntr}\)-encoding \textit{pts} gene has been shown to play a role in poly-\(\beta\)-hydroxybutyrate metabolism and respiratory protection of nitrogenase under carbon-limiting conditions (18). In \textit{K. pneumoniae}, \textit{P. aeruginosa}, and \textit{R. eii}, IIA\(^{Ntr}\) has been shown to function in capacities similar to those demonstrated for \(E.\ \textit{coli}\) (12–14, 41). \(E^{Ntr}\), NPr, and IIA\(^{Ntr}\) have all been identified in the fully sequenced genome of \textit{P. aeruginosa}. As for \(E.\ \textit{coli}\), \(E^{Ntr}\) \textit{Pseudomonas} proteins presumably comprise a phosphoryl transfer chain that functions in parallel with EI, HPr, and various IIA\(^{sugar}\) proteins with entirely different physiological consequences (see Fig. 7). Phylogenetic data have shown that NPr is a distant homolog of HPr (10), whereas \(E^{Ntr}\) is a distant homolog of EI (17). The mechanistic implications of our biochemical observations are that specific residues in EI versus \(E^{Ntr}\) and/or HPr versus NPr must control the interactions and phosphoryl transfer reactions between these proteins. Alignments of two segments of all sequenced \(E^{Ntr}\)s (Fig. 6A, top) with representative EIs (Fig. 6A, bottom) as well as of recognized NPrs (Fig. 6B, top) and HPrs (Fig. 6B, bottom) revealed such candidate residues. Residues that are fully conserved among all homologous proteins are presented in bold print, whereas residues that are fully conserved in one group, but of a different type in the other group, are shaded. In comparing \(E^{Ntr}\) with EI, we found that residues conserved in one of these two sets of proteins but not the other were scattered unevenly throughout the alignment.

The greatest abundance of such residues was found to immediately surround the active site histidine (Fig. 6A, top), a region of catalytic importance. This fact is particularly significant as the active site region is well conserved among either the EIs or the \(E^{Ntr}\). The second region of the multiple alignment exhibiting a high frequency of residues conserved in only one set of these two proteins occurred far downstream of the active site histidine in a region of unknown function (Fig. 6A, bottom). Neither of these two regions is at the EI-HPr interface (42). In the region that defines the EI-HPr interaction surface, two residues were markedly different in the EIs versus the \(E^{Ntr}\). These residues are the (A/G)H dipeptide in the EIs, which corresponds to the (V/L)Y dipeptide in the EI \(N^{tr}\).
Enzyme $F_{\text{Ntr}}$ from E. coli

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