THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 15, Issue of April 15, pp. 15356–15361, 2005
Printed in U.S.A.

^{31}P NMR and Genetic Analysis Establish hinT as the Only *Escherchia coli* Purine Nucleoside Phosphoramidase and as Essential for Growth under High Salt Conditions*[S]

Received for publication, January 13, 2005
Published, JBC Papers in Press, February 9, 2005, DOI 10.1074/jbc.M500434200

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Eukaryotic cells encode AMP-lysine (AMP-N-ϵ-(*N*-α-acetyl lysine methyl ester) 5′-phosphoramidate) hydrolases related to the rabbit histidine triad nucleotide-binding protein 1 (Hint1) sequence. Bacterial and archaeal cells have Hint homologs annotated in a variety of ways, but the enzymes have not been characterized, nor have phenotypes been described due to loss of enzymatic activity. We developed a quantitative ^{31}P NMR assay to determine whether *Escherichia coli* possesses an adenosine phosphoramidase activity. Indeed, soluble lysates prepared from wild-type laboratory *E. coli* exhibited activity on the model substrate adenosine 5′-monophosphoramidate (AMP-NH_2). The *E. coli* Hint homolog, which had been comprehensively designated *ycfF* and is here named *hinT*, was cloned, overexpressed, purified, and characterized with respect to purine nucleoside phosphoramidate substrates. Bacterial hinT was several times more active than human or rabbit Hint1 on five model substrates. In addition, bacterial and mammalian enzymes preferred guanosine *versus* adenosine phosphoramidates as substrates. Analysis of the lysates from a constructed *hinT* knock-out strain of *E. coli* demonstrated that all of the cellular purine nucleoside phosphoramidase activity is due to *hinT*. Physiological analysis of this mutant revealed that the loss of hinT results in failure to grow in media containing 0.75 M KCl, 0.9 M NaCl, 0.5 M NaOAc, or 10 mM $MnCl_2$. Thus, cation-resistant bacterial cell growth may be dependent on the hydrolysis of adenylylated and/or guanylylated phosphoramidate substrates by hinT.

Histidine triad enzymes are a superfamily of nucleoside monophosphate hydrolases and transferases containing an active site motif related to His-*X*-His-*X*-His-*X*-*X*, where *X* is a hydrophobic amino acid (1). The Hint branch, named for similarity with rabbit Hint1,[1] is the most ancient branch, having representatives in all forms of life (1, 2). The best biochemical substrates of these enzymes are model compounds containing a adenylylated-lysine residue from which Hint hydrolyzes AMP, leaving unmodified lysine (3–5). Deletions or point mutations that target the enzymatic activity of *Saccharomyces cerevisiae* homolog *hnt1* result in cells that are temperature-sensitive on galactose media and are hypersensitive to mutations in components of the general transcription factor transcription factor II H (namely Kin28, Ccl1, Tfb3) and to mutations in the cyclin-dependent kinase-activating kinase Cak1 (3). Birds encode a typical *HINT* gene on the Z chromosome, such that males have two doses, whereas avian *ASW* genes, which may function as dominant negative inhibitors of Hint via Gln-127, are repeated 40 times on the female-specific W chromosome (5, 6). The *hint1* homolog of mouse is a nonessential gene (7), the loss of which predisposes to an increased incidence of tumors (8). Two less typical members of the Hint branch of the superfamily are Aprataxin, which is mutated in ataxia-oculomotor apraxia1 (9, 10), and the scavenger-decapping enzyme, Dcps (11–13).

The second and third branches of the HIT superfamily are homologs of the Fhit tumor suppressor protein and enzymes related to galactose-1-phosphate uridylyltransferase (1). Fhit and homologs are diadenosine polyphosphate hydrolases, but Fhit function as a tumor suppressor is not limited by substrate hydrolysis but rather substrate binding (14). Enzymes in the GalT branch of the superfamily are nucleoside monophosphate transferases for a variety of unusual nucleotide substrates (1). Galactosemia in humans is usually caused by mutations that inactivate GalT activity (1), consistent with the idea that Hint branch enzymes and GalT branch enzymes have a conventional relationship between loss of enzymatic and biological function.

Although Hint hydrolases have been associated only recently with adenosine phosphoramidase activity, nucleoside phosphoramidase activity has been observed in partially purified extracts from rabbit liver (15, 16), cells and extracts of the human T-lymphoblast leukemia cell line, CEM (17), peripheral blood mononuclear cells (18, 19), and green monkey Vero cells (20). Part of the reason for continued interest in phosphoramidates has been their demonstrated utility as prodrugs of antiviral and anticancer nucleoside monophosphates or as pronucleo-

* This work was supported by U. S. Public Health Service Grant CA55334 (to C. R. W.) and CA075954 (to C. B.) from the National Cancer Institute and a development grant in drug design from the Department of Medicinal Chemistry, University of Minnesota. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains five supplemental figures and two supplemental tables.

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[1] The abbreviations used are: Hint, histidine triad nucleotide-binding protein; AMP-NH_2, adenosine 5′-monophosphoramidate; AMP-lysine, AMP-N-ϵ-(*N*-α-acetyl lysine methyl ester) 5′-phosphoramidate; AMP-Mor, adenosine 5′-monophosphomorpholidate; GMP-lysine, GMP-N-ϵ-(*N*-α-acetyl lysine methyl ester) 5′-phosphoramidate; GMP-Mor, guanosine 5′-monophosphomorpholidate; Cdk7, cyclin-dependent kinase 7; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DTT, dithiothreitol; Fhit, fragile histidine triad; GalT, galactose-1-phosphate uridylyltransferase; TMP, trimethyl phosphate.

tides (21–25). Typically, therapeutic nucleosides must be converted to the corresponding mono-, di-, and triphosphates before exhibiting biological activity. Nevertheless, many nucleosides are poor substrates for endogenous nucleoside kinases. To overcome this hurdle, several pronucleotide approaches have been investigated, including the use of nucleoside monophosphoramidates (24, 26).

Although the enzyme responsible for phosphoramidate hydrolysis has not been determined, direct evidence of intracellular P-N bond hydrolysis by a putative phosphoramidase has been demonstrated by studies of the intracellular metabolism of fluorodeoxyuridine phosphoramidates with permeabilized cells (17) and O^{18}-labeled 3′-azido-3′-deoxythymidine tryptophan methyl ester phosphoramidate with capillary liquid chromatograph negative mode electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (25). Recently, Bieganowski *et al.* (3) reported that both *S. cerevisiae* Hnt1 and rabbit Hint1 hydrolyze AMP-NH_2, AMP-lysine, AMP-Mor, and AMP-alanine and suggested that Hint hydrolases might be the enzymes responsible for nucleoside monophosphoramidate prodrug activation *in vivo* (3). Consequently, in addition to gaining an understanding of their natural function, the study of Hint hydrolases may facilitate the design of tissue- and species-specific pronucleotides of potential therapeutic utility.

In this study, we developed a simple ^{31}P NMR assay capable of detecting total phosphoramidase activity in cell lysates. We scanned the *Escherichia coli* genome (27) for a Hint homolog and discovered an open reading frame designated *ycfF* at the 16-min position (161090–1161467) on the *E. coli* genetic map with 47% amino acid sequence identity to rabbit and human Hint1. Testing the hypothesis that *ycfF* encodes a *E. coli* Hint homolog (here named hinT) that is responsible for observed cellular phosphoramidase activity, we cloned, purified, and characterized the activity of hinT *in vitro* and also constructed a mutant strain in which the *hinT* gene was disrupted. Supporting the view that the *hinT* gene is entirely responsible for nucleoside monophosphoramidate hydrolysis, such substrates were completely stable in extracts of *E. coli hinT* knock-out mutants. Further characterization demonstrated that bacterial hinT is homodimeric and capable of hydrolyzing adenosine and guanosine 5′-phosphoramidate monoesters significantly faster than mammalian Hint (3). In addition, evaluation of an *E. coli hinT* knock-out mutant established a requirement for protein expression when grown on NaCl, KCl, $MnCl_2$, and NaOAc.

EXPERIMENTAL PROCEDURES

Evaluation of Phosphoramidase Activity in E. coli Cell Lysates—AMP-NH_2 was used to investigate the phosphoramidase activity in *E. coli* cell lysates because it was earlier shown to be a specific substrate for rabbit and yeast Hint hydrolases (3). Phosphoramidase activity in cell lysates was measured by the ^1H-decoupling mode of ^{31}P NMR spectroscopy (Varian VAC-300 spectrometer), which can clearly distinguish the substrate, AMP-NH_2, and the product, AMP. NMR spectrometry is suitable for quantitation by integration of the area under the peak, which is proportional to the amount of nuclei present. The relative ratio of peak areas can be used to calculate the amount of turnover based on a standard curve (supplemental data Fig. 1). Chemical shift information is described in Fig. 1 of the supplemental data. Cell lysates from *E. coli* Tuner(DE3)pLacI cells (Novagen) were obtained by treatment with lysozyme (1 mg/ml), and the DNA was precipitated by protamine sulfate followed by centrifugation to remove cell debris. The supernatant was dialyzed extensively against Buffer A (20 mM Tris, pH 7.0; 1 mM EDTA; 1 mM DTT, 0.01 mM phenylmethylsulfonyl fluoride) to remove cellular phosphates and nucleotides. Phosphate-free cell lysates (0.75 mg of total protein) were incubated with 5 mM AMP-NH_2 in 0.5 ml of reaction buffer (0.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2) at 37 °C for intervals of 15 min, 30 min, and at 22 °C for 12 h. Reactions were terminated by snap-freezing in a dry ice/acetone slush bath and lyophilized for 5 h. Samples were resuspended in NMR buffer (600 μl, 50 mM EDTA, 20 mM HEPES, pH 7.2, and 1 mM TMP (internal standard)), and the insoluble material was removed by centrifugation at 12,500 × *g* for 5 min. A ^{31}P NMR spectrum was then collected for the supernatant. For the low pH control experiment, 10 mM AMP-NH_2 in reaction buffer (500 μl, pH 2.0) was incubated at 37 °C for 12 h, and the ^{31}P signal was detected under the same conditions.

Genetic Disruption of E. coli hinT—The *E. coli hinT* gene in strain BW25113 was disrupted as described (28). Primers 7024 and 7025 (sequences in Supplemental Table I) were used for amplification of the chloramphenicol resistance marker (Cm^r) in plasmid pKD3. Stable chloramphenicol-resistant transformants of BW25113 were tested by PCR with primers 7026 and 7027 to confirm correct recombination of the Cm^r marker into the *hinT* locus. A strain that tested positive by PCR was named BB1. Strain BB3 was constructed by removing the antibiotic resistance marker using Red recombinase as described previously (28).

Plasmid Constructions—The *hinT* gene was amplified from the chromosomal DNA of *E. coli* strain BW25113 with primers 7328 and 7329. The product of this reaction was cloned into Bluescript SK+ using EcoRI and BamHI sites included in the sequences of the primers. The resulting plasmid was named pB429. Mutagenesis (29) of phagemid DNA with primer 7330 resulted in plasmid pB431 containing *hinT* with an H101A substitution. Plasmids pB432 and pB433 were obtained by cloning HindIII-EcoRI fragments containing wild-type and H101A mutated alleles of *hinT* from plasmids pB429 and pB431 into plasmid pACYC184. The expression construct pPH70D contains the *E. coli* dihydrofolate reductase (DHFR) gene followed by a thrombin cleavage site and the protein of interest (30). The fusion proteins are purified with a methotrexate affinity chromatography followed by cleavage with human thrombin to release the native protein. Plasmids were constructed by replacing the NAT2 (hamster polymorphic *N*-acetyltransferase 2) cDNA in pPH70D with the desired open reading frames. After PCR amplification from the chromosomal DNA of the *E. coli* K12 strain, EMG2, with the primers 101 and 102 containing XhoI and XbaI sites, the product was cloned into the T/A cloning vector, pSTBlue 1 (Novagen). Double digestion of pTFCY-TA followed by subcloning into pPH70D resulted in the plasmid, pTFCF15DmY. The plasmid pJLCF15DmH, containing human *HINT1* cDNA was obtained by a similar procedure after PCR amplification with primers 103 and 104 from cDNA prepared from the human T-lymphoblast leukemia cell line, CEM. *E. coli* hinT-pSAG02 was subjected to site-directed mutagenesis using primer 105 and 106 to create *E. coli* hinT-H101A mutant.

Expression and Purification of Recombinant Proteins—The cell growth and cell lysate extraction were described previously except that 0.5 mM isopropyl-β-D-thiogalactopyranoside was used for induction (30). Bacterial or human Hint-DHFR fusion proteins were purified by methotrexate-agarose (Sigma) using a 12.5-ml column, washed with 40 column volumes of buffer A, 60 column volumes of buffer A with 1 M NaCl, and followed by folate elution with 5 mM folate, 32 mM Tris, pH 9.0, 1 mM EDTA, 1 mM DTT, and 0.01 mM phenylmethylsulfonyl fluoride. Fractions (8 ml) were collected at a flow rate 3 ml/min, and an aliquot (10 μl) of each fraction was assayed for protein concentration with the Bradford dye reagent (Bio-Rad). Fractions containing more than 0.1 mg/ml protein were analyzed by 12% SDS-PAGE, and DHFR activities were determined (31, 32). The standard DHFR assay mixture contained 50 μM DHF, 100 μM NADPH, and 1 mM DTT in the assay buffer (50 mM 2-morpholinoethanesulfonic acid, 25 mM tris(hydroxymethyl)aminomethane, 25 mM ethanolamine, and 100 mM NaCl, pH 7.0) and the enzyme in a final volume of 1.0 ml. The reaction was started by the addition of DHF (32). Fractions containing pure Hint-DHFR fusion proteins were pooled and concentrated to 2 mg/ml, and the buffer was exchanged with 50 mM Tris, pH 8, 100 mM NaCl, and 2.5 mM $CaCl_2$. The fusion protein was digested at 4 °C for 19 h with 8 units of human thrombin (Sigma)/mg of protein. To separate Hint hydrolases from DHFR, reaction mixtures were applied onto a 10-ml AMP-agarose affinity column (Sigma) and washed with 16 column volumes of buffer A with 1 M NaCl and 4 column volumes of buffer A and then eluted with adenosine buffer (2 mM adenosine, 20 mM Tris, pH 7.0; 1 mM EDTA; 1 mM DTT). Fractions (8 ml) were collected at a flow rate of 3 ml/min, and aliquots (10 μl) from each fraction were used to determine the protein concentration. Fractions containing more than 0.1 mg/ml protein were analyzed by 18% SDS-PAGE. Fractions containing pure Hint protein were pooled and concentrated to 2.6 mg/ml. Rabbit Hint1 and *E. coli* hinT-H101A were purified by AMP-agarose affinity chromatography as described previously (3, 33) with the exception that expression was carried out in the *E. coli hinT* disruption mutant (BB1) described in this study.

Substrate Specificity—AMP-NH_2, AMP-Mor, and GMP-Mor (10 mM) were incubated with human Hint1-DHFR, *E. coli* hinT-DHFR, human

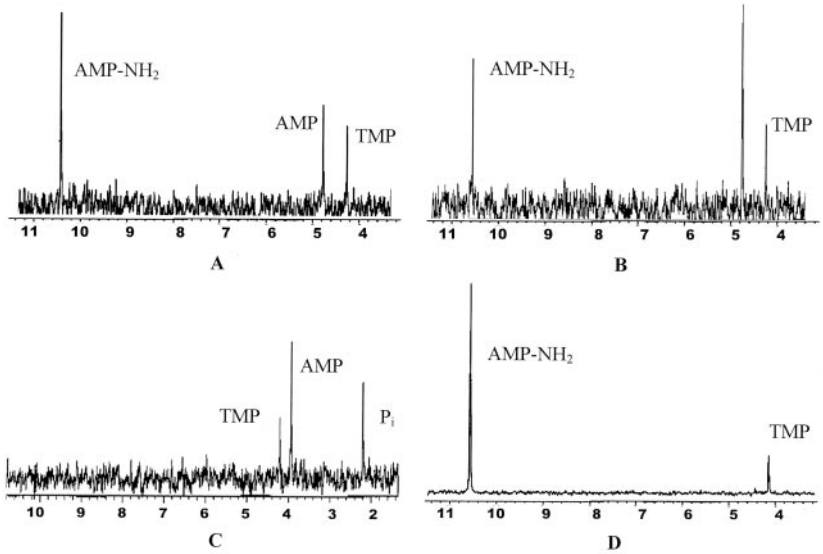

FIG. 1. **Adenosine monophosphoramidase activity in *E. coli* cell lysates.** Tuner(DE3)pLacI, a modified *E. coli* BL21 strain, was used to determine phosphoramidase activity in cell lysates. *A*, AMP-NH_2 (5 mM) incubated with *E. coli* cell lysates (0.75 mg of total protein) at 37 °C for 15 min; *B*, for 30 min; *C*, at 22 °C for 12 h, which showed completely conversion of AMP-NH_2 to AMP. The inability to completely control the pH of the lysates reaction resulted in an observed up field shift of the AMP resonance relative to TMP. The addition of exogenous AMP confirmed the identity of the AMP resonance (data not shown); *D*, control, AMP-NH_2 (10 mM) incubated with pH 2.0 buffer at 37 °C for 12 h.

Hint1, rabbit Hint1, and *E. coli* hinT (2.5 or 10 μg) in the reaction buffer (500 μl, 0.5 mM $MgCl_2$, 20 mM HEPES, pH 7.2) at 22 °C for intervals of 36 and 66 min. To determine the residual phosphoramidase activity in *E. coli* hinT-H101A, AMP-NH_2 (10 mM) was incubated with *E. coli* hinT-H101A (1200 and 2700 μg) in the reaction buffer at 22 °C for 14 h. Reactions were quenched by adding 5 M NaOH (20 μl) followed by snap-freezing in a dry ice/acetone slush bath and then lyophilized. AMP-lysine and GMP-lysine (2.5 mM) were incubated with *E. coli* hinT (0.25 μg) and human Hint1 (0.625 μg) for 20 and 40 min. Reactions were stopped by snap-freezing in a dry ice/acetone slush bath and then lyophilized. Sample preparation for ^{31}P NMR measurement was as described for the cell-lysate experiment except that the pH was adjusted to 7.0 by the addition of 12 μl 6 M HCl before NMR spectra were collected.

Synthesis of AMP-Lysine and GMP-Lysine—AMP-N-ϵ-(*N*-α-acetyl lysine methyl ester) and GMP-N-ϵ-(*N*-α-acetyl lysine methyl ester) 5′-phosphoramidates were prepared in a single-step condensation reaction employing the carbodiimide-mediated coupling method of monophosphate to amine with minor modification (34). To synthesize AMP-lysine, 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide HCl (0.24 g, 1.25 mmol) was added to the flask containing AMP (0.15 g, 0.42 mmol), *N*-α-acetyl-L-lysine methyl ester (0.2 g, 0.84 mmol), and H_2O (15 ml, pH ~7.0, adjusted with triethylamine). The reaction mixture was stirred and heated to 60 °C for 7 h. After cooling to room temperature, water was then removed under reduced pressure. The residue was dissolved in a mixture of chloroform/methanol/water (5:3:0.5, v/v) with 0.5% ammonium hydroxide and subjected to chromatography on a silica gel column. The column was eluted first with the same solvent mixture and followed by increasing the polarity to a solvent mixture of chloroform/methanol/water (5:4:0.5, v/v) with 0.5% ammonium hydroxide. The product was isolated as a white solid (90 mg, yield 39%). ^1H NMR (D_2O): δ 8.33 (1H, s), 8.05 (1H, s), 5.95 (1H, d), 4.36 (1H, m), 4.20 (1H, m), 3.94 (1H, m), 3.84 (2H, m), 3.51 (3H, s), 2.45 (2H, m), 1.82 (3H, s), 1.40–0.80 (6H, m). ^{31}P NMR (D_2O): δ 10.37. ESI-MS *m/z* $[M+H]^+$ 532.2 and $[M-H]^-$ 530.1.

To synthesize GMP-lysine, 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide HCl (0.15 g, 0.78 mmol) was added to the flask containing GMP (0.1 g, 0.25 mmol), *N*-α-acetyl-L-lysine methyl ester (0.19 g, 0.78 mmol), and H_2O (10 ml, pH ~7.0 adjusted with triethylamine). The product was isolated as described above (40 mg, yield 29%). ^1H NMR (D_2O): δ 7.97 (1H, s), 5.77(1H, d), 4.56 (1H, m), 4.16 (1H, m), 3.98 (1H, m), 3.83 (2H, m), 3.55 (3H, s), 2.48 (2H, m), 1.84 (3H, s), 1.42–0.85 (6H, m). ^{31}P NMR (D_2O): δ 10.5 (supplemental data, Fig. 2). ESI-MS *m/z* $[M+Na]^+$ 570.17 and $[M-H]^-$ 546.2.

RESULTS

E. coli Cell Lysates Possess Adenosine Phosphoramidase Activity—AMP-NH_2 hydrolysis by *E. coli* cell lysates (pH 7.2, 37 °C) was observable by ^{31}P NMR with a rate of 76.3 (± 20.9) nmol mg^{-1} min^{-1} over the course of 30 min (Fig. 1, *A* and *B*). Partial conversion of the hydrolysis product, AMP, to P_i, presumably by *E. coli* phosphatases, could be detected after incubation at 22 °C for 12 h (Fig. 1*C*). To verify that the formation of AMP was due to an enzymatic process and not chemical hydrolysis, AMP-NH_2 was incubated for 12 h under acidic conditions (pH 2.0, 37 °C) for 12 h (Fig. 1*D*). The pH 2.0 test conditions were chosen due to the reported acid lability of the phosphorous-nitrogen bond (35). AMP-NH_2 and TMP signals were visible by ^{31}P NMR, with no detectable AMP or P_i, confirming that AMP-NH_2 is stable at pH 2.0 for at least 12 h.

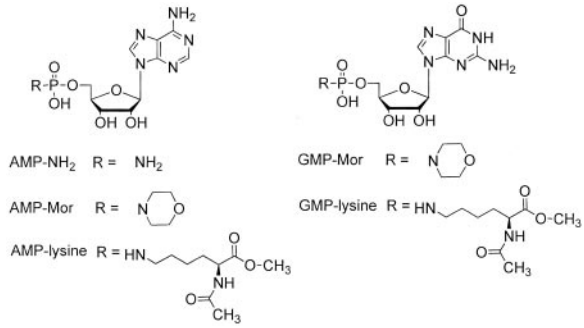

FIG. 2. **Structures of substrates for recombinant bacterial and mammalian phosphoramidases.**

hinT Is the Only Bacterial Source of Adenosine Phosphoramidase Activity—To determine whether *hinT* is solely responsible for the observed cellular phosphoramidase activity, a mutant strain, BB1, in which the *hinT* gene was replaced with the chloramphenicol resistance marker, was constructed. The phosphoramidase activities of the wild-type *E. coli* BL21 Star strain and the mutant strain lysates were determined (supplemental data, Fig. 3). When using AMP-NH_2 as a substrate, the specific activity of BL21 Star wild-type cell lysates was found to be 20.1 (± 7.7) nmol mg^{-1} min^{-1} (pH 7.2, 22 °C). However, no AMP formation could be observed with lysates derived from the *hinT* disruption strain, BB1 (supplemental data, Fig. 3 and Table II). Therefore, we conclude that *hinT* is fully responsible for the soluble adenosine phosphoramidase activity of *E. coli*.

TABLE I
Summary of the specific activity (nmol nmol^{-1} min^{-1}) of the recombinant purified Hint hydrolases
ND, not determined.

Substrate	*E. coli* hinT-DHFR	Human Hint1-DHFR	*E. coli* hinT	Human Hint1	Rabbit Hint1
AMP-NH_2	753 ± 2	388 ± 45	526 ± 27	196 ± 33	70.0 ± 1.3
AMP-Mor	411 ± 4	43.4 ± 4.0	360 ± 1	45.0 ± 0.1	26.6 ± 0.6
GMP-Mor	675 ± 17	87.2 ± 11.8	669 ± 1	78.7 ± 1.3	48.6 ± 0.4
AMP-lysine	ND	ND	529 ± 7	102 ± 19	ND
GMP-lysine	ND	ND	836 ± 262	232 ± 68	ND

Nucleoside and Leaving Group Specificity of Bacterial and Mammalian Hint Phosphoramidases—Both *E. coli* hinT and human Hint1 were purified to homogeneity as DHFR fusion proteins by methotrexate affinity chromatography (see supplemental data, Fig. 4, *lanes 1*, *2*, and *6*). Removal of the DHFR affinity handle by thrombin digestion (supplemental data, Fig. 4, *lane 7*) and AMP affinity chromatography afforded the purified recombinant enzymes (supplemental data, Fig. 4, *lanes 3–5*). The apparent molecular sizes of recombinant purified *E. coli* hinT and *E. coli* hinT-DHFR fusion protein were analyzed by gel filtration chromatography on a Superdex G-75 size exclusion column and strongly indicated, as has been observed for rabbit and human Hint1, the formation of a stable homodimer in solution (supplemental data, Fig. 5) (2, 36). Based on our ^{31}P NMR assay, we found that *E. coli* hinT and human Hint1 are adenosine phosphoramidases with specific activities of 526 (±27) and 196 (±33) nmol nmol^{-1} min^{-1}, respectively. The representative ^{31}P NMR spectra of *E. coli* hinT incubation with AMP-NH_2 are shown in Fig. 3*A*.

The co-crystal structure of rabbit Hint1 with GMP (2) and the fact that GMP can elute rabbit Hint1 from an adenosine affinity column (33) imply that guanosine phosphoramidates may also be substrates for Hint hydrolases. To evaluate the substrate specificity with respect to the different purine nucleoside phosphoramidates, the purified Hint hydrolases were evaluated with AMP-Mor and GMP-Mor (Fig. 2). AMP-Mor has previously been shown to be a reasonable substrate for rabbit Hint1 and yeast Hnt1 (3). All three Hint hydrolases were fully capable of utilizing both compounds as substrates with a 2-fold preference for the GMP-Mor. Since AMP-lysine was shown to be a substrate for rabbit Hint1, yeast Hnt1, and chicken Hint (3, 5), both AMP-lysine and GMP-lysine were also evaluated as substrates for *E. coli* hinT and human Hint1. As observed for the morpholino compounds, GMP-lysine was approximately a 2-fold better substrate than AMP-lysine for both enzymes (4). Hydrolysis rates for the three Hint hydrolases with each substrate are summarized in Table I.

Determination of Phosphoramidase Activity for E. coli hinT-H101A Mutant—Phosphoramidase activity could not be observed in cell extracts expressing the *E. coli* hinT-H101A mutant (data not shown). However, when the protein was purified and sufficiently concentrated, enzyme concentration-dependent activity for the *E. coli* hinT-H101A mutant (0.017 (±0.002) nmol nmol^{-1} min^{-1}) was determined (Fig. 3*B*). These results are consistent with previous studies of yeast Hnt1 and rabbit Hint1, demonstrating that at least 99.99% of the phosphoramidase activity is dependent on the strictly conserved residue His-101 (3).

hinT Is Required for Growth under High Salt Conditions—Earlier a *Synechococcus* strain was constructed in which the Hint-homologous gene was deleted and a slow growth phenotype was reported (37). Using the BB3 strain, we constructed a stain with an *E. coli hinT* disruption and carried out a screen for mutant phenotypes. The *E. coli hinT* knock-out strain was transformed with the control plasmid, pACYC, a plasmid expressing *E. coli hinT*, pACYC-hinT, or a plasmid expressing the catalytically inefficient hinT His-101 to Ala mutant, pACYC-hinT-H101A, and screened for the phenotypic consequences of hinT mutations. As shown in Fig. 4, when transformed with pACYC, the mutant was sensitive to 0.9 M NaCl, 0.75 M KCl, 0.5 M NaOAc, and 10 mM $MnCl_2$. However, when the mutant strain was transformed with either pACYC-hinT or pACYC-hinT-H101A, phenotypic rescue was observed. Consequently, the expression of hinT with as little as 0.003% catalytic activity appears to be required by *E. coli* for growth under elevated salt conditions.

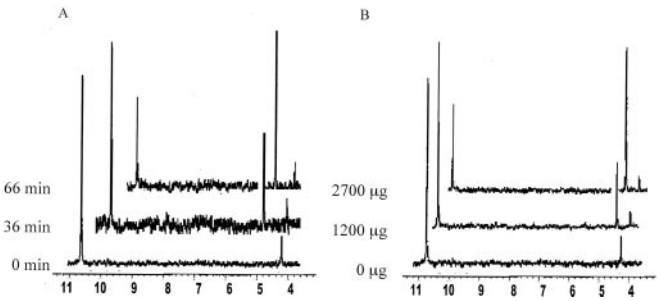

FIG. 3. **Phosphoramidase activity of the wild-type *E. coli* hinT and H101A mutant by ^{31}P NMR Assay.** *A*, *E. coli* hinT protein (2.5 μg) incubated with AMP-NH_2 (10 mM) at 22 °C for 36 and 66 min; *B*, *E. coli* hinT-H101A, 1200 and 2700 μg were incubated with AMP-NH_2 (10 mM) at 22 °C for 14 h.

DISCUSSION

Nucleoside phosphoramidates have been exploited for the delivery of nucleoside monophosphate antiviral and anticancer agents. Extensive analysis of their metabolism by cell extracts and whole cells has provided supporting evidence that the hydrolysis of the P-N bond necessary for subsequent phosphorylations is enzyme-mediated. Recently, yeast Hnt1, rabbit Hint1, and chicken Hint have been shown to be AMP-lysine hydrolases, suggesting that this class of enzymes may be responsible for nucleoside phosphoramidase activities (3–5). A test of this hypothesis required development of an assay that could measure total cellular nucleoside monophosphoramidate hydrolase activity and a genetic system to knock out candidate genes. Here we accomplished this in *E. coli*.

The ^{31}P NMR assay requires little sample preparation and is capable of accurately determining the concentration of substrates and products because the chemical shifts of the phosphoramidate and the monophosphate are generally separated by 4–6 ppm. In addition, we have demonstrated that this assay is independent of the structure of either the nucleoside or the amine that compose the nucleoside phosphoramidate.

Applying our assay to the investigation of the phosphoramidate hydrolase of bacteria cells, we found that AMP-NH_2 was rapidly hydrolyzed by *E. coli* cell extracts. Purified recombinant *E. coli* hinT and human Hint1 were shown to be adenosine/guanosine phosphoramidases. In addition, rabbit Hint1, known to be an adenosine phosphoramidase, was shown to be a guanosine phosphoramidase as well. We can also conclude, based on gene replacement in the BB1 cell lysate studies, that hinT is the only purine phosphoramidase expressed by *E. coli*. Conversion of the specific activity of the purified *E. coli* hinT (526 nmol/nmol/min) to units used for cellular lysates (39,000

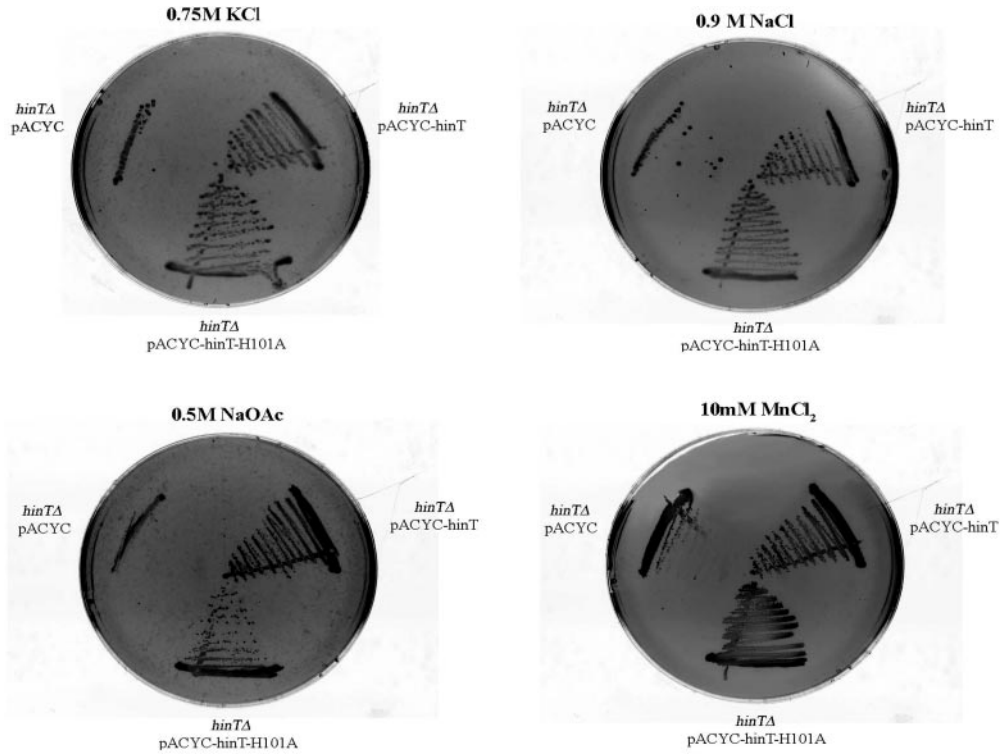

FIG. 4. ***E. coli* hinT is required for growth on the LB-agar media containing 0.75 M KCl, 0.9 M NaCl, 0.5 M NaOAc, and 10 mM $MnCl_2$.** The *E. coli hinT* knock-out strain BB3 (*hinT*Δ) was transformed with an empty vector (*hinT*Δ pACYC), a plasmid expressing *E. coli* hinT (*hinT*Δ pACYC-*hinT*), or a plasmid expressing the His-101 to Ala mutant (*hinT*Δ pACYC-*hinT*-H101A). Colonies were observed for BB3 cells transformed with pACYC-*hinT* (*hinT*Δ pACYC-*hinT*) and pACYC-*hinT*-H101A (*hinT*Δ, pACYC-*hinT*-H101A) when grown with LB-agar media containing 0.75 M KCl, 0.9 M NaCl, 0.5 M NaOAc, and 10 mM $MnCl_2$.

nmol/mg/min) indicates that the lysate (20 nmol/mg/min) contains hinT as ~1 part in 2000 of total lysate protein.

Evaluation of the substrate specificity of the *E. coli* hinT and human Hint1 revealed that GMP-lysine was a 3-fold better substrate for human Hint1 than GMP-Mor but only modestly preferred by *E. coli* hinT. A similar conclusion was observed AMP-NH_2 and AMP-Mor. These results suggest that the two enzymes differ in their capacity to accommodate bulky leaving groups. The preference of all three Hint proteins for the guanosine may result from hydrogen bonding between the N2 amino group of guanine and the backbone carbonyl oxygen of an active site residue, as observed in the GMP-bound rabbit Hint1 crystal structure (2). However, although *E. coli* hinT has almost 50% sequence identity with human and rabbit Hint1, the overall greater catalytic activity of the bacterial enzyme suggests that key differences do exist between these enzymes. Ongoing structural and enzymatic analysis of these enzymes should provide the insights necessary to develop a rationale for this discrepancy.

The physiological role of mammalian Hint proteins remains controversial, with a mixture of conflicting evidence and isolated experiments. An interaction between human Hint1 and Cdk7 was observed in a yeast-two hybrid assay (38). However, studies with transgenic mice, in which the *hint1* gene was deleted, failed to reveal a phenotype consistent with the regulation of Cdk7 (7). On the other hand, evidence from *S. cerevisiae* indicates that Hnt1 functions as a positive regulator of the Cdk7-homologous Kin28 kinase (3). Our observation of the association of *E. coli* hinT with growth under high salt conditions is the first suggestion of the possible physiological role of these enzymes in bacteria. Although wild-type hinT is 30,000-fold more active than the H101A mutant, expression of either protein rescued the knock-out strain from the cation-dependent phenotype. Given the wide distribution of orthologous genes in bacteria, the expression of these proteins may be a general requirement for cation-resistant bacterial cell growth.

A possible unique role for Hint proteins has been proposed in regulating the nucleotidylation of protein substrates (1, 3). The nucleotidylation post-translational modification has been observed for the regulation of the glutamine synthetase activity by two enzymes: uridylyl transferase and adenylyl transferase (39). Our data also indicate that both AMP-lysine and GMP-lysine are excellent substrates for *E. coli* hinT and human Hint1 (Table I). Based on these observations, it is likely that Hint proteins maybe involved in reversing either protein adenylylation and/or protein guanylylation. Ongoing studies should define the natural substrates and physiological role of Hint proteins and their relationship to cation resistance. In addition, the potential differences in substrate specificity observed between the bacterial and mammalian enzymes suggest that the design of bacteria-specific pronucleotides may be achievable.

Acknowledgement—We gratefully thank Jonathan Carlson for critical comments on this manuscript.

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