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Biochemical, Crystallographic, and Mutagenic Characterization of Hint, the AMP-Lysine Hydrolase, with Novel Substrates and Inhibitors*

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Hint, histidine triad nucleotide-binding protein, is a universally conserved enzyme that hydrolyzes AMP linked to lysine and, in yeast, functions as a positive regulator of the RNA polymerase II C-terminal domain kinase, Kin28. To explore the biochemical and structural bases for the adenosine phosphoramidate hydrolase activity of rabbit Hint, we synthesized novel substrates linking a *p*-nitroaniline group to adenylate (AMP-*p*NA) and inhibitors that consist of an adenosine group and 5′-sulfamoyl ($AdoOSO_2NH_2$) or *N*-ethylsulfamoyl ($AdoOSO_2NHCH_2CH_3$) group. AMP-*p*NA is a suitable substrate for Hint that allowed characterization of the inhibitors; titration of each inhibitor into AMP-*p*NA assays revealed their K_i values. The *N*-ethylsulfamoyl derivative has a 13-fold binding advantage over the sulfamoyl adenosine. The 1.8-Å cocrystal structure of rabbit Hint with *N*-ethylsulfamoyl adenosine revealed a binding site for the ethyl group against Trp-123, a residue that reaches across the Hint dimer interface to interact with the alkyl portion of the inhibitor and, presumably, the alkyl portion of a lysyl substrate. Ser-107 is positioned to donate a hydrogen bond to the leaving group nitrogen. Consistent with a role in acid-base catalysis, the Hint S107A mutant protein displayed depressed catalytic activity.

Histidine triad proteins are a superfamily of enzymes that act as hydrolases and transferases on substrates that contain a nucleoside monophosphate group linked to an amino group, a nucleotide, or a phosphorylated sugar (1). The first branch of the histidine triad superfamily is the most ancient and contains enzymes related to rabbit Hint (2). The crystal structure of Hint demonstrated that the protein is a dimer with two identical AMP/GMP-binding sites for each dimer formed by the most highly conserved amino acids in the superfamily (2). By screening a large number of compounds, the adenosine monophosphoramidates AMP-NH_2, AMP-*N*-ϵ-(*N*-α-acetyl lysine methyl ester), and AMP-*N*-alanine methyl ester were identified as rabbit Hint and yeast Hnt1 substrates (3). Building on the observation that the RNA polymerase II C-terminal domain kinase Cdk7/Kin28 may be functionally linked to Hint/Hnt1 (4), it was demonstrated that, in yeast, Hnt1 enzymatic activity acts as a positive regulator of Kin28 function (3). Based on the observation that Hint and Hnt1 hydrolyze AMP linked to lysine (3), we developed the hypothesis that Hint homologs may hydrolyze adenylyl-modified proteins to regulate their functions *in vivo* (1).

The mouse homolog of rabbit Hint, termed *hint1*, has been knocked out and found to be encoded by a nonessential gene (5) that may protect the forestomach from carcinogenesis induced by *N*-nitrosomethylbenzamine (6). In birds, Hint-related genes are sex-linked. Male birds are homogametic (ZZ), whereas females are heterogametic (ZW). A typical *HINT* gene is located on the Z chromosome (7), whereas an extremely unusual *HINT*-related gene, termed *ASW* was found repeated ~40 times on the female-specific W chromosome (7, 8). Although every Hint-related sequence examined to date conserved the 15 amino acids surrounding the AMP-binding site more than any other amino acids (2), these residues were specifically altered in Asw, apparently to eliminate or modify catalytic activity (9). Molecular modeling indicated that the Asw protein has conserved the amino acids required to heterodimerize with avian Hint and, potentially, to insert across the dimer interface a nonconserved Gln-127 in the vicinity of the Hint active site to depress or alter Hint specificity in a putative Hint-Asw heterodimer (9).

Additional interest in Hint-related hydrolases was generated by the observation that human ataxia-oculomotor apraxia 1, the second most common of the autosomal recessive ataxias, is caused by loss of a gene on 9p13 that encodes Aprataxin, a Hint-related hydrolase with an N-terminal FHA domain and a C-terminal sequence reminiscent of a zinc finger (10, 11). The physical association of Aprataxin with DNA repair proteins Xrcc1 and Xrcc4 suggests that a repair deficiency may underlie the ataxia-telangiectasia-like neurological symptoms of ataxia-oculomotor apraxia.[1]

Although Hint genes are found in all organisms, reasonable

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The atomic coordinates and structure factors (code 1RZY) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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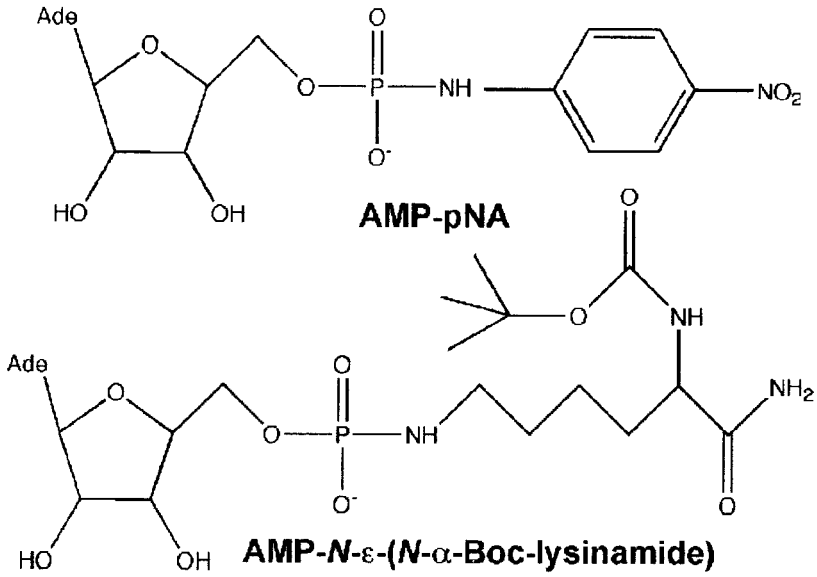
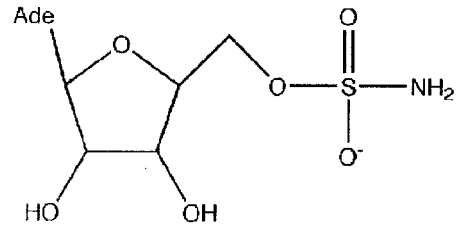
[1] P. M. Clements, C. Breslin, E. D. Deeks, P. J. Byrd, L. Ju, P. Bieganowski, C. Brenner, M.-C. Moreira, A. M. R. Taylor, and K. W. Caldecott, submitted manuscript.





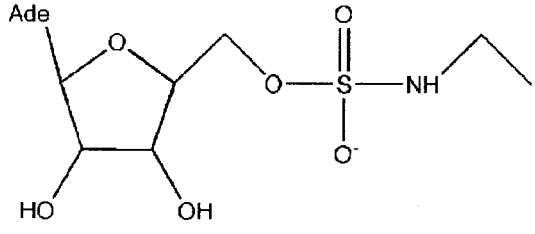


FIG. 1. **Novel Hint substrates and inhibitors.** Chemical structures of Hint substrates AMP-*p*NA and AMP-*N*-ε-(*N*-α-Boc-lysinamide) and Hint inhibitors, sulfamoyl adenosine and *N*-ethylsulfamoyl adenosine

Hint substrates were only identified (3) and a catalytic mechanism proposed (1) in 2002. To study the mechanism and specificity of Hint hydrolases, here we developed adenosine 5′-*O*-*p*-nitrophenylphosphoramidate (AMP-*p*NA)[2] as a novel spectroscopic substrate (see Fig. 1). Using methods we established for analysis of Fhit with the fluorogenic substrate Gpp-pBODIPY (12), we titrated nonlabeled inhibitors into assays of wild-type and mutant Hint enzymes to determine their equilibrium inhibitory binding constants. Biochemical and crystallographic analysis of wild-type Hint with newly synthesized adenosine sulfamoyl inhibitors indicated and located a binding site for an alkylamino leaving group. The inhibited Hint crystal structure also provides information about hydrogen bonding that suggests that the carbonyl oxygen of Gly-105 may assist the side chain hydroxyl of Ser-107 as the acid-base catalyst, in contrast to the earlier suggestion of His-114 as the acid-base catalyst (1). Indeed, biochemical characterization of the S107A mutant indicates that the Ser hydroxyl plays a facilitative role in catalysis, although in its absence, the Gly-105 carbonyl may assist a water molecule bound by the S107A enzyme to provide residual activity for protonation of the leaving group and activation of the hydrolytic water.

[2] The abbreviations used are: AMP-*p*NA, adenosine 5′-*O*-*p*-nitrophenylphosphoramidate; DBU, 1,8-diazabicyclo-(5.4.0)-undec-7-ene; FAB-MS, fast atom bombardment mass spectroscopy; HPLC, high pressure liquid chromatography; ESMS, electrospray MS; HRMS, high resolution MS.

EXPERIMENTAL PROCEDURES

Synthesis of Substrates—AMP-*p*NA was prepared in a three-step procedure employing oxathiaphospholane ring opening condensation chemistry (13). First, to a solution of *p*-nitroaniline (0.276 g, 2 mmol) in dry pyridine (10 ml), elemental sulfur (0.128 g, 4 mmol) was added followed by a dropwise addition of 2-chloro-1,3,2-oxathiaphospholane (0.314 g, 2.2 mmol). The reaction mixture was stirred at room temperature for 12 h. Solvent was then removed *in vacuo*, and the residue was triturated with 15 ml of acetonitrile. Excess sulfur was filtered off, and the filtrate was concentrated *in vacuo*. The residue was dissolved in 2.5 ml of chloroform and applied to a silica gel column (2.5 × 18 cm). The column was eluted with methanol in chloroform (0→1%). Appropriate fractions were combined and evaporated to give *N*-(2-thiono-1,3,2-oxathiaphospholanyl)-*p*-nitroaniline (0.348 g, 63%), ^{31}P NMR (CD_3OD) δ: 91.09 ppm, FAB-MS *m/z* (M−1) 275. Second, to a solution of *N*-(2-thiono-1,3,2-oxathiaphospholanyl)-*p*-nitroaniline (0.276 g, 1 mmol) in dry acetonitrile (10 ml), N^6,N^6,$O^{2'}$,$O^{2'}$-tetrabenzoyladenosine (0.683 g, 1 mmol) was added followed by 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) (0.182 g, 1.2 mmol). The reaction mixture was stirred for 12 h at

TABLE I
Steady state characterization of Hint substrates and inhibitors

Compound	k_{cat}	K_m	K_{cat}/K_m	K_i
	s^{-1}	μM	$M^{-1}\ s^{-1}$	μM
AMP-*p*NA	0.00187 ± 0.00006	134. ± 11	14.0	
AMP-*N*-ε- (*N*-Boc-Lys-NH_2)	0.234 ± 0.011	0.472 ± 0.057	496,000	
Ado-SO_2-NH_2				16.1 ± 0.2
Ado-SO_2-NH-C_2H_2				1.26 ± 0.28

TABLE II
Crystallographic data collection and refinement statistics for Hint-ethylsulfamoyl adenosine

Space group	$P4_32_12$
Unit cell dimensions (Å)	$a = b = 39.88$, $c = 142.3$
Reflections (measured/unique)	95,355/10,698
Resolution limits (Å)	23–1.8
Completeness (%)	93.9 (88.3)[a]
R_{sym} (%)[b]	6.5 (14.3)[a]
I/σ	9.2 (5.1)[a]
Multiplicity	8.4 (3.4)[a]
R_{work} (%)[c]	21.6 (29.9)[a]
R_{free} (%)[d]	24.2 (28.1)[a]
Protein and nucleotide nonhydrogen atoms	961
Water molecules	72
Root mean square difference, bond lengths (Å)	0.013
Root mean square difference, bond angles (°)	1.4
Average *B* factor (Å^2)	16.9

[a] Numbers in parentheses refer to data in the 1.86–1.80-Å shell.
[b] $R_{sym} = \Sigma|I - \langle I\rangle|/\Sigma\langle I\rangle$ in which I is a measured intensity and $\langle I\rangle$ is the average intensity from multiple measurements of symmetry-related reflections.
[c] $R_{work} = \Sigma|F_o - F_c|/\Sigma F_o$.
[d] R_{free} was calculated as for R_{work} using a test set of reflections (7%) not used for refinement.

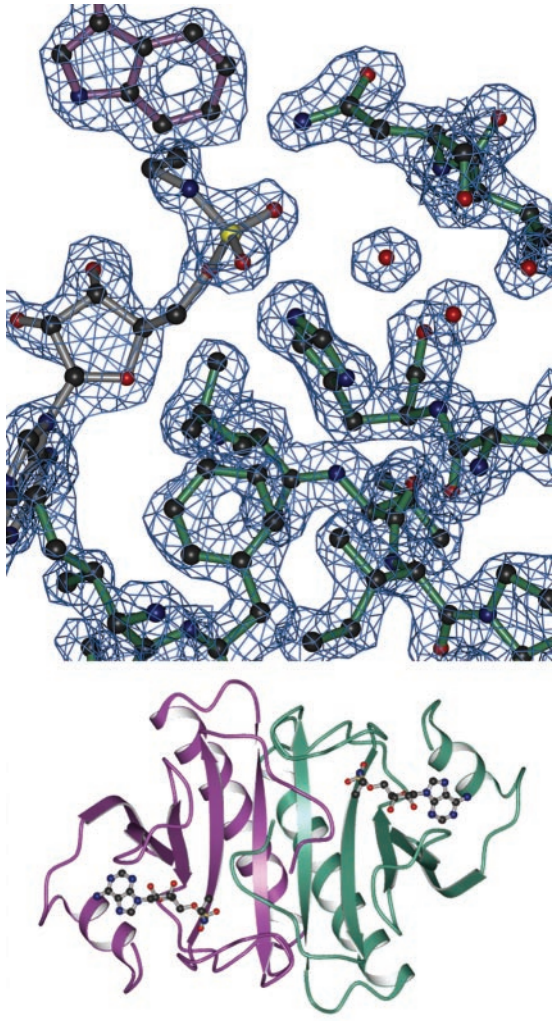


FIG. 2. **Hint-ethylsulfamoyl adenosine complex: electron density and overall structure.** One Hint monomer is depicted in *green*, the second Hint monomer in *purple* and the *N*-ethylsulfamoyl adenosine in *gray*. *Top*, representative $2F_o - F_c$ electron density, contoured at 1 σ and used to fit the coordinates. Note the position of Trp-123 in *purple* as proximal to the nucleotide-binding site of the *green* monomer. *Bottom*, the dimeric Hint-ethylsulfamoyl adenosine crystal structure. Ethylamino groups are visible protruding from the α-sulfates of the bound inhibitors in the Hint homodimer. Molecular graphics were generated as described (2).

room temperature at which time solvent was evaporated, and the residue was dissolved in a mixture of chloroform/methanol (50:1, v/v) and chromatographed on a silica gel column. The column was eluted with methanol in chloroform (2→10%). The appropriate fractions were combined and evaporated to give N^6,N^6,$O^{2'}$,$O^{2'}$-tetrabenzoyladenosine-5′-*O*-*p*-nitrophenylphosphoramidothioate (0.557 g, 62%), ^{31}P NMR (CD_3CN) δ: 49.66 and 49.93 ppm, FAB-MS *m/z* (M-1) 898. Third, a solution of N^6,N^6,$O^{2'}$,$O^{2'}$-tetrabenzoyladenosine-5′-*O*-*p*-nitrophenylphosphoramidothioate (0.449 g, 0.5 mmol) in acetonitrile (3 ml) was added a solution of oxone (Dupont Chemicals, 0.41 g, 0.65 mmol) in sodium citrate (pH 6.2, 7 ml). The reaction mixture was stirred for 16 h at room temperature. Aqueous Na_2SO_3 (1.0 ml) was then added, and a solid precipitate was removed by filtration. The filtrate was extracted with three 2-ml volumes of chloroform, and the solvent was removed from the filtrate. Residual solid was suspended in 10 ml of 20% aqueous ammonia and left for 24 h at room temperature in a tightly closed vial. Ammonia was then evaporated, and the residue was dissolved in a mixture of methanol and water (6:1, v/v) and purified on a Sephadex A-25 column, which was eluted with a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) from 0.05 to 0.5 M. The appropriate fractions were combined and evaporated to yield adenosine 5′-*O*-*p*-nitrophenylphosphoramidate (0.047 g, 20%), ^{31}P NMR (D_2O) δ: 0.76 ppm, FAB-MS *m/z* (M−1) 466.

AMP-*N*-ϵ-(*N*-α-Boc-lysinamide) was obtained in a three-step procedure. First, a reaction of *N*-α-Boc-lysine methyl ester with one molar equivalent of 2-chloro-1,3,2-oxathiaphospholane in the presence of pyridine and elemental sulfur gave *N*-ϵ-(2-thiono-1,3,2-oxathiaphospholanyl)-*N*-α-Boc-lysine methyl ester in 51% yield (^{31}P NMR (CD_3OD) δ: 97.04 and 96.99 ppm, FAB-MS *m/z* (M−1) 397). This compound in reaction with a molar equivalent of N^6,N^6,$O^{2'}$,$O^{2'}$-tetrabenzoyladenosine in acetonitrile solution, in the presence of DBU, provided N^6,N^6,$O^{2'}$,$O^{2'}$-tetrabenzoyladenosine-5′-*O*-(*N*-ϵ-(*N*-α-Boc-lysinamide))-phosphoramidothioate (^{31}P NMR (CD_3CN) δ: 59.62 and 59.45 ppm, FAB-MS *m/z* (M−1)1019), which subsequently under treatment with oxone followed by aqueous ammonia was converted into AMP-*N*-ϵ-(*N*-α-Boc-lysinamide) (yield 26%, ^{31}P NMR (D_2O) δ: 9.94 ppm, FAB-MS *m/z* (M−1) 573).

Synthesis of Inhibitors—5′-*O*-Sulfamoyladenosine was synthesized by modification of the methods of Shuman *et al*. (14). 2′,3′-*O*-Isopropylidene-5′-*O*-sulfamoyladenosine was first purified as follows. A mixture of 2′,3′-*O*-isopropylidene adenosine (3.00 g, 9.76 mmol) and hexabutyldistannoxide (11.50 g, 20 mmol) in anhydrous benzene was heated to reflux under argon for 2 h. The resulting clear solution was cooled to 5 °C, and a solution of sulfamoyl chloride (4.50 g, 39 mmol), synthesized as described (15), in dioxane (80 ml) was added dropwise. After stirring at room temperature for 30 min, the solvent was evaporated, and the residue was extracted with two 50-ml volumes of hexane. The insoluble residue was treated with a dilute solution of methanolic ammonia (50 ml), evaporated and purified by SiO_2 column chromatography using 9 volumes of chloroform to 1 volume of methanol as the mobile phase. The

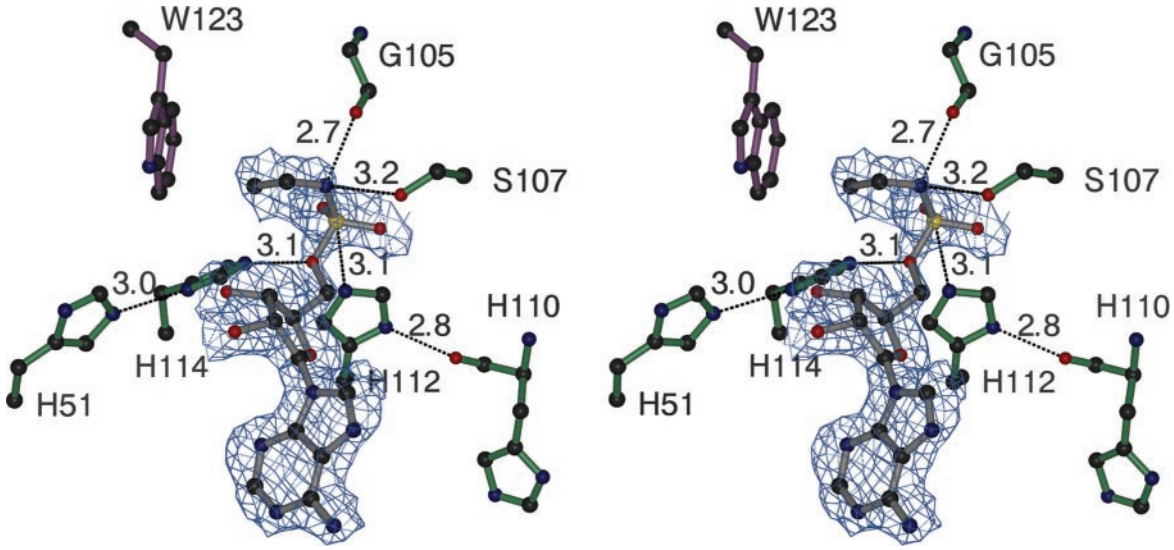


FIG. 3. **Active-site geometry of Hint.** Stereo view depicting the refined *N*-ethylsulfamoyl adenosine inhibitor inside 1.5 σ $F_o - F_c$ difference electron density calculated from protein and water coordinates alone. As in Fig. 2, the Hint monomer binding the inhibitor is in *green* and Trp-123 from the other monomer is in *purple*. Atomic distances (Å) are shown for the carbonyl oxygen of Gly-105 and the γ-oxygen of Ser-107 to the nitrogen of *N*-ethylsulfamoyl adenosine, the hydrogen bond network connecting His-51, His-114, and the 5′-oxygen of the inhibitor, and for the two interactions of the adenylylating His-112.

appropriate fractions were evaporated to give pure 2′,3′-*O*-isopropylidene-5′-*O*-sulfamoyl adenosine (2.27 g, 60%) as a white foam. ^{1}H NMR (Me_2SO-d_6) δ 1.31 (s, 3H, CH_3-isopropylidene), 1.50 (s, 3H, CH_3-isopropylidene), 4.20 (m, 2H, H-5′,5″), 4.36 (m, 1H, H-4′), 5.05 (dd, 1H, H-3′), 5.45 (dd, 1H, H-2′), 6.20 (d, 1H, H-1′, J_{1-2} = 2 Hz), 7.35 (br s, 2H, NH_2, D_2O-exchangeable), 7.60 (br s, 2H, SO_2-NH_2, D_2O exchangeable), 8.15 (s, 1H, H-2), 8.30 (s, 1H, H-8). 2′,3′-*O*-Isopropylidene-5′-*O*-sulfamoyl adenosine (386 mg, 0.10 mmol) was dissolved in a solution of aqueous trifluoroacetic acid (90%, 20 ml) and stirred at room temperature for 30 min. The solution was then evaporated to dryness, and the residue was dissolved in 15 ml of aqueous methanolic ammonia and evaporated to dryness leaving a white residue crystallized from water yielding pure 5′-*O*-sulfamoyl adenosine (246 mg, 72%) as white needles. ^{1}H NMR (Me_2SO-d_6) δ 4.25 (m, 4H, H-5′,5″, H-4′, and H-3′), 5.51 (m, 1H, H-2′), 5.45 (d, 1H, OH, D_2O-exchangeable), 5.65 (d, 1H, OH, D_2O-exchangeable) 5.90 (d, 1H, H-1′, J_{1-2} = 4 Hz), 7.30 (s, 2H, SO_2-NH_2, D_2O-exchangeable), 7.65 (s, 2H, NH_2-6, D_2O-exchangeable, H 8.15 (s, 1H, H-2), 8.30 (s, 1H, H-8). ESMS (*m/z*) 347 (M+1, 100%), 338 (10%), 308, 280, 250.

5′-*O*-Ethylsulfamoyl adenosine was synthesized as follows. Adenosine (3.26 g, 12.2 mmol) was dissolved in dry dimethylformamide (40 ml), heated to 50 °C, and the resulting solution was treated with excess imidazole (1.66 g, 24.4 mmol). Ethylsulfamoyl chloride (1.75 g, 12.2 mmol), purified by modification of the method of Weiss and Schultz (16), was added at 0 °C, and the solution was stirred for 30 min at room temperature. The resulting oil was dissolved in dichloromethane and methanol (9:1), then solvent-evaporated, and the oily product was decanted from a solid residue, which was twice extracted with dichloromethane. The crude oily product crystallized on standing. The dichloromethane extracts and the solid product were dissolved in methanol, the solvent was evaporated, and the residue was taken up in dichloromethane and methanol (9:1) for silica chromatography, eluting with a gradient of methanol (10–20%) in dichloromethane. Appropriate fractions were identified by TLC, pooled, and evaporated to give 5′-*O*-ethylsulfamoyladenosine as a white solid, further purified by reverse phase HPLC. ^{1}H NMR (Me_2SO) δ 0.95 (t, 3H, Me), 2.85 (q, 2H, CH_2), 4.10–4.25 (m, 4H, H-3′,4′,5′,5″), 4.65 (dd, 1H, H-2′), 5.45 (d, 1H, OH, D_2O-exchangeable), 5.65 (d, 1H, OH, D_2O-exchangeable), 5.90 (d, 1H, H-1′, J_{1-2} = 4 Hz), 7.30 (bs, 2H, NH_2-6, D_2O-exchangeable), 7.85 (bs, 1H, SO_2NH, D_2O-exchangeable), 8.15 (s, 1H, H-2), 8.35 (s, 1H, H-8). HRMS (FAB) found 375.1072, calculated for $C_{12}H_{19}N_6O_6S$ 375.1087. Substrates and inhibitors are shown in Fig. 1.

Mutagenesis and Enzymology—Wild-type rabbit Hint was expressed and purified in bacteria using pSGA02-*HINT* as described (3). The S107A mutant of rabbit Hint was generated by site-directed mutagenesis of the wild-type expression vector using primer 7129 (5′-ATGAACGTGATAGACGGCCTGTCCACCATCGGA) to generate plasmid pB415, which was used to produce homogeneous mutant Hint enzyme as above. AMP-*p*NA substrate at concentration of 1 mM was incubated with homogeneous rabbit Hint enzymes in reactions at 30 °C containing 20 mM Na-HEPES, pH 7.2, and 0.5 mM $MgCl_2$. Reaction samples were spotted on silica TLC plates (Merck). Plates were developed in 2-propanol:NH_4OH:1,4-dioxane:H_2O (50:35:8:7). Developed plates were imaged by epi-UV illumination on a Bio-Rad Fluor S instrument. Initial rate assays for AMP-*p*NA were performed in spectrophotometric cuvettes. Premixes containing AMP-*p*NA (50, 100, 300, 400, or 700 μM), 20 mM Na-HEPES, pH 7.2, and 0.5 mM $MgCl_2$ were equilibrated at 30 °C and then reactions were initiated with addition of 97.5–239.3 pmol of rabbit Hint. To determine kinetic parameters for AMP-*N*-ε-(*N*-α-Boc-lysinamide), initial rate reactions were completed in the same buffer at 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, and 20 μM concentration of substrate, and the products were analyzed by HPLC on a HQ column (Applied Biosystems) with NH_4HCO_3, pH 8, as the mobile phase. The pH dependence of k_{cat}/K_m was determined at six pH values (5, 5.5, 6, 6.5, 7, and 7.25) with four or five concentrations of AMP-*p*NA (from 12 to 400 μM) for the wild-type and mutant enzyme. These reactions were performed in 66 mM sodium/potassium phosphate buffers with 0.5 mM $MgCl_2$ using 964 pmol of wild-type Hint, or 1776 pmol of S107A mutant Hint.

K_i values for sulfamoyl and N-ethylsulfamoyl adenosine were obtained by titrating the inhibitors (0, 5, 10, 40, 60, and 100 μM) into complete digests of 100 μM AMP-*p*NA. Using a known amount of enzyme (96 pmol), first order decay curves for remaining substrate were used to calculate k_{cat}/K_m and the inhibitor concentration dependence of reduction of k_{cat}/K_m (apparent) was calculated as described (12).

X-ray Crystallography—Rabbit Hint crystals were grown as described (2). Crystals were soaked in a well solution saturated with either sulfamoyl or *N*-ethylsulfamoyl adenosine for 2 h. Cryoprotectant solutions were prepared from the well solution containing 13% glycerol and saturated with the inhibitor. Crystals were scooped from the soak solution and briefly submerged in cryoprotectant solution just prior to freezing in liquid nitrogen. Hint-sulfamoyl adenosine data were collected in the cryostream at −180 °C at the X-25 beamline of the National Sychrotron Light Source at Brookhaven National Laboratory. Hint-ethylsulfamoyl adenosine data were collected on the Kimmel Cancer Center home source. We employed CNS (17) for model refinement and calculation of electron density maps. Model building was carried out using O (18). Collection and refinement statistics are provided in Table II. Coordinates and structure factors have been deposited with the Research Collaboratory for Structural Bioinformatics Protein Data Bank.

RESULTS AND DISCUSSION

Biochemical Evidence for an Alkylamine-binding Site in Rabbit Hint—To study the enzymatic activity of the Hint AMP-lysine hydrolase, we synthesized AMP-*p*NA (Fig. 1). To test whether Hint would tolerate *p*NA as a leaving group, we incubated Hint with AMP-*p*NA and analyzed the progress of the

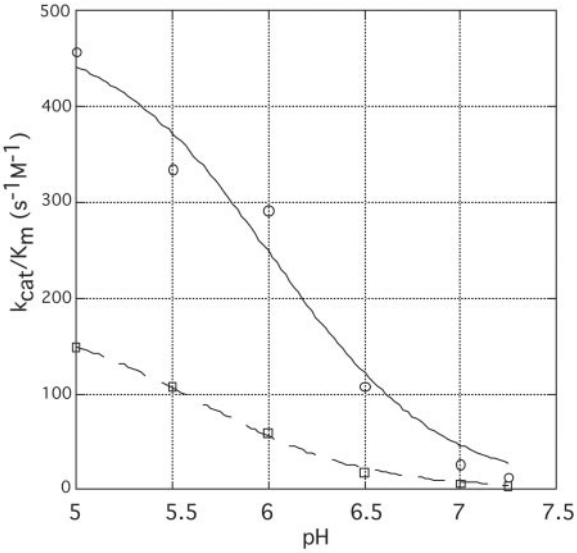


FIG. 4. **pH-dependence of wild-type (*o*) and S107A (□) forms of Hint.** Enzyme specificity constants (k_{cat}/K_m) with AMP-*p*NA were plotted as a function of pH and fitted to k_{cat}/K_m (observed) = (k_{cat}/K_m (protonated))10 (pK_a − pH)/(1 + 10 (pK_a − pH)).

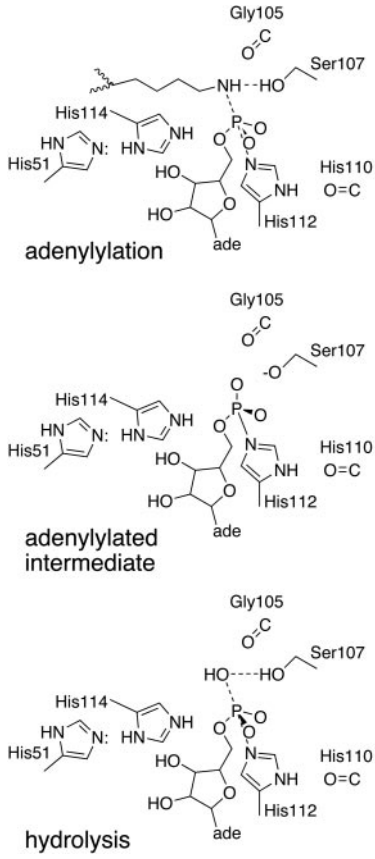


FIG. 5. **Proposed catalytic mechanism for Hint hydrolases.** In the adenylylation step, we propose that Ser-107 donates a proton to the leaving group such that the adenylylated intermediate, with inverted configuration at phosphorus, contains a serine alkoxide ion. The serine alkoxide would then abstract a proton from the attacking water to hydrolyze the adenylylated intermediate, returning the phosphorus to its original configuration. As shown in Fig. 3, the reaction geometry is supported by additional hydrogen bonds. In particular, the leaving group and hydrolytic geometry is thought to be enforced by the carbonyl group of Gly-105. Note that this reaction would proceed in an identical manner with a S107A enzyme containing a water molecule bound in place of the γ-hydroxyl group.

reaction by TLC and HPLC. Both assays made it clear that Hint cleaves this molecule generating one equivalent of AMP and *p*NA (data not shown). Release of the chromophore allowed the new substrate to be followed spectroscopically at 410 nm. To compare AMP-*p*NA with a more biologically authentic substrate, AMP-*N*-ϵ-(*N*-α-Boc-lysinamide) was prepared and assayed by HPLC. As shown in Table I, consistent with rabbit Hint as an AMP-lysine hydrolase, the enzyme liberated AMP from AMP-*N*-ϵ-(*N*-Boc-lysinamide) with a k_{cat} of 0.23 s^{-1} and a K_m of 470 nm. In contrast, the convenience of the continuously and spectroscopically monitored substrate came at the cost of a 280-fold higher K_m and about 100-fold lower k_{cat} presumably because the enzyme tolerates the bulky leaving group very poorly. As the hydrolytic step for both of these substrates is expected to proceed identically through adenylylated His-112 (1), the *p*NA leaving group is expected to inhibit adenylylation, *i.e.* the productive covalent attack of the α-phosphate by His-112.

Despite the relatively poor kinetic values, the AMP-*p*NA substrate is reasonably suited for assays of competitive inhibitors and determination of their K_i values with methods we established for GpppBODIPY and Fhit (12). We synthesized new Hint inhibitors consisting of adenosine and 5′-sulfamoyl or *N*-ethylsulfamoyl groups (Fig. 1). Both were titrated into AMP-*p*NA assays and competitive K_i values were obtained by calculating the inhibitor concentration-dependence in reduction of k_{cat}/K_m (apparent) for substrate hydrolysis (12). As shown in Table I, the sulfamoyl adenosine inhibited Hint with a K_i value of 16.1 μM, whereas addition of the ethyl group to sulfamoyl adenosine resulted in an inhibitor with a K_i value of 1.25 μM. The 13-fold advantage in equilibrium binding conferred by an addition of the ethyl group suggests a favorable interaction with an alkylamine leaving group such as a lysine or protein-lysine.

Crystallographic Identification of the Alkylamine-binding Site in rabbit Hint—Previously, the most informative crystal structures of Hint have been bound to GMP, 8-Br-AMP (2), and adenosine tungstate (19). The GMP and 8-Br-AMP crystal structures can be considered product complexes that represent the form of the enzyme bound to a nucleoside monophosphate following hydrolysis of a putative nucleoside monophosphoramidate substrate, whereas the adenosine tungstate structure can be considered to be an analog of the transition state. An additional crystal structure of human Hint bound to adenosine α,β-methylenediphosphonate was described as an analog of a substrate complex (19), but as ADP is an extremely poor substrate of Hint (3), it is not clear that this complex can serve as a model of a good enzyme-substrate complex.

To examine active site geometry with amine-containing nucleotides, we soaked rabbit Hint crystals (2) with the two inhibitors synthesized in this study. Both soaks resulted in crystals that diffracted to 2.0 Å or higher resolution. When the difference electron density of the sulfamoyl adenosine-soaked crystal was examined, we could easily identify the adenosine density and place the sulfur atom in the position of the 5′-phosphorus but we could not interpret which two of the sulfur ligands were oxygen and which one was the amino group (not shown). In contrast, the ethyl group of *N*-ethylsulfamoyl adenosine allowed unequivocal assignment of every nonhydrogen atom at 1.8 Å resolution. Because this inhibitor was shown to possess a 13-fold binding advantage over the sulfamoyl adenosine, we suggest that the alkyl portion of this inhibitor is bound in a substrate-analogous fashion. Data collection and refinement statistics for this crystal structure are provided in Table II. Representative electron density and the overall structure of the Hint dimer bound to *N*-ethylsulfamoyl adenosine

are shown in Fig. 2. Difference electron density for the inhibitor is shown in Fig. 3.

As observed earlier in crystal structures of rabbit Hint bound to GMP and 8-Br-AMP (2), the most highly conserved amino acids in Hint homologs and, indeed among all histidine triad enzymes, surround the purine base, the ribose, and the 5′-phosphate groups. Recently, we observed that an additional amino acid is highly conserved in Hint hydrolases. The amino acid was missed in our initial analysis because one monomer is not in contact with the nucleotide bound in that monomer but rather makes a close approach to the nucleotide across the dimer interface (9). This amino acid is one of a remarkable 15 residues that are sexually dimorphic in avian Hint-related genes; it is Trp-123 in the typical Hint polypeptide encoded on the Z chromosome, and it is Gln on the unusual and potentially inhibitory Asw polypeptide that is encoded in a tandem array of ~40 repeats on the female-specific W chromosome (9). The present biochemical studies establish a binding site for the alkyl portion of the alkylamine leaving group of Hint substrates. As shown in Figs. 2 and 3, that site is located across the Hint dimer interface against Trp-123.

Evidence That Ser-107 Is the Acid-Base Catalyst for Hint—Earlier we argued that for Hint hydrolases, whose phosphoramidate substrates necessarily have an amine leaving group, protonation of the phosphoramidate nitrogen may be an important kinetic component of the adenylylation step in catalysis (1). The crystal structure of Hint bound to *N*-ethylsulfamoyl adenosine allowed us to analyze how conserved features of Hint hydrolases interact with an analog of the 5′-phosphoramidate nitrogen, equatorial oxygens, and the alkylamine leaving group.

Although we considered His-114 to be the candidate hydrogen bond donor (1) extrapolating from product complexes (2), this structure suggests that the Ser-107 hydroxyl is positioned for proton donation with alignment that is assisted by the carbonyl oxygen of Gly-105. In the present structure the ϵ-nitrogen of His-114 is 3.1 Å from the ribose 5′-oxygen and 3.3 Å from one of the equatorial oxygen atoms of α-sulfur and not in hydrogen-bonding distance or orientation with the sulfamoyl nitrogen. In contrast, the sulfamoyl nitrogen is 2.7 Å from the carbonyl oxygen of Gly-105 and 3.2 Å from the Ser-107 γ-oxygen. Thus, we propose that Ser-107 donates a hydrogen bond to the phosphoramidate nitrogen of a Hint substrate.

To test the hypothesis that Ser-107 plays a role in catalysis, we performed a site-directed alteration of this residue to Ala. Rabbit Hint-S107A was expressed and purified in *Escherichia coli* and characterized with respect to AMP-*p*NA hydrolysis. The pH-dependence of k_{cat}/K_m was determined at six pH values for the wild-type and mutant enzymes. When, as shown in Fig. 4, the specificity constant, k_{cat}/K_m was plotted as a function of pH, we were able to model Hint enzymatic activity on AMP-*p*NA as being controlled by a single titratable group with a pK_a of 6.0 ± 0.1 and maximal activity in the protonated form. The S107A mutant, in the same assays, exhibited ~3-fold reduced activity at all pH values with little alteration (a 0.4 unit drop) in pK_a. Given the position of the Ser-107 hydroxyl, the reduced activity of this mutant was expected, although one might have expected the magnitude of the defect to be greater than 3-fold. To test whether a larger catalytic defect of the S107A mutant was masked by the AMP-*p*NA substrate, we determined k_{cat} and K_m for AMP-*N*-ϵ-(*N*-Boc-lysinamide). Although the K_m of the mutant was unaffected, the k_{cat} was depressed by 4-fold.

In the absence of the Ser-107 hydroxyl, Hint clearly has the ability to adenylylate and hydrolyze substrates at a reduced rate. We hypothesize that the carbonyl oxygen of Gly-105 assists the function of Ser-107 as the acid-base catalyst for Hint. In the absence of Ser-107, we propose that a water molecule bound to the S107A enzyme functions as the acid-base catalyst and that the cost of loss of the protein-positioned hydroxyl group is the observed 3–4-fold-reduced activity of the mutant enzyme. In GalT, a member of the distinctive branch three-histidine triad nucleoside monophosphate transferases (1), the corresponding Ser-161 also functions on the leaving group side (20). However, rather than donating a hydrogen bond to the α-β-bridging oxygen in a UDP-hexose substrate, which would be analogous to donating a hydrogen bond to the phosphoramidate nitrogen in a Hint substrate, Ser-161 donates to a β-phosphate equatorial oxygen (20).

Conclusions—We have used organic synthesis, enzymology, x-ray crystallography, and mutagenesis to develop novel substrates and inhibitors of the Hint adenosine monophosphoramidate hydrolase. Consistent with our view that adenylylated lysine residues are natural substrates, this work establishes that Hint kinetics are superior with an alkylamine substrate such as AMP-*N*-ϵ-(*N*-α-Boc-lysinamide) *versus* AMP-*p*NA and that the ethyl group of an ethylsulfamoyl adenosine inhibitor confers a 13-fold binding advantage over the nonderivatized sulfamoyl adenosine. The crystal structure of the *N*-ethylsulfamoyl adenosine inhibitor identified the location of the alkylamine-binding site against conserved residue Trp-123, which interacts with the inhibitor across the dimer interface and also identified Ser-107 as the candidate acid-base catalyst for the enzyme. pH titration of wild-type and mutant enzymes establishes that Hint functions with a protonated titratable group and that Ser-107 may donate and receive back a proton in a manner that can be substituted by bound water in adenosine monophosphoramidate hydrolysis. Our proposed catalytic mechanism for Hint hydrolases is provided in Fig. 5.

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