Histidine Triad Nucleotide-binding Protein 1 (HINT-1) Phosphoramidase Transforms Nucleoside 5'-O-Phosphorothioates to Nucleoside 5'-O-Phosphates

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Nucleoside 5'-O-phosphorothioates are formed in vivo as primary products of hydrolysis of oligo(nucleoside phosphorothioate)s (PS-oligos) that are applied as antisense therapeutic molecules. The biodistribution of PS-oligos and their pharmacokinetics have been widely reported, but little is known about their subsequent decay inside the organism. We suggest that the enzyme responsible for nucleoside 5'-O-monophosphorothioate ((d)NMPS) metabolism could be histidine triad nucletotide-binding protein 1 (Hint-1), a phosphoramidase belonging to the histidine triad (HIT) superfamily that is present in all forms of life. An additional, but usually ignored, activity of Hint-1 is its ability to catalyze the conversion of adenosine 5'-O-monophosphorothioate (AMPS) to 5'-O-monophosphate (AMP). By mutagenetic and biochemical studies, we defined the active site of Hint-1 and the kinetic parameters of the desulfuration reaction (P-S bond cleavage). Additionally, crystallographic analysis (resolution from 1.08 to 1.37 Å) of three engineered cysteine mutants showed the high similarity of their structures, which were not very different from the structure of WT Hint-1. Moreover, we found that not only AMPS but also other ribonucleoside and 2'-deoxyribonucleoside phosphorothioates are desulfurated by Hint-1 at the following relative rates: GMPS > AMPS > dGMPS ≥ CMPS > UMP > dAMP > dCMP > dTMP, and during the reaction, hydrogen sulfide, which is thought to be the third gaseous mediator, was released.

5'-O-Phosphorothioates of ribonucleosides (NMPS) (1, R1 = OH, X = S) or 2'-deoxyribonucleoside phosphorothioates (dNMPS, 1, R1 = H, X = S) are sulfur-substituted, isoelectric analogs of 5'-O-phosphates of natural nucleosides (NMP, 1, R1 = OH, X = O) or 2'-deoxyribonucleosides (dNMP, 1, R1 = H, X = O), respectively (Fig. 1).

NMP's and dNMP's (together denoted (d)NMPS) are formed during the enzymatic hydrolysis of oligo(nucleoside phosphorothioate) (PS-oligos) that contain a sulfur atom attached in non-bridging positions to the phosphorus atom at each or selected internucleotide bond(s). Synthetic PS-oligos have been developed as antisense probes for genomic research and medicinal applications (1, 2). These oligonucleotides are promising therapeutic molecules because they are much more stable against nucleolytic degradation in blood and various cellular systems than their natural, unmodified counterparts (3–5). Their hydrolysis in plasma, kidney, and liver proceeds mainly from the 3' end, resulting in the appearance of the mononucleoside 5'-phosphorothioates identified in urine from PS-oligo-injected animals (6, 7). (d)NMPS may exert cytotoxic effects affecting cell proliferation, DNA or RNA synthesis, and other unknown processes (8, 9). Recently, the phosphorothioate DNA segments have been identified in bacterial DNA (10), which makes investigations into PS-oligo metabolism even more important.

Although several reports have been published on the biodistribution of PS-oligos, little is known about the metabolism of the products of their degradation in vivo. It has been suggested that extracellular dNMPS and dNMP can be converted to the corresponding nucleoside by 5'-nucleotidase (ecto-5'-NT) (9). This membrane-bound enzyme preferably releases adenine from extracellular AMP, but other purine and pyrimidine 5'-nucleotides are also dephosphorylated by this protein (11). Alkaline phosphatase has also been shown to be able to hydrolyze O-phosphorothioate monoesters, although much more slowly than their phosphate analogs (12, 13). It is possible that nucleoside phosphorothioates are also substrates for this enzyme.

Other classes of compounds that can be transformed to (d)NMPS and (d)NMPS in vivo are nucleoside phosphorami- dates (2, Fig. 1, X = O) and phosphoramidothioates (2, X = S) carrying an N-alkyl residue, most often derived from amino acids (14–16). In the prodrug approach, such molecules are converted intracellularly to the corresponding nucleoside 5'-monophosphates (17). It is believed that the enzyme responsible for hydrolysis of the P-N bond in both classes of nucleotide phosphorami- dates is histidine triad nucleotide-binding protein 1 (Hint-1) (14, 18). The Hint-1 protein is a member of the histidine triad (HIT) protein superfamily. The family
amp-toward several synthetic phosphoramidate substrates, such as (19). Interestingly, Hint-1 exerts its phosphoramidase activity chemistry of rabbit Hint-1-assisted hydrolysis of the reoisomers of adenosine 5-phoramidothioate (AMPS-Trp, AMP-(H114D and H114N) and compared their activities toward C84A), a serine mutant (S107A), and two histidine mutants neered three cysteine mutants (C38A, C84A, and C38A/HIT motif (His-114 for rHint-1). For that purpose, we engi-nective enzyme-assisted removal of the sulfur atom from AMPS, ess of the P-N bond cleavage in AMPS-Trp was followed by ef-tryptophanyl residue), we observed that the stereoretentive proc-FIGURE 1. Structure of ribonucleoside 5'-O-phosphates (1, R1 = OH, X = O), 5'-O-phosphorothioates (1, R1 = OH, X = S), ribonucleoside 5'-O-phosphoramidates (2, R1 = OH, X = O), ribonucleoside 5'-O-phosphoramidothioates (2, R1 = OH, X = S), and deoxyribonucleoside 5'-O-phosphoramide (2, R1 = H, X = O), and ribonucleoside 5'-O-phosphoramidothioates (2, R1 = H, X = S).members act as hydrolases and transferases and contain an amino acid sequence motif, HXHXXHXXX (H, histidine resi-du, X, hydrophobic amino acid residue), at their C termini (19). Interestingly, Hint-1 exerts its phosphoramidase activity toward several synthetic phosphoramidate substrates, such as AMP-N-ε-lysine, AMP-N-alanine, AMP-N-morpholine, and adenosine 5'-phosphoramidate (AMP-NH2) (20). Although the cellular function and biochemical relevance of that activity remains unknown, it has been suggested that mammalian Hint-1 has some tumor suppressor activity (21–23) and is involved in regulating apoptosis (24).

Crystallographic studies on human and rabbit Hint-1 revealed that the HIT motif is involved in nucleotide substrate binding and hydrolysis (25, 26). The phosphoramidase activity of Hint-1 strongly depends on the presence of the conserved middle histidine of the histidine triad motif (His-112 for rHint-1) (20). In contrast to earlier suggestions, Ser-107 but not His-114 acts as the acid-base catalyst (27).

Another activity of Hint-1, the ability to hydrolyze adeno-sine 5'-O-phosphorothioate (AMPS), was initially described by Bieganowski et al. (20). During our studies on the stereochemistry of rabbit Hint-1-assisted hydrolysis of the D-piaste-reoisomers of adenosine 5'-O-[N-(trytophanylamide)phosphoramidothioate (AMPS-Trp, 2, Fig. 2, X = S, RNH = tryptophanyl residue), we observed that the stereoretentive pro cess of the P-N bond cleavage in AMPS-Trp was followed by ef-fective enzyme-assisted removal of the sulfur atom from AMPS, resulting in the formation of AMP (18).

In this report, we describe mutagenetic and biochemical studies executed to define the substrates, active site, and ki-netic parameters of the P-S bond cleavage catalyzed by Hint-1, which is the putative enzyme responsible for (d)NMPS metabolism. In the catalysis reaction, we analyzed the participation of two cysteine residues (Cys-38 and Cys-34 for rHint-1), Ser-107 and the conserved third histidine of the HIT motif (His-114 for rHint-1). For that purpose, we engineered three cysteine mutants (C38A, C84A, and C38A/HIT motif (His-114 for rHint-1). For that purpose, we engineered three cysteine mutants (C38A, C84A, and C38A/HIT motif (His-114 for rHint-1). For that purpose, we engineered three cysteine mutants (C38A, C84A, and C38A/C84A), a serine mutant (S107A), and two histidine mutants (H114D and H114N) and compared their activities toward AMP-N-ε-lysine (a substrate for the Hint-1 phosphoramidase P-N bond cleavage activity) and AMPS (a substrate for the Hint-1 desulfurase P-S bond cleavage activity). Additionally, due to differences in their enzymatic activities, the crystal structures of the three cysteine mutants were compared. Moreover, the analysis of Hint-1 activity toward new substrates indicated that not only AMPS but also GMPS, CMPS, UMP, and corresponding phosphorothioate 2'-deoxyribo-nucleosides are desulfurated by Hint-1.

EXPERIMENTAL PROCEDURES

Synthesis—AMPS (adenosine 5'-O-phosphorothioate), AMP-N-ε-lysine (AMP-N-ε-(N-α-Boc-lysinamide), and other tested compounds were synthesized as described elsewhere (28).

Mutagenesis and Purification of Proteins—Mutants of rab-bit Hint-1 were generated by site-directed mutagenesis of the WT protein expression vector pSGA02-HINT (20) using the QuiChange Site-directed Mutagenesis kit (Stratagene) and appropriate pair of primers (LP and RP): C38A-LP, 5'-CGAG-GATGACCCAGGCCGCTTCTCTTTTCTATGACTTT-CCTCCAAGC; C38A-RP, 5'-GCAAGGGGAAAATGCTA-TGGAAAAAGCAAGCGCCTGTGCTACCCAG; C84A-LP, 5'-GCTCCCCTAGGTGGCAAGACGAAATTCTGC-TAAAGGGGAGATTCCG; C84A-RP, 5'-CGAATGCCCGTTTTCTTTCGAGTGACATGAGGCC; H114D-LP, 5'-GTGCCGGTATCACGTTTCATCAACGTTCGTTTG-GGG; H114D-RP, 5'-CAAGGAAGGATGGTGGCGTAAACGC-TGATACCGGAC; H114N-LP, 5'-GTGCGGTGTTACGCGCGTTCCTATGACAGCGTTC-GGG; and H114N-RP, 5'-CCCAAGAACATCGAGATGACCTACCGACCGGAC.

To generate the C38A/C84A double mutant of rabbit Hint-1, site-directed mutagenesis was performed with primers, C84A on the C38A mutant, as described above. The sequences of all obtained plasmid constructs were confirmed by DNA sequencing (Institute of Biochemistry and Biophysics PAS, Warsaw, Poland).

The proteins were expressed from plasmids pSGA02-HINT, pSGA02-C38A, pSGA02-C84A, pSGA02-H114D, pSGA02-H114N, and pB415-S107A (18) in the Escherichia coli BL-21* strain and purified according to a published procedure (20) using AMP-agarose (Sigma) affinity chromatography. Homogenous enzyme preparations were dialyzed against a buffer containing 20 mm Tris (pH 7.5) and 150 mm NaCl, concentrated, and stored at −80 °C. The purity of WT and all generated mutants was assessed by SDS-PAGE (4–20% Precise Protein Gel; ThermoScientific), followed by staining with the PAGE Blue Protein Staining Solution (Fermentas, Lithuania) (Fig. 2).

Enzymatic Assays—For the enzymatic digestion, solutions of substrates at 200 μM (AMP-N-ε-lysine, NMPS, and dNMPS) or 1 mm (dCMPs, TMPS, AMPS(OMe), and dinucletide (dApsA)) were prepared in 20 mm Na-HEPES buffer (pH 7.2) or 66 mm sodium/potassium phosphate buffer (pH 7.2) containing 0.5 mm MgCl2 and the enzyme (0.1–2.0 μg) in a 10-μ1 total volume. The samples were incubated for 0.1–24 h at 30 °C. The reaction mixtures were quenched by cooling on ice and analyzed by RP-HPLC on a BDS Hypersil C18 col umn (5 μm, 250 × 4.6 mm; Thermo Electron Corporation) with a mobile phase, a gradient of acetonitrile in 0.1 m triethylammonium bicarbonate (pH 7.4) from 0 to 20% over 24 min at a flow rate of 1 ml/min. The specific activity of the Hint-1 enzyme was calculated from the reactions in which hydrolysis
did not exceed 10%. Each experimental point represents the mean ± S.E. from measurements performed in triplicate.

Kinetic Assay—To determine the kinetic parameters of the AMPS desulfuration by rHint-1, the initial rate assays were completed in a buffer containing 0.5 mM MgCl₂ and 20 mM Na-HEPES (pH 7.2) at 8 substrate concentrations, ranging from 50 μM to 1 mM. The products were analyzed by RP-HPLC as described above. To calculate \( K_m \) and \( k_{cat} \) values, Lineweaver-Burk plots were used.

Circular Dichroism Measurements—The circular dichroism (CD) spectra were recorded on a CD6 dichrograph (Jobin-Yvon, Longjumeau, France) using cuvettes with a 0.1-mm path length, 2 nm bandwidth, and 1 to 2-s integration time. The protein samples (1 mg, molecular mass of 13,693 Da, 126 residues) were dissolved in 0.4 ml of buffer containing 20 mM Tris (pH 7.5) and 150 mM NaCl. The spectra were recorded in a range of 200 to 350 nm at 25 °C and smoothed with a 25-point algorithm included in the manufacturer’s software, version 2.2.1. The data in Table 1 were calculated using the algorithm K2D (a web server Computational Biology Group of European Molecular Biology Laboratory, Heidelberg, Germany), which uses the experimental CD spectrum obtained as described above.

Crystallization and X-ray Data Collection—C38A, C84A, and C38A/C84A rHint-1 mutants were crystallized as described for WT Hint-1 (25, 26) by the vapor diffusion method in a hanging drop variant at 5 °C using 10 mg/ml of protein solution and a precipitant solution containing 100 mM sodium cacodylate buffer (pH 6.5), 20 to 30% (w/v) PEG 8000, both with or without the addition of 100 mM sodium acetate. Crystallization drops were set up by mixing 2 μl of the protein solution with 2 μl of the precipitant solution and suspended over precipitant solution (1 ml). Crystals of typical dimensions (0.1 × 0.2 × 0.6 mm) and rhombic shape appeared after 48 to 72 h.

Crystals of rHint-1 cysteine mutants were flash-frozen by transferring them into 25% (v/v) PEG 400 (used as a cryoprotectant) followed by freezing in a nitrogen stream at −173 °C. The diffraction data were collected using synchrotron radiation with a Rayonics MX-220 CCD detector on beamline MX-14-1 at BESSY, Berlin, Germany (C38A, C38A/C84A) and with an MAR CCD 165-mm detector on beamline I911-2 at the MAX-Lab Synchrotron, Lund, Sweden (C84A). The data were processed, integrated, and scaled with MOSFLM (29) and SCALA (30) from the CCP4 program package (31) or with DENZO and SCALEPACK from the HKL2000 program package (32). The data collection and processing statistics are given in Table 3.

Structure Determination, Refinement, and Analysis—All datasets were analyzed using programs from the CCP4 package. The structures were solved by the molecular replacement method using the program MOLREP (33). A search model for rHint-1 cysteine mutants was the structure of wild-type rHint-1 solved previously at 1.1-Å resolution (Protein Data Bank code 3LLJ). One monomer was identified and placed in the asymmetric unit of the crystal in each of the datasets. The structures were completed using alternate cycles of manual building, including main and side chain rebuilding, adding alternative residue conformations, loop fragments, and solvent molecules in COOT (34) and refinement in REFMACS (35). All refinement steps were monitored using \( R \) and \( R_{free} \) values. The quality of each model was judged using the program PROCHECK (36). Values of mean temperature factors for the protein main and side chains as well as water molecules were calculated using the program BVERAGE from the CCP4 program suite. Protein structures were compared using the program LSQKAB (37). Visualization of electrostatic potential surfaces was generated using PyMol (38).

RESULTS AND DISCUSSION

H₂S Evolution from the AMPS to AMP Transformation Reaction—The Hint-1-assisted removal of the sulfur atom from AMPS resulted in the formation of AMP (18) and release of H₂S into the reaction mixture; the characteristic rotten egg smell was sensed organoleptically (Fig. 3). The evolution of H₂S to the reaction mixture containing rHint-1 and AMPS was confirmed by a highly sensitive (10 nmol) analytical reaction with AgNO₃, leading to the formation of a black deposit of Ag₂S. In an independent experiment, HEPES buffer, used in the AMPS to AMP transformation reaction, was excluded as a source of sulfur. Therefore, one could conclude that the produced H₂S carried the sulfur atom originally present in the 5’-O-phosphorothioate moiety.

Recent studies suggest that hydrogen sulfide (H₂S) is the third gaseous mediator, in addition to nitric oxide (NO) and carbon monoxide (CO), in mammalian cells (39). In most tissues, as well as in plasma, the physiological concentration of H₂S is about 50 μM, but it can be three times higher in the brain (40). Notably, the toxic concentration is only two times higher than the physiological concentration. H₂S is involved in the regulation of many physiological processes, and its defi-
ciency as well as its excess can cause physiological and even pathological effects (39, 41, 42).

It is a widely accepted opinion that the P-S bond in phosphorothioate monoesters and diesters is quite stable under physiological conditions. In principle, the P-S bond in (d)NMPS can be converted into the P-O bond in (d)NMP by the following reactions: 1) oxidation with the participation of an R-SH group to form a disulfide P-S–S-R moiety, followed by hydrolysis; 2) Lewis acid-assisted hydrolysis; 3) alkylation-promoted hydrolysis; or 4) radical oxidation. For enzymatic conversion, the first two mechanisms should be considered more plausible. A sulfhydryl group of a cysteine residue may be involved in the initial oxidation, whereas the positively charged or proton-donating amino acid residues (e.g. Arg, Lys, and Ser) may act as the Lewis acid. The two other mechanisms seem to be much less likely, yet not impossible. Moreover, there are known enzymes (e.g. cysteine desulfurase) that catalyze the conversion of L-cysteine to L-alanine and sulfide via the formation of a protein-bound cysteine per sulfide intermediate on a conserved cysteine residue (43, 44). They are engaged in the biogenesis of iron-sulfur proteins. Due to their intrinsic redox properties, proteins with Fe/S clusters are ubiquitous in biological electron transport systems.

Cysteine Mutant Proteins: CD Spectra and Activity toward AMP-N-e-lysine and AMPS—To determine whether the desulfuration process occurs via the formation of a disulfide intermediate (path 1) with the participation of the sulfur atom of any of two cysteine residues present in the polypeptide chain of rHint-1, two single mutants, C38A and C84A, and one double mutant, C38A/C84A, were engineered. Although CD measurements showed that both single mutants had remarkably altered global secondary structures (the molar ellipticity decrease by ∼20% in the 200 to 240 nm region of the spectrum) as compared with WT rHint-1 (Table 1, Fig. 4), their activities remained virtually unchanged. The recombinant proteins exerted phosphoramidase activity toward AMP-N-e-lysine and converted AMPS to AMP at rates comparable with those of the WT rHint-1 (Table 2).

The data in Table 1 were calculated using the K2D algorithm (see “Experimental Procedures”), which deconvolutes the CD data. The results did not show any participation of the β-sheet in the rHint-1 structure. This finding is not compatible with the data derived from the crystal structure of the rHint-1 protein, where the presence of an antiparallel 10-stranded β-sheet has been demonstrated (26). Unfortunately, our samples could not be analyzed in a wavelength range of 185 to 200 nm, preventing the use of more accurate algorithms (e.g. Fasman-Johnson) for the secondary structure calculations of these proteins.

Designed by CD analysis, the secondary structure of the double mutant C38A/C84A was not very different from the

![Figure 4. CD spectra (200–250 nm) for WT rHint-1 and rHint-1 mutants. The protein samples (1 mg) were dissolved in buffer containing 20 mM Tris (pH 7.5) and 150 mM NaCl. Data were collected at 25 °C.](image-url)
structures of the single mutants (Table 1, Fig. 4). Notably, it retained its activities for both substrates (AMPS and AMP-N-ε-lysine), albeit at levels about 13 and 7 times lower, respectively, than those of WT rHint-1 and both single cysteine mutants (Table 2). However, this reduced activity is obscure because of small differences in the predicted structures of the three cysteine mutants designated on the base of the CD spectra.

**Crystallographic data and refinement statistics**

**TABLE 3**

| Protein | AMP-N-ε-lysine | AMPS |
|---------|----------------|------|
| WT rHint-1 | 1.388 ± 0.321 | 0.202 ± 0.0067 |
| Hint C38A | 1.107 ± 0.083 | 0.232 ± 0.0031 |
| Hint C84A | 0.977 ± 0.035 | 0.218 ± 0.034 |
| Hint C38A/C84A | 0.102 ± 0.0068 | 0.029 ± 0.0073 |
| Hint H114D | 0.043 ± 0.006 | 0.005 ± 0.0008 |
| Hint H114N | 0.170 ± 0.020 | 0.05 ± 0.0059 |
| Hint S107A | 0.815 ± 0.027 | 0.0356 ± 0.0006 |

**TABLE 2**

| Protein AMP-N-ε-lysine | AMPS |
|-------------------------|------|
| WT rHint-1 1.388 ± 0.321 | 0.202 ± 0.0067 |
| Hint C38A 1.107 ± 0.083 | 0.232 ± 0.0031 |
| Hint C84A 0.977 ± 0.035 | 0.218 ± 0.034 |
| Hint C38A/C84A 0.102 ± 0.0068 | 0.029 ± 0.0073 |
| Hint H114D 0.043 ± 0.006 | 0.005 ± 0.0008 |
| Hint H114N 0.170 ± 0.020 | 0.05 ± 0.0059 |
| Hint S107A 0.815 ± 0.027 | 0.0356 ± 0.0006 |

**TABLE 3**

| Crystallographic data and refinement statistics | Values in parentheses are for the outermost shell. |
|-------------------------------------------------|-----------------------------------------------|
| **Unit cell parameters** | **P4_{21}2_{1}** | **P4_{21}2_{1}** | **P4_{21}2_{1}** |
| a (Å) | 39.96, 141.70 | 39.77, 141.41 | 39.79, 142.63 |
| b (Å) | 14.14-1.08 | 1.00-1.08 (1.10-1.08) | 26.17-1.30 (1.37-1.30) |
| c (Å) | 48.86 | 49.845 | 29.103 |
| α (°) | 26.3 (5.0) | 16.15 (2.88) | 14.2 (4.1) |
| β (°) | 0.066 (0.556) | 0.110 (0.628) | 0.103 (0.680) |
| γ (°) | 19.9 (14.3) | 10.7 (7.3) | 12.4 (12.3) |
| Rmerge (%) | 46.067 | 47.232 | 27.588 |
| Completeness (%) | 96.7 (93.6) | 99.6 (97.9) | 99.8 (98.8) |
| Rn (%) | 13.55/17.16 | 19.08/21.29 | 12.19/17.58 |
| No. protein atoms | 1,083 | 1,072 | 811 |
| No. water molecules | 240 | 148 | 294 |
| No. sodium ions | 6 | 0 | 0 |
| No. adenosine molecules | 1 | 1 | 0 |

**Root mean square deviations from ideal geometry**

| Bond lengths (Å) | 0.030 | 0.030 | 0.024 |
| Bond angles (°) | 2.385 | 2.649 | 2.076 |

**Ramachandran plot statistics for non-proline and non-glycine residues (%)**

| Most favored regions | 94.7 | 87.2 |
| Additional favored regions | 11.7 | 5.3 | 12.8 |

**Root mean square deviations in B values (Å²)**

| Main chain atoms | 14.065 | 16.687 | 12.106 |
| Side chain atoms | 17.165 | 19.697 | 15.554 |
| Average B values for water molecules (Å²) | 50.749 | 33.049 | 43.025 |
binding pocket of the C38A/C84A protein, water molecules are noticeable instead of the adenosine observed for both single mutants.

The crystal structure comparison did not provide any hint as to the explanation for the lowered activity of the C38A/C84A mutant protein toward both substrates. However, this observation eliminated a mechanism of desulfuration, based on the formation of the disulfide intermediate and the contribution of the cysteines’ methylene-mercapto residue in this reaction, because in all three cysteine mutants, the mutations changed the phosphoramidase and desulfurase activity to a similar extent. The above results allow us to argue for the participation of the same amino acids in the desulfuration reaction and in the phosphoramidase reaction, which indicates that at both activities, the histidine triad motif is engaged.

Histidine and Serine Mutant Proteins: Structure and Activity—Because the His-112-mutant protein was expected to be completely inactive in the hydrolysis process (20), the conserved third histidine was mutated to investigate participation of the HIT motif in the desulfuration process. Two histidine mutants, H114D and H114N, were constructed. The CD measurements showed that for both mutants, the molar ellipticity in the 200–240 nm region was lowered by about 11%, compared with WT rHint-1 (Table 1, Fig. 4). The changes in the global secondary structures seem to be smaller than for cysteine mutants C38A, C84A, and C38A/C84A. However, the activities of mutants H114D and H114N toward both substrates were substantially reduced (Table 2), up to 32% compared with WT rHint-1 (Table 1, Fig. 4). The participation in the “holding” of the substrate during the hydrolysis of both substrates in the same binding pocket, a result of Ser-107 was similarly engaged in the binding and catalysis of both substrates in the same binding pocket, a reduction to AMP-N-ε-lysine and AMPS, respectively, was observed. Such a low activity of the Hint-H114D mutant can be partially caused by a non-full homogeneous preparation (a small amount of impurities in the solution of the protein, Fig. 2). During the purification step, lower affinity of this mutant to the AMP resins compared to other studied proteins was noticed, and was revealed as additional proof of His-114 participation in the “holding” of the substrate during the hydrolysis. These results indicate that His-114 plays similar roles in phosphoramidase and desulfurase activities, which may suggest a common mechanism of action.

To test the hypothesis that Ser-107 plays a similar role in the desulfuration and phosphoramidase reactions, we used a rabbit S107A mutant protein and characterized its activity toward AMP-N-ε-lysine and AMPS (Table 2). If the hydroxyl function of Ser-107 was similarly engaged in the binding and catalysis of both substrates in the same binding pocket, a reduction of both activities should be observed. Indeed, the S107A mutant exerted ~2 times reduced activity toward AMP-N-ε-lysine and ~5 times toward AMPS in comparison to WT Hint-1. Thus, Ser-107 seems to serve as a hydrogen bond donor to both the sulfur atom in AMPS and the phosphoramidate nitrogen in the tested substrates.

Based on our results and crystal structure of rHint-1 (26, 27), we propose a scheme for possible interactions between AMPS and amino acid residues engaged in hydrolysis (Fig. 5). The desulfuration of AMPS is expected to proceed through adenylated His-112 (Fig. 6). Presumably, the nitrogen atom of the imidazole group of His-114 forms a hydrogen bond with the bridging 5′-oxygen atom of the substrate (N-O distance ~0.312 nm, on the basis of the rHint-1 crystal structure from Ref. 27, PDB ID 1RZ0 or 0.307 nm, on the basis of the rHint-1 crystal structure from Ref. 26, PDB code 3RHN). Within this frame, only one orientation of the sulfur atom allows the reaction to occur because the position of the leaving group should be “in line” with the attacking His-112 residue (the first step). The distance requirement to form the hydrogen bond S—H—O—Ser-107 (N-O distance ~0.32 nm (27) or 0.281 nm (26), where the nitrogen atom is replaced by a larger sulfur atom) can be fulfilled; thus, the sulfur atom, after protonation, becomes a good leaving group and hydrogen sulfide is released upon attack of His-112. The resulting intermediate product (with adenylated His-112) is identical to that of the phosphoramidate substrate, so the next hydrolytic step should be identical. Thus, we suggest that the mechanism of the “loss of sulfur” in AMPS is similar to cleavage of the P-N bond in AMP-N-ε-lysine and involves two discrete steps, with participation of Ser-107 (rHint-1) acting as the acid-base catalyst (Fig. 6).

Kinetic Parameters of the AMPS Hydrolysis—Kinetic parameters (kcat and Km) were determined for the rHint-1-catalyzed desulfuration of AMPS and compared with those for the hydrolysis of AMP-pNA and AMP-N-ε-lysine (27). The kcat/Km ratio for the desulfuration (81.0 M−1 s−1) was more than 5 times higher than for the hydrolysis of AMP-pNA (14.0 M−1 s−1). On the other hand, Hint-1 converted AMPS to AMP with 500 times higher Km and 10 times lower kcat compared with AMP-N-ε-lysine (Table 4). The higher Km values are reasonable because sulfur has a larger atomic radius and lower electronegativity than oxygen; thus, its H-bonding ability is weaker relative to the latter, and therefore, AMPS demonstrates weaker affinity to the enzyme than AMP-N-ε-lysine and AMP-pNA.

NMPS and dNMPS as the Substrates for Hint-1—We have found that the desulfuration process is not specific toward AMPS. Also, treatment of the 5′-O-phosphorothioylated derivatives of guanosine, cytidine, and uridine as well as those of deoxyadenosine, deoxyguanosine, deoxyctydine, and thymidine with rHint-1 leads to the corresponding 5′-O-phosphates. However, the cleavage rate of the P-S bond in deoxyribonucleotide phosphorothioates is lower than for the corresponding ribonucleotide analogs (Table 5). The data indicate that the rates of desulfuration decrease in the following order: GMPS > AMPS > dGMPS ≡ CMPS > UMPS > dAMPS ≫ dCMPS > TMPS.

It has been demonstrated for the phosphoramidase activity of Hint-1 that the enzyme prefers purine-based substrates rather than pyrimidines and that a hydroxyl group at the ribose 2′ position of the substrate is essential for interactions with the γ-oxygen of Asp-43 (rHint-1) of the enzyme in the binding pocket (45). We observed a similar tendency for rHint-1 desulfurating activity. However, our results show that the purine deoxyribose derivative, dAMPS, is a worse substrate than both pyrimidine ribose analogs, CMPS and UMPS. On the other hand, we noticed that dGMPS was an equally
good substrate as CMPS. These data indicate that the preference of the enzyme substrate pocket to purine residues (such as dGMPS) is balanced by the presence of the 2'-OH group in CMPS, but the affinity to purines in dAMPS appears less important than the influence of 2'-OH group of the ribose pyrimidine analogs. All desulfuration reactions catalyzed by rHint-1 occurred at greater rates than the spontaneous loss of sulfur from AMPS (Table 5).
We also examined whether phosphate diester derivatives could be the substrates for Hint-1. In these reactions, neither AMPS(OMe) nor dinucleotide d(ApsA) were hydrolyzed by the enzyme (Table 5), and no products of desulfuration were observed.

We found that the Fhit protein, another enzyme of the HIT superfamily, which usually acts as an Ap3A and Ap4A hydro-lase (19), can also desulfurate AMPS, although at a rate ~50

![Table 4](image)

**FIGURE 6. Tentative mechanism of the desulfuration reaction of AMPS catalyzed by Hint-1.** The possible hydrogen bonds are marked as broken lines. The atomic distances (Å) for the rHint-1 crystal structure are taken from PDB codes 3RHN (26) and 1RZY (27).
times lower than that caused by Hint-1 (Table 5). Recently, Hint enzymes from other organisms (Hint from *E. coli* and Hint-1 from *Arabidopsis thaliana*) have been shown to be able to desulfurate the AMPS (46).

**Conclusion**—The discovery of the first phosphorothioate backbone modification of DNA in *vivo* (10) and the use of synthetic PS-oligos in medicinal applications give rise to questions about the function and metabolism of this modification. Nucleoside 5′-O-phosphorothioates are formed as primary products of the hydrolysis of oligo(nucleoside phosphorothioates). The enzymes responsible for the degradation of phosphorothioate oligonucleotides in *vivo* are well known (7, 47) (e.g. 3′-exonuclease from plasma (48)); however, the mechanism of their subsequent conversion to (d)NMP is still obscure. In this paper we have shown that under *in vitro* conditions, Hint-1 is able to catalyze hydrolysis of the 5′-O-phosphorothiolated ribonucleosides (A, G, C, U) and deoxyribonucleosides (dA, dG, dC, dT) by a mechanism similar to the P-N bond cleavage in phosphorimidate substrates. Because it has been found that Hint-1 protein homologues are present in all forms of life (26), this enzyme seems to be a good candidate responsible for the desulfuration of PS-substrates in *vivo*. Nonetheless, its ability to execute P-S to P-O conversion inside the cell requires confirmation, and further studies are in progress.

Furthermore, the release of H$_2$S within cells during the desulfuration reaction catalyzed by Hint-1 may have remarkable biological consequences, and the regulation of the production of H$_2$S may be of therapeutic value. Thus, PS-oligo and (d)NMPs can be considered a source of H$_2$S in *vivo*, although their usefulness in this field requires further studies.

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