Recognition of different nucleotidyl-derivatives as substrates of reactions catalyzed by various HIT-proteins†‡

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Proteins that have a histidine triad in their active sites belong to the HIT-protein superfamily. They are ubiquitous, are involved in the metabolism of different nucleotides and catalyze their hydrolysis and/or phosphorolysis liberating either the corresponding 5′-NMP or 5′-NDP, respectively. We studied substrate specificity of nine recombinant HIT-proteins with adenosine 5′-phosphosulfate (**1**), adenosine 5′-phosphoramidate (**2**), adenosine 5′-phosphorothioate (**3**), adenosine 5′-phosphorofluoride (**4**), diadenosine 5′,5‴-P^1,P^3-triphosphate (**5**), di(7-methylguanosine) 5′,5‴-P^1,P^3-triphosphate (**6**) and adenosine 5′-hypophosphate (**7**). Preferences for the recognition of these compounds as substrates by individual proteins differed. All the proteins hydrolyzed (**1**) but the *Arabidopsis thaliana* Hint1 did it very slowly. None of the proteins cleaved (**7**). Only *A. thaliana* Hint1 and *Escherichia coli* HinT hydrolyzed (**3**). Three proteins known as dinucleoside triphosphatases, human and *A. thaliana* Fhit-proteins and *Trypanosoma brucei* HIT-45, cleaved (**1**), (**2**), (**4**), (**5**) and (**6**). *Caenorhabditis elegans* decapping protein DcpS degraded (**1**), (**5**), (**6**) and poorly (**4**). *A. thaliana* aprataxin-like protein and Hint4 hydrolyzed only (**1**), (**2**) and (**4**), in that order of efficiency. Velocities of those reactions and some K_m values were determined. Applicability of this study to the metabolism of certain nucleotidyl-derivatives is discussed.

Introduction

Proteins that have a histidine triad in their active sites belong to the HIT-protein superfamily. Sequence alignment of amino acid fragments that contain a histidine-triad-motif of the proteins used in this study and phylogenic relations of these proteins are depicted in Fig. 1A and B, respectively.[1] HIT-proteins are ubiquitous and interact with different mono- and dinucleotides. The two-step mechanism according to which HIT-proteins (HIT-enzymes) act involves first the formation of an enzyme-nucleotidyl and then splitting of this intermediate. In the first step, one (central) of the triad histidines participates in a covalent nucleotidyl phosphohistydyl intermediate P–N bond formation and in the second, the bond is cleaved either by water, in the case of Fhit-proteins/dinucleoside-triphosphatases (EC 2.6.1.29)[2,3] and nucleoside phosphoramidases,[2,4] or by phosphorylated sugar, in the case of galactose-1-phosphate uridylyltransferase (EC 2.7.7.10).[2] Also dinucleoside tetraphosphate phosphorylase (EC 2.7.7.53) probably functions according the same mechanism. One of its histidine residues occurs in the region that resembles the active sites of the enzymes mentioned above. Formation of the enzyme-adenylate has been proved experimentally[5,6] and splitting of that intermediate could occur in the presence of phosphate (yielding ADP, ppA)[7] or ATP (yielding Ap_4A, AppppA).[8]

This study stemmed from the investigation of substrate specificity of Fhit-proteins (from fragile histidine triad)[9] that form one of at least four branches of the HIT-protein superfamily. Fhit-proteins, were known primarily as typical dinucleoside triphosphatases.[10,11] However, our recent study showed that these proteins possess also activities which had been assigned to nucleoside phosphoramidases, adenylylsulfatases and phosphodiesterases. The investigated human and *Arabidopsis thaliana* Fhit-proteins effectively catalyzed liberation of 5′-AMP (pA) from such naturally occurring nucleotides as adenosine 5′-phosphoramidate (NH_2-pA) and adenosine 5′-phosphosulfate (SO_4-pA, commonly known as APS), as well as from unnatural nucleotidyl-derivative, adenosine 5′-phosphofluoride (F-pA).[9] Earlier, similar enzymatic activities have been demonstrated for some other HIT-proteins. Hint-proteins exhibited adenosine phosphoramidase activity[2,4,12,13] and rabbit Hint1 was shown to catalyze liberation of 5′-NMP (pN) from nucleoside

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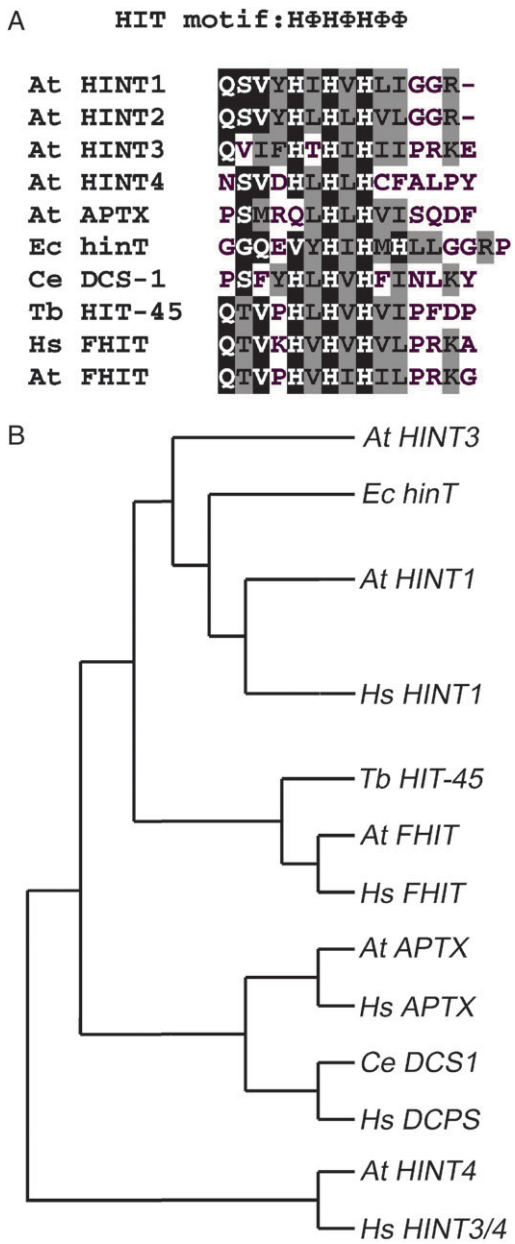

Fig. 1 (A) Sequence alignment of amino acid fragments that contain histidine triad motif of the proteins used in this study. Abbreviations are as follows: At: *Arabidopsis thaliana*, Ec: *Escherichia coli*, Ce: *Caenorhabditis elegans*, Hs: *Homo sapiens*, Tb: *Trypanosoma brucei*. Φ represents a hydrophobic amino acid. (B) Phylogenetic tree of the HIT-proteins used in this study. It was plotted using Clusta1X and Treeview software according to Thompson *et al.*[1] The "tree" shows also positions of human congeners of some HIT-proteins which were not investigated but are closely related to the studied ones.

5′-*O*-phosphorothioates (S-pNs) with concomitant release of H_2S.[14,15] In addition to Hint- and Fhit-proteins, two other enzymes that belong to the HIT family were recently described. One of them is aprataxin—an enzyme that removes AMP from DNA breaks. Such an adenylylated DNA results from aborted DNA ligation.[16] The other enzyme, DcpS/DCS1, functions as a "decapping" enzyme that hydrolyzes the cap structure during mRNA degradation.[17] We wondered if among the different nucleotides there is at least one that could be recognized as a substrate by all tested HIT-proteins, and if among these proteins there is/are enzyme(s) that exhibit rather narrow substrate specificity. We have collected for this study nine recombinant HIT-proteins representing four branches of the HIT superfamily: three Fhit-proteins (human HsFhit,[9] *Arabidopsis thaliana* AtFhit,[9] and *Trypanosoma brucei* TbHIT-45[18]), four Hint-proteins (AtHint1, AtHint3 and AtHint4 from *A. thaliana*, and EcHinT from *Escherichia coli*), one decapping protein from *Caenorhabditis elegans*, CeDcpS,[19] and aprataxin-like protein from *A. thaliana*, AtAtpx. Their hydrolytic activity was tested using the following seven nucleotides: adenosine 5′-phosphosulfate (**1**), adenosine 5′-phosphoramidate (**2**), adenosine 5′-phosphrothioate (**3**), adenosine 5′-phosphoro-fluoride (**4**), diadenosine 5′,5‴-P^1,P^3-triphosphate (**5**), di(7-methylguanosine) 5′,5‴-P^1,P^3-triphosphate (**6**) and adenosine 5′-hypophosphate (**7**). Their structures are shown in Fig. 2. The results of these studies are presented and the potential role of the HIT-proteins in the metabolism of nucleotide pro-drugs is discussed.

Results and discussion

At the beginning of this study, we performed pilot experiments aimed to identify, amidst the nucleotidyl-derivatives, **1–7**, potential substrates for the reactions catalyzed by nine HIT-proteins. Each protein was incubated with 1 mM nucleotide in 50 mM potassium phosphate (pH 6.8) containing 5 mM $MgCl_2$. For the qualitative assays the protein concentration was sufficient to obtain an enzymatic activity that allowed the progress of each reaction to be monitored over a reasonable range of time by analyzing reaction mixtures on thin-layer chromatograms. Such an approach allowed us to determine if a given nucleotide was recognized as a substrate by a particular protein and to select protein/enzyme dilution for quantitative measurements (see below). As shown in Fig. 3A–D, all four HIT-proteins that recognize dinucleoside triphosphates (human and *Arabidopsis* Fhit-proteins, *T. brucei* HIT-45 and *C. elegans* DcpS) easily split ApppA (**5**) to AMP (pA) and ADP (ppA). They also hydrolyzed another dinucleoside triphosphate tested, m^7Gpppm^{7}G (**6**), which is an analog of the eukaryotic mRNA 5′ end (cap structure). Whereas HsFhit and TbHIT-45 hydrolyzed the latter compound 3–5-fold more slowly than ApppA, the AtFhit cleaved m^7Gpppm^{7}G about 4-fold faster than ApppA (Table 1). In this respect AtFhit resembled the yellow lupin nucleoside triphosphate hydrolase whose substrate specificity had been described in 1996. Generally, the capped, *i.e.* the m^7G-containing dinucleoside triphosphates were much better substrates of the lupin enzyme than ApppA or GpppG and of all 11 dinucleotides tested m^7Gpppm^{7}G was hydrolyzed with the highest velocity; 4.5-fold faster than ApppA.[20] In contrast to the aforementioned Fhit-proteins and CeDcpS, neither the four investigated Hint-proteins (Fig. 3E–H) nor aprataxin-like protein (Fig. 3I) hydrolyzed ApppA or m^7Gpppm^{7}G. However, the AtHint4 catalyzed slow phosphorolysis rather than hydrolysis of ApppA and this yielded ADP as the reaction product (Fig. 3G). The Hint4 exhibited most clearly its dual catalytic activity when incubated with adenosine 5′-phosphosulfate (**1**).

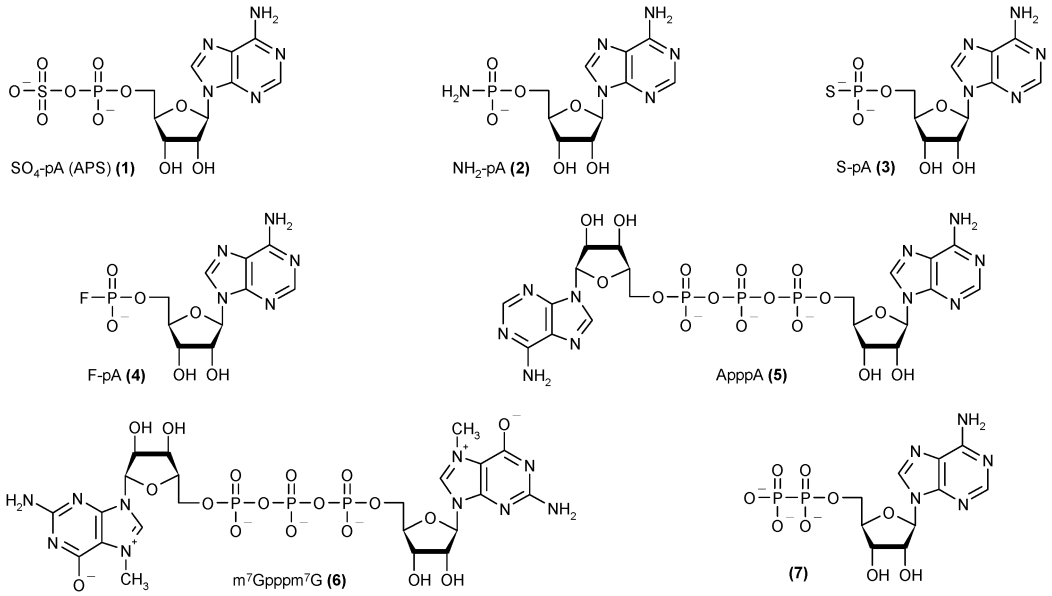

Fig. 2 Structures of the nucleotidyl-derivatives used in this study.

Under the assay conditions, *i.e.* in the presence of 0.1 M orthophosphate, AtHint4 catalyzed both the hydrolysis of (**1**) (forming AMP) and the phosphorolysis (forming ADP). The catalytic duality of AtHint4 was less visible with (**4**) and (**2**) (Fig. 3G). CeDcpS also exhibited catalytic duality but to a lesser extent than the AtHint4 (see enzyme incubated with (**1**), Fig. 3D). Very recently, more details on the dual catalytic activities of these two HIT-proteins have been described elsewhere.[21] All nine HIT-family proteins hydrolyzed adenosine 5′-phosphosulfate (**1**) and all except CeDcpS cleaved adenosine 5′-phosphoramidate (**2**). The latter property distinguishes CeDcpS from other Npp-pN-cleaving enzymes which eagerly hydrolyzed (**2**). Compound (**1**) appeared to be a rather good substrate for all tested proteins, except AtHint1 which hydrolyzed (**1**) very slowly (Table 1) (Fig. 3E). Only AtHint1 and EcHinT catalyzed desulfuration of adenosine 5′-phosphrothioate (**3**) (Fig. 3E and H[1]). However, in case of AtHint1 the hydrolysis of that compound proceeded at least 25-fold more slowly than hydrolysis of adenosine 5′-phosphoramidate (**2**) and in case of EcHinT 100-times more slowly (Table 1). To show that slow reaction, two EcHinT concentrations, differing 50-fold, were used; compare Fig. 3H[1] and H[2].

Initial velocities of the catalyzed reaction have been estimated for each nucleotide-substrate by the use of HPLC (see Experimental). Table 1 summarizes results of these measurements. For each HIT-protein, the percent hydrolysis of a given substrate was expressed relative to the hydrolysis of the compound that was degraded with the highest velocity. These were: NH_2-pA (**2**) for HsFhit, AtHint1, AtHint3 and EcHinT, m^7Gpppm^7G (**6**) for AtFhit, and SO_4-pA (**1**) for TbHIT-45, CeDcpS, AtHint4 and AtAptx. Using radio-labeled adenosine-8-^{3}H 5′-phosphoramidate, we have estimated the K_m values for this nucleotide which, as mentioned above, appeared to be a good substrate for all the HIT-proteins except CeDcpS. As shown in Table 2, most of the K_ms for NH_2-pA fall in the micromolar range. The same concerns App-pA; the K_ms estimated earlier for that compound were 1.2 μM for HsFhit[10]

Table 1 Initial velocities of hydrolysis of different nucleotides catalyzed by different HIT-proteins

	HIT-proteins and relative initial velocities of degradation (%)[a]								
Nucleotide (no.)	HsFhit	AtFhit	TbHIT-45	CeDcpS	AtHint1	AtHint3	AtHint4	EcHinT	AtAptx
SO_4-pA (**1**)	65	48	100	100	0.1	33	100	11	100
NH_2-pA (**2**)	100	64	96	0	100	100	33	100	13
S-pA (**3**)	0	0	0	0	4	0	0	1	0
F-pA (**4**)	25	19	97	2	70	2	12	9	4
App-pA (**5**)	58	27	72	8	0	0	0	0	0
m7Gpp-pm7G (**6**)	8	100	1	10	0	0	0	0	0

[a] The initial velocities of the hydrolysis were measured by the HPLC analysis of reaction mixture withdrawn at various time intervals. Incubation was performed at 30 °C and the mixture (0.075 ml final volume) contained 50 mM potassium phosphate (pH 6.8), 5 mM $MgCl_2$, 1 mM substrate and appropriately diluted HIT-protein. To stop the reaction, 10 μl aliquots were transferred to the test-tubes preheated to 95 °C and kept in that temperature for 3 min. Then the samples were brought to 0.1 ml water and 25 μl of the resulted solution subjected to HPLC under the conditions described in Experimental. For each HIT-protein, the values have been related to the value obtained for substrate that was hydrolyzed with the highest velocity. The corresponding numbers were as follows: 22 nmol NH_2-pA/min μg^{-1} HsFhit; 17.6 nmol m^7Gpppm^7G/min μg^{-1} AtFhit; 0.8 nmol SO_4-pA/min μg^{-1} TbHIT-45; 11.5 nmol SO_4-pA/min μg^{-1} CeDcpS; 8.35 nmol NH_2-pA/min μg^{-1} AtHint1; 4.4 nmol NH_2-pA/min μg^{-1} AtHint3; 3.3 nmol SO_4-pA/min μg^{-1} AtHint4, 56 nmol NH_2-pA/min μg^{-1} EcHinT, and 0.85 nmol SO_4-pA/min μg^{-1} AtAptx.

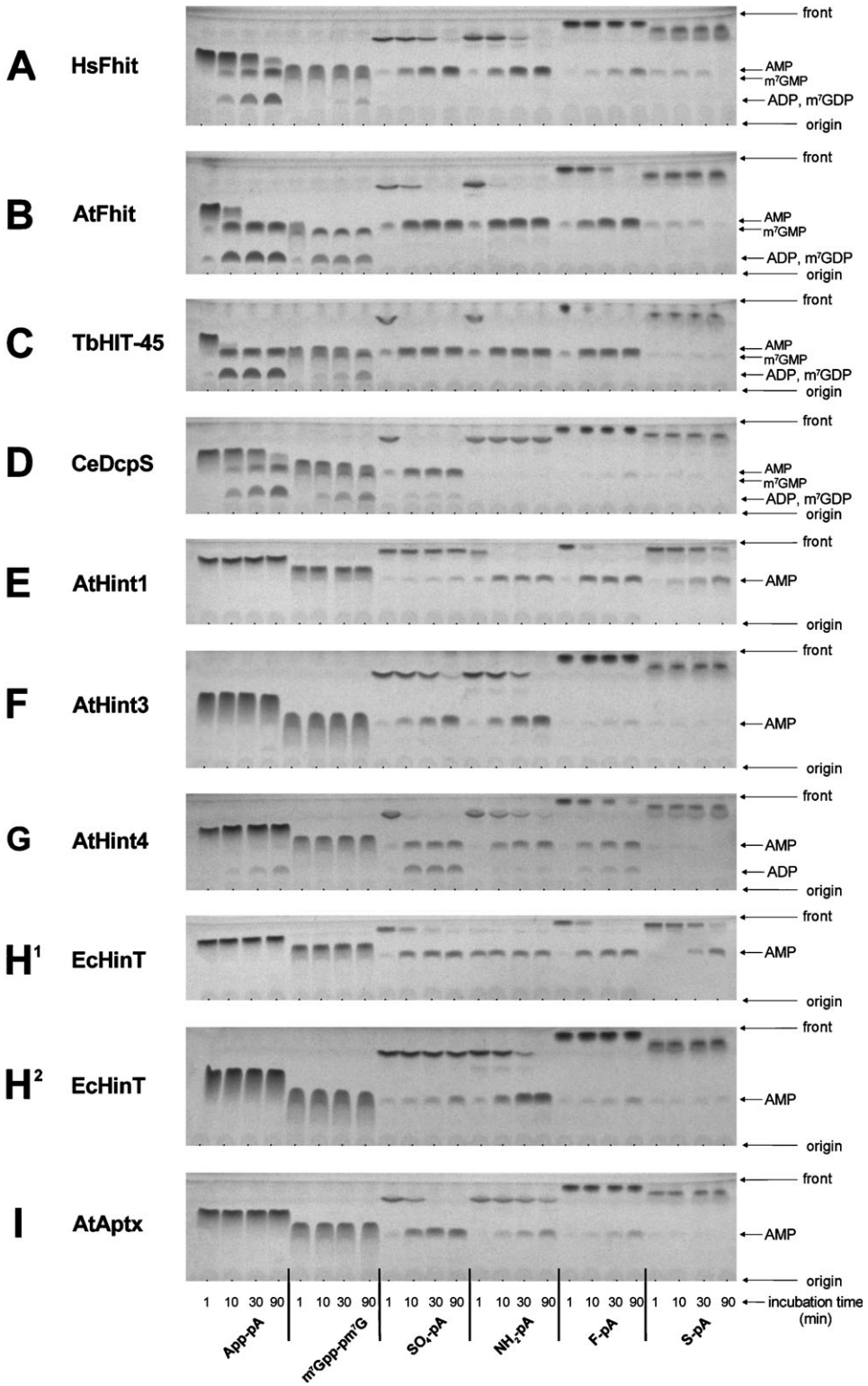

Fig. 3 Susceptibility of certain (di)nucleotides to cleavage catalyzed by different HIT-proteins. The reaction mixture (25 μl) contained 0.1 M potassium phosphate (pH 6.8), 5 mM $MgCl_2$, 1 mM (di)nucleotide indicated and the following amounts of the analyzed HIT-protein: (A) 0.085 μg of human Fhit (HsFit); (B) 0.11 μg of *Arabidopsis thaliana* Fhit (AtFhit); (C) 10 μg of *Trypanosoma brucei* HIT-45 (TbHIT-45); (D) 0.2 μg of *Caenorhabditis elegans* DcpS (CeDcpS); (E) 0.24 μg of *A. thaliana* Hint1 (AtHint1); (F) 0.5 μg of *A. thaliana* Hint3 (AtHint3); (G) 0.3 μg of *A. thaliana* Hint4 (AtHint4); (H^1) 1 μg of *Escherichia coli* HinT (EcHinT); (H^2) 0.02 μg of *E. coli* HinT (EcHinT); (I) 1.6 μg of *A. thaliana* aprataxin-like protein (AtAptx). Incubation was carried out at 30 °C. At time intervals (1, 10, 30 and 90 min) 3 μl aliquots were spotted on the origin and chromatogram developed in dioxane : 25% ammonia : water (6 : 1 : 4, by volume) for 40 min. Spots of the substrates effectively separated from corresponding products were visualized on the chromatograms under short-wave UV light and photographed. In each case the substrate (not pointed at by an arrow) migrated faster than the reaction product(s).

Table 2 The K_m values estimated for adenosine 5′-phosphoramidate with some HIT-proteins

HIT-protein	K_m for NH_2-pA (μM)
AtHint1	1
AtHint3	12
AtHint4	135
AtAptx	28
EcHinT	0.4
TbHIT-45	15
CeDcpS	N/A
HsFhit[a]	3
AtFhit[a]	3

N/A: Not applicable, given nucleotide is not a substrate. [a] The K_m values for HsFhit and AtFhit were published earlier.[9]

and 6.5 μM for TbHIT-45.[18] This suggests that different HIT-proteins exhibit high affinity at least for those two naturally occurring nucleotides.

Regarding newly synthesized adenosine 5′-hypophosphate (**7**), none of the HIT-proteins investigated cleaved its P-P bond and liberated pA. Compounds with such a bond do not occur in living systems and proteins probably do not recognize this structure. Earlier, we found that (**7**) was also refractory to other hydrolases (bacterial alkaline phosphatase, lupin apyrase and snake venom phosphodiesterase) and that (**7**) did not inhibit these enzymes. Only snake venom 5′-nucleotidase slowly hydrolyzed this compound, releasing adenosine (A. Guranowski, unpublished observations).

Except for AtHint1, the HIT-proteins also did not release pA from such a common metabolite as ADP (ppA). AtHint1 was the only protein that catalyzed noticeable release of pA from ppA but velocity of that reaction was more than 250-fold lower than that of the hydrolysis of adenosine 5′-phosphoramidate (**2**).

Conclusions

This study extends our earlier observations concerning Fhit proteins,[9] AtHint4 and CeDcpS,[21] and confirms that different HIT-proteins may be involved in the metabolism of SO_4-pA and hence affect the sulfur metabolism in different organisms. Also, the results presented in this communication should be useful for those who study fates of nucleotide prodrugs delivered to target cells. Exchange of the oxygen atom in the nucleotide phosphate moiety to such residues as -F, -S, -NH_2, -SO_4 or -ppA apparently lowers the nucleotide's polarity. (Compare, for example, markedly lower chromatographic mobility of adenine nucleotides with the mobility of the adenylyl-derivatives; Fig. 3.) Therefore such synthetically modified nucleotidyl-derivatives may serve as model compounds for prodrugs which may undergo hydrolysis in cellular environment, *e.g.* due to activity of different HIT-proteins, and liberate the corresponding 5′-NMPs which easily enter common metabolic pathways.

Special attention should be given to nucleoside 5′-phosphorothioates, *e.g.* compound (**3**). Their administration to animals or humans can appear beneficial. Desulfuration of those thiocompounds catalyzed by certain HIT-proteins (rabbit Hint1 described earlier[15] and AtHint1 and EcHinT reported in this paper) releases hydrogen sulfide; a new gaseous signaling molecule. In addition to nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) has emerged recently as a potentially important mediator of cardiovascular homeostasis, cytoprotection and signal transduction in the central nervous system.[22–24]

Experimental

Chemicals

AMP (pA), ADP (ppA), ApppA (**5**), NH_2-pA (**2**), S-pA (**3**) (commonly abbreviated as AMPS) and most of the bulky reagents were from Sigma, St. Louis, MO, USA. F-pA (**4**) was synthesized as described.[25] Radio-labeled adenosine-8-^{3}H 5′-phosphoramidate was purchased from Moravek Biochemicals, Brea, CA, USA. Syntheses of adenosine 5′-phosphosulfate (**1**)[21] and of di(7-methylguanosine) 5′,5‴-P^1,P^3-triphosphate[26] (**6**) were described previously. Adenosine 5′-hypophosphate (**7**) was synthesized according to Setondji *et al.*:[27] Tri-n-butylammonium hypophosphate (tetrahydrate) (0.41 g, 1 mmol) and 2′,3′-isopropylideneadenosine (76 mg, 0.25 mmol) were dissolved in 5 ml of dimethylformamide (DMF). The mixture was cooled down to 4 °C and a solution of dicyclohexylcarbodiimide (DCC) (0.29 g, 1.37 mmol) in DMF (0.4 ml) was added. The reaction mixture was kept at 4 °C for 1 h and the stirring continued for 12 h at room temperature. Then, 50 ml of water was added and the mixture filtered through a Schott funnel. 1 M HCl (5 ml) was added to the filtrate and the reaction to remove the isopropylidene group was continued for 2 h. The product was isolated on a Sephadex A-25 column eluted with a gradient of 0.05–0.5 M triethylammonium bicarbonate. The appropriate fractions were collected and concentrated to dryness. To remove the residual buffer, methanol (5 ml) was added and the sample concentrated to yield *ca.* 95 mg of the adenosine 5′-hypophosphate (triethylammonium salt, M.W. 725; 0.13 mmol, 52% yield). Then, using Dowex cation-exchanger, the product was quantitatively converted into the trisodium salt, concentrated and lyophilized yielding 80 mg; (M.W. 477, δ ^{31}P NMR (D_2O) 17.09 ppm (d), 4.93 ppm (d), $^1J_{P\text{-}P}$ = 660 Hz; MALDI TOF MS *m*/*z* 410.0 M^-).

Recombinant proteins

Origin of the recombinant HIT-proteins and procedures used for their purification were described previously.[9,18,19,21]

Chromatographic systems

Thin-layer chromatography was performed on aluminium plates precoated with silica gel containing fluorescent indicator (Merck). The chromatograms were developed in dioxane : 25% ammonia : water (6 : 1 : 5, by volume) for 30–40 min. Two HPLC systems were used. Most of the reaction mixtures were chromatographed on a reverse phase column (Teknokroma C_{18}, 150 mm × 4.6 mm; 5 μm). The column was eluted with linear gradient of 50 mM triethylamine buffer (pH 7.4 adjusted with CO_2) : acetonitrile (97 : 3, v/v) (solvent A) and solvent A : acetonitrile (60 : 40, v/v, solvent B); 0–15 min, 20% solvent B, at a flow-rate of 1 ml min^{-1}. In that system the retention times (min) were as follows: AMP (pA) 3.5, NH_2-pA 4.0, ADP (ppA) 4.5, S-pA 4.6, SO_4-pA 5.5, Ap_3A (App-pA) 9.8 and

F-pA 12. Reaction mixtures containing m^7Gpppm^7G were chromatographed on an anion exchange column (Hamilton PRP-X100). Two solutions A and B were prepared. Solution A was 25 mM citric acid pH 3.5 and solution B was A : acetonitrile (17 : 3). The column was eluted for 1 min with A and then 0–5 min with linear gradient 0–50% B. The retention times (min) were 1.1 for m^7pG, 2.1 for m^7ppG, and 4.7 for m^7Gpppm^7G.

Enzyme assays

The reaction mixture (usually 50 µl) contained 0.1 M potassium phosphate (pH 6.8), 5 mM $MgCl_2$, 1 mM di(nucleotide) indicated and appropriately diluted protein solution. Incubations were carried out at 30 °C and at time intervals 3 or 10 µl aliquots were analyzed; either by TLC or HPLC, respectively. Incubation time and protein concentrations were appropriately adjusted to obtain reliable values of the initial velocities. For more details see the legend to Fig. 3 and Table 1.

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