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Fhit proteins can also recognize substrates other than dinucleoside polyphosphates

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This work is dedicated to Professor Antonio Sillero on the occasion of his 70th birthday and to Professor Maria Antonia Günther Sillero.

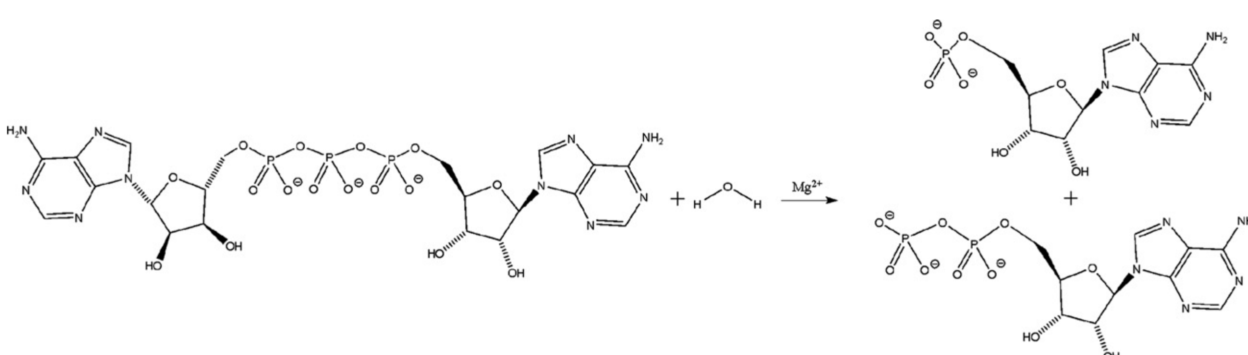
Abstract **We show here that Fhit proteins, in addition to their function as dinucleoside triphosphate hydrolases, act similarly to adenylylsulfatases and nucleoside phosphoramidases, liberating nucleoside 5′-monophosphates from such natural metabolites as adenosine 5′-phosphosulfate and adenosine 5′-phosphoramidate. Moreover, Fhits recognize synthetic nucleotides, such as adenosine 5′-*O*-phosphorofluoridate and adenosine 5′-*O*-(γ-fluorotriphosphate), and release AMP from them. With respect to the former, Fhits behave like a phosphodiesterase I concomitant with cleavage of the P–F bond. Some kinetic parameters and implications of the novel reactions catalyzed by the human and plant (*Arabidopsis thaliana*) Fhit proteins are presented.**

Keywords: Fhit protein; Dinucleoside triphosphatase activity; Nucleoside phosphoramidase activity; Adenylylsulfatase activity; Phosphodiesterase I activity; P–F bond cleavage

1. Introduction

Cells contain various minor nucleotides. Among these are the dinucleoside 5′,5‴-P^1,P^n-polyphosphates (Np$_n$N′s, where N and N′ are 5′-*O*-nucleosides and *n* represents the number of phosphate residues in the polyphosphate chain that esterifies N and N′ at their 5′ position) [1]. Np$_n$N′s accumulate as a result of the activity of certain ligases and transferases that catalyze transfer of a nucleotidyl moiety onto various acceptors containing a pyrophosphate residue, including NTPs (pppNs) and NDPs (ppNs), from a variety of donors. For reviews see [2,3]. Np$_n$N′s play different intracellular and extracellular functions [4–6]. The cellular level of Np$_n$N′s can be controlled by various hydrolases or phosphorylases [7]. Among specific hydrolases is the dinucleoside triphosphatase (EC 3.6.1.29) that preferentially hydrolyzes NpppN′ to an NMP and N′DP (see Reaction 1):

This enzyme was first discovered in Silleros' laboratory in extracts of rat liver [8] and subsequently in extracts of yellow



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Abbreviations: APS or S-pA, adenosine 5′-phosphosulfate; NH_2-pA, adenosine 5′-phosphoramidate; ATP-F or F-pppA, adenosine 5′-*O*-(γ-fluorotriphosphate); HPLC, high performance liquid chromatography; TLC, thin layer chromatography

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lupin seeds [9], *Saccharomyces cerevisiae* [10], *Artemia* [11], and green algae [12]. The intriguing finding that *FHIT* (from fragile histidine triad), a putative human tumor suppressor gene, encodes a typical dinucleoside triphosphatase [13] focused much attention on this particular enzyme. *FHIT* genes occur in different eukaryotic organisms [14] and homogeneous Fhit proteins have been obtained by overexpression of *FHIT* from human [15], yeast [16] and *Arabidopsis thaliana* (present study).

Work described here stemmed from the investigations of the regiospecificity of the hydrolysis of Np_nN's and in particular from a study on the mechanism of action of Fhit/Np_3N' hydrolases. Preference for Np_3N's and a unique mode of substrate cleavage are features that distinguish Np_3N' hydrolases from other specific hydrolases acting on Np_nN's. Using $H_2{}^{18}O$ and mass spectrometry of the isolated reaction products, it was first shown that the Np_3N' hydrolase from yellow lupin exclusively hydrolyzes the anhydride bond of its substrates (ApppA, AppppA, $AppCH_2pA$ or AppCHFpA) between P^{α} and P^{β}, incorporating ^{18}O only into AMP [17]. It was subsequently shown by Abend et al. that human Fhit/Np_3N' hydrolase acts in the same fashion [18]. Moreover, they demonstrated that cleavage proceeds with overall retention of configuration at phosphorus, implying a double inversion mechanism, and they postulated that all three histidines of the histidine triad (His94, His96 and His98) are involved in the formation of an intermediate in which His96 first undergoes adenylation and then deadenylation by water. Pursuing that issue, Frey et al. showed that Fhit and specifically mutated Fhit variants catalyzed the hydrolysis of adenosine 5′-phosphoimidazolide (APS or S-pA) [19] and, poorly, of *p*-nitrophenyl-AMP [20]. Thus, Fhits behave like a nucleoside phosphoramidase and phosphodiesterase, respectively.

We demonstrated that two naturally occurring AMP derivatives: sulfoadenylate (S-pA), a key metabolite on the sulfate assimilation pathway, and adenosine 5′-phosphoramidate (NH_2-pA, aminoadenylate), a less known natural metabolite [21] originating from APS by enzymatic displacement of its sulfate moiety by ammonia [22], *are* Fhits' substrates. We also showed that both human and plant (*Arabidopsis*) Fhits liberate AMP from such synthetic AMP congeners as fluoro-adenylate (F-pA) and fluoro-ATP (ATP-F).

2. Materials and methods

2.1. Materials

Adenosine 5′-*O*-fluorophosphate (F-pA) was synthesized according to [23] and adenosine 5′-*O*-(γ-fluorophosphate) (Fpp-pA) according to procedure developed for the synthesis of GTPγF [24]. Other adenine (di)nucleotides were from Sigma, St. Louis, MO, USA. NH_2-pA was custom-labeled with tritium at its C-8 by Moravek Biochemicals, Brea, CA, USA. Amino-[8-^{3}H]inosylate (NH_2-p[8-^{3}H]I) was obtained by deamination of NH_2-p[8-^{3}H]A catalyzed by adenosine phosphate deaminase (EC 3.5.4.17) from *Helix pomatia* [25].

Primers 5′CGACGCATATGTCGTCTACTTGTTCTTCG and 5′CGGCTCGAGCTAGCAATCGAAAAGAGATCTG were used to amplify the coding sequence of *A. thaliana* FHIT. The PCR product obtained with *A. thaliana* cDNA was cloned using NdeI and XhoI restriction sites included in the primer sequences into pSG02 vector [26]. Plasmid for the human Fhit expression was described earlier [27]. *A. thaliana* and human Fhit proteins were expressed in *Escherichia coli* strain BL21. Cells were lysed by sonication in buffer A, containing 100 mM NaCl, 20 mM Tris–HCl, pH 7.5 and 2 mM dithiothreitol. Nucleic acids were precipitated using polyethyleneimine at 0.1% concentration. Insoluble debris was removed by centrifugation and remaining proteins were precipitated with ammonium sulfate added to 70% saturation. Precipitated proteins were resuspended in buffer A and ammonium sulfate precipitation was repeated. The resulting protein pellet was resuspended in buffer A and loaded onto an AMP-agarose column. Unbound proteins were washed out with buffer A and Fhit proteins were eluted with buffer A supplemented with 1 mM adenosine. This procedure yielded proteins that were about 90% pure by SDS–PAGE gel. Molecular mass of the human Fhit monomer is 16 800 Da and *Arabidopsis* Fhit monomer 18 120 Da. Both Fhit samples were then dialyzed against 20 mM Hepes/NaOH, pH 7.5 containing 100 mM NaCl and 5% glycerol, concentrated by ultrafiltration on Microcone filters from Millipore to 1.3 mg/ml in case of the human Fhit and to 1.5 mg/ml in case of the *Arabidopsis* Fhit, and stored at −20 °C.

2.2. Enzyme assays

Hydrolytic activities of the Fhit proteins were assayed in reaction mixture (0.1 ml) containing 50 mM Mes/KOH (pH 6.5), 5 mM $MgCl_2$, 1 mM substrate and rate-limiting amounts of either human or plant Fhit protein. The reactions were carried out at 30 °C. At time intervals (0, 5, 10 and 30 min), 20 μl aliquots were withdrawn and the reaction stopped by heating the samples for 5 min at 96 °C. The samples were chilled, diluted three-fold with 50 mM TEAB (triethylamine buffer, pH 7.4), filtered through ultrafree-MC filters (from Millipore) and 10 μl aliquots subjected to high performance liquid chromatography (HPLC) on a Discovery C18 column (4.6 × 250 mm, 5 μm); Supelco at a flow rate 1 ml/min. The column was eluted with a linear gradient of 50 mM TEAB (pH 7.4) (solvent A) and solvent A:acetonitrile (60:40, v/v) (solvent B); 0–19 min, 40% B. The retention times of the nucleotides are presented in Table 1. At the aforementioned experimental conditions there was a linear dependence between time and AMP, the reaction product, peak areas. This allowed to calculate and compare the rates of the hydrolysis of investigated substrates.

The nucleoside phosphoramidase activity of the Fhits was estimated in a reaction mixture containing 50 mM Mes/KOH (pH 6.5) and appropriate concentration of NH_2-p[8-^{3}H]A. When the K_m values were estimated the radiolabeled substrate concentration varied between 1 and 15 μM. At time intervals, 5 μl aliquots of the reaction mixture were spotted onto thin layer chromatography (TLC) aluminum plates precoated with silica gel containing fluorescent indicator (from Merck), standards of NH_2-pA and AMP applied at the origin and the plates developed for 60 min in dioxane/ammonia/water (6:1:4, by vol.). Spots of the nucleotides were visualized under short-wave UV light and those of the reaction product (AMP) cut out, immersed in a scintillation cocktail, and radioactivity counted.

K_i values of different nucleotides used in competition with NH_2-p[8-^{3}H]A in the nucleoside phosphoramidase reaction were determined in a reaction mixture (50 μl) containing 50 mM Mes/KOH (pH 6.5), 5 mM $MgCl_2$, 2.75 μM NH_2-p[8-^{3}H]A (580 000 c.p.m.) and varied concentrations of one of the following nucleotides: AMP, ADP, ATP, Ap_3A, S-pA, F-pA or adenosine-5′-*O*-(γ-fluoridotriphosphate) (ATP-F or F-pppA). Reaction rates were estimated as described above. K_i values were calculated according [28].

Table 1
Various substrates of human and *Arabidopsis* Fhit proteins

Nucleotide	Retention time (min)[a]	Relative velocities of hydrolysis (%)[b]	
		Human Fhit	*Arabidopsis* Fhit
NH_2-pA	9.7	100	100
S-pA	10.3	65	75
ApppA	11.8	58	51
F-pppA	10.2	43	90
F-pA	13.1	25	62

[a]The conditions of the used HPLC are in Section 2. The retention times for the reactions' product were: 8.8 min for AMP (pA) and 10.3 for ADP (ppA).

[b]The reaction mixture contained 50 mm Mes/KOH (pH 6.5), 5 mM $MgCl_2$, 1 mM substrate and rate-limiting amounts of either human or *Arabidopsis* Fhit protein. The k_{cat} values for the preferred substrate NH_2-pA were 1.26 and 1.27 s^{-1}, respectively.

2.3. NMR spectroscopy

1D ^{31}P NMR spectra were recorded on a Bruker DRX 500 spectrometer with a 5 mm BB probe. 1D ^{19}F NMR spectra were recorded on the same spectrometer using a 5 mm ^{1}H/^{19}F dual probe. Data were processed using Felix (Felix-NMR Inc., San Diego). Standard Bruker referencing was used (1 M phosphoric acid for ^{31}P, and trichlorofluoromethane for ^{19}F).

3. Results

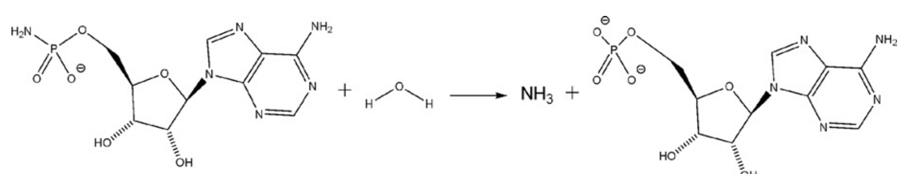
3.1. Identification and cloning of the A. thaliana FHIT gene

BLAST algorithm was used to search for the *A. thaliana* proteins similar to known Fhit proteins. The search identified product of the At5g58240 gene as the most likely candidate. The At5g58240 protein was 50% identical to human Fhit and 46% identical to *S. cerevisiae* Hnt2, whereas its similarity to Hint nucleoside phosphoramidases from the same organisms was much lower (27% and 23% identity to *S. cerevisiae* Hnt1 and human Hint1, respectively). Based on these results and on the catalytic properties we decided that At5g58240 gene encoded genuine Fhit protein. The complete *A. thaliana* FHIT-coding sequence was PCR-amplified, cloned and sequenced. The obtained sequence was identical to the mRNA sequence deposited in GENEBANK under accession number AK228164.

3.2. Fhit proteins behave as nucleoside phosphoramidases

Earlier observations that human Fhit protein effectively hydrolyzed the P–N bond in adenosine 5′-phosphoimidazolide [19] led us to check whether NH_2-pA, the simplest and naturally occurring nucleoside phosphoramidate, is also recognized as substrate by the human and plant Fhits. Analysis of reaction mixtures in which NH_2-pA was the only potential substrate of the Fhits was performed first by TLC (Fig. 1) and then by HPLC (Table 1). It clearly showed that Fhits act as adenosine phosphoramidases and catalyze the Reaction 2:

In order to estimate the kinetic parameters of this reaction by the most direct and sensitive method, we used tritium-labeled NH_2-pA as a substrate to determine pH optimum, metal ion requirements, K_m and k_{cat} values, and also K_is for different nucleotides that act in the phosphoramidase reaction as substrate competitors. The two Fhit proteins investigated catalyzed cleavage of the P–N bond in NH_2-pA most effectively at neutral pH in a reaction independent of Mg^{2+}. K_m values estimated in 50 mM Mes/KOH buffer (pH 6.8) were 3 ± 0.7 μM both for the human and *Arabidopsis* Fhits and k_{cat} values were calculated to be 1.26 and 1.27 s^{-1}, respectively. Using non-specific adenosine phosphate deaminase [25] we converted NH_2-p[8-^{3}H]A into NH_2-p[8-^{3}H]I and demonstrated that the latter was also deaminated to IMP by the Fhits. Cleavage of the P–N bonds in those two substrates (1 mM) proceeded at the same rate. Finally, we estimated the K_i values for the human and *Arabidopsis* Fhits from the effects exerted by nucleotides used by the enzymes either as substrates for other reactions (see below) or as products (Table 2). Generally, human Fhit recognized fluoroadenylates more poorly than did its plant counterpart. Tested as an adenosine phosphoramidase, human Fhit was practically not inhibited by F-pA and F-pppA, whereas *Arabidopsis* Fhit was; with the K_i values approximately one (for F-pA) and two (for F-pppA) orders of magnitude higher than the K_m for NH_2-pA. Both Fhits were inhibited with comparable effectiveness by AMP, one of the reaction products. ADP and ATP, poorly inhibited the plant enzyme and were practically without effect when tested in the reaction catalyzed by the human one. The nucleoside-phosphoramidase reaction catalyzed by Fhits, i.e., the hydrolysis of NH_2-pA or NH_2-pI, was inhibited neither by Ap_3A nor by F-pppA in the absence of Mg^{2+}, which is a co-substrate of the reactions of hydrolysis of Ap_3A or F-pppA (see below). Analysis of the data (Tables 1 and 2) shows that the more effective is the nucleotide as a substrate of Fhit, the stronger is its inhibitory effect on the conversion of NH_2-pA into AMP and NH_3.



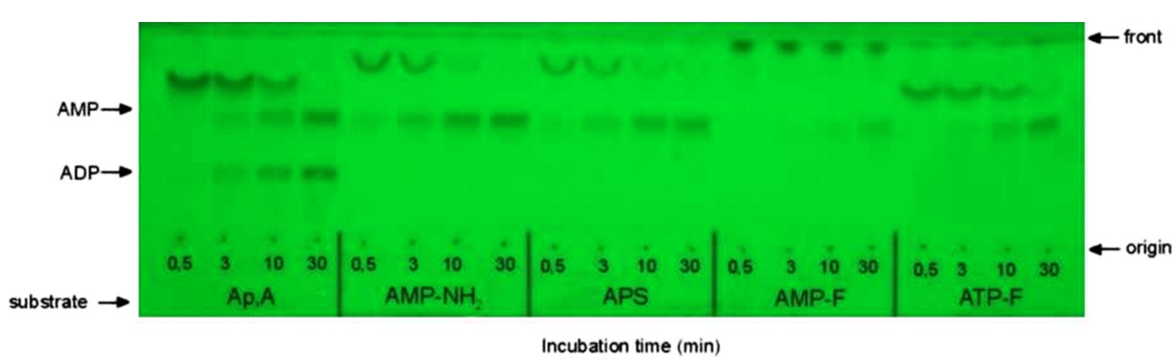
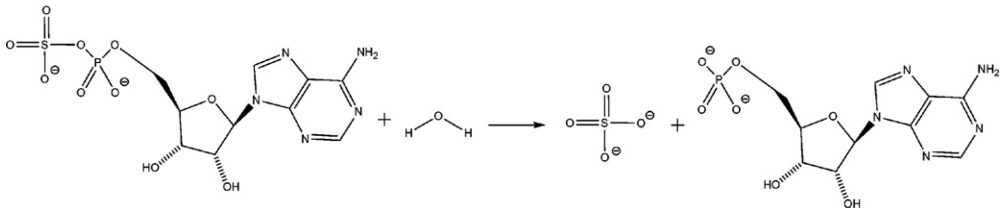


Fig. 1. Hydrolysis of different substrates by the human Fhit analyzed by TLC. The reaction mixture (50 μl final volume) contained 50 mM Mes/KOH (pH 6.5), 5 mM $MgCl_2$, 1 mM indicated substrate and 0.2 μg of recombinant human Fhit. Incubation was carried out at 30 °C. At times indicated, 3 μl aliquots were spotted on a TLC plate (aluminium precoated with silica containing fluorescent indicator, from Merck). The chromatogram was developed in dioxane:ammonia:water (6;1:4, by vol.) and photographed under short-wave UV light.

3.3. Fhit proteins act as adenylyl sulfate sulfohydrolase

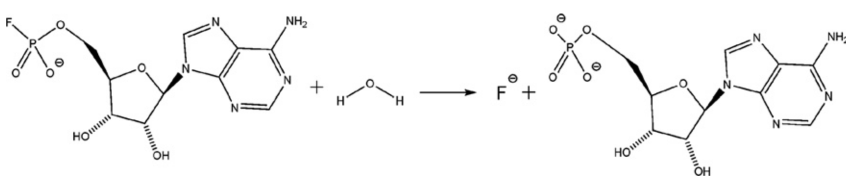
We further tested the promiscuity of Fhits in catalyzing the hydrolysis of different AMP-containing compounds. We found that the mixed anhydride, sulfoadenylate (S-pA), was also recognized by the Fhits as a substrate (Reaction 3):



This liberation of AMP from S-pA did not require Mg^{2+}. At 1 mM substrate concentration the catalytic release of AMP from S-pA proceeded at only slightly lower rates than the release of AMP from NH_2-pA: 1.5-fold lower with the human Fhit and 1.3-fold lower with *Arabidopsis* Fhit (Table 1).

3.4. Fhit proteins catalyze the hydrolysis of P–F bond

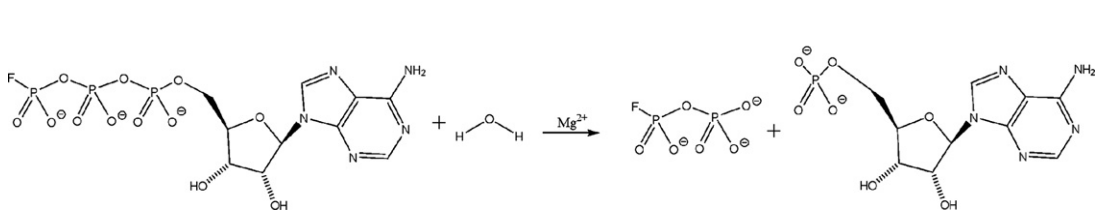
Adenylylfluoride (F-pA), was also tested as a potential substrate of human and plant Fhits. We have found that this non-natural nucleotide is also recognized as substrate for which P–F cleavage does not depend on Mg^{2+} (Fig. 1 and Table 1, see Reaction 4):



The rates of release of AMP from F-pA were 4- and 1.6-fold lower for the human and plant Fhits, respectively, than those from NH_2-pA.

3.5. Liberation of AMP from F-pppA

We have also examined the substrate behavior of F-pppA. This ATP analogue is recognized as a substrate of Fhits only in the presence of Mg^{2+} and was cleaved to AMP (Fig. 1) and fluoropyrophosphate (Reaction 5):



The hydrolysis products were identified by ^{31}P NMR. Three major peaks are present in the 1D spectrum of the reaction products (Fig. 2). These correspond to AMP (3.6 ppm, [29]), and fluoropyrophosphate. The $^2J_{PP}$ coupling (17 Hz) and $^1J_{PF}$ coupling (920 Hz) are as expected for fluoropyrophosphate, while fluoromonophosphate would give rise to a double singlet at −3 ppm [30,31].

We also confirmed earlier observations [13] that Fhit proteins do not hydrolyze ATP, ADP and adenosine 5′-tetraphosphate. It can be added here that ADP-ribose was also not cleaved to AMP and that diadenosine pyrophosphate (AppA), a good substrate for phosphodiesterase I, was hydrolyzed at an extremely low rate; more than 10 000-fold slower than the rate of the hydrolysis of NH_2-pA.

4. Discussion

Fhit proteins comprise one of three branches of proteins within the HIT superfamily [14]. Whereas Fhits have been recognized primarily as typical dinucleoside triphosphatases [10,16], their adenosine phosphoramidase activity is known as a feature of a different branch called Hint [14,32,33]. In their elucidation of the mechanism of human Fhit action, Huang et al. showed that the enzyme cleaves adenosine phosphoimidazolide, an analog of the reaction intermediate having a P–N bond. We have used the simplest nucleoside phosphoramidate, NH_2-pA, and established that both human and *Arabidopsis* Fhits function as effective nucleoside phosphoramidases. It has already been shown that two enzymatic activities capable of deaminating NH_2-pA and yielding AMP exist in a higher plant (*Lupinus luteus*). One of them was a feature of the yellow lupin Ap_3A hydrolase and the other a typical nucleoside phosphoramidase that did not exhibit the Ap_3A-ase activity [34]. Those and the present observations concerning the *Arabidopsis* Fhit strongly suggest that the yellow lupin enzyme characterized previously as a dinucleoside triphosphatase [35] is in fact a Fhit protein.

Our current study also shows that Fhit proteins catalyze cleavage of S-pA to AMP and sulfate. Till now that reaction

Table 2
Inhibition of the adenosine phosphoramidase activity of human and *Arabidopsis* Fhit proteins by various nucleotides

Inhibitor	K_i (μM)	
	Human Fhit	*Arabidopsis* Fhit
S-pA	13.6 ± 2.2	7.1 ± 1.6
ApppA/Mg^{2+}	18.0 ± 3.0	2.1 ± 0.2
F-pppA/Mg^{2+}	>1000	235 ± 35
F-pA	>1000	18.2 ± 0.7
pA	110 ± 15	113 ± 18
ppA	>1500	140 ± 20
pppA	>2000	145 ± 22

K_i values are means of three independent determinations. For details see Section 2.2. The K_m values estimated for adenosine 5′-phosphoramidate for the both Fhits were 3 μM.

has been assigned to an adenylyl sulfate sulfohydrolase (EC 3.6.2.1). Such an enzymatic activity was identified in rat liver extracts and it did not hydrolyze ATP [36]; as is the case with Ap_3A hydrolases/Fhits [33, and this study]. Neither ApppA nor NH_2-pA was then tested as potential substrates of that sulfohydrolase.

Because of the nucleoside phosphoramidase and the adenylyl sulfate sulfohydrolase activities identified in this work, Fhits must now be considered as enzymes involved in the metabolism of *many* AMP-containing compounds, a list minimally including diadenosine triphosphate, naturally occurring nucleoside 5′-phosphoramidates and adenosine 5′-phosphosulfate. The influence of Fhits on the metabolism of adenosine 5′-phosphosulfate may be even more important for cells than Fhits regulation of the metabolism of dinucleoside triphosphates. The newly revealed activities should be taken into account particularly in the study of the anti-oncogenic function of Fhit and extend it beyond the binding and/or hydrolysis of dinucleoside polyphosphates [37,38].

It has been known for some time that mutations or deletions of the Fhit genes results in an increased incidence of spontaneous tumor formation in humans [37]. Recently, a similar correlation has been observed in mice having a deletion of the Hint gene [39]. The results of our study suggest that in vivo Fhit may recognize Hint substrates, i.e., at least nucleoside phosphoramidates. The loss of Hint may thereby be alleviated by Fhit enzymatic activity. Therefore, potential defects caused by the loss of HINT may be visible only when FHIT is also inactivated.

Their capacity to liberate AMP from F-pA broadens the catalytic promiscuity of Fhits and shows that, with respect to cleavage of the P–F bond, Fhits act like a phosphodiesterase I (EC 3.1.4.1), as first reported by Wittmann [23]. Subsequently, the latter enzyme was demonstrated to split the P–F bond in uridine 5′-*O*-phosphorofluoridate [40], inosine 5′-*O*-phosphorofluoridate [41] and thymidine 5′-*O*-phosphorofluoridothioate [42].

Liberation of AMP from F-pppA sheds new light on the substrate requirements of Fhits. F-pppA appears to mimic one of the natural substrates of Fhits, ApppA, both nucleotides having three phosphate negative charges. In Fhit-catalyzed reactions, the nucleophilic histidine attacks P^{α} in both nucleotides to displace ADP in case of ApppA but fluoropyrophosphate in the case of F-pppA. In the past, F-pppA has been studied in various enzymatic systems. Haley and Yount reported that the compound was cleaved to AMP and fluoropyrophosphate by snake venom phosphodiesterase [43]. Thus, the latter behaves as the Fhits.

The newly identified properties of Fhits can also be used in studies on the delivery of pronucleotides to target cells or organisms [44]. It seems viable that nucleoside phosphoramidates can be employed as nucleotide prodrugs. The same may be true for nucleoside phosphofluoridates that are also less polar than nucleoside monophosphates and therefore should penetrate the cell membrane better. As mentioned above, the plant-specific phosphoramidase did not hydrolyze ApppA [34]. Whether such specific phosphoramidases can cleave nucleoside phosphofluoridates thereby liberating nucleoside monophosphates from prodrugs is worthy of further study. Our work shows that both the specific nucleoside phosphoramidases (Hint proteins) and Fhits may be important in the metabolism of such prodrugs.

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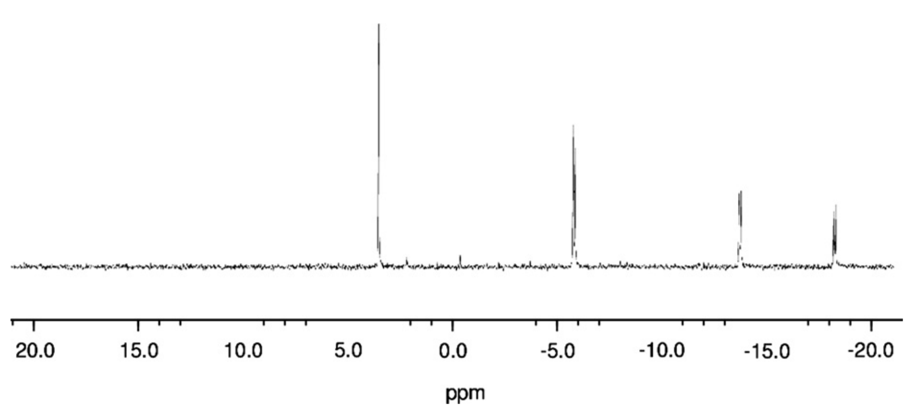

Fig. 2. ^{31}P NMR spectrum of the products of the reaction of ATP-γF with human Fhit. The reaction mixture (0.1 ml total volume) contained 50 mM Hepes/KOH (pH 7.8), 5 mM $MgCl_2$, 1 mM F-pppA and 1 μg of the human Fhit. The reaction was carried out at 25 °C and its completion confirmed by TLC. Peaks are assigned as follows: 3.6 ppm (singlet) AMP; −5.8 ppm (d, $^2J_{PP}$ = 17 Hz), fluoropyrophosphate (FPP); −16 ppm (dd, $^1J_{FP}$ = 920 Hz, $^2J_{PP}$ = 17 Hz), fluoropyrophosphate (F**P**P). Spectra were recorded with 32 K scans using a spectral width of 12136 Hz and 4 K points resulting in 338 ms acquisition time. Couplings are accurate to 3 Hz. Assignments made in comparison to those published [30,45].

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