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Dual activity of certain HIT-proteins: *A. thaliana* Hint4 and *C. elegans* DcpS act on adenosine 5′-phosphosulfate as hydrolases (forming AMP) and as phosphorylases (forming ADP)

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ABSTRACT

Histidine triad (HIT)-family proteins interact with different mono- and dinucleotides and catalyze their hydrolysis. During a study of the substrate specificity of seven HIT-family proteins, we have shown that each can act as a sulfohydrolase, catalyzing the liberation of AMP from adenosine 5′-phosphosulfate (APS or SO_4-pA). However, in the presence of orthophosphate, *Arabidopsis thaliana* Hint4 and *Caenorhabditis elegans* DcpS also behaved as APS phosphorylases, forming ADP. Low pH promoted the phosphorolytic and high pH the hydrolytic activities. These proteins, and in particular Hint4, also catalyzed hydrolysis or phosphorolysis of some other adenylyl-derivatives but at lower rates than those for APS cleavage. A mechanism for these activities is proposed and the possible role of some HIT-proteins in APS metabolism is discussed.

1. Introduction

Recently, Fhit proteins have been shown to catalyze liberation of 5′-AMP not only from diadenosine triphosphate (App-pA), a known substrate since 1996 [1], but also from other natural and synthetic adenylyl-containing nucleotides, such as adenosine 5′-phosphoramidate (NH_2-pA), adenosine 5′-phosphosulfate (SO_4-pA, commonly abbreviated as APS), adenosine 5′-phosphofluoride (F-pA) and adenosine 5′-(γ-fluorotriphosphate) (Fpp-pA) [2]. Fhit proteins belong to the histidine triad (HIT) superfamily and possess a HIT motif in their active sites. The HIT genes occur in different phyla and Hit proteins have been shown to be involved in the metabolism of different nucleotides, such as dinucleoside polyphosphates including mRNA 5′ caps [3–5], and in the interaction with some transcription factors [6]. In addition to the previously studied human and *Arabidopsis thaliana* Fhits [2], we have now examined the substrate specificity of six other recombinant HIT-proteins (Table 1) and found that SO_4-pA is a substrate for each of them. The proteins readily cleaved SO_4-pA to AMP and sulfate in Hepes buffer (pH 6.8). However, when the assays were performed in potassium phosphate buffer at pH 6.8, two proteins, *A. thaliana* Hint4 (whose function has not been established), and the scavenger mRNA decapping enzyme DcpS from *Caenorhabditis elegans* [3,4], converted SO_4-pA not only to AMP but also to ADP. Some other adenylyl-containing compounds were also substrates in these two types of reaction, particularly in the case of *A. thaliana* Hint4 that exhibited stronger catalytic duality and broader substrate specificity than DcpS. Here, we present data on that serendipitous catalytic duality, propose a potential mechanism for it, and speculate on presumable role of Hint4 in the APS metabolism in plants.

2. Materials and methods

2.1. Chemicals

AMP (pA), ADP (ppA), ATP (pppA), Ap_3A (ApppA), Ap_4A (AppppA), NH_2-pA and most bulk reagents were from Sigma, St.

Abbreviations: APS or SO_4-pA, adenosine 5′-phosphosulfate; IPS or SO_4-pI, inosine 5′-phosphosulfate; HIT, histidine triad; HPLC, high performance liquid chromatography; TLC, thin layer chromatography

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Table 1
Origin of the recombinant HIT-proteins and procedures used for their purification.

Recombinant HIT-protein	Origin	GeneBank entry	Vector/*E. coli* strain	Final purification (affinity resin/eluting agent)	Ref.
Fhit	*H. sapiens*	NP 002003	pSG02/BL21	AMP-agarose/1 mM adenosine	[2]
Hint1	*A. thaliana*	AT3G56490	pSG02/BL21	AMP-agarose/1 mM adenosine	This paper
Hint3	*A. thaliana*	AT5G48545	pSG02/BL21	AMP-agarose/1 mM adenosine	This paper
Hint4	*A. thaliana*	AT4G16566	pSG02/BL21	AMP-agarose/1 mM adenosine	This paper
GST-aprataxin like	A. *thaliana*	AT5G01310	pGEX-5X1/BL21	Glutathione-agarose/10 mM glutathione	This paper
HinT	*E. coli*	NC 012892	pSG02/BL21	AMP-agarose/1 mM adenosine	This paper
His-DcpS (DCS-1)	*C. elegans*	NP 507876	pET-16b/BL21	Ni^{2+}-agarose/250-300 mM imidazole	[3]

Louis, MO, USA. APS (SO_4-pA) was synthesized by coupling of 5′-AMP imidazolide with sulphate (VI) triethylammonium salt in dimethylformamide in the presence of excess $ZnCl_2$, adapting methodology previously developed for the synthesis of nucleoside 5′-(2-thiodiphosphates) [7]. The reaction mixture was chromatographed on DEAE-Sephadex A25 (HCO_3^- form) and the product converted to the sodium salt using Dowex Wx8 (200–400 mesh) resin. The structure and homogeneity of the compound were confirmed by reverse phase high performance liquid chromatography (HPLC) analysis, electrospray mass spectrometry in negative mode (ESI-MS), ^{1}H NMR and ^{31}P NMR spectroscopy. Complete synthesis details and discussion about the utility of the chemical method will be published elsewhere. This preparation was much purer and more stable than any commercially available APS. Inosine 5′-phosphosulfate (IPS or SO_4-pI) was obtained by deamination of APS catalyzed by adenosine phosphate deaminase from *Helix pomatia* [8]. Carrier-free [^{33}P]-phosphoric acid (30 μM) was purchased from Hartmann Analytic GmbH, Braunschweig, Germany.

2.2. Recombinant proteins

Using human HIT-proteins as a template in a BLAST homology search, we identified five different proteins containing HIT motif in *A. thaliana* genome. We named them Hint1, 2, 3, 4 and Aptx. Note that human Hint3 and Hint4 proteins are in fact product of the same gene represented by two different GeneBank entries, whereas *Arabidopsis* Hint3 and Hint4 are products of two separate genes. *Arabidopsis* Hint3 is more similar to human Hint1 and Hint2 (27% and 29% identity, respectively), whereas *Arabidopsis* Hint4 is 39% identical to human Hint3 or Hint4. We were not able to obtain proteins Hint2 in *Escherichia coli* cells. Origin and purification procedures employed to obtain other *Arabidopsis* HIT-proteins and three other HIT-proteins used in this study are summarized in Table 1. The proteins showed at least 90% purity by SDS–PAGE and their electrophoretic mobility agreed with predicted molecular mass.

2.3. Enzyme assays

Hydrolytic and/or phosphorolytic activities of the proteins were assayed in reaction mixtures (50 μl) containing 1 mM substrate, enzyme protein and one of the following buffers: 0.1 M Hepes/KOH buffer (pH 6.8), 0.1 M potassium phosphate (pH 6.8), 0.1 M KH_2PO_4 (pH 4.2) or 0.1 M K_2HPO_4 (pH 8.2). The reactions were carried out at 30 °C. Qualitative visualization of the reactions was performed by thin layer chromatography (TLC). At time intervals, 3 μl aliquots of the reaction mixture were spotted on to an aluminum plate precoated with silica gel containing fluorescent indicator (from Merck), developed for 40 min usually in dioxane/25% ammonia/water (6:1:5, by volume), dried and visualized/photographed under short-wave UV light in the gel visualization system (G:Box, Syngene).

Quantitative measurements were performed either by HPLC or, in the case of phosphorolysis, with labeled [^{33}P]-orthophosphate which was separated from the reaction product [β-^{33}P]ADP by TLC. (In the chromatographic system used, orthophosphate moves only slightly from the origin.)

HPLC analysis allowed estimation of the initial rates of degradation of APS and other nucleotides based on peak areas. Five microlitres aliquots of the reaction mixture were added to 95 μl of preheated (95 °C) water and kept at that temperature for 3 min, then chilled and kept frozen prior to chromatography. Each sample was first diluted twofold with 50 mM triethylamine buffer (TEAB) (pH 7.4 adjusted with carbonate) and 20-μl aliquots applied onto the reverse-phase column (Teknokroma C_{18}, 150 mm × 4.6 mm; 5 μm). The column was eluted with a linear gradient of 50 mM TEAB (pH 7.4): acetonitrile (97:3, v/v) (solvent A) and solvent A:acetonitrile (60:40, v/v) (solvent B); 0–15 min, 20% B, at a flow-rate of 1 ml/min. Retention times (min) were as follows: pA 3.5; NH_2-pA 4.0; ppA 4.5; SO_4-pA 5.5; Appp-pA 8.1; App-pA (Ap_3A) 9.8 and F-pA 12. Modifications to reaction mixtures introduced for particular experiments are described in the legends to relevant figures.

2.4. Determination of K_m values

Two examples are described in detail. The K_m value for orthophosphate determined for phosphorolysis catalyzed by DcpS was estimated in a 25 μl reaction mixture containing 3.6 mM APS, 0.17 μg DcpS protein and various concentrations of [^{33}P] KH_2PO_4 (20–300 mM). Incubation was carried out at 30 °C. To estimate initial velocities, 3-μl aliquots were transferred on to the origin of a TLC plate after 3, 6, 9 and 12 min. Amounts of radioactivity in the 3-μl aliquots varied between 60 000 and 960 000 cpm. Each spot also included 5 nmol ADP standard. The chromatogram was developed as described above and the nucleotide spots visualized under short-wave UV light. The ADP areas were cut out, immersed in 5 ml of scintillation fluid and radioactivity counted in Beckman LS 3801 counter.

With Hint4, the K_m for APS was estimated in a 25 μl reaction mixture containing 175 mM [^{33}P] KH_2PO_4, 0.05 μg Hint4 protein and various concentrations of APS (0.45–3.6 mM). Estimation of the initial velocities was conducted as described above. In this experiment, 3-μl aliquots of each reaction mixture contained 1 200 000 cpm.

3. Results

3.1. Discovery of dual catalytic activity of Hint4 and DcpS

Following our studies on the substrate specificity of the Fhit proteins [2], we investigated six other HIT-family proteins. In Hepes buffer (pH 6.8), all of them displayed adenylylsulfate sulfohydrolase (EC 3.6.2.1) activity [9], efficiently converting APS to AMP (see Fig. 1, reaction 1). The same activity was displayed by all when assayed in potassium phosphate buffer (pH 6.8). However two of the proteins, *A. thaliana* Hint4 and *C. elegans* DcpS, produced ADP in addition to AMP, thus behaving as ADP-sulfurylases (EC

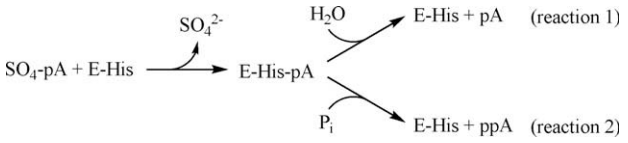

Fig. 1. Possible alternative reactions catalyzed by the *Arabidopsis* Hint4 protein in 0.1 M potassium phosphate (pH 6.8).

2.7.7.5) (Fig. 1, reaction 2 and Fig. 2). (The term "ADP-sulfurylase" is actually misleading as these enzymes catalyze the *irreversible* displacement of sulfate from APS by orthophosphate and so should be called "adenylylsulfate:phosphate adenylyltransferase" [10].) After this pilot experiment, we focused on the two HIT-proteins (*A. thaliana* Hint4 and *C. elegans* DcpS) that exhibited dual activities. We characterized their phosphorolytic and hydrolytic activities as well as substrate specificities.

3.2. Effect of pH

The phosphorylase activities of both proteins (Hint4 and DcpS) were enhanced in acidic pH values (0.1 M KH_2PO_4, pH ≈ 4.2), whereas high pH (0.1 M K_2HPO_4, pH ≈ 8.2) converted them exclusively into hydrolases, although each behaved somewhat differently. Whereas at pH 4.2 Hint4 acted almost exclusively as an APS phosphorylase, DcpS still exhibited dual activity (Fig. 3). Hint4 degraded APS phosphorolytically twice as fast at pH 4.2 than at pH 6.8. Based on this observation, further characterization of the phosphorolytic activities was conducted at pH 4.2 and the hydrolytic activities at pH 8.2. None of other five HIT-proteins assayed in 0.1 M KH_2PO_4 catalyzed phosphorolysis of APS.

3.3. Substrate specificity of the phosphorolytic reaction

Hint4 and DcpS were tested for their ability to recognize nucleotides other than APS as substrates. IPS and APS were converted to IDP and ADP, respectively, at the same rate. Results of preliminary experiments performed with Hint4 and monitored by TLC are shown in Fig. 4. We also compared rates of degradation of different adenylyl-containing compounds. While APS was the most effective substrate, Hint4-catalyzed with lower rates the phosphorolysis of NH_2-pA, F-pA, App-pA and Appp-pA (Figs. 5 and 6).

Using $^{33}P_i$, we quantitatively compared the rates of the [β-^{33}P]ADP formation by incubating Hint4 with different substrates. At 0.1 M [^{33}P] KH_2PO_4 the relative reaction rates (percent) were as follows: APS 100, NH_2-pA 22, F-pA 8, Ap_3A 7 and Ap_4A 2. The enzyme also catalyzed slow ADP ↔ P_i exchange with a rate relative to the fastest reaction mentioned above (displacement of sulfate from SO_4-p A by phosphate) of approximately 2.

DcpS protein had a narrower substrate specificity than Hint4. In the mixtures containing 0.1 M [^{33}P] KH_2PO_4 and 1 mM substrate, DcpS produced ADP 20 times faster from APS than from App-pA. Neither Appp-pA, NH_2-pA nor F-pA underwent both phosphorolysis or hydrolysis regardless of pH (Table 2).

K_m values were estimated only in the monobasic potassium phosphate at pH 4.2 (see Section 2.4). The K_m values for orthophosphate were 600 ± 100 mM for Hint4 and 46 ± 6 mM for DcpS protein. Both proteins function as homodimers and the calculated k_{cat} value of the phosphorolytic reaction for Hint4 was 36.2 s^{-1} and for DcpS 2.6 s^{-1}. The K_ms for APS were 1 ± 0.2 mM and 26 ± 4 mM, respectively.

When assayed in 0.1 M KH_2PO_4 with APS, neither enzyme could use pyrophosphate or tripolyphosphate as potential adenylate

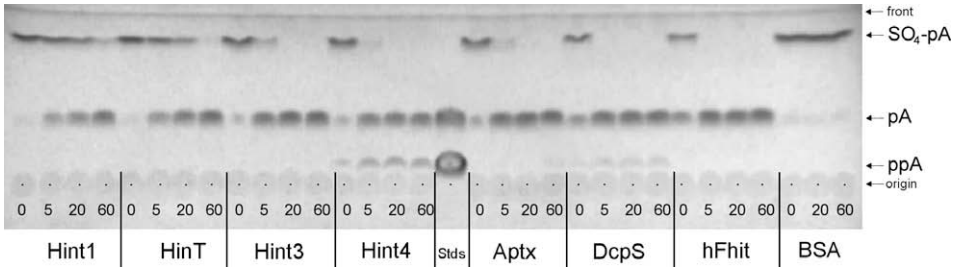

Fig. 2. Cleavage of adenosine 5′-phosphosulfate by different HIT-proteins. The reaction mixture (25 μl) contained 0.1 M potassium phosphate (pH 6.8), 1 mM APS and approximately 1 μg of the indicated HIT-protein or bovine serum albumin (BSA) used as a control. Incubation was carried out at 30 °C. At time intervals (0, 5, 20 and 60 min) 3 μl aliquots were spotted on the origin and the chromatogram developed in dioxane:25% ammonia:water (6:1:5, by volume). Standards of authentic AMP and ADP were also chromatographed.

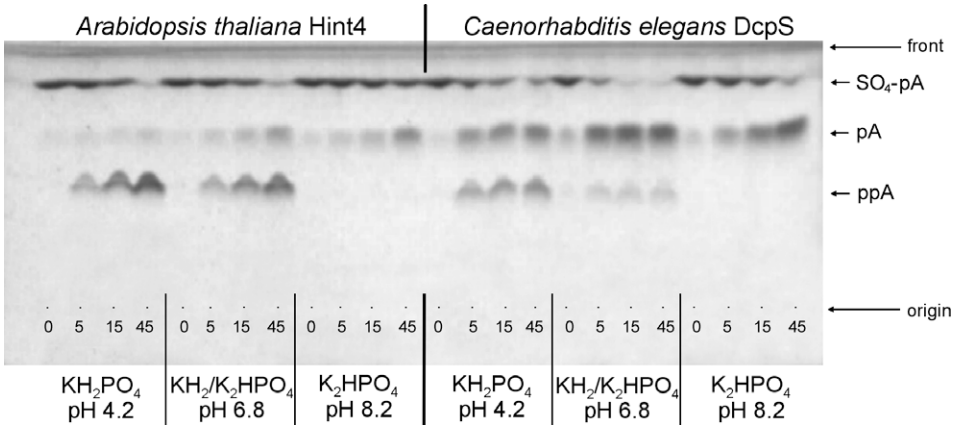

Fig. 3. Cleavage of APS by *A. thaliana* Hint4 and *C. elegans* DcpS proteins in different phosphate solutions. The reaction mixtures (25 μl) contained 0.2 M potassium phosphate as indicated, 1 mM APS and either 0.1 μg Hint4 or 0.45 μg of DcpS. Incubation and analysis of the reaction mixtures were performed as described in the legend to Fig. 1.

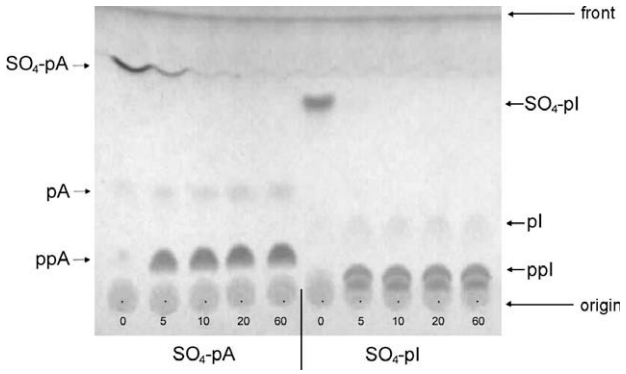

Fig. 4. Time-course of phosphorolysis of APS and IPS catalyzed by the *A. thaliana* Hint4 protein. The reaction mixture contained 0.2 M KH_2PO_4, 1 mM APS or IPS and 0.4 μg Hint4 protein. Chromatogram was developed in dioxane:25% ammonia:water (6:1:5, by volume).

acceptors in place of orthophosphate; neither ATP (pppA) nor adenosine 5′-tetraphosphate (ppppA) was formed in the reaction mixtures.

3.4. Substrate specificity of the hydrolytic reactions

The relative rates of the adenylate release catalyzed by Hint4 in 0.1 M K_2HPO_4 (pH ≈ 8.2) from different adenylyl-containing compounds were: APS 100, NH_2-pA 42, F-pA 17 and p-pA (ADP) 3. (It should be noted that ADP is stable for at least 2 h in all 0.1 M phosphate solutions used in this study.) No AMP release was observed either from App-pA or Appp-pA when incubated with Hint4 at pH 8.2. The products of all these reactions were analyzed by HPLC. Also at this higher pH DcpS exhibited much narrower substrate specificity than did Hint4. Of the compounds studied, only APS and App-pA were substrates for DcpS. Hydrolysis of the former proceeded in 0.1 M K_2HPO_4 20–25 times faster than of the latter (Table 2).

The dual catalytic ability of DcpS was observed only at low pH. At neutral and higher pH APS was converted exclusively to AMP. In 0.1 M K_2HPO_4, App-pA was hydrolyzed to AMP plus ADP and in the latter case DcpS behaved as a typical dinucleoside triphosphatase. (App-pA was hydrolyzed at the highest rate at neutral pH.) Interestingly, DcpS catalyzed degradation of App-pA in the absence of exogenous Mg^{2+}. In addition, 1 mM EDTA did not affect the reaction rate. In this respect DcpS differs from the Fhit proteins that strictly require divalent cations for their dinucleoside triphosphatase activity.

3.5. Effects of additions to the reaction mixtures

None of the following ions or compounds affected the APS-degrading activities of the Hint4 and DcpS proteins: $MgCl_2$ (up to 5 mM), dithiothreitol (up to 1 mM), EDTA (up to 10 mM), NaF (up to 10 mM) and imidazole (up to 20 mM). We also checked whether the rates of APS phosphorolysis catalyzed by Hint4 differed in the presence of the phosphate counter-cations such as K^+, Na^+ and NH_4^+

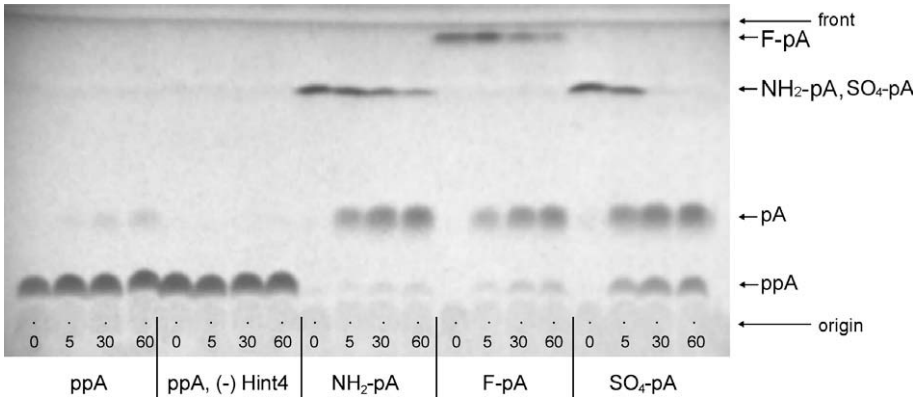

Fig. 5. Degradation of different adenylates by the *A. thaliana* Hint4 protein. The reaction mixtures contained 0.1 M potassium phosphate buffer pH 6.8, 2 mM substrate or ADP and 0.8 μg Hint4 protein. At the indicated times, 3-μl aliquots were spotted on to the origin and chromatographed as described in Section 2.3.

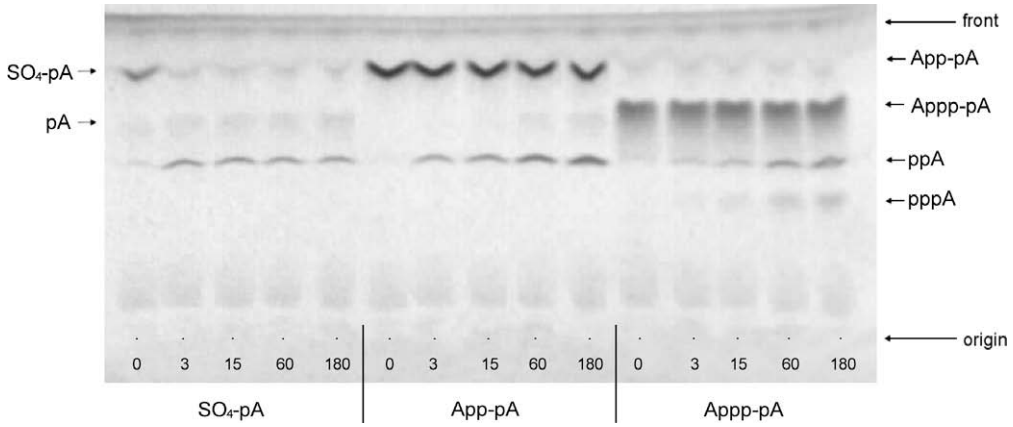

Fig. 6. Comparison of degradation of APS and two diadenosine polyphosphates Ap_3A and Ap_4A by the Hint4 protein. The reaction mixture contained 0.2 M KH_2PO_4 (pH 4.2), 1 mM substrate and 2 μg Hint4 protein. At the indicated times 3-μl aliquots were spotted on to the origin and chromatographed as described in Section 2.3.

Table 2
Relative velocities of the phosphorolysis or hydrolysis of different adenylyl-derivatives catalyzed by Hint4 of *Arabidopsis thaliana* and DcpS of *Caenorhabditis elegans*.

Substrate (1 mM)	Relative velocities (%)[a]			
	Phosphorolys		Hydrolysis	
	Hint4	DcpS	Hint4	DcpS
SO_4-pA (APS)	100	100	100	100
NH_2-pA	22	0	42	0
F-pA	8	0	17	0
App-pA	7	5	0	4
Appp-pA	2	0	0	0

The reactions of phosphorolysis (yielding ADP) were conducted in 0.1 M [^{33}P] KH_2PO_4 (pH 4.2) and the reactions of hydrolysis (yielding AMP) in 0.1 M K_2HPO_4 (pH 8.2). Other details are provided in Section 2.

[a] The velocities are related to the velocity obtained in each case for 1 mM APS.

but no difference in velocity was observed when the reaction was carried out either in 0.1 M KH_2PO_4, NaH_2PO_4 or $NH_4H_2PO_4$.

4. Discussion

4.1. On HIT-proteins and APS metabolism

Capability of different HIT-proteins to degrade APS (SO_4-pA), either hydrolytically or phosphorolytically ([2] and this work) should not be ignored by those who study sulfur metabolism and the fates of APS, in particular. This nucleotide has been known as a natural metabolite since 1950-ties, when Lipmann and co-workers demonstrated its formation in the following reaction catalyzed by the yeast ATP-sulfurylase (EC 2.7.7.4) [11]:

$$\mathrm{ATP} + \mathrm{SO_4^{2-}} \rightleftarrows \mathrm{SO_4\text{-}pA} + \mathrm{PP_i} \qquad \text{(reaction 3)}.$$

This enzyme was then demonstrated in many phyla, including mammals [12] and plants [12–14]. Its role is to introduce sulfate anion into the cell and to sulfur-metabolic pathways. Since the equilibrium of reaction 3 favors the conversion of SO_4-pA to ATP, under physiological conditions the former metabolite undergoes an instant phosphorylation catalyzed by specific adenylylsulfate kinase (EC 2.7.1.25) that yields 3′-phosphoadenosine 5′-phosphosulfate (PAPS). PAPS is an "active sulfate" and serves as a sulfate donor in biological systems. We speculate that *Arabidopsis* Hint4 or its plant ortholog, due to functioning in acidic pH as an absolute APS phosphorylase, can affect APS metabolism in the following way: as has been demonstrated by plant physiologists, P_i adsorption acidifies the cytoplasm [14]. This and local availability of orthophosphate with simultaneous accumulation of APS (due to possible unavailability of ATP or malfunctioning of APS kinase) could enhance the Hint4-catalyzed conversion of APS to ADP.

4.2. On the discovered catalytic duality of some HIT-proteins

To the best of our knowledge, enzymes that catalyze the degradation of nucleosides or nucleotides have been classified either as hydrolases or phosphorylases (transferases). For example, purine nucleosides (adenosine, guanosine and inosine) are cleaved hydrolytically to ribose and the corresponding base by more or less specific nucleosidases (EC 3.2.2.x) that occur in plants, fungi, protozoa and bacteria [15]. Animals, including humans, possess purine nucleoside phosphorylases (EC 2.4.2.1) specific for guanosine and inosine which catalyze phosphorolytic cleavage of the *N*-glycosidic bond yielding ribose-1-phosphate and guanine or hypoxanthine, respectively [16]. Dinucleoside tetraphosphates undergo either hydrolysis, catalyzed by asymmetrically (EC 3.6.1.17) or symmetrically (EC 3.6.1.41) acting hydrolases, or phosphorolysis catalyzed by specific phosphorylases (EC 2.7.7.53) [17]. We know of no condition under which these phosphorylases could act as hydrolases or vice versa. Therefore, we were surprised to observe that, in addition to possessing hydrolytic activity like all other HIT-proteins, two of seven investigated proteins catalyzed phosphorolysis of different adenylates, degrading APS at the highest rate.

Phosphorolysis of APS yielding ADP was observed only in the phosphorolytic direction in crude yeast extract over half a century ago [11]. Later, work with homogeneous Ap_4A phosphorylase from the yeast *Saccharomyces cerevisiae* showed that this enzyme exhibits a property attributed to ADP-sulfurylase; the estimated k_{cat} was 58 s^{-1} [18]. More recently, the same irreversible reaction leading from APS to ADP was demonstrated for homogeneous protein isolated from *Thiobacillus denitrificans*. Based on the presented data, we calculated the k_{cat} as 2916 s^{-1} [10]. The two HIT-enzymes described in this communication extend the list of identified "ADP-sulfurylases". However, neither the yeast Ap_4A phosphorylase nor the adenylylsulfate:phosphate adenylyltransferase from *Thiobacillus* showed hydrolytic cleavage of APS in addition to phosphorolysis as do the *A. thaliana* Hint4 and *C. elegans* DcpS proteins. All the HIT-proteins investigated here catalyzed hydrolysis of APS but only two of them displayed dual activity. We propose the following explanation for the action of orthophosphate: the fact that phosphorolysis catalyzed by Hint4 and DcpS is promoted at acidic pH suggests that there may be some acidic amino acid residues within the enzyme active sites whose negative charge is eliminated or reduced at pH 4.2 resulting in a loss of repulsion between the negatively charged dihydrogen phosphate that prevails at this pH ($H_2PO_4^-$) and these residues. Under these conditions the anion can displace the adenylyl-moiety from the presumed enzyme:AMP intermediate. (See Ref. [10] for the proposed ping-pong mechanism according to which the reaction probably proceeds.) Evaluation of this hypothesis will require detailed conformational studies supported by site-directed mutagenesis. In case of the *C. elegans* DcpS protein, two amino acid residues, D179 and E159, might be first candidates to be checked whether they are involved in the catalytic duality. Comparison of the amino acid sequences of the *C. elegans* DcpS and human DcpS [19] shows that those two acidic amino acid residues are involved in substrate binding.

At this stage the reported catalytic duality of two HIT-family proteins is only a test-tube curiosity and any biological implication remains to be determined.

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