ACTA ABP BIOCHIMICA POLONICA

Vol. 60, No 2/2013
249–254
on-line at: www.actabp.pl

Regular paper

Hint2, the mitochondrial nucleoside 5′-phosphoramidate hydrolase; properties of the homogeneous protein from sheep (*Ovis aries*) liver

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Adenosine 5′-phosphoramidate (NH_2-pA) is a rare natural nucleotide and its biochemistry and biological functions are poorly recognized. All organisms have proteins that may be involved in the catabolism of NH_2-pA. They are members of the HIT protein family and catalyze hydrolytic splitting of NH_2-pA to 5′-AMP and ammonia. At least five HIT proteins have been identified in mammals; however, the enzymatic and molecular properties of only Fhit and Hint1 have been comprehensively studied. Our study focuses on the Hint2 protein purified by a simple procedure to homogeneity from sheep liver mitochondrial fraction (OaHint2). Hint1 protein was also prepared from sheep liver (OaHint1) and the molecular and kinetic properties of the two proteins compared. Both function as homodimers and behave as nucleoside 5′-phosphoramidate hydrolases. The molecular mass of the OaHint2 monomer is 16 kDa and that of the OaHint1 monomer 14.9 kDa. Among potential substrates studied, NH_2-pA appeared to be the best; the K_m and k_{cat} values estimated for this compound are 6.6 μM and 68.3 s^{-1}, and 1.5 μM and 11.0 s^{-1} per natively functioning dimer of OaHint2 and OaHint1, respectively. Studies of the rates of hydrolysis of different NH_2-pA derivatives show that Hint2 is more specific towards compounds with a P-N bond than Hint1. The thermostability of these two proteins is also compared.

Key words: histidine triad nucleotide binding proteins, nucleoside 5′-phosphoramidase, Hint1, Hint2, purification to homogeneity

Received: 28 March, 2013; revised: 04 June, 2013; accepted: 10 June, 2013; available on-line: 14 June, 2013

INTRODUCTION

Adenosine 5'-phosphoramidate (NH_2-pA) is believed to occur in all organisms. However, only one paper by Frankhauser and coworkers (1981a) has reported the detection of this compound among cellular nucleotides purified from the green alga *Chlorella pyranoidosa*. The same authors showed that NH_2-pA is a product of the following reaction catalyzed by adenylylsulfate:ammonia adenylyltransferase (EC 2.7.7.51):

$$SO_4\text{-pA} + NH_4^+ \rightarrow NH_2\text{-pA} + SO_4^{2-} + 2H^+.$$

They demonstrated this transferase activity in *Ch. pyranoidosa, Euglena gracilis, Dictyostelium discoideum, Escherichia coli*, and in higher plants such as spinach and barley (Frankhauser *et al.*, 1981b). Recently, we have found the same activity in mammalian tissues (Wojdyła-Mamoń & Guranowski, unpublished). The supposition that NH_2-pA is a ubiquitous compound and that its concentration is enzymatically controlled may be supported by the existence of various proteins that catalyze the cleavage of NH_2-pA to ammonia and either 5'-AMP (by hydrolysis) (Rossomando & Hadjimichael, 1986; Kuba *et al.*, 1994; Bieganowski *et al.*, 2002; Guranowski *et al.*, 2008; 2010a; 2010b; 2011) or 5'-ADP (by phosphorolysis) (Guranowski *et al.*, 2010a). All these enzymes probably belong to the histidine triad (HIT) superfamily and have a HIT motif in their active sites. HIT genes occur in various phyla, where the HIT proteins can control the metabolism of mononucleotides such as the aforementioned NH_2-pA and SO_4-pA (Guranowski *et al.*, 2008; 2010a; 2010b) and of dinucleoside polyphosphates, *e.g.*, ApppA (Barnes *et al.*,1996) and mRNA caps (Cohen *et al.*, 2004; Banerjee *et al.*, 2009).

Our knowledge about the biological functions of nucleoside phosphoramidates and different HIT proteins is stile obscure. One of these proteins, Hint1, have been shown to mediate the activities of protein kinases (Bieganowski *et al.*, 2002) and to interact with certain transcription factors (Korsisaari & Mäkelä, 2000; Carmi-Levy *et al.*, 2008; Weiske & Huber, 2005). Hint2, which is expressed in mitochondria, has also been demonstrated to act as a nucleoside phosphoramidase and, in addition, to sensitize hepatocarcinoma cells to apoptosis (Martin *et al.*, 2006) and to affect steroidogenesis in H295R cells, a classical model commonly used for investigating adrenal steroid biosynthesis (Lenglet *et al.*, 2008). The Hint and other HIT proteins (Fhit, aprataxin) have attracted particular interest due to the considerable evidence for their actions as tumor suppressors; see Martin *et al.* (2011) for review. Yet the detailed molecular mechanisms by which these proteins regulate carcinogenesis await elucidation. So far, there is no evidence that enzymatic activities of Hint proteins are essential for their biological role (Korisaari *et al.* 2003; Weiske & Huber, 2006). However, the HIT protein superfamily is very old and evolution-

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Abbreviations: Aptx, aprataxin; Fhit, fragile histidine triad protein; HIT, histidine triad; Hint, histidine triad nucleotide binding protein; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; ApppA, diadenosine 5′, 5‴-P^1,P^3-triphosphate; NH_2-pA, adenosine 5′-phosphoramidate; NH_2-pC, cytidine 5′-phosphoramidate; SO_4-pA, adenosine 5′-phosphorosulfate; SO_4-pC, cytidine 5′-phosphorosulfate; S-pA, adenosine 5′-phosphorothioate; S-pG, guanosine 5′-phosphorothioate; F-pA, adenosine 5′-phosphorofluoride.

arily conserved. The amino acid conservation concerns mainly the active center and this might point to the biological significance of the considered enzymatic activity. One can also speculate that a very early function of the HIT proteins was to remove nucleoside phosphoramidates and dinucleoside polyphosphates — compounds that could form under prebiotic conditions (Lohrmann, 1997). Since these compounds can be also side-products of certain enzymatic reactions, the aforementioned putative function of HIT proteins may still be valid. Conditions that favor formation of these uncommon nucleotides in the cells of contemporary organisms have been studied and reviewed (McLennan, 2000).

Recently, we studied the substrate specificity of nine recombinant HIT-proteins with seven nucleotides – their potential substrates (Guranowski *et al*., 2010b). The general conclusion of the study was that the individual proteins differ in their preferential recognition of these compounds as substrates. However, they appeared to fall into two distinct groups. One comprises the human, plant (*Arabidopsis thaliana*) and protozoan (*Trypanosoma brucei*) Fhit proteins, which exhibit quite a broad substrate specificity and catalyze efficient degradation of both dinucleoside polyphosphates (ApppA, m^7Gpppm^7G) and mononucleotides (SO_4-pA, NH_2-pA and F-pA). The second group consists of AtHint1, AtHint3, AtHint4, AtAptx (*A. thaliana* aprataxin-like protein) and EcHinT proteins, which did not catalyze hydrolysis of dinucleoside polyphosphates but efficiently degraded NH_2-pA. At the time we performed those studies (Guranowski *et al*., 2010a; 2010b), none of the Hint2 proteins was available. Based on the information that Hint2 is predominantly expressed in liver and pancreas and is localized to mitochondria (Martin *et al*., 2006; Lenglet *et al*., 2008), we have developed a purification procedure which yields homogeneous Hint2 from sheep liver mitochondria. A kinetic and molecular characterization of this protein is presented below, which supplements existing data on the substrate specificity of the HIT-proteins.

MATERIALS AND METHODS

Chemicals. Common nucleotides, adenosine 5'-phosphoramidate (NH_2-pA), adenosine 5'-phosphorothioate (S-pA) and most of the general reagents were purchased from Sigma, St. Louis, MO, USA. Adenosine 5'-phosphorosulfate (SO_4-pA) and cytidine 5'-phosphorosulfate (SO_4-pC) (Kowalska *et al*., 2012), guanosine 5'-phosphorothioate (S-pG) (Mukaiyama & Hashimoto, 1971), and adenosine 5'-phosphorofluoride (F-pA) (Wittman, 1963) were prepared as described previously. Cytidine 5'-phosphoramidate (NH_2-pC) was prepared as described by Chambers and Moffatt (1958). Briefly, cytidine 5'-phosphate (0.1 mmol) was dissolved in a mixture of 2 M ammonium hydroxide (250 μL) and dimethylformamide (170 μL) and into this solution dicyclohexylcarbodiimide (100 mg, 0.5 mmol) dissolved in *t*-butyl alcohol (650 μL) was added. The two-phase reaction mixture was heated at 80°C for 8 hours and then allowed to stand overnight at room temperature. The precipitated dicyclohexylurea was removed by filtration and washed 3 times with water. The *t*-butyl alcohol was removed under reduced pressure and the aqueous dimethylformamide solution extracted 3 times with ether. Then water was removed under reduced pressure and the desired product was isolated using ion-exchange chromatography (DEAE-Sephadex A-25) with triethylammonium bicarbonate buffer (from 0.0 to 0.4 M, pH 7.5) as the eluent. NH_2-pC was obtained with 93% yield and its identity confirmed by ^{31}P-NMR (D_2O) and fast atom bombardment mass spectrometry analyses. Structures of the nucleotides used as substrates are shown in Fig. 1.

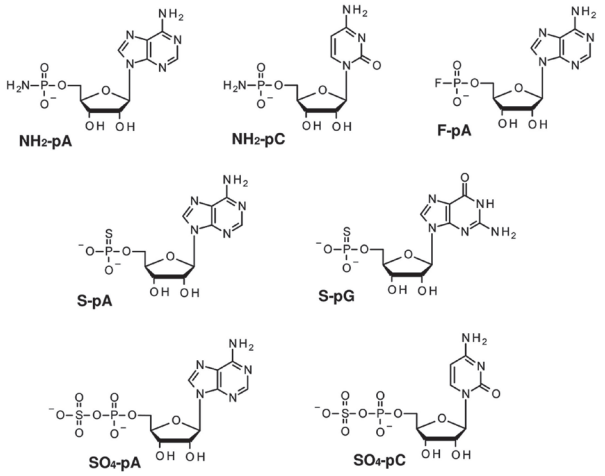

Figure 1. Structures and abbreviations of compounds used in this study.

Radiolabeled [8-^{3}H]adenosine-5'-phosphoramidate was purchased from Moravek Biochemicals, Brea, CA, USA.

Chromatographic systems. Analyses of the hydrolysis of NH_2-pA and its analogs were performed on TLC aluminum plates precoated with silica gel containing fluorescent indicator (Merck KGaA, Darmstadt, Germany, cat. no. 5554) which were developed in dioxane:25% ammonia:water (6:1:4, v/v/v). Ion-exchange chromatography and gel filtration were performed on DEAE-Sephacel, Sephadex G-75 superfine, and Sephadex G-100 columns (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Affinity elution of the sheep Hint proteins was performed from 6-AH-AMP-agarose (Jena Bioscience, Jena, Germany, cat. no. AC-145L) in which the AMP ligand is attached to the agarose support via an aminohexyl spacer at its N^6 atom.

Buffers. Buffer A: 10 mM Tris/HCl (pH 8.8) containing 5% glycerol and 1 mM 2-mercaptoethanol. Buffer B: 50 mM potassium phosphate (pH 6.8) containing 5% glycerol and 1 mM 2-mercaptoethanol. Buffer C: 10 mM Tris/HCl (pH 7.4) containing 0.25 M sucrose, 0.1 mM EGTA and 0.2% bovine serum albumin.

Analytical methods. Protein concentration was estimated by the turbidimetric tannin method (Mejbaum-Katzenelenbogen, 1955). MALDI-TOF mass spectrometric analysis was performed at the proteomics facility of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Peptides that corresponded to the protein of the highest score were analyzed in the MASCOT Search Program. Molecular masses of the native enzymes were estimated by gel filtration on a Sephadex G-100 column (Andrews, 1964) and of the denatured proteins by SDS/PAGE.

Enzyme assay. Qualitatively, during purification of the proteins nucleoside phosphoramidate hydrolase activity was measured in a reaction mixture (50 μL) containing 50 mM potassium phosphate (pH 6.8), 1 mM substrate, bovine serum albumin (0.2 mg/mL), other additions (if any), and a rate-limiting amount of either OaHint1 or OaHint2. When the K_m values were being estimated, the NH_2-p[8-H^3]A concentration varied between 1.9 and 31.2 μM and the mixture contained either 45 pg OaHint1 or 26 pg OaHint2. At time intervals,

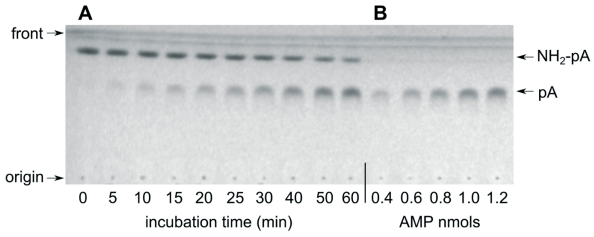

Figure 2. Time-course of hydrolysis of NH_2-pA catalyzed by sheep liver Hint1.
It was analyzed by thin-layer chromatography (**A**) and quantified with AMP standard (**B**). Part A: The reaction mixture (50 µL) contained 50 mM potassium phosphate buffer (pH 6.8), 1 mM NH_2-pA and 0.22 µg OaHint1. The reaction was carried out at 37 °C. At time intervals 2-µL aliquots were spotted onto the TLC plate origin. Part B: Indicated amounts of 5′-AMP (pA) were spotted onto the origin. The chromatogram was then developed in dioxane:25% ammonia:water (6:1;4, by volume) for 30 min. The chromatogram was dried and the spots of substrate and product/standard were visualized under short-wave UV light and photographed. Densitometric analysis of the AMP standard spots, within the range shown in B, revealed perfect linearity of the dependence optical density *versus* nucleotide amount.

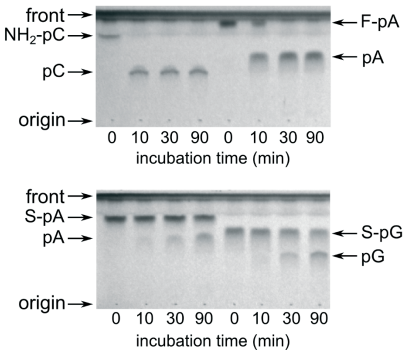

Figure 3. Cleavage of indicated substrates by OaHint2.
The reaction mixture (50 µL) contained 50 mM potassium phosphate buffer (pH 6.8), 1 mM substrate and 0.2 µg OaHint2. The incubation and TLC analysis were performed as described in the legend to Fig. 2.

3-µL aliquots of the reaction mixture were spotted onto TLC plates alongside standards of NH_2-pA and AMP (pA) and the plate developed for 45 min in the solvent system described above. This system effectively separated nucleoside phosphoramidates from corresponding nucleoside monophosphates, the reaction products (see Figs. 2 and 3 for examples). During kinetic studies, spots of the nucleotides were visualized under short-wave UV light and those of the reaction product (pA) cut out, immersed in scintillation cocktail, and the radioactivity determined. The rates of hydrolysis of unlabeled substrates were analyzed by TLC and densitometry of the reaction product spots by the use of the G:Box Syngene system and the Gene Tools program.

RESULTS

Hint1 purification

All operations during purification of OaHint1 and OaHint2 were carried out at 4°C. Fresh sheep liver (100 g) was chopped, suspended in 400 mL buffer A and homogenized. The homogenate was centrifuged (20 000 × *g* for 10 min) and the clear supernatant applied to a DEAE-Sephacel column (200 ml) equilibrated with the same buffer. The column was washed with 1 L buffer A and then a linear (0–0.5 M NaCl in the buffer) gradient was applied (total volume 2 L). The nucleoside phosphoramidase activity appeared at 80–100 mM NaCl. Active fractions were pooled and the enzyme concentrated by ammonium sulfate precipitation (70% saturation). The precipitated proteins were collected by centrifugation (30 000 × *g* for 15 min), resolubilized in 5 mL buffer B and applied to a Sephadex G-75 superfine column (2.5 × 94 cm) equilibrated with the same buffer. Active fractions, which emerged at $V_e/V_o = 1.26$, were pooled and applied to an AMP-agarose column (1 mL) equilibrated with buffer B. After washing the column with five volumes of buffer B, Hint1 was eluted with the same buffer containing 5 mM 5'-AMP. The eluting ligand was removed by dialyzing the fraction against buffer B. Finally, the sample was concentrated by dialysis against dry Sephadex G-200 and stored at –20°C for further analysis. The procedure yielded 225 µg of a homogeneous protein identified as OaHint1.

Hint2 purification

Another protein that exhibited adenosine phosphoramidase activity was purified from the mitochondrial fraction of fresh sheep liver. This fraction was obtained by differential centrifugation as follows: tissue (100 g) was chopped, suspended in 400 mL buffer C and gently homogenized in a laboratory knife blender. The debris was removed by centrifugation (800 × *g* for 10 min) and the mitochondria collected from the supernatant by centrifugations at 8 600 × *g* for 10 min. The resulting pellet was resuspended in buffer C, centrifuged again and the procedure repeated once more. The mitochondrial pellet obtained after the third centrifugation (14.5 g) was suspended in 50 mL buffer A and subjected to sonication. The homogenate was clarified by centrifugation (20 000 × *g* for 15 min) and the debris suspended again in 10 mL buffer A, sonicated and the homogenate centrifuged. The two supernatants were combined and applied to a DEAE-Sephacel column. From this stage the purification followed the procedure described above for Hint1, with the mitochondrial nucleoside phosphoramidase emerging from the Sephadex G-75 superfine column at $V_e/V_o = 1.14$. This procedure yielded 30 µg of a homogeneous protein identified as OaHint2.

Molecular characterization of OaHint1 and OaHint2

Molecular mass of the native proteins was estimated by gel filtration on a Sephadex G-100 column calibrated with molecular mass standard proteins (Fig. 4). The re-

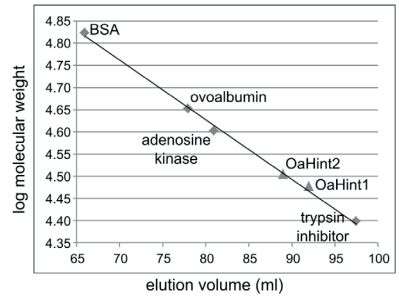

Figure 4. Estimation of molecular masses of native sheep liver OaHint1 and OaHint2.
Gel filtration was performed on a Sephadex G-100 column (1.6 x 74 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.8) containing 5% glycerol and 1 mM 2-mercaptoethanol. The following standards were used: bovine serum albumin (67 kDa), ovalbumin (44 kDa), adenosine kinase from yellow lupin seeds (38 kDa) (Guranowski, 1979) and soybean trypsin inhibitor (21.1 kDa).

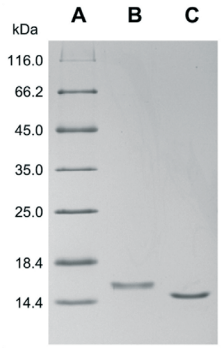

Figure 5. Electrophoresis in the presence of SDS of Hint1 and Hint2 proteins purified from sheep liver.
Lane A, molecular mass standard proteins (Fermentas, cat. no. SMO 0431); **lane B**, 0.3 μg OaHint2; lane C, 0.6 μg OaHint1.

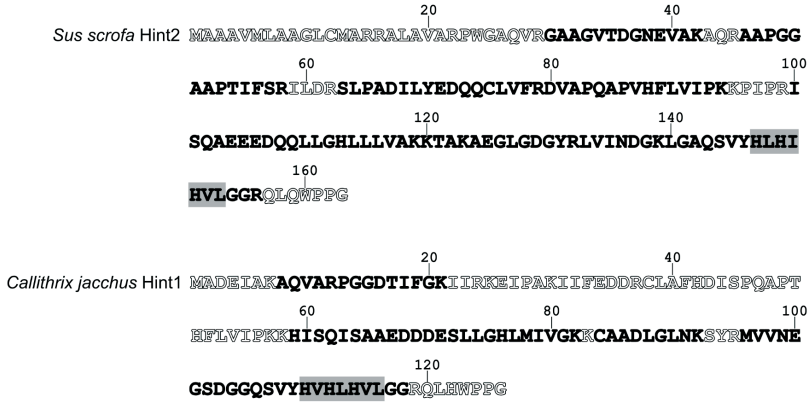

Figure 6. Identification of peptides of the sheep liver Hint proteins by MASCOT Search Program.
The peptides with the best scores for each of these proteins are shown as examples. For OaHint1 the sequence coverage was 56% and for OaHint2 70%. Matched peptides are shown in black bold and the histidine triad motifs in shadowed boxes.

sults of exclusion chromatography under non-denaturating conditions and SDS/polyacrylamide gel electrophoresis (Fig. 5) showed that sheep Hint1 and Hint2 exist as homodimers of about 30 kDa and 32 kDa, respectively. Mass spectrometric analysis performed on the homogeneous proteins cut out from the SDS gels revealed their closest similarities to annotated mammalian Hint proteins — OaHint1 to Hint1 proteins of white-tufted-ear marmoset (*Callithrix jacchus*) and cow (*Bos taurus*), and OaHint2 to Hint2 proteins of pig (*Sus scrofa*) and naked mole rat (*Heterocephalus glaber*). Peptide identification of the sheep proteins is presented in Fig. 6.

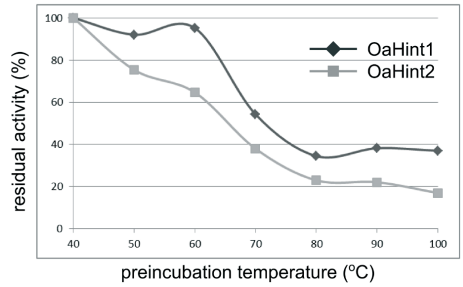

Figure 7. Thermostability of the sheep Hint proteins.
The preincubation mixture (50 μL) contained 25 mM Tris/HCl (pH 7), 10 μM dithiothreitol, 5% glycerol, bovine serum albumin (0.1 mg/mL) and 0.11 μg of OaHint1 or 0.02 μg of OaHint2. Preincubation was carried out for 5 min at the indicated temperature. The mixture was then chilled on ice and a 10-μL aliquot withdrawn and assayed for remaining adenosine phosphoramidase activity, as described in the experimental procedures.

Kinetic parameters

The pH optima for sheep liver Hint1 and Hint2 were around 7. Thus, hydrolysis of NH_2-pA and its analogs was routinely measured in 50 mM potassium phosphate (pH 6.8). Divalent metal ions were apparently not required for catalysis and the rate of the reaction was not affected by 10 mM EDTA. The hydrolytic cleavage of the P-N bond in NH_2-pA followed Michaelis-Menten kinetics. The apparent K_m values calculated from Eadie-Hofstee plots (*v versus v*/[S]) were 6.6 μM for OaHint2 and 1.5 μM for OaHint1. The k_{cat} values estimated for the natively functioning dimers were 68.3 s^{-1} and 11.0 s^{-1}, respectively.

Substrate specificity

Both sheep Hint proteins were tested mainly with the same uncommon (di)nucleotides that had been shown to be substrates for other HIT-proteins (Guranowski *et al.*, 2008; 2010b). Diadenosine triphosphate, ApppA, one of the preferred substrates of Fhit proteins, was hydrolyzed neither by OaHint1 nor by OaHint2, while another good Fhit substrate, adenosine 5'-phosphosulfate (SO_4-pA) was a very poor substrate for both sheep proteins. Its rate of hydrolysis catalyzed by OaHint2 was 5000-fold and by OaHint1 333-fold lower than that for NH_2-pA. As has been shown previously, these features are typical for Hint1 proteins and for their ortholog from *Escherichia coli* (EcHinT) (Bieganowski *et al.*, 2002; Guranowski *et al.*, 2010b; 2011). The rate of hydrolysis of SO_4-pC, a pyrimidine analog of SO_4-pA, was at the very limit of detection, being one order of magnitude slower than cleavage of SO_4-pA. OaHint1 and Hint2 slowly catalyzed the hydrolysis of the P-S bond in nucleoside 5'-phosphorothioates, S-pA and S-pG (Fig. 3). This property was also previously shown to be typical of Hint1 proteins (Guranowski *et al.*, 2010b; 2011; Ozga *et al.*, 2010). Among those compounds, NH_2-pA appeared to be hydrolyzed at the highest rate. Its pyrimidine analog NH_2-pC was cleaved at about half this rate. A synthetic adenylyl-derivative, F-pA, also appeared to be a good substrate for the two sheep Hint proteins (Figs. 2 and 3). Results of these studies are summarized in Table 1. Neither OaHint1 nor OaHint2 exhibited the catalytic duality that had been observed earlier for two other HIT-proteins, *Arabidopsis thaliana* Hint4 and *Caenorhabditis elegans* DcpS (Guranowski *et al.* 2010a), i.e., they did not degrade their substrates phosphorolytically.

Thermostability

It is known that nucleoside phosphoramidate hydrolases exhibit remarkable thermostability (Kuba *et al.*, 1994; Guranowski *et al.*, 2010b). Therefore we checked if this was the case with the sheep liver enzymes and whether OaHint1 and OaHint2 differed in this respect from each other. As shown in Fig. 7, the former enzyme was more stable than the latter. OaHint1 retained

Table 1. Hydrolysis of adenosine 5′-phosphoramidate (NH_2-pA) and some other nucleotidyl-derivatives catalyzed by sheep liver OaHint1 and OaHint2.
The reaction mixtures were prepared as described in the experimental procedures. For poor substrates (bottom four nucleotides), the protein concentration and/or time of incubation was adjusted to get enough products for the densitometric analyses. The k_{cat} values are means of three independent measurements; standard errors did not exceed 5%.

Nucleotide	Activity of OaHint1		Activity of OaHint2	
	k_{cat} (s^{-1})	rel. vel. (%)	k_{cat} (s^{-1})	rel. vel. (%)
NH_2-pA	11.00	100	68.30	100
NH_2-pC	4.98	45.30	28.65	41.90
F-pA	7.90	71.80	12.6	18.40
S-pA	0.18	1.60	0.43	0.60
S-pG	0.29	2.60	0.70	1.00
SO_4-pA	0.03	0.30	0.012	0.02
SO_4-pC	0.004	0.04	<0.002	<0.002

full activity after a 5-min preincubation at 60°C, whereas OaHint2 lost one third. Moreover, OaHint1 preincubated at 80, 90 or 100°C retained almost 40% of its original activity while, under the same conditions, OaHint2 retained only approximately 20%.

DISCUSSION

Here we describe a procedure that allowed us to obtain (i) native (non-recombinant) Hint2 protein in an electrophoretically homogeneous form for the first time and (ii) homogeneous Hint1 protein from the same biological material (sheep liver). The crucial step of this procedure that separated the sheep liver Hint2 from Hint1 was first to prepare the mitochondrial fraction. Interestingly, the crude cytosolic extract, i.e., the $20\,000 \times g$ supernatant, from whole sheep liver was apparently free of Hint2. This confirms the mitochondrial localization of Hint2 and its tight association with mitochondrial membranes, which has been reported recently (Lenglet *et al.*, 2008).

Purification of the two proteins was monitored simply by following their nucleoside phosphoramidase activity by the use of thin-layer chromatography. The final and most effective purification step was affinity chromatography on AMP-agarose, as described earlier by Bieganowski and coworkers (2002). Slight modifications of their procedure were: the use of 6-AH-AMP-agarose, an extremely selective resin purchased from Jena Bioscience, and 5 mM AMP, instead of adenosine, as the enzyme-eluting agent.

OaHint1 and OaHint2 exhibited many similarities to their native counterparts from rat liver (Kuba *et al.*, 1994) and yellow lupin (Guranowski *et al.*, 2008) and to the recombinant proteins from rabbit (rHint1) (Bieganowski *et al.*, 2002; Ozga *et al.*, 2010), yeast (Hnt1) (Bieganowski *et al.*, 2002) and *Arabidopsis thaliana* (AtHint1) (Guranowski *et al.*, 2010b). Both the rat liver enzyme (Kuba *et al.*, 1994) and the sheep liver Hints studied here function as homodimers of approximately 30 kDa. They hydrolyzed NH_2-pA with 2–3 fold higher rates than they did NH_2-pC. OaHint2 seemed to be slightly more specific than OaHint1 with respect to the P-N bond. A comparison of the substrate specificity of these enzymes shows that OaHint2 is closely related only to Hint1 proteins. This is in line with the very recently published structural similarity and phylogeny of human Hint1 and Hint2 (Maize *et al.*, 2013). Although Hint2 has features common to all HIT proteins such as the HIT motif, it recognizes only certain nucleotides and would not be able to substitute for Fhits, Hint3 or Hint4 in the catabolism of natural metabolites such as ApppA or SO_4-pA. Among the other capabilities of OaHint2 common to Hint1 proteins are the relatively efficient cleavage of the P-F bond in F-pA and the P-S bond in S-pNs. The latter conversion is accompanied by the liberation of H_2S (Ozga *et al.*, 2010), easy to smell when one works with these compounds.

The results of this study supplement our knowledge about HIT proteins, in particular their substrate specificity. The new information about native Hint2 should be taken into account during investigations of the biological function of this mitochondrial protein. In addition to its possible function as a regulatory protein (Lenglet *et al.*, 2008; Martin *et al.*, 2001), it can affect the basic metabolism of certain nucleotides, e.g., NH_2-pA and SO_4-pA. Hint2 may play the same role in mitochondrial metabolism as does Hint1 in the cytosolic metabolism. From a broader perspective, one can consider nucleoside phosphoramidates and the enzymes degrading them as an example of the "metabolite damage and its repair" concept; see recent review on that issue by Linster and coworkers (2013). Finally, understanding of the metabolism of nucleoside phosphoramidates is important for those who investigate pro-drugs containing P-N bonds (Romanowska *et al.*, 2011 and references therein).

Acknowledgements

This work was supported by the National Science Center (grant 2012/05/B/NZ1/00025).

We thank Dr. Jarosław Zimny (Poznań University of Life Sciences, Poland) for providing sheep liver mitochondria. We are grateful to Professor Alexander G. McLennan (University of Liverpool, UK) for linguistic help in preparation of final version of this paper.

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