Characterization, expression and localization of S-adenosylhomocysteine hydrolase from amphioxus Branchiostoma belcheri tsingtaunese

Yuan WANG*, Bosheng ZHAO*†, Shicui ZHANG† and Xiaojuan QU*

*School of Life Sciences, Shandong University of Technology, Zibo, 255049, People’s Republic of China, and †Department of Marine Biology, Ocean University of China, Qingdao, 266003, People’s Republic of China

Synopsis
A cDNA clone encoding AmphiSAHH [amphioxus SAHH (S-adenosylhomocysteine hydrolase)] protein was isolated from a cDNA library from the gut of Branchiostoma belcheri tsingtaunese. It contained a 1305 bp open reading frame corresponding to a deduced protein of 434 amino acid residues, with a predicted molecular mass of approx. 47.8 kDa. Phylogenetic analysis showed that AmphiSAHH and sea-urchin SAHH joined together and positioned at the base of the vertebrate SAHH clade, suggesting that both AmphiSAHH and sea-urchin SAHH might share some characteristics of the archetype of vertebrate SAHH proteins. The genomic DNA sequence of AmphiSAHH contained eight exons and seven introns, which was similar to B. floridae and sea-urchin SAHH exon/intron organization. Sequence comparison suggested the evolutionary appearance of the ten exon/nine intron organization of SAHH genes after the split of invertebrates and vertebrates, after which it has been highly conserved. AmphiSAHH has been successfully expressed in Escherichia coli and purified. Western blotting confirmed that the enzyme has a native molecular mass of approx. 48 kDa, and the catalytic activities and NAD+/NADH binding affinity of recombinant AmphiSAHH were measured. Immunohistochemistry analysis showed that SAHH was strongly expressed in hepatic caecum, gill, spermary and ovary of amphioxus.

Key words: amphioxus Branchiostoma belcheri, enzyme activity, genome analysis, immunohistochemistry, S-adenosylhomocysteine hydrolase (SAHH), Western blotting

INTRODUCTION

SAHH (S-adenosylhomocysteine hydrolase) is an enzyme which catalyses the reversible conversion of SAH (S-adenosylhomocysteine) into Hcy (homocysteine) and Ado (adenosine) [1]. It plays a central role in regulation of the level of SAH in tissue. SAH is the product and the competitive inhibitor of all biomethylation reactions in which SAM (S-adenosylmethionine) acts as a methyl donor, transferring methyl groups to nucleic acids, proteins, phospholipids and other small molecules [2–6]. The enzyme also plays an important role in the process of trans-sulfuration and purine metabolism [7,8]. SAHH is the only enzyme involved in SAH metabolism and the reaction it catalyses is reversible. However, under physiological conditions, the removal of Ado and Hcy is sufficiently rapid to allow the reaction to proceed in the direction of hydrolysis [9,10]. Inhibition of SAHH leads to the accumulation of SAH, increasing the ratio of SAH and SAM, and accompanies the subsequent inhibition of SAM-dependent trans-methylation reactions in most organisms except for some prokaryotes, such as Escherichia coli [5,6,11,12]. Furthermore, this inhibition results in various pharmacological effects. Therefore SAHH is an attractive target for the design of antiviral, antitumour and antiparasitic agents etc. [10,12,13–15]. There are many inhibitors of SAHH which have been synthesized and identified previously [16–19].

All SAHHs identified so far are oligomeric proteins with subunits of a molecular mass of 45–50 kDa. Vertebrate SAHHs consist of four subunits of approx. 48 kDa, and they tightly bind one NAD⁺ molecule per subunit, which is essential for catalysis [20–22]. Ado reduces the tightly bound NAD⁺ to NADH and inhibits the catalytic activity of SAHH [23,24]. cAMP competes with Ado for the same binding site on the enzyme, but it does not inhibit, but stimulates, SAHH [25–27]. The crystal structure of SAHH has been described previously by several groups [9,28]. This enzyme has been isolated from a variety of sources, including

Abbreviations used: Ado, adenosine; DIG, digoxigenin; Hcy, homocysteine; IPTG, isopropyl β-D-thiogalactoside; LB, Luria-Bertani; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; AmphiSAHH, amphioxus S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine, UTR, untranslated region.

The nucleotide sequence data reported will appear in GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AY278950.

†To whom correspondence should be addressed (email zhaobosheng@sdut.edu.cn).
humans, rats, plants, yeasts, protozoa and prokaryotes, and a majority of them also have been cloned. Amphioxus, a cephalochordate, has long been regarded as the extant invertebrate most closely related to the proximate ancestor of vertebrates, which is of great significance in understanding the origin and evolution of vertebrates [29,30]. However, little is known to date about SAHH in this evolutionarily important organism. Therefore the aim of our study was to characterize the SAHH gene from the amphioxus Branchiostoma belcheri, then express and purify the recombinant protein and to examine its localization in adult amphioxus tissues.

MATERIALS AND METHODS

Animals

Amphioxus (B. belcheri tsingtaunese) were collected from the sandy bottom of the sea near Shazikou, Qingdao, China, and cultured in containers with continuous aeration. They were starved for 3 days in sterilized filtered seawater to remove all food in the gut. Amphioxus were then rinsed three times with DEPC (diethyl pyrocarbonate)-treated water and then frozen immediately in liquid nitrogen until use.

Cloning and sequence analysis of cDNA

The gut cDNA library of adult amphioxus was constructed using a SMART cDNA library construction kit (Clontech) according to a method published previously [31]. By large-scale sequencing of the amphioxus gut cDNA library with a ABI PRISM 377XL DNA sequencer, more than 2000 clones were analysed for coding probability using the DNATools program [32]. Comparison against the GenBank® Entrez Protein database was performed using the BLAST network server at the National Center for Biotechnology Information (NCBI) [33]. Multiple protein sequences were aligned by the CLUSTAL method using the MegAlign program in the DNASTAR software package [34]. A phylogenetic tree was constructed by the neighbour-joining and maximum-parsimony methods within the PHYLIP 3.5c software package [35] using 1000 bootstrap replicates.

Northern blot analysis

Total RNAs were prepared using TRIZol (Gibco) from adult amphioxus ground in liquid nitrogen. An aliquot of RNA (5 μg) was electrophoresed and blotted onto nylon membranes (Amresco, Solon, OH, U.S.A.). The DIG (digoxigenin)-labelled AmphisAHH (amphioxus SAHH) riboprobes of approx. 1600 bp were synthesized in vitro from linearized plasmid DNA using DIG-UTP following the manufacturer’s instructions (Roche). The blots were hybridized at high stringency with 1 μg/ml AmphisAHH riboprobes at 58°C for 16 h, and washed twice in 2× SSC buffer (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) with 0.1% SDS at 25°C for 5 min each and twice in 0.1× SSC buffer with 0.1% SDS at 65°C for 20 min each. They were subsequently incubated in a blocking solution containing 100 mM maleic acid (pH 7.5), 150 mM NaCl and 1% blocking reagent (Roche) for 1 h (25°C) and then incubated in blocking solution with an alkaline-phosphatase-conjugated anti-DIG antibody (1:10000 dilution; Roche) at room temperature (25°C) for 2 h. After washing with 100 mM maleic acid buffer (pH 7.5) containing 150 mM NaCl and 0.3% Tween 20 and then with 100 mM Tris/HCl buffer (pH 9.5) containing 100 mM NaCl, the hybridized bands were visualized using BM-Purple (Roche).

Genomic amplification

In order to amplify the putative introns of AmphisAHH, PCR was performed with a pair of specific primers (forward primer, 5′-CATCTCATGGGTGATCTCAGCT-3′, and reverse primer, 5′-CTTTTTATTGAGGTAGTCT-3′) using approx. 100 ng of genomic DNA as the template DNA (94°C for 5 min, and 35 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 3 min). The PCR products were detected by agarose-gel electrophoresis, purified and ligated into pMD18-T (TaKaRa) and then transformed into E. coli JM109 cells. They were DNA-sequenced on both strands, with at least two runs performed in each direction for the complete sequence.

Construction of the expression vector

The open coding region of the amphioxus SAHH gene was amplified using Ex Taq™ HS DNA polymerase (TaKaRa) using the upstream primer 5′-GGATCCTGCCCCGTTTCTT-3′ (BamHI site is underlined) and the downstream primer 5′-GAATTCTGATTTAAGTTTCACTCAGGGGTAGTCTCACGCT-3′ (HindIII site is underlined). The reaction was carried out under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 40 s at 55°C and extension at 72°C for 2 min. The PCR product was digested with BamHI and HindIII and sub-cloned into the pET28a expression vector (Novagen) digested previously with the same restriction enzymes. The identity of the insert was confirmed by DNA sequencing, and the expression construct was designated pET28a-AmphisAHH.

Expression and purification of recombinant protein

E. coli BL21 cells were transformed with pET28a-AmphisAHH. A single colony was cultured overnight in LB (Luria–Bertani) broth containing kanamycin (50 μg/ml). The culture was diluted 1:100 in LB broth and subjected to further incubation at 37°C for 5 h. Expression and purification of the recombinant protein was performed as described by Fan et al. [36]. The eluted samples were analysed by SDS/PAGE (12% gels) and staining with Coomassie Brilliant Blue R-250.

Western blot analysis

The cell lysates of induced E. coli BL21 cells transformed with the pET28a-AmphisAHH expression vector and the purified fusion protein His-SAHH were resolved by SDS/PAGE (12% gels, with a 5% spacer gel). The cell lysates of uninduced E. coli BL21 cells transformed with the expression vector were also analysed as a negative control. The gels were incubated for 5 min in transfer buffer [15.6 mM Tris/HCl, 120 mM glycine and 20% (v/v) methanol (pH 8.3)], and proteins on the gels were blotted onto to PVDF membranes (Amresco). The membranes were blocked in 20 mM...
PBS containing 50 mM NaCl and 5% (w/v) BSA at room temperature for 1 h, and incubated with rabbit anti-(human SAHH) antibody (1075-2-AP, 1:2000 dilution; Proteintech Group, Chicago, IL, U.S.A.) at room temperature for 90 min. After washing in PBST buffer [10 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, 2 mM KH2PO4 and 0.05% Tween 20 (pH 7.4)] three times, the membrane was incubated with alkaline-phosphatase-conjugated sheep anti-rabbit IgG antibody (1:300 dilution; Sigma) for 30 min at room temperature. After three washes with PBST buffer, the membrane was incubated in the substrate solution (0.1 M PBS containing 0.06% 4-dimethylaminobenzene and 0.03% H2O2). The reaction was quenched with distilled water after the bands were visible [37].

**Assay of recombinant AmphiSAHH activity**

SAHH activity in the synthetic direction was measured as the absorption at 258 nm as described previously [38]. The rate of Hcy formation was then coupled to the generation of the intensely yellow Nbs22+ from DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)]. The dissociation constant (Kd) was determined spectrophotometrically by measuring the absorbance at 412 nm using the molar absorption coefficient (ε) of Nbs22+ (13 600 M−1·cm−1). SAHH activity in the synthetic direction was measured as the rate of SAH production from Hcy and using HPLC. SAH was separated by use of a reverse-phase HPLC C-18 column with UV detection at an absorbance of 258 nm as described previously [39,40].

**Binding of NAD+/NADH to recombinant AmphiSAHH**

The enzyme activities of recombinant AmphiSAHH were measured in the synthetic direction as described above in the presence of 0 to 100 μM NAD+ in the incubation medium [13,40]. The dissociation constant (Kd) for NAD+ (Kd,NAD) was calculated by fitting the data to a one-site model using the following equation and the Microcal Origin program:

\[
\text{Enzyme activity} = \frac{V_{\text{max}}[\text{NAD}^+]}{(K_d + [\text{NAD}^+])}
\]

The activities of recombinant AmphiSAHH were measured in the hydrolytic direction as described above in the presence of 100 μM NAD+ with different concentrations of NADH (0–10 μM) in the incubation medium [13,40]. The Kd for NADH (Kd,NADH) was calculated by fitting the data to the following equation using the Microcal Origin program:

\[
\text{Activity} = \frac{\text{Activity}_{\text{max}}}{(K_d,[\text{NADH}]/K_d,[\text{NADH}] + 1)}
\]

**Immunohistochemistry**

Adult amphioxus were cut into three to four pieces and fixed in freshly prepared 4% (w/v) paraformaldehyde in 100 mM PBS at 4°C for 24 h. After serial dehydration, these samples were embedded in paraffin and sectioned at 7 μm thickness. The sections were mounted on to slides and dried at 42°C for 36–48 h. The immunohistochemical staining was performed as described by Liang et al. [41]. After the chromogenic reaction was achieved, the sections were mounted in neutral balsam, observed and photographed using a Zeiss Axioskop-40 microscope.

**RESULTS AND DISCUSSION**

**Sequence and phylogenetic analysis of AmphiSAHH**

The cDNA clone (GenBank® accession numberAY278950) obtained from the gut cDNA library of *B. belcheri* is 1531 bp long, and its longest open reading frame consists of 1305 bp, encoding a deduced protein of 434 amino acids with a predicted molecular mass of approx. 47.8 kDa. The start codon (ATG) was flanked by a purine base at positions −3 and +4, matching the Kozak consensus sequence [42] and is suggested to optimize translational efficiency. The 5′-UTR (untranslated region) is 68 bp in length and the 3′-UTR is 158 bp long, with a polyadenylation signal (AATAAA) and a polyadenylation tail. Therefore the cDNA encodes a full-length protein. Initial BLASTP searches at the NCBI revealed that the protein encoded by the amphioxus cDNA shared 82.1% identity (354 out of 429 amino acids) with *Strongylocentrotus purpuratus* SAHH at the amino-acid level. It was further compared with other representative members of the SAHH family (GenBank® Entrez Protein accession number in parentheses), including human (NP_000678), monkey (BAE87650), cow (NP_001029487), rat (NP_058897), mouse (EDL06108), dog (XP_534388), chicken (XP_417331), fish (NP_954688), fruitfly (AAM27497), honey bee (XP_391917) and so on. The showed that the deduced AmphiSAHH protein has 67.3–82.1% amino-acid identity with its homologues from a variety of organisms, including microbes, invertebrates and vertebrates. The significant homology noted between different categorized groups clearly suggests that SAHHS are conserved proteins in almost all species, so we consider the proteins to be very similar and to have very important functions.

In addition, searching the CDD (Conserved Domain Database) at the NCBI demonstrated the presence of a conserved domain typical of amphioxus SAHH, including active sites (His57, Thr59, Gln61, Asp134, Glu159, Thr160, Lys189, Asp193 and His355) which can combine with SAH, Hcy, Ado and cAMP in the process of reversible catalysis or to inhibit hydrolysis. The NAD+ -binding sites of AmphiSAHH (Thr160, Thr161, Thr162, Asn194, Gly223, Gly225, Asp226, Val227, Thr245, Glu246, Ile247, Ala278, Thr279, Ile284, Ile302, Gly303, His304, Leu346, Asn348 and His355) can change the catalytic activity through alternatively binding to NAD+ and/or NADH. There are two sites (Thr160 and His355) which appear to be not only in the active site, but also in the NAD+ -binding site, and some of the residues (Glu159, Thr160, Thr162, Asp193 and Asn348) are neighbouring (Figure 1). These results are very similar to those from rat and human SAHH [9,28]. Therefore the cDNA encodes AmphiSAHH protein.
The sequence of known SAHH proteins from representative species, including amphioxus, was used to construct a phylogenetic tree by neighbour joining using *Saccharomyces cerevisiae* SAHH as the outgroup. It was found that AmphiSAHH formed an independent cluster with sea-urchin SAHH, which is positioned at the base of vertebrate SAHH proteins (Figure 2). This reflects the established phylogeny of the chosen organisms, and suggests that both AmphiSAHH and sea-urchin SAHH share some characteristics of the archetype of vertebrate SAHH proteins.

**Genomic organization of AmphiSAHH**

A PCR-based strategy method was used to understand the genomic organization of AmphiSAHH. The product of genomic DNA amplification was obtained using primers corresponding to the 5′- and 3′-UTRs in AmphiSAHH cDNA and amphioxus genomic DNA as the template, and the resultant product was DNA sequenced. The fragment named AmphiSAHH1 is 6063 bp long. Comparing the sequence of AmphiSAHH1 with that of AmphiSAHH cDNA showed that the coding region of genomic AmphiSAHH is composed of eight exons interrupted by seven introns. There are eight exons of 98, 192, 229, 114, 210, 200, 197 and 278 bp, interspaced with seven introns of 1087, 618, 243, 346, 1105, 566 and 580 bp (Figure 3). All seven introns obey the GT–AG rule, containing sequences which were thought to be necessary for correct RNA splicing of various other eukaryotic genes [43].

A search of the genome of *B. florideae* revealed the presence of a Florida AmphiSAHH cDNA and its genomic DNA sequence (http://genome.jgi-psf.org//Brafl1). Sequence comparison demonstrated that AmphiSAHH shared 95.6% similarity at the amino-acid level to the deduced protein encoded by Florida amphioxus SAHH gene, suggesting that SAHH is highly conserved between species. Analysis of the genomic DNA structure exhibited that the Florida AmphiSAHH gene is composed of eight exons and seven introns. The eight exons of 89, 191, 229, 113, 208, 203, 195 and 286 bp were interspaced with seven introns of 1072, 560, 320, 416, 725, 483 and 356 bp (Figure 3), all of which begin with the typical GT dinucleotide and end with the AG dinucleotide. The exons in AmphiSAHH shared more than 80% identity at the amino-acid level with the exons in the
Florida amphioxus. However, through comparing *B. belcheri* with *B. floridae* SAHH, we found that there are differences not only in the size, but also in the homology, between their relevant introns, with the percentage identity of the relevant introns at the nucleic-acid level not more than 20%. This discrepancy may suggest that the two species have previously undergone interspecific differentiation because of dissimilar evolutionary environments. It is notable that SAHH genes in deuterostomes, such as human, mouse and zebrafish SAHH genes, contain ten exons and nine introns and the sea-squirt SAHH gene has nine exons and eight introns, whereas amphioxus and sea-urchin SAHH genes encompassed eight exons and seven introns respectively (Figure 3). It is therefore apparent that the gene exon–intron structure is not conserved throughout deuterostome evolution. Homologue sequence comparisons suggested that sea-squirt SAHH was closer to vertebrates than to its sister group amphioxus [44–46]. The vertebrate SAHH exons 5, 6, 7 and 8 may arise from an ancestral sea-squirt and AmphiSAHH homologue by exon–splitting events, based on their size and identity, which is in agreement with other reported genes [47]. This indicates that the

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**Figure 1** Alignment of the amino-acid sequences of SAHH proteins using the MegAlign program (DNASTAR) by the CLUSTAL method

Shaded (solid black) residues indicate the amino acids that match the consensus sequence. Gaps introduced into sequences to optimize alignment are represented by -. Active sites were marked by * and the NAD binding sites were marked by #. SAHH amino acid sequences were obtained from the NCBI (species name and GenBank® Entrez Protein accession number): *Saccharomyces cerevisiae* (NP_010961), *Volvariella volvacea* (AAZ95180), *Dictyostelium discoideum* (XP_647635), *Leishmania major* (XP_001686974), *Trypanosoma cruzi* (XP_001686974), *Drosophila melanogaster* (AAM27497), *Bombyx mori* (NP_001093271), *Apis mellifera* (XP_391917), *Strongylocentrotus purpuratus* (XP_780172), *Tetraodon nigroviridis* (CAF95753), *Danio rerio* (NP_954688), *Gallus gallus* (XP_417331), *Equus caballus* (XP_001501233), *Canis familiaris* (XP_534388), *Bos taurus* (NP_001029487), *Sus scrofa* (NP_001011727), *Rattus norvegicus* (NP_058897), *Mus musculus* (EDL06108), *Macaca fascicularis* (BAE87650), *Homo sapiens* (NP_000678).
Figure 2  Phylogenic tree of SAHH proteins including AmphiSAHH constructed by the neighbour-joining method within the PHYLIP 3.5c software package
The numbers refer to 1000 bootstrap value replicates. Sequences used were obtained from the GenBank® Entrez Protein database (see Figure 1 for details). *Xenopus laevis (GenBank® Entrez Protein accession number NP_001089040).

Figure 3  Diagram of the genomic structure and organization of SAHH genes from human, mouse, zebrafish, sea squirt, Florida amphioxus and sea urchin
Black boxes represent the exons and the joining lines indicate the sequences of introns. The values above the lines and the boxes indicate the size of the introns and exons respectively.

Evolutionary emergence of the ten exon/nine intron organization of the SAHH genes occurred after the split of invertebrates and vertebrates, and since the split the organization has been highly conserved.

Expression and purification of recombinant AmphiSAHH protein and Western blotting
In order to further understand the function of AmphiSAHH, an expression vector, including the entire open reading frame of AmphiSAHH and a 5'-additional tag of pET28a was constructed, and this resulted in the original N-terminal methionine residue in the recombinant protein replaced by Met-Gly-Ser-Ser-(His)$_6$-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met. The individual clone was cultured until it reached an attenuation at 600 nm ($D_{600}$) of 0.6, after which expression was induced by IPTG (isopropyl $\beta$-D-thiogalactoside). The expression efficiency and intracellular solubility of SAHH were detected by SDS/PAGE. The recombinant AmphiSAHH protein was expressed and purified by affinity chromatography on a Ni$^{2+}$-NTA (Ni$^{2+}$-nitrilotriacetate) resin and Desalt column. The purified recombinant AmphiSAHH with the His$_6$ (hexahistidine) tag yielded a single band of approx. 48 kDa on SDS/PAGE (12% gels) detected by Coomassie Brilliant Blue staining (Figure 4), which exactly matched the predicted molecular mass deduced from the AmphiSAHH gene.
Figure 4  SDS/PAGE and Western blot analysis of AmphiSAHH
Lanes 1 and 5, total cellular extracts from IPTG-induced E. coli BL21 cells transformed with pET28a-AmphiSAHH; lanes 2 and 6, recombinant AmphiSAHH purified on a Ni²⁺-NTA (Ni²⁺-nitrilotriacetate) resin column; lanes 3 and 7, total cellular extracts from E. coli BL21 cells transformed with pET28a-AmphiSAHH before induction; lanes 4 and 8, amphioxus extracts. The molecular mass of AmphiSAHH is indicated in kDa (KD).

Western blotting showed that the primary antibodies reacted with amphioxus extracts, purified fusion protein and the supernatant of the cell lysate of IPTG-induced E. coli BL21 cells transformed with pET28a-AmphiSAHH, illustrated by the presence of a single positive band with an apparent molecular mass of approx. 48 kDa, corresponding to the molecular mass predicted by AmphiSAHH cDNA. The band was not detected in the supernatant of the cell lysate of E. coli BL21 cells transformed with the expression vector before induction by IPTG (Figure 4). This showed that the rabbit antiserum has a conspicuous antigen-specific reactivity.

Figure 5 Relationship between enzyme activity and concentration of NAD⁺ in the incubation mixture
The activity of recombinant AmphiSAHH was assayed in the synthetic direction. The dissociation constants (Kₐ) were estimated by fitting the data to a one-site model using the Microcal Origin program. uM, μM.

Figure 6 Northern blotting to detect AmphiSAHH RNA
Lane 1, the blot was hybridized with DIG-labelled AmphiSAHH RNA probe. The arrow indicates the position of the fragment equivalent to 1600 bp. Lane 2, total RNA of amphioxus. 28 S and 18 S rRNA bands are indicated.

Substrate kinetic properties of the recombinant AmphiSAHH
The catalytic activity of recombinant AmphiSAHH was assayed in both the hydrolytic and synthetic directions. For both assays, a final concentration of 100 μM NAD⁺ was included in the assay buffer. The Kₐ values for Ado, Hcy and SAH were 3.4 μM, 85 μM and 16.1 μM. The kinetic constants for human placental SAHH have been reported previously [40]. Compared with human SAHH, recombinant AmphiSAHH has a similar Kₐ value for Hcy, whereas it has slightly higher Kₐ values for Ado and SAH.

Figure 5 shows that the activity of recombinant AmphiSAHH is dependent on the concentration of the cofactor NAD⁺. The curve was fit for the one-site model (r > 0.97), suggesting that the four NAD⁺-binding sites of the tetrameric enzyme have similar affinities for this cofactor. The dissociation constant (Kₐ) was calculated to be 3.4 ± 0.2 μM. Binding of NADH to recombinant AmphiSAHH produced an inactive form of the enzyme.
Figure 7 Localization of SAHH transcripts in different tissues of adult amphioxus was detected by immunohistochemistry

(A) A micrograph shows the presence of SAHH transcripts in the male amphioxus. (B) Transverse section through gill (g) and hepatic caecum segments of female amphioxus and positive expression of AmphiSAHH was found in gill, hepatic caecum and ovary (o). (C) Enlargement of the box in (A), showing the presence of AmphiSAHH in hepatic caecum (hc), gill and spermary (t). (D) Transverse section through hind-gut (hg), where no strong signal was detected. (E) Section processed and hybridized in parallel using BSA instead of the antibody. No signal is seen in the control. m, muscle; nc, notochord; nt, neural tube. Scale bar, 100 μm.

The $K_d$ for NADH was estimated to be $0.2 \pm 0.1 \mu M$, which is approx. 17 times higher than the binding affinity of the enzyme for NAD$^+$. The $K_d$ value of human SAHH is $120 \pm 20 \text{nM}$ for both NAD$^+$ and NADH [13]. Release of the cofactors from the human enzyme is not normally observed, owing to the very slow dissociation rates. In contrast, it is loosely observed for recombinant AmphiSAHH binding to NAD$^+$ ($K_d = 3.4 \pm 0.2 \mu M$). However, recombinant AmphiSAHH has an affinity ($K_d = 0.2 \pm 0.1 \mu M$) for NADH that is similar to that of the human enzyme.

Tissue-specific expression of AmphiSAHH in adult amphioxus

Northern blotting was conducted to assess the presence and size of the AmphiSAHH transcript. As shown in Figure 6, a AmphiSAHH transcript of approx. 1600 bp in size was detected. Immunohistochemical staining using the anti-SAHH antibody demonstrated that AmphiSAHH was most abundant in the hepatic caecum, gill, spermary and ovary, whereas it was present at a low level in other tissues, including notochord, muscle and hind-gut (Figure 7). SAHH is a highly conserved enzyme and is ubiquitously distributed in all animal tissues. In the rat, the highest level of activity was found in the liver and pancreas, followed by kidney, adrenal gland and brain. Low enzyme activity was reported for heart, muscle, prostate, spleen and lung [48,49]. The results showed that AmphiSAHH was variously expressed in a tissue-specific manner, in contrast with the widespread expression pattern of other animals, and suggested that AmphiSAHH might play a fundamental but tissue-specific role in spermiogenesis, oogenesis and various biochemical reactions by the pathway of intracellular AdoHcy, AdoMet, Hcy and Ado metabolism [25,50].

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In conclusion, SAHH from was cloned for the first time. AmphiSAHH and sea urchin SAHH might be the archetype of the vertebrate SAHH protein in the phylogenetic tree. Sequence comparison suggested that the evolutionary appearance of the ten exon/nine intron organization of SAHH genes occurred after the split of invertebrates and vertebrates. Western blotting for recombiant AmphiSAHH protein and amphioxus extracts reveals the presence of a single positive band with an apparent molecular mass of approx. 48 kDa. Purified recombinant AmphiSAHH exhibited differences in cofactor binding and catalytic efficiency compared with the human enzyme. Finally, we observed that AmphiSAHH was strongly expressed in the hepatic caecum, gill, spermary and ovary.

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