Identification of two human dimethylarginine dimethylaminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases

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Methylarginines inhibit nitric oxide synthases (NOS). Cellular concentrations of methylarginines are determined in part by the activity of dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18). We have cloned human DDAH and identified and expressed a second novel DDAH isoform (DDAH I and II respectively). DDAH I predominates in tissues that express neuronal NOS. DDAH II predominates in tissues expressing endothelial NOS. These results strengthen the hypothesis that methylarginine concentration is actively regulated and identify molecular targets for the tissue and cell-specific regulation of methylarginine concentration.

Key words: rapid amplification of cDNA ends, symmetrical dimethylarginine, transcription and PCR.

INTRODUCTION

Arginine residues in proteins are methylated by a family of protein arginine N-methyltransferases (PRMTs) [1]. These enzymes catalyse the methylation of guanido nitrogens of arginine to produce N°-monomethyl-L-arginine (L-NMMA), N°,N°-dimethyl-L-arginine (asymmetrical dimethylarginine; ADMA) and N°,N°-dimethyl-L-arginine (symmetrical dimethylarginine; SDMA). Proteolysis of proteins containing these residues releases free methylarginines [2]. Although the biological role of methylarginine residues in proteins is unclear, free L-NMMA and ADMA, but not SDMA, are inhibitors of all three isoforms of nitric oxide synthase (NOS) and might alter NOS activity in health or disease [3]. Free methylarginines are found in cell cytosol, plasma and tissues; their concentrations differ between tissues and between regions within a single tissue or organ [3–5]. Elevated concentrations of ADMA have been detected in endothelial cells repopulating blood vessels damaged by balloon injury [6], in the plasma of patients or experimental animals with hyperlipidaemia [7], renal failure [3] or atherosclerosis [8], and in patients with schizophrenia [9] or multiple sclerosis [10]. Altered biosynthesis of nitric oxide (NO) has been implicated in the pathogenesis of all of these conditions and it is possible that the accumulation of endogenous ADMA underlies the inhibition of NO generation. Conversely, a decrease in the plasma concentration of methylarginine, which is correlated with a decrease in blood pressure, has been observed in normal pregnancy [11].

The production of methylarginines is probably an obligatory step in protein turnover; rates of production can show tissue-specific and temporal variations [12] that might lead to fluctuations in their intracellular concentrations. However, L-NMMA and ADMA, but not SDMA, are actively metabolized to citrulline and methylamines by the action of dimethylarginine dimethylaminohydrolase (DDAH) [13]. Certain tissues that express NOS also express DDAH [14]. The pharmacological inhibition of DDAH increases the concentration of ADMA in endothelial cells and inhibits the NO-mediated endothelium-dependent relaxation of blood vessels [4]. These observations suggest that DDAH activity ensures that the local concentration of ADMA does not normally rise sufficiently to affect NO generation and that changes in DDAH activity could alter NOS activity. However, we and others have also found that DDAH activity and DDAH protein expression are not always correlated; this led us to speculate that there might be more than one isoform of the enzyme.

In this paper we report the cloning of the human DDAH cDNA and present its derived amino acid sequence. In addition, we have discovered a novel DDAH isoform, which we have named DDAH II. Recombinant expression of DDAH II confirms that this protein is a functional homologue of DDAH (now termed DDAH I). The DDAH I and II isoforms show distinct tissue distributions with some relationship to NOS isoforms. The existence of two isoforms of DDAH with different tissue distributions suggests that the regulation of methylarginine levels is of considerable biological importance.

During the preparation of this paper a sequence identical with that of DDAH II has been entered into the Genbank database (accession number AF070667) and identified as a putative DDAH homologue.

MATERIALS AND METHODS

Database searching and cDNA cloning

The cDNA sequence of human DDAH I was obtained by a combination of database searching, specific reverse-transcriptase-mediated PCR and 5′/3′ rapid amplification of cDNA ends (RACE). A BLAST search of the database of expressed sequence tags with the rat DDAH I sequence (accession number d86041 [15]) identified a single human cDNA sequence comprising 161 bp
of human DDAH I cDNA fused downstream of 160 bp of unknown sequence (accession number aa376335). Human kidney poly(A)\(^+\) RNA was reverse-transcribed from an oligo(dT) primer, after which human DDAH I cDNA was PCR amplified in two PCR reactions incorporating either HDDAH I.1 and RDDAH I.1 or HDDAH I.2 and RDDAH I.2 (Table 1). For 5’ RACE, human kidney poly(A)\(^+\) mRNA was reverse-transcribed with primer HDDAH I.3. RNA was digested with RNase H and cDNA was purified with a HighPure DNA purification kit (Boehringer). Purified cDNA was poly(A)-tailed by incubation with terminal transferase in the presence of dATP and used directly in PCR reactions incorporating Oligo(dT) Anchor and HDDAH I.4. For 3’ RACE, human poly(A)\(^+\) RNA was primed with Oligo(dT) Anchor and reverse-transcribed before PCR with oligonucleotides HDDAH I.5 and Anchor. PCR products were cloned into pCRTOP2.1 (Invitrogen) and inserts were sequenced on both strands. For expression of recombinant human DDAH II, bacterial cell lysate was incubated at 37 °C for 60 min with 50 μl of 100 mM Na\(_2\)HPO\(_4\), pH 6.5, containing 0.02 mCi of L-[\(^{14}C\)]-citrulline content by vortex-mixing with 1 ml of 50 % (v/v) Dowex 50X8-400 and centrifugation at 10000 g for 5 min; 500 μl of the supernatant was then mixed with 5 ml of liquid-scintillation fluid and the \(^{14}C\) content was determined by liquid-scintillation counting. For colorimetric assays, 50 μl of bacterial cell lysate was incubated at 37 °C for 60 min with 50 μl of 100 mM Na\(_2\)HPO\(_4\), pH 6.5, containing 8 mM L-NAME or a colorimetric assay for citrulline production. For the radiochemical assay, 50 μl of bacterial cell lysate was incubated at 37 °C for 60 min with 50 μl of 100 mM Na\(_2\)HPO\(_4\), pH 6.5, containing 0.02 mCi of L-[\(^{14}C\)]-citrulline content by vortex-mixing with 1 ml of 50 % (w/v) Dowex 50X8-400 and centrifugation at 10000 g for 5 min; 500 μl of the supernatant was then mixed with 5 ml of liquid-scintillation fluid and the \(^{14}C\) content was determined by liquid-scintillation counting. For colorimetric assays, 50 μl of bacterial cell lysate was incubated at 37 °C for 60 min with 50 μl of 100 mM Na\(_2\)HPO\(_4\), pH 6.5, containing 8 mM L-NAME or a colorimetric assay for citrulline production. After incubation, samples were prepared for determination of \(^{14}C\)citrulline content by vortex-mixing with 1 ml of 50 % (w/v) Dowex 50X8-400 and centrifugation at 10000 g for 5 min; 500 μl of the supernatant was then mixed with 5 ml of liquid-scintillation fluid and the \(^{14}C\) content was determined by liquid-scintillation counting. For colorimetric assays, 50 μl of bacterial cell lysate was incubated at 37 °C for 60 min with 50 μl of 100 mM Na\(_2\)HPO\(_4\), pH 6.5, containing 8 mM L-NAME or a colorimetric assay for citrulline production.
Isoforms of human dimethylarginine dimethylaminohydrolase blot (Clontech). Probes were produced by PCR amplification of oligo(dT)-primed human kidney poly(A)* mRNA with oligonucleotide primer pairs HDDAH I 4 and 5, HDDAH II 3 and 4, HENOS 1 and 2 and HNNOS 1 and 2. After PCR, reaction products were resolved on 2% (w/v) agarose gels, isolated from the gel and labelled with a random-primed labelling kit (Boehringer). Labelled probes were purified on Nick columns (Pharmacia) and hybridized to filters in accordance with the manufacturer’s instructions. Signals were detected by autoradiography. For quantification, membranes were exposed overnight to a Fuji BASStation PhosphorImager screen.

RESULTS
Cloning of human DDAH I and DDAH II
By using a combination of reverse-transcriptase-mediated PCR and RACE, a cDNA encoding the entire ORF of human DDAH I was assembled. The 858 bp ORF is 90% identical with rat DDAH I ORF (results not shown) and encodes a polypeptide of 285 residues that is 95% identical with the rat protein (Figure 1).

Figure 1 Amino acid alignment of rat and human DDAH I with human DDAH II
The derived amino acid sequences of rat and human DDAH I and human DDAH II were aligned by using the CLUSTAL program. Amino acid identities are indicated by asterisks, highly conservative substitutions by colons and conservative substitutions by full stops.

Figure 2 Recombinant expression of human DDAH II
Aliquots of E. coli transfected with either empty vector (lanes 1 and 3) or vector containing human DDAH II cDNA (lanes 2 and 4) were resolved by SDS/PAGE (15% (w/v) gel). Gels were either stained for total protein with Coomassie Blue (lanes 1 and 2) or processed for Western blotting (lanes 3 and 4) as described in the Materials and methods section. The filled arrow indicates the recombinant protein that is specifically recognized by the anti-PentaHis antibody. The migration of molecular mass markers is indicated at the left.

Figure 3 Tissue distribution of human DDAH and NOS isoforms
Labelled probes specific for human DDAH I, DDAH II, neuronal NOS, endothelial NOS and β-actin were sequentially hybridized to a commercially available multiple-tissue Northern blot. The migration of molecular mass markers is indicated at the left.

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Table 2  Quantification of DDAH isoform expression in human tissues

A human multiple Northern blot was probed sequentially with probes specific for human DDAH I and DDAH II (see Figure 3). Hybridization signals were quantified by phosphorimaging and corrected for β-actin signals to correct for RNA loading. Signals are expressed as percentages of the maximum level of expression for each isoform.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DDAH I</th>
<th>DDAH II</th>
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<tbody>
<tr>
<td>Heart</td>
<td>15</td>
<td>100</td>
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<tr>
<td>Brain</td>
<td>56</td>
<td>19</td>
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<tr>
<td>Placenta</td>
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<td>Pancreas</td>
<td>47</td>
<td>34</td>
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Identification of DDAH-related proteins

To identify proteins with significant primary sequence homology to DDAH I/II we performed a search of the SwissProt database with both the human DDAH I and DDAH II protein sequences. This search revealed significant homology between both DDAH sequences and the sequences of arginine deiminase enzymes from several microbial species. The highest degree of homology was found with the sequence of arginine deiminase from Pseudomonas putida (accession no. p41142). The overall similarity to human DDAH I and DDAH II was 48% and 31% respectively; however, within a 72-residue domain (residues 123–194 of DDAH I and 121–192 of DDAH II) the similarity increased to 70%, and amino acid identity was 20% (Figure 4). In this domain, DDAH I and DDAH II are 80% identical. A comparison of the sequences of human DDAH I and DDAH II with other arginine-utilizing or arginine-producing enzymes, such as peptidyl-arginine deiminase, arginase, argininosuccinate lyase, arginine decarboxylase and nitric oxide synthase, revealed no significant amino acid homology.

DISCUSSION

Multiple isofoms of DDAH enzymes exist in mammals

In this study we report the cloning of the human cDNA encoding DDAH I and identify a second DDAH isoform. DDAH I was originally identified by Ogawa et al. as being responsible for the metabolism of ADMA (but not SDMA) residues in rat kidney [13]. Further studies by these authors reported first the purification of the enzyme and subsequently the cloning of the rat DDAH I cDNA [15] and, while our experiments were in progress, the cloning of human DDAH I cDNA [16]. We have expressed rat DDAH I cDNA in E. coli and confirmed that this cDNA does indeed encode an enzyme that metabolizes ADMA (J. M. Leiper, unpublished work).
Leiper, unpublished work). The cDNA and deduced amino acid sequences that we obtained for human DDAH I agree with those published by Kimoto et al. [16] and confirm that the rat and human proteins are 95% identical, indicating a relatively high degree of conservation throughout evolution.

We also identified a protein with approx. 62% overall amino acid sequence similarity to DDAH I. This protein and its cDNA are highly conserved between mammalian species with 98% amino acid identity between the sequences of the mouse and human proteins (J. M. Leiper and Joanne Santa Maria, unpublished work). Recombinant expression showed that the protein metabolizes ADMA and 1-NNMA to citrulline but does not metabolize SDMA or arginine. Thus, like DDAH I, the enzyme metabolizes only asymmetric methyl arginines, permitting its designation as an asymmetric methyl arginine methylaminohydrolase. At a single high concentration of substrate the enzyme metabolizes more ADMA than 1-NNMA, although we have not definitively established its substrate preference. On the basis of the sequence similarity and functional results we have named the enzyme DDAH II, although in due course the name asymmetric methyl arginine methylaminohydrolase might be considered to be more appropriate for both enzymes. Further studies of purified DDAH II will be required to characterize its activity fully in comparison with that of DDAH I.

**Distinct tissue distribution of human DDAH isoforms**

We have previously suggested that the concentration of ADMA residues might have a role in the regulation of NOS activity in certain situations [4,11,17]. ADMAs compete with arginine for NOS and can thereby alter the apparent \( K_m \) of NOS for arginine [8]. Recent studies have revealed a family of PRMT enzymes that differ in their tissue distributions, intracellular localizations, substrate specificities and regulation [12]. The action of these enzymes could produce both tissue-specific and temporal changes in the level of protein arginine methylation that would result, via proteolysis, in changes in the concentration of intracellular free methylarginine. Uncontrolled fluctuations in free methylarginine concentration might then cause inappropriate inhibition or activation of arginine-utilizing enzymes such as NOS. Our present finding that there are at least two DDAH isoforms with markedly different tissue distributions raises the possibility that methylarginine concentrations are highly regulated in a tissue-specific manner. Indeed, we suggest that recent observations that tumour necrosis factor \( \alpha \) and oxidized low-density-lipoprotein increase the accumulation of ADMA in the conditioned medium of human endothelial cells without any apparent change in DDAH I protein [18] might now be explained by the down-regulation of DDAH II in these cells. Preliminary results from our laboratory indicate that the mRNA for both DDAH I and DDAH II isoforms is present in human endothelial cells (J. M. Leiper and Joanne Santa Maria, unpublished work). The \( K_m \) of DDAH II for 1-NNMA is 510 \( \mu \)M, which is comparable to 360 \( \mu \)M for DDAH I [13]. It is unclear why the \( K_m \) values are so much higher than the reported intracellular concentrations of methylarginines, although it is possible that high local concentrations are reached in certain circumstances.

Taken together with the observation that neuronal NOS is found in tissues that express predominantly DDAH I, whereas endothelial NOS is found in tissues with high levels of DDAH II, a mechanism of isoform-specific regulation of NOS via modulation of methylarginine concentration becomes a possibility. However, it is also clear that some tissues that express DDAH do not express either neuronal NOS or endothelial NOS. The reasons for this are not yet known but methylarginines might have important functions other than, or in addition to, the regulation of NOS. Consistent with this possibility is our recent identification of functional homologues of DDAH in several microbial species that do not express NOS [19].

**Mammalian DDAHs are homologous with bacterial arginine deiminases**

When we compared the amino acid sequence of human DDAH I and DDAH II with the sequence of enzymes known to be involved in the synthesis or metabolism of arginine, we found no homology with any mammalian enzymes. However, we identified significant homology between DDAH isoforms and arginine deiminases, a family of enzymes hitherto described only in prokaryotic organisms and the primitive eukaryotic organism *Giardia intestinalis* [20]. Arginine deiminase is the first enzyme of the arginine dihydrolase pathway, an important source of energy and nitrogen in microbes. Arginine deiminase catalyses the hydrolysis of arginine to ammonia and citrulline, a reaction that closely resembles the hydrolysis of methylarginine to methylamine and citrulline that is catalysed by DDAH [13]. DDAH I and DDAH II are 80% identical over a region of 72 residues (Figure 4), which also has high homology with bacterial deiminases and contains two of the three domains that are highly conserved between all arginine deiminases [20]. It has been suggested that these domains might constitute a substrate-binding/catalytic site in arginine deiminase; the finding that this region is highly conserved in DDAHs suggests that it might be relevant to substrate binding or the catalytic activity of these enzymes.

The generation of methylarginines occurs in a wide range of cells and tissues; the asymmetrical methylation catalysed by type 1 PRMT enzymes leads to the formation of compounds that have the capacity to compete with arginine and inhibit NOS activity. The discovery of a second DDAH isoform with a novel tissue distribution and the cloning and sequencing of the two DDAH isoforms mean that it should now be possible to perform the molecular, cellular and clinical studies necessary to elucidate the roles of endogenous methylarginines in health and disease. The existence of a second DDAH explains some of the discrepancies in the literature in which DDAH activity and expression do not coincide. We have also identified a likely functional domain that is not present in other mammalian arginine-utilizing or arginine-producing enzymes; this provides a useful lead for discovering pharmacological agents with specific actions on DDAH.

This work was supported by British Heart Foundation Programme Grant RG9004.

Received 6 April 1999/7 July 1999; accepted 28 July 1999