FEBS Letters 544 (2003) 258–261

FEBS 27318

Identification and characterization of plant agmatine iminohydrolase, the last missing link in polyamine biosynthesis of plants

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Received 4 April 2003; revised 5 May 2003; accepted 5 May 2003

First published online 16 May 2003

Edited by Ulf-Ingo Flügge

Abstract The cloning, expression and characterization of plant agmatine iminohydrolase (AIH, also known as agmatine deiminase, EC 3.5.3.12) is described. Recombinant AIH of *Arabidopsis thaliana* forms dimers and catalyzes the specific conversion of agmatine to *N*-carbamoylputrescine and ammonia. Biochemical data suggested that cysteine side chains are involved in catalysis. However, site-directed mutagenesis of the two highly conserved cysteine residues of AIH showed that these cysteines are important but not essential for activity, arguing against a thioester substrate–enzyme intermediate during catalysis. This work represents the completion of the cloning of the arginine decarboxylase pathway genes of higher plants.

Key words: Agmatine iminohydrolase (agmatine deiminase); *N*-Carbamoylputrescine; Polyamine biosynthesis; Putrescine; *Arabidopsis thaliana*

1. Introduction

Polyamines are small organic compounds found in all living organisms. Throughout the kingdoms of life, different pathways for the biosynthesis of these essential compounds have evolved (for review see [1,2]). The basic polyamine, which is also the precursor of higher polyamines, is putrescine (1,4-diaminobutane, H_2N-$(CH_2)_4$-NH_2). In plants, putrescine is synthesized by two different pathways (Fig. 1): in the arginine decarboxylase (ADC) pathway arginine is first decarboxylated by ADC to form agmatine which is subsequently converted to putrescine by the combined action of agmatine iminohydrolase (AIH, also known as agmatine deiminase, forming *N*-carbamoylputrescine from agmatine) and *N*-carbamoylputrescine amidohydrolase (CPA, forming putrescine from *N*-carbamoylputrescine). In the ornithine decarboxylase (ODC) pathway putrescine is the direct product of ornithine decarboxylation by the action of ODC. The ADC pathway is also known from some bacteria, while the ODC pathway is the dominant pathway for putrescine biosynthesis in animals and fungi. The completion of the *Arabidopsis thaliana* genome project revealed that in this plant no ODC gene exists; in addition, no enzymatic conversion of ornithine was found in extracts from *A. thaliana* [3]. This means that *A. thaliana* is totally dependent on the ADC pathway for putrescine biosynthesis. Genes for ADCs have been cloned from several plants, including *A. thaliana* (for review see [1,2]). Although AIH and CPA activities in plants have been known for a long time [4,5] and AIHs have been purified to homogeneity from different sources [6–9], amino acid sequences or the genes for these enzymes were unknown. Recently, Nakada and co-workers, analyzing agmatine utilization mutants of *Pseudomonas aeruginosa*, identified the genes for CPA and AIH of this bacterium [10]. Based on the published sequence of *P. aeruginosa* CPA we were successful in identifying the CPAs from *A. thaliana* and *Lycopersicon esculentum* [11]. Here we report on the cloning, expression, and biochemical characterization of AIH from *A. thaliana*. This work represents the completion of the cloning of the ADC pathway genes of higher plants.

2. Materials and methods

2.1. Synthesis of N-carbamoylputrescine

N-Carbamoylputrescine was synthesized from putrescine and cyanate as described by Smith and Garraway [4]. Purification of the reaction product *N*-carbamoylputrescine from side products is described in Piotrowski et al. [11].

2.2. Cloning and expression of A. thaliana AIH

The EST clone 106O4T7 (GenBank accession number T22747) was received from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH, USA). After sequencing the whole insert, the proposed open reading frame was amplified by polymerase chain reaction (PCR) with the following primers: 5′-TATCATATGGAGGAGTCACGAGAATCGC-3′ (AtAIHForw) and 5′-TATCTCGAGGTGGCCATTTTCGGCGACGG-3′ (AtAIHHis), thereby introducing a *Nde*I site at the 5′-end including the start codon and a *Xho*I site at the 3′-end, omitting the stop codon. The PCR product was cloned into the *Nde*I/*Xho*I sites of the pET-21b(+) vector (Novagen, Madison, WI, USA), resulting in a C-terminal translational fusion with the His_6 tag. Expression was done in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA, USA) and the His_6-tagged protein was purified by Ni^{2+}-chelate affinity chromatography.

2.3. Blue native polyacrylamide gel electrophoresis (PAGE)

Blue native PAGE was carried out as described by Schägger et al. for soluble proteins using a 5–18% acrylamide gradient gel [12].

2.4. Activity measurements

The activity of AIH was determined in a total volume of 1 ml containing 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM dithiothreitol, 1–4 mM substrate and 1 μg of protein. Reactions were carried out in triplicate at 30°C (for determination of kinetic parameters) or at 37°C for 10–30 min against a control of heat-denatured protein. Released ammonia was quantified using the Berthelot reac-

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Abbreviations: ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00515-5

tion as described earlier [13]. Qualitative and semi-quantitative measurements of produced *N*-carbamoylputrescine were done by thin layer chromatography as described in [11]. Collision-induced dissociation MS/MS spectra of authentic *N*-carbamoylputrescine and of the reaction product were recorded with a Q-TOF2 mass spectrometer (Micromass, Manchester, UK).

2.5. Inhibitor experiments

Enzymes were pre-incubated in *N*-ethylmaleimide (1 mM) or iodoacetamide (2 mM) for 30 min in the dark in the presence or absence of agmatine (2 mM, added 5 min before the inhibitor). Elimination of inhibitor and agmatine was done by gel filtration on PD-10 columns (Amersham Biosciences) equilibrated in 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM dithiothreitol. Controls without inhibitor were performed in parallel. The eluted protein was then used for activity measurements as described above.

2.6. Site-directed mutagenesis

Site-directed mutagenesis of Cys180 and Cys366 to alanines was performed using the GeneEditor in vitro Site-Directed Mutagenesis System according to the manufacturer's protocols (Promega, Mannheim, Germany).

3. Results and discussion

3.1. Cloning and expression of A. thaliana AIH

In 2001 Nakada and co-workers [10] reported the identification of two genes from *P. aeruginosa*, which encode AIH (*AguA*) and CPA (*AguB*) involved in agmatine utilization of this bacterium. Interestingly, *P. aeruginosa* AIH shows 59% amino acid identity to a putative protein from *A. thaliana*. We received the cDNA for this putative *At*AIH from the Arabidopsis Biological Resource Center. Sequencing the cDNA revealed that it contained a proposed complete open reading frame of 1152 bp (encoding 383 amino acids with a total molecular mass of 43 kDa) which is identical to the proposed mRNA sequence derived from the genome data (At5g08170, annotated as putative protein, GenBank accession number NM_120900). The protein was expressed as a C-terminal His_6 tag fusion protein and purified by Ni^{2+}-chelate affinity chromatography to apparent homogeneity as judged by Coomassie-stained sodium dodecyl sulfate (SDS)–polyacrylamide gels (Fig. 2A). Using blue native PAGE (Fig. 2B) the molecular mass of native *At*AIH was determined to be 81 kDa, showing that the native enzyme is a homodimer. The molecular mass of the monomer as well as the organization of native AIH as dimer fit well with published data about the AIH from maize [6,9].

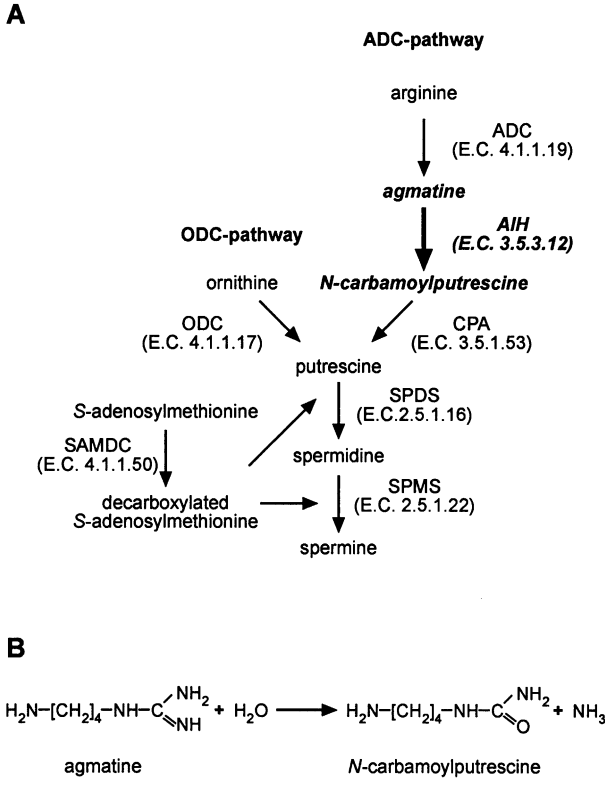

Fig. 1. Polyamine biosynthesis in plants. In A the two pathways of putrescine biosynthesis occurring in plants are shown. B shows the reaction catalyzed by AIH in detail. SAMDC: *S*-adenosylmethionine decarboxylase, SPDS: spermidine synthase, SPMS: spermine synthase.

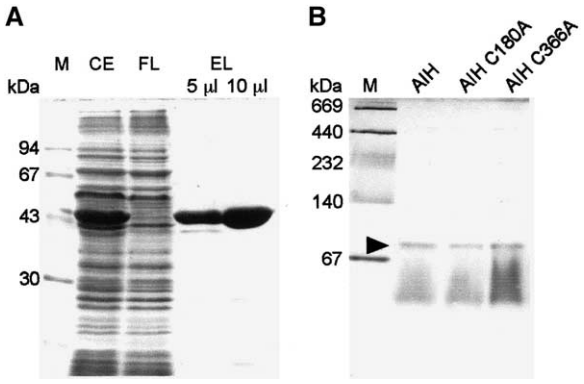

Fig. 2. Expression and purification of recombinant *At*AIH (A) and determination of its native molecular mass (B). A: A Coomassie-stained SDS–polyacrylamide gel is shown. Ten µl of the soluble bacterial crude extract (CE), 10 µl of the column flow-through (FL), and 5 or 10 µl of the combined eluate (EL) were loaded. B: Blue native PAGE of unmodified *At*AIH, *At*AIH C180A, and *At*AIH C366A. Ten µl each were loaded. The band representing the dimer is indicated by an arrowhead. M: molecular mass marker.

3.2. Conversion of agmatine to N-carbamoylputrescine by AtAIH

The recombinant *At*AIH converted agmatine to *N*-carbamoylputrescine and ammonia as shown by thin layer chromatography, mass spectrometry, and the Berthelot reaction (Fig. 3). We tested a battery of other substances, namely the guanidino compounds guanidinoacetic acid, β-guanidinopropionic acid, γ-guanidinobutyric acid; the amino acids L-arginine, L-citrulline, L-glutamine, L-ornithine; the *N*-carbamoyl compounds *N*-carbamoyl-β-alanine and *N*-carbamoyl-D,L-aspartic acid; the polyamines putrescine, spermine and spermidine; and the nitriles allylcyanide, β-cyano-L-alanine, indole-3-acetonitrile, and 3-phenylpropionitrile. None of them was a substrate for *At*AIH.

3.3. Biochemical characterization of AtAIH

The biochemical characterization of *At*AIH resulted in the following data: the optimal activity was reached at pH 7 and between 35 and 40°C. Half-maximal velocity was reached at an agmatine concentration of 112 µM and maximal velocity was 440 nkat/mg protein.

3.4. Involvement of cysteine side chains in catalysis

Thiol-reducing agents like dithiothreitol, 2-mercaptoethanol and L-cysteine could be used to activate the enzyme (not

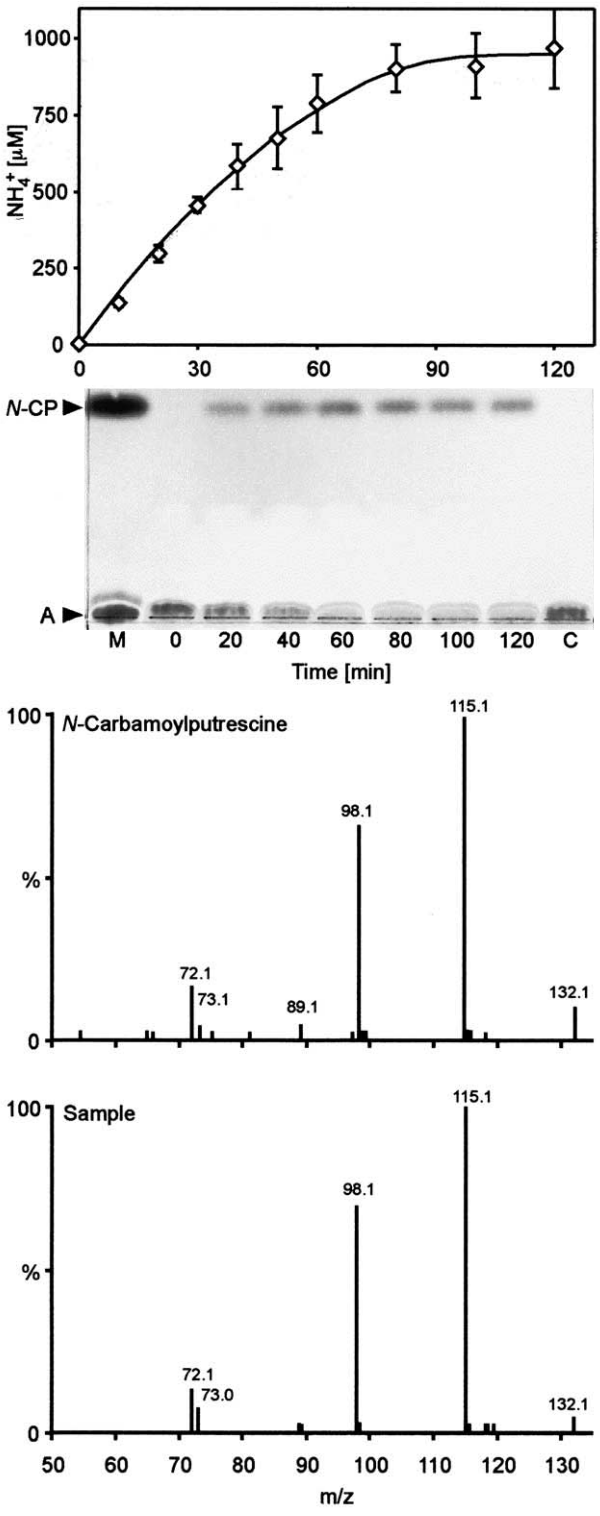

Fig. 3. Hydrolysis of agmatine to *N*-carbamoylputrescine and ammonia by *At*AIH. *At*AIH (1 μg) was incubated with agmatine (1 mM) as described in Section 2. After the indicated times, released ammonia was quantified (first panel) and aliquots were subjected to thin layer chromatography (second panel). C: heat-denatured control, M: mix of *N*-carbamoylputrescine (*N*-CP) and agmatine (A). After completion of the reaction, the reaction product was analyzed by ESI-MS/MS (fourth panel) against authentic *N*-carbamoylputrescine (third panel).

shown). In addition, the enzyme was strongly inhibited by the thiol-modifying agents *N*-ethylmaleimide (not shown) and iodoacetamide (Fig. 4A). The sensitivity to thiol-modifying agents was also described for AIHs analyzed from different sources [5–9,14]. Inhibition of *At*AIH by iodoacetamide could be largely abolished by simultaneous incubation of the enzyme with its substrate agmatine (Fig. 4A). These data can be explained assuming that at least one cysteine residue is located in or near the catalytic pocket and is thus protected by the substrate from alkylation. Alignment of the *At*AIH sequence with 17 homologous sequences obtained from GenBank revealed the existence of two strongly conserved cysteine residues, namely Cys180 (numbering refers to *At*AIH) and Cys366, which are conserved in 16 and 17, respectively, of the 17 sequences (data not shown). Both cysteines were exchanged to alanines by site-directed mutagenesis. The mutated enzymes were still able to form dimers (Fig. 2B) indicating that no major structural changes have occurred due to the mutation. *At*AIH C180A and *At*AIH C366A displayed about 35% and 15%, respectively, of the activity of the unmodified protein (Fig. 4B). This shows that both cysteines are important for activity but are not essential. Both mutated enzymes were still inhibited further by iodoacetamide and this inhibition was still diminished by parallel incubation with agmatine (Fig. 4A). So in each mutant at least one additional cysteine is substrate-protected from the alkylating agent. Mutating two

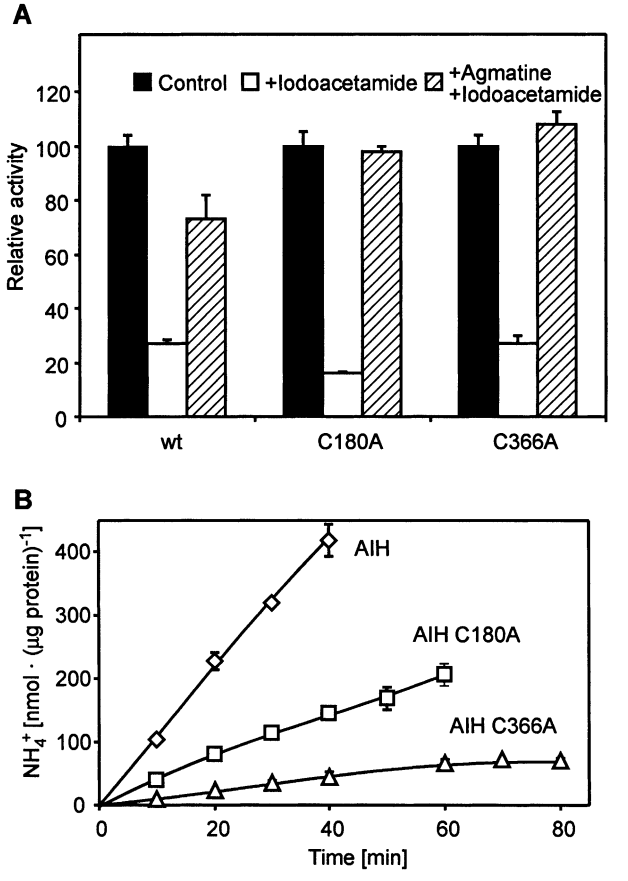

Fig. 4. Involvement of cysteine residues in catalysis. A: The enzymes were pre-incubated in iodoacetamide, iodoacetamide plus agmatine, or without iodoacetamide, before activity was analyzed. For details see Section 2. B: Activity of unmodified *At*AIH, *At*AIH C180A, and *At*AIH C366A.

other cysteines of *At*AIH, Cys229 and Cys230, which are conserved in four and seven of the 17 sequences, respectively, did not result in reduction of activity (data not shown). We therefore propose that both cysteines, Cys180 and Cys366, are located in the substrate pocket of the enzyme. So alkylation of Cys366 results in further inhibition of activity of *At*AIH C180A and vice versa.

3.5. Conclusions

The putative protein encoded by the *A. thaliana* gene At5g08170 represents a functional AIH. Together with the recent identification of the CPA of the same plant [11], all enzymes of the arginine-derived polyamine biosynthesis pathway of *A. thaliana* have now been identified and their genes have been cloned. AIH represents a member of a new family of *C-N*-hydrolases. Although some experiments indicate that AIH, like the members of the nitrilase superfamily, possesses a catalytically active cysteine residue, our experiments have shown that Cys180 and Cys366 may be near or in the catalytic center of the enzyme but neither one of them is essential for catalysis. This argues against a role of one of these cysteines to form a thioester bond to the substrate.

Acknowledgements: The authors wish to thank the Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH, USA) for providing EST clone 106O4T7 and Prof. Elmar W. Weiler for supporting this project.

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