Property comparison of recombinant amphibian and mammalian allantoicases

Davide Vigetti, Loredano Pollegioni, Claudio Monetti, Mariangela Prati, Giovanni Bernardini, Rosalba Gornati*

Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi dell'Insubria, Via J.H. Dunant 3, 21100 Varese, Italy

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Abstract Allantoicase is an enzyme involved in uric acid degradation. Although it is commonly accepted that allantoicase is lost in mammals, birds and reptiles, we have recently identified its transcripts in mice and humans. The mouse mRNA seems capable of encoding a functional allantoicase, therefore we expressed the *Xenopus* and mouse allantoicases (MAlc and XAlc, respectively) in *Escherichia coli* and characterized the recombinant enzymes. The two recombinant allantoicases show a similar temperature and pH stability but, although XAlc and MAlc share a 54% amino acid identity, they differ in sensitivity to bivalent cations, in substrate affinity and in the level of expression in tissues (as revealed by means of Western blot analysis). We propose that the loss of allantoicase activity in mouse is due to a low substrate affinity and to a reduced expression level of the enzyme.

Key words: Purine degradation; Uric acid; Uricolysis; Animal evolution

1. Introduction

Allantoicase (allantoate amidohydrolase, EC 3.5.3.4) is an enzyme of the uricolytic pathway that catalyzes the hydrolysis of allantoate to ureidoglycolate (Fig. 1). Allantoicase most likely appeared very early during evolution as its activity is present in all five kingdoms [1,2]. For bacteria and fungi, the characterization of the enzymatic activity was complemented by the molecular cloning of the allantoicase gene [3,4]. In contrast, the evidence for allantoate-degrading enzymes in plant is ambiguous: the presence of allantoicase was proposed by Shelp and Ireland [5], but there is much more evidence for allantoate amidohydrolase (EC 3.5.3.9) [6–12]. All evidence is indirect, no real enzyme activity in cell extracts was measured. Allantoicase activity is widespread in the animal kingdom [13], but the protein sequences were unknown until recently, when we cloned allantoicase in the anuran *Xenopus laevis* [14].

Interestingly, allantoicase activity appears to have been lost just during vertebrate evolution [15]; it has been conserved in fishes and amphibians but not in tetrapods. Reptiles, birds and Hominoidea excrete uric acid, whereas mammals other than Hominoidea possess uricase and excrete allantoin [16].

Although it is generally accepted that mammals do not demonstrate any allantoin-degrading enzyme, Fujiwara and Noguchi [17] found a functional ureidoglycolate lyase in mammal liver. More recently, we have identified an allantoicase transcript in mice as well as in humans [18,19]. Notably, a mouse allantoicase transcript has been identified only in testis and it corresponds to the complete open reading frame (ORF) encoding a hypothetical 46 kDa polypeptide that shares 54% of identity with *Xenopus* allantoicase (Fig. 2). Therefore, also the mouse allantoicase gene could encode a functional enzyme. To investigate this possibility, we have expressed both *Xenopus* and mouse allantoicases in *Escherichia coli* and compared their properties and expression in vivo.

2. Materials and methods

2.1. DNA manipulations

Cloning and transformation techniques were performed essentially as described by Sambrook et al. [20]. *Xenopus* and mouse expression constructs were generated by polymerase chain reaction (PCR). Briefly, the *Xenopus* allantoicase ORF (accession number AF153230) was amplified by PCR, starting from a previously characterized clone 32 DNA [14] using Tli DNA polymerase (Promega) and the following primers: GGAATTCCATATGCACCATCATCATCATCACATGTTTGCTCATCCAAAAGAA and GCGGGATCCTTAAAGTAGAGGCCGAGGAA. The mouse allantoicase ORF (accession number AF278712) was amplified starting from the plasmid DNA of EST clone number 515776 [18] as a template using Advantage 2 polymerase mix (Clontech) and the following primers: GGAATTCCATATGCACCATCATCATCATCACATGGCTGATACTCCAAA and GCGGGATCCTTAAAGGTTCGCCCTGAATCC (the sequences corresponding to *Nde*I and *Bam*HI recognition sites are underlined and the sequence encoding the six histidine tag is double underlined). The amplified products were digested with *Nde*I and *Bam*HI (New England Labs) and cloned into the *Nde*I–*Bam*HI linearized pT7-7 plasmid. These constructs (pHisXAlc or pHisMAlc) were then sequenced; they encode the full-length XAlc (purified His-tagged *Xenopus* allantoicase) and MAlc (purified His-tagged mouse allantoicase) proteins, respectively, both having an additional His-tag sequence at the N-terminus.

2.2. HisXAlc and HisMAlc overexpression

For protein expression, the plasmids pHisXAlc and pHisMAlc were transferred to the host BL21(DE3)pLysS *E. coli* strain (Promega). *E. coli* cells carrying the recombinant plasmids were cultivated at 37°C in LB medium containing ampicillin and chloramphenicol (100 μg/ml and 34 μg/ml final concentration, respectively) and, after an

*Corresponding author. Fax: (39)-332-421300.
E-mail address: rosalba.gornati@uninsubria.it (R. Gornati).

Abbreviations: XAlc, purified His-tagged *Xenopus* allantoicase; MAlc, purified His-tagged mouse allantoicase; ORF, open reading frame; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside

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overnight growth ($OD_{600\,nm} > 2.5$), isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM. The temperature was reduced to 30°C and the cells collected after 24 h. Crude extracts were prepared by French press lysis (four cycles at 1000 psi) of the cell suspension in 50 mM sodium phosphate buffer, pH 7.0, containing 10 μg/ml RNase and DNase. The lysate was then centrifuged at $39\,000 \times g$ for 40 min at 4°C and the precipitate discarded.

2.3. XAlc and MAlc purification and antibodies production

To improve the specificity of interaction in the subsequent affinity chromatography step, 1 M NaCl, 20 mM imidazole and 5% glycerol (all final concentrations) were added to the crude extract obtained as reported above. The enzyme solution was then applied to a HiTrap Chelating affinity column (Pharmacia Biotech) equilibrated with 50 mM sodium pyrophosphate buffer, pH 7.2, containing 1 M NaCl, 20 mM imidazole and 5% glycerol, using an ÄKTA FPLC system (Pharmacia Biotech). The bound protein was subsequently eluted with 50 mM sodium pyrophosphate buffer, pH 7.2, containing 500 mM imidazole and 5% glycerol. The fractions containing allantoicase activity were pooled and concentrated using a Centricon Plus-20 (Amicon). The purified enzymes were stored in 50 mM potassium phosphate buffer, pH 7.0, by desalting on a Sephadex G25 PD10 column (Pharmacia Biotech) equilibrated in the same buffer.

Rabbit anti-XAlc antibodies were produced by Davids Biotechnologie (Regensburg, Germany) using the purified XAlc as antigen.

2.4. Allantoicase assay

The allantoicase activity assay was always performed in triplicate according to the procedure reported by Piedras et al. [21] with minor modifications. Briefly, the reaction mixture contained 15 mM allantoic acid in 20 mM potassium phosphate buffer, pH 7.0. The mixture was incubated at 25°C and the reaction was initiated by the addition of 10 μg of XAlc or 30 μg MAlc. The amount of ureidoglycolate formed was determined by withdrawing aliquots (0.2 ml) of the assay solution at different reaction times and stopping the reaction by adding 0.2 ml of 0.5 N NaOH (which transformed ureidoglycolate into glyoxylate). After 10 min of incubation at room temperature, 0.8 ml of 0.4 M phosphate buffer, pH 7.0, and 0.2 ml of 25 mM phenylhydrazine were added and the solution was further incubated for 10 min at room temperature. Finally, the absorbance was determined at 535 nm after the addition of 1 ml of 12 N HCl and 0.2 ml of 50 mM potassium ferricyanide. A titration curve was obtained with the same procedure using different concentrations of glyoxylate ($\Delta\varepsilon_{535\,nm} = 3025\ mM^{-1}\ cm^{-1}$). One allantoicase unit is defined as the amount of enzyme that converts 1 μmol of allantoic acid in product in 1 min at 25°C. The steady-state kinetic parameters of XAlc and MAlc were determined measuring the enzyme activity using different allantoic acid concentrations, at 25°C and pH 7.0.

2.5. Determination of pH, temperature optima and stability

The phenylhydrazine spectrophotometric assay was used. Activity was assayed over the 25–70°C temperature range and 4.8–9.0 pH range. To determine temperature stability, enzyme samples were incubated at pH 7.0 and at 25, 30 and 40°C and aliquots taken periodically. For assessing the pH stability, activity was measured using the standard assay after incubation of enzyme samples at 25°C for different periods of time in a multi-buffer system at various pH values (from 5 to 8) containing 15 mM Tris, 15 mM H_3PO_4, 15 mM Na_2CO_3 and 250 mM KCl and brought to the indicated pH with a fixed volume of NaOH/HCl. A high KCl concentration was used as a buffer against minor changes in the ionic strength at different pH values.

2.6. Effect of bivalent metals

To assess the effect of bivalent ions and chelating agents on allantoicase activity, samples were preincubated for 10 min and then assayed using the phenylhydrazine spectrophotometric assay in the presence of 10 and 100 nM, 1 μM, 1, 5 and 10 mM of Ca^{2+}, Mg^{2+}, EDTA or EGTA.

3. Results

3.1. Overexpression and purification of recombinant allantoicases

His-tagged *Xenopus* and mouse allantoicases were expressed in *E. coli* BL21(DE3)pLysS cells transformed with pHisXAlc and pHisMAlc expression plasmids. Experiments were performed to optimize the expression conditions, analyzing the effect of temperature, time of induction and collection, and IPTG concentration on allantoicase expression. The best conditions were obtained using *E. coli* cells grown overnight at 37°C, induced with 1 mM IPTG, cultured at 30°C and harvested 24 h after induction. The recombinant allantoicases overexpressed under these conditions were completely soluble and thus fully recovered in the crude extract. The specific allantoate-degrading activity in the crude extract of uninduced *E. coli* BL21(DE3)pLysS was about 0.3 U/mg (due to endogenous *E. coli* allantoate amidohydrolase), whereas the specific activity of IPTG-induced crude extract was 35 U/mg for XAlc and 1 U/mg for MAlc.

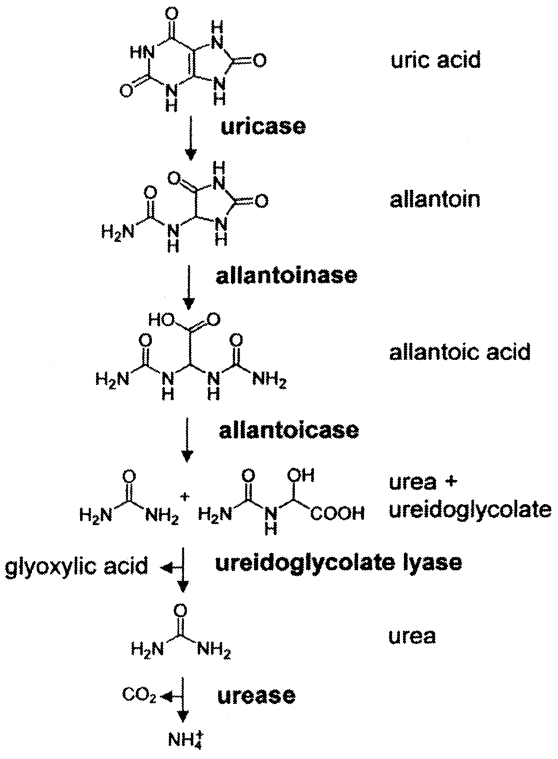

Fig. 1. Purine degradation pathway in animals.

Chromatography of the crude extract from IPTG-induced *E. coli* cells was performed on a HiTrap Chelating affinity column. Under the elution conditions described in Section 2, XAlc and MAlc were selectively eluted as a single resolved peak, whereas the contaminant proteins did not selectively bind to the resin (data not shown). The final preparation was at least 90% homogeneous for both XAlc and MAlc as judged by SDS–PAGE analysis (data not shown). After the affinity chromatography step, specific activity (determined at fixed, 15 mM, allantoic acid concentration) increased to 350 and 50 U/mg protein for XAlc and MAlc, respectively. An overall yield of about 4 mg of purified allantoicase starting from 6 l of *E. coli* culture (about 45 g of cells) was produced for both XAlc and MAlc.

3.2. Chemico-physical properties of recombinant allantoicases

Xenopus and mouse allantoicase show a high degree of amino acid homology (54%; Fig. 2). Their calculated molecular

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Xenopus  MFAHPKENIALPVPEFLQMNNLACESVGGKVLFATDDWFAPAEHLLKKTEPEFKVGLFTE 60
Mouse    MADTPKEGKLTRFLDFTQLIDLASECVGGKVLFATDDFFGPAENLIKSDSPTFKEHEYTE 60
         *  ***.  .:* *:.:**.*.***********:*.***:*:*:.:* **  :**

Xenopus  FGKWMDGWETRRKRIPGHDWCIIQLGVPGIIHGFEADTRFFTGNYAPRISIQAACLKPEE 120
Mouse    FGKWVDGWETRRKRIPGHDWCVIQLGIQGIIRGIDVDISYFSGNHAPRMSIQAANLSEED 120
         ****:*****************:****: ***:*:::* :*:**:***:***** *. *:

Xenopus  IT--LQPREDKIGTAASDEEFKAADKLKSEKWSHLLKMTELKPGYAESSHSYFQVNSKER 178
Mouse    TMSNIPPRGVRMGTAATPEEFEAVTELKSHSWDYLVPMSELKLGDPDSSHNYYFVNSQQR 180
           : **  ::****: ***:*. :***.:*.:*: *:*** * .:***.*: ***::*

Xenopus  WTHLRLNIYPDGGIARFKVYGIGQRDWTSCGPNDFEDLLSMVNGGVCLGFSDAHYGHPRN 238
Mouse    WTHIRLNIFPDGGVARLRVYGTGQRDWAALGSTEPVDLVAIAFGGVCVGFSNAHFGHPNN 240
         ***:****:****:**::*** *****:: *.:  **:::. ****:***:**:***.*

Xenopus  LIGNGRACDMGDGWETARRLDRPPILKADSKGILQVPGFEWAVLKLGHPGLVTHIEIDTN 298
Mouse    MIGVGEPKSIADGWETARRLDRPPVLEASENGLLLVPGCEWAVFRLAHPGVITQIEIDTK 300
         :** *.. .::**************:*:*.::*:* *** ****::*.***::*:*****:

Xenopus  HFKGNSPNSCKIDACALKPTEQEEVKGHGNFEQGYNWKPLLPVTQIHPHKRHFMESTSLA 358
Mouse    YFKGNCPNSCKVDGCILTTLEEEDMIRHNWNLPAHKWKSLLPVTKLIPNQNHLLDSLTLE 360
         :****.*****:*.* *.. *:*:: *.  .::**.*****:: *::.*:::* :*

Xenopus  LHQVISHVKITIAPDGGVSRIRLWGFP------RPLP---------- 389
Mouse    LQDVITHAMITIAPDGGVSRLRLKGFPSSICLLRPLREKPLLRFSLKTGFRANL 414
         *:.**:*. ***********:** ***     ***
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Fig. 2. Alignment of *Xenopus* (GenBank accession number AF153230) and mouse (GenBank accession number AF278712) allantoicase protein sequences using ClustalW program. Dashes indicate gaps introduced for optimal alignment; asterisks indicate identical or conserved residues in all sequences; semicolons indicate conserved substitutions; and dots, semi-conserved substitutions.

mass is 44 718 and 47 159 Da, respectively. The two purified recombinant enzymes, thus, show similar electrophoretic mobility in SDS–PAGE analysis ($M_r = 43.7 \pm 1.8$ kDa and 46.5 ± 2.3 kDa for XAlc and MAlc, respectively), demonstrating that both cDNAs encode the full-length polypeptide.

Purified XAlc and MAlc possess a similar extinction coefficient at 280 nm (a value of 1.763 and 1.714 was obtained for a 1 mg/ml solution, respectively).

Preliminary experiments showed that the allantoicase activity was easily lost during long-term storage. The optimal storage conditions were obtained when the purified allantoicases were stored at −20°C in 50 mM potassium phosphate buffer, pH 7.0. A significant and rapid loss of activity occurs when XAlc and MAlc are stored at temperatures higher than 4°C. The stability of allantoicases was investigated following the time course of XAlc and MAlc activity during incubation at 25, 30 and 40°C. The results indicate that the two enzymes (which show a similar pattern of inactivation, see Fig. 3A at 40°C) are quite sensible to thermal inactivation. Interestingly, in both cases the loss of activity is not complete, reaching about 30–40% of the initial activity after 120 min of incubation. This suggests that the inactivation should be due to a change in the conformation-aggregation state of the two proteins. Since allantoicases are known to be influenced by metal-ions [22,23], another possibility is that the thermal stability of XAlc and MAlc (Fig. 3A) depends on the presence of two different forms of the enzyme with respect to metal binding. However, this may not be the case since the addition of 5 mM EDTA did not affect the thermal stability pattern of XAlc and MAlc (data not shown). The different behavior of XAlc and MAlc in presence of exogenous chelators or bivalent ions (Fig. 5) is indeed a peculiar effect.

The temperature-dependence profile of the activity clearly distinguishes XAlc from MAlc. Plotting XAlc activity, over the 25–70°C temperature range, shows an activity maximum of 40–45°C, whereas MAlc showed a maximum at about 50–55°C (see Fig. 3B). A sharp decrease in enzyme activity due to protein instability is evident at higher temperatures for both proteins.

The effects of pH on the XAlc and MAlc activity was investigated using the enzymatic assay at pH values ranging from 4.8 to 9 (Fig. 4). At pH values lower than 4.8 the activity assay we used is unreliable because of the fast degradation of the substrate allantoate. Both enzymes were active over a rather broad range of pH. The activity decreased at pH values higher than 8 and an apparent $pK_a = 8.8 \pm 0.2$ was estimated

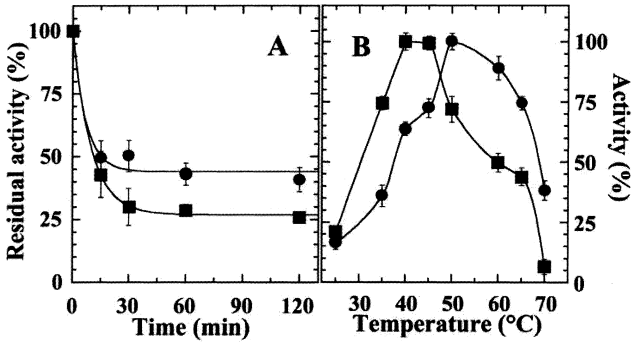

Fig. 3. A: Effect of temperature on the stability of recombinant XAlc and MAlc. The residual activity of XAlc (■) and MAlc (●) samples incubated at 40°C was determined at different times, using the phenylhydrazine spectrophotometric assay. Error bars indicate the standard deviation of three determinations. B: Effect of temperature on the activity of recombinant XAlc and MAlc. A total of 10 μg of XAlc (■) and 30 μg of MAlc (●) were assayed using the spectrophotometric assay at the reported temperatures; 100% corresponds to the higher value determined for each enzyme. Error bars indicate the standard deviation of three determinations.

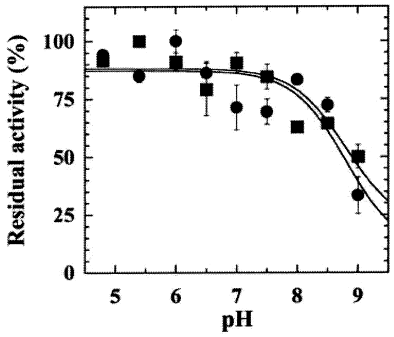

Fig. 4. Effect of pH on the activity of recombinant XAlc and MAlc. A total of 10 μg of XAlc (■) and 30 μg of MAlc (●) were diluted in multi-buffer and assayed at pH values reported in the graph using the phenylhydrazine spectrophotometric assay; 100% corresponds to the higher value determined for each enzyme. The fit of the experimental points was obtained using the equation for a single ionization. Error bars indicate the standard deviation of three determinations.

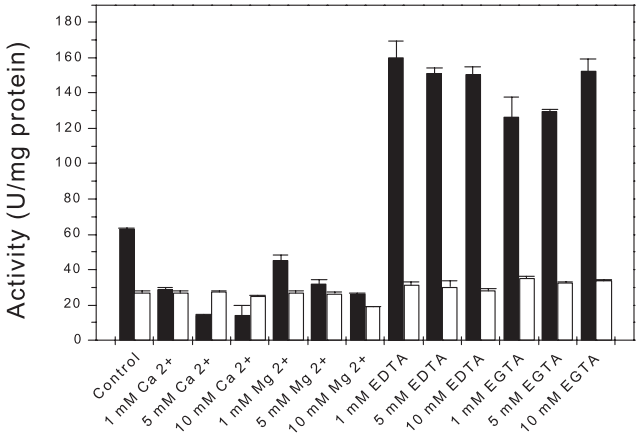

Fig. 5. Effects of bivalent ions on allantoicase activity. A total of 10 μg of XAlc (black bars) and 30 μg of MAlc (white bars) were assayed using the standard phenylhydrazine assay (15 mM allantoic acid, pH 7.0) in the presence of the indicated concentrations of Ca^{2+}, Mg^{2+}, EDTA and EGTA. Standard deviations of three different experiments are also reported.

for both enzymes. This effect is specific on the activity since the enzyme is fully stable during the time of the assay at 25°C. The two allantoicases also showed a similar stability in a wide range of pH values; after 120 min of incubation at pH ranging from 5 to 8, 50% of the initial activity was recovered when the pH was brought back to 7.0 for the activity assay (data not shown).

3.3. Kinetic properties of recombinant allantoicases

A preliminary investigation of the optimal conditions for assaying the allantoicase activity was carried out using the purified recombinant XAlc. Thus, the activity was assayed at pH 7.0 (a decrease in activity is observed at higher pH values, see Fig. 4) and 25°C. The activity of previously characterized allantoicases depends on the presence of bivalent ions [22,23]. XAlc and MAlc activity was thus assayed at different Ca^{2+} and Mg^{2+} concentrations. As clearly shown in the histogram of Fig. 5, XAlc activity is strongly inhibited by Ca^{2+} and Mg^{2+} whereas MAlc activity is not altered by the presence of bivalent ions. Analogously, XAlc activity is strongly enhanced by the presence of chelating agents such as EDTA and EGTA, while no effect on MAlc activity is observable. Interestingly, the effect of EDTA on XAlc activity was not evident up to 1 μM (result not shown), a concentration similar to that of the enzyme in the assay solution. Both recombinant enzymes contain the His-tag sequence, but bivalent cations and EDTA affect only the activity of XAlc, this observation indicates that the effect on XAlc is specific. Moreover, the inhibition of XAlc activity by bivalent ions is fully reversible since it can be recovered by incubation in the presence of an excess of EDTA or EGTA of XAlc samples previously treated with Ca^{2+} or Mg^{2+}. A similar effect was observed on crude extracts from *Xenopus* liver (data not shown).

The steady-state kinetic parameters of XAlc and MAlc have been determined in the absence of EDTA and both purified proteins showed hyperbolic kinetics with allantoate as the substrate (Fig. 6A,B). The two enzymes strongly differ in the affinity for substrate (K_m values of 4.5 mM and 250 mM for XAlc and MAlc, respectively), although they possess similar V_{max} values (i.e. 450 and 640 μmol/min/mg protein for XAlc and MAlc, respectively). These results suggest that during evolution the ability of the enzymes to bind the substrate was largely modified but their turnover efficiency was left untouched. In the presence of 1 mM EDTA, about a two-fold increase in XAlc kinetic parameters was evident ($V_{max} = 900$ μmol/min/mg protein and $K_m = 10$ mM, data not shown).

3.4. In vivo detection of allantoicase expression

The purified XAlc has been used to raise anti-XAlc rabbit antibodies. In Western blot analysis anti-XAlc antiserum was

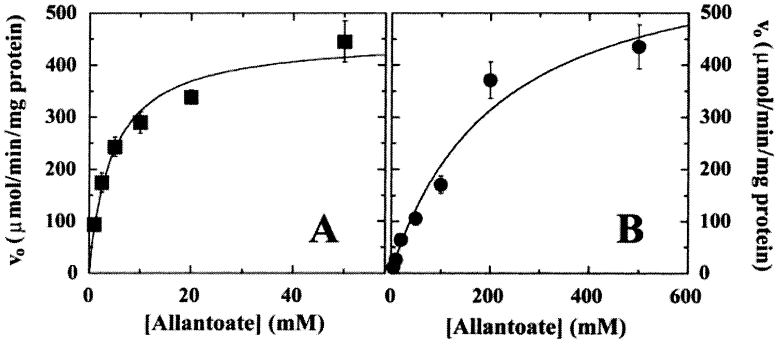

Fig. 6. Kinetics of XAlc (A) and MAlc (B) with allantoate as substrate. Measurements were taken at 25°C and pH 7.0, using 7.5 μg and 21.5 μg of purified XAlc and MAlc, respectively. Error bars indicate the standard deviation of three determinations.

able to detect up to 0.1 μg and 0.3 μg of pure XAlc and MAlc, respectively. A panel of *Xenopus* tissues (lung, muscle, intestine, kidney, testis, ovary, brain and liver) was tested for the presence of allantoicase with anti-XAlc antiserum by Western blot; only liver and kidney showed the 45.5 ± 3.0 kDa allantoicase band. Corresponding experiments were performed on mouse testis extracts and no signal was identified (data not shown). Accordingly, we conclude that the expression of allantoicase in mouse is less than 0.5% of the total proteins in the crude extract.

4. Discussion

The purine catabolism that brings to the formation of uric acid is common to all vertebrates, while its further degradation can vary from species to species [15]. The causes of extinction of uricolytic enzymes are poorly understood; nonsense mutations inactivating uricase gene product were found only in Hominoidea [24–26]. Allantoicase is one of the enzymes involved in uricolysis and its activity is absent in reptiles, birds and mammals. Recently, we cloned vertebrate allantoicase cDNA from different sources, i.e. the amphibian *X. laevis* [14], the mammals *Mus musculus* [18] and *Homo sapiens* [19]. The human allantoicase transcript only contains information to encode the C-terminal portion of the enzyme, which could be the cause of the loss of activity in humans. On the other hand, the mouse allantoicase transcript, specifically expressed in testis, possesses all the information required to produce a functional enzyme. In fact, it contains a complete ORF encoding a polypeptide which shows a 54% identity with the *Xenopus* enzyme (Fig. 2) and which retains the four structural motifs that characterize the functional allantoicases from bacteria to *Xenopus* [18].

To determine whether mouse allantoicase cDNA encodes a functional enzyme, we constructed two expression vectors (pHisXAlc and pHisMAlc) to produce both *Xenopus* and mouse allantoicases tagged with a six histidine sequence at the N-terminus for the rapid purification procedure. These recombinant proteins were expressed in *E. coli*, purified and characterized. XAlc and MAlc are properly folded since both purified enzymes are competent in catalysis and because the K_m value for allantoate determined using recombinant XAlc is similar to that determined using crude extract from *Xenopus* kidney [14]. Therefore, we conclude that the mRNA expressed in mouse testis encodes a protein (homologue to *Xenopus* allantoicase) capable of catalyzing allantoate hydrolysis.

The biochemical properties of both MAlc and XAlc were investigated. They showed a similar pattern of stability at different temperatures, as well as at different pH values in the 4.5–9.0 pH range, as also previously reported for *Chlamydomonas reinhardtii* allantoicase [22]. The two recombinant allantoicases can be distinguished from each other by their optimal temperature for activity, i.e. 40°C for XAlc and 55°C for MAlc (see i.e. Fig. 3B). These relatively high temperatures are not surprising since a previously characterized allantoicase from *C. reinhardtii* possesses an optimum temperature activity of 60°C [22].

From a kinetic point of view, the two enzymes show similar V_{max} values in the absence of EDTA. In contrast, a main difference between XAlc and MAlc is represented by the affinity for the substrate; in fact, the K_m of MAlc is about 50-fold higher than that determined for XAlc. The high value of K_m (about 250 mM) determined for mouse allantoicase renders it very improbable that the enzyme is effective under in vivo conditions. This hypothesis is further supported by the evidence that the functional allantoicases characterized so far (from microorganisms to fishes) possess K_m values ranging from 4 to 9 mM [27–29].

XAlc and MAlc also distinguish from each other by the effect of bivalent cations (i.e. Ca^{2+} and Mg^{2+}) and chelating agents (i.e. EDTA and EGTA) on enzyme activity. Our experiments showed that XAlc (as well as the native enzyme from *Xenopus* liver) is sensitive to Ca^{2+} and Mg^{2+} (the presence of such ions decreases the allantoicase activity), whereas treatment with chelating agents produces an increase in enzyme activity. In contrast, MAlc activity is not modified by the presence of bivalent metals. Similar experiments performed on purified allantoicase from the microalgae *C. reinhardtii* [22], showed that EDTA treatment causes a loss of allantoicase activity that can be fully recovered by adding Mn^{2+}.

In conclusion, we have demonstrated that allantoicase mRNA from mouse, which is specifically expressed in testis, is capable of encoding a functional allantoicase. The absence of allantoicase activity detectable in mouse cells [18] is the result of different events. Western blot experiments using anti-XAlc antibodies indicated that the level of allantoicase in mouse testis, if present, is lower than 0.5% of the total protein content in the crude extract. Furthermore, and according to the high K_m value of MAlc for allantoic acid, enzyme activity in vivo should be very low.

Why is the allantoicase gene conserved in mouse and its mRNA specifically expressed in testis? What is its role? Allantoicase might indeed have a different function in mammals. A similar hypothesis was suggested by Fujiwara and Noguchi [17] for ureidoglycolate lyase, another enzyme of the uricolytic pathway; in fact, although it is generally accepted that this enzyme is lost in mammals, these researchers have found ureidoglycolate lyase in the liver of mammals. A detailed analysis of the evolution of purine degradation pathway in vertebrates could provide us with surprises.

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