Journal of Inorganic Biochemistry 132 (2014) 92–95

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Further insight into the inhibitory action of a LIM/double zinc-finger motif of an agmatinase-like protein

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ARTICLE INFO

Article history:
Received 17 May 2013
Received in revised form 13 December 2013
Accepted 16 December 2013
Available online 25 December 2013

Keywords:
Agmatine
LIM-domain
Agmatinase-like protein

ABSTRACT

Agmatine is a precursor for polyamine biosynthesis also associated to neurotransmitter, anticonvulsant, antineurotoxic and antidepressant actions in the brain. It results from decarboxylation of L-arginine by arginine decarboxylase and it is hydrolyzed to urea and putrescine by agmatinase. Recently, we have described a new protein which also hydrolyzes agmatine although its sequence greatly differs from all known agmatinases. This agmatinase-like protein (ALP) contains a LIM-like double Zn-finger domain close to its carboxyl terminus, whose removal results in a truncated variant with a 10-fold increased k_{cat}, and a 3-fold decreased K_m value for agmatine. Our proposal was that the LIM-domain functions as an autoinhibitory, regulatory entity for ALP. Results in this report provide additional support for the postulated inhibitory effect. The purified isolated LIM domain was shown to be competitively inhibitory to a truncated variant ALP (lacking the LIM-domain), but not to the wild-type species. The C453A variant was shown to be a Zn^{2+}-free enzyme with kinetic parameters similar to those of the truncated-ALP. A molecular dynamic simulation of a modeled LIM-domain 3D structure showed that, as a consequence of C453A mutation, the coordination of the zinc ion is broken and the structure of the zinc finger is melted. The inhibitory action of the LIM/double Zinc-finger motif was associated to a significant conformational change, as detected by tryptophan fluorescence studies, but was not related to changes in the association of the enzyme with the catalytically essential Mn^{2+}.

1. Introduction

Agmatine, produced by decarboxylation of L-arginine, binds to various receptors and exhibits neurotransmitter actions in the brain [1]. At physiological concentrations, agmatine is inhibitory to the neuronal nitric oxide synthase [2] and participates in modulation of insulin release from pancreatic cells and renal sodium excretion [3]. There is also evidence for anticonvulsant, antinociceptive, anxiolytic and antidepressant like actions associated to agmatine [4]. A fine control of agmatine levels is evidently required, thus justifying the interest in enzymological aspects of agmatine synthesis and degradation in mammals.

Agmatine results from decarboxylation of L-arginine by arginine decarboxylase and it is hydrolyzed to urea and putrescine by agmatinase [5]. The human agmatinase has been cloned but only poorly characterized, due to the considerably low activity exhibited by the obtained recombinant species. However, the functionality of the cloned human gene was deduced from a functional complementation test with a yeast strain which contains a disruption in the gene encoding ornithine decarboxylase and thus requires exogenous polyamines for growth [6]. The amino acid sequence of the human agmatinase is about 30% and <20% identical to bacterial agmatinases and mammalian arginases, respectively [7]. Moreover, ligands for binding of the metallic cofactor Mn^{2+} in bacterial agmatinase and other members of the arginase superfamily are fully conserved in human agmatinase.

Recently, we have described a new protein, immunohistochemically detected in the hypothalamic region and hippocampal astrocytes and neurons of the rat brain, which also hydrolyze agmatine although its sequence greatly differs from all known agmatinases and arginases [8,9]. The predicted amino acid sequence of the rat brain protein, designated as agmatinase like protein (ALP) resulted to be remarkably different to the sequences of all cloned agmatinases and the evolutionary related arginases, including the localization of residues which are strictly conserved and critical for catalysis by these enzymes [8]. One particularly interesting finding was a LIM-like domain closes the carboxyl terminus of the ALP (Fig. 1), whose removal results in a truncated variant with a 10-fold increased k_{cat}, and a 3-fold decreased K_m value for agmatine [10]. As expected for a LIM protein, zinc was detected only in the wild-type ALP and amounted to 2 Zn^{2+}/monomer [10].

The LIM domain is a zinc-coordinating domain, consisting of two tandemly repeated zinc fingers, generally involved in cellular differentiation and control of cellular growth, gene expression, interactions with cytoskeleton, auto-inhibitory effects and possibly as biosensors that mediate communication between the cellular and nuclear compartments [11,12]. The classic LIM consensus sequence includes a CX2CX16–23-HX2CX2CX2CX16–21CX2 (CH/D) sequence, associated with a highly

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http://dx.doi.org/10.1016/j.jinorgbio.2013.12.006

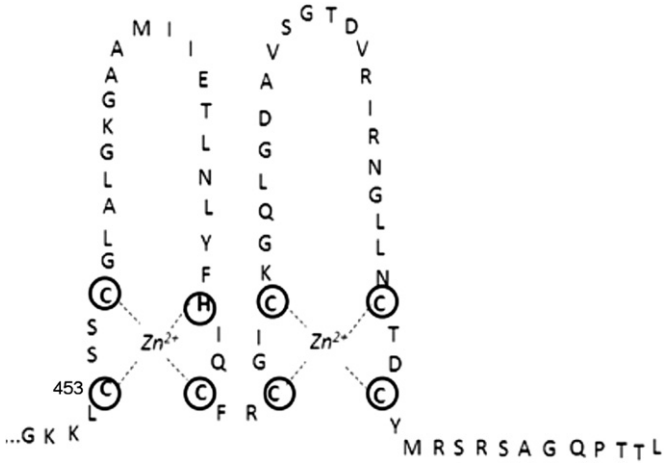

Fig. 1. Proposed schematic representation of a LIM-domain of ALP. Postulated ligands to the zinc ions are encircled and cysteine 453 is indicated [10].

variable sequence in the remainder of the domain that confers functional specificity [13]. Our proposal was that the LIM-domain functions as an auto inhibitory, regulatory entity for ALP and that inhibition is reversed by interaction of the domain with some yet undefined brain protein. Similar results have been reported for the LIM-kinase [14].

We have now examined the interaction of ALP with the catalytically required Mn^{2+} and the LIM-associated Zn^{2+}, by using mutagenic and kinetic approaches. The present report provides additional evidence for the postulated effect of the LIM domain on the agmatinase activity expressed by the protein ALP. The effect is shown to be strictly related to the Zn^{2+} ions, which are required for proper folding of the LIM domain, and not to the interaction of the enzyme with the catalytically essential Mn^{2+}.

2. Materials and methods

2.1. Materials

Agmatine, glycine, Tris, SDS and all other reagents were of the highest quality commercially available (most from Sigma Aldrich Chemical Co. Louis, MO, USA). Restriction enzymes, as well as enzymes and reagents for PCR, are obtained from Invitrogen Co (Carlsbad, CA, USA). The synthetic nucleotide primers were obtained from the Fermelo Biotec Co. (Santiago, Chile).

2.2. Enzyme preparations

All species, including the wild-type, LIM-truncated species, C453A-ALP variant and also the isolated LIM-domain, were directionally cloned into the histidine-tagged pQE60 *Escherichia coli* expression vector, and the histidine-tagged proteins were expressed in *E. coli* strain JM109, following induction with 0.05 mM isopropyl-β-D-thiogalactopyranoside. All protein variants were purified by ion exchange chromatography on a DEAE-cellulose column and affinity chromatography on NTA–Ni^{2+} columns. The purity of all preparations was ~90%.

C453A-ALP variant was obtained by using the QuikChange® Site-Directed Mutagenesis Kit of Stratagene, with the plasmid H_6pQE60-29,4 containing the ALP cDNA as template. The presence of the desired mutation and the absence of unwanted changes were confirmed by automated DNA sequence analysis.

2.3. Enzyme and protein assays

Routinely, agmatinase activities were determined by measuring the formation of urea (product) from 80 mM agmatine in 50 mM glycine–NaOH (pH 9.0) and 2 mM $MnCl_2$. All the assays were initiated by adding the enzyme to the substrate, buffer and $MnCl_2$ solution previously equilibrated at 37 °C, and urea was determined by a colorimetric method with α-isonitrosopropiophenone [15], measuring the absorbance at 540 nm.

Initial velocity and inhibition studies were performed in duplicate and repeated three times. The inhibitory patterns were initially determined by double reciprocal plots and replots of intercepts versus inhibitor concentrations. Kinetic parameters were obtained by fitting the experimental data to the appropriate Michaelis–Menten equation ($v_i = V_{max}S / K_m + S$) by using nonlinear regression with Graph Pad Prism version 5.0 for Windows (Graph Pad Software Inc., San Diego, CA, USA). Protein concentration was determined by means of the standard Bio-Rad protein assay (Bio-Rad, CA, USA) with bovine serum albumin as standard.

2.4. Enzyme–metal interactions

The manganese and zinc content of ALP and mutant species was determined by total reflection X-ray fluorescence analysis on a S2 Picofox Bruker spectrometer equipped with an X-ray metal–ceramic tube and a molybdenum target, working at 50 W of maximum power, at 50 kV and 1 mA and air-cooled. This technique is based in that when materials are excited with high-energy, short wavelength radiation (e.g., X-rays), they become ionized. If the energy of the radiation is sufficient to dislodge a tightly-held inner electron, the atom becomes unstable and an outer electron replaces the missing inner electron. When this happens, energy is released due to the decreased binding energy of the inner electron orbital compared with an outer one. The emitted fluorescent radiation is of lower energy than the primary incident X-rays. Because the energy of the emitted photon is a characteristic of a transition between specific electron orbitals in a particular element, the resulting fluorescent X-rays can be used to detect the abundances of elements in the sample [16]. The stoichiometry calculations were based on a previously determined subunit molecular mass of ALP 58 kDa [8].

The affinity of Mn^{2+} binding ($ALP + Mn^{2+} \leftrightarrow ALP - Mn^{2+}$),was evaluated by following the manganese reactivation of fully inactivated species, obtained by incubation with 50 mM EDTA in 50 mM Tris–HCl (pH 7.5) for 30 min at room temperature and then dialyzed for 6–8 h against ultrapure water. For reactivation, the enzymes were incubated for 15 min at 37 °C with varying concentrations of Mn^{2+} in 10 mM Tris–HCl (pH 8.5), 50 m MKCl and 10 mM nitrilotriacetic acid as a metal ion buffer. Then, agmatinase activities were determined in 50 mM Tris–HCl, pH 8.5. The studies were performed in duplicate and repeated two times. Dissociation constants (K_d) were estimated from the hyperbolic dependence of agmatinase activity on free-Mn^{2+} concentrations, with nonlinear regression using Graph Pad Prism 5.0. Free Mn^{2+} concentrations were calculated using a dissociation constant of 3.98×10^{-8} M and a pK_a value of 9.8 for nitrilotriacetic acid [17].

2.5. Fluorescence spectra

Fluorescence measurements were made at 25 °C on a Shimadzu RF-5301 spectrofluorimeter. The protein concentration was 40–50 μg/ml and emission spectra were measured with the excitation wavelength at 295 nm. The slit width for both excitation and emission was 1.5 nm, and spectra were corrected by subtracting the spectrum of the buffer solution in the absence of protein. The buffer solution (pH 7.5) contained 5 mM Tris–HCl and 250 mM KCl. In the fluorescence quenching experiments, the acrylamide concentrations varied from 0 to 100 mM.

2.6. Molecular modeling and molecular dynamic simulation

A model of the LIM-domain of ALP was generated with MODELLER [18]. Even though its sequence identity is lowered to