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Evidence for an inhibitory LIM domain in a rat brain agmatinase-like protein

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ABSTRACT

We recently cloned a rat brain agmatinase-like protein (ALP) whose amino acid sequence greatly differs from other agmatinases and exhibits a LIM-like domain close to its carboxyl terminus. The protein was immunohistochemically detected in the hypothalamic region and hippocampal astrocytes and neurons. We now show that truncated species, lacking the LIM-type domain, retains the dimeric structure of the wild-type protein but exhibits a 10-fold increased k_{cat}, a 3-fold decreased K_m value for agmatine and altered intrinsic tryptophan fluorescent properties. As expected for a LIM protein, zinc was detected only in the wild-type ALP (~2 Zn^{2+}/monomer). Our proposal is that the LIM domain functions as an autoinhibitory entity and that inhibition is reversed by interaction of the domain with some yet undefined brain protein.

Introduction

Agmatine, a decarboxylated derivative of L-arginine, has been associated to several important biological processes in mammals, including neurotransmitter, anticonvulsant, antineurotoxic and antidepressant actions in the brain [1]. This primary amine is packed into synaptic vesicles in the brain and spinal cord and acts on transmembrane receptors (α2-adrenergic, imidazoline and gultamatergic NMDA receptors) [2]. At physiological concentrations, agmatine is inhibitory to the neuronal nitric oxide synthase [3] and participate in modulation of insulin release from pancreatic cells [4,5] and renal sodium excretion [6]. A fine control of agmatine levels is evidently required, thus justifying the interest in the enzymological aspects of agmatine synthesis and degradation in mammals.

Agmatine result from decarboxylation of arginine by arginine decarboxylase [7,8] and it is converted to putrescine and urea by agmatinase [9]. With regard to agmatinase, there are reports establishing its presence in the brain [9,10], although the study of their enzymic properties has been complicated by the lack of appropriately active preparations of the enzyme. This includes two reported recombinant forms of human agmatinase which are very poorly active under *in vitro* conditions [11,12]. In one of these reports, 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 [11]. In contrast with these reports, we have recently cloned and expressed a rat brain protein which is significantly active as agmatinase *in vitro*, although its amino acid sequence is only about 12% identical with the human and bacterial enzymes [13]. In any case, certain degree of structural homology was revealed by its reaction with a polyclonal antibody raised against *Escherichia coli* agmatinase. We refer to this protein as agmatinase-like protein (ALP).

Like *E. coli* agmatinase, ALP was shown to require Mn^{2+} for catalytic activity and to be maximally active at pH 9–9.5 [13]. By RT-PCR and immmunohistochemical methods, the protein was detected in the hypothalamus in glial cells and arcuate nucleus neurons, and also in hippocampus astrocytes and neurons, but not in brain cortex [14]. Considering that, in general, this localization coincides with that described for its substrate agmatine, ALP was suggested to be involved in the regulation of intracellular concentrations of this neurotransmitter/neuromodulator.

One especially interesting finding was a terminal segment that includes a LIM-type domain in the sequence of ALP [13]. 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 [15,16]. The classic LIM consensus sequence includes a $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_2$(CH/D) sequence, associated with a highly variable sequence in the remainder of the domain that confers functional specificity [17].

As a part of a continuing effort aimed to characterize the molecular and functional aspects of ALP, we have now turned our attention to the LIM-type domain, by examining the consequences of its

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removal from the ALP sequence. Our suggestion is that the domain acts as an autoinhibitory entity and that inhibition is reversed by interaction of the domain with some yet undefined brain protein.

Methods

Materials

All reagents were of the highest quality commercially available (most from Sigma, St. Louis, MO, USA) and were used without further purification. Restriction enzymes, as well as enzymes and reagents for the polymerase chain reaction (PCR) were obtained from Invitrogen Co. (Carlsbad, CA, USA). The plasmid pE-PHO was generously donated by Dr. Juan Olate (Universidad de Concepción, Chile) and the *Saccharomyces cerevisiae* strain TRY104Δspe1, was generously provided by Dr. Herbert Tabor (National Institutes of Health, Bethesda, USA).

Enzyme and protein assays

Routinely, agmatinase activities were determined by measuring the formation of urea from 80 mM agmatine in 50 mM glycine-NaOH (pH 9.0). All the assays were initiated by adding the enzyme to the substrate buffer solution previously equilibrated at 37 °C. 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. Data were then fitted to the appropriate equations by using nonlinear regression with GraphPad Prism version 5.0 for Widows (GraphPad Software Inc., San Diego, CA, USA). Urea was determined by a colorimetric method with α-isonitrosopropiophenone [18] and protein by means of the standard Bio-Rad protein assay (Bio-Rad, CA, USA) with bovine serum albumin as standard.

LIM domain deletion and expression of ALP

The gene of ALP lacking the sequence corresponding to the LIM-domain was amplified with the high-fidelity Pfx polymerase, from vector H6PQE60-29, two primers used were 5′ cac ggt gcc cat ggt gac acc cag gcc 3′ and 5′ gcc gga agc ttt cac tac cac agg agg agc aca g 3′ which included specific restriction sites (NcoI and Hind III respectively). The PCR fragment was then directionally cloned into the histidine-tagged pQE60 *E. coli* expression vector, and the histidine-tagged enzyme was expressed in *E. coli* strain JM109, following induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The bacterial cells were disrupted by sonication on ice (5 × 30 s pulses) in a buffer solution containing 100 mM Tris–HCl (pH 7.5), 2 mM $MnCl_2$, 2 mM putrescine, 2 mM DTT, 0,1 mM PMSF and 100 mM KCl. After centrifugation for 20 min at 96,000g, the supernatant was applied to a Ni^{2+}-NTA column equilibrated with 20 mM Tris–HCl (pH 7.9), containing 5 mM imidazole and 500 mM NaCl. Proteins non specifically bound to the resin were removed by washings with a buffer solution containing 20 mM Tris–HCl (pH 7.9), 12 mM imidazole and 500 mM NaCl, until the absorbance of the eluates at 280 nm dropped to 0.01. This was followed by elution with a solution containing 20 mM Tris–HCl (pH 7.9), 100 mM imidazole and 500 mM NaCl to obtain ALP. The purity of the enzyme was assessed by SDS–PAGE (12%); only one band, with an apparent molecular mass of about 60 kDa was detected after staining with Coomassie blue R-250. The theoretical molecular weights of the recombinant wild-type and truncated proteins were calculated using Compute pI/Mw from ExPASy Proteomic Server (http://www.expasy.ch/tools).

Molecular weights determinations

Molecular weights were determined by gel filtration on a calibrated column of Sephadex G-200. The column was equilibrated and eluted with a buffer solution containing 10 mM Tris–HCl (pH 7.5) and 50 mM KCl.

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 2 mM $MnCl_2$. In the fluorescence quenching experiments, the acrylamide concentrations varied from 0 to 100 mM.

Zinc contents

The zinc contents of wild-type and truncated preparations of ALP were determined by atomic absorption on a Perkin Elmer 1100 atomic absorption spectrometer equipped with a graphite furnace and a deuterium arc background corrector. Recovery was nearly 100%. For analysis, the purified preparations were incubated with 10 mM EDTA in 10 mM Tris–HCl (pH 7.5), followed by dialysis with two changes of 10 mM Tris–HCl (pH 7.5).

Yeast strains transfection

With the use of appropriate restriction sites and standard procedures for subcloning, the wild-type and truncated species of ALP were inserted into the EcoRI site in the yeast expression plasmid pE-PHO, which contains the ura3 selectable marker and gene expression is induced in a medium low in phosphate. A yeast strain containing a disruption of the spe1 gene encoding ODC (strain TRY104Δspe1), was used for complementation studies essentially as described [19]. The specific yeast strains designated in results were transformed with pE-PHO-ALP or pE-PHO-truncated ALP by the lithium acetate procedure, and transformants were selected on medium lacking uracil. The transfected yeast were grown for 48 h at 30 °C to deplete the endogenous pool of polyamines in low phosphate YMM medium with amino acid supplement and kanamycin 25 μg/mL under agitation. 1 mL of this inoculum was added to 50 mL of YMM low phosphate, supplemented with amino acid, kanamycin 25 μg/mL and 10 μg/mL agmatine. As a control, non transfected strain and the minimal medium containing agmatine and uracil was used. Grow was estimated by nephelometry at 600 nm. When values were higher than 0.5, the sample was diluted by 10-fold.

Results and discussion

To gain some insight into a possible role for the LIM-like domain in agmatine hydrolysis by the rat brain agmatinase-like protein (ALP), the structural and functional consequences accompanying its removal were examined.

Truncated species were catalytically active and retained the ability to discriminate between agmatine and its precursor L-arginine; both the wild-type and mutant variants were totally inactive on L-arginine. Interestingly, truncated species exhibited a 10-fold increased k_{cat} value, a 3-fold decreased K_m for agmatine and increased affinity for the product putrescine (Table 1). As previously described for *E. coli* agmatinase [20,21], product inhibition

Table 1
Kinetic parameters of wild-type and truncated species of ALP.

Variant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_i^{Put} (mM)
wt	0.9 ± 0.2	3.0 ± 0.2	300	5.0 ± 0.4
Truncated	10 ± 1	1.2 ± 0.5	9091	1.3 ± 0.2

Values, derived from three experiments in duplicate, represents the means ± SD. *Put* refers to the product putrescine.

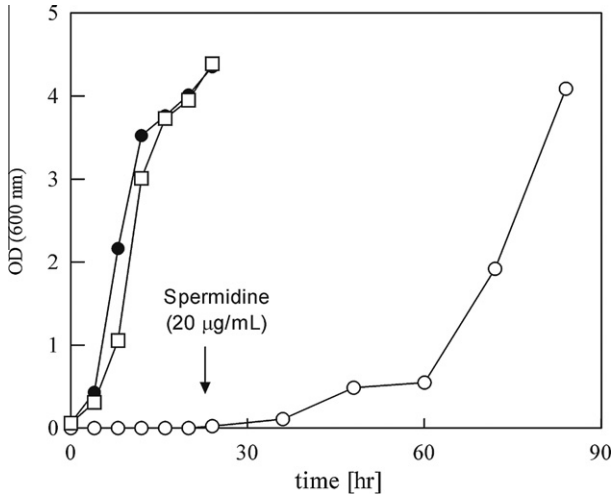

Fig. 1. Time course of growth of yeast strains TRY104Δspe1 deficient in L-ornithine decarboxylase, transfected with wild-type ALP (□) or truncated species lacking the LIM domain (●). The control (○) indicates the growth of the deficient, non transfected yeast cells. In all cases, the yeast cells were grown in a minimal medium containing agmatine (10 μg/ml).

by putrescine was linear competitive. Thus, upon truncation, the catalytic efficiency (k_{cat}/K_m value), of ALP was increased by about 30 times. The significantly higher k_{cat} value indicates that agmatine is more efficiently positioned for optimal hydrolysis in the truncated variant. Considering the information which is available for arginase and *E. coli* agmatinase, the most extensively kinetically characterized agmatinase [20,21], agmatine hydrolysis by ALP is expected to involve a nucleophilic attack by a manganese-bound hydroxide on the guanidine carbon of agmatine. With available information, the K_m value for agmatine cannot be equated to the thermodynamic dissociation constant of the enzyme–substrate complex and, therefore, $1/K_m$ cannot be unequivocally taken as a direct measure of substrate affinity. However, considering identical binding areas for putrescine and the nonguanidino portion of the substrate, a higher affinity for agmatine may be envisioned for the truncated variant. The k_{cat} values for the wild-type and truncated variants of ALP are considerably lower than the 120 ± 10 s^{-1} previously reported for *E. coli* agmatinase; however, the K_m value for agmatine and the K_i value for putrescine inhibition are in order of those for the bacterial enzyme [20,21].

The functionality of the truncated species was also examined by the functional reconstitution of the polyamine biosynthetic pathway in *S. cerevisiae* strain TRY104Δspe1. This yeast strain lacks ornithine decarboxylase, the first and rate-limiting step of polyamine biosynthesis and it is, therefore, strictly dependent of an exogenous source of polyamines for growth [19]. Genes for the wild-type and truncated variants of ALP were transfected to the yeast strain and, as shown in Fig. 1, both kind of transformed yeast cells were able to grow in the presence of agmatine, whereas addition of spermidine was required in the case of the nontransformed cells. The requirement for ALP to be transcribed by the cell was indicated by the inability of its substrate agmatine to restore the normal growth of the nontransformed cells.

The altered kinetic parameters for the truncated variant of ALP are indicative of a conformational alteration accompanying deletion of the LIM-like sequence. To probe any tertiary structure alteration, the tryptophan exposure was investigated by fluorescence studies, including quenching by acrylamide [22]. The uncharged quencher acrylamide was used because of its potential ability to penetrate into the interior of the protein and, thus, to quench partially buried tryptophan residues [23]. As shown in Fig. 2, together with a small blue shift of the maximum emission wavelength, from 339 to 336 nm, there was an important change from linear to hyperbolic Stern–Volmer plots. The hyperbolic plot indicates the presence of at least two classes of tryptophan residues with different accessibilities to acrylamide in the truncated variant of ALP, thus revealing a significant conformational difference between the variants. The deduced conformational change would explain the increased catalytic efficiency of the truncated variant. Whatever the exact nature and extension of the change, it had no consequences on the dimeric structure of the protein. From gel filtration, calculated molecular weights were 117 and 90 kDa for wild-type and truncated species, respectively. The difference is explainable by the absence of the last 66 amino acids, which include the LIM domain, in the truncated variant. The expected theoretical values for the dimeric wild-type and truncated variants were about 118 and 104 kDa. The divergence between the experimental and theoretical data for the mutant would be another evidence for a conformationally altered state.

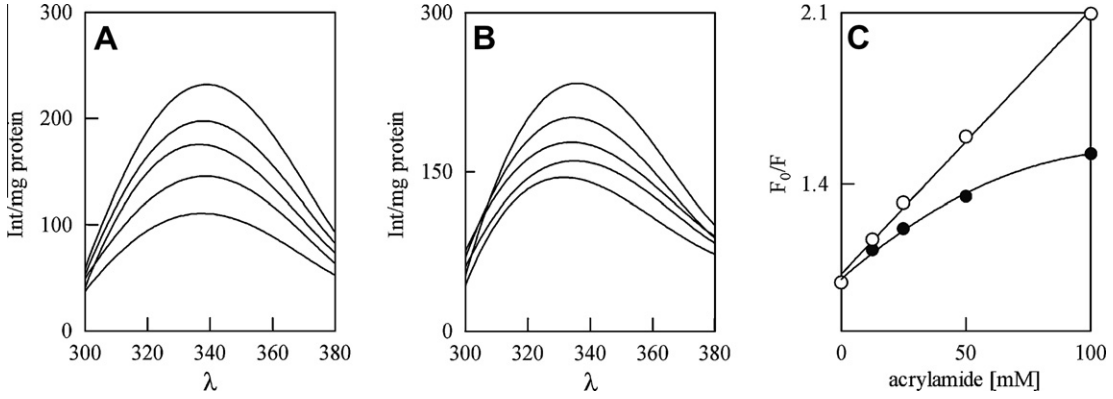

Fig. 2. Intrinsic tryptophan fluorescence and acrylamide quenching of wild-type ALP (○) and truncated species lacking the LIM domain (●). Quenching was analyzed by Stern–Volmer plots; F_0 and F indicates the areas under the curves in the absence and presence of acrylamide, respectively. The experiment was repeated for three times with equivalent results.

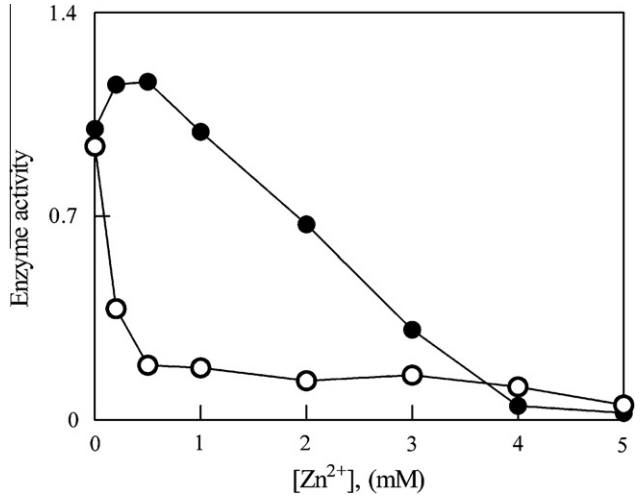

Fig. 3. Efect of Zn^{2+}on the catalytic activity of the the truncated variants of ALP. The effect was examined both in the absence (○) and presence (●) of 5 mM Mn^{2+}. Values are from one representative experiment repeated for three times.

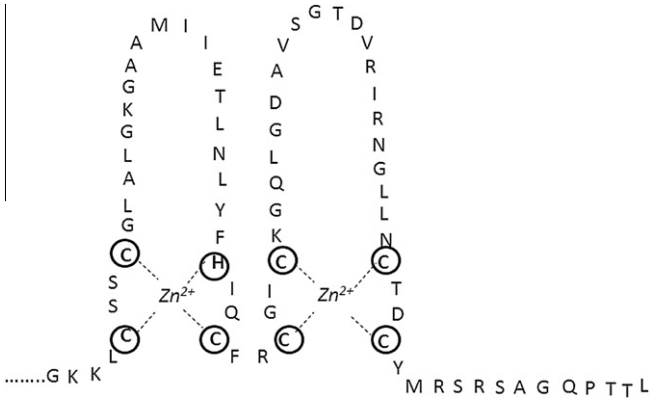

Fig. 4. Proposed schematic representation of a LIM domain in ALP. Postulated ligands to the zinc ions are encircled.

By definition, a LIM domain corresponds to a specific zinc-binding protein domain, which comprises two tandemly repeated zinc fingers [15]. Concordant with this, atomic absorption metal analysis revealed the presence of 1.9 ± 0.1 Zn^{2+}/subunit of wild-type species of ALP and the absence of zinc ions in the truncated variant. Thus, in addition to fulfilling the general distinguishing features of a LIM domain containing protein [15], our results indicates that Zn^{2+} is not required for agmatine hydrolysis by ALP. Moreover, zinc ions were inhibitory to both the wild-type and truncated variants. The results in Fig. 3, specifically corresponding to the truncated variant, were not significantly different to those obtained in the case of the wild-type species. Considering that Mn^{2+} is essential for catalysis by ALP [13], and the results obtained in the presence of 5 mM Mn^{2+}, the inhibition may be explained by replacement of the metallic cofactor by Zn^{2+} in the active site of ALP. The proposed LIM domain in the structure of ALP is schematically shown in Fig. 4.

Further studies are evidently required to unequivocally define the exact role of the LIM domain in the biological function of ALP. However, a working hypothesis may be advanced on the basis of previously demonstrated participation of this kind of domains in protein–protein interactions [16,17]. Based on the increased catalytic efficiency of truncated species, our suggestion is that the LIM domain acts as an autoinhibitory entity and that inhibition is reversed by interaction of the domain with some yet undefined brain protein. A mechanism of this kind was demonstrated for a LIM-kinase; similarly to what observed here, removal of the LIM domain also resulted in activation of this enzyme [24].

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