Glu-256 is a main structural determinant for oligomerisation of human arginase I

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Abstract One determinant that could play a role in the quaternary structure of human arginase is the pair of salt links between the strictly conserved residues R255 from one monomer and E256 from every adjacent subunit. In this work, the ionic interaction between monomers was disrupted by expressing a human arginase where Glu-256 had been substituted by Gln. Biochemical analyses of the mutant protein showed that: (i) it shares the wild-type kinetic parameters of the arginine substrate; (ii) E256Q arginase behaves as a monomer by gel filtration; (iii) it is drastically inactivated by dialysis in the presence of EDTA, an inhibitory effect which is reversed by addition of Mn^{2+}; and (iv) the mutant enzyme loses thermal stability. The lack of oligomerisation for E256Q arginase and the conservation of E256 throughout evolution of the protein family suggest that this residue is involved in the quaternary structure of arginases.

Key words: Arginase monomer; Quaternary structure; Site-directed mutagenesis; Mn^{2+} cofactor

1. Introduction.

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is a binuclear manganese metalloenzyme that catalyses the hydrolysis of L-arginine to L-ornithine and urea [1,2]. The biological importance of arginase is related to its role in the control of cellular levels of arginine and ornithine, since these amino acids are required for various critical metabolic processes, including protein synthesis, L-proline biosynthesis [2] and production of polyamines [3] and nitric oxide [4–6].

The enzyme is widely distributed throughout the evolutionary spectrum in organisms as diverse as bacteria, fungi, plants and animals. Two isoenzymes of arginase are found in mammals, which differ in tissue distribution and physiological function. Isoenzyme I is mainly localised in the liver, whilst isoenzyme II is extrahepatic [7].

The crystal structure of rat arginase I reveals a homotrimeric protein with a binuclear manganese cluster located at the bottom of a 15 Å deep active site cleft in each monomer [8]. This structure led to the proposal of a mechanism of hydrolysis in which a metal-activated water molecule initiates the nucleophilic attack on the guanidinium carbon of arginine [8,9].

All arginases are oligomeric proteins, trimers are found in eukaryotes, whereas prokaryotic enzymes are mainly hexameric [7]. Rat arginase I has an unusual S-shaped motif in the last 19 amino acids (residues 304–323) of the C-terminal end, which shares the majority of inter-monomer contacts, and thus, it was proposed as the oligomerisation determinant of the protein. In fact, a salt link between R308 and D204 from adjacent monomers was predicted from the 3D structure [8], and a recent report showed that a monomeric rat arginase results from a single mutation of R308 [10]. Moreover, deletion of the last 14 residues of human arginase I produced a mutant enzyme that was still trimeric [11], with R308 as the C-terminus of the protein, reinforcing the role of this structural determinant for oligomerisation of mammalian arginases [11]. Nevertheless, this picture is complicated by the fact that most non-mammalian arginases lack this residue [12], including a member of the protein family from the eubacterium *Bacillus caldovelux*, which is an oligomeric enzyme organised as a dimer of trimers [13].

In this work, we conducted a search for evolutionarily conserved residues with the potential of being involved in ionic bonds among subunits of human arginase I. A salt link at the trimer interface has been identified between R255 and E256, and its role in oligomerisation has been analysed by expressing a mutant arginase upon site-directed substitution of E256 by Q, which resulted in an active and monomeric enzyme.

2. Materials and methods

2.1. Plasmid construction and site-directed mutagenesis

All DNA manipulations were carried out using standard procedures [14]. The full-length cDNA of human arginase I [11] was inserted into the pBS(KS) vector (Stratagene) as a *Bsp*HI/*Bam*HI fragment.

The mutant E256Q was obtained by inverse PCR [15] using pBluescript II KS containing the cDNA of human arginase I as template, and oligonucleotides GLUP1: 5′-cAgGGTCTCTACATCACAGAAGAA-3′ and GLUP2: 5′-CCTGTATGTCAGACCTCCCAC-3′ (lower-case letters are the bases altered to introduce the desired mutation, and the underlined base introduces a silent mutation to avoid internal hybridisations). Before ligation, the PCR product was phosphorylated with polynucleotide kinase, treated with Pfu polymerase in order to obtain filled-in fragments, and digested with *Dpn*I to eliminate the template. The enzyme used in the inverse PCR was Elongase enzyme mix (Gibco BRL). After ligation, plasmids containing coding sequences for wild-type (WT) and E256Q arginases were transformed in

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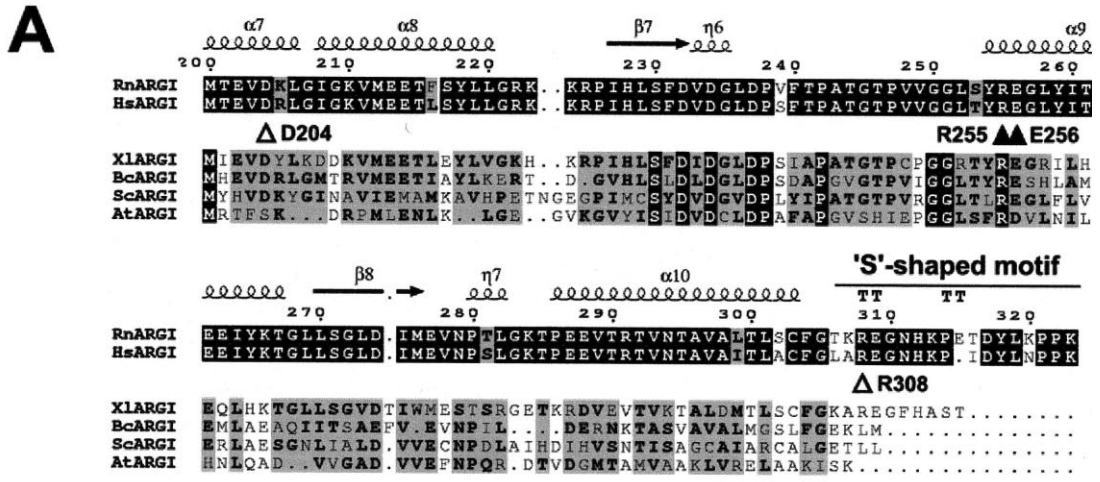
Escherichia coli XLI-blue (Stratagene), double-digested with *Bsp*HI/*Bam*HI and ligated into pET 11d (Clontech), which were transformed in *E. coli* BL21 (Clontech). The full lengths of both WT and E256Q arginases were verified by sequencing.

2.2. Human arginase I and E256Q mutant production and purification

Expression of the WT and E256Q arginases from the pET vector and preparation of soluble protein extracts from transformed bacteria were the same as described previously for the expression from the



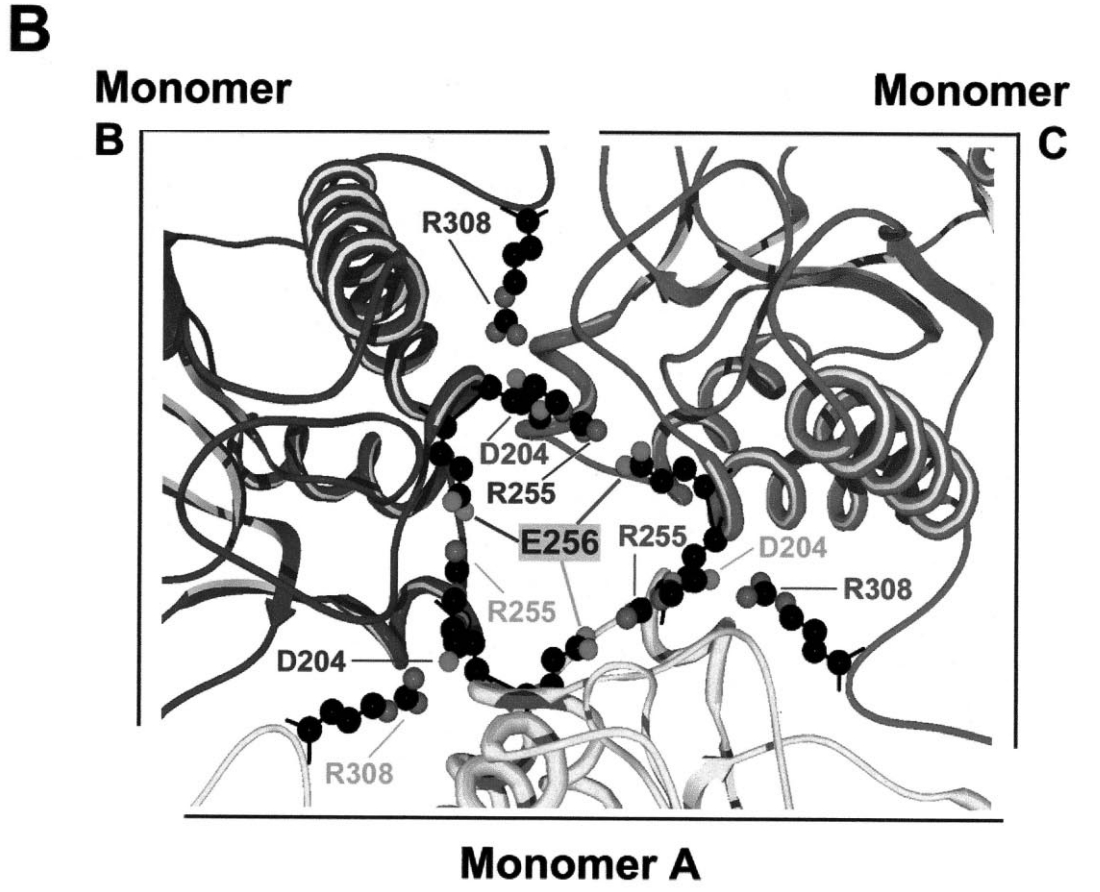


Fig. 1. Determinants for quaternary structure of arginase. A: Sequence alignment and comparison of arginase I from *Rattus norvegicus* (RnARGI; SwissProt ID: P07824), *Homo sapiens* (HsARGI; P05089), *Xenopus laevis* (XlARGI; P30759), *Saccharomyces cerevisiae* (ScARGI; P00812), *Bacillus caldovelox* (BcARGI; P53608) and *Arabidopsis thaliana* (AtARGI; P46637). Secondary structure for rat arginase I was determined by 3D model ([8]; PDB ID: 1RLA), and secondary structure elements are indicated above their amino acid sequences (α-helices, η[3.10]-helices, β-strands, T-turns), including the so-called S-shaped motif. Identity between both mammalian sequences, or among all arginases, is represented by black boxes, whilst residue similarity and global frame similarity are indicated by bold characters and grey boxes, respectively. Putative critical determinants for quaternary structure are indicated by inverted triangles (black, conserved; white, non-conserved). B: Predicted structure of the central core of trimeric human arginase I, as deduced by homology-based 3D modelling, using the atomic co-ordinates from the rat enzyme (1RLA), and the sequence alignment shown in A.

pKK 223-3 vector [11] with minor modifications; 1 mM of IPTG was used in the induction during 4 h at 37°C.

Purification of WT and E256Q arginases was performed by two heating steps exploiting the heat stability of this protein [16]. Soluble protein extracts were heated at 50°C for 20 min. After cooling at 4°C for 10 min, the sample was heated again at 65°C for 20 min, cooled and centrifuged at 11 000×*g* for 10 min at 4°C. At this point, the supernatant contains arginase at more than 90% homogeneity as estimated by Coomassie blue staining of SDS–PAGE [11].

2.3. Bioinformatic tools for protein analysis

Homology modelling was performed using program facilities at the EXpert Protein Analysis SYstem proteomics server (EXPASY; http://www.expasy.ch) of the Swiss Institute of Bioinformatics (SIB). Espript v1.9. and SwissModel v3.6 were used for sequence alignment and homology-based 3D modelling, respectively [17].

2.4. Other protein analysis

Protein concentration was determined by the method of Bradford [18].

Arginase activity was performed as indicated by Schimke [19] in a reaction buffer containing 0.5 M arginine–NaOH (pH 9.7) and 1 mM $MnCl_2$. Mn^{2+} was removed from WT and E256Q arginases by dialysing twice against 500 volumes of buffer 10 mM Tris–HCl (pH 8), 150 mM NaCl and 1 mM EDTA for 2 h at 4°C each time. Then, the dialysed protein was used for activity assays or thermal stability studies. When the recovery of arginase activity by Mn^{2+} or its inhibition by Ca^{2+} were measured, these ions were included in the reaction buffer at the desired concentrations.

Molecular mass calculations for WT and E256Q arginases were performed by gel filtration through Sephacryl S-300HR in a buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM $MnCl_2$ and 150 mM KCl [11]. The following standards were used for calibration: alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa).

For thermal stability studies, purified arginase was diluted at 0.2 μg/μl in 10 mM Tris–HCl (pH 7), 150 mM NaCl and 5 mM $MnCl_2$, and was heated at the desired temperature in a thermocycler for 20 min. After cooling for 10 min at 4°C, the sample was centrifuged at 11 000×*g* for 10 min at 4°C. Supernatant containing the soluble protein was used for arginase activity. T_m was calculated as the temperature at which the effect was half maximal.

3. Results

3.1. Sequence alignments and homology modelling

The predicted structure of the central core of human arginase I (Fig. 1B) reveals a network of cross-salt links involving residues D204, R255, E256 and R308 from every monomer. A direct role in oligomerisation has been demonstrated for the D204–R308 ionic bond [10], which is conserved in mammalian and amphibian arginases (Fig. 1A). Beside that, a second ionic interaction involves R255 and E256 from every adjacent monomer, and since R255 and E256 are strictly conserved in all oligomeric arginases, including Glu for Asp substitution in a plant enzyme, these observations suggest that E256–R255 salt links could play a role in all oligomeric arginases.

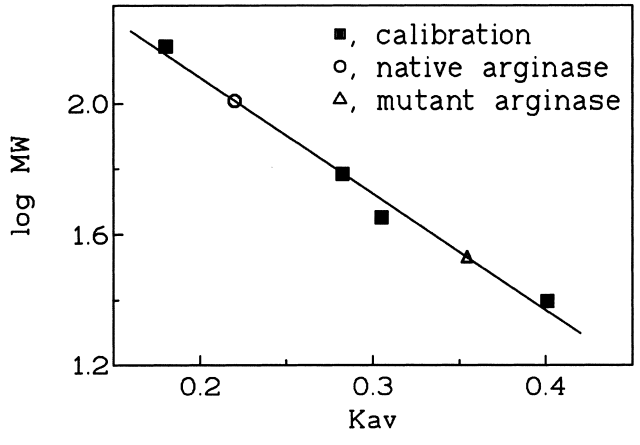


Fig. 2. Molecular mass determination by size exclusion chromatography. Standard proteins (■) are indicated in Section 2. Other symbols: WT arginase (○) and E256Q mutant (△).

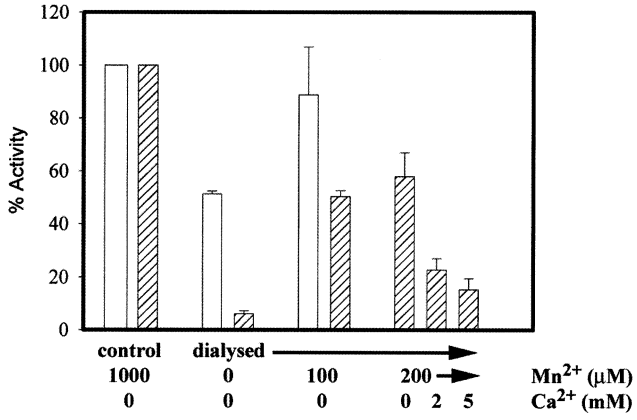


Fig. 3. Effect of EDTA on arginase activity and reversion by Mn^{2+}. Open bars correspond to WT arginase and hatched bars correspond to mutant E256Q.

3.2. Kinetic parameters and oligomerisation state

A single replacement of E256 by Q was performed by site-directed mutagenesis in the coding sequence for human arginase I (see Section 2).

Native and mutant E256Q arginases were evaluated for catalytic properties. Under standard conditions and saturating Mn^{2+} concentration (1 mM), purified samples of WT and E256Q arginases showed very similar specific activities (536 and 561 U/mg, respectively). In addition, K_m and k_{cat} of both WT and mutant enzymes did not differ significantly, with K_m 26 and 22 mM, and k_{cat} 447 and 467 s^{-1}, respectively. These results indicate that E256Q retains the WT catalytic properties.

The oligomerisation state of WT and E256Q arginases was analysed by gel filtration chromatography through Sephacryl S-300HR. Fig. 2 shows the calibration for determination of apparent molecular mass and K_{av} for WT and mutant forms of human arginase I. The molecular masses obtained for both proteins were 102 and 34 kDa, respectively, which correlate well with trimeric and monomeric 35-kDa polypeptide subunits [11]. The elution profiles for both proteins (results not shown) revealed only one peak, without shoulders, indicating the lack of additional oligomerisation (secondary) forms. This result suggests that the E256Q mutation abolishes interactions among subunits, indicating that the predicted salt link R255–E256 from adjacent monomers is an important determinant for the trimeric state of human arginase. In addition, since the E256Q monomeric protein was active, a situation also found for the R308 mutants expressed from rat arginase [10], it might be concluded that trimerisation is not critical for arginase activity.

3.3. Inhibition by EDTA and activation by Mn^{2+}

When human arginase I was dialysed in the presence of EDTA, a 50% of inhibition of enzyme activity was observed

(Fig. 3). In contrast, dialysis of E256Q arginase in the presence of EDTA produced a full inactivation of the mutant enzyme. Both inhibitory effects seem to be mediated by the loss of Mn^{2+} from the proteins, since metal addition restored almost all arginase activity of WT (89%) and about 50% of E256Q activity (Fig. 3). Moreover, the recovery by Mn^{2+} of E256Q arginase activity was inhibited by other divalent cations, Mg^{2+} (results not shown), and notably by Ca^{2+}, which produced a concentration-dependent decrease of enzyme activity, an effect that was not observed for WT protein (Fig. 3). Taken together, these results suggest that Mn^{2+} binds reversibly to human arginase I. Complete inactivation by dialysis of E256Q arginase and inhibition by Ca^{2+} on Mn^{2+} activation of the mutant enzyme indicate that it might bind Mn^{2+} with a lower affinity than WT protein.

3.4. Thermal stability

As previously reported, WT arginase is relatively thermostable [22], with a T_m of about 78°C estimated by its relative solubility in heated samples (Fig. 4A). However, E256Q arginase was less stable, with a T_m of about 71°C, a result which is consistent with the loss of thermal stability of monomeric rat arginase obtained by R308 mutation [10]. Moreover, the activity of native arginase increases by heating to over 55°C in the presence of Mn^{2+} (Fig. 4A), an effect that was explained previously by the occupation with Mn^{2+} of site A, the lowest-affinity binding site for the metal [20,21]. The monomeric mutant lacks this effect (Fig. 4A), suggesting a different behaviour of E256Q against Mn^{2+}. In fact, dialysis in the presence of EDTA drastically reduced the thermal stability of E256Q arginase, whilst native arginase remained stable at 70°C (Fig. 4B). These results also support that E256Q mutant could bind Mn^{2+} with a lower affinity than WT protein, a behaviour which is similar to rat arginase proteins in which active site His were specifically mutated, an alteration of the binding site for the catalytic Mn^{2+} that produced an inactive enzyme with a reduced thermal stability [22].

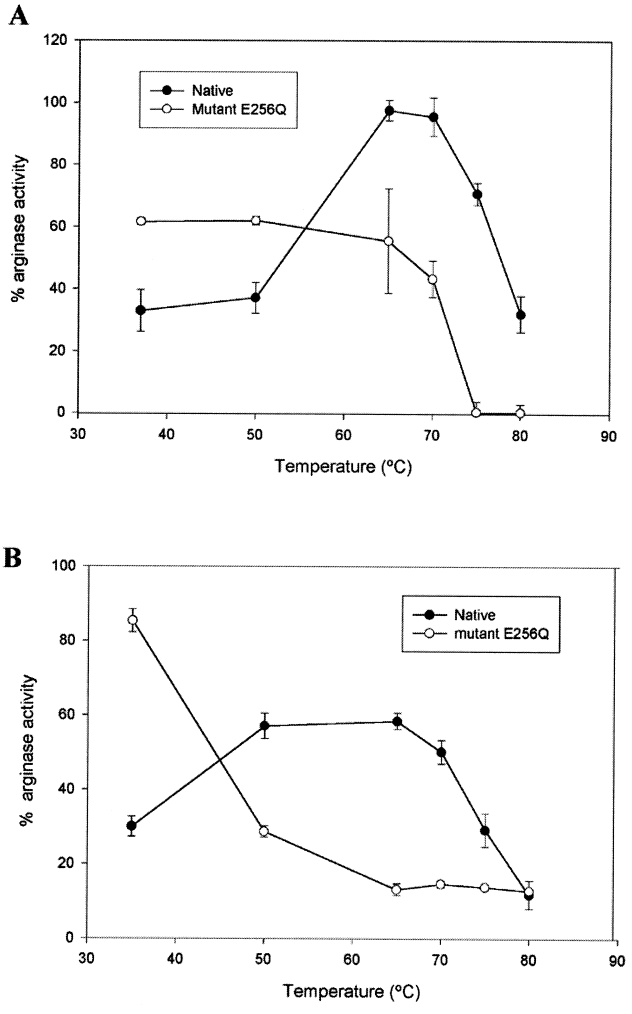


Fig. 4. Thermal stability of WT and mutant E256Q arginases. A: Arginase activity of soluble protein after heating for 20 min at the indicated temperatures. B: Arginase activity of dialysed arginases after heating for 20 min at the indicated temperatures. The activity was performed in a reaction buffer containing 1 mM $MnCl_2$. Symbols: WT arginase (●) and E256Q mutant (○). Maximal activity (100%) was the activity of WT arginase after heating at 65°C.

4. Discussion

The structure of rat arginase suggested a role in oligomerisation of the S-shaped motif at the carboxy-end of the protein [8]. Among the several interactions localised in that region, a cross-salt link between R308 and D204 is the main determinant for quaternary structure of rat arginase, since point mutations in that residue produce monomeric proteins [10], and a deletion mutant that harbours R308 at the carboxy-end remains trimeric [11]. Upon further investigation of the trimer interface, a number of inter-monomer salt links were identified that involved residues conserved in all arginases. One such residue was E256 (Fig. 1). In this work we demonstrate, by gel filtration studies, that point mutation of this residue produces an active monomer. This result indicates the important role of E256 and its inter-monomer salt link with R255, an interaction that stabilises the quaternary structure of human arginase. Thus, this contribution and a previous work [10] provide biochemical evidence to show the involvement of R308 (D204) and E256 (R255) as structural determinants for oligomerisation of mammalian arginases.

E256 and R255, but not R308 and D204, are also present in other oligomeric arginases (Fig. 1A), and in another related protein, agmatine ureohydrolase from *E. coli* [12], where the glutamic residue is substituted by an aspartic residue (D173–R174). Strict conservation of these amino acids among remotely related members of the protein family reinforces their role as structural determinants for enzyme function.

At saturating Mn^{2+} concentration (1 mM), E256Q arginase is fully active, with catalytic constants similar to WT; however, the mutant protein is almost totally inactivated by dialysis with 1 mM EDTA whilst WT was 50% inhibited (Fig. 3). Considering that treatment of arginase crystal with metal chelators resulted in the dissociation of one Mn^{2+} [16], and that two Mn^{2+}/subunit are required for activity of arginase, since the mononuclear sites are inactive [22], the inhibition of E256Q arginase by EDTA could be interpreted as a loss of Mn^{2+} affinity in the mutant protein. This possibility is reinforced by the observed inhibition of E256Q activity with other divalent cations, such as Ca^{2+} (Fig. 3), and by the lowering of the thermal stability of the E256Q mutant when it is dialysed with EDTA (Fig. 4B), a behaviour also observed in rat arginase mutants in which Mn^{2+} binding centres were mutated (H101N or H126N) [22]. In this regard, Lavulo et al. [10]

have shown that one monomeric arginase (R308K) retains only 1 Mn^{2+}/subunit, a result that could reflect a lower affinity of the mutant protein to Mn^{2+}. Interestingly, also R308K rat arginase loss thermal stability [10], a behaviour which correlates with the biochemical properties described for monomeric protein from human arginase I (Fig. 4). These and previous results [10] show the involvement of E256 and R308 in maintaining the trimeric state of mammalian arginases, and suggest that quaternary structure could play a role in thermal stability and affinity to Mn^{2+} of arginases.

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