Arginine Metabolism: Enzymology, Nutrition, and Clinical Significance

Structure and Function of Arginases[1,2]

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ABSTRACT The arginases catalyze the divalent cation dependent hydrolysis of L-arginine to produce L-ornithine and urea. Although traditionally considered in terms of its role as the final enzyme of the urea cycle, the enzyme is found in a variety of nonhepatic tissues. These findings suggest that the enzyme may have other functions in addition to its role in nitrogen metabolism. High-resolution crystal structures have been determined for recombinant rat liver (type I) arginase and for recombinant human kidney (type II) arginase, their variants, and complexes with products and inhibitors. Each identical subunit of the trimeric enzyme contains an active site that lies at the bottom of a 15 Å deep cleft. The 2 essential Mn(II) ions are located at the bottom of this cleft, separated by ~3.3 Å and bridged by oxygens derived from 2 aspartic acid residues and a solvent-derived hydroxide. This metal bridging hydroxide is proposed to be the nucleophile that attacks the guanidinium carbon of substrate arginine. On the basis of this proposed mechanism, boronic acid inhibitors of the enzyme have been synthesized and characterized kinetically and structurally. These inhibitors display slow-onset inhibition at the pH optimum of the enzyme, and are found as tetrahedral species at the active site, as determined by X-ray diffraction. The potent inhibition of arginases I and II by these compounds has not only delineated key enzyme-substrate interactions, but has also led to a greater understanding of the role of arginase in nonhepatic tissues. J. Nutr. 134: 2760S–2764S, 2004.

KEY WORDS: • *arginase* • *manganese* • *protein structure* • *enzyme inhibitors* • *boronic acids*

The arginases catalyze the divalent cation-dependent hydrolysis of L-arginine to form the nonprotein amino acid L-ornithine and urea (**Fig. 1**). In the liver, this reaction constitutes the final step in urea biogenesis. The flux through this reaction is considerable, as the average adult excretes ~10 kg of urea/y. The urea cycle arginase (arginase I or liver arginase) is cytosolic, and is the best characterized of the mammalian arginases. A second isozyme, arginase II or kidney arginase, is mitochondrial in location. The human type I and type II arginases are related by 58% sequence identity (1), and are immunologically distinct. The comparative properties of the 2 arginase isozymes are discussed in a number of recent reviews (2–4). Arginase activity has been detected in a number of nonhepatic tissues that lack a complete urea cycle; the reaction is thought to provide a source of ornithine, the biosynthetic precursor of proline and the polyamines. For example, in lactating mammary gland, arginase activity rises to about 25% that found in liver in order to supply the proline required for milk protein biosynthesis (5). Ornithine is also a biosynthetic precursor of the polyamines (6), and myometrial arginase activity increases ~25-fold during pregnancy to supply the rapidly growing fetus with polyamines to facilitate cell proliferation (7). The requirement of rapidly dividing tissues for enhanced polyamine biosynthesis is apparently met by increased arginase activity as found in gastric cancers (8–10) and in breast cancer (11). Recently, arginase activity has been detected in certain human colon cancer and human breast cancer cell lines (12,13).

Metal activation and kinetic properties

A common feature of all arginases thus far studied, whether of eukaryotic or prokaryotic origin, is a requirement of divalent cations for activity. Mn^{2+} is the physiologic activator, although the divalent cation requirement for some arginases has been reported to be satisfied by Co^{2+} and Ni^{2+} (14–16) and in some instances by Fe^{2+}, VO^{2+}, and Cd^{2+} (17,18). Mn(II) binding experiments in this laboratory have shown that fully Mn-activated arginase contains 2 Mn(II)/subunit and that these Mn^{2+} ions form electron paramagnetic resonance (EPR)[4] spin-coupled binuclear centers (19). Detailed analyses of the temperature dependence of the Mn(II) EPR properties indicate an Mn(II)-Mn(II) separation of 3.36–3.57 Å in the native enzyme (20).

Similarly, most arginases have an alkaline pH optimum,

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[4] Abbreviations used: ABH, 2(*S*)-amino-6-boronohexanoic acid; BEC, *S*-(2-boronoethyl)-L-cysteine; EPR, electron paramagnetic resonance; NO, nitric oxide.

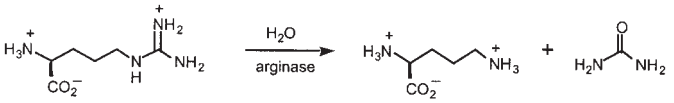


FIGURE 1 The arginase reaction.

with maximal velocities observed in the range of pH 9.0–9.5 (21). For the rat liver enzyme, plots of log V_{max} versus pH provide a pK value of ~7.8–8.0 (22), while pK values of 7.8 and 7.9 have been reported for the human liver (23) and mouse liver (24) enzymes. These pK values are consistent with the pK expected for the ionization of a solvent molecule bridging the Mn(II)-Mn(II) center (25), and it is thought that the resultant metal bridging hydroxide is the catalytic nucleophile.

Substrate specificity of the arginases depends on *1*) the presence of an intact guanidinium group, *2*) the proper length and hydrophobicity of the side chain, and *3*) the stereochemistry and nature of substituents at Cα (26). For example, D-arginine and guanidinobutyrate are not substrates for the enzyme, and alterations about Cα (L-argininamide, L-argininic acid, and agmatine) result in 170–54,000-fold reductions in k_{cat}/K_M (26). Such exquisite substrate specificity suggests that a well-defined constellation of hydrogen bond donors must be present at the active site.

Structural properties of arginase

High resolution crystal structures are currently available for rat liver type I arginase (27), a truncated form of the recombinant human kidney type II arginase (28), and the arginase from the extreme thermophile *Bacillus caldovelox* (29). Additional structures are available for variants of the recombinant rat liver enzyme in complexes with products and a variety of inhibitors (30–34). Liver arginase is a 105 kDa homotrimer and each 35 kDa subunit contains a binuclear Mn(II) center that is critical for catalytic activity The overall fold of each subunit belongs to the α/β family, consisting of a parallel, 8 stranded β-sheet flanked on both sides by numerous α-helices. The binuclear Mn(II) center is located at the bottom of an ~15 Å-deep active site cleft (27). The Mn(II)-Mn(II) separation is 3.3 Å, a value that is consistent with that determined by EPR measurements (20). Mn_A, the more deeply situated metal ion, has square pyramidal coordination; His101 and As128 are terminal ligands and Asp124 (bidentate), Asp232 (monodentate) and hydroxide ions are bridging ligands (**Fig. 2**). Mn_B is coordinated with distorted octahedral geometry by terminal ligands His126 and Asp234 (bidentate), and bridging ligands Asp124 (bidentate), Asp232 (monodentate) and hydroxide ion. A hydrogen bond is donated by metal-bridging hydroxide to the noncoordinating oxygen of Asp128. Interestingly, residues analogous to Asp128 that coordinate a metal ion and hydrogen bond to a bridging hydroxide have been identified at the active sites of bimetallic hydrolases such as phosphotriesterase (35), dihydroorotase (36), isoaspartyl dipeptidase (37), aminopeptidase P (38), and prolidase (39). The polypeptide fold of human arginase II is topologically identical to the α/β fold of rat arginase I and the hexameric arginase from *Bacillus caldovelox* (28). The structure of the binuclear Mn(II) cluster of human arginase II is nearly identical to that of rat arginase I, a finding that is not surprising considering that all metal ligands are conserved between the 2 sequences.

Catalytic mechanism

The arginase mechanism proposed by Kanyo and colleagues (27) is most consistent with available biochemical, enzymological, and structural data (**Fig. 3**). Key features of this mechanism include *1*) a precatalytic binding side chain for arginine in which the side chain of Glu277 plays a role in substrate recognition, *2*) attack of a nucleophilic metal-bridging hydroxide ion, *3*) formation of a neutral tetrahedral intermediate that is stabilized by the binuclear Mn(II) center, and *4*) a possible role for His141 as a proton shuttle in mediating proton transfer between the active site and bulk solvent. His141 resides about halfway out of the active site cleft, and it has been proposed that this residue is a proton shuttle, analogous to His64 of carbonic anhydrase II, which is known to facilitate proton transfer between zinc-bound water and bulk solvent in order to regenerate the catalytically active zinc-bound hydroxide (40). Additionally, His141 is extraordinarily reactive towards histidine modifying reagents such as diethylpyrocarbonate, and the modified enzyme has only about 10% residual activity (22).

Inhibition by N^{ω}-hydroxy-L-arginine and boronic acids

The discovery of nitric oxide (NO) synthase, which catalyzes the oxidation of arginine to form NO and citrulline, has generated significant interest in the interplay between the NO synthase and arginase pathways. A number of tissues express both type I and type II arginases as well as nitric oxide synthase, and because these enzymes compete for a common substrate, L-arginine, the coexpression of these enzymes raises interesting questions concerning the regulation of arginine flux through the competing pathways (**Fig. 4**). N^{ω}-hydroxy-L-arginine, an intermediate in the synthesis of NO, is a moderately potent competitive inhibitor of arginase with a K_i value in the range of 10–42 μmol/L (41–43), compared to the K_M value of 1 mmol/L for arginine (26). Significant amounts of the N^{ω}-hydroxy-L-arginine intermediate dissociate from the NOS active site to serve as an endogenous arginase inhibitor in both macrophages (43,44) and endothelial cells (45).

The reciprocal nature of the regulation of NOS and arginase activity has only recently been appreciated and, in part, stems from the development of potent boronic acid-based inhibitors of type I and type II arginases (28,33,46,47). The boronic acids, 2(*S*)-amino-6-boronohexanoic acid (ABH) and

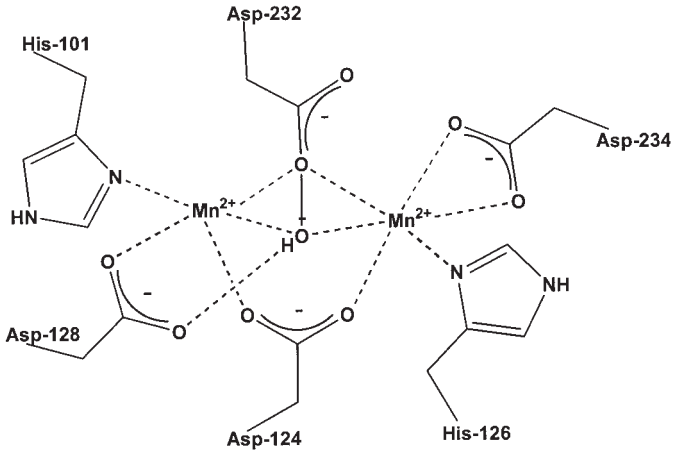


FIGURE 2 Binuclear center of rat liver arginase I. Adapted from reference 27.

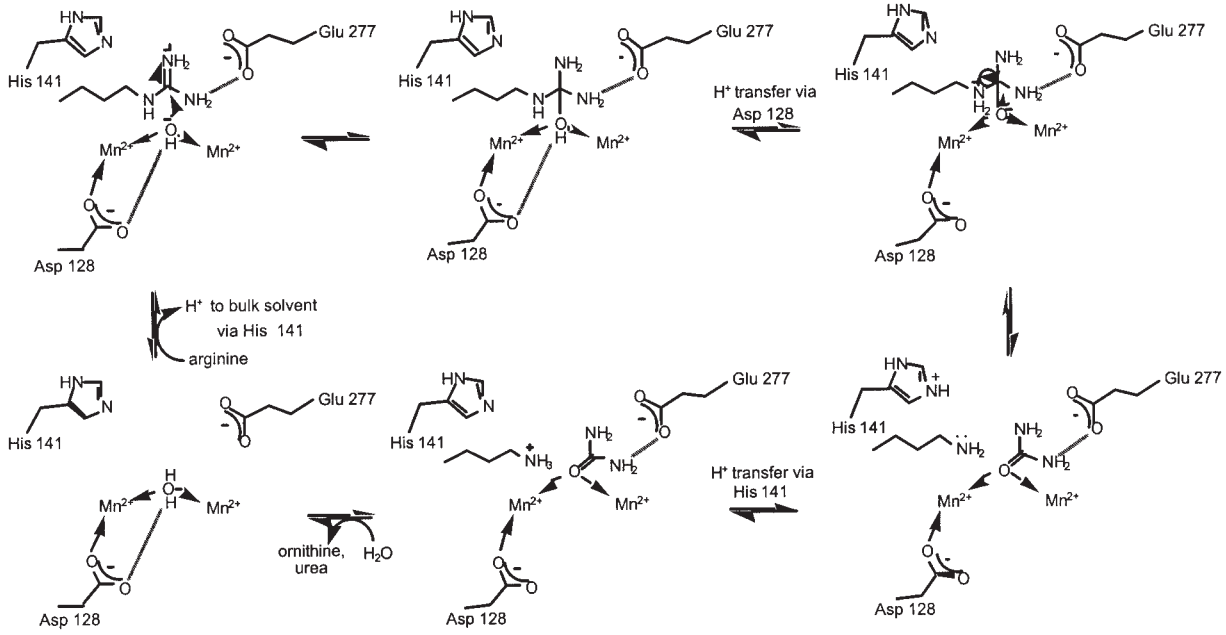


FIGURE 3 Proposed mechanism for the hydrolysis of arginine based on the crystal structure of liver arginase (27). The α-amino and α-carboxylate groups of the substrate are omitted for clarity.

S-(2-boronoethyl)-L-cysteine (BEC), were designed as potential mimics of the tetrahedral intermediate that is formed upon attack of the metal-bridging hydroxide on the guanidinium carbon of substrate arginine. The trigonal boronic acid could be converted to the tetrahedral boronate either by an analogous attack of the metal-bridging hydroxide at the active site or by direct binding of the tetrahedral species to the enzyme. Indeed, the boronic acids are potent inhibitors of both arginase I and arginase II (33,46–48); kinetic data are presented in **Table 1** for inhibition of arginase II. The boronic acids are classic, competitive inhibitors at physiologic pH, with binding constants of ~0.3 μmol/L. The K_M for arginine at this pH is 5 mmol/L, and hence the inhibitors bind with roughly 20,000-fold higher affinity than does substrate. At the pH optimum of 9.5, the boronic acids display slow-binding behavior as evidenced by nonlinear progress curves. Such curves are indicative of a rapid formation of a reversible enzyme-inhibitor complex, followed by a slow isomerization or conformational change to yield the final inhibitory complex. Analysis of these progress curves yields K_i values in the 10–30 nmol/L range, compared to a K_M value of 0.3 mmol/L for arginine at this pH (48). Interestingly, a slight isozyme selectivity for inhibition of the arginases by the boronic acids is observed, with arginase II showing somewhat higher affinity for these compounds. This finding suggests that it may be possible to design isozyme selective inhibitors of the enzyme.

Structures have been determined for complexes of arginase I and arginase II with the boronic acid inhibitors (28,33,47). In all complexes, the tetrahedral boronate is bound at the active site, with 1 of the boronate hydroxyl groups bridging the 2 Mn(II) ions, occupying the site normally populated by the bridging hydroxyl group in the free enzyme. Additionally, 1 of the boronate hydroxyl groups is now coordinated to the vacant coordination site on Mn_A. Although the binuclear Mn(II) cluster of human arginase II is nearly identical to that of rat liver arginase I in its complex with BEC, a number of notable

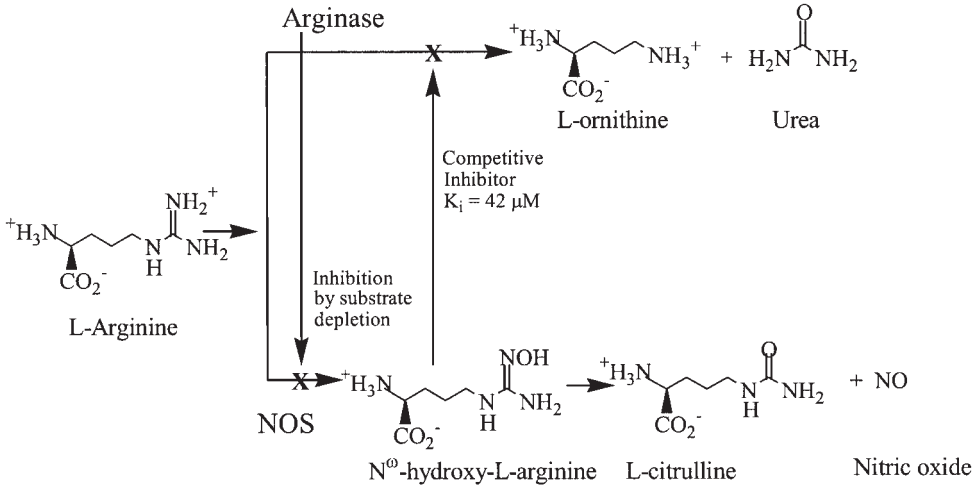
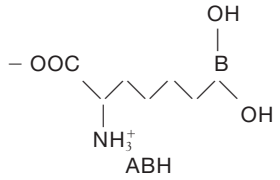
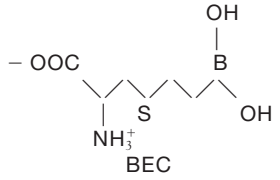
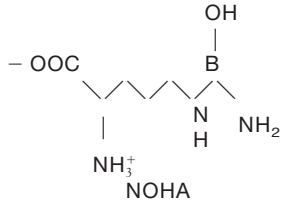


FIGURE 4 The relationship between arginase activity and NO synthase activity. The synthesis of N^{ω}-hydroxy-L-arginine by NO synthase requires O_2 as a substrate.

TABLE 1

Potent competitive inhibitors of type II arginase[1]

Inhibitor	K_i	Type of Inhibition
 ABH	K_i at pH 9.5 = 8.5 nM	slow binding
	K_i at pH 7.5 = 0.25 μM	competitive
 BEC	K_i at pH 9.5 = 30 nM	slow binding
	K_i at pH 7.5 = 0.31 μM	competitive
 NOHA	K_i at pH 9.5 = 2 μM	competitive
	K_i at pH 7.5 = 1.6 μM	competitive

[1] Adapted from (48).

differences are observed. First, the hydrogen bond between a boronate hydroxyl group and Glu277 is consistent with the proposal that Glu277 hydrogen bonds with the η_1-NH_2 group of the substrate and stabilizes the tetrahedral transition state. Second, in the arginase II–BEC complex, Asp232 undergoes changes in Mn_B coordination to hydrogen bond with another boronate hydroxyl group. These results suggest that Asp232 could move to hydrogen bond with the η_2-NH_2 group of substrate and tetrahedral intermediate.

Because the boronic acids ABH and BEC are not inhibitors of NO synthase, they have been used to probe the role of arginase in the regulation of NO-mediated smooth muscle relaxation in the gastrointestinal tract (46), as well as in penile (33,47) and clitoral (28) corpus cavernosum tissues. Both ABH and BEC enhance relaxation of these smooth muscle tissues in ex vivo organ bath experiments. Inhibitors of arginase activity thus enhance L-arginine concentrations for NO biosynthesis and NO-dependent smooth muscle relaxation. Recent studies have shown that type I and type II arginases are located extrahepatically [for a review, see (49)], and therefore both enzymes may be involved in the regulation of NO production in mammals. For example, type II arginase and NOS are induced early in lipopolysaccharide-induced mouse macrophages, while the type I isozyme is induced much later. It is believed that in this system the elevated type II arginase activity is involved in producing ornithine for the production of proline and the polyamines, necessary for wound healing, while expression of the type I isozyme prevents the overproduction of toxic NO (50,51). The development of isozyme selective inhibitors would be advantageous in delineating the precise biological roles of these enzymes in mammalian tissue.

Concluding remarks

Despite the wealth of structural and biochemical information available, there remain a number of intriguing questions concerning the structure and function of the arginases. Although the enzyme can be activated by a number of metals in vitro, Mn(II) is the physiologically relevant metal ion. The molecular basis for the selection of this trace metal to serve as a cofactor in a hydrolytic reaction is unclear. Similarly, the role of His141 has remained enigmatic. This highly conserved residue is extraordinarily reactive towards chemical modification, yet its replacement by site-directed mutagenesis results in enzymes with considerable catalytic activity. Finally, and perhaps most important from the standpoint of understanding the biologic roles of the enzyme, can isozyme selective inhibitors be developed? The boronic acid-based inhibitors show modest selectivity for arginase II; however, the conservation of active site structures for arginase I and arginase II presents a significant challenge to the development of such isozyme selective inhibitors.

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