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INHIBITION OF RAT LIVER ARGINASE BY AN INTERMEDIATE IN NO BIOSYNTHESIS, N^G-HYDROXY-L-ARGININE: IMPLICATIONS FOR THE REGULATION OF NITRIC OXIDE BIOSYNTHESIS BY ARGINASE

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SUMMARY: N^G-hydroxy-L-arginine, an intermediate in the biosynthesis of nitric oxide (NO), has been found to be a uniquely potent competitive inhibitor of rat liver arginase. Among previously reported inhibitors of arginase and the eight arginine analogs tested herein, only N^G-hydroxy-L-arginine was found to be strongly inhibitory. Significantly, the K_i (42 μM) for inhibition of rat liver arginase by N^G-hydroxy-L-arginine was found to be 20-40-fold lower than the K_M (1-1.7 mM) for its natural substrate, L-arginine. Since N^G-hydroxy-L-arginine is the only known intermediate in the biosynthesis of NO from L-arginine, this finding may have significant implications for the regulation of NO levels in tissues or cells, such as liver or macrophages, which synthesize both NO and contain arginase.

The metabolic fate of L-arginine follows two major and distinct pathways: 1) hydrolysis by the enzyme arginase (EC 3.5.3.1) to form L-ornithine and urea and 2) oxidation by the enzyme nitric oxide synthase (NOS) to give nitric oxide (NO) and L-citrulline. In spite of the fact that these two pathways share a common substrate, the interaction of the pathways or possible regulation of one enzyme by the other have not been considered or investigated. The physiological utility of these two individual enzyme systems is a topic of significant recent interest. The physiologic functions of NO are varied and ubiquitous, as NO plays

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a role in the vascular system, in immune system response and in the central and peripheral nervous systems (1). Nitric oxide synthases have been purified and characterized from a number of sources (2). Besides its obvious role in the urea cycle, arginase has also been implicated in modulating some aspects of the immune response (3) as well as in the production of L-ornithine for the synthesis of proline (4) and the polyamines (5).

Isolated rat liver arginase contains tightly bound Mn(II), and the enzyme is further activated upon the reversible binding of additional Mn(II) (6). Studies in this laboratory have shown that the fully Mn-activated enzyme contains 6 Mn(II) ions per trimer and that the Mn(II) ions form a novel binuclear metal center that appears to be essential for catalytic activity (7,8). The development of potent arginase inhibitors would facilitate ongoing spectroscopic and X-ray diffraction (9) studies of the enzyme, and provide important insights into the chemical mechanism of this enzyme. In addition, such inhibitors might also provide insight into the relationship between arginase and NOS. L-Ornithine, one of the products of arginine hydrolysis, is the most potent reported competitive inhibitor of arginase, with a K_i (1 mM) comparable to the K_M of 1 - 1.7 mM for L-arginine (10,11). Boric acid is a non-competitive inhibitor of the enzyme with K_{ii} of 0.26 mM and K_{is} of 1 mM (10). In the present study we have evaluated the inhibition of rat liver arginase by a series of arginine analogs. Potent inhibition of arginase activity is observed with N^G-hydroxy-L-arginine, an intermediate in the biosynthesis of nitric oxide from L-arginine (12-14).

MATERIALS AND METHODS

Chemicals: L-[guanido-^{14}C]-Arginine, specific activity 2.5 GBq mmole^{-1}, was from NEN/DuPont. N^G-Hydroxy-L-arginine was synthesized as described previously (12) or purchased from Alexis Biochemicals. N^G-amino-L-arginine was synthesized according to published procedures (15). The synthesis of the other arginine analogs shown in Table 1 was accomplished using the general procedures of Patthy *et al.* (16) and Corbin and Reporter (17), and will be described elsewhere.

Purification and Assay of Arginase: Arginase was purified to homogeneity from fresh rat livers as described previously (7). The enzyme was activated with $MnCl_2$ (6) and assayed by a modification of the method of Rüegg and Russell (18). Assays were performed in 100 mM Bicine at pH 9, the pH optimum for enzyme activity. Reactions were initiated by the addition of enzyme to a 1 mL solution that contained the Bicine buffer, 0.1 - 20 mM L-arginine, and ~1 x 10^5 cpm L-[guanido-^{14}C]-arginine. At various times, 50 μL

of the assay mixture was added to 200 μL of a stop solution containing 0.25 M acetic acid, 7 M urea at pH 4.5. [^{14}C]-Urea was separated from unreacted L-[guanido-^{14}C]-arginine by treatment of this solution with 200 μL of a 1:1 v/v slurry of Dowex 50W-X8 in water, and quantitated by adding 200 μL of the supernatant from the Dowex treatment to 3 mL Liquiscint (National Diagnostics) for liquid scintillation counting. The effect of N^G-hydroxy-L-arginine on arginase activity was evaluated using this assay and varying the concentration of N^G-hydroxy-L-arginine over the range of 20-140 μM. The kinetic data for N^G-hydroxy-L-arginine were fit to the equation for competitive inhibition using the computer programs of Cleland (19). Inhibition constants for weak inhibitors ($K_i \geq 4$ mM) were determined by titrating standard reaction mixtures containing 1 mM L-arginine with increasing concentrations of inhibitor. The K_i was estimated from the equation for competitive inhibition, even though complete inhibition patterns were not determined.

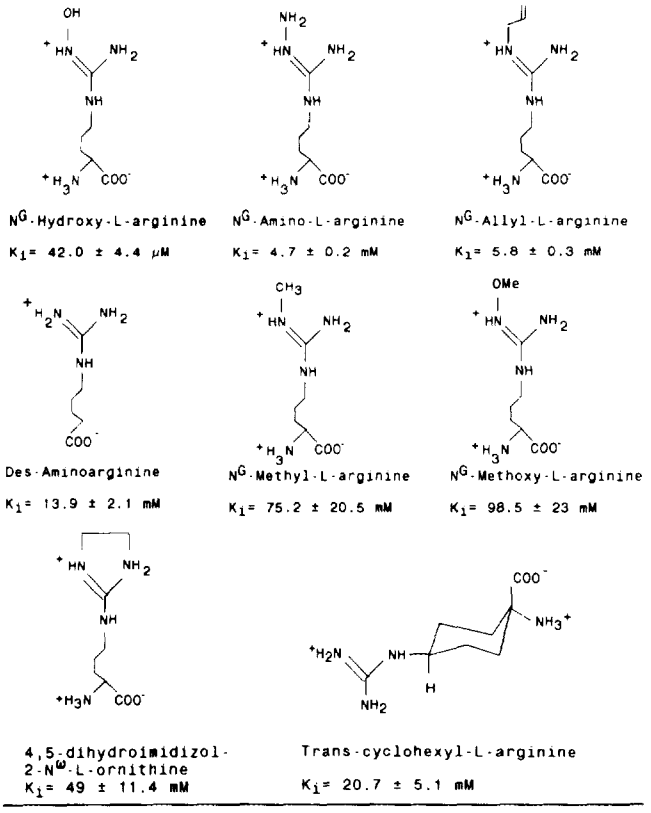
Des-aminoarginine was evaluated as an alternate substrate with a colorimetric assay for arginase (11) that incorporates the ammonia produced via the hydrolysis of urea by urease into indophenol, which is measured spectrophotometrically at 590 nm. Assay mixtures of 1 mL contained 10 mM concentrations of L-arginine or des-aminoarginine in 5 mM sodium bicarbonate at pH 9.5. The hydrolysis of N^G-hydroxy-L-arginine can be detected with this assay; however, quantitation of rates is not possible due the time-dependent inactivation of urease during turnover of hydroxyurea (20).

Alternate substrate activity of N^G-hydroxy-L-arginine and N^G-amino-L-arginine was evaluated by ^{1}H NMR spectroscopy. Samples for NMR were prepared in aqueous solution containing 5 mM Tris-KOH at pH 9 and 5-10 mM of L-arginine or the arginine analog. The solvent was removed with a Speed-vac, and the samples were redissolved in 99.9% D_2O. ^{1}H NMR spectra were recorded on a Bruker WM-300 spectrometer with a sweep width of 1500 Hz, an acquisition time of 3 s, a relaxation delay of 4 s, and a 45° flip angle. After recording a control spectrum, the reactions were initiated by the addition of 0.33 to 33 μg of arginase to the 0.5 mL sample. The hydrolysis reaction is readily monitored by the change in chemical shift of the δ-protons of L-arginine (3.2 ppm) upon conversion to L-ornithine (2.9 ppm).

RESULTS AND DISCUSSION

N^G-hydroxy-L-arginine, an intermediate in the biosynthesis of nitric oxide, is a potent competitive inhibitor of rat liver arginase, with a K_i of 42 μM (Table 1). In contrast, the other N^G-substituted arginine analogs such as N^G-amino-L-arginine shown in Table 1 are poor inhibitors of the enzyme, with K_i values in the millimolar range. The relatively high uncertainties associated with the estimated K_i values for N^G-methyl-L-arginine, N^G-methoxy-L-arginine, and 4,5-dihydroimidazol-2-N^ω-L-ornithine result from the limited practical concentration ranges for these compounds due to their poor inhibitory properties. Des-aminoarginine has a K_i of approximately 14 mM, and although this analog has an unsubstituted guanidino group it is not a substrate for rat liver arginase.

Table I: Inhibition Constants for Analogs of L-Arginine



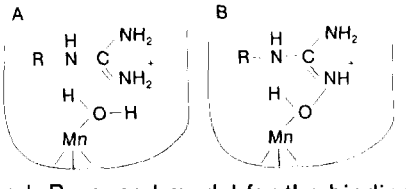
Previous studies in this laboratory have shown that the corresponding des-carboxyarginine (agmatine) is a very poor substrate for arginase (10). Thus, arginase shows specificity for the presence of both the α-amino and α-carboxyl groups, and since D-arginine is not a substrate for the enzyme, strict stereochemical constraints also apply.

Previous studies have shown that the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, and aminoguanidine are very poor inhibitors of rat liver arginase in crude extracts (21). Similarly, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine, and N^G-tosyl-L-arginine have virtually no effect on the activities of the arginases from bovine liver or rat macrophages (22). In contrast, in the present study we have shown potent competitive inhibition of purified rat liver arginase by N^G-hydroxy-L-arginine. The K_i value for N^G-hydroxy-L-arginine is approximately 40 μM, compared to the K_M of 1 mM for L-arginine and

the K_i of 1 mM for the product of the arginase reaction, L-ornithine. Thus, the inhibition data indicate that N^G-hydroxy-L-arginine is bound to the enzyme with 25-fold higher affinity than L-ornithine. Preliminary binding studies at physiologic pH have shown that the K_d for N^G-hydroxy-L-arginine is ~25 μM, while the K_M for L-arginine is in the range of 2-5 mM. Both N^G-hydroxy-L-arginine and N^G-amino-L-arginine are hydrolyzed upon prolonged incubations with arginase. Under the conditions of the NMR experiments, N^G-hydroxy-L-arginine was hydrolyzed at a rate approximately 10^{-4} of that observed for L-arginine, while the rate of N^G-amino-L-arginine hydrolysis was ~5 x 10^{-4} of that for L-arginine. The relative activity of the enzyme with N^G-amino-L-arginine represents a lower limit, since unlike N^G-hydroxy-L-arginine, this compound was not present at saturating levels in the experiments.

A model for the arginase catalyzed hydrolysis of L-arginine involves attack at the substrate guanidino carbon by a water molecule that is bound to one or both of the Mn(II) ions in the binuclear metal center (Scheme 1A). Inhibition by N^G-hydroxy-L-arginine is readily accommodated in this model if the hydroxyl group of the inhibitor displaces the metal bound water and enters the first coordination sphere of one of the metal ions as shown in Scheme 1B. In this scheme N^G-hydroxy-L-arginine is best considered as a bisubstrate analog for the arginase reaction. The low substrate activity of N^G-hydroxy-L-arginine may result from an alternate binding mode in which the hydroxyl group is not coordinated to the metal ion.

A mechanism for regulating the competing pathways of L-arginine metabolism is likely to exist in those tissues that possess both arginase and NOS activities. The disparate K_M values for L-arginine of NOS (K_M in the micromolar range (2)) and arginase (K_M of approximately 1 mM) may provide one level of regulation,



Scheme I. Proposed model for the binding of L-arginine (A), and N^G-hydroxy-L-arginine (B) to arginase.

dependant upon the relative amounts of the two enzymes and their corresponding k_{cat} values. The unique and relatively potent inhibition of arginase by the biosynthetic intermediate in NO generation, N^G-hydroxy-L-arginine, suggests an alternate means of regulation. For example, in tissues or cells (such as liver or macrophages) that contain both NOS and arginase, it is possible that the inhibition of arginase by N^G-hydroxy-L-arginine may ensure an adequate supply of substrate L-arginine for NOS. Since the level to which N^G-hydroxy-L-arginine accumulates during NO synthesis *in vivo* is not known, it is difficult to evaluate this possible means of regulation. However, it is interesting and provocative that N^G-hydroxy-L-arginine appears to be unique in its ability to potently inhibit arginase activity.

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