N^G-Allyl- and N^G-Cyclopropyl-L-arginine: Two Novel Inhibitors of Macrophage Nitric Oxide Synthase

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N^G-Methyl-L-arginine has recently been shown to inactivate the inducible murine macrophage nitric oxide (•NO) synthase (Olken, N. M.; Rusche, K. M.; Richards, M. K.; Marletta, M. A. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 828–833). N^G-Allyl-L-arginine and N^G-cyclopropyl-L-arginine were synthesized as potential mechanism-based enzyme inhibitors to exploit the chemistry presumed to occur at the active site. N^G-Cyclopropyl-L-arginine was found to be a potent reversible inhibitor with a K_i = 7.7 μM. N^G-Allyl-L-arginine was found to be both a potent reversible (K_i = 2.1 μM) and irreversible inhibitor of the enzyme. This irreversible inhibition demonstrated pseudo-first-order inactivation kinetics with k_{inact} = 0.026 min^{-1} and K_I = 3.4 μM. Stereospecific protection of the inactivation was afforded by L-arginine, and saturability of the inactivation rate was observed. Our studies indicate that both reversible and irreversible inhibition of the inducible •NO synthase can be achieved with relatively simple modifications of the substrate L-arginine.

Introduction

The manipulation of the recently discovered mammalian pathway of L-arginine oxidation to nitric oxide (•NO) has the potential of broad therapeutic application.[1] •NO produced in the vascular endothelium is critical to the homeostatic regulation of blood pressure and has an antiaggregatory effect on platelets.[2] •NO liberated by immunostimulated macrophages has been shown to be crucial to the cytotoxic and cytostatic function of these activated cells.[3] The •NO synthases are a family of enzymes which synthesize •NO by the oxidation of one of the two terminal guanido nitrogens of L-arginine (1) (Scheme I). Both endothelial cells[4] and immunostimulated macrophages[3,5,6] have been shown to generate [^{15}N]•NO following treatment with [^{15}N-*guanido*-N_2]-L-arginine. Incubation of the macrophage enzyme with [^{18}O]O_2 results in ^{18}O incorporation into the ureido group of citrulline, the other reaction product.[7] Marletta and colleagues originally proposed[5,6] that hydroxylation of L-arginine to N^G-hydroxy-L-arginine (2) was the first enzymatic step in this reaction. In support of this hypothesis, incubation of macrophage •NO synthase with chemically synthesized [^{15}N-OH]N^G-hydroxy-L-arginine was found to produce [^{15}N]•NO exclusively.[8,9] Furthermore, N^G-hydroxy-L-arginine has been shown to have vasoactive properties indistinguishable from those of L-arginine.[10] Analogous to oxidations performed by the cytochromes P450[11] and the pterin-dependent amino acid hydroxylases,[12] the mechanism of this hydroxylation may involve single electron oxidation of a terminal guanido nitrogen followed by radical recombination with a metal–oxygen complex at the active site.

We have recently reported that N^G-methyl-L-arginine (L-NMA, 3) is a mechanism-based enzyme inhibitor of murine macrophage •NO synthase.[13] Typical pseudo-first-order inactivation kinetics were found, with k_{inact} = 0.050 min^{-1} and K_I = 4.2 μM at 37 °C. We speculated that either single electron oxidation or the N^G-methylated nitrogen followed by deprotonation or direct oxidation of the methyl group could produce a carbon-centered free radical capable of enzyme alkylation (Scheme II, R = H). Subsequent hydroxylation of the methyl group would result in the formation of a carbinolamine at the active site of the •NO synthase. This carbinolamine would then be in equilibrium with an electrophilic iminium species and the products of N-dealkylation, namely arginine and formaldehyde. Indeed, cytochrome P450 has been shown to dealkylate methylguanidine to guanidine and formaldehyde.[14] To explore these mechanistic possibilities and

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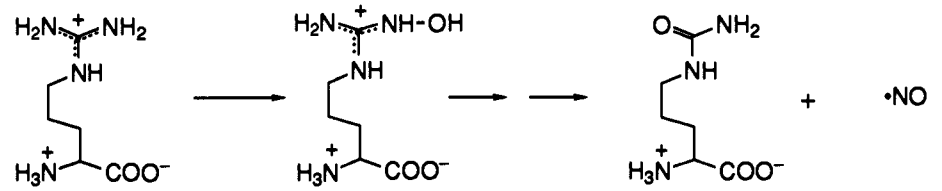
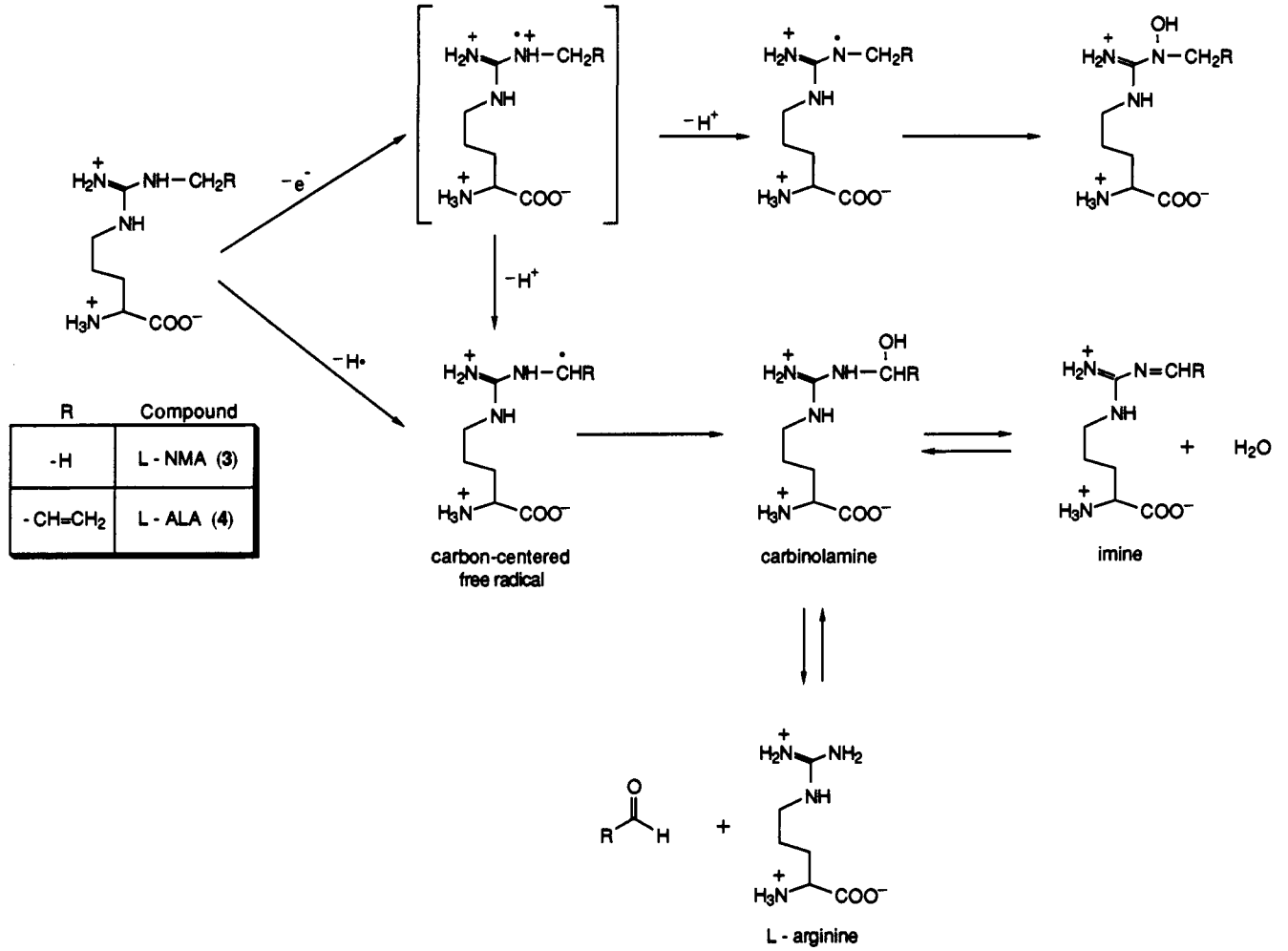
Scheme I. Reaction Catalyzed by •NO Synthase[a]



[a] The oxidation of L-arginine (1) to citrulline and nitric oxide catalyzed by •NO synthase, showing the reaction intermediate N^G-hydroxy-L-arginine (2).

Scheme II. General Rationale for the Inactivation of the •NO Synthase by N^G-Alkyl-substituted-L-arginines[a]



[a] Illustrated are potential routes of oxidation of N^G-alkyl-substituted-L-arginine derivatives at either the alkyl-bearing guanido nitrogen or at the α-carbon position directly. Nitrogen oxidation followed by radical recombination with activated oxygen results in N-hydroxylation. Hydrogen atom abstraction at carbon or nitrogen oxidation followed by deprotonation would result in a carbon-centered radical capable of covalent enzyme inactivation. Subsequent radical recombination with activated oxygen results in hydroxylation at carbon. This carbinolamine could then dehydrate to produce an imine-like Michael acceptor, or decompose to liberate an aldehyde at the active site, also a Michael acceptor.

to increase our understanding of the substrate binding site of the macrophage •NO synthase, we prepared N^G-allyl- and N^G-cyclopropyl-L-arginine.

N^G-Allyl-L-arginine (L-ALA, 4) was designed to capitalize on the potentially facile oxidation of an allylic position if hydrogen atom abstraction of an N^G-alkyl group were the initial step in enzyme inactivation. Alternatively, initial N-oxidation could be followed by deprotonation to generate the same allylic radical, a potential covalent enzyme inactivator (Scheme II, R = CH=CH_2). Subsequent hydroxylation would produce a carbinolamine which would be in equilibrium with two potential Michael acceptors: an electrophilic iminium species and/or the dealkylation product acrolein. N^G-Cyclopropyl-L-arginine (L-CPA, 5) was designed to inactivate the •NO synthase if single electron oxidation of the guanido nitrogen bearing the cyclopropyl ring is the first step in the enzymatic mecha-

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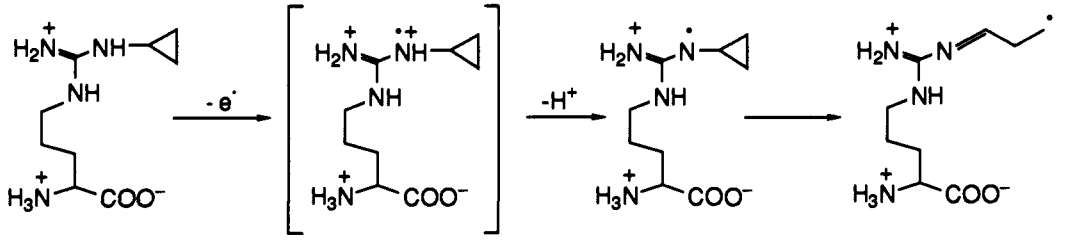
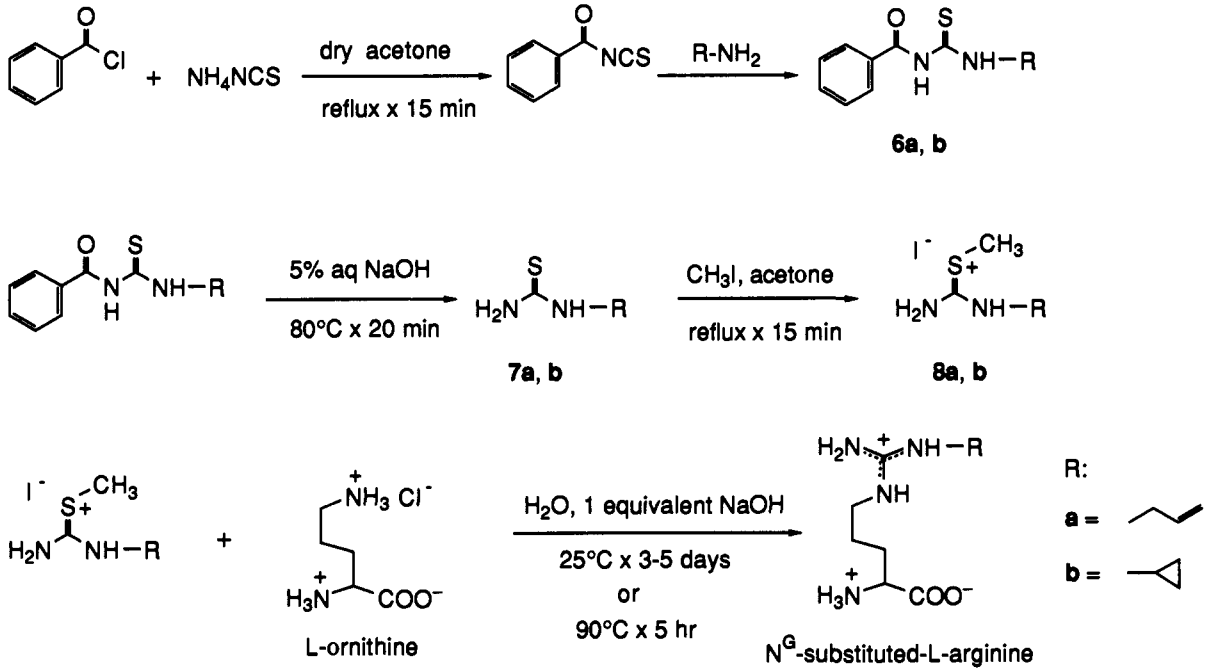
Scheme III. Rationale for Inactivation of the •NO Synthase by L-CPA[a]



N^G-cyclopropyl-L-arginine (**5**)

[a] Oxidation of the cyclopropyl-bearing guanido nitrogen by •NO synthase would produce a homoallylic free radical following cyclopropyl ring opening.

Scheme IV. Synthesis of N^G-Substituted-L-arginines[a]



[a] Synthesis of monosubstituted pseudothiouronium salts from the corresponding amines. These salts react regiospecifically at the δ-nitrogen of L-ornithine to produce N^G-substituted-L-arginines in modest yields.

nism. This oxidation would be expected to result in rapid cyclopropyl ring opening and covalent enzyme modification, similar to cyclopropylamine-based inhibitors of cytochrome P450[15,16] and monoamine oxidase B[17] (Scheme III).

Chemistry

N^G-Allyl- and N^G-cyclopropyl-L-arginine (**4**, **5**) were synthesized from L-ornithine and either *N*-allyl- or *N*-cyclopropyl-*S*-methylpseudothiouronium iodide (**8a,b**), respectively (Scheme IV). TLC of the reaction mixtures typically indicated only 50–75% conversion of L-ornithine to the N^G-substituted-L-arginine product. Although careful optimization was not performed, neither prolonged heating at 90–100 °C nor a 2- or 3-fold molar excess of the pseudothiouronium salts drove the reaction further. Purification was therefore complicated by the relatively large amounts of unreacted cationic starting materials. Both N^G-substituted-L-arginines were purified by repeated cation-exchange chromatography. Many attempts failed to crystallize the arginine derivatives as acetate salts and the hydrochloride salts were quite hygroscopic. Thus the free base of both products was found to be the most conveniently isolated form. L-CPA was determined to be analytically pure following chromatography, while L-ALA required recrystallization of the monoflavianate salt from water.

N-Allyl- and *N*-cyclopropylthioureas (**7a,b**) were synthesized by modification of a recently reported method for the preparation of arylthioureas.[18] This method was selected because of the reported ease of isolation and relative purity of the thioureas following precipitation from water. However, neither thiourea precipitated from ice-cold water following hydrolysis of the benzoyl group, and extraction into ethyl acetate was therefore required. These thioureas could be recrystallized readily from ethyl acetate and

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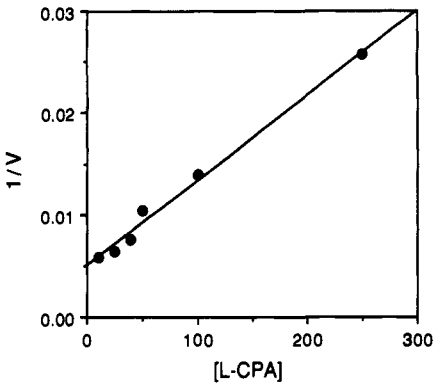

Figure 1. Reversible inhibition of macrophage •NO synthase by L-CPA. A Dixon plot of $1/V$ vs [L-CPA] (μM) at a constant concentration of 50 μM L-arginine where V is expressed in nmol of •NO mg^{-1} h^{-1}.

hexane. The pseudothiouronium salts which resulted from the S-methylation of the substituted thioureas also proved difficult to recrystallize. Thus the pseudothiouronium salts (**8a,b**) were used directly following rotary evaporation of acetone and unreacted methyl iodide.[19]

The synthesis of N^G-substituted-L-arginines has most frequently been accomplished as shown in Scheme IV, and requires the nucleophilic attack by the δ-amino group of L-ornithine on the methylthiocarbonyl of a substituted pseudothiouronium salt. This reaction has been used to prepare N^G-methyl-,[20-22] N^G,N^G-dimethyl-,[21] N^G,N'^G-dimethyl-,[21] N^G,N^G,N'^G-trimethyl-,[21] and N^G-ethyl-L-arginine.[19] Some authors have used the Cu(II) complex of L-ornithine to ensure attack by the δ- and not the α-nitrogen,[19,20] while others have not.[21,22] Only recently has a report emerged which specifically examined the regioselectivity of this reaction.[22] Ferrario and colleagues synthesized N^G-methylarginine from L- and D-ornithine without Cu(II). For comparison, they prepared both N^G-methyl-L- and D-arginine by a method which unambiguously guanidinated the δ-nitrogen of ornithine, and which was mild enough to minimize the possibility of racemization at the α-carbon of L- or D-ornithine. They demonstrated that the reaction is in fact completely regiospecific for the δ-nitrogen, and that no racemization occurs.

Results

L-CPA or L-ALA as Substrates for •NO Synthase. Neither 1 mM L-CPA nor 1 mL L-ALA generated detectable levels of •NO when incubated with cofactors (1 mM Mg^{2+}, 167 μM DTT, 100 μM NADPH, and 12 μm BH_4) and enzyme in the presence of 2 μM oxyhemoglobin (oxyHb).

Inhibition of •NO Synthase by L-CPA. A K_i of 7.7 μM for L-CPA was derived from a Dixon plot of $1/V$ vs [L-CPA] at 50 μM L-arginine (Figure 1). The K_m of L-arginine is 7.4 μM under these conditions. L-CPA was studied to determine if it was capable of time-dependent inactivation of macrophage •NO synthase activity. However, pre-incubations with cofactors and up to 100 μM L-CPA did not result in loss of enzyme activity significantly different from a control over 180 min at 37 °C.

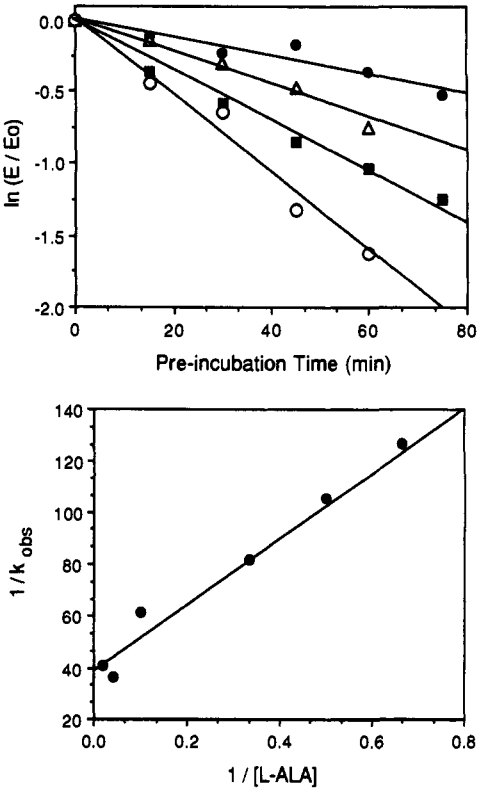

Figure 2. (A) Time- and concentration-dependent inactivation of macrophage •NO synthase by L-ALA. The vertical axis is the natural log of the ratio of enzyme activity remaining at the end of the pre-incubation time to the enzyme activity measured after no pre-incubation (t = 0 min). All pre-incubations were done at 37 °C with 0 (●), 3.0 (△), 10 (■), or 25 (○) μM L-ALA, 1 mM Mg^{2+}, 833 μM DTT, 100 μM NADPH, and 60 μM BH_4. (B) Kitz and Wilson replot. The inactivation kinetics shown in part A were replotted according to Kitz and Wilson.[23] The reciprocal of the k_{obs} (min^{-1}) obtained from the inactivation kinetic experiments have been plotted against the reciprocal of L-ALA concentration (μM). This graph also includes data from inactivation experiments at 1.5, 2.0, or 50 μM L-ALA not shown in Figure 2A.

Inhibition of •NO Synthase by L-ALA. A K_i of 2.1 μM for L-ALA was derived from a Dixon plot of $1/V$ vs [L-ALA] at 250 μM L-arginine (not shown). In addition, L-ALA produced time- and concentration-dependent loss of macrophage •NO synthase activity (Figure 2A). Preincubations containing enzyme, cofactors and 0, 1.5, 2, 3, 10, 25, and 50 μM L-ALA were assayed for activity at 0, 15, 30, 45, 60, and in some cases 75 min. A Kitz and Wilson replot[23] (Figure 2B) of $1/k_{obs}$ vs 1/[L-ALA] gave a k_{inact} = 0.026 min^{-1} and a K_I = 3.4 μM.

Irreversibility of Macrophage •NO Synthase Inactivation by L-ALA. To determine if the inhibition by L-ALA was irreversible, an enzyme preparation [383 nmol of •NO (mg of protein)$^{-1}$ h^{-1}] was pre-incubated with cofactors and 50 μM L-ALA at 37 °C for 60 min. In accordance with an expected k_{obs} = 0.024 min^{-1}, 22% [85 nmol of •NO (mg of protein)$^{-1}$ h^{-1}] of the enzyme activity remained. This pre-incubation was next subjected to desalting to remove all unbound inhibitor, and the protein was then re-assayed for activity. The specific activity of the protein following desalting was 103 nmol of •NO (mg

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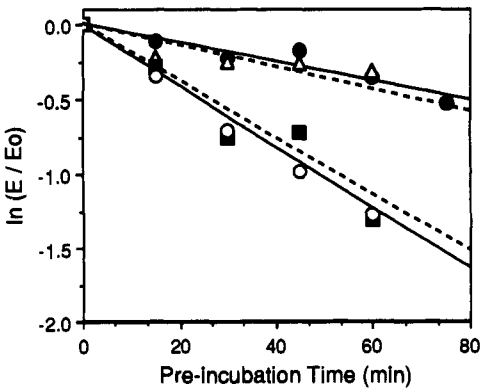

Figure 3. Stereospecific substrate protection by L-arginine and saturability of the inactivation process. A control pre-incubation with enzyme and the required cofactors (●, solid line) was assayed for activity at 15-min intervals as described in Methods. Pre-incubation with enzyme, the required cofactors, and 500 μM L-arginine and 25 μM L-ALA (△, dashed line) demonstrated a similar rate of loss of enzyme activity. A pre-incubation mixture with enzyme, the required cofactors, and 500 μM D-arginine and 25 μM with L-ALA (○, dashed line) demonstrated a similar rate of loss of activity as a pre-incubation with enzyme, the required cofactors, and 250 μM L-ALA alone (■, solid line).

of protein)$^{-1}$ h^{-1}. Therefore, the inactivation was essentially irreversible within the time frame of this experiment (~2 h).

Saturability of Macrophage ·NO Synthase Inactivation by L-ALA. Pre-incubations which contained cofactors and 50 or 250 μM L-ALA (250 μM, Figure 3) were assayed for activity at 0, 15, 30, 45, and 60 min. The k_{obs} were 0.024 and 0.020 min^{-1}, respectively. Thus, at the higher concentrations of L-ALA that were studied, the values of k_{obs} approach the maximal value (k_{inact} = 0.026 min^{-1}) predicted by the Kitz and Wilson replot.

Substrate Protection by L-Arginine of Time-Dependent Inactivation by L-ALA. Preincubation with cofactors, 500 μM L-arginine, and 25 μM L-ALA resulted in a near-complete absence of time-dependent loss of ·NO synthase activity. Pre-incubation with cofactors, 500 μM D-arginine, and 25 μM L-ALA did not protect ·NO synthase activity against inactivation (Figure 3).

Discussion

Initial characterizations of the ·NO synthases from different cell types suggest that two distinct forms exist: a constitutively expressed Ca^{2+}/calmodulin-dependent form and a cytokine inducible, calmodulin-independent form. The inducible form of ·NO synthase, characterized initially in macrophages, plays an important function in cell-mediated immunity.[3,24,25] We have recently purified the inducible murine macrophage ·NO synthase and find it to be a homodimer of 130 000 Da per subunit, with 1 equiv each of flavin adenine dinucleotide (FAD) and flavin monophosphate (FMN) bound per subunit.[26] The constitutive ·NO synthases from rat cerebellum[27,28] and porcine cerebellum have also been purified,[29] and are single polypeptides of 150 000 Da on SDS-PAGE. The gene for the rat cerebellar ·NO synthase has recently been cloned, and it shows significant sequence homology with cytochrome P450 reductase, a flavoprotein containing 1 equiv each of FAD and FMN.[30] In cytochrome P450 reductase these two flavins serve to shuttle electrons from NADPH to the active-site heme iron of cytochrome P450, thereby enabling the reductive activation of molecular oxygen.[31]

The mechanism(s) of the ·NO synthases are currently unknown. Both forms of ·NO synthase utilize the co-substrates L-arginine and O_2 and require NADPH. The initial step in the conversion of L-arginine to ·NO is now thought to involve a monooxygenase-like hydroxylation reaction producing N^G-hydroxy-L-arginine[8,9] (Scheme I). We and others have shown that the inducible macrophage form of the enzyme is dependent on the cofactor (6*R*)-tetrahydro-L-biopterin (BH_4).[32,33] The requirement for this cofactor suggests that it might be involved in this initial hydroxylation step in a manner similar to that proposed for the aromatic amino acid hydroxylases.[34] Like other non-heme metal containing monooxygenases,[12,35] the mechanism of hydroxylation may occur in two separable steps: single electron (or hydrogen atom) oxidation of the substrate followed by radical recombination with a metal–oxygen complex. If the macrophage ·NO synthase likewise initiates catalysis of the hydroxylation of L-arginine via a single electron oxidation of the guanido moiety, then appropriate substituents at the guanido position might serve as mechanism-based inhibitors.

The choice of these substituents was suggested by our results with L-NMA (**3**). In those studies we observed significant metabolism of L-NMA while studying its mechanism of inactivation of macrophage ·NO synthase.[36] We speculated that either single electron oxidation of the N^G-methylated nitrogen followed by deprotonation or oxidation of the methyl group directly could produce a carbon-centered free radical capable of covalent enzyme inactivation (Scheme II, R = H). Subsequent hydroxyla-

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tion of the methyl group would result in the formation of a carbinolamine which would be in equilibrium with an iminium species capable of enzymic inactivation or N-dealkylation and formaldehyde production at the active site.[13] This suggested to us that L-ALA (4) and L-CPA (5) might be more efficient inactivators of the inducible •NO synthase if they were processed by the enzyme in a similar manner. This is illustrated in Schemes II and III.

In contrast to our expectations, L-CPA was found to act strictly as a reversible inhibitor. It was relatively potent, with a K_i = 7.7 μM which is essentially the same as the K_m for L-arginine in this system.[13] L-CPA is most likely a competitive inhibitor, although our analysis does not distinguish mixed-type from competitive inhibition. The •NO synthase did not generate any detectable •NO from L-CPA. On the other hand, pre-incubation of the enzyme with L-ALA led to time-dependent, irreversible inactivation of the macrophage •NO synthase activity. Other features of mechanism-based enzyme inhibition were observed as well, including stereospecific substrate protection by L-arginine and not D-arginine, and a saturable rate of inactivation. L-ALA was a relatively efficient irreversible inhibitor with a k_{inact} = 0.026 min^{-1} and a K_I = 3.4 μM. As with L-CPA and L-NMA, the formation of •NO was not observed in reaction mixtures containing L-ALA, enzyme, and cofactors.

The absence of irreversible enzyme inhibition by L-CPA certainly does not rule out single electron oxidation of one of the two terminal guanido nitrogens as the first step in the catalytic cycle of the inducible •NO synthase. L-CPA may simply not serve as a substrate, or may undergo a form of processing by the •NO synthase distinct from that of L-arginine. The irreversible inhibition produced by L-ALA may mechanistically result from either N- or C-oxidation (Scheme II). This mechanistic flexibility was designed into the substrate analogue with the expectation of creating an extremely potent irreversible inhibitor. The k_{inact} of 0.026 min^{-1} for L-ALA is only 52% that of L-NMA, which is 0.050 min^{-1} in the same system. Steric constraints at the active site may make L-NMA a more suitable substrate and these constraints for N^G-substituents may explain why L-ALA and not L-CPA is a time-dependent inactivator. The relative conformational freedom of the N^G-allyl group may allow L-ALA to be processed, leading to •NO synthase inactivation, whereas L-CPA is more rigid and thus more sterically demanding, and may not be processed.

There have been a number of reports of reversible inhibitors of the •NO synthases. Initially, L-NMA was found to inhibit, almost equipotently, the inducible •NO synthase in macrophages[37] and the constitutive •NO synthase in vascular endothelium[38] and the brain.[39] However, while L-canavanine inhibits the inducible •NO synthase in macrophages,[5] it has little or no effect on •NO synthesis in endothelial cells.[38] N^G-Nitro-L-arginine is a potent inhibitor of the constitutive vascular and brain forms of •NO synthase, while being a relatively poor inhibitor of the inducible macrophage enzyme.[40,41] N^G-Amino-L-arginine is also somewhat selective for the endothelial cell •NO synthase.[41,42] These findings showed that small substituents appended to one terminal guanido nitrogen can be readily accommodated into the substrate binding site of both forms of NO synthase. However, evidence such as that presented above suggests that the substrate binding sites of the inducible and constitutive •NO synthases are different and can be exploited in the development of isoform-selective •NO synthase inhibitors. Reports of irreversible inhibition of the •NO synthases have been more limited. In addition to our own work with L-NMA,[13] Moncada and colleagues have reported that N-(iminoethyl)-L-ornithine is an irreversible inhibitor of •NO synthase activity in phagocytes,[43] and Snyder and colleagues have reported that N^G-nitro-L-arginine irreversibly inactivated the rat brain enzyme.[44] Neither of these two reports, however, carried out a formal kinetic analysis of the inactivation process.

The generation of nitric oxide must be finely tuned in order to carry out the important physiological functions ascribed to it. Cellular communication seems to involve low, transient bursts of •NO formation while higher levels synthesized over an extended period of time are generated by the inducible enzyme. However, the sustained and elevated generation of •NO can be deleterious and may be important in the etiology of endotoxic shock,[45] inflammation-related tissue damage,[46] and N-nitrosamine-induced carcinogenesis.[47] The inducible •NO synthase, best characterized in the murine macrophage, has been implicated as the source of this sustained •NO formation. Receptor-mediated •NO formation by the constitutive enzyme in response to the excitatory amino acid glutamate has now been implicated in certain types of neurotoxicity.[48] The design of isoform specific inhibitors of different •NO

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synthases could be a key therapeutic advance in the treatment of these and other clinical problems. L-CPA and L-ALA, the two novel arginine analogues described in this report, coupled with our earlier results with L-NMA show that these relatively simple modifications of arginine can lead to dramatic differences in the type of enzyme inhibition. We are investigating these differences in order to understand those features of substrate analogues which make them reversible or irreversible inhibitors of •NO synthase. These features should also help in deciphering the mechanism of the •NO synthase.

Experimental Section

General Biochemical Methods. Analytical Methods. Assays for •NO synthase activity were carried out as we recently reported.[13] Briefly, a reference cuvette was charged with 2 μM oxyhemoglobin (oxyHb) in 15 mM Hepes (pH 7.4) in a final volume of 500 μL. A typical sample assay contained 2 mM L-arginine, 1 mM Mg^{2+}, 167 μM DTT, 100 μM NADPH, 12 μM BH_4, and 2 μM oxyHb in 15 mM Hepes (pH 7.4) in a final volume of 500 μL. All assays were initiated with enzyme. The Hepes buffer was pre-heated to 37 °C prior to use. •NO reacts with oxyHb to yield methemoglobin which can be measured at 401 nm (ϵ = 19 700 M^{-1} cm^{-1}).[49] Protein concentration of desalted supernatant was determined with the Bradford Assay (Bio-Rad) using bovine serum albumin as the standard.[50]

Reagents. L-Arginine, human ferrous hemoglobin A_0, NADPH, DTT, Hepes, and magnesium acetate were purchased from Sigma. (6*R*)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Dr. B. Schircks Laboratories, Jona, Switzerland.

Enzyme Preparation. Macrophage cytosol was prepared from the murine macrophage cell line RAW 264.7 and desalted as described previously.[32] A typical preparation of desalted supernatant had a specific activity of 300 nmol of •NO (mg of protein)$^{-1}$ h^{-1} as determined with the hemoglobin assay.

Inhibition Methods. Reversible Inhibition of Murine Macrophage •NO Synthase by L-ALA and L-CPA. Desalted supernatent (50 μL) was added to an assay cuvette containing L-arginine, 1 mM Mg^{2+}, 167 μM DTT, 100 μM NADPH, 12 μM BH_4, 2 μM oxyHb, and L-ALA or L-CPA in 15 mM Hepes (pH 7.4) at 37 °C, in a final volume of 500 μL. The K_i for L-CPA was determined from a Dixon plot with 50 μM L-arginine and 10, 25, 40, 50, 100, and 250 μM L-CPA. The K_i for L-ALA was determined from a Dixon plot with 250 μM L-arginine and 25, 50, 100, 250, and 500 μM L-ALA.

Time- and Concentration-Dependent Inactivation of Murine Macrophage •NO Synthase by L-ALA. Desalted supernatant (300 μL) (typically ~500 μg of protein) was added to a pre-incubation mixture to produce final concentrations of 1 mM Mg^{2+}, 833 μM DTT, 100 μM NADPH, 60 μM BH_4, and L-ALA in 15 mM Hepes (pH 7.4), in a final volume of 600 μL. Concentrations of L-ALA in the pre-incubation mixture were 1.5, 2.0, 3, 10, 25, or 50 μM. After the addition of enzyme, an initial 100-μL aliquot was removed and assayed for activity as described above. The pre-incubation mixture was then placed in a 37 °C water bath. Aliquots (typically 100 μL) were removed and assayed for activity after 15, 30, 45, 60, and 75 min. Each aliquot was added to a cuvette containing cofactors and oxyHb to produce a final volume of 500 μL and final concentrations of 2 mM L-arginine, 1 mM Mg^{2+}, 167 μM DTT, 100 μM NADPH, 12 μM BH_4, and 2 μM oxyHb in 15 mM Hepes (pH 7.4) at 37 °C.

Saturability and Substrate Protection by L-Arginine of •NO Synthase Inactivation by L-ALA. Saturation kinetics were examined with a pre-incubation which contained 250 μM L-ALA. Aliquots of 50 μL were removed and assayed for activity as above. Substrate protection was examined with pre-incubations which contained 25 μM L-ALA and 500 μM L-arginine or 25 μM L-ALA and 500 μM D-arginine and assayed for activity as above.

Irreversibility of •NO Synthase Inactivation by L-ALA. A pre-incubation was performed at 37 °C for 60 min with 818 μL of desalted supernatant, 1 mM Mg^{2+}, 833 μM DTT, 100 μM NADPH, 60 μM BH_4, and 50 μM L-ALA. After 60 min the pre-incubation was removed from the 37 °C water bath and a 50 μL aliquot was assayed for activity. The remaining pre-incubation mixture was desalted (Fast Desalting Column, Pharmacia). The protein-containing fractions were combined and re-assayed for enzyme activity and protein concentration.

General Chemical Methods. Melting points were determined in open capillary tubes on an electrothermal melting point apparatus and are uncorrected. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. ^{1}H-NMR and ^{13}C-NMR were performed on a Bruker WM-360 in the solvents indicated. ^{1}H-NMR chemical shifts in $CDCl_3$ and DMSO are reported relative to internal TMS. ^{1}H-NMR chemical shifts in D_2O are reported relative to internal TSP. ^{13}C-NMR in D_2O are reported relative to internal 1,4-dioxane (67.4 ppm). Elemental analysis were obtained from M-H-W Laboratories, Phoenix, AZ. Fast atom bombardment mass spectrometry was performed on a VG-Instruments 70-250-S mass spectrometer. Silica gel column chromatography was performed with EM Science silica gel 60 (70–230 mesh). Thin-layer chromatography was carried out on Analtech Silica Gel GHL glass-backed plates, 0.25 mm in thickness. Amino acids were visualized with a ninhydrin/pyridine spray reagent.

Chemical Syntheses. *N*-Allyl-*N'*-benzoylthiourea (6a). To a stirred suspension of ammonium thiocyanate (11.07 g, 145.7 mmol) in 150 mL of acetone at room temperature was added dropwise benzoyl chloride (15.47 mL, 133.0 mmol) over 5 min. The solution was heated to reflux for 15 min and allowed to cool to room temperature. This solution of benzoyl isothiocyanate was then chilled in an ice bath, and allylamine (10.00 mL, 133.0 mmol) was added dropwise over 5 min. This was allowed to warm to room temperature and then stirred for 2 h. The solution was poured onto 500 mL of cracked ice. Crude **6a** was filtered and the precipitate was washed with cold water (21.20 g, 72.36%). This was used without further purification in the next reaction. For analysis, the crude product was eluted from silica gel 60 (70–230 mesh) with 1:1 hexane/ethyl acetate. The product was recrystallized from ethyl acetate and hexane: mp 65–65.5 °C; ^{1}H-NMR ($CDCl_3$) δ 7.84 (d, 2 H, ArH ortho), 7.60 (t, 1 H, ArH para), 7.50 (t, 2 H, ArH meta), 5.95 (m, 1 H, C*H*=CH_2), 5.28 (m, 2 H, CH=C*H*$_2$), 4.36 (m, 2 H, C*H*$_2$CH=CH_2). Anal. ($C_{11}H_{12}N_2OS$) C, H, N.

Allylthiourea (7a). Compound **6a** (10.00 g, 45.39 mmol) was added in one portion to 50 mL of a solution of 5% (w/v) NaOH pre-heated to 70 °C. This solution was stirred for 20 min and was poured onto cracked ice and excess dilute HCl. The resulting solution was titrated to pH 8.2 with concentrated NH_4OH. The crude product was extracted from this aqueous solution with EtOAc (4 × 250 mL). These were combined, and the solvent was removed by rotary evaporation at 40 °C. The resultant brown viscous oil was applied to 50 g of silica gel and **7a** was eluted with 1:1 hexane/ethyl acetate. Pure **7a** was recrystallized from ethyl acetate and hexane (1.70 g, 32.23%): mp 73–73.5 °C; ^{1}H-NMR (DMSO) δ 5.82 (bs, 1 H, C*H*=CH_2), 5.10 (m, 2 H, CH=C*H*$_2$), 4.03 (bs, 2 H, C*H*$_2$CH=CH_2). Anal. ($C_4H_8N_2S$) C, H, N.

***N*-Allyl-*S*-methylpseudothiouronium Iodide (8a).** To a stirred suspension of **7a** (1.00 g, 8.61 mmol) in 10 mL of acetone at room temperature was added dropwise 590 μL (9.54 mmol) of methyl iodide. This was heated to reflux for 15 min and then allowed to cool to room temperature and stir for 1 h. Crude **8a** was obtained as a viscous yellow oil by rotary evaporation of the reaction mixture (2.10 g, 94.60%). For analysis crude **8a** was dissolved in water and chromatographed on Dowex 50X8 (H^+ form) 200–400 mesh. The chloride salt of **8a** was eluted with 1 N HCl. This was concentrated by rotary evaporation and recrystallized from $CHCl_3$ and diethyl ether: mp 91.5–92.0 °C; ^{1}H-NMR (DMSO) δ 5.82 (m, 1 H, C*H*=CH_2), 5.22 (m, 2 H, CH=C*H*$_2$), 4.04 (m, 2 H, C*H*$_2$CH=CH_2), 2.66 (s, 3 H, CH_3). Anal. ($C_5H_{11}ClN_2S$·0.5H_2O) C, H, N.

N^G-Allyl-L-arginine (4). To a solution of L-ornithine hydrochloride (3.70 g, 8.10 mmol) in 8.10 mL of 1 N NaOH was

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added **8a** (2.10 g, 8.10 mmol). This was allowed to stir at room temperature for 72 h. A stream of nitrogen was bubbled through the solution to disperse the methanethiol as it evolved. TLC on silica gel with 2:2:1 $CHCl_3$/MeOH/NH_4OH demonstrated unreacted L-ornithine (R_f = 0.3) and **4** (R_f = 0.5). The pH was adjusted to 4 with concentrated HCl and the solution was applied to 10 mL of Dowex 50X8 (H^+ form) 200–400 mesh. After washing with 50 mL of water, 0.25 N NH_4OH, pH 8.2 (adjusted with HCl), was used to elute first L-ornithine and then **4**. After three successive columns all the L-ornithine had been removed. The free base of **4** was obtained from the hygroscopic hydrochloride salt by loading the pure amino acid onto Dowex 50X8 (H^+ form) and eluting with 0.25 N NH_4OH. Fractions which contained the amino acid were combined, and the water was removed by rotary evaporation at 37 °C. The clear residue which remained was further dried under a vacuum for 3 days at room temperature (94 mg, 5.4%): ^{1}H-NMR (D_2O) δ 5.88 (m, 1 H, C*H*=CH_2 allylic), 5.26 (m, 2 H, CH=CH_2), 3.85 (m, 2 H, CH_2CH allylic), 3.27 (t, 1 H, α C*H*) 3.23 (t, 2 H, δ CH_2), 1.64 (m, 4 H, β CH_2 and γ CH_2); ^{13}C-NMR (D_2O) δ 183.40 (*C*O_2), 156.74 (guanido *C*), 133.34 (*C*H allylic), 117.15 (CH_2*C*H=CH_2), 56.35 (α *C*H), 45.85 (NH*C*H_2 allylic), 41.86 (δ *C*H_2), 32.27 (β *C*H_2), 25.40 (γ*C*H_2); FAB MS (M + 1) 215 (base peak). For elemental analysis the free base of **4** was recrystallized as the monoflavianate salt from water: mp 238–240 °C dec. Anal. ($C_{19}H_{24}N_6O_{10}S$) C, H, N.

***N*-Cyclopropyl-*N'*-benzoylthiourea (6b).** To a stirred suspension of ammonium thiocyanate (13.33 g, 175.1 mmol) in 150 mL of acetone at room temperature was added dropwise benzoyl chloride (20.41 mL, 175.1 mmol) over 5 min. The solution was heated to reflux for 20 min and allowed to cool to room temperature. This solution of benzoyl isothiocyanate was chilled to 0 °C in an ice bath and cyclopropylamine (10.00 g, 175.1 mmol) was added dropwise over 5 min. This was allowed to warm to room temperature and stir for 1 h. The solution was poured onto 700 mL of cracked ice. The crude product was filtered, redissolved in 200 mL of ethanol and treated with activated charcoal. The solution was filtered through celite and dried by rotary evaporation. Nearly pure **6b** was obtained by recrystallization from ethanol (16.87 g, 43.71%). This was used without further purification in the next reaction. For analysis the crude product was chromatographed on silica gel 60 and eluted with 1:1 hexane/ethyl acetate. The product was recrystallized from ethyl acetate and hexane: mp 109–109.5 °C; ^{1}H-NMR ($CDCl_3$) δ 7.82 (d, 2 H, ArH ortho), 7.61 (t, 1 H, ArH para), 7.52 (t, 2 H, ArH meta), 3.22 (m, 1 H, C*H*), 0.96 (m, 2 H, cyclopropyl), 0.78 (m, 2 H, cyclopropyl). Anal. ($C_{11}H_{12}N_2SO$) C, H, N.

Cyclopropylthiourea (7b). Compound **6b** (14.00 g, 63.64 mmol) was added to 150 mL of a solution of 5% (w/v) NaOH and heated to 80 °C. This solution was stirred for 20 min and was then cooled to room temperature in a cold water bath. The resulting solution was titrated to pH 7.9 with concentrated HCl. The crude product was extracted from this aqueous solution with ethyl acetate (4 × 250 mL). These were combined, and the solvent was removed by rotary evaporation at 40 °C. The thiourea was collected as a pale yellow powder (3.30 g, 44.70%) and was used in the next reaction without further purification. For analysis **7b** was chromatographed on 50 g of silica gel 60 and eluted with 1:1 hexane/ethyl acetate. Pure **7b** was recrystallized from ethyl acetate and hexane: mp 142.5–143 °C; ^{1}H-NMR (DMSO) δ 2.38 (bs, 1 H, C*H*), 0.63 (m, 2 H, cyclopropyl), 0.47 (m, 2 H, cyclopropyl). Anal. ($C_4H_8N_2S$) C, H, N.

***N*-Cyclopropyl-*S*-methylpsuedothiouronium Iodide (8b).** To a solution of **7b** (2.00 g, 17.24 mmol) in 35 mL of acetone was added methyl iodide (2.13 mL, 34.48 mmol). This was heated to 55 °C for 1 h. The acetone was removed by rotary evaporation. Crude **8b** (4.10 g, 92.54%) was obtained as an amber yellow oil and was used without further purification: ^{1}H-NMR (DMSO) δ 2.73 (m, 1 H, C*H*), 2.64 (s, 3 H, CH_3), 0.84 (m, 2 H, cyclopropyl), 0.70 (m, 2 H, cyclopropyl).

***N*^{G}-Cyclopropyl-L-arginine (5).** To a solution of L-ornithine hydrochloride (618 mg, 3.9 mmol) in 5 mL of 1 N NaOH was added **8b** (1.00 g, 3.7 mmol). This was heated to 90 °C for 5 h. TLC on silica gel with 2:2:1 $CHCl_3$/MeOH/NH_4OH demonstrated unreacted L-ornithine (R_f = 0.3) and **5** (R_f = 0.5). The pH was adjusted to 4 with concentrated HCl and the solution was applied to 10 mL of Dowex 50X8 (H^+ form) 200–400 mesh. After washing with 50 mL of deionized water, 0.25 N NH_4OH pH 8.2 (adjusted with HCl) was used to elute the amino acids. Fractions judged by TLC to contain **5** were combined and concentrated by rotary evaporation at 40 °C. After three successive columns all unreacted L-ornithine had been removed and the hygroscopic hydrochloride salt of pure **5** was loaded onto Dowex 50X8 (H^+ form) 200–400 mesh. The free base was obtained by elution with 0.25 N NH_4OH. Amino acid containing fractions were combined, and water was removed by rotary evaporation at 40 °C. The clear residue was dried under a vacuum for 48 h at room temperature (60 mg, 7.6%): mp 65–67.5 °C; ^{1}H-NMR (D_2O) δ 3.44 (t, 1 H, α C*H*), 3.23 (t, 2 H, δ CH_2), 2.51 (m, 1 H, C*H* cyclopropyl), 1.75–1.60 (m, 4 H, β and γ CH_2), 0.84 (m, 2 H, cyclopropyl), 0.66 (m, 2 H, cyclopropyl); ^{13}C-NMR (D_2O) 180.97 (*C*O_2), 157.89 (*C* guanidino), 55.96 (α *C*H), 41.60 (δ *C*H_2), 30.97 (β *C*H_2), 25.21 (γ *C*H_2), 22.56 (*C*H cyclopropyl), 7.27 (*C*H_2 cyclopropyl); FAB MS (M + 1) 215 (base peak). Anal. ($C_9H_{18}N_4O_2 \cdot H_2O$) C, H, N.

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