INACTIVATION OF MACROPHAGE NITRIC OXIDE SYNTHASE ACTIVITY BY
NG-METHYL-L-ARGININE

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Summary. \(\cdot\text{N}=\text{O}\) synthase catalyzes the oxidation of one of the two chemically equivalent guanido nitrogens of L-arginine to nitric oxide \(\cdot\text{N}=\text{O}\). NG-Methyl-L-arginine has been previously characterized as a potent competitive inhibitor of both major types of \(\cdot\text{N}=\text{O}\) syntheses. Initial rate kinetics were performed with a spectrophotometric assay based on the oxidation of oxy- to methemoglobin by \(\cdot\text{N}=\text{O}\). NG-Methyl-L-arginine was a competitive inhibitor of \(\cdot\text{N}=\text{O}\) synthase activity derived from activated murine macrophages with a \(K_i\) of 6.2 \(\mu\text{M}\). When the enzyme was pre-incubated in the presence of the required cofactors NADPH and tetrahydrobiopterin, time- and concentration-dependent irreversible inactivation of the activity was observed. At \(37^\circ\text{C}\) the \(k_{\text{inact}}\) was 0.050 \(\text{min}^{-1}\). This inactivation process exhibited substrate protection, saturation kinetics and required the cofactors necessary for enzymatic turnover. These data indicate that NG-methyl-L-arginine acts as a mechanism-based enzyme inactivator of murine macrophage \(\cdot\text{N}=\text{O}\) synthase.

Many cells are now known to synthesize nitric oxide \(\cdot\text{N}=\text{O}\) by the oxidation of one of the two chemically equivalent guanido nitrogens of L-arginine (1). Two types of \(\cdot\text{N}=\text{O}\) syntheses have been identified: a constitutively expressed \(\text{Ca}^{2+}/\text{calmodulin-dependent}\) form isolated from rat cerebella (2) and identified in vascular endothelial cells (3); and a cytokine inducible, calmodulin-independent form isolated from neutrophils (4) and macrophages (5), and identified in a wide variety of other cell types (6). Both forms of \(\cdot\text{N}=\text{O}\) synthase utilize the co substrates L-arginine and molecular oxygen, and require NADPH. Tetrahydrobiopterin \((\text{BH}_4)\) has been shown to be a required cofactor for \(\cdot\text{N}=\text{O}\) synthase activity derived from the supernatant of activated murine macrophages (7, 8).

The inducible form of \(\cdot\text{N}=\text{O}\) synthase isolated from macrophages and neutrophils is expressed in response to various cytokines, such as interferon-\(\gamma\), tumor necrosis factor-\(\alpha\) and bacterial lipopolysaccharide. Upon induction, these cells generate \(\cdot\text{N}=\text{O}\) continuously for as long as

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Abbreviations: L-NMA, NG-methyl-L-arginine; DTT, dithiothreitol; \(\cdot\text{N}=\text{O}\), nitric oxide; \(\text{BH}_4\), \((6\text{R})\)-tetrahydro-L-biopterin; \(\text{BH}_2\), 7,8-dihydro-L-biopterin; NADPH, nicotinamide adenine dinucleotide reduced form; NADP\(^+\), nicotinamide adenine dinucleotide oxidized form; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; oxyHb, ferrous oxyhemoglobin; metHb, ferric hemoglobin.
72 hours (9). This \( \cdot N=O \) clearly serves to augment cell-mediated immunity. Specifically, \( \cdot N=O \) has been shown to cause a pronounced loss of intracellular iron and the inhibition of the iron-sulfur protein aconitase in L10 hepatoma cells (10). The iron-sulfur protein ribonucleotide reductase also appears to be an important target for \( \cdot N=O \) (11). Additionally, the intracellular destruction of Leishmania (12) and Toxoplasma gondii (13) by macrophages has been closely correlated with \( \cdot N=O \) generation.

\( \text{NG-Methyl-L-arginine (L-NMA)} \) was initially reported by Hibbs and colleagues to be a potent inhibitor of macrophage nitrite generation (14). In fact, L-NMA has been reported to be a competitive inhibitor of both forms of the \( \cdot N=O \) synthase (15, 16). We noted a time-dependent loss of \( \cdot N=O \) synthase activity after pre-incubation with L-NMA, NADPH and BH4. This inactivation has now been characterized under initial rate conditions by measuring \( \cdot N=O \) generation directly with a spectrophotometric assay based on the rapid and quantitative oxidation of oxy- to methemoglobin (metHb) by \( \cdot N=O \) (17, 18). L-NMA produces a time- and concentration-dependent irreversible loss of \( \cdot N=O \) synthase activity from activated murine macrophages. This inactivation has all the markings of mechanism-based enzyme inactivation: turnover-dependence, saturation kinetics and substrate protection.

**Materials and Methods**

**Analytical Methods.** Assays for enzyme activity were carried out in disposable semimicro cuvettes (Bio-Rad), on a Perkin-Elmer 553 spectrophotometer equipped with a Perkin-Elmer R100 chart recorder. A reference cuvette was charged with 2 \( \mu \)M oxyhemoglobin (oxyHb) in 15 mM HEPES (pH 7.4), in a final volume of 500 \( \mu \)l. A typical sample assay contained 2 \( \mu \)M L-arginine, 1 mM Mg\( ^{2+} \), 167 \( \mu \)M DTT, 100 \( \mu \)M NADPH, 12 \( \mu \)M BH4, and 2 \( \mu \)M oxyHb in 15 mM HEPES (pH 7.4), in a final volume of 500 \( \mu \)l. All assays were initiated with protein. The HEPES buffer was heated to 37\( ^\circ \)C prior to use, and the cell holders inside the spectrophotometer were kept at 37\( ^\circ \)C with a Thermomix 1460 thermocirulator. Enzyme activity is directly proportional to the increase in absorbance at 401 nm over time. Protein concentration of desalted supernatant was determined with the Bradford Assay (Bio-Rad) using bovine serum albumin as the standard (19).

**Reagents.** \( \text{NG-Methyl-L-arginine, acetate salt (L-NMA)} \) was purchased from Calbiochem. L-arginine, human ferrous hemoglobin A\( _0 \), NADPH, DTT, HEPES, and magnesium acetate were purchased from Sigma. (6R) 5,6,7,8-Tetrahydro-L-biopterin (BH4), and 7,8 dihydro-L-biopterin (BH2) were 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 (7). A typical preparation of desalted supernatant had a specific activity of 300 nmoles \( \cdot N=O \)/mg protein/hour as determined with the hemoglobin assay.

**Competitive Inhibition of Murine Macrophage \( \cdot N=O \) Synthase by L-NMA.** Desalted supernatant (60 \( \mu \)l) was added to an assay cuvette containing \( 50 \mu \)M L-arginine, 1 mM Mg\( ^{2+} \), 167 \( \mu \)M DTT, 100 \( \mu \)M NADPH, 12 \( \mu \)M BH4, 2 \( \mu \)M oxyHb, and 0, 5, 10, 25, or 50 \( \mu \)M L-NMA in 15 mM HEPES (pH 7.4) at 37\( ^\circ \)C, in a final volume of 500 \( \mu \)l.

**Time- and Concentration-Dependent Inactivation of Murine Macrophage \( \cdot N=O \) Synthase by L-NMA.** Desalted supernatant (300 \( \mu \)l) (typically about 500 \( \mu \)g of protein) was added to a pre-incubation mixture to produce final concentrations of 1 mM Mg\( ^{2+} \), 833 \( \mu \)M DTT, 100 \( \mu \)M NADPH, 60 \( \mu \)M BH4 and L-NMA in 15 mM HEPES (pH 7.4), in a final volume of 600 \( \mu \)l. Concentrations of L-NMA in the pre-incubation mixture were 1.5, 3.0, 5, 10, 25 or 50 \( \mu \)M. After the addition of enzyme, an initial 100 \( \mu \)l aliquot was removed and assayed for activity as described below. The pre-incubation mixture was then placed in a 37\( ^\circ \)C water bath. Aliquots
(typically 100 μl) were removed and assayed for activity after 15, 30, 45, 60 and 75 minutes. 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.

Irreversibility of N=O Synthase Inactivation. A pre-incubation was performed at 37° C as described above in the presence of 25 μM L-NMA for 45 minutes. At this time the remaining enzyme activity was measured, the pre-incubation was removed from the 37° C waterbath (the residual activity is unstable at 37° C), placed at room temperature, and 5 mM L-arginine was added. The activity was assayed at 30 minutes intervals for 2.5 hours, as described above.

Cofactor-Dependence of N=O Synthase Inactivation. Pre-incubations and activity assays were run at 22° C in the presence of 25 μM L-NMA, but otherwise as described above. BH$_2$ and NADP$^+$ concentrations were 60 and 100 μM, respectively.

Results

Time- and Concentration-Dependent Inactivation of Murine Macrophage N=O Synthase by L-NMA. Figure 1A shows the time- and concentration-dependent inhibition of murine macrophage N=O synthase by 1.5, 5 and 25 μM L-NMA. The control experiment illustrates the relatively small loss in activity which occurs when the enzyme is pre-incubated with 1 mM Mg$^{2+}$, 833 μM DTT, 100 μM NADPH and 60 μM BH$_4$ in the absence of substrate. In the presence of L-arginine, there is typically almost no loss of activity measured under these conditions after 1 hour (not shown). From a Kitz and Wilson plot (20) of 1/k$_{obs}$ vs. 1/I (figure 2) a k$_{inact}$ of 0.050 min$^{-1}$ and a K$_I$ of 4.2 μM were obtained.

Figure 1A. Time- and Concentration-Dependent Inactivation of Murine Macrophage N=O Synthase by L-NMA. 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. All pre-incubations were done at 37° C with 0 (○), 1.5 (■), 5 (△) or 25 (●) μM L-NMA, 1 mM Mg$^{2+}$, 833 μM DTT, 100 μM NADPH and 60 μM BH$_4$. At 15 minute intervals the remaining activity was assayed as described in Methods.

Figure 1B. Concentration-Dependent Substrate Protection by L-Arginine. L-arginine at 100 (△) or 500 (■) μM concentrations was pre-incubated at 37° C with 25 μM L-NMA and 1 mM Mg$^{2+}$, 833 μM DTT, 100 μM NADPH and 60 μM BH$_4$. The control activity (○) represents a pre-incubation mixture of enzyme and 1 mM Mg$^{2+}$, 833 μM DTT, 100 μM NADPH and 60 μM BH$_4$ without either substrate or inhibitor. Shown for comparison is a pre-incubation with 25 μM L-NMA (●) and no L-arginine run under the same conditions as above.

Figure 2. Kitz and Wilson Replot. The inactivation kinetics shown in figure 1A were replotted according to Kitz and Wilson (20). This graph includes the data from figure 1A as well as that obtained from inactivation experiments with 3, 10 and 50 μM L-NMA (not shown).
Figure 3. Cofactor-Dependence of \( \cdot \)N=O Synthase Inactivation by L-NMA.

Pre-incubation at 22\(^\circ\) C with 25 \( \mu \)M L-NMA in the presence of 1 mM Mg\(^{2+}\), 833 \( \mu \)M DTT, 100 \( \mu \)M NADPH and 60 \( \mu \)M BH\(_4\) (O) or 1 mM Mg\(^{2+}\), 100 \( \mu \)M NADP\(^+\) and 60 \( \mu \)M BH\(_2\) (\( \Delta \)). Each pre-incubation mixture also contained 300 \( \mu \)l of protein in a final volume of 600 \( \mu \)l of 15 mM HEPES (pH 7.4).

Irreversibility and Saturability of \( \cdot \)N=O Synthase Inactivation. Since enzyme activity was determined with a continuous spectrophotometric assay in the presence of 2 mM L-arginine (\( K_m \) 7.4 \( \mu \)M in this system), rapid reactivation of the \( \cdot \)N=O synthase (following inactivation by L-NMA) would have been apparent. However, even after 15-20 minutes of observation no evidence for any increase in the velocity of the enzymatic reaction was noted. In fact, no restoration of enzyme activity was detected when enzyme inactivated over 45 minutes by 25 \( \mu \)M L-NMA was incubated at room temperature (22\(^\circ\) C) with 5 mM L-arginine over 2.5 hours. The inactivation process was shown to be saturable as well, with concentrations of L-NMA greater than 25 \( \mu \)M resulting in very small increases in the rate of enzyme inactivation (not shown).

Substrate Protection. Figure 1B shows the concentration-dependent substrate protection afforded by 100 and 500 \( \mu \)M L-arginine when pre-incubated at 37\(^\circ\) C with 1 mM Mg\(^{2+}\), 833 \( \mu \)M DTT, 100 \( \mu \)M NADPH, 60 \( \mu \)M BH\(_4\) and 25 \( \mu \)M L-NMA.

Cofactor-Dependence of \( \cdot \)N=O Synthase Inactivation. \( \cdot \)N=O synthase activity requires NADPH (21), and, in fact, is quite unstable at 37\(^\circ\) C in the absence of either NADPH or BH\(_4\). Therefore it was impossible to demonstrate the retention of enzyme activity following pre-incubation of the enzyme and L-NMA in the absence of one of these cofactors at 37\(^\circ\) C. At 4\(^\circ\) C a pre-incubation with NADPH, DIT and 25 \( \mu \)M L-NMA showed no significant loss of activity over several hours (not shown). The problem of demonstrating cofactor-dependent loss of \( \cdot \)N=O synthase activity with L-NMA was solved by pre-incubating the enzyme with reduced (NADPH and BH\(_4\)) or oxidized (NADP\(^+\) and BH\(_2\)) cofactors at room temperature (22\(^\circ\) C). Figure 3 shows the time-dependent loss of enzyme activity for a pre-incubation carried out at 22\(^\circ\) C with 100 \( \mu \)M NADPH, 60 \( \mu \)M BH\(_4\), and 25 \( \mu \)M L-NMA. The \( k_{obs} \) is 0.013 min\(^{-1}\), only 30% that observed at 37\(^\circ\) C.

A pre-incubation carried out at 22\(^\circ\) C with the oxidized forms (100 \( \mu \)M NADP\(^+\) and 60 \( \mu \)M BH\(_2\)) of the required cofactors and 25 \( \mu \)M L-NMA also initially demonstrates some enzyme inactivation, which slows after 30 minutes and ceases after about 90 minutes (Figure 3). Although some enzyme inactivation was evident when NADP\(^+\) and BH\(_2\) were used as cofactors, this may
have been due to the \textit{in situ} reduction of NADP$^+$ to NADPH (7). Additionally, the pterin appears to be tightly bound (unpublished observations). For these reasons it was impossible to abolish enzymatic turnover. In fact, a low but detectable initial velocity of \(\cdot\text{N}=\text{O}\) generation was noted with protein, oxidized cofactors, and L-arginine at 22$^\circ$ C.

**Discussion**

In agreement with the initial observations, L-NMA was found to be a relatively potent competitive inhibitor of the \(\cdot\text{N}=\text{O}\) synthase activity in activated murine macrophages. A \(K_I\) of 6.2 \(\mu\)M was derived from a Dixon plot (not shown). For comparison, the \(K_m\) of L-arginine under these conditions was 7.4 \(\mu\)M. As described above and shown in figure 1A, pre-incubation of enzyme with L-NMA, NADPH, and BH$_4$ at 37$^\circ$ C results in a time- and concentration-dependent loss of \(\cdot\text{N}=\text{O}\) synthase activity. From a Kitz and Wilson replot (20) of \(1/k_{\text{obs}}\) vs. \(1/[I]\) (figure 2) a \(k_{\text{inact}}\) of 0.050 min$^{-1}$ and a \(K_I\) of 4.2 \(\mu\)M were obtained. In addition, as shown in figure 1B the substrate L-arginine protects against this inactivation in a concentration-dependent fashion suggesting that the inactivation process occurs at or near the active-site.

The inactivation of \(\cdot\text{N}=\text{O}\) synthase is apparently irreversible. That is, enzyme inactivated during a 45 minutes pre-incubation by 25 \(\mu\)M L-NMA was then incubated at room temperature with 5 mM L-arginine for 2.5 hours. Aliquots removed at 30 minute intervals failed to demonstrate enzyme reactivation. The inactivation process was also shown to be saturable; concentrations of L-NMA greater than 25 \(\mu\)M resulted in very small increases in the rate of enzyme inactivation (not shown).

The enzyme is unstable at 37$^\circ$ C in the absence of either BH$_4$ or NADPH, preventing the study of turnover-dependent inactivation at that temperature. Time-dependent inactivation of \(\cdot\text{N}=\text{O}\) synthase activity also occurs at 22$^\circ$ C when the pre-incubation reaction contained NADPH, BH$_4$, and 25 \(\mu\)M L-NMA (Figure 3). In contrast, also at 22$^\circ$ C, a pre-incubation reaction which contained NADP$^+$, BH$_2$ and 25 \(\mu\)M L-NMA did not demonstrate a time-dependent loss of \(\cdot\text{N}=\text{O}\) synthase activity over 2.5 hours (Figure 3). In this way conditions were found which supported enzymatic turnover in the presence of the required reduced cofactors, and which maintained enzyme stability in their absence.

The inactivation of the enzyme most likely arises from one of three mechanisms (Scheme 1). One possibility is that L-NMA proceeds down the normal reaction pathway generating an N-hydroxylated intermediate that is tightly bound. Thus, L-NMA could be converted to $\text{NG}^\text{-hydroxy-NG}^\text{-methyl-L-arginine}$, a mimic of $\text{NG}$, $\text{NG}^\text{-dihydroxy-L-arginine}$, a proposed reaction intermediate (21). An alternative, covalent mechanism is the formation at the active site of a chemically reactive species such as a carbon-centered free radical or an imine-type Michael acceptor. Thirdly, peroxide formation uncoupled from substrate hydroxylation at the active site is a well-characterized means by which oxygenases like phenylalanine hydroxylase auto-inactivate in the presence of some substrate analogs (22). We have synthesized [3H]-L-NMA and are investigating the actual molecular nature of the inactivation by L-NMA herein reported. In addition, we are extending these findings to a number of arginine analogs.
Scheme 1. Two Mechanisms for Inactivation of \( \cdot \mathrm{N}=\mathrm{O} \) Synthase by L-NMA.

N-hydroxylation produces \( \mathrm{N}^\mathrm{\cdot}=\mathrm{O} \)-hydroxy-\( \mathrm{N}^\mathrm{\cdot}=\mathrm{O} \)-methyl-L-arginine, a potential tight-binding analog of a postulated reaction intermediate, \( \mathrm{N}^\mathrm{\cdot}=\mathrm{O}, \mathrm{N}^\mathrm{\cdot}=\mathrm{O} \)-dihydroxy-L-arginine. Alternatively, C-hydroxylation produces a carbanolamine which dehydrates to yield a Michael acceptor imine-like moiety. This, in turn, could hydrolyze to yield formaldehyde and L-arginine.

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References