Communication

L-Citrulline Production from L-Arginine by Macrophage Nitric Oxide Synthase

THE UREIDO OXYGEN DERIVES FROM DIOXYGEN*

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Previously proposed mechanisms for the production of L-citrulline from L-arginine by macrophage nitric oxide (NO') synthase involve either hydrolysis of arginine or hydration of an intermediate and thus predict incorporation of water oxygen into L-citrulline. Macrophage NO' synthase was incubated with L-arginine, NADPH, tetrahydrobiopterin, FAD, and dithiothreitol in H2O/18O. L-Citrulline produced in this reaction was analyzed with gas chromatography/mass spectrometry. Its mass spectrum matched that of L-citrulline generated in H2O/18O. The base fragment ion of m/z 99 was shown to contain the ureido carbonyl group by using [L-guanidino-13C]arginine as substrate. When the enzyme reaction was performed in H2O/18O2, the base fragment ion shifted to m/z 101 with L-[guanidino-13C]arginine as the substrate and to m/z 102 with L-[guanidino-18C]arginine. These results indicate that the ureido oxygen of the L-citrulline product of macrophage NO' synthase derives from dioxygen and not from water.

Synthesis of nitric oxide (NO') is a recently discovered property of macrophages (2-4), endothelial cells (5, 6), neutrophils (7, 8), some tumor cells (9), hepatocytes (10), and unidentified cells in adrenal gland (11) and cerebellum (12). NO is responsible for some of the cytotoxic effects of macrophages on tumor cells and microbes (13-15) and is a principal endothelium-derived relaxing factor (5, 6). The functions of NO' in the other cell types are not yet clear.

In macrophages and endothelial cells, 15N studies have shown that an N2-guanidino nitrogen of L-arginine is utilized to produce NO', leaving L-citrulline (16-18). NADPH is absolutely required (19). Recently we (20) and others (21) demonstrated that macrophage NO' synthase requires tetrahydrobiopterin as a cofactor. A reductase to reduce biopterins (e.g. dihydropteridine reductase) is necessary for continuous recycling of the cofactor (20). FAD and a reduced thiol such as GSH are necessary for maximal NO' generation by the partially purified macrophage enzyme (19). The requirement for FAD and NADPH suggests involvement of a flavoprotein (19, 22).

Two mechanisms of L-citrulline and NO' formation from L-arginine have been proposed. One involves an enzyme-catalyzed hydrolytic deamination of L-arginine to form L-citrulline and ammonia, with subsequent oxidation of the ammonia to form NO' (3). A second mechanism involves formation of N'-hydroxyl-L-arginine via an NADPH-dependent monooxygenase reaction followed by electron removal and homolytic bond cleavage to yield NO' and an amino acid carbodiimide, which upon hydration forms citrulline (18, 21). Both mechanisms predict incorporation of water oxygen to form citrulline. To test this, we studied L-citrulline production by macrophage NO' synthase in H2O or under an 18O2 atmosphere. The results indicate that the oxygen atom in the ureido group of L-citrulline originates from dioxygen, not water.

EXPERIMENTAL PROCEDURES

Materials—H2O (isotope enrichment, >99%) and 18O2 (>99%) were obtained from Isotec Inc. (Miamisburg, OH). L-[guanidino-13C]Arginine (99%) was from ICON (Mt. Marion, NY). L-[guanidino-15N]Arginine (55 mCi/mmol) was from ICN Radiochemicals (Irvine, CA). Dimethylformamide dimethylacetal (Methyl-g) and N,O-bis(tri-methylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane) was from Pierce Chemical Co. (6R,8S)-5,6,7,8-Tetrahydro-1-biopterin was from Dr. B. Schircks (Jona, Switzerland). L-Arginine, L-citrulline, dithiothreitol, NADPH, and FAD were from Sigma. The murine macrophage-like cell line RAW 264.7 was from the American Type Culture Collection (Rockville, MD).

Preparation of NO' Synthase from Macrophages and Enzyme Reaction Conditions—NO' synthase was partially purified from cytosol of interferon-γ/lipopolysaccharide-activated RAW 264.7 cells by 2',5'-ADP affinity column chromatography (19). Reaction of the enzyme was performed in the presence of 4 μM tetrahydrobiopterin, 4 μM FAD, 3 mM dithiothreitol (substituting for dihydropteridine reductase (19, 23, 34)), and 2 mM NADPH in 40 mM Tris-HCl, pH 8.0, for 5 h at 37 °C. L-Arginine or L-[guanidino-13C]Arginine (2 mM) was used for the substrate, as indicated. The activity of the enzyme was measured by determining nitric oxide as previously described (54, 55) using high performance liquid chromatography (19).

The enzyme reaction in 18O2 was performed as follows. 18O2 was removed from the reaction mixture (1 ml) excluding enzyme by bubbling at 4 °C with humidified N2 for at least 2 h in a closed test tube (16 × 100 mm) equipped with a narrow needle (23 gauge) vent. Head space N2 of the reaction chamber was replaced with 25% 18O2 in N2, the solution shaken, and then enzyme (50 μg, <10%, v/v) was added using a gas-tight syringe before placing the reaction tube in a 37 °C water bath. The reaction chamber was kept under positive pressure throughout the incubation and purged with fresh 25% 18O2 in N2 every 30 min. For the enzyme reaction in H2O, 1 ml of 40 mM Tris-HCl, pH 8.0, was dried under vacuum and then reconstituted.

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with H$_2^{18}$O and various reaction components before the addition of enzyme. The reaction was performed as above but in an O$_2$ atmosphere.

Gas Chromatography/Mass Spectrometry (GC/MS)—After the incubation, protein from the incubate was removed by filtration through a Centricon-10 microconcentrator (Amicon) at 4°C. The filtrate (~1 ml) was alkalized with 1 ml of 3 M ammonium hydroxide and then loaded onto a column containing the acetate form of Dowex 1-X8 (200-400 mesh, 0.5 ml, Bio-Rad). The NO$^\bullet$ synthase product, citrulline, was eluted with 3 ml of 2 N HCl. The eluate was dried under vacuum, dissolved in 1 ml of water, adjusted to pH 7.5 with 3 M ammonium hydroxide, and then dried under vacuum again. The sample was dissolved in 0.5 ml of water, transferred to a 1-ml reaction vial, dried under N$_2$, and derivatized with 50 μl of Methyl-8-acetamide (3:2:1) as described (26). Less than 2 μl of the derivatized sample was used for a single GC/MS analysis. Monitoring for total ion abundance (m/z 40-400) and the selected ion at m/z 99 and 101 was performed using a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph and a 5970 mass selective detector. Electron impact ionization was achieved at 70 eV and an ion source temperature of 220°C. For gas chromatography, an HP-1 dimethylpolysiloxane column (length 12 m, inner diameter 2 × 10$^{-3}$ m, film thickness 3 × 10$^{-7}$ m) was used. The column temperature was initially 140°C and then increased to 252°C at a rate of 8°C/min after 2 min. The carrier gas was helium at a flow rate of 1.06 ml/min at 160°C. Injector and transfer line temperature was 225°C.

RESULTS

Authentic L-citrulline was derivatized with Methyl-8 and subjected to GC/MS analysis. Three major peaks were eluted from the GC (Fig. 1A). Two peaks eluting at ~4.5 and ~14.4 min showed a similar mass spectrum. The inset of Fig. 1B shows the mass spectrum of the 14.4-min peak, which was similar to a previously published spectrum of L-citrulline dimethylaminomethylene methyl ester (DMAM-citrulline) (26, 27). The spectrum showed the molecular ion (M$^+$) of m/z 299 and a base ion of m/z 99. The base fragment ion was reported to contain the ureido carbonyl group of L-citrulline (27). The 4.5-min peak showed the same base ion of m/z 99 and a similar ion fragmentation pattern (Fig. 1B). However, fragment ions heavier than the ion group at m/z 227 ((M - 72)') were not detected. This substance eluting at ~4.5 min may result from the loss of (CH$_2$)$_2$NCHNH from DMAM-citrulline during derivatization (26).

L-Citrulline produced by NO$^\bullet$ synthase was partially purified with anion exchange chromatography, derivatized with Methyl-8, and analyzed by GC/MS. The 14.4-min peak of DMAM-citrulline was not separated from contaminants. However, the derivatized reaction component eluting at ~4.5 min was well separated, and its mass spectrum displayed a base ion of m/z 99 and an ion fragmentation pattern similar to that of authentic citrulline (compare Fig. 1B and Fig. 2D). When the enzyme reaction was performed in the presence of diphenylenedioiodomethane, a recently discovered NO$^\bullet$ synthase inhibitor (22), disappearance of the 4.5-min peak correlated with inhibition of NO$^\bullet$ synthesis (data not shown). In a similar experiment using N,O-bis(trimethylsilyl)trifluoroacetamide as a derivatizing reagent, an enzyme reaction product showed a retention time and mass spectrum identical to those of authentic L-citrulline. Thus, our GC/MS studies confirm that citrulline is a product of NO$^\bullet$ synthase, as shown previously by other methods (3, 16).

Next, the enzyme reaction was performed in 94% H$_2^{18}$O and various reaction components before the addition of enzyme. The reaction was performed as above but in an O$_2$ atmosphere. The NO$^\bullet$ synthase product, citrulline, was eluted with 3 ml of 2 N HCl. The eluate was dried under vacuum, dissolved in 1 ml of water, adjusted to pH 7.5 with 3 M ammonium hydroxide, and then dried under vacuum again. The sample was dissolved in 0.5 ml of water, transferred to a 1-ml reaction vial, dried under N$_2$, and derivatized with 50 μl of Methyl-8-acetamide (3:2:1) as described (26). Less than 2 μl of the derivatized sample was used for a single GC/MS analysis. Monitoring for total ion abundance (m/z 40-400) and the selected ion at m/z 99 and 101 was performed using a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph and a 5970 mass selective detector. Electron impact ionization was achieved at 70 eV and an ion source temperature of 220°C. For gas chromatography, an HP-1 dimethylpolysiloxane column (length 12 m, inner diameter 2 × 10$^{-3}$ m, film thickness 3 × 10$^{-7}$ m) was used. The column temperature was initially 140°C and then increased to 252°C at a rate of 8°C/min after 2 min. The carrier gas was helium at a flow rate of 1.06 ml/min at 160°C. Injector and transfer line temperature was 225°C.

FIG. 1. GC/MS of authentic L-citrulline derivatized with Methyl-8. Authentic L-citrulline (500 nmol) was derivatized with Methyl-8 and analyzed as described under "Experimental Procedures." Panel A shows an elution profile of the GC. Three major peaks were eluted. The peak intensity was measured by total ion monitoring in the range of m/z 40-400. The mass spectrum of the peak eluting from the GC at ~4.5 min is shown in panel B; the mass spectrum of the 14.4-min peak is displayed in the inset along with the structure of DMAM-citrulline.

FIG. 2. GC/MS of derivatized reaction product of NO$^\bullet$ synthase. NO$^\bullet$ synthase reactions were performed using L-arginine as the substrate in H$_2^{18}$O/H$_2$O (A, D), H$_2$O/H$_2^{18}$O (B, E), or H$_2$O/H$_2^{16}$O (C, F). Nitrite production by each incubate (1 ml) was 33, 29, and 22 nmol, respectively. Amino acid reaction products were partially purified by anion exchange chromatography, derivatized with Methyl-8, and analyzed by GC/MS. Panels A-C show single ion monitoring at m/z 99 (solid lines) and 101 (broken lines) during elution times of 4-5 min. Panels D-F show mass spectra of the 4.5-min peaks.

FIG. 3. Mass spectra of derivatized citrulline products from NO$^\bullet$ synthase-catalyzed reactions using L-[guanidino-$^{13}$C]arginine as substrate. The NO$^\bullet$ synthase reaction was performed using L-[guanidino-$^{13}$C]arginine in an O$_2$ atmosphere (nitrite production by the incubate (1 ml) was 152 nmol). The mass spectrum was obtained from the peak eluting at ~4.5 min. The inset shows DMAM-citrulline double-labeled by performing the enzyme reaction in H$_2$O/H$_2^{18}$O (54 nmol of nitrite was produced in this enzyme reaction).

under aerobic conditions (i.e. in an O$_2$ atmosphere), Citrulline in the reaction product was partially purified, derivatized with Methyl-8, and analyzed with GC/MS as before. In the
single ion monitoring at m/z 99 and 101, DMAM-citrulline showed a peak of m/z 99 at -4.5 min, while no peak of m/z 101 eluted between 4 and 5 min (Fig. 2B). This peak pattern in single ion monitoring was the same as in the sample from the NO\(^-\) synthase reaction in \(\text{H}_2\text{O}/\text{O}_2\) (Fig. 2A). Also, the ion fragmentation patterns were identical for DMAM-citrulline produced under these two different reaction conditions (Fig. 2, compare D and E). These results suggested that water oxygen was not incorporated into citrulline during the enzyme reaction.

When the NO\(^-\) synthase reaction was performed anaerobically, production of citrulline was reduced by >98% (n = 2 experiments), indicating the requirement of oxygen for enzymatic L-citrulline synthesis. Therefore, the enzyme reaction was next performed in the presence of \(^{18}\text{O}_2\). Single ion monitoring at m/z 101 detected a peak eluting from the gas chromatograph at ~4.5 min (Fig. 2C). The 4.5-min peak of m/z 101 was not detected in parallel experiments run in an \(^{16}\text{O}_2\) atmosphere (Fig. 2, A and D). The mass spectrum of the 4.5-min peak from DMAM-citrulline produced in \(^{18}\text{O}_2\) showed shifts of m/z 168 → 170, m/z 227 → 229, and the base peak of m/z 99 → 101 (Fig. 2F) when compared with DMAM-citrulline produced in \(^{16}\text{O}_2\) (Fig. 2, D and E). These results suggested NO\(^-\) synthase catalyzed the incorporation of oxygen to form citrulline during NO\(^-\) synthase.

Since the mass spectrum of the citrulline derivative eluting at ~4.5 min did not show M\(^+\) of m/z 299, it was necessary to confirm the presence of the ureido oxygen in the base fragment of this derivative. This was tested by carrying out the enzyme reaction using L-[guanidino-\(^{13}\text{C}\)]arginine as the substrate. In this case, the mass spectrum of the 4.5 min peak (Fig. 3) revealed a shift in base ion m/z 99 → 100 and a shift in (M – 72) m/z 227 → 228, demonstrating that the base fragment ion of the citrulline derivative eluting at ~4.5 min contained the ureido carbon. When the enzyme reaction with L-[guanidino-\(^{13}\text{C}\)]arginine was performed in \(^{18}\text{O}_2\), the base ion of m/z 101 shifted to 102 (Fig. 3, inset). These results confirmed that dioxygen is incorporated into the citrulline product of NO\(^-\) synthase.

Finally, to exclude the possibility of the incorporation of dioxygen into citrulline from an exchange or a side reaction during incubation in an \(^{18}\text{O}_2\) environment, NO\(^-\) synthase was incubated with \(^{18}\text{O}_2\) and the various cofactors, but L-citrulline (1 mM) replaced L-arginine. L-Citrulline recovered from this incubate displayed the same mass spectrum as that of authentic citrulline not exposed to the enzyme preparation (data not shown).

**DISCUSSION**

These observations make two points. First, they establish that dioxygen is a cosubstrate of macrophage NO\(^-\) synthase. This brings to six the number of cosubstrates and cofactors known to be required for full activity of the partially purified enzyme, namely L-arginine, \(^{18}\text{O}_2\), NADPH, tetrahydrobiopterin, FAD, and thiol. Second, these findings demonstrate that macrophage NO\(^-\) synthase incorporates oxygen to form L-citrulline during NO\(^-\) synthase from L-arginine, excluding reaction mechanisms that hinge on water to provide the ureido oxygen.

NO\(^-\) synthase from a guanidino nitrogen of L-arginine represents a 5-electron oxidation that presumably occurs as a multistep process. In macrophages, NO\(^-\) generation requires tetrahydrobiopterin (20, 21), a known cofactor for phenylalanine, tyrosine, and tryptophan mixed function hydroxylases (20-30). An NADPH-dependent flavoprotein is also involved in macrophage NO\(^-\) generation (19, 22). Thus, oxygen activation by NO\(^-\) synthase could be accomplished through tetrahydrobiopterin- and/or flavoprotein-dependent steps (21-33). Our results imply that NO\(^-\) synthase catalyzes a reaction between activated oxygen and the guanidino carbon of L-arginine or of an arginine-derived intermediate. Identifying the source of the NO\(^-\) oxygen will help determine if \(^{18}\text{O}_2\) is also utilized to hydroxylate an N\(^-\)-guanidino nitrogen, as proposed previously (18, 21).

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'We tried to trap NO\(^-\)' or its oxidation products with morpholine as described (16). The generation of nitrosomorpholine (M\(^+\) = m/z 110) from authentic NO\(^-\) was detectable by GC/MS. In the presence of \(\text{H}_2\text{O}/\text{O}_2\), \(^{18}\text{O}\) enrichment was only 19%, suggesting that side reactions with water would not preclude using this method to identify the source of oxygen in NO\(^-\). However, the trapping was not successful in the reaction with partially purified NO\(^-\) synthase preparations, even in the presence of superoxide dismutase and catalase to prolong the half-life of NO\(^-\) and even though morpholine was not inhibitory to the enzyme reaction (data not shown).
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