Purification of Nitric Oxide Synthase from Rat Macrophages*

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Nitric oxide (NO) synthase (EC 1.14.23) has been purified to apparent homogeneity from rat macrophages. The purification procedure involves affinity chromatography with adenosine 2',5'-diphosphate-agarose and gel filtration chromatography on a Superose 12 HR 10/30 column. The apparent molecular weight is 300,000 by gel filtration. On polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the enzyme migrates as a single protein band with $M_r = 150,000$. The purified enzyme is colorless, and an absorption maximum is observed at 280 nm. The half-life of the enzyme activity is 6 h at pH 7.4 and 4 °C. The enzyme activity required the presence of NADPH, (6R)-5,6,7,8-tetrahydro-L-biopterin, and dithiothreitol. Although the cerebellar and endothelial enzyme require Ca"+ and calmodulin, these are not required by the macrophage enzyme. The macrophage nitric oxide synthase (an inducible enzyme) seems to be different from the cerebellar and endothelial enzyme (a constitutive enzyme).

Nitric oxide is synthesized in macrophages (1), neutrophils (2), endothelial cells (3), cerebellum (4), and hepatocytes (5). Using partially purified macrophage nitric oxide synthase, the reaction mechanism and characteristics of this enzyme have been studied: Nitric oxide is synthesized from a guanidine nitrogen of L-arginine (6). NADPH is absolutely necessary for the reaction (7-10). (6R)-BH$_2$ is also required as a cofactor (8, 9). FAD and thiol are necessary for maximal nitric oxide generation (7). The oxygen atom in the ureido group of L-citrulline originates from dioxygen, not water (10).

Recently, nitric oxide synthase was purified to near homogeneity from cerebellum, and the enzyme activity was found to be dependent on Ca"+ and calmodulin (4, 11). Until now, however, the macrophage enzyme has not been purified to homogeneity. In this study, macrophage nitric oxide synthase was purified to the homogeneity, and some of its characteristics were investigated.

EXPERIMENTAL PROCEDURES

Materials

Peritoneal macrophages were obtained from male B6C3F1 mice (200-220 g) after intraperitoneal injection of Bacillus Calmette-Guerin vaccine (12). Macrophages were placed on 10-cm culture dishes containing Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 10% fetal calf serum (Gibco Laboratories), and 10 ng/ml lipopolysaccharide. Cells were incubated for 24 h in humidified 5% CO$_2$ and 95% air at 37 °C. After incubation, nonadherent peritoneal exudate cells were removed by washing three times with phosphate-buffered saline. The adherent cells were scraped with a rubber policeman and collected.

Macrophages were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM dithiothreitol (DTT), at a cell density of 1 × 10$^6$ cells/ml. The following protease inhibitors were added: phenylmethylsulfonyl fluoride (0.1 mg/ml), trypsin inhibitor (0.01 mg/ml), leupeptin (0.01 mg/ml), antipain (0.01 mg/ml), and pepstatin (0.01 mg/ml).

Cells were then disrupted by sonication for 10 s (Dial 6, UR-200P, Tomy, Tokyo, Japan), and the sonicate was centrifuged at 100,000 $X$ g for 60 min at 4 °C. The supernatant was removed and used as the crude fraction.

Calmodulin antagonists, W5 (N-6-aminohexyl)-1-naphthalenesulfonamide hydrochloride, W7 (N-6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, and W13 (N-4-aminobuty)-5-chloro-2-naphthalenesulfonamide hydrochloride, were obtained from Seikagaku Kogyo (Tokyo, Japan). Trilufoerazine dihydrochloride was obtained from Research Biochemicals Inc. (Natick, MA). Bovine brain calmodulin was obtained from Calbiochem and (6R)-5,6,7,8-tetrahydro-L-biopterin (6R-BH$_2$) was obtained from Research Biochemicals Inc.

All other chemicals were from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified.

Methods

Enzyme Assay—The activity of nitric oxide synthase was determined by measuring the generation of nitrite and nitrate, breakdown products of nitric oxide. The complete reaction mixture (600 µl) contained purified enzyme, 50 mM Tris-HCl (pH 7.4), 1 mM NADPH, 1 mM L-arginine, 1 mM DTT, 0.1 mM (6R)-BH$_2$, and 1 µM FAD. When necessary, 5 mM EGTA was added in the absence of Ca"+. The mixture was incubated for 20 min at 37 °C, and the reaction was stopped by boiling at 100 °C for 30 s. The tube was centrifuged at 15,000 $X$ g for 20 min, and the resulting supernatant was passed through a Millipore TGC filter (Millipore Corp., Bedford, MA). The filtrate was analyzed for nitrite and nitrate. The total recovery of nitrite and nitrate was 90%. Nitrite and nitrate were determined independently. Enzyme activity was expressed as nitrite + nitrate formation/min/mg of protein.

Nitrite (NO$_2$-) Determination—Nitrite in the enzyme reaction mixture was determined by the post-column method using the diazo-coupling reaction (13). NADPH required as a substrate in the enzyme reaction was found to interfere with this reaction. Therefore, we separated nitrite from NADPH using ion-pair chromatography.

Samples (30 µl) were injected through an autosampler (SIL-6B) onto a C-18 reverse phase column (YMC A-301, 100 × 4.6 mm, Kyoto, Japan) using 5 mM tetra-n-butyl ammonium phosphate as the carrier solvent at a constant flow rate of 1.0 ml/min. To column effluents, the following reagents for diazo-coupling reaction were added sequentially by the reaction pump (FII-300N, Nihon Bunko Company, Tokyo, Japan): 1% (w/v) sulfanilic acid in 2 N HCl (0.3 ml/min), and 1% (w/v) N-(1-naphthy1)ethylenediamine dihydrochloride (0.3 ml/min). The wavelength of the detector (SPD-6AV) was 548 nm. All equipment, unless otherwise specified, was from Shimadzu Corp. (Kyoto, Japan). The detection limit and coefficients of variation (n = 10) were 0.1 pmol and 0.5%, respectively.

Nitrate (NO$_3$-) Determination—Nitrate was determined by ion chro-

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‡ The abbreviations used are: (6R)-BH$_2$, (6R)-5,6,7,8-tetrahydro-L-biopterin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.
TABLE I

| Step                  | Total protein | Total activity | Specific activity | Yield % |
|-----------------------|---------------|----------------|-------------------|---------|
|                       | µg            | pmol/min       | nmol/min/mg protein |         |
| Cytosol               | 36,600        | 76,860         | 2.1               | 100     |
| 2',5'-ADP-agarose     | 231           | 40,656         | 176               | 53      |
| Superose 12 HR 10/30  | 21            | 19,824         | 944               | 26      |

Fig. 1. Gel filtration chromatography on a Superose 12 HR 10/30 column. The shaded area shows the active fractions. Molecular weight (native form) was estimated by gel filtration chromatography with molecular standards (inset).

Fig. 2. SDS-polyacrylamide gel electrophoresis of nitric oxide synthase. The Phast system with M, was seen. Silver staining also displayed no additional bands.

Purification of Nitric Oxide Synthase—The purification started from 15 ml of cytosol. The cytosol (7.5 ml) was applied to a column of 2',5'-ADP-agarose (1 x 2 cm, Sigma) equilibrated with 50 mM Tris-HCl buffer at pH 7.4 containing 1 mM DTT. The column was washed with 10 ml of 50 mM Tris-HCl containing 1 mM DTT and 750 mM NaCl and with 10 ml of 50 mM Tris-HCl containing 1 mM DTT, successively. The enzyme was then eluted with 2 ml of 50 mM Tris-HCl containing 1 mM DTT and 1 mM NADPH and was concentrated to 50 µl using a Centricon 10 (Amicon, Danvers, MA). This sample was injected onto Superose 12 HR 10/30, a gel filtration column (300 x 10 mm, Pharmacia LKB Biotechnology Inc., Uppsala, Sweden), which was equilibrated with 50 mM Tris-HCl containing 1 mM DTT. Superose resin is a cross-linked agarose-based medium with average particle size of 10 x 2 x 2 mm. This step was carried out by FPLC system (Pharmacia LKB Biotechnology Inc.) at room temperature. Eluents were fractionated by 0.5 ml.

Sodium Dodecyl Sulfate (SDS)-polyacrylamide Gel Electrophoresis—Electrophoresis was performed using the Phast-System (Pharmacia LKB Biotechnology Inc.) on a 10-15% gel according to the manufacturer's directions. Proteins were stained with Coomassie Blue. Molecular weight standards (Pharmacia LKB Biotechnology Inc.) were: thyroglobulin, 330,000; ferritin, 220,000; albumin, 67,000; catalase, 60,000; and lactate dehydrogenase, 36,000.

Estimation of Molecular Weight by Gel Filtration—Gel filtration chromatography was carried out on a Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 50 mM Tris-HCl (pH 7.4) and 1 mM DTT, at a flow rate of 0.5 ml/min. The eluent was monitored at 280 nm. The standard proteins (Pharmacia LKB Biotechnology Inc.) and their molecular weights were: ferritin, 440,000; catalase, 222,000; and aldolase, 158,000.

Other Determinations—The absorption spectrum of nitric oxide synthase at pH 7.4 was determined using a Beckman DU-65 spectrophotometer with a microcell. The protein concentration was determined by a dye-binding microassay (Bio-Rad). Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Table I shows the purification steps of macrophage nitric oxide synthase.

The molecular weight of the enzyme is 300,000 on Superose 12 HR 10/30 gel filtration chromatography (shaded area in Fig. 1). On SDS-polyacrylamide gel electrophoresis, the active fractions exhibit a single band with M, = 150,000 (Fig. 2). Thus, this protein appears to be a dimeric protein.

The purified enzyme exhibited a half-life of 6 h at pH 7.4 and 4 °C, whereas the cytosol preparation was stable after 24 h (Fig. 3). This might be due to the loss of a stabilizing factor, as reported for a neutrophil nitric oxide synthase (14) or might simply be due to the low protein concentration. The addition of NADPH, (6R)-BH, and FAD did not increase stability. A poor yield during purification is considered to reflect instability of the enzyme due to loss of stabilizing factor. The loss of cofactors during purification may also induce instability of the enzyme. The half-life of the nitric oxide synthase purified from neutrophils was 3 h at pH 7.4 and 4 °C, and the specific activity was 122 nmol/min/mg protein. The specific activity of the macrophage nitric oxide synthase was 944 (Table I). This increased activity may derive from the longer half-life of the macrophage enzyme.

The enzyme was colorless, and an absorption maximum was observed at 280 nm (Fig. 4). A spectrum characteristic of a flavoprotein (maximal absorption at 383 and 442 nm) in the

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FIG. 3. Stability of the purified nitric oxide synthase. The activity of the purified nitric oxide synthase was measured after incubation at 4°C for the times indicated on the abscissa and is expressed as the ratio to the activity determined at time 0. Data are the mean of three experiments performed using different samples. Squares represent cytosol, per se, used as an enzyme source. Open circles, 50 mM Tris-HCl (pH 7.4) and 1 mM DTT; closed circles, 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM NADPH, 0.1 mM (6R)-BH₄, and 1 μM FAD.

FIG. 4. Absorption spectrum of nitric oxide synthase. A sample solution in 50 mM Tris-HCl (pH 7.4) containing 1 mM DTT was measured in a microcell. The visible region was not observed.

EC₅₀ values for cofactors of the purified enzyme are shown in Table II. Table III shows the percent decrease in activity by omitting each of the cofactors. NADPH, (6R)-BH₄, and DTT were necessary for enzyme activity; however, the contribution of FAD was not great.

Calmodulin antagonists (100 μM of W5, W7, W13, and trifluoperazine dihydrochloride) did not inhibit purified enzyme activity. Addition of calmodulin (1 μM) to the purified enzyme did not increase activity. The addition of EGTA to the reaction mixture without Ca²⁺ did not affect enzyme activity. Therefore, the macrophage enzyme was considered not to require Ca²⁺ and calmodulin for activation.

The apparent Kₘ values for arginine and the Vₘₐₓ at 37°C and pH 7.4 are 32.3 mM and 1052 nmol/min/mg protein, respectively (Fig. 5). This enzyme functions optimally at pH 7–8.

TABLE II

| Cofactor     | EC₅₀ (μM) |
|--------------|-----------|
| NADPH        | 100       |
| (6R)-BH₄     | 30        |
| DTT          | 80        |
| FAD          | 90        |

TABLE III

Effect of omission of each of the cofactors from the enzyme reaction mixture on percent inhibition of enzyme activity

| Cofactor   | Percent Inhibition |
|------------|--------------------|
| −NADPH     | 100%               |
| −(6R)-BH₄  | 82%                |
| −DTT       | 96%                |
| −FAD       | 10%                |

In the stoichiometry studies, 10.5 μM arginine consumption led to the formation of 9.7 μM citrulline and 10.5 μM (nitrite + nitrate) formation. Thus, the stoichiometry of arginine to the formation of citrulline and nitrite + nitrate formation seems to be 1/1/1.

In rat, nitric oxide synthase consists of 0.017% of the protein in the 15,000 x g supernatant of cerebellum (4). The content of macrophage nitric oxide synthase is 0.22% of the 100,000 x g supernatant. This may represent the difference in the nature of these enzymes. Nitric oxide synthase of the cerebellum and endothelial cell are constitutive enzymes, but...
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