Identification of Inducible Calmodulin-dependent Nitric Oxide Synthase in the Liver of Rats*

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Sachio Iida†, Hiroshi Ohshima‡§§, Shinobu Oguchi†, Toshio Hata||, Hisanori Suzuki†∗∗, Hiroshi Kawasaki†++, and Hiroyasu Esumi†§§

From the †Biochemistry Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chu-o-ku, Tokyo, Japan; the ‡Department of Obstetrics and Gynecology, Saitama Medical School, Faculty of Medicine, 38 Morohongo, Iruma-gun, Saitama, Japan; and the §§Department of Molecular Biology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo, Japan

A calmodulin-dependent nitric oxide synthase was significantly induced in the liver of rats treated intravenously with heat-killed Propionibacterium acnes and 5 days later with Escherichia coli lipopolysaccharide. The apparent calmodulin-dependent and -independent isozymes were separated by Mono Q column chromatography after their partial purification by 2',5'-ADP-agarose affinity chromatography. Both enzymes had a molecular weight of 125,000 as determined by SDS-polyacrylamide gel electrophoresis and required NADPH, tetrahydrobiopterin, and dithiothreitol as cofactors. Their activities were completely inhibited by the specific nitric oxide synthase inhibitors N'-monomethyl-L-arginine and N'-nitro-L-arginine at 80 and 800 μM, respectively. The peptide maps of these two isozymes with lysylendopeptidase and their reverse-phase column chromatographic profiles were indistinguishable. In the presence of bovine calmodulin, the purified calmodulin-dependent isozyme behaved as a calmodulin-independent isozyme on Mono Q column chromatography. The purified calmodulin-independent isozyme was converted to a calmodulin-dependent isozyme by EDTA and EGTA. Calmodulin blot analysis using [125I]-calmodulin showed that the two isoforms bound calmodulin equally efficiently.

Nitric oxide (NO)1 has recently been shown to be formed from L-arginine by NO synthase (EC 1.14.23), which is present in various tissues and organs (1–4). This NO appears to be responsible for the cytotoxic effects of macrophages and neutrophils (5), for vasodilatation mediated by endothelial cells (6) and for cell-to-cell communication in the nervous system (3). NO activates soluble guanylyl cyclase resulting in increase in cellular cGMP. It has been reported that there are at least two types of NO synthase: a calmodulin-dependent isoform present constitutively in tissues such as the brain (7, 8), endothelial cells (9), platelets (10), and the adrenal gland (11) and a calmodulin-independent isoform that is induced in the liver of rats by treatment with Escherichia coli lipopolysaccharide (LPS) (4, 12) and in macrophages by their activation with LPS or lymphoblasts (13, 14). Here, we report that calmodulin-dependent NO synthase is induced in the liver of rats by their treatment with heat-killed Propionibacterium acnes plus LPS and also provide evidence that at least a part of the apparent calmodulin-independent NO synthase induced in the liver is a calmodulin-bound form of calmodulin-dependent NO synthase.

**EXPERIMENTAL PROCEDURES**

Materials—L-[2,3-3H]Arginine (specific activity, 55 Ci/mmol; 1 Ci = 37 GBq) and [125I]-calmodulin (specific activity, 80 μCi/μg; 1 Ci = 37 GBq) were obtained from Du Pont-New England Nuclear. N'-Monomethyl-L-arginine and (6R)-5,6,7,8-tetrahydro-L-biopterin were from Calbiochem and Dr. B. Schircks Laboratories (Jona, Switzerland), respectively. Other reagents were of analytical grade and were purchased from Sigma or Wako (Osaka, Japan).

Induction of NO Synthase in Vivo—Male Sprague-Dawley rats, weighing between 200 and 300 g, were given heat-killed P. acnes at 75 mg/kg body weight through a tail vein, followed 5 days later by an intravenous dose of LPS at 4 mg/kg body weight (12). Livers were removed 5 h after LPS injection and kept at −80 °C until use.

Partial Purification of NO Synthase—Frozen rat livers were thawed and homogenized with a Polytron homogenizer in ice-cold buffer A (50 mM Tris-HCl, pH 7.4, containing 0.5 mM EDTA and EGTA, 1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) using 3 ml of buffer/g of tissue. All subsequent procedures were carried out at 4 °C. The homogenate was centrifuged at 27,000 × g for 60 min. The resulting supernatant was incubated for 30 min with 2',5'-ADP-agarose gel (0.75 ml/50 ml supernatant) with gentle agitation and centrifuged at 3000 rpm for 10 min. The precipitated 2',5'-ADP-agarose was transferred to a fritted column. The column was washed with 50 volumes of buffer A containing 0.5 M NaCl and then with 30 volumes of buffer A only. NO synthase was then eluted with 10 mM NADPH in buffer A. The fractions containing the activity were pooled and applied to an FPLC Mono Q anion exchange column HR 5/5 (0.5 × 5 cm, Pharmacia LKB Biotechnology Inc.) equilibrated with buffer A and eluted with a gradient of 0–0.5 M NaCl in buffer A.

Assay of NO Synthase—NO synthase activity was measured by determining either the conversion of L-[2,3-3H]arginine to L-[2,3-3H]citrulline essentially based on the method of Bredt and Snyder (7, 8). Initial concentration of L-[2,3-3H]arginine in the reaction mixture was 15 μM. Enzyme activity was also measured by the amount of the formations of nitrite and nitrate, stable oxidized products of NO, with an automated analyzer (Flow Injector Analyser, TCI-NOX 1000, Tokyo Kasei) (15). The levels of nitrite and nitrate were determined.
after deproteinizing samples by adding 60 ml of 0.5 N NaOH and 40 ml of 0.42 M zinc sulfate.

Peptide Mapping—Two types of NO synthase were partially purified by 2',5'-ADP-agarose and Mono Q column chromatographies and were purified further by SDS-PAGE. After brief staining with Coomassie Brilliant Blue, the bands at 125 kDa were excised from the gel and digested completely by overnight incubation with lysylendopeptidase at an enzyme concentration of 2.0 mg/ml. The resulting solubilized material was subjected to reverse-phase chromatography on an ODS column.

Calmodulin Bindings of the Two Enzymes—The partially purified NO synthases were subjected to SDS-PAGE in 6% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked by incubation with 1% bovine serum albumin in TBS/CaMg buffer (50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, 0.5 mM CaCl₂, and 50 mM MgCl₂) for 16 h at 4 °C and then incubated with 10 μCi/ml of [35S]-calmodulin in TBS/CaMg buffer for 3 h at 4 °C. It was then washed three times with TBS/CaMg buffer for 10-min periods and exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) for 10 h. Radioactivity was detected with a Bio-image analyzer (Fuji Photo Film Co., Tokyo, Japan).

RESULTS

Induction of NO Synthase in Liver—NO synthase activity was induced significantly in the liver of rats by treatment with *P. acnes* and LPS. Its level was in the range of 0.15 and 0.35 mmol of NO₃ formed per mg of protein/h. The activity in the liver of untreated rats was negligible. The nitrate level in plasma obtained 5 h after LPS injection was also significantly elevated from 25 ± 1 mM in control rats to 274 ± 28 mM and was well correlated with the NO synthase activity in the liver. Under these conditions, the glutamate-oxaloacetate transaminase and glutamic-pyruvic transaminase levels in the plasma of treated rats also increased about 13- and 6-fold, respectively.

Purification of NO Synthase—Soluble NO synthase was purified from the liver of treated rats as described under "Experimental Procedures." Most of the NO synthase activity in cytosol from the liver of untreated rats was retained on 2',5'-ADP-agarose affinity gel and could be eluted from the gel with buffer containing NADPH. SDS-PAGE analysis showed the presence of an inducible protein with a molecular mass of 125 kDa, which was not present in the liver of untreated rats (15). Material in the eluate from the 2',5'-ADP-agarose column was purified further by FPLC Mono Q anion exchange chromatography. The enzyme in the first peak required calmodulin for activity, no activity being detected in the absence of calmodulin. In contrast, the enzyme in the second peak showed activity in the absence of calmodulin (Fig. 1). Although the activity in the first peak was absolutely calmodulin-dependent, Ca²⁺ requirement was not absolute. Even in the presence of 6 mM EGTA, NO synthase activity was detected at one-third of maximal activity which was observed at calcium acetate concentration of 1.5–12.5 mM in the reaction mixture. A similar calcium requirement was also observed for calmodulin-independent activity in the second peak. Thus the first and second peaks seemed to be due to calmodulin-dependent and -independent NO synthase, respectively. SDS-PAGE analysis of these FPLC fractions showed that the activity was closely associated with a protein of 125 kDa (data not shown). The 125-kDa protein present in these two peak fraction could also be detected by western blot analysis with the antibody raised against the inducible rat liver NO synthase (15). Neither calmodulin-dependent nor-independent NO synthase activity was detected in the liver of untreated rats (data not shown), indicating that these activities were inducible. Both peaks of NO synthase activity were completely inhibited by 80 μM N⁵-monomethyl-L-arginine or 800 μM N⁵-nitro-L-arginine in the presence of 15 mM of L-arginine as substrate.

Peptide Mapping of the Two Isozymes—To characterize the calmodulin-dependent and -independent isozymes of NO synthase, we purified them separately. For this, each isozyme was purified further by SDS-PAGE and subjected to peptide mapping analysis with lysylendopeptidase. As shown in Fig. 2, the peptide maps of the two isozymes were almost identical, indicating close similarity of the two isozymes, despite their difference in requirement for calmodulin.

Calmodulin Bindings of the Two Isozymes—The calmodulin binding of these two isozymes after SDS-PAGE and transfer to a nylon membrane were examined using 125I-bovine calmodulin. Result showed that the two isozymes had similar calmodulin binding abilities (Fig. 3), although one isozyme was calmodulin-independent. The possibility that these two isozymes are interconvertible, suggested from these data, was examined directly. After purification of the calmodulin-dependent isozyme, it was rechromatographed on a Mono Q column with or without preincubation with bovine calmodulin. As shown in Fig. 4, the calmodulin-dependent isozyme was completely converted to the calmodulin-independent form by preincubation with calmodulin in the absence of EDTA or EGTA. Conversely, the calmodulin-independent isozyme was partially, although not completely converted to the calmodulin-dependent form by chromatography on a Mono Q column in the presence of 5 mM EGTA. On rechromatography of calmodulin-independent NO synthase, more than half the activity was converted to the calmodulin-dependent form and even the activities in fractions containing
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8. 600 ng of protein of peak fraction containing calmodulin-independent NO synthase was detected using antiserum against inducible NO synthase after preincubation with calmodulin using buffer A without EDTA and EGTA. This efficient conversion of calmodulin-dependent NO synthase to the calmodulin-independent form by incubation with calmodulin strongly suggested that at least part of the calmodulin-independent NO synthase was derived from the calmodulin-bound form of calmodulin-dependent NO synthase. 3) ¹²⁵I-Bovine calmodulin bound to the calmodulin-dependent and -independent NO synthases equally efficiently. These findings support the idea that the calmodulin-independent NO synthase is derived from the calmodulin-dependent isozyme. Recently we cloned and analyzed the cDNA for an inducible NO synthase from rat liver and found that the putative calmodulin binding site of the enzyme is extremely hydrophobic. The binding of calmodulin with the calmodulin-binding sites of many calmodulin-dependent proteins is known to be due to hydrophobic interaction (17). Therefore, it is probable that rat liver-inducible calmodulin-dependent NO synthase is not readily dissociated from calmodulin by EGTA. This may be the cause of incomplete conversion of calmodulin-independent NO synthase to calmodulin-dependent form by 5 mM EGTA.

Although induced NO synthase was dependent on calmodulin, calcium was not required absolutely. This finding was unexpected. Recently, Geiser et al. (18) made a similar finding that a mutant calmodulin, which does not bind calcium, supports yeast strains growth quite normally. The present finding is well consistent with this observation. Calcium may be required only for the efficient binding of calmodulin to NO synthase by only factor 3. This interpretation is also consistent with the results in Fig. 4, in which calmodulin-independent activity was accelerated by the addition of excess calmodulin. Because calmodulin-independent NO synthase is a calmodulin-bound form of calmodulin-dependent NO synthase, gradual release of calmodulin from NO synthase may happen during Mono Q column chromatography in which 0.5 mM each EDTA and EGTA were present.

Calmodulin-independent NO synthase has been reported by others (12) to be induced in the liver of rats treated in the way used in the present study. In the present work, we showed that calmodulin-dependent NO synthase is induced in the liver in this way. It is noteworthy that this calmodulin-dependent NO synthase could be demonstrated only after Mono Q column chromatography. There is still a possibility that the calmodulin-dependent and -independent forms of the enzyme are different, but we think that after appropriate purification, most of, if not all, the NO synthase activity would be calmodulin-dependent.

Under the present experimental conditions, similar enzyme activity was also induced in the lung, spleen, and colon and the most of activities in these organs were also found to be calmodulin-dependent (19). Recently, calmodulin-independent NO synthase was purified from LPS-stimulated culture murine macrophages and rat granulocytes (13, 14), and we

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found that the inducible NO synthase in cultured murine macrophage-like cells, J774.1, was calmodulin-dependent. Further study is needed to determine which cell type produce calmodulin-dependent NO synthases upon stimulation with P. acnes and LPS in various organs. But the above findings raised the question of whether calmodulin-independent NO synthase is really calmodulin-independent. Further purification and/or molecular cloning of these isozymes is necessary to answer this question.

Addendum—After submission of this manuscript, a similar finding was published by Carl Nathan’s laboratory (20).

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