Vol. 183, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1992 Pages 238-244

PURIFICATION OF NITRIC OXIDE SYNTHASE FROM BOVINE BRAIN: IMMUNOLOGICAL CHARACTERIZATION AND TISSUE DISTRIBUTION

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Received January 21, 1992

SUMMARY: Nitric oxide (NO) synthase (EC 1.14.23) was purified to homogeneity from bovine cerebrum. The molecular weight of NO synthase was estimated to be 150 kDa by both SDS/PAGE and gel filtration at high salt concentration. For activity, the enzyme required NADPH, Ca^{2+}, calmodulin and tetrahydrobiopterin as cofactors. Rabbit polyclonal antibody to bovine brain NO synthase reacted with 150 kDa NO synthase in various bovine and rat organs, including the brain, pituitary and adrenal glands, but not with that in stimulated macrophages, indicating that there are at least two immunologically distinct NO synthases.

Nitric oxide (NO) formed from L-arginine plays an important role in regulating basal blood flow and cell-to-cell communication in various organs and cells (1,2). NO acts as an intra- and inter-cellular signaling molecule to activate soluble guanylate cyclase within the same cell and in specific target cells. The cytotoxic effects of macrophages and neutrophils also appear to be attributable to NO generated from L-arginine by NO synthase (3). NO synthase (EC 1.14.23) from rat and porcine cerebellum has recently been purified and characterized (4-6). In this work we purified and characterized the enzyme from bovine brain and raised antibody against the purified enzyme in rabbits. We then used the antibody to study the distribution of the enzyme in various rat and bovine

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0006-291X/92 $1.50

organs, and activated macrophages by immunoblotting analysis. Unlike in the rat, bovine cerebrum was found to have a higher content of NO synthase than the cerebellum.

MATERIALS AND METHODS

Materials. L-[2,3-^{3}H]Arginine (spec. act. 55 Ci/mmol; 1Ci = 37 GBq) was obtained from NEN (DuPont de Nemours, Wilmington, DE). N^G-Monomethyl-L-arginine acetate and (6R)-5,6,7,8-tetrahydro-L-biopterine (H4BP) were from Calbiochem (La Jolla, CA) and Dr.B.Laboratories (Jona, Switzerland), respectively. Other reagents were of analytical grade and were purchased from Sigma (St. Louis,MO) or Wako (Osaka, Japan).

Enzyme purification. Bovine brains were obtained from a local slaughterhouse. The enzyme was purified from 500g of bovine cerebrum at 4 °C, by the method of Bredt and Snyder (4) with a minor modification. NO synthase was eluted from an 2',5' ADP-agarose affinity column with 50 ml of buffer A (10 mM Tris-HCl pH 7.4, 1 mM EDTA and 5 mM 2-mercaptoethanol) containing 10 mM NADPH. The eluate was then dialyzed overnight against buffer A saturated with ammonium sulfate and centrifuged for 30 min at 105,000 x g. The resulting precipitate was dissolved in a small volume of 50 mM phosphate buffer pH 7.0, containing 100 mM sodium sulfate and 1 mM EDTA and subjected to HPLC gel filtration on a TSKgel G3000 SW glass column (0.8 x 30 cm, Tosoh, Tokyo, Japan) equilibrated with the same phosphate buffer. The fractions containing NO synthase activity was pooled and used for characterization of the enzyme. Protein concentrations were determined by the method of Bradford (7) with bovine serum albumin as a standard.

Assay of NO synthase. NO synthase activity was measured by monitoring the conversion of L-[2,3-^{3}H]arginine to L-[2,3-^{3}H]citrulline by the method of Bredt and Snyder (4). For study of the stoichiometry of the reaction, 500 µl of reation mixture containing 50 mM Hepes pH 7.4, 100 µM L-arginine, 100 µM NADPH, 1 mM EDTA, 1.25 mM calcium acetate, 80 uM H4BP and 10 µg of calmodulin and enzyme was incubated for 1 h at 37 °C. The levels of nitrite/nitrate were determined in an automated analyzer (Flow Injector Analyzer, TCI-NOX 1000, Tokyo Kasei)(8). A 200 µl aliquot of the reaction mixture was deproteinized by adding 60 µl of 0.5 N NaOH and 40 µl of 0.42 M zinc sulfate. o-Phthalaldehyde derivatives of L-citrulline and L-arginine were analyzed as reported previously (9) by HPLC/fluorescence detection. The mixture (200 µl) was added to 200 µl of ice-cold methanol to stop the reaction and centrifuged for 5 min at 10,000 x g. A 50 µl portion of the supernatant was incubated with 50 µl of o-phthalaldehyde reagent for 1 min at room temperature, and 25 µl was applied to a 0.46 x 25 cm TSKgel ODS-80 HPLC column (Tosoh).

Production of antiserum to NO synthase. Antiserum to NO synthase was prepared by the method of Harlow and Lane (10). NO synthase partially purified from bovine cerebrum by DEAE-cellulose and 2',5' ADP-agarose affinity column chromatographies as described above was further separated by

-SDS-polyacrylamide gel electrophoresis (SDS/PAGE) on 7.5% polyacrylamide gel (11). A section of the gel was stained with Coomassie Brilliant Blue, and the 150 kDa band corresponding to NO synthase (about 200 μg) was excised and used as antigen. The antiserum was verified by immunoblotting analysis.

Immunoprecipitation and immunoblotting. Various tissues from oxen and male Sprague-Dawley rats were homogenized in 5 vols of buffer A and centrifuged for 1 h at 105,000 x g. The murine macrophage cell line J774-1 was cultured in the presence of γ-interferon and *E. coli* lipopolysaccharide(LPS) as previously reported (8). A 0.1-0.5 ml portion of the resulting supernatant containing 2 mg of protein was incubated overnight at 4°C with 5 μl of rabbit antiserum to NO synthase. About 1 mg of Protein-A-sepharose CL-4B (Pharmacia) was added the mixture was incubated for 1 h at 4°C with gentle agitation. Western blot analysis was performed by the method of Harlow and Lane (10). The immunoreactive bands were detected with 3,3'-diaminobenzidine as a substrate for peroxidase.

RESULTS AND DISCUSSION

Bovine cerebrum showed higher activity of NO synthase than the cerebellum. This is in contrast with findings in the rat and pig (4-6,12), that the cerebellum contains higher NO synthase activity than the cerebrum. The enzyme was therefore purified from bovine cerebrum by the method of Bredt and Snyder (4), followed by ammonium sulfate precipitation and HPLC gel filtration, as summarized in Table 1. The final preparation of

Table 1. Purification of NO synthase from bovine cerebrum

Fraction	Protein (mg)	Specific activity (nmol/min/mg)	Yield (%)
10000xg supernatant	12480	0.074	100
DEAE-cellulose eluate	2760	0.19	58
2',5'-ADP agarose eluate	3.96	74	32
Ammonium sulfate precipitate	1.55	120	20
Gel filtration eluate	0.15	350	6

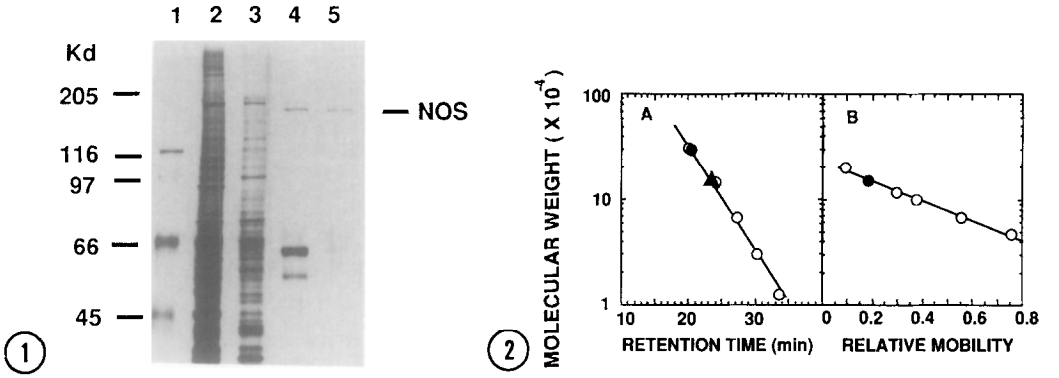

Fig.1. SDS-polyacrylamide gel electrophoresis of fractions at each purification step of nitric oxide synthase from bovine cerebrum. After electrophoresis, the 7.5 % polyacrylamide gel was stained with silver. Lanes: 1, molecular weight markers; 2, crude extract; 3, fraction form a DEAE-cellulose column; 4, fraction from a 2',5' ADP-agarose column, and 5, preparation after HPLC gel filtration. NOS:NO synthase.

Fig.2. Molecular weight of bovine brain nitric oxide synthase.(A) gel filtration on a TSKgel G3000SW column and (B) SDS/PAGE analysis. ●;nitric oxide synthase at 100mM NaCl in the buffer A, ▲; nitric oxide synthase at 300mM NaCl in the buffer A, ○;standard of known molecular weight.

purified NO synthase appeared homogeneous on SDS/PAGE analysis (Fig. 1). The molecular weight of native NO synthase was estimated to be 300 kDa at 100 mM NaCl concentration, but 150 kDa by gel filtration at 300 mM NaCl concentration and by SDS/PAGE (Fig.2). Thus the purified NO synthase seems to be a monomer of 150 kDa that easily associates into a dimer. These findings may explain the discrepancy between the recent report by Schmidt et al.(6) that NO synthase purified from the rat cerebellum is a homodimer of a 155 kDa subunit and the report by Bredt et al.(4) that rat brain NO synthase is a monomer. The recovery of enzyme activity during its purification was very low. This was partly due to the instability of the purified enzyme, the half life of the enzyme activity being 6 h at 4°C (data not shown).

The properties of purified bovine brain NO synthase, including its Vmax and Km values for L-arginine and NADPH, the Ki values for N^G-methylarginine and N^G-nitroarginine and the EC_{50} values for calmodulin and H4BP, are summarized in Table 2. The enzyme requires NADPH, Ca^{2+}, calmodulin and H4BP: omission of any one of these compounds from the reaction mixture essentially

Table 2. Properties of bovine cerebrum NO synthase

Km	(L-arginine)	2.3 μM
Km	(NADPH)	0.4 μM
Vmax		115 *
Ki	(N^G-methyl-L-arginine)	2.5 μM
Ki	(N^G-nitro-L-arginine)	1.5 μM
EC_{50}	(calmodulin)	3 nM
EC_{50}	(H4BP)	50 nM

* nmole of L-citrullin/min/mg protein

abolished the enzyme activity, indicating calmodulin-dependency of the bovine NO synthase, as reported for the rat and porcine enzymes(4,12).

The stoichiometry of the reaction was examined by incubation of reaction mixture containing 100 μM arginine, 100 μM NADPH and other cofactors at 37°C for 1h, as described in "Materials and Methods". Consumption of 4.13 ± 0.50 nmol arginine resulted in formation of 3.88 ± 1.12 and 4.03 ± 0.85 nmol of citrulline and nitrite/nitrate, respectively (n=3). These data indicate that one molecule of NO and one molecule of citrulline are formed at the expense of one molecule of arginine.

Fig. 3 A, B and C show the results of immunoblotting of NO synthase from various bovine and rat tissues and murine macrophages. An immunoreactive band was observed at 150 kDa in immunoprecipitates of extracts of bovine cerebrum, brain stem and adrenal gland, but not of other tissues such as the liver. In the rat, in addition to brain tissues (cerebellum , cerebrum and brain stem), the pituitary and adrenal glands also contained the 150 kDa immunoreactive protein detected with antiserum against bovine brain NO synthase. The occurrence of NO synthase activity in these organs has been reported (13,14). The murine macrophage cell line J774-1 activated with LPS or interferon-γ , or both compounds did not give any immunoreactive

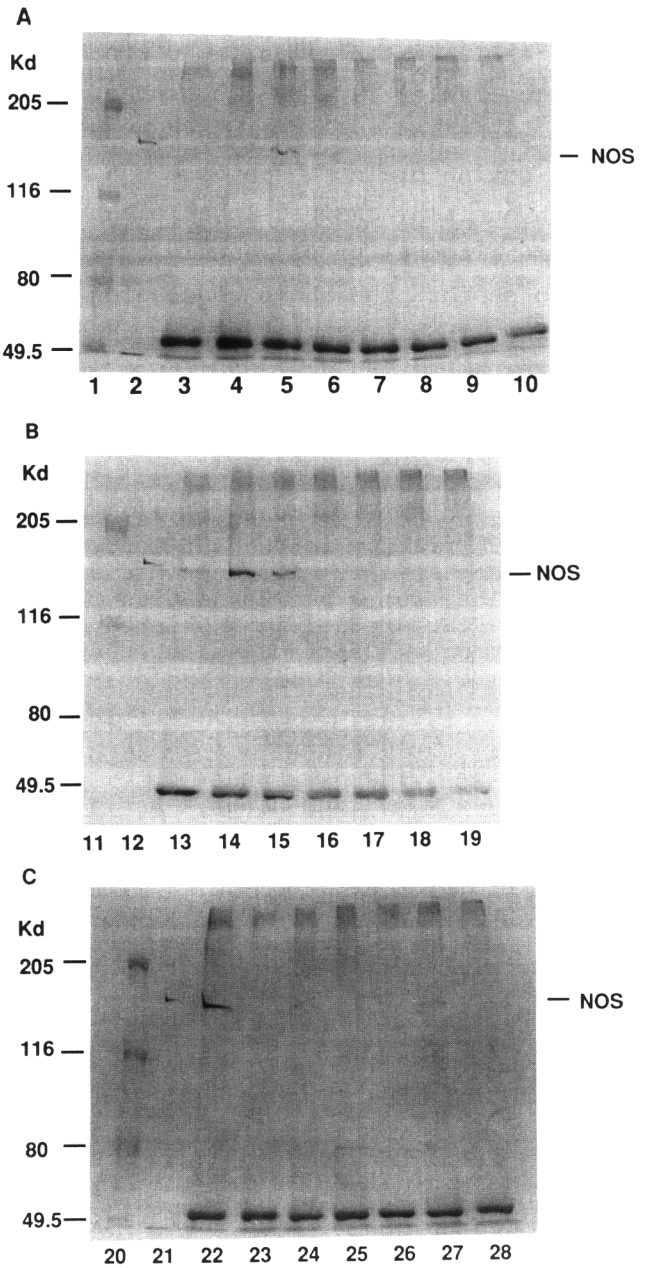

Fig.3. Immunoprecipitation followed by immunoblotting of nitric oxide synthase from (A) various rat organs, (B) rat brain and murine macrophages and (C) bovine tissues. Lanes 1, 11 and 20, molecular weight markers. Lanes 2, 12 and 21, purified bovine brain nitric oxide synthase. Lanes 3-10, immunoprecipitated extracts of rat organs(3; heart, 4; spleen, 5; adrenal glands, 6; pituitary gland, 7; thyroid, 8; kidney, 9; lung, and 10; liver). Lanes 13-19: immunoprecipitated extracts of rat brain tissues and murine macrophages(13; cerebrum, 14;cerebellum, 15; brain stem, 16; untreated macrophages, 17; macrophages cultured with LPS, 18; macrophages cultured with interferon-γ, and 19, macrophages cultured with both LPS and interferon-γ).Lanes 22-28: immunoprecipitated extracts of bovine tissues(22; cerebrum, 23; cerebellum, 24; brain stem, 25,liver, 26; pituitary gland, 27; adrenal glands, and 28; aorta endothelium.

band, although these compounds markedly induced NO synthase activity in these cells (8). Similarly, NO synthase induced in the liver of Sprague-Dawley rats by i.p. injection of *Propionibacterium acnes* and LPS (15) was not detected with antiserum against bovine brain NO synthase (data not shown). We recently raised an antibody against inducible-type NO synthase of rat liver. Immunological cross-reactivity studies using this antibody also support the above conclusion (to be published elsewhere). These results suggest that there are at least two distinct types of NO synthase, that are immunologically distinguishable.

Acknowledgments. This study was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan,a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare and a grant from Japan Foundation for Applied Enzymology. H.O. and H.S. are Foreign Research Fellows of the Foundation for Promotion of Cancer Research, Japan.

REFERENCES

1. Marletta, M.A. (1989) TIBS. 14, 488-492.
2. Moncada,S., Palmer,R.M.J., and Higgs,E.A. (1989) Biochem.Pharmacol. 38, 1709-1715.
3. Hibbs J.B.Jr., Taintor,R.R., Vavrin,Z., and Rachlin,E.M. (1988) Biochem. Biophys. Res. Commun. 157, 87-94.
4. Bredt,D.S., and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. USA 87, 682-685.
5. Mayer, B., John, M., and Böhme, E. (1990) FEBS Lett. 277 215-219.
6. Schmidt, H.H.H.W., Pollock, J.S., Nakane, M., Gorsky, L.D., Förstermann, U., and Murad, F. (1991) Proc. Natl. Acad. Sci. USA 88, 365-369.
7. Bradford,M.M. (1976) Anal. Biochem. 72, 248-254.
8. Ohshima,H., Tsuda,M., Adachi,H., Ogura,T., Sugimura,T., and Esumi,H. (1991) Carcinogenesis 12,1217-1220.
9. Hecker,M., Mitchell,J.A., Harris,H.J., Katsura, M., Thiemermann, C., and Vane, J.R. (1990) Biochem. Biophys. Res. Commun. 167, 1037-1043.
10. Harlow and Lane (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Laemmli,U.K. (1970) Nature 227, 680-685.
12. Förstermann,U., Gorsky,L.D., Pollock,J.S., Schmidt,H.H.H.W., Helle,R.M., and Murard, F.(1990) Biochem. Biophys. Res. Commun. 168, 727-732.
13. Palacios, M., Knowles, R.G.,Palmer, R.M.J., and Moncada, S. (1989) Biochem. Biophys.Res. Commun. 165,802-809.
14. Bredt,D.S., Hawng,P.M., Snyder,S.H. (1990) Nature 347, 768-770.
15. Billiar,T.R., Curran,R.D., Stuehr,D.J., Stadler,J., Simmons,R.L., and Murray,S.A.(1990) Biochem. Biophys. Res. Commun. 168, 1034-1040.