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Biochemistry

Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme

(endothelium-derived relaxing factor/arginine/cGMP)

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ABSTRACT **Nitric oxide mediates vascular relaxing effects of endothelial cells, cytotoxic actions of macrophages and neutrophils, and influences of excitatory amino acids on cerebellar cyclic GMP. Its enzymatic formation from arginine by a soluble enzyme associated with stoichiometric production of citrulline requires NADPH and Ca^{2+}. We show that nitric oxide synthetase activity requires calmodulin. Utilizing a 2′,5′-ADP affinity column eluted with NADPH, we have purified nitric oxide synthetase 6000-fold to homogeneity from rat cerebellum. The purified enzyme migrates as a single 150-kDa band on SDS/PAGE, and the native enzyme appears to be a monomer.**

Endothelium-derived relaxing factor, a labile substance formed by endothelial cells, which mediates vasodilation, has been shown to be identical to nitric oxide (NO) (1–3). In addition to relaxing blood vessels, NO has multiple messenger functions as it has been demonstrated in macrophages (4) and in brain tissue (5–7). NO appears responsible for the cytotoxic effects of macrophages and neutrophils (8). We have obtained direct evidence for NO as a messenger for the influences of the excitatory amino acid glutamate on cGMP in the cerebellum (7). We showed a striking enhancement by glutamate and other excitatory amino acids of the conversion of arginine to NO and the associated formation of citrulline. Moreover we observed that N^{ω}-monomethyl-L-arginine (MeArg), an inhibitor of the enzymatic conversion of arginine to NO, inhibits glutamate-elicited cGMP formation, an influence selectively reversed by excess arginine.

Evidence that NO mediates functions of tissues as diverse as the brain, endothelium, and blood cells suggests a widespread role for NO as a messenger molecule. Localizing NO formation at a cellular level throughout the body would be greatly facilitated by immunohistochemical identification of NO synthetase, the NO-forming enzyme. The mechanism of conversion of arginine to NO, presently unclear (9), would be greatly clarified by purification of NO synthetase. Characterization of this enzyme has been hampered by the complex assays required to assay the enzyme by monitoring NO formation directly. We have shown that NO formation is accompanied by the stoichiometric conversion of arginine to citrulline, permitting a simple, sensitive, and specific enzyme assay measuring the transformation of [^{3}H]arginine to [^{3}H]citrulline (7). Utilizing this assay in the present study, we have purified NO synthetase to homogeneity. We demonstrate that NO synthetase is a calmodulin-requiring enzyme, explaining numerous reports of a crucial role for calcium in endothelium-dependent smooth muscle relaxation.

MATERIALS AND METHODS

Materials. [^{3}H]Arginine (53 Ci/mmol; 1 Ci = 37 GBq) was obtained from NEN/DuPont. All other chemicals were obtained from Sigma.

Assay of NO Synthetase. NO synthetase activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline as described (7). For routine assays, we added 25 μl of enzyme extract and 25 μl of 100 nM [^{3}H]arginine to 100 μl of buffer containing 50 mM Hepes (pH 7.4), 1 mM NADPH, 1 mM EDTA, 1.25 mM $CaCl_2$, 1 mM dithiothreitol, and 10 μg of calmodulin per ml. After incubation for 5 min at 22°C, assays were terminated with 2 ml of 20 mM Hepes, pH 5.5/2 mM EDTA, and were applied to 1-ml columns of Dowex AG50WX-8 (Na^+ form), which were eluted with 2 ml of water. [^{3}H]Citrulline was quantified by liquid scintillation spectroscopy of the 4-ml flow-through.

Purification of NO Synthetase. Eighteen rat cerebella were homogenized in 100 ml of ice-cold buffer A [50 mM Tris·HCl, pH 7.4/1 mM EDTA/antipain (10 mg/liter)/leupeptin (10 mg/liter)/soybean trypsin inhibitor (10 mg/liter)/pepstatin (10 mg/liter)/chymostatin (10 mg/liter)/phenylmethylsulfonyl fluoride (100 mg/liter)], and all subsequent procedures were carried out at 4°C. The homogenate was centrifuged at 20,000 × *g* for 15 min, and the supernatant was loaded at 2 ml/min onto a 20-ml column of diethylaminoethyl (DEAE) equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with a 100-ml linear gradient of 0–400 mM NaCl in buffer A. Fractions (2.5 ml) were assayed for enzyme activity. Fractions containing the first peak of activity from the DEAE column were pooled and added to 2 ml of 2′,5′-ADP agarose equilibrated in buffer B (10 mM Tris·HCl, pH 7.4/1 mM EDTA/5 mM 2-mercaptoethanol). After a 10-min incubation, the suspension was poured into a fritted column, which was washed with 50 ml of buffer B with 0.5 M NaCl and then with 20 ml of buffer B alone. NO synthetase was eluted with 8 ml of buffer B containing 10 mM NADPH.

RESULTS

In our preliminary efforts to purify NO synthetase, we observed that enzymatic activity adheres to a DEAE column and can be eluted by 1 M NaCl. However, with gradient elution of NaCl, enzymatic activity was not recovered in eluate fractions, suggesting the separation during purification of the enzyme from an important cofactor. Since NO formation requires Ca^{2+}, we speculated that calmodulin might be involved. Addition of calmodulin to DEAE eluate fractions restores enzyme activity (Fig. 1). NO synthetase activity elutes in one sharp, major peak followed by a smaller peak of activity, which is observed reproducibly in multiple experiments. Calmodulin is an extremely potent stimulator of NO synthetase activity (Fig. 2*A*). In the presence of 1 μM Ca^{2+}, 50% of maximal stimulation of enzyme activity is apparent with ≈10 nM calmodulin, while no stimulation is observed in the absence of calcium. As observed previously, Ca^{2+} is a potent activator of enzymatic activity (Fig. 2*B*). In the presence of 1 mM NADPH, 50% of maximal stimulation of

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Abbreviations: W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

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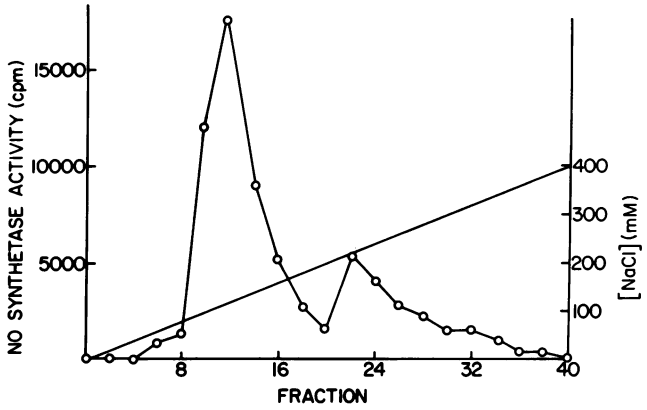

FIG. 1. Chromatography of NO synthetase on DEAE-cellulose. The column was loaded and eluted, and fractions were assayed as described. Data are from a representative purification, which was replicated five times.

enzyme activity is apparent with ≈200 nM Ca^{2+} with maximal enhancement of activity observed at 1 μM Ca^{2+} and some reduction in activity at concentrations exceeding 100 μM Ca^{2+}. In the absence of NADPH, Ca^{2+} fails to stimulate NO synthetase activity.

In crude cerebellar supernatant preparations, calmodulin is not required to demonstrate enzyme activity and added calmodulin (1 μM) has no influence on enzyme activity. However, trifluoperazine, a calmodulin antagonist, inhibits enzyme activity of crude preparations with an IC_{50} of ≈10 μM (Fig. 3*A*). Calmodulin (1 μM) competitively reverses this inhibition, increasing the IC_{50} for trifluoperazine to 45 μM. In other experiments, we have observed the same calmodulin reversible effects of trifluoperazine in crude supernatant preparations of vascular endothelial tissue, indicating that regulation of the endothelial and brain enzymes by calmodulin is similar. Trifluoperazine exerts multiple effects such as blockade of dopamine receptors. The drugs W-5 [*N*-(6-aminohexyl)-1-naphthalenesulfonamide] and W-13 [*N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide] are more selective calmodulin antagonists. In crude brain supernatant preparations, W-5 and W-13 inhibit NO synthetase activity with respective IC_{50} values of 70 and 25 μM (Fig. 3*B*).

For purification of NO synthetase, we have focused on the first, major peak of enzyme activity eluting from the DEAE column, which provides a 5.6-fold purification of enzyme activity with 60% recovery (Table 1, Fig. 1). Since NADPH is a required cofactor for NO synthetase, we conducted

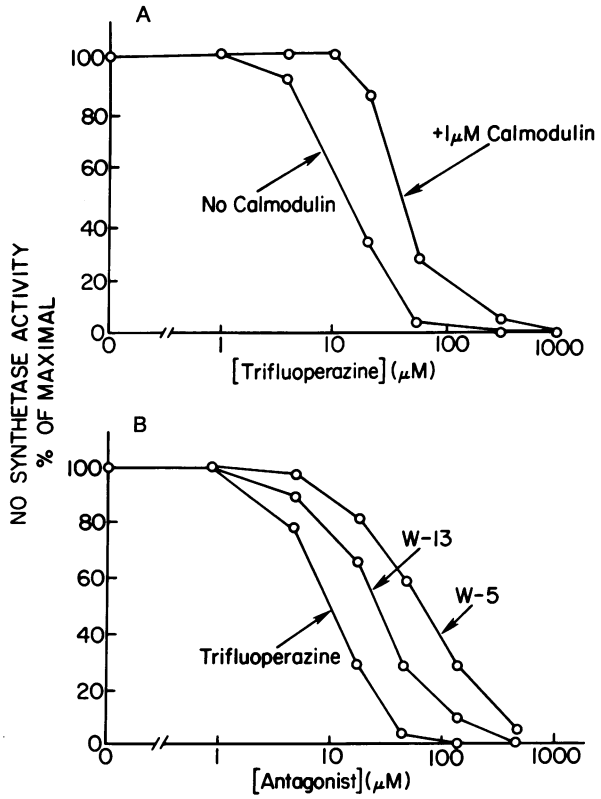

FIG. 3. Inhibition of NO synthetase by calmodulin antagonists. (*A*) Trifluoperazine. (*B*) W-5 and W-13. Enzyme activity in crude cerebellar supernatants with calmodulin and other agents added as indicated was assayed as described. Data are means of triplicate determinations from a representative experiment, which was repeated with similar results.

further purification utilizing affinity chromatography with a 2′,5′-ADP-linked agarose column, which has been used by others for purification of NADPH-requiring enzymes (10). NO synthetase activity adheres to this column and is not eluted by 0.5 M NaCl. After the 0.5 M NaCl wash, NO synthetase activity can be eluted with 10 mM NADPH, providing a 1000-fold purification of enzyme activity in this step. The overall purification of NO synthetase utilizing two steps, DEAE chromatography and 2′,5′-ADP affinity chromatography, affords a 6000-fold purification of enzyme activity with 30% recovery. The purified enzyme eluting from

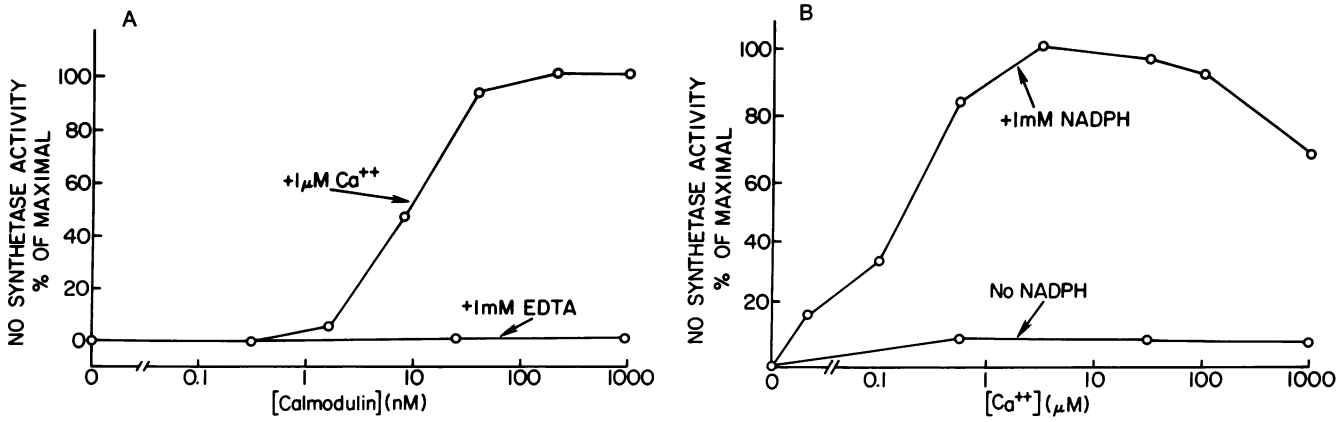

FIG. 2. Calcium/calmodulin dependence of NO synthetase. (*A*) Concentration dependence on calmodulin. (*B*) Concentration dependence on Ca^{2+}. Enzyme activity in a DEAE eluate preparation was assayed as described. Calmodulin (0.5 μM) was added to all samples in *B*. Free Ca^{2+} concentrations were maintained with Ca^{2+}/EDTA buffers and calibrated with a Ca^{2+} electrode. Data are means of triplicate determinations from a representative experiment repeated twice with similar results.

Table 1. Purification of NO synthetase

Fraction	Protein, μg	Recovery, %	Specific activity, nmol·mg^{-1}·min^{-1}	Purification, -fold
15,000 × g supernatant	180,000	100	0.16	1
DEAE eluate	20,000	60	0.9	5.6
2',5'-ADP agarose eluate	9.0	30	960	6000

Enzyme was purified and fractions were assayed as described. Data presented are from a typical purification, which was repeated five times with closely similar results.

the ADP affinity column appears homogeneous, constituting a single band on SDS/PAGE (Fig. 4). The molecular mass of this band is ≈150 kDa. To estimate the molecular mass of the native enzyme, we conducted gel filtration chromatography with a Superose-6 column (data not shown). NO synthetase activity of the purified enzyme emerges from the column as a single peak coincident with the peak of protein with an apparent molecular mass of 200 kDa, similar to the elution of β-amylase whose molecular mass is 200 kDa. Thus, purified NO synthetase appears to be a monomer.

The purified enzyme has high affinity for arginine with a K_m of ≈2 μM, similar to what we observed previously in crude supernatant preparations (7). The V_{max} of the purified enzyme is ≈1 μmol per mg of protein per min, similar to the V_{max} values for other NADPH-requiring oxidative enzymes (11) (Table 2). The K_i for MeArg inhibition of NO synthetase activity in the purified enzyme is ≈1.4 μM, similar to values we observed previously in crude preparations (7). The EC_{50} for calmodulin enhancement of enzyme activity in the pure enzyme, 10 nM, is similar to the value observed in crude preparations. Also, the EC_{50} for calcium stimulation of the purified enzyme is the same in the pure and crude preparations.

The purified enzyme is unstable. When stored at 0°C, 50% of enzyme activity is lost in 2 hr, whereas the crude supernatant preparation loses 50% activity at 0°C in 2 days.

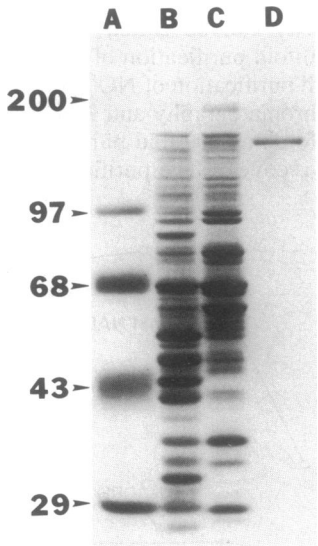

FIG. 4. SDS/PAGE analysis of purified NO synthetase. A 7.5% polyacrylamide gel was stained with Coomassie blue. Lanes: A, molecular mass markers (myosin, 200 kDa); phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; B, 30 μg of 20,000 × g supernatant; C, 30 μg of DEAE eluate; D, 3 μg of purified NO synthetase. Silver staining displayed no additional protein bands with purified NO synthetase.

Table 2. Properties of NO synthetase

Arginine, K_m	1.5 μM
V_{max}	0.96 μmol per min per mg of protein
MeArg, K_i	1.4 μM
Ca^{2+}, EC_{50}	200 nM
Calmodulin, EC_{50}	10 nM
Calmodulin antagonists, IC_{50}	
Trifluoperazine	10 μM
W-5	25 μM
W-13	70 μM

Purified enzyme was assayed as described. Values are means of two to six determinations, which varied by <20%.

Stability is enhanced by storing the enzyme in bovine serum albumin (1 mg/ml)/20% (vol/vol) glycerol at −70°C. When stored in this way, the enzyme loses <50% activity in 7 days.

DISCUSSION

Our ability to purify NO synthetase to homogeneity was made possible by our use of a simple, sensitive, and specific assay monitoring the conversion of arginine to citrulline as well as our discovery that calmodulin is required for activity of partially purified preparations. The requirement of calmodulin for NO synthetase activity can explain numerous observations of a requirement for calcium in the regulation of endothelium-mediated smooth muscle relaxation (12). The requirement of endothelial (13) and brain (6) enzymes for calcium is also consistent with NO synthetase being a calmodulin-dependent enzyme. In macrophages, NO synthetase activity has been reported to be enhanced by magnesium (4). Purification of NO synthetase from multiple tissues may clarify possible differences in ion requirements for the enzyme from different sources. The molecular mechanism for the conversion of arginine to NO is unclear. Two alternative mechanisms proposed are initial hydroxylation of a guanidino nitrogen (4) or deimination followed by oxidation of ammonia (14). Availability of homogeneous enzyme preparations should permit clarification of the mechanism of this reaction. Several properties of purified NO synthetase, such as its V_{max} and its instability when purified, resemble properties of other NADPH oxidative enzymes (11). Molecular cloning to ascertain the amino acid sequence of NO synthetase may permit a detailed comparison with other enzymes.

Antisera to purified NO synthetase can be raised and used for immunohistochemical localization of the enzyme. This should permit a clarification of its role as a messenger for neurotransmitters, hormones, and other regulatory agents throughout the body.

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