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Purification of a distinctive form of endotoxin-induced nitric oxide synthase from rat liver

(endothelium-derived relaxing factor/L-arginine/calmodulin)

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ABSTRACT **An endotoxin-induced form of nitric oxide synthase (EC 1.14.23) was purified to homogeneity from rat liver by sequential anion-exchange chromatography and affinity chromatography using 2',5'-ADP-Sepharose. The enzyme has a subunit molecular mass of 135 kDa as determined by SDS/PAGE, a maximum specific activity of 462 nmol of citrulline formed from arginine per min per mg, and a K_m for arginine of 11 μM. The enzyme was strongly stimulated by the addition of calmodulin with an EC_{50} of 2 nM, but removal of free calcium from the assay medium only reduced activity by 15%. Calmodulin inhibitors significantly reduced the enzyme activity. Tetrahydrobiopterin, FAD, and FMN were all required for full enzyme activity. This form of endotoxin-induced nitric oxide synthase from liver differs from the inducible enzyme found in macrophages and is unusual in that it is stimulated by calmodulin with little dependence on the calcium ion concentration.**

Nitric oxide (NO) plays an important role as a physiological mediator in a number of different systems (reviewed in ref. 1). It is responsible for the biological activity of endothelium-derived relaxing factor, an important mediator of vascular tone (2), and mediates glutamate-induced stimulation of guanylate cyclase activity in the brain (3). It is also produced by activated macrophages (4), where it mediates cytotoxicity against a variety of intracellular pathogens and expression of the ability to inhibit tumor target cell metabolism (5, 6). NO production has also been demonstrated *inter alia* in endotoxin-treated hepatocytes (7, 8), endotoxin-treated smooth muscle cells (9), and neutrophils (10).

Characterization of the enzymes responsible for NO production has broadly classified them into two groups: constitutive NO synthase and inducible NO synthase. A constitutive form of the enzyme with a molecular mass of 150 kDa has been purified from rat cerebellum (11), and its gene has been isolated and sequenced (12). This form of the enzyme is strongly dependent on calcium and calmodulin for its activity. The inducible form of the enzyme from a macrophage cell line (13) and peritoneal macrophages has also been purified (14). This has a subunit molecular mass of 130 kDa in the cell line and 150 kDa in the peritoneal macrophages. In neither case was calcium or calmodulin required for full enzyme activity. An NO synthase that required calcium but not calmodulin for full expression of activity (15) has also been purified from neutrophils. Both of these forms of NO synthase were cytosolic, but in endothelial cells the majority of NO-synthesizing capability appears to be membrane bound (16). This form of the enzyme has also been purified and has a subunit molecular mass of 135 kDa (17). Taken together, these data suggest that there are a number of different isoforms of the enzyme.

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We are interested in the hepatic form of the NO synthase, which is induced by the administration of endotoxin (8). Livers from untreated animals show minimal NO synthase activity. NO production in the liver seems to suppress protein synthesis and protects the liver against damage (18). We have purified this enzyme to homogeneity and show that it is strongly stimulated by calmodulin, whereas its activity is only affected to a small degree by free calcium concentrations—even in the presence of calmodulin. Thus, this hepatic endotoxin-induced NO synthase is distinct from that described in endotoxin-treated macrophages, which does not depend on calmodulin for full enzyme activity (13, 14).

MATERIALS AND METHODS

L-[2,3,4,5-^{3}H]Arginine (specific activity, 35–70 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. (6*R*)-5,6,7,8-Terahydro-L-biopterin was from the laboratory of B. Schirks (Jona, Switzerland). N^{ω}-Methyl-L-arginine (L-NMA) and the D isomer were a gift of the Wellcome Foundation. All other biochemicals were from Sigma.

Animals and Preparation of Liver Homogenates. Male Wistar rats (250–350 g) were injected intraperitoneally with endotoxin (*Klebsiella pneumoniae*, phenol extract; Sigma) at 10 mg/kg and sacrificed by a rising concentration of carbon dioxide after 6 h. The livers were removed and homogenized in 5 vol of ice-cold buffer A [0.32 M sucrose, 50 mM Tris Cl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT), soybean trypsin inhibitor (10 μg/ml), antipain (1 μg/ml), pepstatin (1 μg/ml), aprotonin (1 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 2 μM FAD, 2 μM FMN, and 2 μM tetrahydrobiopterin] with a Teflon pestle in a glass homogenizer. The homogenates were centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant was used for subsequent purification.

Assay of NO Synthase. NO synthase activity was assayed by the conversion of radiolabeled arginine to citrulline as described (11). Citrulline is produced with equimolar amounts of nitric oxide. The reaction buffer contained 50 mM Tris Cl (pH 7.4), 1 mM NADPH, 1 mM DTT, 4 μM FAD, 4 μM FMN, and 50 μM tetrahydrobiopterin. An assay mix was composed of 100 μl of reaction buffer, 25 μl of 100 nM radiolabeled arginine, and 10 μl of the enzyme fraction to be tested. Reactions were allowed to proceed for 15 min at 37°C. In crude liver preparations, endogenous arginase and other urea cycle enzymes were inhibited by including 50 mM valine, 1 mM ornithine, and 1 mM citrulline in the reaction buffer as described (8). In addition, to validate the enzyme activity of the purified preparations, these were also assayed for their ability to form nitrite ions, a stable end product of NO in aqueous solutions. The method for nitrite generation is described in ref. 4; 10 μl of purified enzyme was added to 200 μl of reaction buffer containing 1 mM NADPH, 1.25 mM $CaCl_2$, 0.2 mM arginine hydrochloride, 1 mM DTT, 50 mM

Abbreviations: L-NMA, N^{ω}-methyl-L-arginine; DTT, dithiothreitol.
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Tris Cl (pH 7.4), calmodulin (2.5 μg/ml), 50 μM tetrahydrobiopterin, 4 μM FAD, and 4 μM FMN. Reactions were allowed to proceed for 3 h at 37°C. Nitrite assays were performed as described in ref. 19.

Purification of NO Synthase. All procedures were performed at 4°C. The supernatant from the liver homogenates (two livers) after the ultracentrifugation step was pumped at 2 ml/min onto a 30-ml column of DEAE-cellulose equilibrated with homogenization buffer with no added sucrose. The column was washed with 70 ml of buffer, and then the enzyme was eluted with a 100-ml gradient of 0–0.5 M sodium chloride in the same buffer. Fractions (2 ml) containing the NO synthase activity were pooled and incubated with 2 ml of 2′,5′-ADP-Sepharose equilibrated with buffer B [10 mM Tris Cl (pH 7.4), 1 mM EDTA, 1 mM DTT, 2 μM FAD, 2 μM FMN, and 2 μM tetrahydrobiopterin] for 30 min. The Sepharose was poured into a column, washed with 50 ml of buffer B containing 0.5 M sodium chloride, and then sequentially washed with 6 ml of buffer B containing 10 mM NADH, 20 ml of buffer B containing 5 mM NADP and 5 mM sodium malate, and 20 ml of buffer B containing 1 mM NADPH. Enzyme activity was eluted with 6 ml of buffer B containing 10 mM NADPH. If not used immediately, the NO synthase was stabilized by the addition of bovine serum albumin to 1 mg/ml and glycerol to 10% (vol/vol); this allowed storage at −70°C with minimal loss of activity. Protein concentrations were determined using the Bio-Rad protein dye binding assay with bovine serum albumin as standard. SDS/PAGE was performed according to the method of Laemmli (20).

RESULTS

Previous reports have shown that an hepatic NO synthase is induced 26-fold by endotoxin treatment in rats, with a peak enzyme activity 6 h after injection (8). We have used homogenized livers from rats treated in this fashion to purify this inducible form of NO synthase. Two purification steps were used. First, anion-exchange chromatography on a DEAE-cellulose column was performed. The profile of NO synthase activity eluting from this column is shown in Fig. 1. One major peak of NO synthase activity was eluted, but in addition there was a consistent smaller peak of activity eluting at a lower salt concentration as shown. Subsequent purification steps used only those fractions corresponding to the major peak of NO synthase activity. The second step of purification was affinity chromatography on a 2′,5′-ADP-Sepharose column, to which the enzyme binds as it utilizes NADPH in the formation of NO. Enzyme activity was selectively eluted from this column with 10 mM NADPH. However, a number of contaminating proteins were also eluted from the column by the 10 mM NADPH. One of these is reported to be "malic" enzyme [(*S*)-malate:NADP$^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] (21). Thus, by washing the column with solutions containing the substrates of this enzyme, NADP$^+$ and malate, this contaminant could be selectively removed while still leaving the NO synthase bound to the column. A combination of washes with NADH and 1 mM NADPH removed the other contaminants, which are presumably other nucleotide cofactor-requiring enzymes that are specifically bound to the 2′,5′-ADP-Sepharose column.

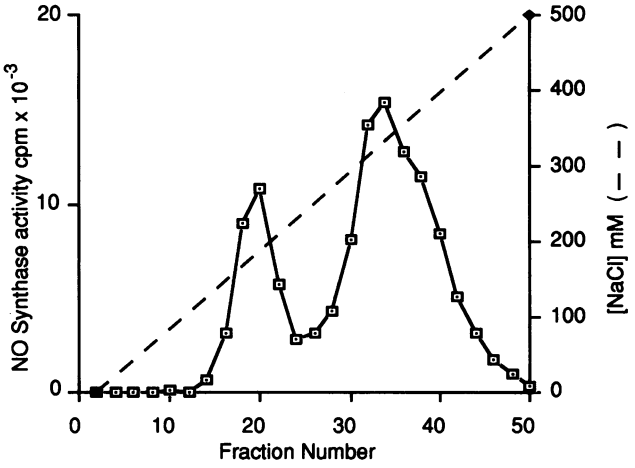

FIG. 1. Profile of NO synthase activity eluting from a DEAE-cellulose column. NO synthase activity is shown as the raw radioactivity of citrulline formed in the assay (□). The concentration of sodium chloride at each point in the gradient is shown on the other *y* axis (---).

The results of a typical purification are shown in Table 1. Yields of the NO synthase were between 3% and 5%, with specific activities ranging from 223 to 462 nmol of citrulline formed per min per mg. As has been noted in the purification of the constitutive isoform of NO synthase, once the enzyme is obtained relatively free of impurities, it is extremely unstable; it loses all of its activity after overnight storage at −70°C. This may account for the rather variable specific activities obtained. As a further verification of the identity of this enzyme as a NO synthase, it was separately assayed for its ability to generate nitrite ions, a stable product of NO in aqueous solution. This gave a value of 190 nmol of nitrite per min per mg. NO produces a varying ratio of both nitrite and nitrate in solution, so it is not possible to compare this directly with the rate observed for the production of citrulline, but it does confirm the ability of the purified enzyme to form nitrite, an expected product of NO formation.

SDS/PAGE of the material eluting from the 2′,5′-ADP-Sepharose column is shown in Fig. 2, lane A. This shows a single major polypeptide band on silver staining, with a molecular mass of 135 kDa. To ascertain whether this band was specific to liver proteins from endotoxin-treated rats, material from similarly treated animals was purified in parallel with liver extracts from untreated control rats. The extra washing steps with NADP/malate and NADH were omitted, to allow a more accurate assessment of the proteins purifying from the control liver homogenates. Material purified (by using this modified protocol) from the endotoxin-treated rats is shown in Fig. 2, lane B and that purified from control rats is shown in lane C. Comparison of these two lanes shows that the 135-kDa band is only present after endotoxin treatment, as would be expected of the inducible NO synthase. In addition, there is a polypeptide of 45 kDa, which is also only seen in the proteins purified from the endotoxin-treated livers. This is

Table 1. Purification of NO synthase from livers of endotoxin-treated rats

Fraction	Total protein, μg	Specific activity, nmol·min^{-1}·mg^{-1}	Yield, %	Purification factor
100,000 × *g* supernatant	1,105,000	0.05	100	1
DEAE-cellulose active fraction	194,000	0.13	51	2.9
2′,5′-ADP-Sepharose effluent	8	223	3.7	4870

The data are representative of three preparations.

almost completely removed in the steps producing the highly purified preparation (lane A). The identity of this 45-kDa component is not known. Fig. 2 also shows the NO synthase purified from rat cerebellum (lane D), according to the method of Bredt and Snyder (11). This is the constitutive form of the enzyme, with a molecular mass of 155 kDa, which is distinct from the endotoxin-induced form shown in lane A.

In the course of purifying this inducible NO synthase, we initially found a marked loss of activity following the affinity purification step. The activity could be recovered, however, by the addition of calmodulin to the reaction buffer. Fig. 3 shows the dependence of the enzyme activity on the concentration of calmodulin, which stimulated the purified NO synthase ≈5-fold, with an EC_{50} of 2 nM. However, stimulation by calmodulin was not particularly dependent on the concentration of calcium in the reaction buffer (Fig. 3). When calcium was omitted from the buffer and free calcium was chelated with 2 mM EGTA, the curve of activation of NO synthase activity against calmodulin concentration was displaced slightly to the right. At the highest calmodulin concentrations tested, the removal of calcium ions inhibited the enzyme ≈15%. This agrees with previous observations on crude NO synthase from endotoxin-treated rat livers, where the NO synthase activity was only marginally depressed by addition of EGTA to the medium (8). The ability of 2 mM EGTA to reduce significantly the level of free calcium was checked by assaying the cerebellar form of the enzyme in the presence and absence of this chelating agent. Under exactly the same conditions as shown in Fig. 3, 2 mM EGTA completely inhibited the cerebellar enzyme activity in the presence of 500 nM calmodulin (data not shown).

The nature of the activation of the enzyme by calmodulin was investigated by a number of additional experiments. Bovine serum albumin, hemoglobin, and soybean trypsin inhibitor (all at final concentrations of 5 μg/ml) were unable to substitute for calmodulin. EDTA at a 2 mM final concentration was also unable to produce any stimulation of the enzyme in the absence of calmodulin, thus making the possibility that calmodulin was activating the enzyme through some nonspecific ability to chelate inhibitory metal ions unlikely.

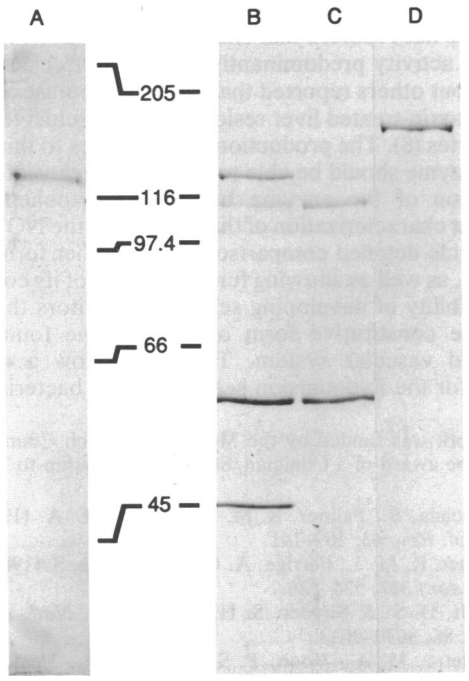

FIG. 2. SDS/PAGE under reducing conditions of various NO synthase preparations performed according to the method of Laemmli in an 8% gel. Lanes: A, 1 μg of 2′,5′-ADP-Sepharose column eluate from endotoxin-treated rats; B, 5 μg of 2′,5′-ADP-Sepharose column eluate from endotoxin-treated rats, omitting the NADH and NADP/malate washes on the column; C, 5 μg of 2′,5′-ADP-Sepharose column eluate from control rats, prepared in the same way as lane B; D, 3 μg of purified rat cerebellar NO synthase. Molecular size markers in kDa are shown between lanes A and B. Lane A was silver stained; the other lanes were stained with Coomassie blue.

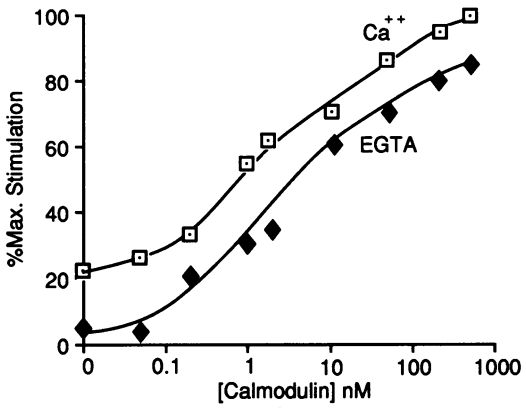

FIG. 3. Effect of calmodulin on the activity of the purified rat liver NO synthase in the presence of Ca^{2+} (□) or with no added Ca^{2+} and the addition of 2 mM EGTA (◆). Results are the means of duplicates, which varied by <10%.

The results of inhibition of the purified enzyme by calmodulin antagonists are shown in Table 2. Both trifluoperazine and the calmodulin-binding protein calcineurin reduced the activity of the enzyme to about 50% of its maximal activity in the presence of 500 nM calmodulin. The residual enzyme activity seen in the absence of calmodulin (Fig. 3) was not further inhibited, however, by the addition of calcineurin to the same final concentration. Also shown in Table 2 is the stereospecific inhibition of the enzyme by L-NMA but not D-NMA in common with all NO synthases described so far.

Full activity of the enzyme is also dependent on the addition of a number of other cofactors as shown in Table 3. Tetrahydrobiopterin stimulated the enzyme 25-fold, with an EC_{50} of 0.1 μM. The enzyme was also stimulated by the flavin nucleotides; FAD and FMN stimulated the enzyme ≈2-fold as shown in Table 3. The K_m of the enzyme for L-arginine was 11 μM, compared with values of 2.8 μM and 32 μM for the macrophage form of inducible NO synthase reported by two separate groups (13, 14).

DISCUSSION

We describe here a method for the purification of a form of NO synthase from rat liver. This form of the enzyme is induced by endotoxin, in common with that seen in a number of other cells. However, it differs in a number of ways from

Table 2. Effects of different inhibitors on the activity of purified hepatic NO synthase

Inhibitor added	NO synthase activity, % control
None (control)	100
Trifluoperazine (200 mM)	56
Calcineurin (0.2 mM)	40
EGTA (2 mM)	85
L-NMA (0.4 mM)	3
D-NMA (0.4 mM)	96

The control incubation was with calmodulin (500 nM) and calcium (1.25 mM). The data are the means of duplicate determinations of NO synthase activity with additions to the standard assay buffer (control incubation) as indicated. Duplicates varied by <10%. In the incubations with EGTA, calcium was omitted from the reaction buffer.

Table 3. Effect of omissions from the reaction buffer on the activity of the NO synthase

Component(s) omitted	NO synthase activity, % control
None (control)	100
Tetrahydrobiopterin	4
FAD	85
FMN	92
FAD and FMN	51

The control incubation was with calmodulin (500 nM) and calcium (1.25 mM). Enzyme was purified with the omission of FAD, FMN, and tetrahydrobiopterin for these determinations. Results are the means of duplicate determinations, which varied by <10%.

the form of endotoxin-induced NO synthase purified from macrophages (13, 14). The main difference is that this hepatic form of the enzyme is stimulated markedly by the presence of calmodulin, although it is not greatly influenced by free calcium concentrations. In contrast, the macrophage isoform was not stimulated by added calmodulin or calcium. The hepatic isoform of NO synthase described here also differs in biochemical characteristics from the constitutive form of the enzyme purified from rat cerebellum (11), which is absolutely dependent on both calcium and calmodulin for its activity. In addition, the hepatic form of the enzyme has a subunit molecular mass of 135 kDa compared to that of 150 kDa for the cerebellar isoform. The lower molecular mass of 135 kDa is however also found for the macrophage isoform of the enzyme purified from a cell line (13). Both groups who have purified the macrophage isoform (13, 14) found it to behave as a homodimer, with loss of activity occurring on dissociation into its subunits. One of the groups who have purified the cerebellar isoform of NO synthase also find it to behave as a dimer (22). We have not been able to determine whether the hepatic enzyme described here is also a dimer.

Although its stimulation by calmodulin with little dependence on the calcium ion concentration clearly differentiates the enzyme from the other known endotoxin-induced NO synthases, the physiological relevance of the stimulation by calmodulin remains unclear. Calmodulin normally provides a means whereby fluctuations in free calcium concentrations can be coupled to the activity of an enzyme. Thus, the activity of the cerebellar isoform of NO synthase is exquisitely dependent on the calcium ion concentration, affording a rapid means of precise control over NO levels. Although a number of proteins that bind calmodulin in a calcium-independent manner have been described (23), it is very unusual for calmodulin to produce a stimulatory effect upon an enzyme in a calcium-independent manner. The only well-documented example is that of an adenylate cyclase purified from *Bordetella pertussis* (24). Since calmodulin is found in large amounts in the liver, it is difficult to see how the calmodulin stimulation of the hepatic NO synthase provides a way of modulating the enzyme activity *in vivo*. The nature of the association of calmodulin with the enzyme remains unknown; cross-linking experiments might enable a complex of calmodulin with the enzyme to be demonstrated. Residual enzyme activity is seen in the absence of calmodulin (Fig. 3), and this is refractory to inhibition by calcineurin; thus, this residual activity does not seem to be due to small amounts of calmodulin remaining bound to the purified enzyme.

The cofactor requirements of this induced hepatic NO synthase are similar to those seen with the induced form of the enzyme purified from macrophages (13, 14). However, the degree of stimulation of the purified enzyme by tetrahydrobiopterin is 25-fold in our experiments (Table 3), which is higher than values of 2.5-fold (13) and 5.6-fold (14) found for the inducible NO synthase from macrophages and 8.3-fold for the cerebellar isoform (25). This suggests that the hepatic isoform of NO synthase has a greater dependency on tetrahydrobiopterin than the other isoforms described, although the values are not directly comparable because they were assayed using different methods and times from those reported here. An alternative explanation is that the hepatic isoform binds tetrahydrobiopterin less avidly, leading to a greater loss during purification and hence a higher stimulation of the purified enzyme by this cofactor. It has been suggested that tetrahydrobiopterin participates directly in the reaction catalyzed by the macrophage isoform of NO synthase and is regenerated by dihydropteridine reductase and/or thiols (26, 27). However, a recent study of the cerebellar NO synthase suggested that tetrahydrobiopterin was not a direct reactant in the reaction catalyzed by this enzyme (28). Further studies are needed to determine if the isoform of NO synthase described here shows similar properties.

The induction of NO synthase by endotoxin and cytokines may be of benefit in cells such as the macrophage, where it has been shown to contribute to the ability of these phagocytes to kill a variety of intracellular parasites (5). In the liver, NO production has been shown to inhibit hepatocyte protein synthesis with minimal cell death (29). Inhibition of NO production by L-NMA in a model of hepatic injury produced by *Corynebacterium parvum* and lipopolysaccharide resulted in increased liver injury, suggesting a protective effect of NO in this instance (18). On the other hand, the excess production of NO induced by endotoxin may contribute to the hypotension characteristic of septic shock, by producing vasodilatation (30). The liver has a key role in Gram-negative sepsis, as the majority of circulating endotoxin localizes to this organ, and considerable hepatic damage is commonly found in those dying from this condition (31).

The cells responsible for the synthesis of the NO synthase purified from the liver in these experiments are not known. One group has reported that endotoxin produces a rise in NO synthase activity predominantly in the Kupffer cell population (7), but others reported that the NO synthase activity of the endotoxin-treated liver resided almost exclusively within hepatocytes (8). The production of antibodies to this isoform of the enzyme should be able to provide a means of accurate localization of the enzyme by immunocytochemistry. A molecular characterization of this isoform of the NO synthase will provide detailed comparisons to the other forms of NO synthase, as well as allowing further studies of its control and the possibility of developing selective inhibitors that do not affect the constitutive form of the enzyme found in the peripheral vascular system. This may allow a means of therapy for the hypotension seen in severe bacterial sepsis.

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