Brief Definitive Report

Calmodulin Is a Subunit of Nitric Oxide Synthase from Macrophages

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Summary

A central issue in nitric oxide (NO) research is to understand how NO can act in some settings as a servoregulator and in others as a cytotoxin. To answer this, we have sought a molecular basis for the differential regulation of the two known types of NO synthase (NOS). Constitutive NOS's in endothelium and neurons are activated by agonist-induced elevation of Ca^{2+} and resultant binding of calmodulin (CaM). In contrast, NOS in macrophages does not require added Ca^{2+} or CaM, but is regulated instead by transcription. We show here that macrophage NOS contains, as a tightly bound subunit, a molecule with the immunologic reactivity, high performance liquid chromatography retention time, tryptic map, partial amino acid sequence, and exact molecular mass of CaM. In contrast to most CaM-dependent enzymes, macrophage NOS binds CaM tightly without a requirement for elevated Ca^{2+}. This may explain why NOS that is independent of Ca^{2+} and elevated CaM appears to be activated simply by being synthesized.

A central question in nitric oxide (NO) research is to understand how diverse physiologic roles can be subserved by the same radical gas acting alternatively as an intercellular servoregulator or a cytotoxin (1–5). In large part this reflects the differential distribution of two major classes of NO synthases (NOS's) distinguished on the basis of their expression and regulation. NOS's of one type, expressed constitutively in endothelial cells or neurons, respond in a cell-type selective manner to agonists that elevate intracellular Ca^{2+}. NO production by these NOS's, like the Ca^{2+} transient itself, lasts for seconds or minutes, and depends on calmodulin (CaM) (6, 7), a protein that transduces Ca^{2+} signals by means of its reversible, Ca^{2+}-dependent binding to target enzymes and their resultant activation (8–10). We have termed these constitutive enzymes cNOS's to signify their dependence on Ca^{2+} and CaM (1, 11). In contrast, catalysis by the immunologically inducible iNOS is independent of Ca^{2+} and elevated CaM (12, 13). Instead, expression of iNOS activity appears to be controlled chiefly at the level of transcription of the gene encoding it (11). Not only is iNOS activated more slowly than cNOS's, but catalysis by iNOS continues for hours or days, leading to the production of much more NO than from an equivalent number of cells expressing cNOS. Accordingly, while cNOS's are key regulators of homeostasis in the circulatory, renal, pulmonary, gastrointestinal, endocrine, and nervous systems, iNOS appears to play an important role in cytotoxic and suppressive functions in the immune system and in toxic states such as septic shock (1–5).

In the present study, we have restated the central question as follows. What is the molecular basis for the differential response to Ca^{2+} and CaM by iNOS and cNOS? The enzymes are ~51% identical at the amino acid level, and each contains a region rich in basic and hydrophobic residues that is structurally similar to known CaM-binding domains in other proteins (11, 14–16). Nonetheless, the activity of purified iNOS is neither enhanced by exogenous Ca^{2+}/CaM, nor inhibited by chelators of divalent cations or drugs that block the binding of CaM to its targets (12, 13). This paradox could be resolved if CaM did not require an elevation in Ca^{2+} to bind tightly to iNOS. Endogenous CaM might then copurify with iNOS, resist displacement by CaM inhibitors, and maintain iNOS in a tonically activated state.

Materials and Methods

Ca^{2+} concentrations were measured using a graphite furnace atomic absorption photometer (Zeeman/3030; Perkin-Elmer, Inc., Norwalk, CT) (17). Other methods are detailed or cited in the legends or text.

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Results

CaM-like Antigen in Purified iNOS. Purified as a 260–290-kD dimer, iNOS migrated on SDS-PAGE as a doublet at 130 kD (Fig. 1, lane *1*), as previously reported (12). No species in the region of CaM (16.8 kD) was visible upon staining with amido black (Fig 1, lane *1*), silver (12) or coomassie blue (H. Cho, et al., unpublished observations). However, CaM is reportedly difficult to visualize by conventional staining methods (18). In our hands, reagent bovine brain CaM (0.5 μg/lane) was undetectable upon staining with amido black (Fig. 1, lane *2*) or coomassie blue, and hard to stain by silver (H. Cho, unpublished observations). Thus, apparently pure iNOS might nonetheless contain CaM.

To look for CaM in pure, enzymatically active iNOS preparations by a more sensitive method, we made use of anti-CaM antibody. Using the COOH-terminal 20 amino acids of CaM coupled to thyroglobulin, Sacks et al. (19) were able to raise a mAb that binds CaM with an affinity of 5×10^8 liter/mol and does not crossreact with the homologous Ca^{2+}-binding proteins troponin C, S100α, S100β, or parvalbumin. This highly specific mAb reacted with two antigens in an immunoblot of purified iNOS (Fig. 1, lane *3*). The lower molecular mass bands comigrated with authentic CaM (Fig. 1, lane *4*). Surprisingly, the iNOS doublet ~130 kD was also reactive.

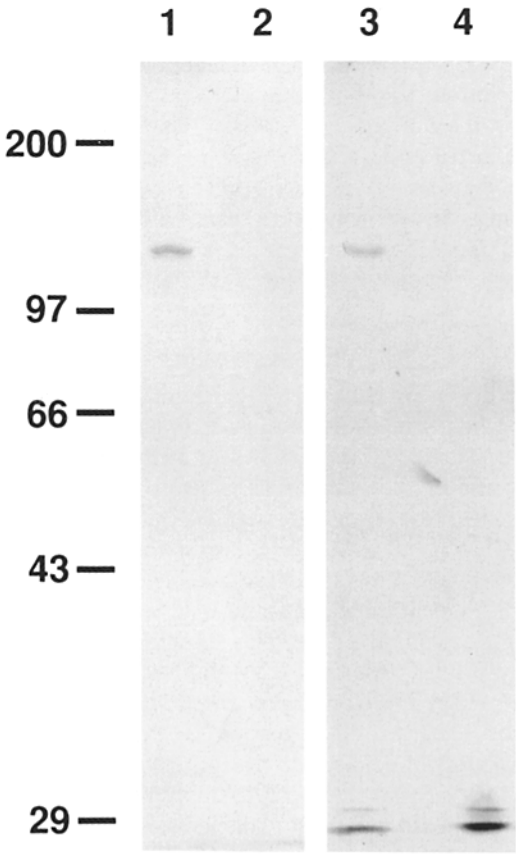

Figure 1. Association of CaM-like antigen with iNOS. Purified iNOS was boiled and subjected to electrophoresis in the presence of 2% SDS (18) in a 16-cm 10% polyacrylamide gel and immunoblotted with anti-CaM mAb (mouse IgG1, 1 μg/ml: Upstate Biotechnology, Lake Placid, NY). Lanes *1* and *3*, iNOS (500 ng); lanes *2* and *4*, bovine brain CaM (500 ng); lanes *1* and *2*, amido black stain; lanes *3* and *4*, immunoblot with anti-CaM mAb. Migration of markers is indicated at the left in kD. Antibody binding was detected with 1:1,000 alkaline phosphatase–conjugated rabbit anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN).

The foregoing results raised three questions. (*a*) Did the CaM-like antigen (that is, the molecule reactive with anti-CAM mAb and migrating with the same apparent molecular mass as CaM) copurify with the iNOS adventiously, or was it tightly bound to the enzyme? (*b*) Did the additional reactivity of anti-CaM mAb with 130-kD iNOS monomer reflect a covalent association of a portion of the CaM with iNOS? And, finally, (*c*) what was the precise molecular identity of the CaM–like antigen? The following experiments addressed each question in turn.

Purified iNOS Contains No Free CaM. The purification of iNOS from activated RAW 264.7 macrophage–like cells involved sequential anion exchange, nucleotide affinity, and size exclusion chromatography (12). These steps should each militate strongly against the adventitious copurification of free CaM, in that iNOS has a predicted pI of 7.7 (11), binds to and elutes specifically from 2′,5′-ADP-Sepharose, and has a mass of ~260–290 kD in its dimeric form (12), while in contrast, CaM has a pI of 4.3, is not expected to bind to or elute specifically from 2′,5′-ADP-Sepharose, and has a mass of 16.8 kD (8–10). The elution profile in the final gel filtration step (Fig. 2) confirms that CaM did not copurify with iNOS as a free molecular species, since iNOS purified through the first two chromatographic steps displayed no peak corresponding to free CaM. As illustrated with reagent CaM, any free CaM that might have been present at that stage would be separated widely from iNOS (Fig. 2).

The binding of CaM to CaM-regulated enzymes usually requires elevated concentrations of Ca^{2+} (8–10). In the present instance Ca^{2+} was not added to any of the buffers

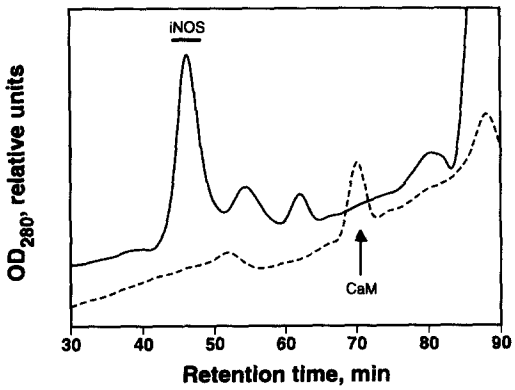

Figure 2. Final step in the purification of iNOS (12) by TSK G3000SW size-exclusion chromatography (Pharmacia LKB, Piscataway, NJ). Partially purified iNOS lacks a CaM peak. The iNOS dimer elutes at ~260–290 kD, well separated from any free CaM (16.8 kD) that may be present below the limit of detection. The latter would elute as shown for bovine brain CaM (Sigma Chemical Co., St. Louis, MO).

or reagents used for purification. As measured by the most sensitive method available, graphite furnace atomic absorption spectroscopy (17), the Ca^{2+} concentration in the buffer used for the final chromatographic step was 39 nM.

Thus, iNOS and a CaM-like antigen coeluted as a 260–290-kD complex at a Ca^{2+} concentration below that found in resting cells.

The CaM-like Antigen Is Noncovalently Attached. Most of the iNOS-associated CaM was displaced from the enzyme by SDS-PAGE. To exclude the possibility that the reactivity of anti-CaM mAb with 130-kD iNOS reflected a covalent attachment of residual CaM, we used the procedure employed by Cohen et al. (20) to demonstrate that CaM is a noncovalently attached subunit of phosphorylase kinase, namely, boiling in the presence of 1 mM EDTA, 15 mM 2-ME, and 200 mM NaCl. Under these conditions, iNOS disappeared from the fluid phase (Fig. 3 *B*, compare lane *2* with lane *1*) and appeared in the precipitate (Fig. 3 *B*, lane *3*), having lost its reactivity with anti-CaM mAb (Fig. 3 *A*, compare lane *3* with lane *1*). We could also remove all detectable CaM-like antigen from iNOS by electrophoresis in 2.5 M urea with 0.9 M acetic acid (pH 3) (H. Cho, unpublished observations). Thus, the association of the CaM-like antigen with iNOS was exclusively noncovalent.

CaM is a heat-resistant protein (8–10). Under the conditions used to boil iNOS, reagent CaM remained partially in solution (H. Cho, unpublished observations). Likewise, when iNOS was boiled with EDTA/2-ME/NaCl, the supernatant as well as the precipitate contained a protein that comigrated with CaM and reacted with anti-CaM mAb (Fig. 3 *A*, lanes *2* and *3*). This provided a source of CaM-like antigen for further characterization.

Identification as CaM. The supernatant generated by boiling purified iNOS in EDTA, as analyzed by reverse phase HPLC, was a pure solution of a molecule that had the same retention time (Fig. 4 *A*), tryptic map, and partial amino acid sequence (Fig. 4 *B*) as authentic CaM (21).

Finally, when pure iNOS was subjected to reverse phase HPLC in 0.1% TFA, two peaks were resolved (Fig. 4 *C*). After protease digestion, the second peak yielded >120 peptides that matched the amino acid sequence deduced from iNOS cDNA (11), but no peptides homologous to CaM (21). In contrast, the first peak, when subjected to electrospray ionization mass spectrometry, gave the series of multiply charged ions expected for CaM, and no other peaks (Fig. 4 *D*). In two such experiments, computer analysis yielded values of 16,791 and 16,793 for the average molecular weight of this pure molecule. This compares to a theoretical molecular weight for N-acetylated, lysine-trimethylated CaM of 16,791.5.

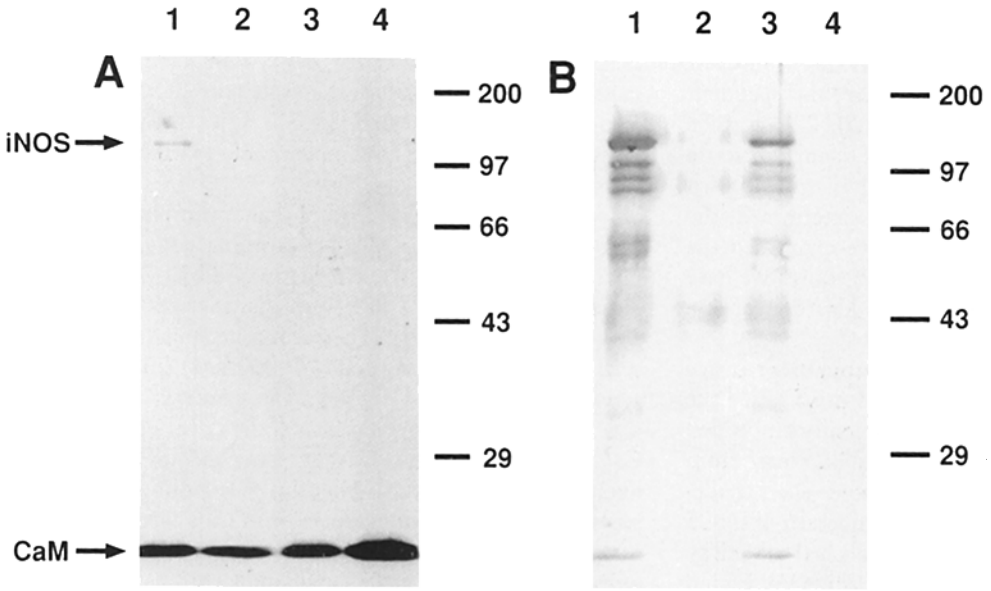

Figure 3. Noncovalent association of a CaM like antigen with iNOS. (*A*) Western blot with anti-CaM mAb after electrophoresis in an 8-cm gel. Lane *1*, pure iNOS (500 ng); lane *2*, supernatant remaining after iNOS was boiled for 2 min in 1 mM EDTA, 15 mM 2-ME, 50 mM Tris, 200 mM NaCl, pH 7.0 (19); lane *3*, precipitate of boiled iNOS; lane *4*, bovine brain CaM (500 ng). (*B*) Western blot with anti-iNOS IgG (1:1,000) (11). The samples are the same as in (*A*). Degradation products that appear on freezing and thawing of purified iNOS are evident in lanes *1* and *3*. Freshly purified iNOS shows only one to three band(s) tightly clustered at 130-kD after staining with amido black (see Fig. 2) or silver (see reference 12). Although one peptide resulting from iNOS degradation migrated near the dye front in a position similar to reagent CaM, this product could clearly be distinguished from CaM, since its migration was different from that of CaM, and since anti-iNOS was completely nonreactive with CaM (lanes *2* and *4*). Antibody binding was detected with 1:1,000 alkaline phosphate–conjugated rabbit anti-mouse IgG (*A*), or 1:1,000 sheep anti-rabbit IgG (*B*) (Boehringer Mannheim Biochemicals).

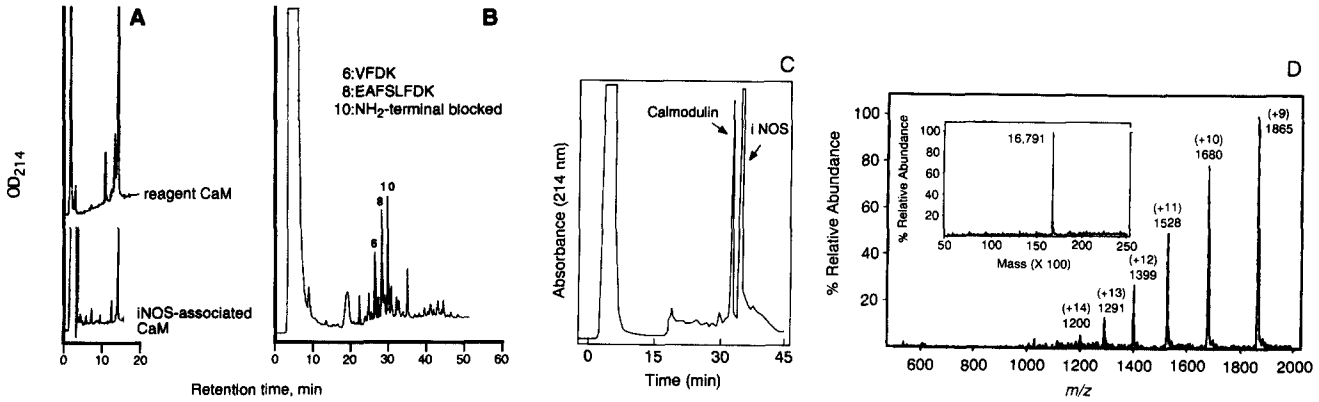

Figure 4. Identification of the CaM-like antigen of iNOS as CaM. (*A* and *B*) Reverse phase HPLC elution profiles of supernatant of purified iNOS boiled in EDTA/2-ME/Tris/NaCl as in Fig. 2. (*A*) Identical retention times (C4 column developed with 0.1% TFA in 15–75% acetonitrile). Upper trace is for bovine brain CaM; lower trace is for iNOS-derived sample. (*B*) Tryptic map of iNOS-derived sample from *A* (C18 column developed with 0.1% TFA in 2–16% acetonitrile). The indicated peaks were sequenced by Edman degradation. Peak 6 corresponds to residues 91–94 from CaM; peak 8 corresponds to residues 14–21; peak 10 is presumed to contain the blocked NH_2-terminus of CaM (20). (*C*) Reverse phase capillary HPLC of nondenatured iNOS (0.16-mm internal diameter C4 column developed with 0.1% TFA in 80% acetonitrile). The eluate passed directly into the mass spectrometer as described in reference 11. (*D*) Electrospray mass spectrum of the first peak in *C*. The spectrum was comprised of a series of ions consistent with CaM bearing from 9 to 14 positive charges, as indicated in parenthesis (*m/z* ratios for the +1 through +8 charge states of CAM would exceed, 2,000, the upper limit of observation). (*Inset*) The observed spectrum was collected and analyzed by Finnegan MAT's BIOMASS data reduction software, yielding a molecular weight of 16,791 (16,793 in a repeat study).

Discussion

Within their host cells, the two known types of NOS (1) produce NO over periods of time and in total quantities that differ by several orders of magnitude. Thus, iNOS and cNOS play strikingly different physiologic roles. This study reveals one aspect of the molecular basis for this dichotomy, namely, the surprising finding that CaM is a subunit of enzymatically active iNOS. CaM binds iNOS tightly but noncovalently, without a requirement for elevation of Ca^{2+} above the concentrations found in resting cells. The molecule associated with iNOS was definitively identified as CaM on the basis of its reactivity with anti-CaM mAb, migration on SDS-PAGE, retention time on reverse phase HPLC, tryptic map, partial amino acid sequence, and exact molecular mass as determined by electrospray ionization mass spectroscopy.

To our knowledge, only three other enzymes bind CaM constitutively, that is, in an apparently Ca^{2+}-independent manner. These are phosphorylase kinase (20, 22), a cyclic nucleotide phosphodiesterase (23), and the adenylyl cyclase of *Bordetella pertussis* (24). While the CaM associated with iNOS was abundant, we have not yet determined the stoichiometry of the complex, nor have we quantified the affinity of iNOS for CaM, since all the procedures we have devised to remove CaM from iNOS have denatured iNOS irreversibly.

Reactivity of the 130 kD region of electrophoregrams of pure iNOS with a highly specific anti-CaM mAb raised the possibility that iNOS might contain a covalently embedded CaM-like domain, as does a recently described kinase from soybean (25). This explanation was eliminated when we determined the nucleic acid and amino acid sequences of iNOS (11) Moreover, two different procedures abolished the ability of anti-CaM mAb to immunoblot iNOS, while preserving the reactivity of the mAb with CaM itself. Thus, there is no CaM-like epitope intrinsic to iNOS. It is difficult to explain why anti-CaM mAb reacted not only with the 16.8-kD species released from iNOS, but also with 130-kD iNOS monomers themselves. Two possibilities can be considered. First, the mAb may have fortuitously crossreacted with an epitope in iNOS other than CaM. This seems unlikely, since boiling iNOS in EDTA abolished its reactivity with anti-CaM mAb, while boiling iNOS in SDS did not. Thus, the putative crossreactive epitope would have to be sensitive to EDTA. Alternatively, a portion of the CaM associated noncovalently with iNOS may have persisted in its association after boiling in SDS and electrophoresis, treatments that normally disrupt noncovalent bonds (26). If so, it is surprising that the apparent mass of the iNOS-CaM complex was the same as the apparent mass of iNOS stripped of its CaM (compare Fig. 3 *A*, lane *1*, with Fig. 3 *B*, lane *3*). Perhaps a conformational change in the complex conferred anomalous electrophoretic behavior.

NOS purified from cells other than macrophages requires 200–400 nM added Ca^{2+} for half-maximal activity (reviewed in reference 1). Similarly, conventional CaM-enzyme complexes typically bind Ca^{2+} with dissociation constants of 400–1,100 nM (27, 28). These concentrations of Ca^{2+} exceed the levels in resting cells (70–100 nM) (8), so that the activity of most CaM-dependent enzymes is regulated by Ca^{2+} transients. In contrast, CaM associates with iNOS at Ca^{2+} concentrations in the range of 39 nM. Thus, CaM is likely to complex with iNOS during or promptly after the translation of iNOS mRNA, even in cells whose Ca^{2+} remains at the resting level. This may explain why iNOS appears to be tonically active once induced.

Mutation studies have shown that CaM can subserve some of its functions in yeast without binding Ca^{2+} (29). A CaM-dependent kinase in neuronal cells can become Ca^{2+} independent after undergoing phosphorylation (30). A neurospecific, nonenzymatic CaM-binding protein binds CaM more tightly in the absence than in the presence of free Ca^{2+} (31). Further work is necessary to determine whether the CaM-iNOS complex binds Ca^{2+}, and if so, whether Ca^{2+} binding plays a role in the formation, stabilization, and/or enzymatic activity of the complex.

In summary, the results of the present study suggest that divergent CaM-binding sequences in iNOS (11) and cNOS (14, 15) may result in radical differences in the regulation of these enzymes, and thus in their physiologic roles.

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