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 Ca2+ and
resultant binding of calmodulin (CaM). In contrast, NOS in macrophages does not require added
Ca2+ 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 add sequence, and exact molecular
mass of CaM. In contrast to most CaM-dependent enzymes, macrophage NOS binds CaM tightly
without a requirement for elevated Ca2+. This may explain why NOS that is independent of
Ca2+ and elevated CaM appears to be activated simply by being synthesized.
**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(16.8 kD) was visible upon staining with amido black (Fig 1, lane I), silver (12) or coomassie blue (H. Cho, et al., unpublished observations).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undetectable upon staining with amido black (Fig. 1, lane 2) or coomassie blue, and hard to stain by silver (H. Cho, unpublished observations).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 $5 \times 10^8$ 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.

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

![Association of CaM-like antigen with iNOS.](image1)

![Final step in the purification of iNOS (12) by TSK G3000SW size-exclusion chromatography (Pharmacia LKB, Piscataway, NJ).](image2)
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 antimouse IgG (A), or 1:1,000 sheep anti-rabbit IgG (B) (Boehringer Mannheim Biochemicals).
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 eNOS 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 spectrometry.

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 adenylly 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 neurenspecific, 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.

We are grateful to Dr. Joseph Graziano and Ms. Nancy Lolaceno (College of Physicians and Surgeons of Columbia University, New York) for atomic absorption photometry, and P. Davies, A. Williamson, J. Schmidt, M. Baum, and K. Elliston (Merck, Sharpe, & Dohme Research Laboratories) for advice. This work was supported by National Institutes of Health grant CA-43610.

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Received for publication 31 March 1992 and in revised form 5 May 1992.

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