Calmodulin-dependent Nitric-oxide Synthase
MECHANISM OF INHIBITION BY IMIDAZOLE AND PHENylimIDAZOLES*

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Calmodulin-dependent nitric-oxide synthase from bovine brain and GH3 pituitary cells is inhibited by imidazole, 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole, with half-maximal inhibition occurring at 200, 25, 160, and 600 μM concentrations of inhibitor, respectively. Imidazole inhibits the maximal velocity of citrulline formation by the enzyme, but does not alter the concentration of arginine, calmodulin, or (6R)-5,6,7,8-tetrahydro-L-biotin required for expression of half-maximal activity. Imidazole, 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole had no effect on calmodulin-dependent reduction of cytochrome c by the enzyme at concentrations up to 50-fold higher than those that inhibited citrulline formation. Imidazole inhibited calmodulin-dependent NADPH consumption by the enzyme with dissolved oxygen as the sole electron acceptor, with half-maximal inhibition occurring at a concentration of 225 μM. These observations are consistent with the proposal that imidazole and phenylimidazoles inhibit citrulline formation and oxygen reduction by acting as a sixth coordination ligand of the heme iron. This interaction prevents the formation of the activated reduced species of oxygen necessary for the formation of citrulline.

Nitric oxide is an effector molecule that mediates endothelium-dependent vascular relaxation and the actions of the excitatory amino acid glutamate in the central nervous system and contributes to the cytotoxic actions of macrophages (1). Nitric oxide is formed from L-arginine and molecular oxygen as the sole electron acceptor, with half-maximal inhibition occurring at a concentration of 225 μM. Recently, White and Marletta (13) have reported that the inducible nitric-oxide synthase purified from murine macrophages undergoes a spectral change in the presence of carbon monoxide consistent with the presence of a heme prosthetic group. Carbon monoxide was found to inhibit citrulline formation by both the inducible and the CaM-dependent enzymes.

While conducting studies of the chemical modification of bovine brain CaM-dependent nitric-oxide synthase, our group has observed that imidazole and phenylimidazoles inhibit citrulline formation by the enzyme. We describe here investigations of the mechanism of this inhibition and propose that these imidazoles exert their actions by binding to the heme iron of the enzyme in place of oxygen. These agents represent a new class of nitric-oxide synthase inhibitors acting by a novel mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—NADPH, imidazole, HEPES, and EGTA were obtained from Sigma. 1-Phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole were obtained from Aldrich. Calmodulin was purified from bovine brain by the procedure of Gopalakrishna and Anderson (14). THB was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). L-[2,3-3H]Arginine (40–70 Ci/mmol) was obtained from Du Pont-New England Nuclear.

Preparation of Nitric-oxide Synthase—GH3 cell nitric-oxide synthase was purified by elution from ADP-agarose as described previously (15). Bovine brain nitric-oxide synthase was prepared as detailed subsequently. Bovine brains obtained on ice within hours of slaughter (2 kg of tissue) were homogenized with a Waring blender at high speed for 1 min in 3 volumes of homogenization buffer containing 50 mM MOPS, pH 7.5, 1 mM EGTA, 1 mM diithiothreitol, and 0.2 mM diisopropyl fluorophosphate. The homogenate was centrifuged for 35 min at 13,000 x g using a Sorval GS-3 rotor. The supernatant (4 liters) was mixed with 2 liters of settled DEAE-cellulose pre-equilibrated with homogenization buffer and stirred with an overhead stirrer for 15 min. The suspension was filtered on a Buchner funnel, the filtrate was discarded, and the resin was washed with 1 volume of homogenization buffer diluted 1:1 with water. The resin was resuspended in wash buffer and packed into a 10 x 60-cm column to a height of 50 cm. The column was developed with a linear gradient generated from 3 liters of homogenization buffer without NaCl and 3 liters of homogenization buffer containing 0.4 M NaCl as limit buffer. Fractions (20 ml) were collected and assayed for nitric-oxide synthase activity in standard incubations. Fractions containing nitric-oxide synthase (eluting from 0 to 0.075 M NaCl) were pooled.

ADP-agarose (10 ml) was added to the pooled DEAE-cellulose eluate in a 1-liter polycarbonate bottle, and the suspension was rocked in the cold room for 1 h. The suspension was centrifuged at 2000 x g

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1 The abbreviations used are: CaM, calmodulin; THB, (6R)-5,6,7,8-tetrahydro-L-biotin; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
for 5 min; the resin was collected in a 40-ml conical centrifuge tube; the supernatants were discarded; and the suspension was washed twice with 30 ml of homogenization buffer containing 0.5 M NaCl, once with 30 ml of homogenization buffer containing 0.1% CHAPS and, finally with 15 ml of homogenization buffer containing 0.1% CHAPS and 50 µM NADPH. The eluate was concentrated with a Centriprep 30 device and either stored frozen at -70 °C or subjected to chromatography on Affi-Gel-calmodulin.

The NADPH eluates from routine preparations exhibited specific activities ranging from 0.3 to 0.6 µmol of citrulline formed per min/mg of protein and represented a 2000-4000-fold purified preparation with respect to its original enzyme fraction. This preparation of enzyme, which was used for the kinetics presented in this report, could be stored at -70 °C for up to 2 months with no detectable loss of activity.

To obtain homogeneous nitric-oxide synthase, the NADPH eluate from ADP-agarose was adjusted with CaCl2 to contain 5 µM free Ca2+, and applied to a 0.9 x 2-cm column of Affi-Gel-calmodulin (2 mg of CaM/ml of gel) equilibrated with homogenization buffer adjusted to 8 µM Ca2+. The column was washed with equilibration buffer containing calcium until A280 nm values <0.01 were obtained. The column was then washed with 25 mM MOPS, pH 7.5, containing 2.5 mM EGTA. Fractions containing protein (A280 nm) were pooled, concentrated, and dialyzed against 10 mM HEPES, 100 mM NADPH, 2 µM CaM (unless otherwise indicated), and 100 µM THB (unless otherwise indicated). In measurements of the arginine concentration dependence of activity, unlabeled arginine was present at the indicated concentrations. All incubations were conducted at 30 °C for 30 min. Citrulline formation was calculated from the known specific activity of arginine. All incubations were conducted in duplicate, and the mean values were calculated. The formation of citrulline by the enzyme under these conditions, was found to be inhibited by imidazole, with half-maximal inhibition observed at 200 µM imidazole. Nitric-oxide synthase has recently been reported to be a hemoprotein (13). Imidazoles of diverse structure have been observed to inhibit the activity of hemoproteins such as aldrid epoxidase with potencies that reflect their association with the heme component as revealed by spectroscopic perturbations. Accordingly, we decided to examine the effect of 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole on citrulline formation by the enzyme. All three phenylimidazoles inhibited citrulline formation by nitric-oxide synthase (Fig. 1B), but with significantly different apparent potencies. Under the measurement conditions, IC50 values of 25, 160, and 640 µM were observed for 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole, respectively.

Reversibility of Effect of Imidazole Drugs—To assure that the effects of imidazoles on citrulline formation were reversible, nitric-oxide synthase was preincubated for 30 min with

**RESULTS**

**Effect of Diverse Imidazoles on Activity of Bovine Brain Nitric-oxide Synthase Activity**—Our initial observation regarding the effect of imidazole on nitric-oxide synthase activity emerged during chemical modification studies using diethyl pyrocarbonate, an agent that acylates the nitric-oxide synthase region of histidine residues within proteins (18). The use of imidazole to terminate the acylation after treatment of nitric-oxide synthase with diethyl pyrocarbonate for specified time intervals revealed that imidazole directly inhibited the formation of citrulline by the enzyme. Accordingly, the effect of increasing concentrations of imidazole on nitric-oxide synthase was determined (Fig. 1A). Citrulline formation by bovine brain CaM-dependent nitric-oxide synthase was found to be inhibited by imidazole, with half-maximal inhibition observed at 200 µM imidazole. Nitric-oxide synthase has recently been shown to be a hemoprotein (13). Imidazoles of diverse structure have been observed to inhibit the activity of hemoproteins such as aldrid epoxidase with potencies that reflect their association with the heme component as revealed by spectroscopic perturbations. Accordingly, we decided to examine the effect of 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole on citrulline formation by the enzyme. All three phenylimidazoles inhibited citrulline formation by nitric-oxide synthase (Fig. 1B), but with significantly different apparent potencies. Under the measurement conditions, IC50 values of 25, 160, and 640 µM were observed for 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole, respectively.

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**DISCUSSION**

The homogeneous preparation catalyzed both NADPH-dependent citrulline formation from arginine and an NADPH-dependent oxidation of cytochrome c. Both activities were stimulated >20-fold by the concurrent presence of Ca2+ and CaM in the incubation. Nitric-oxide synthase-mediated NADPH consumption was eliminated in incubations with diethyl pyrocarbonate, an agent that acylates the nitric-oxide synthase region of histidine residues within proteins (18). The use of imidazole to terminate the acylation after treatment of nitric-oxide synthase with diethyl pyrocarbonate for specified time intervals revealed that imidazole directly inhibited the formation of citrulline by the enzyme. Accordingly, the effect of increasing concentrations of imidazole on nitric-oxide synthase was determined (Fig. 1A). Citrulline formation by bovine brain CaM-dependent nitric-oxide synthase was found to be inhibited by imidazole, with half-maximal inhibition observed at 200 µM imidazole. Nitric-oxide synthase has recently been shown to be a hemoprotein (13). Imidazoles of diverse structure have been observed to inhibit the activity of hemoproteins such as aldrid epoxidase with potencies that reflect their association with the heme component as revealed by spectroscopic perturbations. Accordingly, we decided to examine the effect of 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole on citrulline formation by the enzyme. All three phenylimidazoles inhibited citrulline formation by nitric-oxide synthase (Fig. 1B), but with significantly different apparent potencies. Under the measurement conditions, IC50 values of 25, 160, and 640 µM were observed for 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole, respectively.

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**FIG. 1. Effect of imidazole (A) and 1-phenylimidazole, 2-phenylimidazole, and 4-phenylimidazole (B) on citrulline formation by bovine brain nitric-oxide synthase.** Standard incubations were conducted as described under "Experimental Procedures" with the indicated concentrations of imidazole (A, O) and 1-phenylimidazole (B, @), 2-phenylimidazole (B, □), or 4-phenylimidazole (B, △). Incubations were initiated with 4.6 µg of nitric-oxide synthase, and citrulline formation was measured as described under "Experimental Procedures." Values are expressed as the percentage of control values (4 nmol/min/mg) of incubations containing no added inhibitor.
Imidazole, 1-phenylimidazole, 2-phenylimidazole, or 4-phenylimidazole at concentrations five times their IC₅₀ values as determined when present in normal incubations. Samples of the preincubated enzyme were used to initiate standard incubations already in progress, imidazole produced an immediate inhibition of citrulline formation. The rate of citrulline formation was assessed as described under "Experimental Procedures." Activity is expressed as micromoles of citrulline formed per minute/milligram of protein. The data are plotted in double-reciprocal format.

Effect of Imidazole on Kinetic Properties of Nitric-oxide Synthase—To explore the mechanism by which imidazole interferes with citrulline formation by nitric-oxide synthase, we examined its effect on the kinetic behavior of the enzyme. The arginine concentration dependence of citrulline formation was examined in incubations with or without either 200 or 600 μM imidazole (Fig. 2). Imidazole was found to inhibit citrulline formation noncompetitively versus arginine substrate, decreasing the maximal velocity, but not altering the concentration of arginine providing half-maximal expression of activity (apparent Kₐ).

The CaM concentration dependence of citrulline formation by nitric-oxide synthase was examined in incubations with or without either 300 or 1000 μM imidazole (Fig. 3). Imidazole decreased the maximal velocity of citrulline formation, but did not alter the CaM concentration dependence of activity.

The activity of CaM-dependent nitric-oxide synthase prepared from brains of different mammalian species and by different procedures has been reported to be stimulated by (6R)-5,6,7,8-tetrahydro-L-biopterin to widely different degrees (2, 6, 20). These differences appear to emerge from the observation that THB may dissociate to different extents from a high affinity binding site during enzyme preparation.

In our laboratory, different preparations of the bovine brain enzyme have been found to vary in responsiveness to added THB, from unresponsive to as much as 4-fold stimulated. Recently, our group has identified and characterized a CaM-dependent nitric-oxide synthase from GH₂ pituitary adenoma cells (15). The enzyme isolated from GH₂ cells exhibits activity stimulated from 8- to 50-fold by THB. Due to the marked dependence on THB of the GH₂ enzyme, we decided to examine the effect of imidazole on the THB concentration dependence of citrulline formation by this enzyme (Fig. 4). Imidazole decreased the maximal velocity of citrulline formation, but did not greatly alter the concentration of THB (10 μM) providing half-maximal stimulation of activity.
both a reductase and an oxygenase domain (13). These properties resemble closely those of cytochrome P-450BM.3, an unusually self-sufficient mammalian P-450 enzyme, possessing cytochrome-c reductase activity and possessing extensive homology to both rat and phenylimidazoles. The two domains can be separated by limited proteolysis (22). 

The recent identification of nitric-oxide synthase as a hemoprotein has led to the proposal that this enzyme is a catalytically self-sufficient mammalian P-450 enzyme, possessing both a reductase and an oxygenase domain (13). These properties resemble closely those of cytochrome P-450BM.3, an omega-2 fatty acid hydroxylase found in the cytoplasm of Bacillus megaterium (22–24). Cytochrome P-450BM.3 is a catalytically self-sufficient enzyme containing 1 mol of heme and 1 mol each of bound FAD and FMN. The protein is divided into two discrete domains, one of which contains a heme group, whereas the second domain contains FAD and FMN. The two domains can be separated by limited proteolysis (22). The flavin-containing domain exhibits cytochrome-c reductase activity and possesses extensive homology to both rat liver cytochrome-P-450 reductase and the carboxyl-terminal region of the CaM-dependent nitric-oxide synthase (Refs. 24 and 7, respectively). The work of Vermillion et al. (25) has demonstrated that for rat liver cytochrome-P-450 reductase, the sequence of electron transfer is NADPH to FAD to FMN. The reduced FMN may then reduce the heme-iron of either cytochrome c or cytochrome P-450. Presumably, a similar electron transfer sequence operates for nitric-oxide synthase.

The CaM-dependent nitric-oxide synthase possesses CaM-dependent cytochrome-c reductase activity (15, 21). Both imidazole and phenylimidazoles were without effect on the CaM-dependent reduction of cytochrome c catalyzed by nitric-oxide synthase. This observation is consistent with the proposal that these imidazoles act at a site distal to the reduction of FMN.

Imidazole and phenylimidazoles have been observed to bind to diverse cytochrome P-450 isoforms (26–28). They interact with the heme moiety, producing a Type II difference spectrum (27). 1-Phenylimidazole has been found to bind to the heme, with an association constant several orders of magnitude higher than that observed for the 2- or 4-substituted imidazoles (26). These observations have been interpreted to indicate that both cytochrome P-450 binding and inhibition depend on the accessibility of the noabonded electrons on the nitrogen atom at position 3 of the imidazole ring. These electrons are necessary for imidazoles to act as a sixth coordination ligand for the heme iron. The marked specificity of the 1-phenyl-substituted imidazole compounds in binding to the heme iron has been confirmed using 1-(4-azido-phenylimidazole) (28). This agent has been used successfully as a photosaffinity ligand to derivatize the heme-binding site of diverse hemoproteins (28–30). We note that the inhibition of citrulline formation (IC50) by 1-phenylimidazole occurred at a concentration lower by a factor of 25 than that observed for inhibition by 4-phenylimidazole and at a concentration lower by a factor of 7 than that observed for 2-phenylimidazole. Thus, the order of potency for inhibition of citrulline formation by nitric-oxide synthase is the same as that observed previously for binding to and inhibition of hemoproteins (26).

Porcine brain CaM-dependent nitric-oxide synthase has been shown to exhibit a Ca2+- and CaM-dependent consumption of NADPH in the absence of added electron acceptor (8). NADPH consumption was accompanied by consumption of molecular oxygen from solution as revealed using an oxygen-sensitive electrode. These consumptions were not dependent on the presence of either arginine or THB. Under these conditions, hydrogen peroxide formation was observed, which was reduced in the presence of arginine and abolished when THB was additionally present. These observations support the contention that the generation of a reduced and activated oxygen intermediate occurs independent of the involvement of THB and l-arginine in the mechanism of citrulline formation. Our laboratory has confirmed that CaM-dependent consumption of NADPH in the absence of added electron acceptor is linked to the availability of dissolved oxygen since purging the incubation mixtures with nitrogen eliminated the activity. Activity could be restored by subsequent re-equilibration with air. Imidazole inhibits citrulline formation in a manner noncompetitive with the arginine substrate (Fig. 2) and noncompetitive with THB (Fig. 4), indicating that imidazoles do not interfere with the binding of either of these substances to the enzyme. Imidazole was found to inhibit CaM-dependent NADPH consumption (linked to oxygen reduction), with an apparent $K_c$ (IC50) virtually identical to that observed for inhibition of citrulline formation. These observations are consistent with the proposal that imidazole and,

**DISCUSSION**

The CaM-dependent nitric-oxide synthase possesses CaM-dependent nitric-oxide synthase activity linked to oxygen reduction (15, 27). Both imidazole and phenylimidazoles were without effect on the CaM-dependent reduction of cytochrome c catalyzed by nitric-oxide synthase. This observation is consistent with the proposal that these imidazoles act at a site distal to the reduction of FMN.

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**FIG. 5. Effect of imidazole on CaM-dependent consumption of NADPH by bovine brain nitric-oxide synthase.** Standard incubations of 1 ml were constructed in quartz cuvettes containing 100 mM HEPES, pH 7.5, 50 μM NADPH, 8 μM free Ca2+, 6 μM CaM, and the indicated concentrations of imidazole. Incubations were initiated with 90 μg of bovine brain nitric-oxide synthase. NADPH consumption was assessed from the change of absorbance at 340 nm. Values are expressed as the percentage of control values (1.7 nmol/min/mg) of incubations containing no added imidazole.
presumably, the phenylimidazoles act to inhibit citrulline formation by occupying the sixth coordination site of the nitric-oxide synthase heme iron. The binding of the imidazoles to the site inhibits the binding of oxygen, thus preventing its conversion to the reduced activated species necessary for citrulline formation.

Addendum—Since the original submission of this manuscript, several laboratories have reported that both the CaM-dependent and inducible nitric-oxide synthases are hemoproteins (19, 31, 32).

REFERENCES