Selective Inhibition of Constitutive Nitric Oxide Synthase by L-N^G-Nitroarginine

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Received April 8, 1993; Revised Manuscript Received May 14, 1993

ABSTRACT: L-N^G-Nitroarginine (NA) inhibited both the L-arginine oxidation and the L-arginine-independent NADPH oxidation reactions catalyzed by the calcium/calmodulin-dependent constitutive nitric oxide synthase (cNOS) from bovine brain. NA binding did not require calmodulin, calcium, or NADPH. The onset of inhibition was slow with a second-order association rate constant (k_{on}) of 4.4×10^4 M^{-1} s^{-1}. The dissociation rate constant (k_{off}) was 6.5×10^{-4} s^{-1}. The K_d value (k_{off}/k_{on}) of bovine brain cNOS for NA was 15 nM. L-Arginine was a competitive inhibitor of NA binding with a K_s value of 0.8 μM. The K_m for L-arginine in the cNOS reaction was 1.2 μM. The NA binding sites of cNOS were titrated with NA, which enabled a k_{cat} of 0.7 s^{-1}, for the oxidation of L-arginine, to be calculated. Finally, a brain cNOS–(^{3}H)NA complex was isolated. In contrast to the potent and slow onset of NA inhibition of brain cNOS, NA inhibition of inducible mouse macrophage NOS (iNOS) was weaker (K_i = 4.4 μM) and rapidly reversible. Thus, NA was a 300-fold more potent inhibitor of bovine brain cNOS than mouse macrophage iNOS.

Nitric oxide (NO)[1] is involved in diverse biological functions including regulation of vascular relaxation, long-term potentiation in neurons, penile erection, and the cytotoxic action of macrophages [reviewed by Nathan (1992); Moncada et al., 1991; Dawson et al., 1992]. Nitric oxide synthase (NOS) catalyzes the formation of NO, L-citrulline, and $NADP^+$ from L-arginine, NADPH, and molecular oxygen. NOS also catalyzes the oxidation of NADPH in the absence of L-arginine (Mayer et al., 1991; Dawson et al., 1991; Schmidt et al., 1992).

The two major classes of NOS include constitutively expressed isozymes and inducibly expressed isozymes [reviewed by Stuehr and Griffith (1992) and Forstermann et al. (1991)]. Constitutive NOS is found in the vascular endothelium and in the brain. Both brain and endothelial cNOS are regulated (activated) by calcium and calmodulin; however, they are structurally distinct. The brain enzyme is approximately 150 kDa, and the endothelial enzyme is 135 kDa. Furthermore, the brain enzyme is soluble, whereas the endothelial enzyme is myristoylated (Pollock et al., 1992) and has been found in the particulate fraction of cell extracts. Inducible NOS is found in smooth muscle, macrophages, and liver (Evans et al., 1992; Iida et al., 1992) and is transcriptionally regulated (Xie et al., 1992; Lorsbach et al., 1993; Geller et al., 1993). Although the macrophage and liver enzymes are not regulated by exogenous calcium or calmodulin *in vitro*, they are tightly associated with calmodulin (Cho et al., 1992; Lowenstein et al., 1992; Iida et al., 1992; Xie et al., 1992). One distinguishing feature between the inducible and constitutive enzymes is the difference in Ca^{2+}/calmodulin requirements.

Several NOS cofactor requirements have been identified [reviewed by Stuehr and Griffith (1992)]. The enzyme contains tetrahydrobiopterin, FAD, FMN, and a heme iron (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; Klatt et al., 1992), analogous to those cofactor requirements of cytochrome P450 reductase. In addition, the amino acid sequence of cNOS is homologous to that of cytochrome P450 reductase.

Several L-arginine analogues are inhibitors of NOS, including L-N^G-methylarginine (MA) and L-N^G-nitroarginine (NA) [reviewed by Stuehr and Griffith (1992)]. MA inhibition of mouse macrophage iNOS has been extensively characterized (Pufahl et al., 1992; Feldman et al., 1993). MA is a competitive inhibitor and an inactivator of mouse macrophage iNOS, although no iNOS–MA complex has been isolated. Although the mechanism of NA inhibition is not as well understood, NA has been reported to be an irreversible inhibitor of brain cNOS and reversible for macrophage iNOS (Dwyer et al., 1991). Herein, we further characterize the mechanism of NA inhibition of brain cNOS and macrophage iNOS.

MATERIALS AND METHODS

Materials. Calmodulin (from bovine brain), NADPH, FAD, FMN, adenosine 2′,5′-diphosphate (ADP) agarose resin, calmodulin agarose resin, L-arginine, and L-citrulline were from Sigma (St. Louis, MO), and (6*R*)-5,6,7,8-tetrahydrobiopterin was from B. Schirks (Jona, Switzerland). Centricon 30 microconcentrators were from Amicon. L-N^G-Nitroarginine was from Aldrich (Milwaukee, MI) and [^{3}H]-L-NA from Amersham Life Science (Arlington Heights, IL). [2,3-^{3}H]-L-Arginine and [^{14}C-U]-L-arginine were from Du Pont, New England Nuclear (Beverly, MA), and Sephadex G-50 (DNA grade, fine), adenosine 2′,5′-diphosphate (ADP) Sepharose resin from Pharmacia (Piscataway, NJ). The LiChroCART 250-4 HPLC column (4 × 250 mm, C_{18} resin) was from EM Science (Gibbstown, NJ). Ultrafree MC devices were from Millipore (Bedford, MA), and Scintiverse BD was from Fisher Scientific.

Enzyme Purification. Bovine brain cNOS was prepared according to the procedures of Schmidt et al. (1990), Mayer

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[1] Abbreviations: bovine serum albumin (BSA); ethylenediaminetetraacetic acid (EDTA); nitric oxide synthase (NOS); constitutive NOS (cNOS); inducible NOS (iNOS); L-N^G-nitroarginine (NA); L-N^G-methylarginine (MA); nicotinamide adenine dinucleotide 3′-phosphate reduced form (NADPH); flavin dinucleotide (FAD); flavin mononucleotide (FMN); *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), dithiothreitol (DTT); trifluoroacetic acid (TFA); sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

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et al. (1991), and Bredt and Snyder (1990). All purification steps were at 4 °C. Briefly, three fresh whole bovine brains (1050 g) were homogenized in a Waring blender for 1 min with 2.6 L of cold buffer A [50 mM HEPES, pH 7.5 (pH at 25 °C), 0.5 mM EDTA, 10 mM DTT). The mixture was centrifuged at 13000*g* for 1 h, and the supernatant fluid was removed (2050 mL). Solid ammonium sulfate (365 g, 30% of saturation) was added to the supernatant fluid and stirred slowly for a total of 30 min. The precipitate was collected by centrifugation at 13000*g* for 30 min and was resuspended in 400 mL of buffer A with 4 μM tetrahydrobiopterin, 1 μM FAD, and 1 μM FMN. The solution was centrifuged at 41000*g* for 60 min, and the supernatant fluid was removed, frozen in liquid nitrogen, and stored at −70 °C. After thawing, the supernatant fluid was passed through an ADP–agarose resin column (0.4 g of resin swelled and equilibrated in buffer A) at 4 mL/min. The column was washed with 100 mL of buffer A, 200 mL of buffer A with 500 mM NaCl, 100 mL of buffer A, and, to elute the enzyme, 40 mL of buffer A with 5 mM NADPH. The enzyme solution was made to 15% glycerol, 1 mM $CaCl_2$, 10 μM tetrahydrobiopterin, 0.1% Tween, 1 μM FAD, and 1 μM FMN. The enzyme was absorbed to a 1-mL column of calmodulin agarose resin that was equilibrated in buffer A in 15% glycerol and 1 mM $CaCl_2$. The column was washed with 15 mL of buffer A in 15% glycerol and 1 mM $CaCl_2$ and 15 mL of buffer A in 15% glycerol and 5 mM EDTA, and the enzyme was eluted with 3 mL of buffer A in 15% glycerol, 5 mM EDTA, and 1 M NaCl. The enzyme solution was made to 10 μM tetrahydrobiopterin, 1 μM FAD and FMN, and 0.1% Tween. This solution was concentrated by Centricon 30 to a volume of approximately 700 μL. Typically, 1.8 mg of enzyme with a specific activity of 70 nmol min^{-1} mg^{-1} was isolated and was homogeneous as determined by SDS–PAGE (Laemmli, 1970).

Mouse macrophage iNOS was purified from RAW 264.7 mouse macrophages according to the procedure of Stuehr et al. (1988), and all steps were at 4 °C. Briefly, a crude extract was applied to adenosine 2′,5′-diphosphate (ADP) Sepharose resin equilibrated in TGDB (20 mM Tris, pH 7.5, 10% glycerol, 1 mM DTT, and 2 μM tetrahydrobiopterin). The resin was sequentially washed with TGDB, 0.5 M NaCl in TGDB, and TGDB. iNOS was eluted with 2 mM NADPH in TGDB. BSA was immediately added to a final concentration of 1 mg/mL. The enzyme was purified approximately 100-fold over the crude extract and had a specific activity of 185 nmol mg^{-1} min^{-1} at 37 °C.

Enzyme Assay. Two enzyme activities were measured. The oxidation of L-arginine was monitored by the conversion of [^{3}H]- or [^{14}C]-L-arginine to L-citrulline as described by Schmidt et al. (1991) which separates L-citrulline from L-arginine by Dowex 50X8-200 (Na) chromatography. Typical reaction mixtures (100 μL) contained 50 mM HEPES, pH 7.0, 8 μM tetrahydrobiopterin, 1 mM $CaCl_2$, 0.01 mg/mL calmodulin, 0.5 mM EDTA, 0.4–50 μM [^{14}C]-L-arginine (30 000 cpm), and 100–200 μM NADPH. The cNOS-catalyzed oxidation of NADPH to $NADP^+$ was monitored by the reduction of absorbance at 340 nm with a Kontron 860 spectrophotometer in a volume of 300 μL. All reactions were at 30 °C unless otherwise indicated.

Protein Concentration. Protein concentration was determined according to the method of Bradford (1976) with γ-globulin as a standard.

Isolation of cNOS–NA by Sephadex G-50 Chromatography. A G-50 column 55 × 5 mm was equilibrated in 50 mM HEPES, pH 7.5, 0.5 mM EDTA, 10% glycerol, 10 mg/mL BSA, 10 mM DTT, and 0.1% Tween. Because cNOS required 10 mg/mL BSA in the chromatography buffer for stability, the enzyme could not be detected by a protein assay. Therefore, the elution volume of the enzyme was determined by monitoring catalytic activity. Enzyme (17 μL, 4.4 μM) was mixed with 6 μL of water or 6 μL of [^{3}H]-NA solution (15 μM, 10 000 cpm/μL) and 37 μL of column buffer and incubated at room temperature for 15 min. Enzyme or enzyme–inhibitor solution (50 μL) applied to the column was eluted with 2.4 mL of buffer. The eluant was collected in 200-μL fractions. A 5-μL aliquot of the column fractions was assayed for enzymatic activity with 5 μM L-arginine for 4 min at 37 °C, and a 100-μL aliquot of the fractions was counted for [^{3}H]-NA in 5 mL of Scintiverse BD.

C_{18} Chromatography of [^{3}H]-NA That Dissociated from Reactivated cNOS. In a volume of 5 μL, 2.1 μM cNOS and 3 μM [^{3}H]-NA (690 000 cpm) were incubated at 25 °C for 5 min and then diluted 1:200 as described in the legend of Figure 4 (except that L-arginine was not radiolabeled). This mixture was allowed to incubate at 30 °C for 80 min and was then ultrafiltered at 4 °C by an Ultrafree MC (10 000 MW cutoff) ultrafiltration device. The ultrafiltered solution was diluted 1:1 with 0.6% TFA and 0.1 mM NA, and a 100-μL aliquot was applied to a LiChroCART 250-4 HPLC column (4 × 250 mm, C_{18} resin) and eluted with 0.1% TFA in H_2O. Unlabeled NA was monitored by its UV absorbance at 210 nm. Retention times for NA and L-arginine were 5.8 and 3.5 min, respectively.

Equations for Interpretation of Kinetic Data. The parameters of the competitive inhibition model (eq 1) were fitted to the initial velocity data from the inhibition of product (L-citrulline) formation to obtain K_i values.

$$V = V_{max}[S]/\left[[S] + K_m\left(1 + \frac{[I]}{K_i}\right)\right] \quad (1)$$

The binding of an inhibitor (I) to an enzyme (E) by the mechanism

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}I \quad (2a)$$

is a first-order process with a first-order rate constant k_{obs}

$$k_{obs} = k_{on}[I] + k_{off} \quad (2b)$$

If an inhibitor and substrate bind to the same site, and substrate binding is in rapid equilibrium, then the substrate will reduce the rate constant for the onset of inhibition (k_{obs}) as defined by

$$k_{obs} = k'_{obs}/[1 + ([S]/K_s)] \quad (3)$$

where [S] is the concentration of substrate, K_s is the inhibition constant for reduction of k_{obs}, and k'_{obs} is k_{obs} with no competing substrate ([S] = 0). Further, the association rate constant (k_{on}) is given by

$$k_{on} = k_{on\text{-}app}[1 + ([S]/K_s)] \quad (4)$$

where $k_{on\text{-}app}$ is the apparent association rate constant determined at a defined concentration of competing substrate ([S]).

For an enzyme binding to a competitive inhibitor, F (the fraction of bound enzyme) at a defined [I] (concentration of inhibitor) can be estimated by 1 minus the fraction activity remaining at that [I]. If [E] (the concentration of enzyme) is much larger than K_d (the dissociation constant) for E·I, then [E] and K_d can be estimated by fitting the parameters

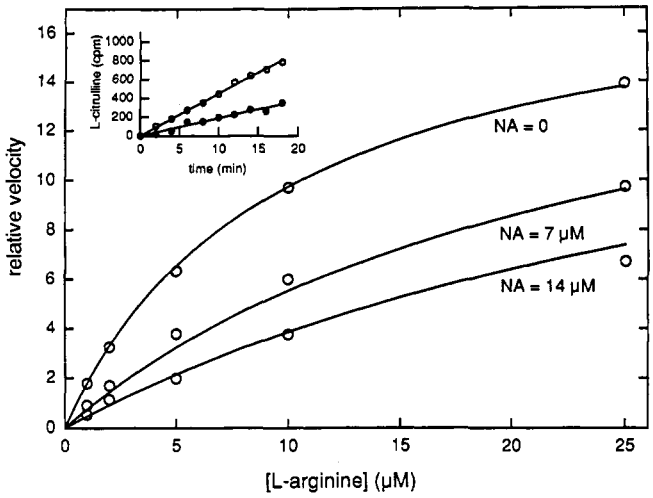

FIGURE 1: Inhibition of mouse macrophage iNOS by NA expressed as relative velocities of L-citrulline production vs the concentration of L-arginine (1–25 μM) at the indicated NA concentrations. The solid line depicts the best fit of the parameters in eq 1 (competitive inhibition model) to the data. (Inset) Progress curve for the mouse macrophage iNOS catalyzed formation of L-citrulline without inhibitor (open circles) or with 10 μM NA (solid circles) at 0.4 μM [^{14}C]-L-arginine in 25 μL.

of eq 5 to F and [I].

$F =$

$$[[I] + [E] + K_d - \{([I] + [E] + K_d)^2 - 4[I][E]\}^{1/2}]/2[E] \quad (5)$$

The parameters of eq 6 were fit to product formation during dissociation of enzyme–inhibitor complex to active enzyme

$$P(t) = Ae^{-k_{off}(t)} + Bt \quad (6)$$

where $P(t)$ was the amount of L-citrulline (product) formed at time t, A was the amplitude of the lag in product formation, B was the final (steady-state) rate of product formation, and k_{off} was the dissociation rate constant. The activity of the uninhibited enzyme decreased over the course of the reactivation experiment. The first-order rate constant for the decay of enzymatic activity (k_{decay}) was $(1.2 \pm 0.1) \times 10^{-4}$ s^{-1}. It was assumed that the inhibited enzyme was similarly unstable. Therefore, the data for both the inhibited and uninhibited enzyme reactions were normalized by k_{decay}, which linearized the data from the uninhibited reaction.

Statistical Analysis. The constants defined by the linear equations were determined by standard or weighted linear regression analysis. The constants defined by the nonlinear equations were estimated with Sigma Plot (Jandel Scientific, Corte Madera, CA). Error estimates were from the error matrix generated during the fitting routine. Error estimates for values calculated from fitted values (for example, the calculated K_d value) were determined by the propagation of error analysis (Bevington, 1969).

RESULTS

NA inhibition of the mouse macrophage iNOS-catalyzed conversion of L-arginine to L-citrulline was rapidly reversible (Figure 1). NA inhibition was competitive with L-arginine with a K_i value of 4.4 ± 0.4 μM and a K_m for L-arginine of 9.5 ± 0.9 μM (Figure 1). A 25-min incubation of macrophage iNOS with 10 μM NA, prior to assaying, did not increase the inhibition (data not shown). This result further indicated that the onset of inhibition was rapid.

In contrast to the rapid binding of NA to macrophage iNOS, NA apparently bound slowly to brain cNOS. The first-order

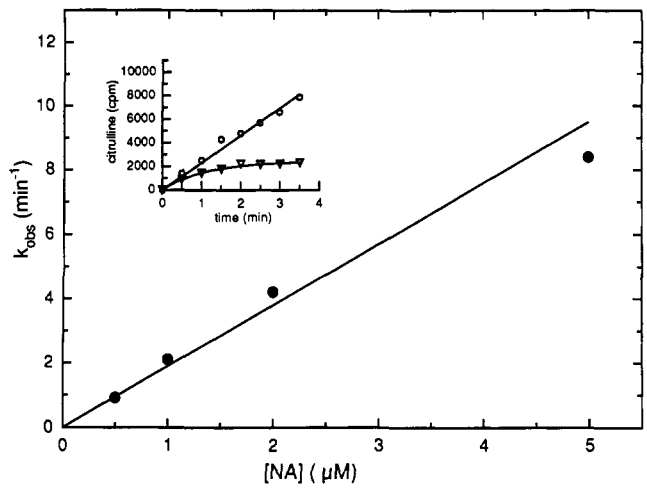

FIGURE 2: Dependence of k_{obs} for NA inhibition on the concentration of NA. k_{obs} for the time-dependent inhibition of cNOS was determined at the indicated NA concentrations. The solid line is the best fit of the parameters in eq 2b to the data. (Inset) The progress curve for L-citrulline formation with 0.4 μM [^{14}C]-L-arginine, 100 μM NADPH, and 0 (circles) or 0.5 μM (triangles) NA in 100 μL.

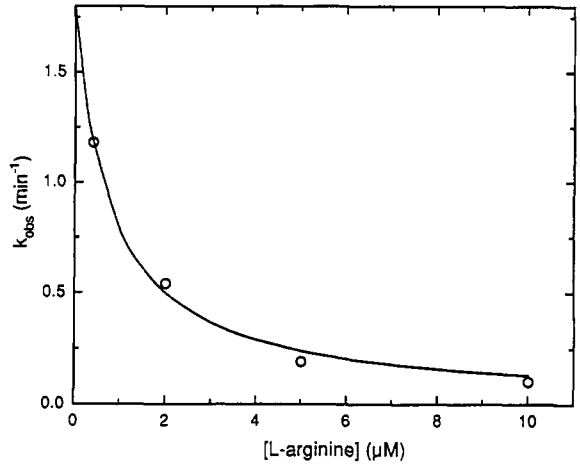

FIGURE 3: Effect of L-arginine on k_{obs} for NA inhibition of cNOS. Assay mixtures contained 100 μM NADPH, 0.5 μM NA, and 0.4–10 μM [^{14}C]-L-arginine. The solid line depicts the best fit of the parameters in eq 3 to the data.

rate constant (k_{obs}) for the onset of inhibition of the conversion of L-arginine to L-citrulline by 0.5 μM NA was 0.015 ± 0.002 s^{-1} (Figure 2). No significant difference in the k_{obs} values for NA inhibition of cNOS was observed when the pH was varied between 6.5 and 8 (data not shown). k_{obs} was linearly dependent on the concentration of NA. Thus, the apparent association rate constant for NA, determined at an L-arginine concentration of 0.4 μM, was $(2.9 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (Figure 2). If L-arginine and NA bound to the same site on cNOS, then L-arginine should competitively inhibit binding of NA with a K_s value equal to its K_m value as a substrate. L-Arginine reduced k_{obs} with a K_s (eq 3) value of 0.8 ± 0.2 μM (Figure 3) that was similar to the K_m value of 1.2 ± 0.2 μM determined by steady-state kinetic analysis. The association rate constant (k_{on}) for NA of $(4.4 \pm 0.4) \times 10^4$ M^{-1} s^{-1} was calculated from the apparent association rate constant at 0.4 μM L-arginine, the K_s for L-arginine, and eq 4.

cNOS catalyzes the oxidation of NADPH in the absence of L-arginine (Mayer et al., 1991; Schmidt et al., 1992). The rate of enzymatic NADPH oxidation was approximately 1.5-fold greater in the absence of L-arginine than it was in the presence of 100 μM L-arginine (data not shown). Greater

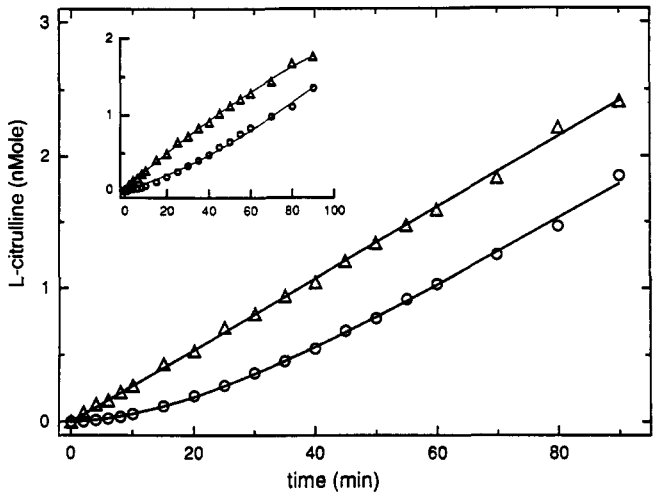

FIGURE 4: Dissociation rate constant for NA and cNOS expressed as progress curves for L-citrulline formation in a 100-μL reaction, 50 μM [^{14}C]-L-arginine, and 200 μM NADPH. cNOS (2.1 μM NA binding sites) was incubated with 5 μM NA (circles) or without NA (triangles) for 10 min at room temperature and then diluted 1:200 into an assay mixture with 50 μM L-arginine. The main figure data are the inset (actual) data normalized as described under Materials and Methods.

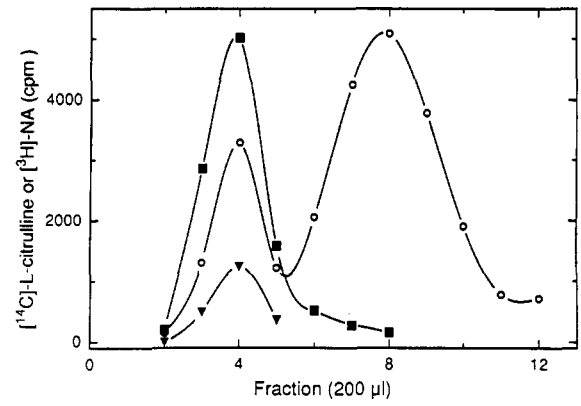

FIGURE 5: Isolation of a cNOS–NA complex by Sephadex G-50 chromatography. Procedure was as described under Materials and Methods. Solid circles and triangles are control and inhibited enzymatic activity, respectively. Open circles are [^{3}H]-NA.

than 90% of the enzyme-catalyzed NADPH oxidation could be inhibited by NA in the absence of L-arginine. k_{on} for NA was determined to be $(3.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} by monitoring cNOS-catalyzed NADPH oxidation. This k_{on} value was similar to the value of $(4.4 \pm 0.4) \times 10^4$ M^{-1} s^{-1} that was determined from the inhibition of L-citrulline formation.

NA binding sites of cNOS were titrated in the absence of NADPH, calcium, and calmodulin. cNOS (0.6 mg/mL) and 0.1–1.25 μM NA were equilibrated for 10 min at 25 °C, in a volume of 10 μL, to inhibit the enzyme. Enzymatic activity was assayed as described under Materials and Methods with 5 μM L-arginine as substrate for 2 min. The parameters of eq 5, which gave E_{total} and K_d, were fit to the fraction of enzyme inhibited and the concentration of NA. The concentration of enzyme was determined to be 1.1 ± 0.1 μM, and the K_d was 10 ± 20 nM. This method was primarily used to determine the concentration of cNOS. An accurate determination of K_d by this method would require a significant amount of free NA. However, in this experiment, the concentration of cNOS was considerably above the K_d, and most of the NA was bound to the enzyme when the concentration of NA was less than the concentration of enzyme. The binding of NA to cNOS did not require NADPH or Ca^{2+}/calmodulin. Since L-arginine was competitive with NA, NA binding sites were assumed to be equal to cNOS active sites. A k_{cat} value of 0.7 ± 0.1 s^{-1} for L-citrulline formation was obtained from the concentration of cNOS and V_{max} for L-citrulline formation.

NA inhibition was slowly reversible. cNOS catalyzed the formation of L-citrulline at a constant rate in the absence of NA (Figure 4). However, when cNOS was incubated with NA prior to initiating catalysis with saturating L-arginine, the rate of L-citrulline formation was initially zero but accelerated to the final steady-state rate of the uninhibited reaction as NA dissociated from cNOS (Figure 4). The apparent first-order rate constant of $(6.5 \pm 0.5) \times 10^{-4}$ s^{-1} for the acceleration to the steady-state rate was the dissociation rate constant (k_{off}) for NA from cNOS (eq 6). The K_d value calculated from k_{off}/k_{on} was 15 ± 2 nM.

To determine whether NA was modified by cNOS, enzyme was incubated with [^{3}H]-NA and allowed to reactivate for 80 min (over 3 half-lives) as described above. All of the radioactive material derived from the reactivated cNOS mixture that was chromatographed on a C_{18} column eluted with the same retention time as authentic NA (see Materials and Methods).

Because k_{off} for NA was small, it was possible to isolate [^{3}H]-NA·cNOS by Sephadex G-50 chromatography. When cNOS was incubated with [^{3}H]-NA and then chromatographed on a column of Sephadex G-50, a peak of radioactivity coeluted with enzyme (Figure 5). Further, NA·cNOS eluted with the same elution volume as native cNOS. NA associated with enzyme was well separated from free NA. The amount of [^{3}H]-NA associated with cNOS was approximately 0.3 equiv of NA binding sites. The enzymatic activity recovered in the uninhibited enzyme control was quantitative, whereas the enzymatic activity of isolated NA·cNOS was 20% of the uninhibited enzyme activity.

DISCUSSION

Minimal kinetic mechanisms described the inhibition of mouse macrophage iNOS and bovine brain cNOS by NA. When enzymatic activity was monitored by the conversion of L-arginine to L-citrulline, inhibition of mouse macrophage iNOS by NA was immediate and competitive with L-arginine with a K_i value of 4.4 μM. In sharp contrast to the rapid inhibition of macrophage iNOS by NA, inhibition of bovine brain cNOS by NA was slow in onset with an association rate constant (k_{on}) of $(4.4 \pm 0.4) \times 10^4$ M^{-1} s^{-1}. The slow onset of NA inhibition of bovine brain cNOS indicated that NA dissociation from NA·cNOS was slow. The k_{off} value, determined from the reactivation of NA-inhibited cNOS, was 6.5×10^{-4} s^{-1} ($t_{1/2}$ = 1500 s). Furthermore, because the compound that was dissociated from NA·cNOS cochromatographed with an authentic sample of NA, it was likely that NA was not chemically modified by cNOS. The K_d value (k_{off}/k_{on}) was 15 nM; therefore, NA was a 300-fold more potent inhibitor of brain cNOS than macrophage iNOS.

L-Arginine competitively reduced k_{obs} for the NA inhibition of cNOS with a K_s value (0.8 μM) that was similar to the K_m value of 1.2 μM for L-arginine in the enzymatic reaction. This result suggested that NA and L-arginine bind to the same site on the enzyme. Thus, a titration of cNOS with NA gave a measurement of the cNOS active site concentration and subsequently permitted a k_{cat} of 0.7 s^{-1} to be calculated.

NA also inhibited the bovine brain cNOS-catalyzed NADPH oxidation (in the absence of L-arginine). The k_{on} value of 3.2×10^4 M^{-1} s^{-1}, determined from inhibition of

NADPH oxidation, was similar to the value of 4.4×10^4 M^{-1} s^{-1}, determined from the inhibition of L-citrulline formation. cNOS-catalyzed oxidation of NADPH in the absence of L-arginine results in the formation of superoxide (Pou et al., 1992). However, in the presence of L-arginine, oxygen reduction (NADPH oxidation) is coupled to the oxidation of L-arginine; therefore, the formation of free superoxide is inhibited (Pou et al., 1992). It is possible that superoxide is still generated in the presence of enzyme-bound L-arginine; however, superoxide would have to react with L-arginine prior to the dissociation of superoxide from the enzyme. This observation suggests that NADPH oxidation (reduction of O_2) is tightly coupled to L-arginine oxidation. The observation that NA can completely inhibit NADPH oxidation was further evidence that the chemistry of the NADPH site was tightly coupled to the L-arginine active site. However, in contrast to L-arginine reaction with the reduced oxygen, enzyme-bound NA prohibited NADPH reduction of oxygen. The methyl ester of NA (1 mM) also inhibits NOS-catalyzed superoxide formation (Pou et al., 1992).

The magnitude of k_{off} (6.5×10^{-4} s^{-1}) made NA·cNOS sufficiently stable to isolate by size exclusion chromatography. Isolated NA·cNOS had 20% of the activity of native cNOS. This activity may be due to dissociation of NA during the enzyme assay or during chromatography. Therefore, it was possible that the isolated NA·cNOS may also contain free cNOS. It is unclear why only 0.3 active site equiv of [^{3}H]-NA was isolated with the enzyme. Since only 20% of the enzyme activity was associated with NA·cNOS, at least 80% of the cNOS should be bound to NA. The discrepancy between the amount of NA bound to cNOS and the amount of cNOS activity recovered with NA·cNOS could be explained if NA·cNOS was not stable to the chromatography and resulted in inactive enzyme.

The second-order association rate constant ($k_{on} = 4.4 \times 10^4$ M^{-1} s^{-1}) for NA and brain cNOS was small compared to typical k_{on} values (10^6–10^8 M^{-1} s^{-1}) of enzyme association with small molecules (Fersht, 1985). Enzyme that binds an ionic form or a conformation of the substrate which is a small fraction of the total concentration could result in a small k_{on}. For example, nitroguanidine has a pK_a of 12 and, assuming cNOS bound only the nitroguanidino anion of NA, then the k_{on} value should be logarithmically dependent on pH between 6.5 and 8 (assuming the enzyme is not significantly altered throughout this pH range). However, k_{obs} values for NA inhibition of cNOS did not vary with pH values between 6.5 and 8. Other explanations for slow k_{on} values include enzyme conformational changes or enzyme/substrate desolvation effects (Fersht, 1985). These mechanisms are considerably more difficult to test.

The results that NA was a 300-fold more potent inhibitor of bovine brain cNOS compared to mouse macrophage iNOS and that k_{off} for cNOS was 6.5×10^4 M^{-1} s^{-1} were consistent with a previous report that NA is an apparently irreversible inhibitor of rat brain cNOS and a rapidly reversible inhibitor of mouse macrophage iNOS (Dwyer et al., 1991). These data suggest that constitutive enzymes may bind NA with kinetics and potency that are considerably different from those characteristics of binding NA by inducible enzymes.[2] Although constitutive and inducible enzymes may be regulated differently, many of the cofactor requirements (heme iron, tetrahydrobiopterin, and FAD) are held in common, as is the marked amino acid sequence homology (Stuehr & Griffith, 1992). These similarities between the constitutive and inducible enzymes make the drastic difference in NA binding a surprise. Therefore, NA may be another useful tool to distinguish between constitutive and inducible NOS.

A selective inhibitor of iNOS as compared to cNOS, the converse of NA, may have a therapeutic application in the treatment of septic shock (Wright et al., 1992). A careful analysis of the kinetic mechanism of inhibition of an NOS inhibitor is clearly required to thoroughly distinguish selectivity. For example, if NA inhibition (slow onset) of cNOS was determined over short times, NA could appear as a less potent inhibitor of cNOS than of iNOS. Although NA was a selective inhibitor of cNOS and therefore had the opposite selectivity required for treatment of septic shock, the difference in selectivity suggests the possibility of another inhibitor with the appropriate selectivity.

[2] A second constitutive enzyme from human placenta was potently inhibited by NA with a k_{on} value of 2.2×10^4 M^{-1} s^{-1} (E. Garvey, unpublished data) that was similar to the k_{on} value of 4.4×10^4 M^{-1} s^{-1} for the bovine brain enzyme. A complete kinetic analysis of NA inhibition was not done due to insufficient amounts of the enzyme.

ACKNOWLEDGMENT

We thank T. Spector for initiating these studies, D. Porter and J. Reardon for helpful comments and discussions during the course of this work, P. Sherman for helpful comments in the preparation of the manuscript, and T. Zimmerman and T. Krenitsky for their continued support.

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