CO binding studies of nitric oxide synthase: effects of the substrate, inhibitors and tetrahydrobiopterin

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Abstract The dissociation constant (K_d) for CO from neuronal nitric oxide synthase heme in the absence of the substrate and cofactor was less than 10^{-3} μM. In the presence of L-Arg, it dramatically increased up to 1 μM. In the presence of inhibitors such as N^G-nitro-L-arginine methyl ester and 7-nitroindazole (NI), the K_d value further increased up to more than 100 μM. Addition of the cofactor, 5,6,7,8-tetrahydrobiopterin (H_4B), increased the K_d value by 10-fold in the presence of L-Arg, whereas it decreased the value to less than one 250th in the presence of NI. Addition of H_4B increased the recombination rate constant (k_{on}) for CO by more than two-fold in the presence of L-Arg or N^6-(1-iminoethyl)-L-lysine, whereas it decreased the k_{on} value by three-fold in the presence of L-thiocitrulline. Thus, the binding fashion of some of inhibitors, such as NI, may be different from that of L-Arg with respect to the H_4B effect.

Key words: Carbon monoxide; Nitric oxide synthase; Hemoprotein; Dissociation constant; Rate constant; Flash photolysis

1. Introduction

Nitric oxide synthase (NOS) produces nitric oxide (NO) for a range of important biological functions ([1–5] and references therein). NOS has an oxygenase domain with a thiol-coordinated heme active site similar to that of cytochrome P450 (P450). For NOS to catalyze efficient monooxygenation reactions, the presence of effectors such as tetrahydrobiopterin (H_4B) and calmodulin (CaM) is a prerequisite [1–5].

CO is often used as an O_2 analogue for binding to the heme iron in order to study the structure of the heme distal site ([6,7] and references therein). Equilibrium CO binding and kinetic studies for ferrous P450s have provided invaluable information about the heme distal structure of the enzyme ([8–10] and references therein). Equilibrium binding constants for axial ligands, as well as for the substrate and other effectors, to NOS can be easily obtained by difference absorption spectroscopy and also give us important information about the heme active site environment [11].

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Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal NOS; P450, cytochrome P450; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; CaM, calmodulin; K_d, dissociation constant; k_{on}, recombination rate constant; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; NMMA, N^G-monomethyl-L-arginine; NAME, N^G-nitro-L-arginine methyl ester; NIL, N^6-(1-iminoethyl)-L-lysine; TC, L-thiocitrulline; DIC, diphenyleneiodinium chloride; NI, 7-nitro-1*H*-indazole

L-Arg analogues such as N^G-monomethyl-L-arginine (NMMA), N^G-nitro-L-arginine methyl ester (NAME), N^6-(1-iminoethyl)-L-lysine (NIL) and L-thiocitrulline (TC) are expected to bind to the same active site as L-Arg and inhibit the monooxidation reaction [12]. Aromatic inhibitors such as diphenyleneiodinium chloride (DIC) and 7-nitro-1*H*-indazole (NI), on the other hand, may bind to NADPH or H_4B binding sites and inhibit the supply of electrons during catalysis [12]. Observing the effect of these NOS inhibitors on the CO binding kinetics of the CO-NOS complex should also provide valuable information on the structure of the heme distal site.

In this study, we have obtained (1) spectral dissociation constants (K_d) for CO from ferrous neuronal NOS (nNOS) with optical absorption spectroscopy and (2) recombination rate constants (k_{on}) for CO to nNOS with flash photolysis in the absence and presence of L-Arg, inhibitors and H_4B. It was found that in contrast to the k_{on} values, the K_d values changed markedly, specifically on addition of the substrate, inhibitors and cofactor. We were able to differentiate between the effects of non-L-Arg analogue inhibitors and those of L-Arg analogue inhibitors on the CO binding equilibrium and kinetics.

2. Materials and methods

2.1. Materials

H_4B was purchased from Schircks (Jona, Switzerland). NIL, TC, DIC and NI were purchased from Dojindo (Kumamoto, Japan). Other reagents, which were from Wako (Osaka, Japan), were of the highest guaranteed grade and were used without further purification.

2.2. Preparation of neuronal NOS

Rat nNOS was expressed in *Saccharomyces cerevisiae* using the acid phosphatase promoter previously used for the expression of cytochrome P450 1A2 [13] and purified as described previously [14]. Purified nNOS was more than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie blue R-250 and by Western blot analysis [14]. The purified and concentrated enzyme was dialyzed against 50 mM Tris-HCl (pH 7.5) buffer containing 4 μM H_4B, 0.1 mM DTT, 0.1 mM EDTA and 10% glycerol. The concentration of nNOS was determined optically from the [CO-reduced]−[reduced] difference spectrum using $\Delta\varepsilon_{444-467nm} = 55\ mM^{-1}\ cm^{-1}$ [14]. The specific activity of the purified enzyme was approx. 70 μmol/min/μmol NOS heme (440 nmol/min/mg NOS) in terms of NO production as determined by the oxyhemoglobin method at 25°C [1]. In order to avoid denaturation of CO-ferrous nNOS during the experiments, we purposely added a minimal amount of H_4B, less than a fifth of nNOS in molar ratio, i.e. 40 nM or 1 μM H_4B for CO equilibrium or flash photolysis studies, respectively. The broad Soret peak in the absorption spectrum of ferric nNOS observed in the presence of these concentrations of H_4B suggests that the enzyme has a heterogeneous heme environment under these conditions and that H_4B binding to the enzyme is negligible. In contrast, the presence of 400 μM H_4B generates a narrow Soret absorption with the iron in the high spin state, suggesting that the heme environment is homogeneous and that the full complement of H_4B is bound to the enzyme.

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2.3. Optical absorption spectral titrations

Spectral titrations were carried out on a Shimadzu UV-2200 spectrophotometer maintained at 25°C by a temperature controller. CO binding studies were conducted in a gas-tight capped cell (10 mm × 2 mm) (10 mm path length) under an argon atmosphere. To ensure that the temperature of the solution was appropriate, the cell was incubated for 10 min prior to spectrometric measurements. For titration studies, each solution was incubated for at least 5 min at 25°C to reach equilibrium after inhibitors or other effectors were added. The concentration of H_4B was adjusted by dialysis against buffer containing an appropriate concentration of H_4B. The concentration of CO in the stock solution was determined spectrophotometrically each time by titrating it with a solution of reduced horse heart myoglobin. The spectral changes caused by CO were fitted to the Michaelis-Menten equation under the assumption that a 1:1 ligand-enzyme complex is formed. Titration experiments were repeated at least twice for each complex. Regression analyses were performed and lines giving an optimum correlation coefficient were selected. Linear least-squares fitting was carried out on a Power Macintosh 6100/60AV personal computer using DeltaGraph software as described previously [13]. Experimental errors were less than 20%.

2.4. Flash photolysis

All flash photolysis reactions were performed in an air-tight cell (10 mm × 1 mm) under argon atmosphere at 25°C. When monitoring the absorption around 444 nm, the photoexcitation of denatured NOS was eliminated by use of two 420 nm cutoff filters. The CO binding reactions were conducted under pseudo-first-order conditions in the presence of excess CO. For titration studies, we kept each solution for at least 5 min at 25°C to reach equilibrium after effectors were added to the solution. The CO-bound nNOS complexes were prepared by addition of an approximately 1.2 mM CO stock solution to solutions of 5 μM nNOS reduced by sodium dithionite.

Flash photolysis experiments were carried out with a homemade flash spectrophotometer. Details of this machine have been described elsewhere [9,15]. Briefly, this spectrometer consists of two xenon flash photolysis lamps (Xenon Corp. N-851C) with a half-duration of 8 μs and with a flash energy of 100 J. Recovery of the nNOS-CO complex was monitored with continuous light (444 nm) by a photomultiplier (Hamamatsu Photonics, R-995). Kinetic data were directly transferred from a digital storage scope (Kawasaki, KDS-102) to an NEC personal computer. First-order plots were calculated on the Power Macintosh personal computer using DeltaGraph software. A total of 700 data points in 1 s were collected; these were fit by a least-squares procedure. Experiments were carried out at least twice for each complex. Experimental errors were less than 20%.

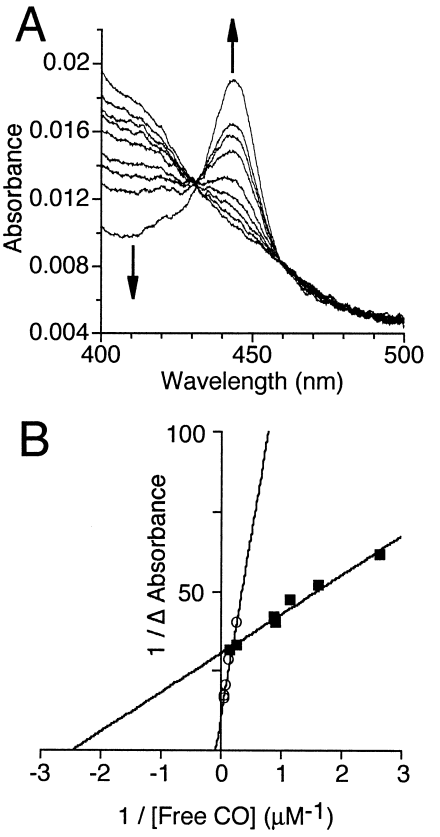

Fig. 1. Absorption spectral changes of nNOS. A: Absorption spectral changes at the Soret region of reduced nNOS caused by addition of CO in the presence of L-Arg. nNOS (0.2 μM) was reduced by sodium dithionite in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM L-Arg, 40 nM H_4B, 1 μM DTT, and 0.1% glycerol. B: Double-reciprocal plots of the absorption intensity change at around 444 nm versus the concentration of free CO in the presence of 10 mM L-Arg and 400 μM H_4B (○) ($R^2 = 0.982$) and 2.5 mM NI and 400 μM H_4B (■) ($R^2 = 0.978$).

3. Results

3.1. K_d values for CO

Fig. 1A shows the typical changes induced in the absorption spectrum of ferrous nNOS on addition of CO to ferrous nNOS. There is an isosbestic point in the CO titration and double-reciprocal plots of the absorption change at 444 nm (the Soret region) versus free CO concentration gave straight lines (Fig. 1B), suggesting that a 1:1 CO-heme complex is formed. Table 1 summarizes the K_d values obtained under various conditions. The affinity of CO for reduced NOS in the absence of L-Arg, inhibitors, or H_4B was very high, with a K_d value much smaller than 10^{-3} μM, thus, estimation of the K_d value was not feasible. Addition of the substrate, L-Arg, substantially increased the K_d value up to the range of μM. Addition of NAME and NI substantially increased the K_d value to more than 10^2 μM. Addition of NMMA and TC also increased the K_d value to similar levels as L-Arg, while addition of NIL and DIC did not increase the K_d value as much.

Table 1
Dissociation constants (K_d) (μM) of CO for nNOS[a]

Compound (concentration)	40 nM H_4B	400 μM H_4B
No substrate	$< 10^{-3}$	$< 10^{-3}$
L-Arg (10 mM)	1.7	18
NMMA (10 mM)	2.1	6.9
NAME (10 mM)	$> 10^2$	$> 10^2$
NIL (10 mM)	0.49	0.22
TC (5 mM)	3.0	2.8
DIC (5 mM)	0.73	0.11
NI (2.5 mM)	$> 10^2$	0.40

[a]Titrations were repeated at least three times, the averaged values are shown. Experimental errors for K_d values were less than 20%. Basal solution: 0.2–0.3 μM nNOS, 50 mM Tris-HCl (pH 7.5), 40 nM H_4B, 1 μM DTT, 0.1% glycerol. In order to avoid denaturation, the presence of 40 nM H_4B (1:5 molar ratio of H_4B to nNOS) was a prerequisite.

For the L-Arg- and NMMA-containing nNOS solutions, in the presence of 400 μM H_4B, the K_d value was 3–10-fold higher than in the presence of 40 nM H_4B. In contrast, the presence of 400 μM H_4B decreased the K_d values for the DIC and NI containing solutions. No substantial effect on the K_d values was observed, when 400 μM H_4B was added to the NAME, NIL or TC-containing solutions.

The K_d values for CO in the presence of 0.12 mM CaM under the same conditions as in Table 1 were also determined

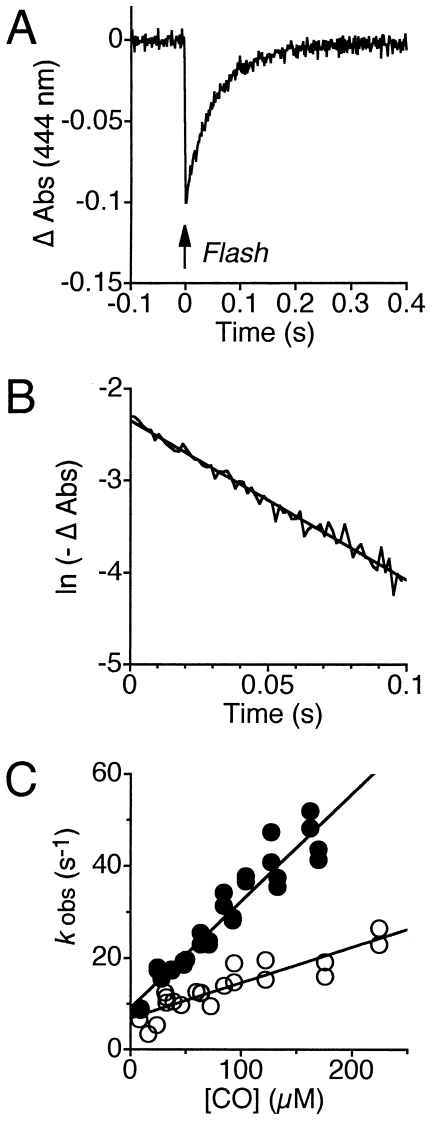

Fig. 2. Flash photolysis of the CO-nNOS complex. A: A flash photolysis time course curve at 444 nm for the CO binding to the reduced nNOS (5 μM) with 0.4 mM NI in the presence of 38 μM CO. B: Semilogarithmic plots of A directly calculated from digitalized data. First-order rate constants (k_{obs}) for CO binding to nNOS were obtained by least-squares fitting to the flash photolysis data. C: Dependence of first-order rate constants for CO binding to reduced nNOS on CO concentration in the presence of 0.4 mM NI (●) ($R^2 = 0.919$) or 10 mM NAME (○) ($R^2 = 0.807$).

(not shown). However, no marked effect on the K_d values was observed in the presence of CaM compared with its absence.

3.2. k_{on} values for CO

Recombination rate constants, k_{on}, for CO binding to ferrous nNOS were obtained by the flash photolysis method (Fig. 2A). The observed time courses for CO binding to NOS were monophasic (at more than 90% absorption) and fitted well to single-exponential expressions (Fig. 2B). The resultant pseudo-first-order rate constants were linearly dependent on CO concentration (Fig. 2C). The k_{on} values obtained are summarized in Table 2. Addition of L-Arg, NMMA, NAME, NIL or TC lowered the k_{on} value more than two-fold, whereas addition of NI had no effect. Addition of DIC inhibited the photo-excited dissociation of CO from ferrous nNOS under these conditions.

Addition of 400 μM H_4B increased the k_{on} values for the L-Arg, NAME or NIL-containing nNOS solutions, but decreased that for the TC-containing solution. However, the k_{on} values for the NMMA, or NI-containing solutions in the presence of 400 μM H_4B were essentially the same as in the presence of 1 μM H_4B.

4. Discussion

L-Arg analogue inhibitors such as NMMA, NIL or TC increased the K_d value for CO in the same way as L-Arg (Table 1). These inhibitors will probably bind to the same site as L-Arg in the enzyme inducing a 'closed' conformation [16], because of their structural similarity to L-Arg, which disrupts CO binding. The binding of such inhibitors may distort the approximate linearity of the Fe-C-O bond formed. NAME has an unusually electron deficient nitrogen atom in its guanidino group due to the presence of NO_2, a strong electron-withdrawing group. The cationic character of this guanidino nitrogen may induce a strong electrostatic interaction with an anionic part of the heme distal site of nNOS, strongly inhibiting CO binding. NI, an inhibitor for L-Arg and H_4B binding in NOS [12], strongly hampered CO binding (Table 1). DIC, which is reported to be an NADPH and flavin antagonist [12], had also disrupted CO binding. Perhaps a global conformational change indirectly caused by NI or DIC distorts the heme distal site space so as to inhibit CO binding.

The addition of 400 μM H_4B increased the K_d values for CO in the presence of L-Arg and NMMA more than threefold. It has been reported that H_4B creates a 'closed' heme distal structure so as to inhibit the binding of axial ligands as

Table 2
Recombination rate contants (k_{on}) ($\mu M^{-1}\ s^{-1}$) of CO to nNOS[a]

Compound (concentration)	1 μM H_4B	400 μM H_4B
No substrate	0.21	0.23
L-Arg (10 mM)	0.0068	0.018
NMMA (11 mM)	0.098	0.089
NAME (10 mM)	0.083	0.12
NIL (10 mM)	0.0052	0.012
TC (10 mM)	0.12	0.038
DIC (5 mM)	no dissociation	no dissociation
NI (0.4 mM)	0.23	0.22

[a]Flash photolysis studies were repeated at least twice for each experiment, the averaged values are shown. Experimental errors for k_{on} values were less than 20%. Basal solution: 5 μM nNOS, 50 mM Tris-HCl (pH 7.5), 1 μM H_4B, 25 μM DTT, 2.5% glycerol, 20 mM sodium dithionite. In order to avoid denaturation, the presence of 1 μM H_4B (1:5 molar ratio of H_4B to nNOS) was a prerequisite.

do L-Arg and L-Arg analogues [16]. In contrast, the addition of 400 μM H_4B decreased the K_d values obtained for the DIC and NI solutions. In particular, the K_d value obtained for the NI-containing solution in the presence of 400 μM H_4B was more than 250-fold lower than that in the presence of 40 nM H_4B. Thus, 400 μM H_4B works in two different ways depending on the effector, although all the effectors more or less inhibit CO binding in a similar way to L-Arg in the presence of 40 nM H_4B.

DIC, an NADPH or flavin binding inhibitor, would be expected to bind to the reductase domain, at a site far from the distal site. The addition of 400 μM H_4B to the DIC solution creates an 'open' heme distal site, which appears to be globally modulated by the distant DIC binding. NI is a competitive inhibitor for H_4B binding in nNOS and thus the presence of 400 μM H_4B may have decreased the effect of NI on the K_d value.

In the presence of 1 μM H_4B, L-Arg and an inhibitor, NIL, are most effective in decreasing the k_{on} value (Table 2) probably because these compounds are properly bound to the heme active site with the optimal orientation to modify the ligand access channel and inhibit CO binding or hinder CO diffusion to the heme iron. NI did not influence the k_{on} value (Table 2). This contrasts with the effect observed on K_d value (Table 1), which was markedly increased by NI. Such a compound may sterically hinder CO binding, but not affect the CO diffusion nor change the conformation/dynamics of the CO access channel of the protein.

The effect of 400 μM H_4B on the k_{on} value for CO in the presence of various effectors was less marked than that on the K_d value. Nevertheless, in the presence of 400 μM H_4B, the k_{on} value with L-Arg, NAME or NIL was noticeably larger than in the presence of 1 μM H_4B (Table 2). Apparently, 400 μM H_4B facilitates CO binding in the presence of these effectors, perhaps by widening the CO access channel. Addition of 400 μM H_4B to nNOS in the presence of L-Arg or NMMA increased the K_d value for CO and disturbs CO binding (Table 1). Thus H_4B seems to have diverse effects on the CO binding. Addition of 400 μM H_4B to an nNOS solution containing TC decreased the k_{on} value, however it did not change the K_d value obtained for CO (Table 1).

In summary, this study suggests that (1) L-Arg, L-Arg analogue inhibitors, and other NOS inhibitors increase the K_d values for CO obtained with nNOS in the presence of 40 nM H_4B; (2) the addition of 400 μM H_4B to nNOS solutions containing most of the effectors further increased the K_d values for CO, although it decreased the K_d values for solutions containing non-L-Arg analogue inhibitors, suggesting that the effect of H_4B on the heme distal site in the presence of non-L-Arg analogue inhibitors is different from that in the presence of the other effectors; (3) the addition of NOS inhibitors in the presence of 1 μM H_4B decreased the k_{on} values for CO binding to nNOS; and (4) the addition of 400 μM H_4B to nNOS in the presence of L-Arg or most of the L-Arg analogues increased the k_{on} value.

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