Reduction of Quinonoid Dihydrobiopterin to Tetrahydrobiopterin by Nitric Oxide Synthase*

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Rat cerebellar nitric oxide synthase (NOS) purified from transfected human kidney cells catalyzes an NADPH-dependent reduction of quinonoid dihydrobiopterin (qBH₂) to tetrahydrobiopterin (BH₄). Reduction of qBH₂ at 25 μM proceeds at a rate that is comparable with that of the overall reaction (citrulline synthesis) and requires calcium ions and calmodulin for optimal activity; NADH has only 10% of the activity of NADPH. The reduction rate with the quinonoid form of 6-methyl-dihydrobiopterin (q6MPH₂) to tetrahydrobiopterin (qBH₂) is recycled to BH₄ by dihydropteridine reductase (10). Whereas it has been proposed that BH₄ functions in NOS in the same manner, neither the oxidation of BH₄ nor the reduction of dihydrobiopterin catalyzed by NOS has been demonstrated (11–14). Giovanelli et al. (15) were unable to detect recycling of added BH₄ and suggested that added BH₄ was not acting as a stoichiometric reactant as it does in the aromatic amino acid hydroxylases. The possibility of recycling of enzyme-bound BH₄ was not excluded (16). On the basis of these results, it was suggested that BH₄ may function as an allosteric effector or to maintain some group(s) on the enzyme in a reduced state for activity (15). This suggestion has been supported by more recent studies showing that BH₄ helps stabilize the active dimeric state of the enzyme (17, 18), maintains a stable heme pocket that is accessible to oxygen (19), and prevents and reverses the inhibition of NOS by nitric oxide (20). Because it is not clear whether BH₄ undergoes redox reactions during NOS catalysis, we have examined directly the reduction by NOS of qBH₂ to BH₄.

EXPERIMENTAL PROCEDURES

Materials—Beef liver catalase was obtained from Boehringer Mannheim, L-1-(2,6-diaminopurine)guanosine (2′-CMP), superoxide dismutase from bovine erythrocytes, glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides, and cytochrome c from horse heart were obtained from Sigma. Diphenyleneiodonium (DPI) was from Aldrich, and 7-nitroindazole (7NI) was from Biomol. CaM from bovine brain was purchased from Calbiochem. BH₄, 7,8-dihydrobiopterin (7,8-BH₂), and (6R)-6-methyl-5,6,7,8-tetrahydropteridin-3-carboxylate (6MPH₃) were obtained from B. Schircks Laboratories (Jona, Switzerland). [1,2,3,4,5,6-[3H]Phenylalanine (50 Ci/mmol), L-[2,3,4,5-3H]arginine (68 Ci/mmol), and L-[U-¹⁴C]Tyrosine (473 mCi/mmol) were obtained from Amersham Corp. qBH₂ was prepared by oxidizing BH₄ with dichlorophenolindophenol (21) at pH 7.6 in 5.5 mM sodium bicarbonate buffer (22), followed by removal of the dye by ether extraction. The qBH₂ solution was flushed with argon to remove the last traces of ether. Quinonoid 6-methyl dihydrobiopterin (q6MPH₃) was prepared from 6MPH₃ using the same method. Details of the methods used to minimize the instability of the quinonoid dihydropterins are described below under “qBH₂ Reduction Assay.”

Enzymes—Recombinant rat phenylalanine hydroxylase (PAH) was prepared by minor modification (23) of the procedure of Citron et al. (24) and had a specific activity of 1.7 μmol min⁻¹ mg⁻¹. Rat brain NOS was purified from transfected human kidney 293 cells (6) by minor modifications (25) of the procedure of McMillan et al. (26). As was done previously (25), dithiothreitol was omitted from the gel filtration procedure. NOS isolated this way had a single band on an SDS-polyacrylamide gel and typically had a specific activity of about 220 nmol min⁻¹.

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‡ The abbreviations used are: NOS, nitric oxide synthase; BH₄, 6-(3-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin (tetrahydrobiopterin); qBH₂, 7,8-dihydrobiopterin; CaM, calmodulin; DPI, diphenyleneiodonium; HPLC, high performance liquid chromatography; 6MPH₃, (6R)-6-methyl-5,6,7,8-tetrahydropteridin-3-carboxylate; NHA, N⁶-hydroxy-L-arginine; 7NI, 7-nitroindazole; NNA, N⁶-nitro-L-arginine; PAH, phenylalanine hydroxylase; qBH₂, quinonoid dihydrobiopterin; q6MPH₃, quinonoid 6-methyl-dihydrobiopterin.
mg⁻¹ as determined by citrulline formation at 25°C essentially as described by Breit and Snyder (27). The BH₄ content of the purified NOS varied between 0.25 and 0.35 mol/mol subunit. Protein was determined by the biuretichromatic acid method (Pierce manual 22325X) with the use of a bovine serum albumin standard.

qBH₂ Reduction Assay—Assay of qBH₂ reduction is complicated by the presence of the pterin. qBH₂ rearranges to 7,8-BH₂ (21), is converted to BH₄, probably by dismutation (28), and is reduced nonenzymatically by NADPH to BH₄ (21). Arrangement to 7,8-BH₂ was minimized by preparing the solution in sodium bicarbonate buffer, pH 7.6 (22) at 0°C and by using this solution within 4 min of its preparation. In agreement with the report of Matsura et al. (28), we observed that nonenzymic dismutation of qBH₂, to BH₄ occurred predominantly during acidification (with perchloric acid). Accordingly, this period was kept as short as possible and constant for all samples. Nonenzymic reduction of qBH₂ to BH₄ was circumvented by decreasing the concentration of NADPH to 1 μmol; the low concentration of NADPH was kept constant by the inclusion of an NADPH regenerating system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase. This low NADPH concentration does not cause a large decrease in the rate of citrulline formation, which is approximately 60% of that measured by the Breit and Snyder assay (27). This is in agreement with the low Km for NADPH of 0.2–0.5 μM (29).²

Reduction of qBH₂ was determined in a standard 20-μl reaction mixture containing 25 mM sodium bicarbonate buffer, pH 7.6, 6.6 μM BH₄, 0.9 mM CaCl₂, 55 μg/ml CaM, 1.5 μM glucose-6-phosphate, 0.12 units of glucose-6-phosphate dehydrogenase, and 1 μM NADPH. Unless stated otherwise, the reaction was started by adding qBH₂ to a concentration of 25 μM immediately after adding NOS (0.5–1.5 μg). The reaction mixture was incubated at 25°C. Blank reactions in which NOS was omitted were used to correct for nonenzymic conversion of qBH₂ to BH₄. Reactions were terminated after 30, 60, and 90 s by adding 4 μl of 1.5 M HClO₄ followed by partial neutralization to pH 3 with 4 μl of approximately 1.4 M KHCO₃. The low pH is important for stability of BH₄ and to permit subsequent assay of the BH₄ content of the partially neutralized solution at a pH of 6.8 (see below). In order to compensate for small variations in concentration of the solutions of HClO₄ and KHCO₃, the exact concentration of the latter solution required to achieve a pH of about 3 should be determined in a separate titration experiment. The precipitate was removed by centrifugation, and 25 μl of the supernatant solution was immediately added to the mix described below in order to determine BH₄. Unless stated otherwise, rates of BH₄ synthesis were determined from an average of the rates determined at 30 and 60 s.

BH₄ formed in the above reaction was determined from the amount of [³H]tyrosine formed in the BH₄-dependent hydroxylation of [³H]phenylalanine catalyzed by rat liver PAH. The reaction mixture (35 μl) contained 0.1 mg/ml cat. catalase, 130 mM potassium phosphate buffer, pH 6.8, 0.1 mM l-tyrosine, 20 μM [³H]phenylalanine (7.5 μCi/ml), and 1.1 μg of PAH. The mixture was incubated for 30 min at 25°C; the reaction was subsequently stopped by adding 20 μl of a solution containing (w/v) trichloroacetic acid, 0.1 mM phenylalanine, and 0.1 mM [¹⁴C]tyrosine (30 nCi), added to correct for losses during subsequent purification. The reaction mixture was immediately added to the mix described below in order to determine [¹⁴C]tyrosine. For [³H]tyrosine less than 5 pmol, the product from the HPLC step was mixed with additional carrier tyrosine and further purified by crystallization (15). The [³H]tyrosine to [¹⁴C]tyrosine ratio was used to calculate the amount of [³H]tyrosine formed. The efficiency of BH₄ determination was assessed with separate BH₄ standards and appropriate corrections were made for calculating amounts of enzymic synthesis of BH₄. The efficiency was usually approximately 70% and somewhat lower for amounts of BH₄ below 25 pmol. These procedures were also used for the determination of the reduction products of 7,8-BH₂ and q6MHPH₂; the reduction product of q6MHPH₂, 6MHPH₄, is also a substrate for PAH (30).

Simultaneous Measurement of qBH₂ Reduction and NOS Activity—Relative rates of conversion of arginine to citrulline and of qBH₂ reduction were determined in a single reaction mixture, the composition of which was identical to that described for assay of qBH₂ reduction except for increasing the volume to 150 μl and adding [³H]arginine (7.5 μCi, 250 μM). The reaction was started by addition of 8.25 μl (54.7 pmol subunit) of NOS, and separate samples of 20 μl were removed after 30, 60, and 90 s for assay of [³H]citrulline or BH₄. Before purification of [³H,¹⁴C]citrulline, [³H]arginine was removed on a column of AG50W-X8 in sodium form (Bio-Rad). [³H]Citrulline was determined by HPLC chromatography on Zorbax 300-SCX (4.6 × 25 mm) as described by Campos et al. (25).

Inhibition by DPI—Because inhibition by DPI is known to be irreversible and time-dependent (31, 32), DPI was first preincubated with NOS for a fixed time, followed by a 10-fold dilution of this preincubation mixture for determination of NOS activity. NOS (2.7 μg) was preincubated at 25°C for 5 min in 2 μl of standard assay mixture in which qBH₂ was omitted and the concentration of CaM was 450 μM and which contained 50 μM DPI and 100 μM NNA. The mixture was then diluted to 20 μl with standard assay mixture containing 100 μM NNA but no qBH₂. Finally, the reaction was started by adding 25 μM qBH₂. Further analysis was performed as described above, except that BH₄ standards contained the same amount of DPI as the reaction mixture.

Inhibition by 7-NI—The effect of 7-NI (100 μM) on qBH₂ reduction was determined in the standard assay mixture modified by the addition of 15 μM arginine. Inhibition of NOS activity (citrulline formation) was determined in the standard assay mixture for qBH₂ reduction modified by the omission of qBH₂ and the addition of 15 μM [¹⁴C]arginine and 1 μM BH₄; incubation was for 15 min.

Stimulation by qBH₂ and BH₄ of NOS Activity and N⁶-Hydroxarginine Synthesis—NOS activity was measured as described previously (15) with the exceptions that the reaction mixture was buffered with 25 mM sodium bicarbonate, pH 7.6, NADPH concentration was decreased to 1 μM in the presence of the NADPH regenerating system described above, and the reaction mixture was incubated for 10 min at 37°C. N⁶-Hydroxarginine (NHA) formation in the absence of added NADPH during an incubation period of 5 min at 25°C was measured as described (25).

Cytochrome c Reduction Assay—Initial rates of cytochrome c reduction were measured spectrophotometrically at 550 nm. Conditions were identical to those for qBH₂ reduction except that qBH₂ was omitted, and the reaction (1 ml) contained 11 μg of CaM and 100 μM cytochrome c.

Results

Conditions for Pterin Reduction—Table I summarizes relative rates of pterin reduction under a variety of conditions. The addition of arginine (100 μM) caused a small (25%) inhibition of the reduction rate, whereas NNA (100 μM), which inhibits electron transfer through heme (33), stimulated the rate between 25 and 50%. Because of the higher reduction rates in the presence of NNA, this compound was included in these assays.

Reduction of qBH₂ was stimulated 4–10-fold by Ca²⁺/CaM. Replacement of NADPH by NADH decreased the activity to 10%. Of the limited number of pterins tested, q6MHPH₂ was the most active, showing twice the activity compared with qBH₂, 7,8-BH₂ was neither active itself, nor did it inhibit qBH₂ reduction. Added BH₄ also did not inhibit qBH₂ reduction. Because large amounts of superoxide can be generated in the absence of arginine (34), we examined the effects of superoxide dismutase (1000 units/ml) plus catalase (20 μg/ml) on the reduction; little (<10%) inhibition was observed. NADPH-cytochrome c reductase activity of NOS showed a similar stimulation (8-fold) by Ca²⁺/CaM and complete insensitivity to superoxide dismutase plus catalase at the concentrations de-
Comparative Rates of qBH₂ Reduction and Citrulline Formation—As shown in Fig. 1, the rate of reduction of qBH₂ at a concentration of 25 \( \mu \text{M} \) catalyzed by NOS is comparable with that of citrulline formation measured under the same conditions. In contrast to citrulline formation, qBH₂ reduction was not linear with time, probably as a result of the decrease in qBH₂ concentration caused by its enzymic reduction and by its marked instability under the assay conditions.

The Effect of Different qBH₂ Concentrations on Its Rate of Reduction—The qBH₂ reduction rate increased with qBH₂ concentration measured in the range of 2.0–50 \( \mu \text{M} \) (Fig. 2). The effects of higher concentrations of qBH₂ were not examined, because they resulted in unacceptably high blank values; therefore, a precise \( K_m \) was not determined. However, the data show that the concentration of qBH₂ required for a half-maximal rate of reduction of this pterin (EC₅₀) exceeds 20 \( \mu \text{M} \) and is thus some 2 orders of magnitude greater than the concentration of BH₄ required for half-maximal activation of NOS (Fig. 3).

Effects of DPI, 7NI, and Methotrexate—The effects of three potential inhibitors were studied. Preincubation of NOS for 5 min with the flavoprotein inhibitor DPI (31, 32) at a concentration of 50 \( \mu \text{M} \) inhibited qBH₂ reduction by 85%. 7NI is believed to bind to heme and interferes with BH₄ binding (14, 35). This compound stimulated qBH₂ reduction 2-fold at a concentration (100 \( \mu \text{M} \)) that inhibited the formation of citrulline from arginine by 80%. Methotrexate, a pterin analogue that is known to inhibit a variety of enzymes involved in dihydrobiopterin reduction (see “Discussion”), had no effect on qBH₂ reduction at a concentration of 100 \( \mu \text{M} \).

Stimulation of NOS Activity by BH₄ and qBH₂—Fig. 3 compares the relative stimulation of the NOS reaction (citrulline synthesis) by added qBH₂ or BH₄. The stimulation curve with qBH₂ is similar to that with BH₄ except that it is shifted to higher concentrations and shows a decrease at higher concentrations of qBH₂. This decrease in stimulation may be due to the presence of 7,8-BH₂ formed nonenzymically from qBH₂ under the assay conditions. 7,8-BH₂ has been shown to attenuate BH₄ stimulation (14, 16). qBH₂ had little or no stimulatory effect on the NOS-catalyzed conversion of arginine to NHA measured in the absence of added NADPH (data not shown).

DISCUSSION

The novel method described here allows for the sensitive and specific determination of BH₄ in the presence of relatively large amounts of qBH₂. BH₄ formed during reduction of qBH₂ is coupled to the PAH-catalyzed conversion of an equivalent amount of \([\text{H}]\)phenylalanine to \([\text{H}]\)tyrosine. The procedure proved to be reproducible and easy to use and avoids HPLC of unstable BH₄ as required in other methods (36, 37).

A proposed scheme for qBH₂ reduction by NOS is presented in Fig. 4. The strong inhibition of qBH₂ by DPI demonstrates a role for flavin. Three enzymes, one of which is also a flavoprotein, have previously been reported to catalyze the pyridine-nucleotide-linked reduction of dihydrobiopterin. They are dihydropteridine reductase (38, 39) and the flavoprotein...
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The reductase or C-terminal domain (left) contains the NADPH, FAD-, and FMN-binding sites, whereas the oxygenase or N-terminal domain (right) contains the heme, BH$_4$, and arginine-binding sites (4–8). CaM binding activates by increasing the rates of electron transfer between NADPH and flavins and also facilitates electron transfer to heme (46). DPI is a flavoprotein inhibitor (31, 32). 7NI is believed to bind to heme and is competitive with BH$_4$ binding (14, 35). NNA also inhibits electron transfer through heme (33). The symbol $e^-$ denotes electrons. Oxygenation of arginine predominantly to NHA is catalyzed by substrate amounts of NOS in the absence of added NADPH (25). Oxygenation is supported by an endogenous reductant that was shown not to be NADPH (25). Added BH$_4$ stimulates each of the two partial reactions involving NHA as an intermediate (25, 50–52).

What is the site of qBH$_2$ reduction? Does reduction proceed directly at the flavoprotein NADPH “diaphorase” site of NOS, or are electrons transferred from flavin to the high affinity BH$_4$-binding site involved in the activation of NOS (14, 15)? Two main findings indicate that the flavoprotein diaphorase is the site of qBH$_2$ reduction. First, BH$_4$ and 7,8-BH$_2$, two compounds with a high affinity for the BH$_4$-binding site (14, 15), have no effect on qBH$_2$ reduction. Second, 7NI, which interferes with BH$_4$ binding to the high affinity site (14, 35), stimulates qBH$_2$ reduction. This stimulation, as well as that by NNA, can be tentatively ascribed to the inhibition of reactions proceeding through heme (14, 33, 35), thereby increasing the availability of reducing equivalents for qBH$_2$ reduction. The high $EC_{50}$ (Fig. 2) for qBH$_2$ reduction compared with the relatively low $K_m$ values for binding and activation with BH$_4$ (Fig. 3, (13–15)) is consistent with qBH$_2$ reduction proceeding at the diaphorase site, rather than the high affinity BH$_4$-binding site.

The NADPH diaphorase component of NOS is known to catalyze the oxidation of NADPH with a range of electron acceptors including cytochrome c, ferricyanide, and various dyes (11, 46–49). Reduction of q6MPH$_2$ at a rate of approximately twice that of qBH$_2$ is consistent with this reported low specificity; the product of q6MPH$_2$ reduction, 6MPH$_4$, shows little or no activation of NOS at concentrations optimal for activation by BH$_4$ (13, 15).

There are conflicting data on whether superoxide is an intermediate in the NADPH-cytochrome c reductase activity of NOS (35, 46, 48, 49). It was therefore of interest to examine the effects of superoxide dismutase on this reaction and also on the NOS-catalyzed reduction of qBH$_2$. Our finding that superoxide dismutase had no effect on the NADPH-cytochrome c reductase activity of NOS is in agreement with other reports (35, 46, 48). Furthermore, the small inhibition of qBH$_2$ reduction by superoxide dismutase further suggests that superoxide is not involved in this reaction.

Our studies show that the reduction of both qBH$_2$ and cytochrome c by NOS is strongly stimulated by Ca$^{2+}$/CaM. Other workers have also reported a strong stimulation of the reduction of the latter substrate by Ca$^{2+}$/CaM; by contrast, for unexplained reasons, the NADPH-dependent reduction of ferricyanide and 2,6-dichlorophenolindophenol by NOS is stimulated to only a small extent or not at all by Ca$^{2+}$/CaM (46, 48, 49). With respect to dependence on Ca$^{2+}$/CaM, qBH$_2$ reduction therefore more closely resembles cytochrome c reduction than ferricyanide or 2,6-dichlorophenolindophenol reduction.

Under the conditions used in our earlier experiment (15), where the PAH reaction would have been expected to generate a steady-state concentration of about 0.05 $\mu$M qBH$_2$ from the added 0.05 $\mu$M BH$_4$ and the limits of detection were 2.5 pmol, there was no detectable regeneration of BH$_4$ in the presence of added NOS and NADPH. Because this result appears to contrast with our present data, it is important to understand why the earlier experiment did not detect any reduction of qBH$_2$. The explanation, in all probability, is that in the earlier experiment, the amount of qBH$_2$ converted to BH$_4$ was below the limits of detection. Thus, from the data in Fig. 2, it can be estimated that 2 pmol of BH$_4$ would have been synthesized during the 30-min incubation period used in the earlier experiment; this amount would be considerably less if the instability of qBH$_2$ and the lack of linearity of BH$_4$ synthesis with time (Fig. 1) are taken into account.

The significance of the qBH$_2$ reduction described here to the essential role of BH$_4$ in NOS remains to be clarified. Our results indicate that the relative high concentration of 25 $\mu$M qBH$_2$ reduction can proceed at a rate comparable with that of the overall reaction (citrulline synthesis) and that the efficiency of added qBH$_2$ in stimulating NOS activity approaches that of added BH$_4$. qBH$_2$ seems to stimulate by first being converted to BH$_4$ and subsequent binding to the BH$_4$-binding site. This postulate is supported by the observation that added qBH$_2$ causes little or no stimulation of NOS-catalyzed oxygenation of arginine to NHA (Fig. 4), a reaction that proceeds in the absence of added NADPH and consequently precludes qBH$_2$ reduction.

A central feature of the model proposed in Fig. 4 is that qBH$_2$ reduction proceeds on the C-terminal (reductase) domain, remote from the high affinity BH$_4$-binding site of the N-terminal (oxidase) domain. Implicit in this model is the notion that if BH$_4$ recycling does occur, it may proceed by a pathway in which qBH$_2$ reduction occurs predominantly at the flavin site, followed by transfer of the BH$_4$ so formed to the high affinity BH$_4$-binding site. A redox cycle would be completed by transfer of qBH$_2$ back to the flavin reduction site. Transfer of pterins between the flavin and BH$_4$-binding sites may proceed either by dissociation from the enzyme or by a direct interdomain transfer. Our present studies also do not eliminate the possibility that cycling of pterin occurs exclusively at the BH$_4$-binding site on the oxygenase domain. These possibilities are currently under investigation.

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