Macrophage Nitric Oxide Synthase: Relationship between Enzyme-Bound Tetrahydrobiopterin and Synthase Activity†

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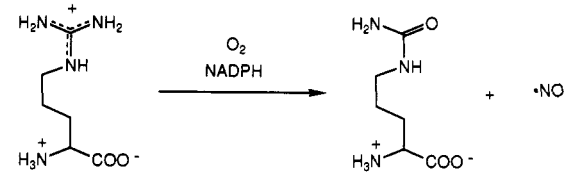
ABSTRACT: Nitric oxide synthase (NOS) (EC 1.14.23) catalyzes the oxidation of L-arginine to citrulline and nitric oxide. The complex reaction carried out by NOS, which involves NADPH, O_2, and enzyme-bound FAD, FMN, and tetrahydrobiopterin (BH_4), has only recently begun to be elucidated. Herein we report the characterization of the pterin requirement of murine macrophage NOS. Although purified NOS activity was not dependent on BH_4, activity was significantly enhanced by BH_4 in a concentration-dependent fashion. NOS purified in the absence of added BH_4 was found to contain substoichiometric concentrations of enzyme-bound pterin, where increased concentrations of bound pterin correlated with an increase in activity when assayed in the absence of exogenous BH_4. However, NOS purified in the presence of BH_4 followed by gel filtration exhibited a 1 mol of pterin:1 mol of NOS 130-kDa subunit stoichiometry and activity that was essentially independent of exogenous BH_4. Experiments to probe a redox role for the pterin were carried out using pterin analogues. 6(*R,S*)-Methyltetrahydropterin was found to increase NOS activity in enzyme purified in the absence of BH_4. However, the deaza analogue, 6(*R,S*)-methyl-5-deazatetrahydropterin, was not only incapable of supporting enzymatic turnover but also inhibited citrulline formation in a concentration-dependent manner. Overall, these results support a role for BH_4 in the NOS reaction that involves stabilization of the enzyme and redox chemistry wherein a 1:1 stoichiometry between bound pterin and NOS subunit results in maximum activity.

Nitric oxide synthase (NOS)[1] catalyzes the five-electron oxidation of L-arginine to citrulline and nitric oxide (•NO) at the expense of molecular oxygen and NADPH (Scheme I). The •NO synthesized is involved in important physiological activities such as vasodilation and regulation of normal vascular tone, inhibition of platelet aggregation, neuronal transmission, and cytostasis [for reviews, see Garthwaite (1991); Marletta et al. (1990), and Ignarro (1990)]. It appears that altered levels of •NO are also involved in hypotension and endotoxic shock (Kilbourn et al., 1990), hypertension and atherosclerosis (Luscher, 1990), and inflammatory response-induced tissue injury (Mulligan et al., 1990). Consistent with the diverse set of activities that is attributed to •NO is the fact that NOS has been found in several different kinds of tissues. Over the past 2 years NOS has been purified from the brain (Bredt & Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991; Schmidt & Murad, 1991), endothelium (Pollock et al., 1991), neutrophils (Yui et al., 1991a), and macrophages (Hevel et al., 1991a; Stuehr et al., 1991a; Yui et al., 1991b).

All of the purified NOSs characterized thus far are flavoproteins containing one FAD and one FMN per subunit (Hevel et al., 1991; Mayer et al., 1991), which in the case of the brain enzyme (Bredt et al., 1991) exhibits partial sequence homology to P-450 reductase, suggesting that reducing equivalents from NADPH may be shuttled into the active site via the flavins. Originally it was thought that only the macrophage NOS required BH_4, but recent evidence (Busse & Mulsch, 1990; Mayer et al., 1990; Pollock et al., 1991; Yui et al., 1991a) including the identification of NOS-bound pterin in brain NOS (Mayer et al., 1991) suggests that all of the NOSs utilize BH_4. The exact function of the pterin is, however, unclear.

Scheme I: Reaction Catalyzed by Nitric Oxide Synthase (NOS)



The first step in the synthesis of •NO by NOS is a monohydroxylation of L-arginine to N^G-hydroxy-L-arginine (Stuehr et al., 1991b; Pufahl et al., 1992) that is chemically reminiscent of the monohydroxylations carried out by the aromatic amino acid hydroxylases (Shiman, 1985; Kaufman, 1987). In these hydroxylases BH_4 is directly involved in hydroxylating the aromatic substrate. When the involvement of BH_4 in the NOS reaction was found (Tayeh & Marletta, 1989; Kwon et al., 1989), an analogous role for BH_4 was proposed. In contrast to the direct role BH_4 plays in the aromatic amino acid hydroxylases, Kaufman and co-workers (Giovanelli et al., 1991) have recently suggested that in the case of the brain NOS the pterin acts as an allosteric regulator of enzyme activity or a stabilizing agent. This conclusion was based on (1) the observation that BH_4 did not increase the initial rate of citrulline formation, (2) the catalytic effect of BH_4 (i.e., each mole of added BH_4 supported the formation of >15 mol of citrulline), and (3) the absence of pterin recycling by NOS.

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[1] Abbreviations: •NO, nitric oxide; NOS, nitric oxide synthase; BH_4, 6(*R*)tetrahydro-L-biopterin; *q*-BH_2, quinonoid dihydrobiopterin; MPH_4, 6(*R,S*)-methyltetrahydropterin; $DZPH_4$, 6(*R,S*)-methyl-5-deazatetrahydropterin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol.

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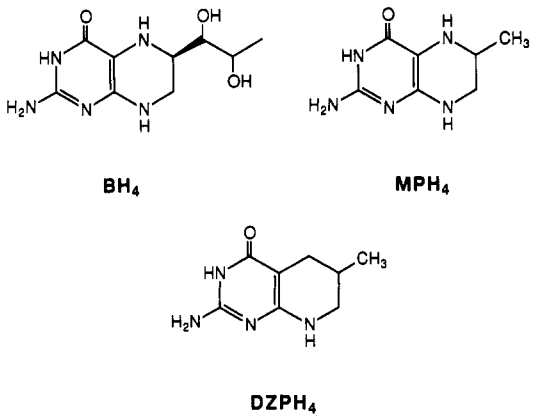

FIGURE 1: Structures of pterin analogues: 6(*R*)-5,6,7,8-tetrahydro-L-biopterin, BH_4; 6(*R,S*)-methyltetrahydropterin, MPH_4; 6(*R,S*)-methyl-5-deazatetrahydropterin, $DZPH_4$.

In this paper we present some of the results of our ongoing investigation into the role BH_4 plays in the macrophage NOS reaction. While addressing the observations of Kaufman and co-workers, we demonstrate that purified NOS contains bound pterin and show that 6(*R,S*)-methyl-5-deazatetrahydropterin ($DZPH_4$) (Figure 1), which is inert to molecular oxygen and redox stable, does not support citrulline formation. This deazapterin analog of 6(*R,S*)-methyltetrahydropterin (MPH_4) has been utilized by Benkovic and co-workers (Moad et al., 1978, 1979a) to probe the mechanism of phenylalanine hydroxylase and has been determined to be incapable of supporting hydroxylation of the aromatic amino acid substrate. Our results, therefore, are consistent with a redox role for BH_4 in the NOS reaction.

MATERIALS AND METHODS

Materials and General Methods. 6(*R,S*)-Methyl-5-deazatetrahydropterin was synthesized as described previously (Moad et al., 1979b) and was used as the monotrifluoroacetate salt. All other pterins were purchased from Dr. B. Schircks Laboratory (Jona, Switzerland) and were prepared in 15 mM Hepes (pH 7.5) containing 100 mM DTT. Oxyhemoglobin (human A_0, ferrous), Hepes, glycerol (molecular biology grade), DTT, NADPH, $NADP^+$, and L-malic acid were purchased from Sigma Chemical Co. 2′,5′-ADP–Sepharose 4B was purchased from Pharmacia-LKB Biotechnology Inc. DEAE-Bio-Gel A, Bio-Gel P-DG, Coomassie Blue R-250, and Bradford protein dye reagent were purchased from Bio-Rad. L-[U-^{14}C]Arginine monohydrochloride (specific activity = 319 mCi/mmol) was purchased from Amersham Corp. Ecolume scintillation cocktail was purchased from ICN-Flow. Gel filtration columns were treated with AquaSil siliconizing fluid from Pierce.

Purification of NOS. Cell culture procedures, induction of murine macrophage NOS activity, and the preparation of 100000*g* supernatant were carried out as described previously (Tayeh & Marletta, 1989). Purification of NOS was carried out as described previously (Hevel et al., 1991a) with the following modifications. Unless stated otherwise, none of the purification buffers contained BH_4. The 100000*g* supernatant (~30 units; a unit is defined as that amount of NOS required to produce 1 μmol of $^{\bullet}NO$/h) was applied to a 2′,5′-ADP–Sepharose 4B column (1 g of resin) equilibrated with 10 mM K_2HPO_4 containing 10% glycerol and 0.5 mM L-arginine at pH 7.5 (buffer A). The column was washed with 25 mL of buffer A containing 0.5 M NaCl, 3.0 mM L-malic acid, and 0.15 mM $NADP^+$ followed by 10 mL of buffer A. NOS activity was eluted directly onto a DEAE-Bio-Gel A column (1.5 mL of resin) with 25 mL of buffer A containing 3 mM NADPH, 0.75 mM $NADP^+$, and 15 mM NaCl. The Bio-Gel A column was then washed with 4 mL of 10 mM K_2HPO_4 containing 10% glycerol (buffer B) followed by 4 mL of buffer B containing 80 mM NaCl. NOS activity was eluted from the column with buffer B containing 120 mM NaCl and concentrated with either Amicon Centricon 30 microconcentrators or an Amicon ultrafiltration system (PM-30 membrane). During the concentration step the phosphate buffer was exchanged for 15 mM Hepes (pH 7.5). Protein was determined by the Bradford protein microassay using bovine serum albumin as a standard. NOS purified by this method was greater than 98% pure as judged by SDS–PAGE stained with Coomassie Blue R-250.

NOS Activity Assay. NOS activity was measured by one or both of the following methods: (A) The hemoglobin assay was employed to measure $^{\bullet}NO$ formation as described previously (Olken et al., 1991). (B) Citrulline formation was followed using [^{14}C]arginine as described previously (Bredt & Snyder, 1990) with several modifications. Enzyme assays (300 μL) containing 100 μM L-[U-^{14}C]arginine (specific activity = 3.3 μCi/μmol), 100 μM NADPH, 20 μM BH_4 (0.27 mM DTT), and NOS in 15 mM Hepes (pH 7.5) were incubated at 37 °C for 5 min. Sets of experiments with the deazapterin analog were performed in 200 mM Hepes (pH 7.5). After 5 min, the entire reaction mixture either was immediately applied to a 1-mL AG 50W-X8 cation exchange column (Bio-Rad, sodium form) which was washed with 3 mL of H_2O directly into 15 mL of Ecolume scintillant or was terminated with 4% TCA, placed on ice, and chromatographed later. Under these conditions the synthesis of [^{14}C]citrulline was linear >7.5 min whether or not BH_4 was added to the enzyme reaction mixture. NOS activities were corrected for the amount of [^{14}C]citrulline formed at t = 0 min. Above concentrations of 12 μM, tetrahydropterins interfere with the hemoglobin assay. Therefore, experiments involving pterin above 12 μM were done with the citrulline assay.

Pteridine Oxidation. Reduced pterins in a 100-μL sample of NOS were oxidized in the dark in 2.3 mM KI/I_2 and 9.0 mM NaOH (final concentrations) (Werner et al., 1987) for 5 min at 100 °C to denature the enzyme and for 55 min at room temperature to complete the oxidation. Samples were neutralized and analyzed as described below. Authentic BH_4 was also oxidized under identical conditions to determine the efficiency of the oxidation procedure. The pterin concentration of each NOS sample was then corrected for recovery (75–98%). Stoichiometric pterin:NOS subunit values was calculated on the basis of an M_r = 130 000 and are the result of at least duplicate measurements.

HPLC/Fluorescence Detection. Biopterin and pterin were detected by the method described by Werner and co-workers (Werner et al., 1987) with the following modifications. Samples were applied to an Altex Ultrasphere-ODS column (25 cm × 4.6 mm) fitted with a μBondapak C-18 guard column (Waters) at a flow rate of 1.2 mL/min and detected at an λ_{ex} = 343 nm and λ_{em} = 438 nm using a Waters 470 scanning fluorescence detector. The detection limit for pteridines using this method was <0.5 pmol/50-μL injection.

BH_4 Preloading of NOS. A 500-μL sample of NOS was incubated with 31.25 μL of a solution containing 6.8 mM BH_4 and 100 mM DTT (final concentrations were 400 μM and 5.4 mM, respectively) at 4 °C for 10 min. An aliquot of the mixture (500 μL) was then passed over a Bio-Gel P-DG gel filtration column (24 cm × 0.7 cm) equilibrated with 15

Table I: Effect of BH_4 on Initial Rate

	activity[a]		% of
	$-BH_4$	$+BH_4$	max activity[b]
100000*g* supernatant	111.8	121.6	92
NOS (Bio-Gel A)	9.9	22.1	45
concentrated NOS	4.4	21.2	21

[a] Activity measured by the hemoglobin assay, expressed as nanomoles of •NO per hour. [b] Maximum activity is defined as that amount of activity observed in the presence of 12 μM BH_4.

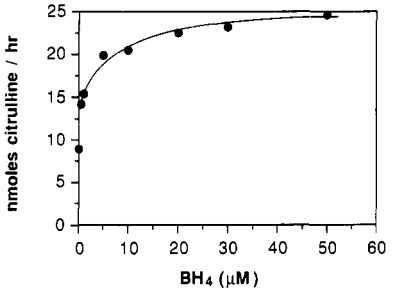

FIGURE 2: Concentration-dependent increase in NOS activity by BH_4. Assays were done in 200 mM Hepes (pH 7.5) using the citrulline assay as described under Materials and Methods and contained 1.6 μg of NOS.

mM Hepes, pH 7.5. The fraction containing NOS activity was analyzed for pterin concentration, protein concentration, and enzyme activity in the presence and absence of exogenous BH_4. In an independent experiment, a 500-μL sample of 500 μM biopterin was passed over the column under the same conditions and the fractions were analyzed by HPLC to ensure separation of free pterin from NOS in the above experiment.

RESULTS

Identification and Quantification of NOS-Bound Pterin. Unlike macrophage 100000*g* supernatant activity, which showed almost no dependence on exogenous BH_4, purified NOS activity was greatly enhanced in the presence of BH_4 as measured by the hemoglobin (Table I) or [^{14}C]citrulline assay (data not shown). The amount of enhancement afforded by BH_4 increased with each purification step and varied with each preparation. The increase in NOS activity resulting from the addition of BH_4 was concentration-dependent and reached maximum velocity at ~50 μM (Figure 2). The substantial rate that was observed in the absence of added BH_4 in the purified NOS, in conjunction with the increased effect BH_4 had on activity as the enzyme was purified, suggested that the pterin was bound to NOS and that some portion was lost during the purification.

HPLC/fluorescence analysis of NOS oxidized with KI/I_2 under alkaline conditions demonstrated the presence of enzyme-bound pteridine (Figure 3) in a ratio of 0.06–0.27 mol of pteridine/130-kDa subunit. While this oxidation method measures the total amount of enzyme-bound pteridine by converting BH_4 and *q*-BH_2 to pterin, we have also looked specifically for enzyme-bound BH_4 with HPLC analysis and electrochemical detection and confirmed the presence of NOS-bound BH_4 (Hevel et al., 1991b). The total pterin:NOS stoichiometry varied among different preparations as did the activity in the absence of exogenous BH_4. To determine if there was a relationship between bound pterin and activity, five separate preparations were carried out and analyzed for pterin content and NOS activity. From the results (Figure 4) it is clear that the percent of activity the enzyme exhibited in the absence of exogenous pterin was directly proportional to the percent of NOS subunit which contained bound pterin.

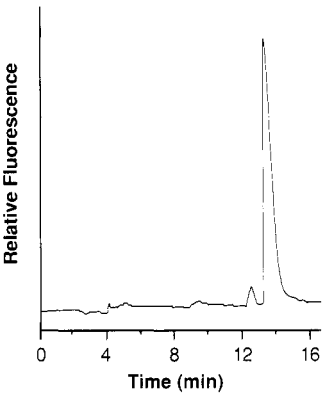

FIGURE 3: HPLC chromatogram of macrophage NOS. A sample of purified NOS was oxidized in base with a KI/I_2 solution and applied to a reversed-phase column, and pterins were detected with fluorescence as described under Materials and Methods. The peaks at 12.5 and 13.5 min correspond to biopterin and pterin, respectively. Under basic conditions, BH_4 is oxidized to pterin.

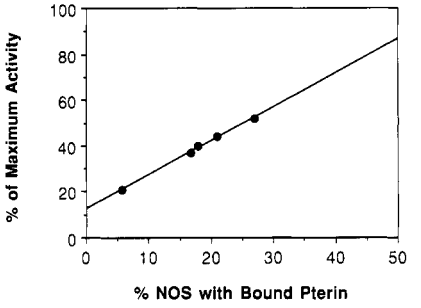

FIGURE 4: Correlation of activity and NOS-bound pterin concentration. Each point represents an average value from duplicate measurements obtained from a single purification and was calculated as follows. The values on the *x* axis were calculated by determining the stiochiometry of the pterin:NOS subunit (described under Materials and Methods) and are expressed as a percentage. The values on the *y* axis were determined using the hemoglobin assay and are expressed as the ratio of activity present in the absence of added BH_4 to the amount of activity present when 12 μM BH_4 is added to the reaction mixture.

Table II: Preloading of NOS with BH_4[a]

		specific activity[b]		stoichiometry (mol of
		$-BH_4$	$+BH_4$	pterin:mol of subunit)
	NOS Purified in the Absence of BH_4			
expt 1	before preloading	12.0	27.1	0.21:1.0
	after preloading	20.2	36.5	0.43:1.0
	NOS Purified in the Presence of BH_4			
expt 2	after preloading	80.8	90.1	1.1:1.0
expt 3	after preloading	69.9	82.5	0.85:1.0

[a] All values are the average of duplicate measurements. [b] Activity measured by the hemoglobin assay. Specific activity is expressed as micromoles of •NO per hour per milligram. Concentration of BH_4 was 12 μM.

The concentration of NOS-bound pteridine could be increased by incubating the NOS with BH_4 (preloading) followed by gel filtration to remove free BH_4. Table II (experiment 1) shows that an increase in bound pterin was accompanied by an increase in the specific activity of NOS regardless of whether the enzyme was assayed in the absence or presence of added BH_4. Figure 4 indicates a direct relationship between bound pterin and percent of maximum activity, which would predict that a 2-fold increase in NOS-bound pterin would lead to approximately 1.8 times more activity. When the NOS was subjected to the preloading procedure, a 2-fold increase in the concentration of bound

Table III: Effect of BH_4, MPH_4, and $DZPH_4$ on NOS Citrulline Formation

pterin added	activity[a]	pterin added	activity[a]
none	3.14 ± 0.1	170 μM MPH_4	5.55 ± 0.09
20 μM BH_4	6.32 ± 0.1	190 μM $DZPH_4$	2.19 ● 0.03

[a] All values are the average of duplicate measurements. Assays were done in 200 mM Hepes (pH 7.5) as described under Materials and Methods and contained 0.4 μg of NOS. Activity is expressed as nanomoles of citrulline per hour.

pterin was associated with a 1.7-fold increase in specific activity when assayed in the absence of added BH_4 (12.0 vs 20.2 μmol of •NO h^{-1} mg^{-1}). However, preloaded NOS displayed only 55% of maximum activity (20.2 vs 36.5 μmol of •NO h^{-1} mg^{-1}) in the absence of BH_4 instead of the 75% predicted by Figure 4. This apparent deviation is related to the unexpected increase in activity that is also seen when the preloaded NOS is assayed in the presence of BH_4 (from 27.1 μmol of •NO h^{-1} mg^{-1} before preloading to 36.5 μmol of •NO h^{-1} mg^{-1} after preloading). In a another experiment using the [^{14}C]citrulline assay to determine activity, similar results were observed (not shown). The following control experiments were carried out to discount the effect of nonspecific reducing equivalents or the removal of interfering substances in the purified preparation. When the NOS was directly applied to the gel filtration column without any incubation or added pterin or when the NOS was incubated with a concentration of DTT equivalent to that found in the BH_4 preloading experiments (5.4 mM), the specific activity decreased by 50% (data not shown).

The results of the preloading experiments suggested that although the pterin:NOS ratio could be increased, saturating levels of bound pterin were not achieved as evidenced by the exogenous BH_4-dependent increase in activity. In an attempt to prevent the loss of pterin during the purification, and thus increase the likelihood of observing the maximum amount of NOS-bound BH_4, the NOS was purified in the presence of 5 μM BH_4. Concentration of the NOS in the presence of 2 μM BH_4 was then followed by a 10-min incubation with 400 μM BH_4 at 4 °C followed by gel filtration. As can be seen in Table II (experiment 2), this NOS demonstrated approximately a 1:1 stoichiometry (69.9 nM pterin:63.4 nM NOS subunit) and exhibited 90% of maximum activity, i.e., 80.8 μmol of •NO h^{-1} mg^{-1} in the absence of exogenous BH_4 vs 90.1 μmol of •NO h^{-1} mg^{-1} with BH_4 present at 12 μM. The fractions following the protein peak off the gel filtration column in this experiment were also analyzed for pterins. These fractions demonstrated less than 5 nM pterin, indicating that the gel filtration procedure was successful at separating NOS-bound and free pterin. Another experiment (Table II, experiment 3) resulted in a 0.85:1 stoichiometry (160 nM pterin:189 nM NOS subunit) and NOS activity that was 85% of maximum rate (69.9 vs 82.5 μmol of •NO h^{-1} mg^{-1}). In addition, the specific activity of the NOS in these two experiments was much higher than in the previous experiment, where the NOS was purified in the absence of BH_4, illustrating the instability of the NOS at 4 °C in the absence of BH_4.

Effect of Pterins on NOS Activity. The effects of BH_4 and the analog 6(*R*,*S*)-methyl tetrahydropterin (MPH_4) on the initial rate of citrulline formation are shown in Table III. As with BH_4, the MPH_4-dependent increase in activity was concentration-dependent (data not shown). However, MPH_4 was not as effective as BH_4 at supporting citrulline formation. Since the final concentration of DTT in an assay containing 50 μM BH_4 is 0.7 mM, experiments were performed to rule out nonspecific reducing equivalents as the source of the NOS activity enhancement. Both ascorbate and DTT (1 and 5 mM) were each added to NOS reaction mixtures, and citrulline formation was measured. Neither reducing agent at either concentration was capable of increasing NOS activity (data not shown). Unlike MPH_4, the deazapterin, $DZPH_4$, at an equivalent concentration did not support citrulline formation (Table III). Including 190 μM $DZPH_4$ in the reaction mixture when no other pterin was present inhibited citrulline formation by 30%. In addition, $DZPH_4$ consistently inhibited citrulline formation in a concentration-dependent fashion when NOS was assayed in the absence of exogenous BH_4 (not shown). The deazapterin also inhibited the reaction when redox active pterins were added. Table IV shows that $DZPH_4$ inhibited citrulline formation when NOS was assayed in the presence of either 1 μM BH_4 or 170 μM MPH_4, resulting in 22 and 30% inhibition of activity, respectively. $DZPH_4$ is isolated as the trifluoroacetate salt; however nonspecific inhibition by sodium trifluoroacetate at 190 μM was not seen (data not shown).

Table IV: Inhibition of Citrulline Formation by $DZPH_4$

pterin added	activity[a]
none	9.47 ± 0.6
1 μM BH_4	16.0 ± 0.3
1 μM BH_4 + 190 μM $DZPH_4$	12.5 ± 0.05
170 μM MPH_4	17.3 ± 0.5
170 μM MPH_4 + 190 μM $DZPH_4$	12.1 ● 0.1

[a] All values are the average of duplicate measurements. Assays were done in 200 mM Hepes (pH 7.5) as described under Materials and Methods and contained 1.6 μg of NOS. Activity is expressed as nanomoles of citrulline per hour.

DISCUSSION

The involvement of a number of cofactors and prosthetic groups attests to the complexity of the chemistry involved in the reaction catalyzed by NOS. The constitutive form of NOS, as typified by that isolated from the brain, is strictly regulated by Ca^{2+} and calmodulin, while the macrophage-isolated inducible form shows no such control. However, the chemical steps in the catalysis are likely to be the same because of the cofactor requirements. Because of the sequence homology to cytochrome P-450 reductase (Bredt et al., 1991) and the presence of NOS-bound FAD and FMN (Hevel et al., 1991; Mayer et al., 1991; Stuehr et al., 1991a), it is likely that the flavins serve to shuttle the NADPH reducing equivalents into the active site. In light of the variable, but substantial, NOS activity found in the absence of added BH_4 and the fact that NOS potentially contains its own reductase, we speculated that if the pterin did function in some redox role, then it might be tightly bound to the NOS (Hevel et al., 1991b). This is in contrast to the use of the BH_4:*q*-BH_2 couple with the aromatic amino acid hydroxylases, where the pterin recycling is carried out by another separate reductase (Bailey & Ayling, 1983; Shiman, 1985; Kaufman, 1987).

Results from a number of studies that have sought to determine the necessity of the pterin have been contradictory. While there was initially some question about whether or not the brain NOS required BH_4 for maximum activity, it is now clear that BH_4 has an enhancing effect on •NO formation regardless of the type of NOS. Studies into the molecular details of the function of BH_4 have illustrated some apparent differences in the effect of BH_4 on the inducible vs the constitutive NOS. For example, in some cases BH_4 has had little effect on the velocity of the reaction, while in others,

most notably the macrophage enzyme, BH_4 causes a significant increase in initial rate. In this paper we demonstrate that a difference in the amount of bound pterin may explain these conflicting results. In addition, we present evidence supporting a redox role for BH_4 in the NOS reaction.

Our results demonstrate that although crude macrophage NOS showed very little dependence on added BH_4, the BH_4-dependent increase in activity was significantly greater in the NOS purified in the absence of BH_4 (Table I). This result is consistent with the conclusion that BH_4 is relatively tightly bound to the NOS and is removed from the protein during the course of the purification. In fact, we found that NOS (purified in the absence of BH_4) from several independent preparations demonstrated enzyme-bound pterin in ratios ranging from 0.06 to 0.27 mol of pterin:1 mol of NOS subunit. As seen in Figure 4, the amount of bound pterin was directly proportional to the amount of activity the NOS displayed when assayed in the absence of pterin, suggesting that the rate of $^{\bullet}NO$ formation observed in the absence of added BH_4 was due to bound pterin. Even though extrapolation of Figure 4 also implies that without any pterin present $\sim$10% of maximum activity should be obtained, a few uncertainties prevent overinterpretation of the graph. The NOS samples analyzed in this experiment were all purified in the absence of any BH_4, a condition in which the enzyme is less stable, resulting in specific activities (assayed in the presence of BH_4) often 2–3 times lower than specific activities of NOS purified in the presence of BH_4. In plotting the data in Figure 4, there was no way of determining how much of the total protein was inactive and if this portion of inactive NOS was capable of binding pterin. Nevertheless, the graph does show that the amount of $^{\bullet}NO$ formed is dependent upon the amount of NOS-bound pterin.

The highest reported stoichiometry of pterin binding to date has been from Murad and co-workers (Schmidt et al., 1992), who demonstrated a 0.5 mol of pterin:1 mol of brain NOS subunit. Although the amount of macrophage NOS-bound pterin observed in our initial preparations was much lower, preincubation of the purified NOS with BH_4 (preloading) followed by gel filtration resulted in an increase in the concentration of bound pterin and a corresponding increase in activity when assayed in the absence of exogenous BH_4. However, NOS preloaded with BH_4 was still dependent upon the addition of BH_4 for maximum velocity (Table II). As an alternative to optimizing the experimental conditions for preloading, we attempted to prevent the loss of pterin during the purification. We found that by including BH_4 in all of the purification buffers, followed by an additional short incubation with BH_4, and then quickly desalting the purified macrophage NOS, we could achieve essentially a 1:1 ratio of pterin to NOS subunit (Table II). More importantly, the activity of this preparation was essentially independent of exogenous BH_4, implying that a 1:1 ratio represents the limit of pterin binding. As shown above, the method by which the NOS is purified has a dramatic effect on the amount of enzyme-bound pterin. This finding would explain the results of previous investigations from which it was concluded that NOS from the brain and endothelium did not require BH_4 for maximum activity (Dwyer et al., 1991; Forstermann et al., 1991) and the results of Giovanelli et al. (1991) which showed that with the brain enzyme BH_4 had no effect on the initial rate of the reaction. Furthermore, our preliminary findings suggest that there is an effect of BH_4 on the initial rate of arginine turnover by a partially purified preparation of the brain NOS (Olken and Marletta, unpublished results). Consequently, the method by which the NOS is purified is likely to result in varying amounts of bound pterin in purified NOS preparations and must be considered when the effect of BH_4 in the reaction is examined. Likewise, the presence of bound pterin precludes the determination of a K_m value for BH_4. In addition, experiments aimed at determining a K_D for the pterin are at present not possible due to the instability of NOS in the absence of BH_4.

The question of the function of this tightly bound pterin is difficult to answer. As we mentioned above, there was reason to believe that the BH_4 might function in a redox capacity, and therefore we carried out experiments to answer this mechanistic question. The initial rate of both citrulline and $^{\bullet}NO$ formation was increased in a concentration-dependent fashion by the addition of BH_4 or MPH_4. The observation that MPH_4 supports citrulline formation is important for the conclusions reached with the deazapterin studies. The structures of MPH_4 and $DZPH_4$ differ only in the absence of the N-5 nitrogen in the deaza analogue. Because of the aromaticity of the A ring and the similarity between the sp^3 methylene group and N-5, the B ring of the deazapterin would be expected to adopt a conformation similar to that of the B ring of MPH_4. The deazapterin lacks a potential hydrogen-bond donor, the effects of which on NOS activity are unknown and possibly could be important for the function of the pterin. However, it is unlikely that the loss of a hydrogen bond would result in the complete loss in function since there are many other potential recognition sites on the molecule. Both compounds, MPH_4 and $DZPH_4$, are racemic, which may in part explain the need for such a high concentration of MPH_4 to increase activity and of $DZPH_4$ to inhibit activity, especially with BH_4. Although difficult to analyze due to the background activity from NOS-bound pterin, the inhibition exhibited by $DZPH_4$ is most easily seen when assayed with MPH_4. The MPH_4-dependent rate can be calculated by subtracting the rate due to NOS-bound pterin. In this case (Table IV), 66% of the MPH_4-dependent rate is inhibited. The observation that $DZPH_4$ is incapable of supporting citrulline formation by itself and that $DZPH_4$ inhibits citrulline formation vs both BH_4 and $DZPH_4$ strongly suggests that NOS activity requires redox chemistry of the pterin during catalytic turnover.

The exact function of NOS-bound BH_4 in the catalytic cycle of the reaction is not known. However, the studies reported here clearly show that BH_4 binds with relatively high affinity to the NOS and that the enzyme activity is maximal when the pterin:130-kDa subunit ratio is 1:1. Our results also show that BH_4 not only stabilizes the NOS but also acts to increase enzyme activity. Lastly the results with the deaza analog implicate a redox role for this bound pterin. At least one step in the conversion of L-arginine to citrulline and $^{\bullet}NO$ is a hydroxylation to form N^G-hydroxy-L-arginine. As with L-arginine, NOS-mediated turnover of this intermediate to form products requires NADPH, and we have speculated that this may mean that a second hydroxylation takes place. Therefore, the results presented here are consistent with the idea that this bound pterin functions in either one of the two postulated hydroxylation steps in a manner similar to that observed with the amino acid hydroxylases. Because this pterin is tightly bound, it would appear to function catalytically and would be recycled on the NOS and not in solution. Consequently, typical inhibitors of dihydrobiopterin reductases such as methotrexate may not inhibit NOS. This theory gains support not only from the observations of Kaufman and colleagues (Giovaneli et al., 1991) but also from preliminary results in our laboratory which show that

neither dihydrofolate reductase nor dihydropteridine reductase increases NOS activity when NOS is assayed under conditions where bound-BH_4 concentrations are suboptimal (Hevel and Marletta, unpublished results). On the other hand, all of the results obtained so far are also consistent with BH_4 functioning in a redox fashion to initially activate the NOS, perhaps similar to the activation of phenylalanine hydroxylase by the first BH_4 equivalent (Marota & Shiman, 1984; Wallick et al., 1984), or to reactivate the NOS after an occasional inactivation event that may occur during turnover. Our continuing studies are directed toward distinguishing between these possibilities.

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