Tetrahydrobiopterin-Deficient Nitric Oxide Synthase Has a Modified Heme Environment and Forms a Cytochrome P-420 Analogue†

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ABSTRACT: Optical absorption and resonance Raman spectra of neuronal nitric oxide synthase (b-NOS) isolated in the absence of tetrahydrobiopterin demonstrate that the enzyme preparation is very unstable. This unstable form of the enzyme has properties analogous to those of cytochrome P-420$_{cam}$, an inactive form of cytochrome P-450$_{cam}$. Although cysteine is preserved as the proximal ligand in both the ferric and ferrous forms of unstable b-NOS, the lack of tetrahydrobiopterin significantly increases the hexacoordinate low-spin fraction of the heme content, resulting in a loss of the enzymatic activity. Upon the addition of CO, the unstable b-NOS converts from a species exhibiting a Soret absorption maximum at 443 nm, as reported for the CO adducts of stable b-NOS and cytochrome P-450$_{cam}$, to a species with a Soret maximum at 421 nm. The resonance Raman spectrum of the 421-nm form is the same as those of CO-bound myoglobin at low pH and CO-bound cytochrome P-420$_{cam}$. The heme in this form of the enzyme is coordinated by a weaker ligand than thiolate; histidine coordination in the CO-bound form of the P-420-like species of NOS is consistent with all of the available data. A similar unstable form of the macrophage (i-NOS) enzyme was also detected. Not only does the heme pocket of NOS have the same coordination as cytochrome P-450 in its stable form, but the partially denatured form has the same properties as cytochrome P-420, the inactive form of cytochrome P-450. Among other possible roles, tetrahydrobiopterin may play a significant role in the stabilization of the active enzyme.

Nitric oxide, shown to play many physiological roles, is formed from the catalytic oxidation of arginine by enzymes termed nitric oxide synthases (NOSs) (Bredt et al., 1991; Culotta & Koshland, 1992; Lancaster, 1992; Stamler et al., 1992; Snyder & Bredt, 1992; Stuehr & Griffith, 1992; Feldman et al., 1993). The neuronal enzyme from rat brain (b-NOS) is constitutive and contains several cofactors such as NADPH, FAD, and FMN, located in its reductase domain, and tetrahydrobiopterin (H_4biopterin), heme, and its substrate, L-arginine, residing in its oxygenase domain. The enzyme is activated by binding calcium−calmodulin (Ca^{2+}−CAM) between the two domains (Bredt et al., 1991), which, by recent findings, triggers the electron transfer from NADPH to the heme catalytic center (Abu-Soud & Stuehr, 1993). Macrophage (inducible) NOS (i-NOS) also requires the tight binding of CAM to carry out the heme iron reduction. The properties of the heme, only recently discovered, are related to those of the cytochrome P-450 class of enzymes in which the heme is coordinated to the apoprotein through a thiolate axial ligand (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan et al., 1992; Wang et al., 1993; Chen et al., 1994). The active site has additional similarities to cytochrome P-450 involving the sensitivity of the spectral properties of bound exogenous ligands to the presence of substrate (Wang et al., 1994). However, the full extent of the analogy to cytochrome P-450 is yet to be determined, and as recently pointed out, NOS may be only distantly related to the large class of P-450 enzymes (Marletta, 1994).

The cytochrome P-450 class of proteins catalyze the monooxygenation reactions of a variety of structurally unrelated substrates including foreign chemicals and physiologically occurring biomolecules (Ortiz de Montellano, 1986). The extensive family shares remarkably similar physical properties despite a wide diversity in the type of substrate and type of reaction. One of the common features of this class of enzymes is that they can be converted to a species exhibiting a characteristic Soret maximum of the CO-bound form at 420 nm by incubation in acetone (Gunsalus et al., 1974; Champion et al., 1978; Lipscomb, 1980) or exposure to high temperature or pressure (Hui Bon Hoa et al., 1989; Martinis, 1990; Wells et al., 1992) and thus this inactive form of the enzyme has been termed cytochrome P-420. In an effort to understand the structural basis causing the conversion, Stern and Peisach (1974) and Collman and Sorrell (1975) found that replacement of the anionic thiolate ligand by a protonated thiol group in CO-bound model heme complexes resulted in a Soret maximum shift from ~450 nm to the 413−422-nm range, which were supported by results of iterative extended Huckel calculations (Hanson et al., 1976). Chang and Dolphin (1975) further suggested that a deprotonated cysteine can serve as the proximal ligand in the ferric, deoxy and ferrous CO-bound forms but not in the oxygenated form of P-450, thus first raising the possibility

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of switching the mercaptide to a non-sulfur-containing ligand in cytochrome P-450s. In the recent characterization on the cytochrome P-420_{cam} (the hemeprotein from *Pseudomonas putida* that hydroxylates camphor) by optical and resonance Raman techniques, Champion and Sligar and co-workers (Wells et al., 1992) convincingly proved that exchange of the proximal anionic thiolate ligand (cysteine) to a histidine, instead of a neutral thiol group, is responsible for the shift of the Soret band from ~450 to 420 nm. The conclusion was established by the remarkable similarity between the spectra from CO adducts of P-420_{cam} and those of low-pH myoglobin (Mb) and by the detection of the Fe−histidine stretching vibrational mode when CO is photolyzed from heme of CO-bound P-420_{cam}.

Here we report the spectroscopic characterization of a neuronal NOS isolated in the absence of H_4biopterin. We find that this form of NOS is unstable and converts from a pentacoordinated high-spin heme to a hexacoordinated low-spin heme, which in turn inhibits the enzyme's activity by blocking the oxygen binding site at the heme. Furthermore, we find that the CO-bound form of native i-NOS also converts to a P-420-like species with time. Thus, both classes of NOS share with the P-450s a conversion from an active species with an absorption maximum near 450 nm, when CO-bound, to an inactive form with an absorption maximum near 420 nm.

MATERIALS AND METHODS

Generation and Purification of H_4Biopterin-Deficient b-NOS. Stably transfected R293 kidney cells expressing neuronal NOS were grown in the presence of 3 mM, 2,4-diamino-6-hydroxypyrimidine, which inhibits biosynthesis of H_4biopterin (Gross & Levi, 1992). NOS was purified by sequential chromatography on 2′,5′-ADP−Sepharose and Mono Q anion-exchange resin as described previously (Stuehr & Ikeda-Saito, 1992), except that H_4biopterin was omitted from all column buffers. The purified NOS preparations were ~90% pure as judged by SDS−PAGE and silver staining. The protein content was assayed with the Bio-Rad kit, using bovine serum albumin as a standard.

Generation of Macrophage i-NOS. Mouse macrophage i-NOS was purified in the presence of 4 μM H_4biopterin as previously described (Stuehr & Ikeda-Saito, 1992) from cultures of RAW 264.7 cells that had been induced to express i-NOS by treatment with interferon γ and *Escherichia coli* lipopolysaccharide.

Quantitation of NO Synthesis. Aliquots from column fractions were assayed at 37 °C in the presence or absence of 4 μM H_4biopterin using a 1.5-h microplate assay for nitrite as described previously (Stuehr & Ikeda-Saito, 1992). The specific activity of the concentrated purified NOS was determined at 37 °C using a spectrophotometric oxyhemoglobin assay for NO as described (Abu-Soud & Stuehr, 1993).

Raman Measurements. The methods to measure the resonance Raman spectra (Rousseau & Ondrias, 1984) of this protein have been reported elsewhere (Wang et al., 1993, 1994). The NO adduct in the oxidized form was prepared by flushing NO gas (Matheson, >99% purity) over the pre-degassed resting enzyme (Wang et al., 1994). The reduced enzyme was prepared by anaerobically adding dithionite solution to the previously degassed proteins, from which the CO adducts were generated by exposure of the samples to either a natural $^{12}C^{16}O$ (Matheson, >99% purity) or a stable isotope $^{13}C^{18}O$ atmosphere (ICON, 99%,99% labeled). The $Fe^{2+}-CN^-$ complex was generated by anaerobically adding 15-fold molar excess dithionite solution to the pre-degassed $Fe^{3+}-CN^-$ sample. All of the samples were buffered by 40−100 mM Hepes, pH 7.4, except for the Mb−CO (100 mM citrate/200 mM phosphate, pH 3.9). Typically, the sample aliquots (140 μL, ~20 μM) sealed in a rotating cell were irradiated at low laser power (<4 mW) with an excitation wavelength selected to optimize the adduct under study as specified in the figure captions. The scattered light was dispersed by a 1.25-m monochromator and detected by a CCD camera. The spectra displayed here are baseline-corrected but unsmoothed. Optical absorption data were recorded both prior to and after each Raman measurement to probe the spectral and structural modifications caused by time delay or laser irradiation.[1]

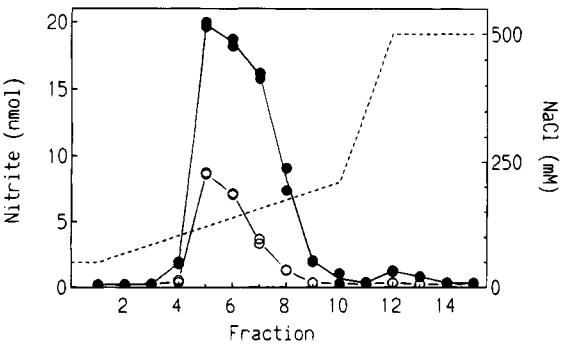

FIGURE 1: H_4biopterin dependence of neuronal NOS following chromatography on Mono Q resin. Neuronal NOS that had been partially purified by 2′,5′-ADP−Sepharose chromatography was loaded onto the Mono Q column and eluted with a salt gradient (- - -). Aliquots of each 2-mL column fraction were assayed in duplicate in the presence (●) or absence (○) of 4 μM H_4biopterin. NO synthesis was measured as nitrite accumulated over the 1.5-h assay period.

RESULTS

The NO synthase activity of neuronal NOS as it eluted from the Mono Q column is shown in Figure 1. Addition of H_4bioptern to the assay buffer increased each fraction's activity by an average of 4-fold, indicating that NOS had lost a significant portion of its bound H_4biopterin during purification (Mayer et al., 1990). The specific activity of the concentrated NOS preparation when assayed in the presence or absence of H_4biopterin was 70 versus 22 nmol of NO min^{-1} mg^{-1}, indicating a 68% dependence on exogenous H_4biopterin. The NOS preparation also displayed an A_{275}/A_{395} absorbance ratio of 5.3, which is significantly higher than the 3.7 ratio observed for neuronal NOS purified in the presence of H_4biopterin (Stuehr & Ikeda-Saito, 1992). The higher ratio indicates that bound heme was partially lost from the enzyme during purification in the absence of H_4-biopterin (Abu-Soud et al., 1994). To measure the heme content, we analyzed the optical spectra by comparing the contributions from the flavins to those of the hemes. It was determined that the heme content in our H_4biopterin-deficient preparation was ~20% of that for preparations done in the presence of the biopterin. This loss of heme thereby accounts for the enzyme having a very low specific activity[1] even when assayed in the presence of H_4biopterin.

[1] The specific activity of neuronal NOS purified in the presence of H_4biopterin typically falls between 400 and 1000 nmol min^{-1} mg^{-1}.

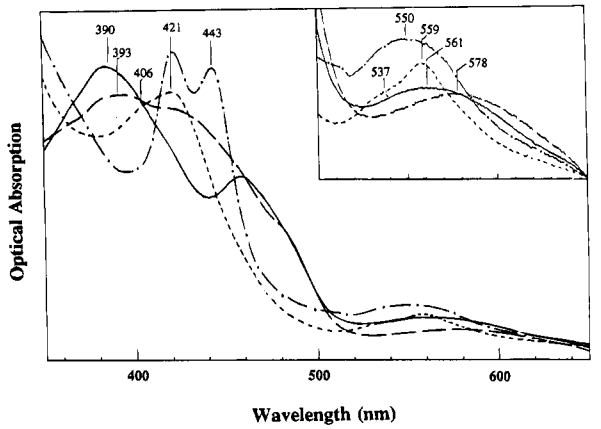

FIGURE 2: Optical absorption spectra of brain nitric oxide synthase, isolated in the absence of H_4biopterin, in the oxidized form before the Raman measurements (—), in the oxidized form after the Raman measurements (— —), in the dithionite-reduced form after the Raman measurements (- - -), and in the CO-bound form (—•—). The data were recorded on the same sample used for the Raman measurements in the sealed Raman spinning cell. The inset is a vertical expansion of the visible region of the spectra in which the spectra were displaced so as to overlap at 650 nm.

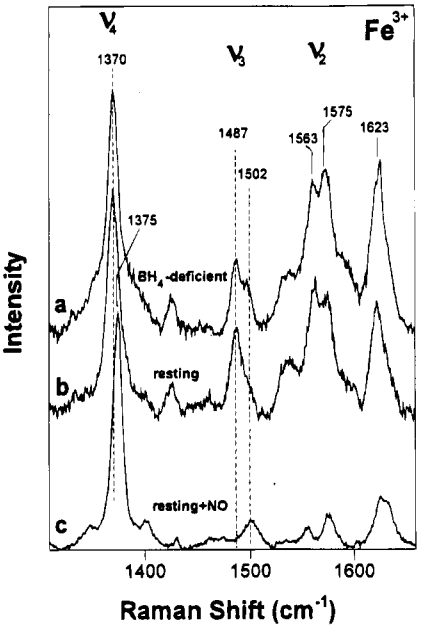

FIGURE 3: Resonance Raman spectra in the high-frequency region of b-NOS, isolated in the absence (a) and presence (b, c) of H_4biopterin in the oxidized forms (pH 7.4). Trace c is the NO adduct of resting enzyme. No exogenous ligands were added to the samples used for spectra a and b. Spectra a and b were excited at 406.7 nm, and spectrum c at 441.6 nm, to optimize the resonance effect. All the spectra are baseline-corrected but not smoothed.

In contrast to our findings with neuronal b-NOS isolated in the presence of H_4biopterin (Wang et al., 1993), we find that various states of the H_4biopterin-deficient b-NOS change with time and also are changed by exposure to the low-power laser probe beam used to obtain the resonance Raman spectra. This is illustrated in Figure 2, in which the optical absorption spectra of various forms of the enzyme are reported. The optical spectrum of the resting H_4biopterin-deficient enzyme (solid line) is similar to that of stable native enzyme prior to laser exposure. However, as seen in Figure 2, after laser exposure (broken line) there is a large and broad increase in intensity in the region centered at about 430 nm.

The resonance Raman spectrum of fully oxidized H_4-biopterin-deficient b-NOS (trace a) is compared to spectra of the resting b-NOS isolated in the presence of H_4biopterin (trace b) and its ferric NO-bound complex (trace c) (Wang et al., 1994) in Figure 3. The frequencies of the major lines are summarized in Table 1. The heme in the resting native form is pentacoordinate high-spin (5C/HS), evident by a single line in the ν_3 region, a spin- and ligation-state marker, at 1487 cm^{-1} (Wang et al., 1993). The lines from ν_4 at 1370 cm^{-1} and ν_2 at 1563 cm^{-1} are also consistent with this conclusion. Coordination of NO to the sixth ligand position (hexacoordinate low-spin, 6C/LS) substantially shifts ν_3 to 1502 cm^{-1}, as well as ν_4 and ν_2 to 1375 and 1575 cm^{-1}, respectively. Ferric H_4biopterin-deficient enzyme shown in trace a is characteristic of a ferric heme species but somewhat different from that of stable b-NOS (trace b). Specifically, in the region of ν_3, we find a splitting into two components located at 1487 and 1502 cm^{-1}. The line at 1487 cm^{-1} coincides with that from the stable enzyme and is characteristic of a species which is five-coordinate high-spin as discussed previously. We assign the component at 1502 cm^{-1} as originating from a contribution from a species with a 6C/LS configuration as it appears at the same frequency as those reported for NO-coordinated ferric b-NOS (trace c) and NO-bound (substrate-free) cytochromes P-450_{cam} (Hu & Kincaid, 1991). However, the five-coordinate high-spin configuration is still the major form present in the oxidized state of the unstable enzyme.

The absorption spectrum of the dithionite-reduced form of the H_4biopterin-deficient enzyme subsequent to obtaining the resonance Raman spectrum is shown in Figure 2. The maximum in the Soret region of the absorption spectrum at 421 nm is shifted from that at 412 nm in the stable b-NOS (Wang et al., 1993). The resonance Raman spectrum of H_4-

Table 1: Resonance Raman Data from b-NOS Isolated in the Presence and Absence of H_4Biopterin[a]

	ν_4 (cm^{-1})	ν_3 (cm^{-1})	ν_2 (ν_{11}, ν_{37}, ν_{19}, ν_{38}) (cm^{-1})	ν_{10} (ν_{vinyl}) (cm^{-1})
oxidized				
b-NOS(+3)	1370	**1487**	1562/1575	**1623**
b-NOS(+3)−NO	1375	**1502**	1575	1626/1634
BH_4-deficient b-NOS(+3)	1371	**1487/1502**	1562/1575	1623
reduced				
b-NOS(+2)	**1347**/1360	**1466**	1553/1584	**1600**/1617
b-NOS(+2)−CN^-	**1360**	**1492**	1553/1580	1615
BH_4-deficient b-NOS(+2)	**1360**	**1467/1490**	1553/1580	1615
reduced-CO				
b-NOS_{450} (native b-NOS−CO)	1369	1495	1561/**1573**	1619/1628
b-NOS_{420} (BH_4-deficient)	1370	1496	1558/**1581**	1619/1628

[a] Modes exhibiting significant shifts are shown in boldface type.

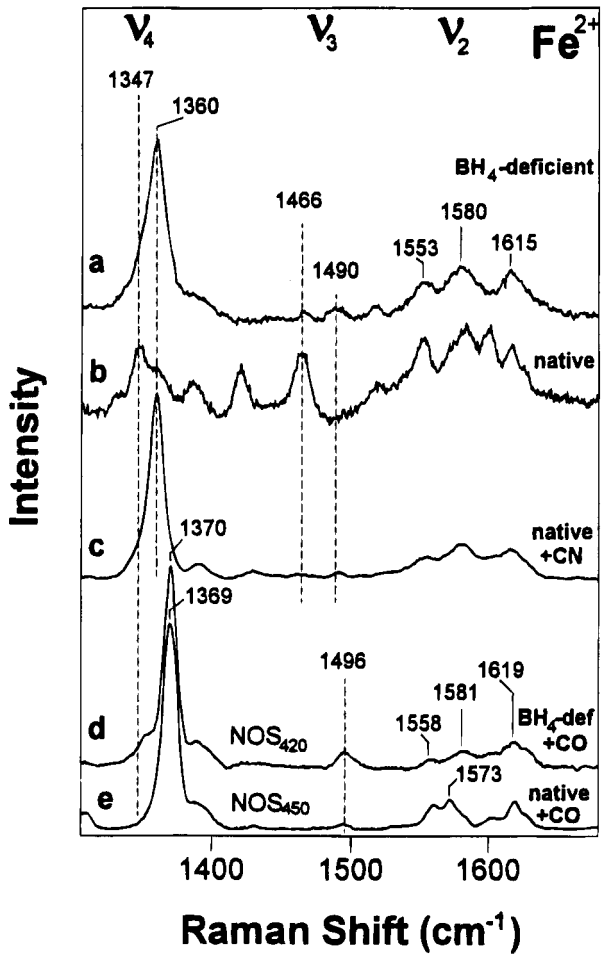

FIGURE 4: Resonance Raman spectra in the high-frequency region of b-NOSs, isolated in the absence (a, d) and in the presence (b, c, e) of H_4biopterin in the dithionite-reduced forms (pH 7.4). Trace c is the CN^- derivative of the reduced enzyme and spectra d and e are the CO derivatives. No exogenous ligands were added to the samples used in spectra a and b. Spectra b and d were excited at 413.1 nm, spectrum a at 406.7 nm, and spectra c and e at 441.6 nm to optimize the resonance effect. All the spectra are baseline-corrected but not smoothed.

biopterin-deficient b-NOS(+2), as shown in trace a of Figure 4, is substantially different from that of stable b-NOS(+2) (trace b). Specifically, in the ν_4 region the line at 1347 cm^{-1} is absent and the line at 1360 cm^{-1} is very strong. Similarly, in the ν_3 region the line at 1466 cm^{-1} is greatly diminished. Several differences are also evident in the ν_2 region. In the stable enzyme, strong lines from ν_3 and ν_{10} (a mode sensitive to the porphyrin core size), located at 1466 and 1600 cm^{-1}, respectively, are characteristic of a 5C/HS heme as suggested previously for reduced b-NOS (Wang et al., 1993) and cytochrome P-450s (Wells et al., 1992). In addition, the appearance of a weak ν_4 at 1347 cm^{-1} is also characteristic of a 5C/HS heme with an electron-donating thiolate coordinated in the proximal side, as reported in cytochrome $P\text{-}450_{cam}$ (Wells et al., 1992), chloroperoxidase (CPO) (Remba et al., 1979), and model compounds (Anzenbacher et al., 1989). To determine the origin of the spectral changes in the ligand-free ferrous form of the unstable enzyme, we examined the cyanide adduct. Addition of the CN^- to the ferrous ligand-free stable b-NOS generates a 6C/LS heme, exhibiting significant shifts of ν_3 to 1490 cm^{-1}, ν_{10} to ~1620 cm^{-1}, and ν_4 to 1360 cm^{-1} with a dramatic intensification (trace c of Figure 4). In comparing the H_4biopterin-deficient b-NOS(+2) without any added ligands (trace a) to the native enzyme (trace b) we observed almost identical spectral alterations as caused by coordination of CN^- to the stable b-NOS(+2), indicating a conversion from 5C/HS to 6C/LS. The differences between the unstable (H_4biopterin-deficient) and the stable forms of the dithionite-reduced b-NOS are similar to those seen in the comparison between cytochrome $P\text{-}420_{cam}$ and cytochrome $P\text{-}450_{cam}$ (Wells et al., 1992). These results clearly show that when b-NOS is isolated in the absence of H_4biopterin, the heme is partially converted to a 6C/LS species. On the basis of the scattering efficiency of the different forms, we estimate that the conversion of the reduced enzyme to the 6C/LS form is near complete (~90%). The changes that we detect in the optical spectrum in which the maximum in the Soret region shifts from 412 nm for the stable enzyme to 421 nm for the unstable enzyme reflect a spin-state transition which was also reported in the $P\text{-}450_{cam}$ case, where a change from 408 to 423 nm was detected upon conversion to the P-420 form.

Despite near-complete conversion of the ligand-free reduced enzyme to a six-coordinate form characteristic of the inactive cytochrome $P\text{-}420_{cam}$, by flushing CO over the reduced unstable enzyme the immediate product obtained was dominated by a species with a Soret maximum at 443 nm (see solid line in Figure 5A) and is thus the P-450 form of the enzyme. However, CO-bound H_4biopterin-deficient b-NOS is unstable and converted to a species with a Soret maximum at 421 nm, a P-420-like form, with time and by laser irradiation (dashed line in Figure 5A). In cytochrome $P\text{-}420_{cam}$ the Soret transition of the CO-bound form is detected at 423 nm (Wells et al., 1992).

To determine if inducible NOS from macrophage also forms a P-420-like species, we examined an enzyme preparation of i-NOS generated in the presence of H_4-biopterin. We found that the optical and the resonance Raman spectra of resting and reduced i-NOS were similar to those of the corresponding forms of native b-NOS (data not shown), indicating similar coordination states and heme environments for these two isoforms. However, the CO-bound derivative of i-NOS was not stable and converted to a P-420-like form (see Figure 5B) during our data acquisition ($i\text{-}NOS_{420}$).

The resonance Raman spectrum in the high-frequency region of the CO-bound enzyme, obtained with 413-nm excitation to enhance the 421-nm species of H_4biopterin-deficient b-NOS ($b\text{-}NOS_{420}$; trace d in Figure 4), confirms that this species is CO-bound as indicated by the line in the ν_4 region which appears at 1370 cm^{-1}. The frequencies and the relative intensities of the lines we detect in the spectrum are very different from those which we found in the stable form of b-NOS-CO ($b\text{-}NOS_{450}$; trace e) but very similar to those of CO-bound myoglobin at low pH and cytochrome $P\text{-}420_{cam}$ (Wells et al., 1992). In particular, a clear shift of ν_2 from 1573 to 1581 cm^{-1}, as well as an intensification of ν_3 at 1496 cm^{-1}, is associated with the conversion from $b\text{-}NOS_{450}$ to $b\text{-}NOS_{420}$. A similar spectrum was obtained from i-NOS.

The spectral difference of $b\text{-}NOS_{420}$ from that of $b\text{-}NOS_{450}$ is more evident in the low-frequency resonance Raman spectra (Figure 6). As reported previously (Wang et al., 1993), the $b\text{-}NOS_{450}$ form (trace a) is characterized by an Fe−C−O bending mode (δ_{Fe-C-O}) at 563 cm^{-1} and a broad signal from the Fe−CO stretching mode (ν_{Fe-CO}), which was

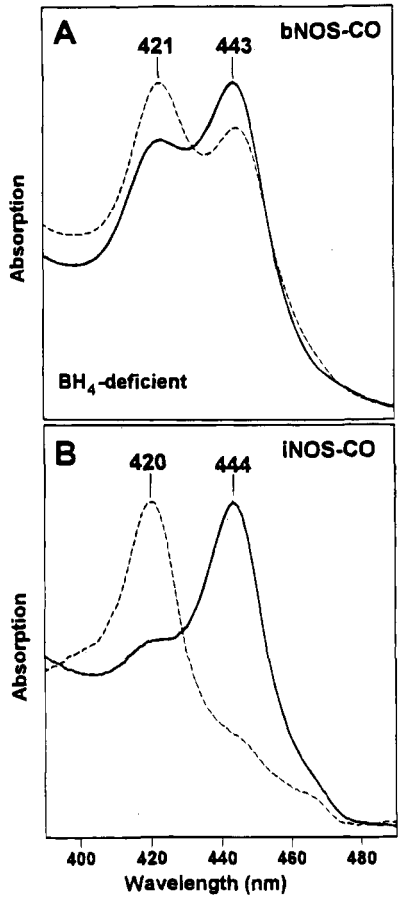

FIGURE 5: Optical absorption spectra of CO adducts of rat brain constitutive b-NOS isolated in the absence of H_4biopterin (A) and inducible i-NOS from macrophage (B). (A) the spectra were obtained immediately after (—) and 8 h after (- - -) the addition of CO to the dithionite-reduced H_4biopterin-deficient b-NOS. (B) The spectra were obtained immediately after (—) and 6 h after (- - -) the addition of CO to the dithionite-reduced i-NOS. The data were recorded on the same sample used for the Raman measurements in the sealed Raman spinning cell. The Raman measurements were made on the samples prior to measuring the 8-h delay (H_4biopterin-deficient b-NOS) and the 6-h delay (i-NOS) absorption spectra.

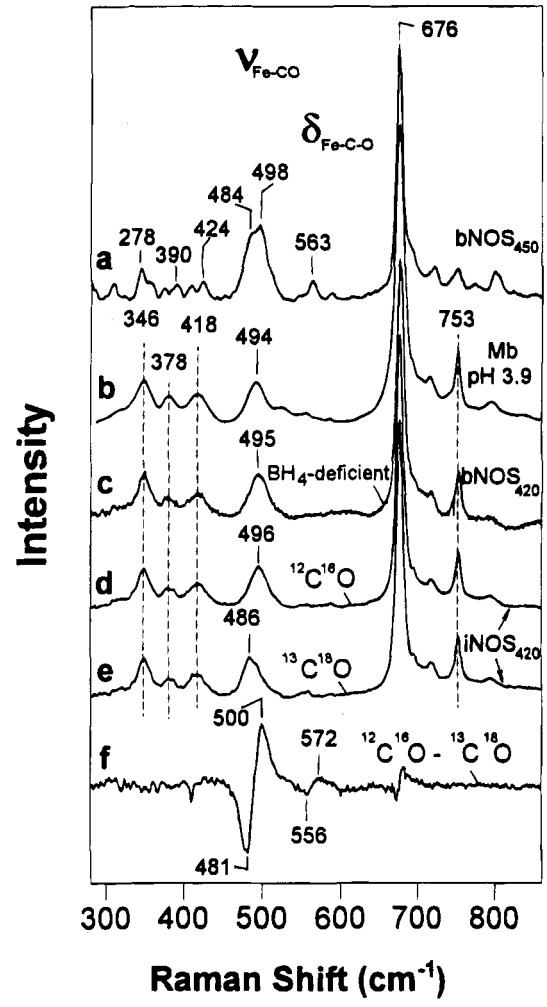

FIGURE 6: Resonance Raman spectra in the low-frequency region of the CO adducts from dithionite-reduced brain NOS isolated in the presence (a) and absence (c) of H_4biopterin and inducible NOS from macrophage (d, e) at pH 7.4. The CO-bound form of reduced myoglobin obtained at pH 3.9 (b) is presented here as a reference for the P-420-like NOS where a histidine was identified as its proximal ligand. Spectra a–d and e were from the $^{12}C^{16}O$-bound and $^{13}C^{18}O$-bound forms, respectively. Trace f is the difference spectrum of (d) minus (e) ($^{12}C^{16}O - {}^{13}C^{18}O$), where the contributions from porphyrin modes balance out, revealing the isotopic shifts from the Fe–CO stretching (~500 cm^{-1}) and Fe–C–O bending (572 cm^{-1}) modes. All the spectra were recorded with an excitation wavelength of 413.1 nm except trace a, where excitation of 441.6 nm was employed. Some of the spectra were baseline-corrected but none were smoothed.

deconvoluted into two components centered at 484 and 498 cm^{-1}, respectively. In the b-NOS_{420} form (trace c) we observed a much narrower line at 495 cm^{-1} and no evidence in the spectrum for a bending mode at 563 cm^{-1}. In addition to these changes, the porphyrin modes of b-NOS_{420} are also quite different, including the increase in the intensity of ν_8 at 753 cm^{-1} and extensive changes in the 300–430-cm^{-1} region, where there are many weak lines in the native enzyme but only three lines in the 420 form. The spectrum of b-NOS_{420} (trace c) becomes identical to that of Mb–CO observed at pH 3.9 (trace b) and that of cytochrome P-420_{cam} reported by Champion and co-workers (Wells et al., 1992). In both cases, the proximal ligand has been proven to be a histidine residue by the detection of a Fe–His stretching mode (ν_{Fe-His}) at 218 cm^{-1} from the photogenerated transient (5C/HS) species in the CO forms of P-420_{cam} and Mb.

The spectrum of i-NOS_{450} (data not shown) is the same as that of b-NOS_{450} (trace a) when excited at 441.6 nm in the "fresh" i-NOS_{450} prior to its conversion to i-NOS_{420}. The resonance Raman spectrum of i-NOS_{420} (trace d), probed with an excitation wavelength of 413.1 nm after the conversion, is almost identical to those from b-NOS_{420} (trace c), P-420_{cam} (Wells et al., 1992), and low-pH Mb–CO (trace b). Substitution of $^{12}C^{16}O$ by the $^{13}C^{18}O$ isotope in i-NOS_{420} substantially shifts the line at 496 cm^{-1} to 486 cm^{-1} as evident in trace e and in the isotope difference spectrum (trace f). In addition, in the difference spectrum a mode is detected at 572 cm^{-1} for $^{12}C^{16}O$ that shifts to 556 cm^{-1} for $^{13}C^{18}O$. The isotope shifts at 496 and 572 cm^{-1} confirm the assignment of these two modes as the iron–CO stretching mode (ν_{Fe-CO}) and the iron–carbon–oxygen bending mode (δ_{Fe-C-O}), respectively. It is noteworthy that the frequency of ν_{Fe-CO} (495–496 cm^{-1}) and the frequency (572 cm^{-1}) and low intensity of δ_{Fe-C-O} exhibited by NOS_{420} species are characteristic of heme proteins with an unconstrained heme pocket and in which the iron atom is coordinated to a neutral imidazole (Yu & Kerr, 1988).

DISCUSSION

The optical absorption and the resonance Raman spectra of an unstable form of b-NOS generated by isolating the enzyme in the absence of H_4biopterin are remarkably similar to those of cytochrome P-420$_{cam}$, the inactive form of cytochrome P-450$_{cam}$. In b-NOS, when the heme is in the ferric oxidation state, the spectra show a mixture of spin and coordination states, unlike the stable form of NOS, which is high-spin and most probably five-coordinate (Wang et al., 1993), but more definitive evidence is needed to rule out a contribution from a six-coordinate high-spin configuration. Prior to the Raman measurements the optical spectrum of the resting form of the H_4biopterin-deficient enzyme was the same as that of the resting form of the native enzyme, and thus we infer that the coordination (thiolate) is the same as that in the native form of the enzyme. However, the heme pocket lacks the stability that it has in the enzyme prepared in the presence of H_4biopterin, allowing the coordination of a residue or exogenous ligand to the sixth position on the heme iron, thus resulting in a contribution from a hexacoordinate low-spin form. A similar transition of the ligation state (from 5C/HS to 6C/LS) was also observed in the substrate-bound cytochrome P-450$_{cam}$ by a shift of the Soret maximum from 390 to 420 nm upon the conversion to P-420 as well as by changes in the resonance Raman spectrum (Wells et al., 1992). The formation of the six-coordinate species does not require exchange or modification of the thiolate ligand, as was proposed for cytochrome P-420$_{cam}$, since the stable NO-bound form of the ferric native enzyme (trace c in Figure 3) has resonance Raman lines similar to those which we attribute to the six-coordinate form of the unstable enzyme (trace a in Figure 3). Also, we do not detect a band in the Soret region at 370 nm which was attributed to the dissociation of the proximal thiolate ligand in cytochrome P-420. Thus, we conclude that the ferric form of H_4biopterin-deficient b-NOS still possesses a proximal thiolate ligand in spite of its increase of population in hexacoordinate low-spin heme.

Upon reduction of the enzyme, the fraction of 6C/LS heme in H_4biopterin-deficient b-NOS increases dramatically. This is evident by the disappearance of lines characteristic of pentacoordination, such as ν_3 at 1466 cm^{-1}, ν_{10} at 1600 cm^{-1}, and ν_4 at 1347 cm^{-1}, and the remarkable similarity of its spectrum to that of CN^--bound stable b-NOS, which is 6C/LS. Similar behavior was also reported in the reduced form of P-420$_{cam}$ and was attributed to the replacement of the proximal thiolate ligand by a histidine residue due to the coincidence of the position of its ν_4 line (1361 cm^{-1}) with that from proteins with proximal nitrogenous ligands (Wells et al., 1992). However, in NOS we conclude that an anionic thiolate residue is still the proximal ligand, as (1) an optical spectrum from the "normal" thiolate-ligated P-450-like form is obtained immediately after the addition of CO to the reduced H_4biopterin-deficient b-NOS sample and (2) coordination of strong field ligands such as CN^- to the stable b-NOS(+2) yields a spectrum (trace c in Figure 4) similar to that of the H_4biopterin-deficient ligand-free enzyme (trace a in Figure 4).

The electronic coupling between axial ligands and the porphyrin ring occurs through the central iron atom since the iron d_π orbitals have the same symmetry as that of the porphyrin π^* orbital (Li & Spiro, 1988). The observation of ν_4 with an anomalously low frequency in the reduced (5C/HS) forms of cytochrome P-450$_{cam}$ (1345 cm^{-1}; Wells et al., 1992), chloroperoxidase (1348 cm^{-1}; Remba et al., 1979) and b-NOS (1347 cm^{-1}; Wang et al., 1993) are the consequences of electron donation from the electron-rich proximal cysteine to the porphyrin π^* antibonding orbitals. However, when ligands possessing empty π^* orbitals such as CO, CN^-, NO, and O_2 bind on the distal side, the competition between the π^* orbitals from these ligands and those from the porphyrin strongly favors the axial ligands and thus the electron population in the porphyrin π^* orbital created by the proximal thiolate ligand is removed (Anzenbacher et al., 1989). This is evident in the various ligand-bound forms of stable b-NOS (such as CO and NO) (Wang et al., 1993, 1994). Moreover, the frequency of ν_4 (1360 cm^{-1}) in the CN^- adducts of reduced b-NOS (trace c in Figure 4) and CPO (Remba et al., 1979) is similar to that from proteins with nitrogenous ligands. We conclude that the frequencies of the low-spin ferrous form of the unstable NOS are fully consistent with thiolate remaining as the proximal ligand and do not require its protonation, as has been proposed for cytochrome P-420 (Stein & Peisach, 1974; Collman & Sorrell, 1975). Furthermore, just as in the case of the oxidized enzyme, it is also not necessary to invoke replacement of the thiolate ligand to account for the spectral properties of the ligand-free reduced form of b-NOS$_{420}$; it is only necessary for another ligand to coordinate to the distal side. We cannot exclude the possible coordination of a histidine residue to the distal side of the heme as suggested by Sono and Dawson (1986) in a study of a P-420-like species from reduced chloroperoxidase generated at alkaline pH. The results reported here show that when NOS becomes unstable, either an endogenous ligand (an amino acid residue) or an exogenous ligand such as OH^- or H_2O can move close to the heme and coordinate in the distal position, thereby converting the heme to a 6C/LS species.

Addition of CO to reduced unstable b-NOS yields an immediate product which has thiolate as the fifth ligand, confirmed by the absorption maximum at 443 nm, but gradually converts to a b-NOS$_{420}$ form with a Soret maximum at 421 nm. Similarly, a time-dependent conversion from the NOS$_{450}$ form to a NOS$_{420}$ species was recorded in CO-bound i-NOS during our data acquisition. The resonance Raman spectra from b-NOS$_{420}$ and i-NOS$_{420}$ are nearly identical to those of CO-bound myoglobin at low pH and CO-bound cytochrome P-420$_{cam}$. On the basis of the frequencies of the heme modes and the frequency of the Fe−CO stretching modes (see Table 2), in both of those proteins the fifth ligand has been assigned as a histidine (Wells et al., 1992). The detection of the ν_{Fe-His} line at 218 cm^{-1} from the photoproduced transient ligand-free species of CO-bound Mb and P-420$_{cam}$ was taken as solid evidence establishing the above assignment.

Owing to the significant difference between the resonance Raman spectra of b-NOS$_{420}$ and i-NOS$_{420}$ from those of the NOS$_{450}$ isoforms (see Figures 4 and 6 and Table 1) and the remarkable similarities of the heme modes and ν_{Fe-CO} frequencies of b-NOS$_{420}$ and i-NOS$_{420}$ to those of low-pH Mb−CO and cytochrome P-420$_{cam}$ (see Figure 6 and Table 2), we make a similar assignment for the CO-bound NOS$_{420}$. We conclude that the gradual changes in the optical absorption spectra are a direct consequence of the ligand exchange from thiolate to neutral imidazole. In addition, the frequency

Table 2: Dependence of Frequencies of ν_{Fe-CO} and δ_{Fe-C-O} on the Identity of the Proximal Ligand in Heme Proteins

proximal ligand	ν_{Fe-CO} (cm^{-1})	δ_{Fe-C-O} (cm^{-1})	ν_{C-O} (cm^{-1})	ν_{Fe-L} (L = His/Cys) (cm^{-1})	proteins[a]	ref
histidine (imidazolate)	528−547	578−590	1905−1955	234−258	CcP, HRP, LPO	*b*−*d*
histidine (neutral)	494−524	574−579	1930−1970	203−230	Mb, Hb, CcO	*d*−*f*
cysteine (thiolate)	464−484	556−558	1932−1963	351	P-450s	*g*, *h*
cysteine (thiolate)	486	560	1958	347	CPO	*g*, *i*
cysteine (thiolate)	484/498	563			b-NOS_{450}	*j*
histidine (neutral)	494−496		1966	218	P-420_{cam}	*k*, *l*
histidine (neutral)	495−496	572			b-NOS_{420}, i-NOS_{420}	*i*

[a] CcP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; Mb, myoglobin; Hb, hemoglobin; CcO, cytochrome *c* oxidase; P-450, cytochrome P-450; CPO, chloroperoxidase; NOS, nitric oxide synthase. [b] Spiro et al. (1990). [c] Hu et al. (1993). [d] Kitagawa (1988). [e] Rousseau et al. (1993). [f] Song et al. (1993). [g] Champion (1988). [h] Yu and Kerr (1988). [i] This work. [j] Wang et al. (1993). [k] Wells et al. (1992). [l] O'Keeffe et al. (1978).

of the Fe−C−O bending mode (δ_{Fe-C-O}) is very sensitive to the identity of the proximal ligand as shown in Table 2. The disappearance of the δ_{Fe-C-O} line at 563 cm^{-1} and the occurrence of a new δ_{Fe-C-O} line at 572 cm^{-1} is an additional confirmation that a switch from a proximal cysteine to a histidine residue is the major structural change associated with the conversion from the CO adduct of NOS_{450} to that of NOS_{420}. The exchange of a neutral histidine for thiolate as the proximal ligand in the CO adduct of NOS is not unexpected as the binding constant of alkylimidazoles to the heme increases by about 4 orders of magnitude (from $\sim 10^4$ to $\sim 10^8$) upon coordination of CO to the heme (Traylor & Sharma, 1992). As a result, at low pH, model porphyrin complexes which lack any axial ligands despite the availability of imidazole do not coordinate the imidazole unless CO binds to the heme (Rougee & Brault, 1975; Geibal et al., 1978). In general, anionic ligands stabilize ferric heme groups and destabilize ferrous heme groups. Thus, for example, the redox potential of cytochrome P-450 is in the −350 to −170 mV range (Peterson & Proguh, 1986), whereas that of myoglobin is +60 mV (Varadarajan et al., 1989). In mutant hemoglobins in which tyrosines are in the heme vicinity, tyrosinate coordinates to the heme in the ferric protein and neutral histidine coordinates in the ferrous form (Nagai et al., 1991). Thus, when NOS becomes unstable, the coordination of the CO and the associated electron repulsion within the d_{z^2} orbital of the iron favors release of the thiolate ligand, a release which does not occur when the heme pocket is constrained by a stable protein structure.

The results reported here clearly indicate not only that the heme pocket of the catalytically active stable form of NOS is very similar to that of cytochrome P-450 but also that purification in the absence of H_4biopterin leads to a form of NOS with properties similar to those of cytochrome P-420_{cam}, the inactive form of cytochrome P-450_{cam}. The similarity between the spectra of the CO-bound H_4biopterin-deficient NOS and P-420 also shows that at least one histidine residue is accessible for coordination to the heme in NOS and a ligand exchange from Fe−Cys to Fe−His is initiated by CO binding. However, it must be pointed out that many P-420 forms of cytochrome P-450 have been reported in which there is no histidine near the heme in the native forms (Omura & Sato, 1964; Poulos et al., 1985; Donaldson & Luster, 1991). Incubation of P-450s in acetone or exposure to high temperature or pressure may induce a large-scale protein conformational alteration which could affect the heme environment, as its native proximal ligand is in a helix on the molecular surface of the enzyme (Poulos, 1986). Thus, the presence of histidines on the protein surface may be sufficient for P-420 formation. Although we cannot exclude the possibility that protonation of the anionic thiolate to form a neutral thiol can account for the conversion from a P-450-like to a P-420-like form observed in the CO adducts of cytochrome P-420_{cam}, CPO, and NOS, all of the spectroscopic markers are consistent with a histidine as the proximal ligand. Comparison of the resonance Raman spectra from the models reported by Stern and Peisach (1974) and Collman and Sorrell (1975) to those from P-420_{cam} (Wells et al., 1992) and the NOS_{420} reported here should give a definitive answer to this question.

Our present work demonstrates that the binding of H_4-biopterin plays a critical structural role in NOS by maintaining a stable heme pocket with a 5C/HS configuration of the reduced heme which is accessible to the incoming physiological ligand, O_2. As mentioned above, a proximal thiolate ligand stabilizes ferric but not ferrous hemes. Consequently, the coordination of the sixth ligand may be favored in the ferrous case to remove the extra electron population donated by the electron-rich proximal cysteine group. This is consistent with the large increase in the fraction of 6C/LS heme content form in the unstable b-NOS upon heme reduction (traces a in Figures 3 and 4). However, binding of H_4biopterin constructs a unique pocket that prohibits the coordination of the low-spin ligand upon heme reduction, as found for stable b-NOS (traces b in Figures 3 and 4). Furthermore, in the stabilized protein the proximal ligand is the electron-rich thiolate residue, which is necessary for the functional activation of O_2. As the binding sites for heme and H_4biopterin are believed to be close to the interface surface of the monomers within the homodimer, deficiency in H_4biopterin may initiate a global protein conformational rearrangement in the heme vicinity, allowing direct coordination of a ligand to the distal binding site, thereby converting the heme to a hexacoordinate low-spin species. This has a profound effect in inhibiting the enzyme's activity by diminishing the availability of the heme active site to the catalytic ligands such as O_2, a similar inhibitory mechanism as reported by our recent investigations on inhibition of NOS by reagent and enzymatically generated NO (Wang et al., 1994). Moreover, the structural perturbations which take place in the absence of H_4biopterin destabilize the protein so as to allow the ligand exchange from thiolate to histidine coordination to be triggered by the binding of CO. These results do not rule out additional functional roles for H_4-biopterin, which are currently being investigated in our laboratories. Finally, the observation that both *i*-NOS and b-NOS can be converted to a P-420 form points to the need

for care in handling the enzymes so as to avoid inadvertent inactivation.

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