We studied catalysis by tetrahydrobiopterin (H4B)-free neuronal nitric-oxide synthase (nNOS) to understand how heme and H4B participate in nitric oxide (NO) synthesis. H4B-free nNOS catalyzed Arg oxidation to \( \text{N}^\bullet \)-hydroxy-L-Arg (NOHA) and citrulline in both NADPH- and \( \text{H}_2\text{O}_2 \)-driven reactions. Citrulline formation was time- and enzyme concentration-dependent but was uncoupled relative to NADPH oxidation, and generated nitrite and nitrate without forming NO. Similar results were observed when NOHA served as substrate. Steady-state and stopped-flow spectroscopy with the H4B-free enzyme revealed that a ferrous heme-NOS complex built up after initiating catalysis in both NADPH- and \( \text{H}_2\text{O}_2 \)-driven reactions, consistent with formation of nitroxyl as an immediate product. This differed from the H4B-replete enzyme, which formed a ferric heme-NOS complex as an immediate product that could then release NO. We make the following conclusions. 1) H4B is not essential for Arg oxidation by nNOS, although it helps couple NADPH oxidation to product formation in both steps of NO synthesis. Thus, the NADPH- or \( \text{H}_2\text{O}_2 \)-driven reactions form common heme-oxy species that can react with substrate in the presence or absence of H4B. 2) The sole essential role of H4B is to enable nNOS to generate NO instead of nitroxyl. On this basis we propose a new unified model for heme-dependent oxygen activation and H4B function in both steps of NO synthesis.
H4B. In addition, it is still unclear how NOHA oxidation by H4B-bound NOS generates NO rather than NO2.

To address these issues, we extensively investigated Arg and NOHA oxidation by H4B-free neuronal NOS (nNOS). Our results reveal that nNOS does indeed catalyze oxidation of both Arg2 and NOHA in the absence of H4B. This result, together with stopped-flow data, enable us to propose a simple, unified mechanism for heme-based oxygen activation, reactivity, and H4B participation in both steps of NO synthesis.

EXPERIMENTAL PROCEDURES

Materials—H4B was purchased from Schirks Laboratory (Jona, Switzerland) and stock solutions prepared in 3 mM diethiothreitol (DTT). 2′,5′-ADP Sepharose 4B was purchased from Alexis Corp. NOHA was a gift from Dr. Bruce King (Wake Forest University, Winston-Salem, NC). All other reagents and materials were obtained from Sigma or from sources reported previously (28).

Expression and Purification of nNOS—Full-length nNOS containing a six-histidine tag at its N terminus was overexpressed in Escherichia coli using the PCWori vector and purified as reported previously (28). The protein as isolated was free of Arg and H4B and was low spin as judged by a Soret peak at 420 nm. The nNOS concentration was estimated based on the absorbance of its ferrous heme-CO adduct as previously reported (28). The nNOS oxygenase domain (nNOSoxy; amino acids 1–720) containing a six-histidine tag at its C terminus was also expressed in E. coli and purified in the absence of H4B as described previously (29).

NO Synthesis and NADPH Oxidation—The initial rate of NO synthesis by nNOS was quantitated at 25 °C using the oxyhemoglobin assay for NO (28). nNOS was added to a cuvette containing 40 mM EPPS, pH 7.6, 150 μg/ml CaM, 0.62 mM CaCl2, 10 units/ml superoxide dismutase, 0.3 mM DTT, 5 mM Arg, 4 μM each of FAD and FMN, 10 units/ml catalase, and 10 μM oxyhemoglobin. NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time at 401 nm.

H4B was purchased from Schirks Laboratory (Jona, Switzerland) and stock solutions prepared in 3 M dithiothreitol (DTT). Critical versus Non-essential Roles for H4B in Catalysis

For ferrous H4B-free nNOS containing 2 mM H2O2. In some cases the enzyme solution also contained 10 μM H4B. Experiments with O2-dependent reactions involved mixing an anaerobic solution of 16 μM ferrous nNOSoxy containing 20 μM H4B and 2 mM NOHA with aerobic buffer solution.

Results—NADPH-dependent Citrulline Production by H4B-free nNOS—H4B-free nNOS generated detectable amounts of citrulline and nitrite plus nitrate from Arg when the reactions contained relatively high concentrations of enzyme (Fig. 1, Table I). NOHA was also detected as a product, indicating that it forms as an intermediate in the reaction catalyzed by H4B-free enzyme. When NOHA was used in place of Arg as a substrate it also was converted to citrulline and nitrite plus nitrate by H4B-free nNOS (Fig. 1, Table I), as shown previously with inducible NOS (26). Citrulline formation from Arg or NOHA was 10 or 15%, respectively, with the H4B-replete enzyme4 in reactions run under otherwise identical conditions (Table I). A somewhat greater proportion of nitrate was generated in reactions catalyzed by the H4B-free enzyme. Control reactions run in the absence of substrate, nNOS, or NADPH did not generate detectable citrulline or nitrite plus nitrate. Including catalase and superoxide dismutase did not diminish product formation in any case. We conclude that H4B is not essential for nNOS to oxidize either Arg or NOHA to nitrite plus nitrate in the NADPH-driven reaction. Our results are the first to show that the Arg reaction can occur in an H4B-free NOS.2 As shown in Fig. 2, NADPH-driven citrulline formation from Arg or NOHA was time-dependent (left panel) and gave initial rates of 2 and 5 min-1, respectively.

2 While this manuscript was in review, Gacchui and colleagues (43) reported that the H4B-free nNOS oxygene domain converted Arg to citrulline and nitrite in an H4B-supported reaction.

3 Under the conditions of our HPLC analysis, any cyanoornithine produced would have been detected as citrulline.

4 The activity of our H4B-reconstituted nNOS was 25 ± 2 min-1 at room temperature when assayed by the oxyhemoglobin assay. This represents 50% of the activity we observe for nNOS purified in the presence of H4B.
The reaction was also dependent on nNOS concentration until it reached a point where all NADPH was exhausted within the time of assay (above 500 nM nNOS, right panel). Importantly, citrulline production was associated with no detectable NO synthesis, even under assay conditions where a single NO per heme would have been detected. This implies the H4B-free nNOS formed a nitrogen oxide product other than NO that could oxidize to nitrite and nitrate.

We next determined the stoichiometric relationship between NADPH consumption and citrulline formation by H4B-free nNOS (Fig. 3). Reactions contained different amounts of NADPH, and the total citrulline produced was determined after all the NADPH was consumed. The slopes indicate that conversion of 1 mol of Arg or NOHA to citrulline was associated with oxidation of 16 and 6 mol of NADPH, respectively. For the same nNOS preparation made replete with H4B, the values were approximately 2 NADPH oxidized per citrulline from Arg, and 0.5 NADPH oxidized per citrulline from NOHA (data not shown), which are close to the theoretical minimum values (7). The uncoupling seen under H4B-free conditions was not due to structural changes, because H2B, which mimics all the structural effects of H4B (17, 30), did not enhance coupling (data not shown). Our analysis indicates that NADPH oxidation in H4B-free nNOS is uncoupled from either Arg or NOHA oxidation to citrulline in a multiple turnover setting.

**Partitioning of the H4B-free nNOS during Catalysis**—To investigate the mechanism of the H4B-independent reaction and identify the nitrogen oxide product, we utilized spectroscopy to observe the enzyme during catalysis. In Fig. 4 spectra of H4B-free nNOS were recorded before or after initiating its aerobic NADPH oxidation at 25 °C in the absence or presence of substrate. Sequential scans were recorded of the CaM-free resting ferric enzyme, after adding NADPH to reduce the flavins, and after adding Ca$^{2+}$ to trigger CaM binding, heme reduction, and catalysis. In the absence of substrate (panel A), the light absorbance spectrum of the CaM- and H4B-free ferric NOS was a mixture of high and low spin heme with a prominent heme Soret band at 420 nm, consistent with its lack of bound H4B (31). Adding NADPH caused losses in visible absorbance between 360 and 420 nm, 440 and 520 nm, and 560 and 680 nm, and buildup of a broad absorbance centered near 365 nm, consistent with reduction of nNOS flavins. After adding Ca$^{2+}$ to trigger steady state NADPH oxidation, we observed some decrease in absorbance between 390 and 420 nm and at 650 nm, consistent with heme reduction occurring in the substrate- and H4B-free nNOS (15). The inset of panel A also shows significant heme reduction occurred as is evidenced by buildup of a 444-nm ferrous-CO species after the reaction was given CO gas.5 As expected, the spectrum taken of the substrate- and H4B-free enzyme during steady state NADPH oxidation showed no evidence for buildup of a six-coordinate ferric or ferrous heme-NO complex, which display Soret absorbance bands at 436 and 440 nm, respectively (32). In the presence of Arg or NOHA (Fig. 4, panels B and C), the heme Soret band of the initial H4B-free ferric nNOS was broader with maximum at 398 nm, consistent with substrate binding and greater high

![FIG. 1. NADPH-dependent Arg or NOHA oxidation by H4B-free nNOS.](image)

**Table 1**

| Experiment       | Citrulline produced | NOHA produced | NO$_2$ + NO$_3$ produced |
|------------------|---------------------|---------------|--------------------------|
|                  | mol/mol nNOS        | mol/mol nNOS  | mol/mol nNOS             |
| E + Arg + NADPH  | 44 ± 4              | 28 ± 2        | 45 ± 5                   |
| E + Arg + H4B + NADPH | 458 ± 30          | 82 ± 8        | 352 ± 30                 |
| E + NOHA + NADPH | 108 ± 10            | NA            | 70 ± 6                   |
| E + NOHA + H4B + NADPH | 745 ± 60          | NA            | 554 ± 50                 |
| Arg + H4B + NADPH | ND                  | ND            | ND                       |
| E + H4B + NADPH  | ND                  | ND            | ND                       |
| E + H4B + Arg    | ND                  | ND            | ND                       |

*E, nNOS; NA, not applicable; ND, not detectable.*
The spectra recorded just after initiating steady-state oxidation of Arg or NOHA showed a small buildup of heme-NO complex in both cases (data not shown). The proportion of this species grew over time as the O2 was consumed in the cuvette. Spectra taken at 10 min (Fig. 4, panels B and C) clearly show the presence of six-coordinate ferrous-NO complex in the Arg and NOHA reactions, as evidenced by the shoulder near 436 nm and single broad absorbance peak near 570 nm (32). The nature of the heme-NO species is further defined by the difference spectra in panels B and C, which show absorbance maxima at 436 and 570 nm. Together, our results suggest that the immediate inorganic product of Arg or NOHA oxidation is nitroxide (NO2), which binds to the ferric heme to form a ferrous heme-NO complex.

We next examined the kinetics of ferrous-NO complex formation in the H4B-free nNOS and its effect on the NADPH oxidation rate. The left panels of Fig. 5 depict ferrous-NO complex formation during the initial phase of Arg or NOHA oxidation at 15 °C. The reactions were started by rapid mixing a solution of Ca2+ with a solution containing CaM, nNOS, substrate, EDTA, and excess NADPH. In both cases, the absorbance increase at 436 nm was best fit to a two-exponential equation, giving apparent rate constants that are listed in Table II. These values indicate that the heme-NO complex formation was biphasic and had the same kinetics whether Arg or NOHA serve as substrate in the H4B-free enzyme. The rates obtained for the H4B-free nNOS are similar to the kinetics of heme-NO complex buildup in H4B-saturated nNOS (33, 34), but differ in two ways. First is the magnitude of absorbance gain at 436, which when normalized on a per heme basis indicate that the proportion of enzyme that forms the heme-NO complex during Arg or NOHA oxidation is small for H4B-free nNOS (approximately 10%) compared with H4B-saturated nNOS (approximately 70%; Ref. 33). Second, the relative absorbance change due to the fast phase of complex buildup in the H4B-free enzyme differs from the H4B-saturated enzyme (33, 34). As shown in the right panels of Fig. 5, rates of NADPH oxidation were not slowed by ferrous-NO complex buildup in H4B free nNOS, consistent with the small proportion of complex that is observed (34).

Critical versus Non-essential Roles for H4B in Catalysis

H2O2-dependent Catalysis by H4B-free nNOS—We next examined if H4B-free nNOS would oxidize Arg to citrulline in a H2O2-driven reaction. In this system, H2O2 was added to a reaction mixture containing Ca2+, nNOS, substrate, EDTA, and excess NADPH. The rates of citrulline formation were monitored as a function of H2O2 concentration. The results indicate that H4B-free nNOS is capable of catalyzing the oxidation of Arg to citrulline in a H2O2-driven reaction.

FIG. 2. Time- and concentration-dependent activities of H4B-free nNOS. Panel A depicts citrulline accumulation during NOHA or Arg oxidation. Panel B shows rates of citrulline formation and NO synthesis from Arg at different concentrations of enzyme. Assay conditions were identical to Fig. 1 except these reactions were started by adding 0.2 mM NADPH and were terminated after 5 min by adding 0.6 N hydrochloric acid. Citrulline was measured by a fluorometric HPLC method, and NO synthesis was measured by the oxyhemoglobin assay. Each point is the mean of three measurements.
Critical versus Non-essential Roles for H4B in Catalysis

**FIG. 5. Kinetics of NADPH-dependent ferrous-NO complex formation in H4B-free nNOS.** Reactions were initiated by rapid mixing of a buffered solution containing 40 mM EPPS, pH 7.6, 2 μM nNOS, 0.4 mM DTT, 1 mM Arg or NOHA, 4.0 μM CaM, 80 μM NADPH, and 0.5 mM EDTA with an equal volume of buffer solution containing 3 mM CaCl2 at 15 °C. The two left panels contain spectral traces recorded 0 to 1.6 s after mixing for the Arg (A) and NOHA (B) reactions. Arrows show the direction of spectral change. Insets show the kinetics of absorbance change at 436 nm to indicate ferrous-NO complex formation. The two right panels show the kinetics of absorbance change at 340 nm to indicate NADPH oxidation in the Arg (C) and NOHA (D) reactions. The insets show traces from replica experiments where data collection took place over a shorter time frame. Smooth lines drawn through the kinetic traces are the calculated lines of best fit.

**TABLE II**

| Experiment | Rate of ferrous-NO complex formation |
|------------|--------------------------------------|
| nNOS + Arg + H4B + NADPH | 11 (46%) 2.2 (54%) |
| nNOS + Arg + NADPH | 10 (75%) 3.0 (25%) |
| nNOS + NOHA + H4B + NADPH | 11 (88%) 3.0 (12%) |
| nNOS + NOHA + NADPH | 11 (44%) 2.8 (56%) |

heme to directly form reactive heme-oxyster species. Although this system’s ability to convert NOHA to citrulline is well established (35), it was reported not to work with Arg (26, 27) until quite recently (43). Fig. 6 shows that H4B-free nNOS generated detectable amounts of citrulline and NOHA from Arg in a reaction run at 25 °C. Nitrite was also detected as a product in the Arg reaction (data not shown). Products were not observed in controls that were missing H2O2, enzyme, or Arg (data not shown). These results are consistent with H4B-free NOS catalyzing NADPH-dependent Arg oxidation, and suggest a similar mechanism operates in the H2O2- and NADPH-driven reactions. Table III compares product formation from Arg or NOHA by nNOS in the absence or presence of H4B. Added H4B caused only a 1.8–2-fold increase in product formation in all cases. Similar small increases with H4B were observed previously in studies that used NOHA as substrate in the H2O2-driven reaction (35).

To better understand the mechanism, we utilized rapid-scanning stopped-flow spectroscopy to follow heme transitions during H2O2-driven NOHA oxidation by nNOSoxy under anaerobic conditions (Fig. 7). Ferric nNOSoxy was rapid-mixed with 2 mM H2O2 at 10 °C. In both the presence and absence of H4B (panels A and B), a product species with absorbance peak at 436 nm formed immediately. The difference spectra show a trough at 393 nm, peak at 436 nm, and a broad visible peak near 570 nm, identifying it as the ferrous-NO complex. This did not form in the absence of NOHA (panel C). Moreover, its spectrum clearly differs from the ferric-NO complex that forms as an immediate product during H4B-dependent aerobic oxidation of NOHA under single-turnover conditions (36), which displays a difference spectrum with peaks at 442, 550, and 580 nm (panel D). Thus, during H2O2-driven oxidation of Arg and NOHA the nNOSoxy forms a ferrous heme-NO complex in the presence or absence of H4B.

**DISCUSSION**

Despite progress in NOS structure-function (11–13), the essential role of H4B in catalysis has remained elusive. Our work with H4B-free nNOS shows that it can catalyze Arg oxidation in both NADPH- and H2O2-supported reactions, without generating any detectable NO. This leads us to conclude the following: 1) H4B is not essential for Arg hydroxylation or NOHA oxidation, although it improves coupling between NADPH oxidation and product formation in both cases. 2) The key function of H4B is to enable generation of NO rather than NO−. These conclusions are surprising, and their implications are discussed below.

**H4B-independent Arg Oxidation**—Although H4B-free NOS is known to oxidize NOHA in both NADPH- and H2O2-driven reactions, Arg hydroxylation in the NADPH-driven reaction was reported to absolutely require H4B (26, 27). A discrepancy exists between the old and our new data primarily because we used nNOS at concentrations that were sufficient to clearly detect products even though the reaction is uncoupled with respect to NADPH oxidation. Our data help explain why nNOS maintains a residual Arg to citrulline activity in the presence of H4B antagonists (44). The different NOS isoforms also appear to catalyze H4B-independent reactions with varying efficiency. For example, we have observed Arg hydroxylation and citrulline synthesis with H4B-free iNOS in the NADPH-driven re-
action, but these occur at about one-tenth the rate observed with nNOS.

Mechanisms for NOS Arg hydroxylation are modeled after cytochrome P450 monoxygenase chemistry (Fig. 8). Transfer of an electron to the heme enables O\textsubscript{2} binding and formation of a detectable ferrous-dioxy species (I) (20, 24, 36, 37). This species then obtains a second electron to form an iron-peroxo species (II), which decays to yield a perferryl FeO species (III) that is thought to hydroxylate the guanidino nitrogen of Arg. As shown, H\textsubscript{2}O\textsubscript{2} can be an alternative source of two electron-reduced O\textsubscript{2} (35) and enables FeO formation without provision of electrons to the heme. H\textsubscript{4}B cannot directly participate in hydroxylation of Arg (11–13), and given that H\textsubscript{4}B is not required for heme reduction (15), it has been puzzling why this cofactor should be essential. Our current results show that H\textsubscript{4}B is in fact not essential, because the NOS heme hydroxylates Arg even in the total absence of H\textsubscript{4}B when provided with two NADPH-derived electrons or with H\textsubscript{2}O\textsubscript{2}. This leaves us to consider how NADPH oxidation becomes more coupled to Arg hydroxylation when H\textsubscript{4}B is bound.

When H\textsubscript{4}B-free nNOS catalyzes Arg hydroxylation, both electrons needed to generate the FeO species must transfer directly to the heme from the reductase domain. However, in H\textsubscript{4}B-bound nNOS this is not necessarily the case (Fig. 8). It is clear that the reductase domain gives the first electron to the heme irrespective of bound H\textsubscript{4}B (15), but the second electron could come either from the reductase domain or from H\textsubscript{4}B (Fig. 8). Our current work shows that the reductase domain can provide the second electron when H\textsubscript{4}B is missing, but this leads to uncoupling between NADPH oxidation and product formation. Recent work suggests that bound H\textsubscript{4}B can also furnish an electron to the ferrous-dioxy complex when the reductase do-

**Fig. 6.** H\textsubscript{2}O\textsubscript{2}-dependent Arg or NOHA oxidation by H\textsubscript{4}B-free nNOS. Middle and lower traces show product formation relative to authentic standards (top panel). Reactions contained 1 mM Arg or NOHA, 0.6 \( \mu \)M nNOS, 1.0 mM DTT, 25 units/ml superoxide dismutase, 0.5 mM EDTA, and 40 mM EPPS buffer, pH 7.6. Reaction were started by adding 30 \( \mu \)M H\textsubscript{2}O\textsubscript{2} and stopped after 10 min by adding of 1300 units/ml catalase. Samples were derivatized with OPA prior to fluorometric HPLC analysis as described under “Experimental Procedures.” Traces are representative of at least 10 independent experiments.

**Fig. 7.** Rapid scan stopped-flow detection of product complexes formed from NOHA by nNOSoxy. In panels A–C, anaerobic ferric nNOSoxy (6 \( \mu \)M) was rapidly mixed at 10 °C with an anaerobic H\textsubscript{2}O\textsubscript{2} solution (2 mM) under various conditions: 1 mM NOHA plus 10 \( \mu \)M H\textsubscript{4}B (A); NOHA without H\textsubscript{4}B (B); H\textsubscript{4}B without NOHA (C). Consecutive scans were collected between 0 and 1 min after mixing. In panel D, anaerobic ferrous nNOSoxy (16 \( \mu \)M) containing 2 mM NOHA and 10 \( \mu \)M H\textsubscript{4}B was rapidly mixed at 4 °C with an air-saturated buffer. The scan marked 416 nm, and the scan marked 439, 550, and 580 nm were collected 0 and 40 ms after mixing, respectively. Insets of all panels show the difference spectra between the initial and last scans. Data in each panel are representative of 7–10 individual reactions.
main is missing (25). Electron transfer from H4B in this type of experimental system is associated with hydroxylation of between 0.2 and 0.8 mol of Arg per heme (24, 25, 36, 45), implying that electron donation from H4B is at least partly coupled to Arg hydroxylation.

It is important to emphasize that the NOS ferrous-dioxy complex is unreactive toward Arg or NOHA (8, 17, 24) and will consequently decay to superoxide (which is unstable) and ferric enzyme if a second electron is not provided in a timely manner (Fig. 8). Thus, one way that H4B could improve coupling between NADPH oxidation and product formation is to provide an electron to the ferrous-dioxy species more quickly than can the reductase domain. Indeed, the uncoupling we observe between NADPH oxidation and product formation from Arg in H4B-free nNOS is at least partly couple to Arg hydroxylation.

H4B-free nNOS suggests that the reductase domain is not efficient in providing the second electron. Consider that the nNOS reductase domain transfers an electron to the ferric heme at a rate of about 3 s⁻¹ under anaerobic conditions at 10 °C (38), and the rate is not changed in the absence of H4B (17). Thus, heme reduction by the reductase domain is somewhat slower that the estimated rate of H4B radical buildup during reaction of an H4B-bound ferrous iNOS oxygenase with O₂ (11–20 s⁻¹; Ref. 25). This rate of H4B radical formation is also similar to the decay rate of the ferrous-dioxy complex in H4B-bound nNOS oxygenase (10 s⁻¹ at 10 °C), which otherwise decays slower in the absence of H4B (20). Thus, the available kinetic data already suggest that electron transfer from H4B can be connected to ferrous-dioxy reduction, and may be faster than electron transfer from the reductase domain. Once formed, the H4B radical is apparently quite stable in NOS (25), and therefore would have time to be reduced back to H4B by an electron from the reductase domain before the next round of oxygen activation. That H4B can circumvent a kinetic problem in heme reduction during stepwise oxygen activation is an attractive possibility, because it would explain how H4B increases coupling between NADPH oxidation and product formation in NOS.

**Critical versus Non-essential Roles for H4B in Catalysis**

Table III shows that only NOHA can generate NO from Arg in the absence of H4B, whereas NOHA plus H4B can generate NO from Arg in the presence of H4B. This finding is particularly damning in light of recent data showing that the structure of a NOS oxygenase dimer contains a ferrous-dioxy complex only when H4B is present.

**A New Model for H4B Function**—Given that H4B is not required for Arg or NOHA oxidation, we conclude its sole essential function is to ensure that NOS produces NO rather than NO²⁻. However, how is this accomplished? NO⁻ is one electron reduced relative to NO; therefore, H4B somehow enables NOS to retrieve an electron from NOHA or a downstream product during catalysis of the second step (4, 7, 8). This explains why H4B-bound NOS only oxidizes 0.5 NADPH to generate NO from NOHA even though it represents a three-electron oxidation of NOHA. Fig. 9 shows a mechanism for H4B function in the second step of NO synthesis that is consistent with all findings to date. It has H4B donating an electron to the ferrous-dioxy species as a central feature, because this generates an H4B radical that acts as an electron acceptor at a later point in the reaction to ensure release of NO instead of NO²⁻. Electron donation by H4B in the second step is consistent with NOHA oxidation being uncoupled from NADPH oxidation in the H4B-free enzyme (see Table I and Fig. 9). Although NOHA was initially proposed to reduce the ferrous-dioxy complex instead of H4B (4, 7, 24), arguments against this possibility exist (42). Moreover, NOHA does not donate an electron to the ferrous-dioxy complex of either H4B-free or H2B-bound NOS (8, 17, 24, 25). This finding is particularly damning in light of recent data showing that the structure of a NOS oxygenase dimer containing NOHA and H2B is essentially identical to a dimer containing NOHA and H4B (30). Thus, there is no structural basis for why the enzyme would allow NOHA to reduce the ferrous-dioxy complex only when H4B is present.

A second essential feature of our model is that a reaction product downstream from NOHA donates an electron back to the H4B radical (Fig. 9). Although NOHA in principle could serve as the reductant, recent evidence suggests it cannot be-

---

**Table III** Comparative activities of nNOS in H₂O₂-driven reactions at 25 °C

| Experiment                      | Citrulline production | NOHA production | NO₂⁻/NOS⁻ production |
|---------------------------------|-----------------------|-----------------|----------------------|
|                                 | nmol/nmol nNOS        | nmol/nmol nNOS  | nmol/nmol nNOS       |
| E + Arg + H₂O₂                 | 2.6 ± 0.4             | 3.3 ± 0.5       | 2.6 ± 0.3            |
| E + Arg + H₂O₂ + NOHA         | 4.7 ± 0.3             | 1.3 ± 0.1       | 5.0 ± 0.5            |
| E + NOHA + H₂O₂               | 56 ± 4                | NA              | 52 ± 5               |
| E + NOHA + H₂B + H₂O₂         | 91 ± 5                | NA              | 86 ± 4               |
| Arg + H₂B + H₂O₂              | ND                    | ND              | ND                   |
| E + Arg + H₂B                | ND                    | ND              | ND                   |
| E + H₂B + H₂O₂               | ND                    | ND              | ND                   |

* C.-C. Wei, Z. Wang, Q. Wang, A. L. Meade, C. Hemann, R. Hille, and O. J. Stuehr, submitted for publication.
and step enables it to perform its essential function as an electron acceptor at a later point during NOHA oxidation, thus insuring that the enzyme generates NO instead of NO$^\cdot$.

**REFERENCES**

1. MacMicking, J., Xie, Q., and Nathan, C. (1997) *Annu. Rev. Immunol.* 15, 323–350
2. Craven, S. E., and Bredt, D. S. (1998) *Cell* 93, 495–498
3. Michel, T., and Peron, O. (1997) *J. Clin. Invest.* 100, 2417–2423
4. Marletta, M. A., Hurshman, A. R., and Rusche, K. M. (1998) *Curr. Opin. Chem. Biol.* 2, 656–663
5. Stuehr, D. J. (1999) *Biochim. Biophys. Acta Bioenerg.* 1411, 217–230
6. Breyer, J., Xie, Q., Hutchinson, N., Cho, H., Wolfe, G. C., and Nathan, C. (1996) *J. Biol. Chem.* 271, 22679–22686
7. Griffith, O. W., and Stuehr, D. J. (1995) *Annu. Rev. Physiol.* 57, 707–736
8. Abu-Soud, H. M., Presta, A., Mayer, B., and Stuehr, D. J. (1997) *Biochemistry* 36, 10811–10816

9. Pou, S., Keaton, L., Surichamorn, W., and Rosen, G. M. (1999) *J. Biol. Chem.* 274, 9573–9580
10. Klatt, P., Schmidt, K., Uray, G., and Mayer, B. (1993) *J. Biol. Chem.* 268, 14761–14767
11. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) *Science* 279, 2121–2126
12. Fischmann, T. O., Hruza, A., DaViux, X., Fonsetta, J. D., Lonn, C. A., Dolphin, E., Proengay, A. J., Paul, R., Landell, D. J., Nurialu, S. K., and Weber, P. C. (1999) *Nat. Struct. Biol.* 6, 233–242
13. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. B., and Poole, T. M. (1998) *Cell* 95, 939–950
14. Mansuy, D., and Renaud, J. P. (1995) in *Cytochrome P450 Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed.) pp. 537–574, 2nd Ed., Plenum Press, New York
15. Presta, A., Weber-Main, A. M., Stankovich, M. T., and Stuehr, D. J. (1998) *J. Am. Chem. Soc.* 120, 8469–8485
16. Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., and Mayer, B. (1995) *EMBO J.* 14, 3687–3695
17. Presta, A., Siddhanta, U., Wu, C., Senequeuir, N., Huang, L., Abu-Soud, H. M., Erzurum, S., and Stuehr, D. J. (1998) *Biochemistry* 37, 298–310
18. McMillan, K., and Masters, B. S. S. (1993) *Biochemistry* 32, 9875–9880
19. Klatt, P., Schmidt, M., Lesgold, E., Schmidt, K., Werner, E., and Mayer, B. (1994) *J. Biol. Chem.* 269, 13861–13866
20. Abu-Soud, H. M., Gachhui, R., Raschel, P. M., and Stuehr, D. J. (1997) *J. Biol. Chem.* 272, 17349–17353
21. Nishida, C. R., and Ortiz de Montellano, P. R., (1998) *J. Biol. Chem.* 273, 5566–5571
22. Kappock, T. J., and Caradonna, J. P., (1996) *J. Bio. Chem. 271*, 23133–23220
23. Huang, L., Abu-Soud, H. M., Hille, R., and Stuehr, D. J. (1999) *Biochemistry* 38, 1912–1920
24. Crane, B. R., Arvai, A. S., Ghosh, S., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (2000) *Biochemistry* 39, 4688–4692
25. Roman, L. J., Sheta, E. A., Martasek, P., Gross, S. S., Lin, Q., and Masters, B. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8428–8432
26. Wang, J., Rousseau, D. L., Abu-Soud, H. M., and Stuehr, D. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 10512–10516
27. Abu-Soud, H. M., Wu, J., Rousseau, D. L., Fukuto, J., Igarro, L. J., and Stuehr, D. J. (1995) *J. Biol. Chem.* 270, 22997–23006
28. Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J. (1999) *J. Biol. Chem.* 274, 23133–23220
29. Wang, L., Abu-Soud, H. M., Hille, R., and Stuehr, D. J. (1999) *Biochemistry* 38, 1912–1920
30. Harris, C., Gachhui, R., Raschel, P. M., and Stuehr, D. J. (1997) *Biochemistry* 36, 14465–14473
31. Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J. (1999) *J. Biol. Chem.* 274, 23133–23220
32. Baggs, S., Huang, L., and Stuehr, D. J. (2000) *Biochemistry* 39, 15502–15508
33. Clague, M. J., Wisnok, J. S., and Marletta, M. A. (1997) *Biochemistry* 36, 14465–14473
34. Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J. (1999) *J. Biol. Chem.* 274, 23133–23220
35. Marletta, M. A. (1999) *Biochemistry* 38, 15503–15512
36. Wang, J., Rousseau, D. L., Abu-Soud, H. M., and Stuehr, D. J. (1995) *J. Biol. Chem.* 270, 22997–23006
37. Adak, S., Wang, Q., and Stuehr, D. J. (2005) *J. Biol. Chem.* 275, 14734–14739
38. Fufahi, R. A., Wisnok, J. S., and Marletta, M. A. (1995) *Biochemistry* 34, 1939–1941
39. Gagdhi, C., and Tainer, J. A. (2000) *Biochemistry* 39, 4688–4692
40. Baggs, B., Huang, L., and Stuehr, D. J. (2000) *Biochemistry* 39, 33142–33149
41. Sato, H., Sagunni, L., Daff, S., and Shimizu, T. (1998) *Biochemistry* 37, 77–264
42. Liao, M., Groehn, V., Kotsonis, P., La, M., Ko¨ster, S., Meinecke, M., Bernhardt, M., Gachhui, R., and Tainer, J. A. (2000) *Biochemistry* 39, 4688–4692
43. Gachhui, R., Abu-Soud, H. M., Ghosh, D. K., Presta, A., Blazing M. A., Mayer, B., George, S. E., and Stuehr, D. J. (1998) *J. Biol. Chem.* 273, 5451–5454
44. Fukuto, J. M., Stuehr, D. J., Feldman, P. L., Bova, M. L., and Wang, P. (1993) *J. Med. Chem. 36*, 217–230
45. Bormelmann, H. M., Reif, A., Frublich, L. G., Frey, A., Hofmann, H., Marek, D. M., Groehn, V., Kotsinson, P., La, M., Koester, S., Meinecke, M., Bernhardt, M., Weiger, M., Ghisla, S., Prestwich, G. D., Pfeifer, W., and Schmidt, H. H. T. (1998) *J. Biol. Chem.* 273, 33142–33149

**Critical versus Non-essential Roles for H4B in Catalysis**

Our work with H4B-free nNOS reveals a simple unified model for heme and H4B function in both steps of NO synthesis. The three essential facets are as follows. 1) Heme-based oxidants that are generated in the NADPH- or H$_2$O$_2$-driven reaction are apparently identical, and are competent to react with either Arg or NOHA in the absence of H4B. 2) H4B likely functions in both steps of NO synthesis as a kinetically preferred donor of the second electron required for oxygen activation. This enables the enzyme to couple product formation to NADPH oxidation. 3) Electron donation from H4B in the sec-