A Conserved Val to Ile Switch near the Heme Pocket of Animal and Bacterial Nitric-oxide Synthases Helps Determine Their Distinct Catalytic Profiles*

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Nitric oxide (NO) release from nitric oxide synthases (NOSs) is largely dependent on the dissociation of an enzyme ferric heme-NO product complex (FeIIINO). Although the NOS-like protein from *Bacillus subtilis* (bsNOS) generates FeIIINO from the reaction intermediate N-hydroxy-L-arginine (NOHA), its NO dissociation is about 20-fold slower than in mammalian NOSs. Crystal structures suggest that a conserved Val to Ile switch near the heme pocket of bsNOS might determine its kinetic profile. To test this we generated complementary mutations in the mouse inducible NOS oxygenase domain (iNOSoxy). Mutations did not significantly alter the electronic properties of the heme or various heme-ligand complexes. Stopped-flow spectroscopy was used to study heme transitions during single turnover NOHA reactions. I224V bsNOS displayed three heme transitions involving four species as typically occurs in wild-type NOS, the beginning ferrous enzyme, a ferrous-dioxy (FeIIO2) intermediate, FeIIINO, and an ending ferrous enzyme. The rate of each transition was increased relative to wild-type bsNOS, with FeIIINO dissociation being 3.6 times faster. In V346I INOSoxys we consecutively observed the beginning ferrous, FeIIO2, a mixture of FeIIINO and ferric heme species, and ending ferrous enzyme. The rate of each transition was decreased relative to wild-type iNOSoxy, with the FeIIINO dissociation being 3 times slower. An independent measure of NO binding kinetics confirmed that V346I INOSoxy has slower NO binding and dissociation than wild-type. Citrulline production by both mutants was only slightly lower than wild-type enzymes, indicating good coupling. Our data suggest that a greater shielding of the heme pocket caused by the Val/Ile switch slows down NO synthesis and NO release in NOS, and thus identifies a structural basis for regulating these kinetic variables.

Nitric oxide synthases (NOSs) are flavoheme enzymes that generate nitric oxide (NO) from L-arginine (Arg) (1, 2). The overall reaction consumes 1.5 NADPH and 2 O2 and involves two steps, the first being Arg hydroxylation to form N-hydroxy-L-Arg (NOHA), and the second being NOHA oxidation to form citrulline and NO (see Scheme 1).

The NOS heme is located in a catalytic "oxygenase" domain that also contains the binding sites for Arg and the essential redox cofactor (6R)-tetrahydrobiopterin (H4B) (3, 4). The heme is ligated to a cysteine thiolate (5–7) and catalyzes a reductive activation of molecular oxygen in conjunction with H4B in each of the two steps of NO synthesis (8–11). The NOS heme also binds self-generated NO as an intrinsic feature of catalysis (12–14). Each molecule of NO generated by NOS has a high probability of binding to the heme before it is released from the enzyme. The resulting ferric heme-NO product complex (FeIIINO) has been observed to build up as a transient species during NOHA oxidation reactions catalyzed by NOS oxygenase domains (NOSoxy) when run in a stopped-flow spectrophotometer under single turnover conditions (9, 13, 14). These experiments also provided spectral and kinetic information regarding the formation of an initial ferrous heme-dioxygen species (FeIIO2), whose disappearance then coincides with kcat and formation of the transient FeIIINO product complex (see Scheme 2). Because dissociation of the FeIIINO product complex is required for NO to escape from the enzyme, it is an essential step for the biologic functions of animal NOSs. In fact, this dissociation rate is one of three key kinetic parameters that together determine the overall catalytic behavior of a given NOS (15, 16). Slower rates of NO dissociation dispose the FeIIINO product complex toward its reduction during catalysis, which then places the ferrous heme-NO enzyme species into a futile cycle that does not release NO (Fig. 1). Interestingly, there is only a modest variation in FeIIINO dissociation rates among the three mammalian NOS isoforms, which range from 2 to 5 s−1 when measured in NOHA single turnover reactions at 10 °C (16).

Given the above, we were surprised to find that a NOS-like enzyme expressed in *Bacillus subtilis* (bsNOS) generated NO from NOHA in a single turnover reaction, but the enzyme exhibited a dissociation rate for its FeIIINO product complex that was 10–20-fold slower than its mammalian NOS counterparts (17). The crystal structure of bsNOS (18) revealed that it
contains an amino acid switch (Val to Ile) that creates greater shielding of its heme pocket compared with that in the mammalian enzymes (Fig. 2). Interestingly, the Ile is conserved among other bacterial NOS-like enzymes, whereas the use of Val at this position is conserved among animal NOSs. We hypothesized that this substitution may help to regulate the dissociation rate of the FeII-NO product complex. We therefore generated complementary mutations in mouse inducible NOSy (iNOSy, V346I) and in bsNOS (I224V), and characterized the kinetics and extent of their NO synthesis from NOHA and their NO release kinetics. Our findings suggest that the difference in heme pocket shielding caused by the Val/Ile switch creates kinetic differences that impact both NO synthesis and NO release in NOSs.

Fig. 1. Kinetic model for NO synthesis by NOS. The enzyme molecules engage in a productive cycle that releases free NO and in a futile cycle that releases a higher oxide of nitrogen. Reduction of ferric enzyme to ferrous (k\(_{\text{cat}}\)) enables the heme to bind O\(_2\) and initiates both Arg hydroxylation (k\(_{\text{out}}\), l) and NOHA oxidation (k\(_{\text{out}}\), 2). After NO is made, an immediate product of catalysis is the ferric heme-NO complex (FeII-NO), which can either release NO (k\(_{\text{obs}}\)) or become reduced (k\(_{\text{rub}}\)) to generate a ferrous heme-NO complex (FeII-NO). The ferrous heme-NO complex dissociates extremely slowly and instead regenerates the active ferric enzyme by reacting with O\(_2\) (k\(_{\text{rub}}\)). Adapted from Refs. 15 and 16.

EXPERIMENTAL PROCEDURES

Materials—All reagents and materials were obtained from Sigma, Aldrich, Alexis, or sources described previously (19).

Mutagenesis—Site-directed mutagenesis of mouse iNOSy DNA in the pCWo1i expression plasmid (coding for amino acids 66–498 plus a His\(_6\) tag at the C terminus) and bsNOS DNA in the pET15b expression plasmid (coding for amino acids 1–370 plus a six-His tag at the N terminus) were performed using the QuikChange site-directed mutagenesis kit from Stratagene. The mutation codon (bold and underlined) and a silent restriction site (italic, NaeI and XhoI for V346I and I224V mutations, respectively) was incorporated into the primers as follows: V346I, 5’-GCA CTG CCG GCC ATG ACG ATG CTA CGC TGC-3’; I224V, 5’-CAG TAG CAT GTC CAG TGC GGC CAG TGC TGC-3’; I224V, 5’-GCC GTC GCA ATT GTT TCF GAT ATG ACG CTC GAT GGC GC-3’; I224V, 5’-GCC ACC ACC TCG AGT ATC TCA GAA ACA ATT GCC AC-3’. The mutations were confirmed at the molecular biology core facility of the Cleveland Clinic by sequencing ~500 consecutive base pairs including the mutation sites. No other mutations were observed.

Protein Expression and Purification—Wild-type and mutant enzymes were overexpressed in Escherichia coli BL21 and purified using Ni\(^{2+}\)-nitrilotriacetate affinity chromatography as reported previously (17, 20). Concentrations of NOSy enzymes were determined from the 444 nm absorbance of the ferrous-CO complex, using an extinction coefficient of 76 mM\(^{-1}\) cm\(^{-1}\) (21). The ferrous-CO complex dissociates extremely slowly and instead regenerates the active ferric enzyme by reacting with O\(_2\) (k\(_{\text{rub}}\)). Adapted from Refs. 15 and 16.

Imidazole and Arg Binding Affinity Measurement—Binding affinities were measured by perturbation difference spectroscopy as reported previously (20). In general, enzymes were incubated with 200 μM H\(_2\)B and then titrated first with imidazole. Spectra were recorded at room temperature after each addition. Double reciprocal plots of the peak to trough absorbance difference versus the imidazole concentration gave the apparent binding constant of imidazole (K\(_{\text{b}}\)). Binding affinity of Arg was then measured in the same way except that substrate Arg was added gradually to enzyme solutions that contained H\(_4\)B and imidazole. The K\(_{\text{b}}\) value of Arg was then calculated using equation 1, K\(_{\text{b}}\) = K\(_{\text{cat}}\)/k\(_{\text{obs}}\) (imidazole), in which K\(_{\text{b}}\) is the apparent binding constant determined for Arg.

Peroxide Assay—H\(_2\)O\(_2\)-dependent NOHA oxidation assays were performed as described previously (20). In short, enzymes were incubated at room temperature with NOHA, dithiothreitol, and different concentrations of H\(_2\)B in 96-well plates. Reactions were initiated by adding 30 mM H\(_2\)O\(_2\) and stopped after 10 min by adding catalase. Griess reagent solution was then added to enable the detection of nitrite production as the absorbance change at 550 nm. Nitrite was quantitated based on NaNO\(_2\) standard solutions.

Single Turnover NOHA Reactions—NOHA oxidation reactions were carried out in a Hi-Tech SF-61 stopped-flow apparatus equipped for anaerobic work and coupled to a Hi-Tech MG-6000 diode array detector, as reported previously (14). An anaerobic solution that contained the dithionite-reduced enzyme at concentrations indicated in the text, 40 mM Hepes, 0.5 or 0.2 mM NOHA, and 0.2 mM H\(_2\)B was transferred into the stopped-flow instrument and rapidly mixed with air-saturated Hepes buffer at 10 °C. Ninety-six spectral scans were obtained after each mixing. Sequential spectral data were fit to different reaction models using the Specfit global analysis program (provided by Hi-Tech Ltd.), which could calculate the number of different enzyme species, their spectra, and their concentrations versus time during the single turnover reactions. Data from six to eight sequential reactions were averaged to obtain the final traces.

Citrulline Analysis—Amino acids in aliquots taken from single turnover reactions were derivatized with o-phthalaldehyde and then...
analyzed by reverse-phase HPLC with fluorescence detection (19). Samples were filtered through an Amicon Centricon device (10,000 milliwatt cut-off) prior to derivatization. Samples were injected onto a Hewlett-Packard ODS-Hypersil column that was eluted with a gradient of buffer A (5 mM ammonium acetate, pH 6.0, 20% methanol) and buffer B (100% methanol). Retention times and concentrations of amino acids were calculated based on NOHA and citrulline standard solutions.

**Kinetics of NO Binding to Ferric Enzymes**—The procedure was as detailed previously (22). Anaerobic buffered solutions containing 2 μM ferric enzyme, 400 μM H₂B, 1.2 mM dithiothreitol, and 400 μM NOHA were rapidly mixed at 10 °C in the stopped-flow instrument with a buffered solution containing different concentrations of NO. Six to eight sequential reactions were run at each NO concentration and then averaged to obtain the final traces. The NO solutions were made by diluting a chilled saturated NO solution in chilled anaerobic buffer and assuming a NO concentration of 2 mM for a saturated solution at 10 °C.

### RESULTS

Because the complementary Val and Ile mutations are located near the NOS heme, we first examined whether they altered the heme environment or the binding properties of the heme or the oxygenase domain. Table I summarizes some spectral properties and binding affinities of the purified mutant and wild-type enzymes. The maximal absorbance values for the Soret peak that we observed in the absence of Arg and H₄B indicate that the mutants each mimicked their wild-type counterpart in that the V346I iNOSoxy had its ferric heme poised in a predominantly low spin state, whereas the I224V bsNOS had its heme poised in a predominantly high spin state. Their Soret peak positions became similar to each respective wild-type enzyme in the presence of Arg and/or H₄B, or when dithiothreitol, CO, or imidazole was bound to the heme as a sixth ligand. V346I iNOSoxy displayed a similar binding affinity toward imidazole as it did the wild-type iNOSoxy but had poorer affinity toward Arg. This is consistent with results obtained for an analogous mutant of neuronal NOS (V567L) that had an altered substrate recognition profile (39). The I224V bsNOS displayed a lower affinity toward imidazole but an increased affinity toward Arg. In an H₂O₂-driven NOHA oxidation assay the H₂B concentration dependence of V346I iNOSoxy was somewhat enhanced. We can conclude that the mutations did not greatly alter NO binding of Arg, H₂B, or small heme ligands, or greatly alter the electronic properties of the heme or various heme-ligand complexes. These results are consistent with wild-type iNOSoxy and bsNOS enzymes also being mostly similar in these regards despite their containing either a Val or Ile at the same position.

We next determined how the amino acid substitutions might influence the kinetics and extent of catalysis. We utilized stopped-flow spectroscopy to study the heme transitions that were associated with catalysis of NOHA oxidation by the mutants in a single turnover reaction. Solutions of ferrous enzymes containing NOHA and H₂B were rapidly mixed with O₂-containing buffer in a stopped-flow spectrophotometer equipped with a rapid-scanning diode array detector, and the collected spectral data were subject to global analysis using software provided by the instrument manufacturer. We have done this type of analysis previously (14, 17) for the NOHA reactions of wild-type iNOSoxy and bsNOS. In those cases the spectral data best fit to an A→B→C→D model with three
consecutive monophasic transitions that together discern four spectrally distinct species. These are in order of appearance: the beginning ferrous enzyme, a ferrous-dioxy (FeIIO₂) intermediate, a ferric-NO (FeIIINO) intermediate, and the ending ferric enzyme.

In the case of I224V bsNOS, we observed the same three transitions involving the same four species (Fig. 3A). The Soret maxima for the FeIIO₂ and FeIIINO intermediates as calculated by global analysis were 427 and 439 nm, respectively, as compared with Soret values of 429 and 440 nm as taken from the collected absorbance traces. Fig. 3B depicts the calculated concentrations of each species during the first 150 ms of the single turnover reaction, whereas Fig. 3C depicts the calculated concentrations of the FeIIINO and ferric heme species over a longer reaction time period to indicate their complete transition. The maximal concentrations indicated for the FeIIO₂ and FeIIINO species during the reaction (Fig. 3B) imply that practically all of the mutant enzyme molecules participated in a productive reaction to generate NO. Indeed, the reaction generated 0.7 citrulline/heme (Table II), which was slightly lower than the product yield for wild-type bsNOS.

The I224V mutation altered the kinetic profile of the single turnover reaction. The calculated rates for the three heme transitions were each faster in I224V bsNOS relative to those of wild-type bsNOS (Table III). The FeIIO₂ formation rate in I224V bsNOS even exceeded that found in wild-type iNOSoxy. Thus, for I224V bsNOS the general progression and product yield of the reaction was normal, but the kinetics of each transition became faster and thus were more like iNOSoxy.

We performed an identical stopped-flow analysis of the NOHA reaction catalyzed by the complementary V346I iNOSoxy mutant. As shown in Fig. 4A, the calculated spectra were typical for the ferrous, FeIIINO (Soret maxima at 429 nm), and ferric heme species. However, the calculated spectrum of the FeIIINO intermediate was different. It had two Soret peaks with maxima at 412 and 440 nm. A closer examination of the visible region (Fig. 4A, inset) showed that there were absorbance peaks at 545, 584, and 645 nm in the spectrum of this species, which strongly suggest that it is a mixture of FeIIINO and ferric heme species. An inspection of the actual spectral traces that were recorded during this transition confirmed that there was no buildup of a pure FeIIINO species during the reaction (Fig. 4B). The recorded spectrum contains two Soret peaks at 413 and 440 nm, which match well with the calculated spectrum in Fig. 4A and confirm that there was a concurrent formation of a FeIIINO and a ferric heme species during this period of the NOHA reaction catalyzed by V346I iNOSoxy. A replica reaction that contained twice the concentration of NOHA gave identical spectral and kinetic results (data not shown) indicating that incomplete NOHA

### Table II

<table>
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<th>Enzyme</th>
<th>Cit substrates</th>
<th>Cit product</th>
<th>Heme product</th>
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<tr>
<td>bsNOS</td>
<td>0.98 ± 0.06</td>
<td>0.78 ± 0.14</td>
<td>0.84 ± 0.21</td>
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<tr>
<td>I224V bsNOS</td>
<td>0.70 ± 0.18</td>
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* Cit, citrulline.

### Table III

<table>
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<tr>
<th>Enzyme</th>
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<tr>
<td>FeIIO₂ formation rate (s⁻¹)</td>
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<tr>
<td>bsNOS</td>
<td>28.7 ± 0.9</td>
</tr>
<tr>
<td>I224V bsNOS</td>
<td>61.8 ± 9.5</td>
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<tr>
<td>iNOSoxy</td>
<td>47.3 ± 3.7</td>
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<tr>
<td>V346I iNOSoxy</td>
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</table>

**Fig. 3.** Stopped-flow analysis of heme transitions during NOHA oxidation by I224V bsNOS. Ferrous enzyme (4 μM) was mixed with an air-saturated buffer in the presence of NOHA (0.5 mM) and H₂B (0.2 mM) at 10 °C and diode array spectra were collected. *A* shows the four spectral species that were detected during the reaction as calculated by global analysis of the spectral data, whereas *B* and *C* show their concentration profiles versus time in a shorter and longer time range, respectively.
binding was not a factor. Indeed, we found that 0.78 citrulline/heme was generated in the NOHA reaction (Table II). This yield is 80% of that of the wild-type reaction and indicates that catalysis was relatively well coupled to product formation in V346I iNOSoxy.

Fig. 4 shows the calculated concentrations of the heme species during the NOHA reaction catalyzed by V346I iNOSoxy. In the case of the third species, as noted above, its concentration trace actually represents a mixture of FeIIINO species. The calculated rates for the three heme transitions were all slower relative to wild-type iNOSoxy, and the rate of FeIIO2 formation was slower than that in bsNOS (Table III). Thus, our analysis of the V346I iNOSoxy reaction suggested that the product yield was good, the general progression was altered somewhat with respect to a smaller buildup of the FeIIINO intermediate, and the kinetics of each transition became slower and more like bsNOS.

To obtain an independent estimate for the dissociation rates of the FeIIINO product complexes, we focused on the absorbance change at 650 nm versus time in each of the NOHA reactions. The absorbance gain at 650 nm during the final transition represents the buildup of the ferric enzyme species and provides an independent estimate of the dissociation rate of the FeIIINO product complex. Absorbance traces recorded during the appropriate time periods from each of the four reactions are shown in Fig. 5. They all fit well to a single exponential equation (dotted line).

Fig. 5. Kinetics of ferric heme recovery at the end of each NOHA oxidation reaction. Data were taken from the reactions run as described in the legends to Figs. 3 and 4. Actual absorbance values at 650 nm versus time were extracted from the diode array data and plotted for each reaction. The starting time for each trace was 0.45 s (I224V bsNOS), 0.6 s (WT bsNOS), 0.019 s (WT iNOSoxy), and 0.361 s (V346I iNOSoxy). The absorbance changes were fit to a single exponential equation (dotted line).
rates of Fe IIINO formation consistent with NO binding being reversible. The estimated when fit to a linear function both plots gave a positive intercept. The plots of the apparent rate constants that we observed at each monitored at 438 nm. The absorbance gain was monophasic and fit are indicated. Fig. 6 contains the neuronal NOS and endothelial NOS FeIIINO product complexes to study NO rebinding kinetics within the heme pocket (25, 26). These studies report that most of the photolysed NO (Fig. 6 qualitatively confirms our other measures of NO release from intact Bacillus cells has yet to be demonstrated. Clearly, the amino acid residues that define heme pockets or active-site channels can impede NO release from most hemeproteins (Table V). Flash photolysis experiments have been done with the neuronal NOS and endothelial NOS FeIIINO product complexes to study NO rebinding kinetics within the heme pocket (25, 26). These studies report that most of the photolysed NO (>80%) undergoes very rapid recombination with the ferric heme (within picoseconds) with much of the remaining NO binding to the heme within nanoseconds. This amounts to a highly efficient geminate or

titatively similar to the rates that were determined by global analysis (see Table III) and provide independent confirmation that the I224V mutation speeds dissociation of the FeIIINO product complex. Formation of the enzyme FeIIINO product complex was followed as an absorbance gain at 437 nm. A and B, plots of the observed rates of FeIIINO formation versus NO concentration for wild-type iNOSoxy and V346I iNOSoxy, respectively. The calculated lines of best fit are indicated.

**TABLE IV**

<table>
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<tr>
<th>Protein</th>
<th>$k_{\text{on}}$</th>
<th>$k_{\text{off}}$</th>
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<tr>
<td>iNOSoxy</td>
<td>0.27 ± 0.03</td>
<td>20.7 ± 3.0</td>
</tr>
<tr>
<td>V346I iNOSoxy</td>
<td>0.033 ± 0.003</td>
<td>1.9 ± 0.3</td>
</tr>
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Kinetics of NO binding to ferric enzymes at 10 °C

**DISCUSSION**

Factors that control heme-NO dissociation are particularly important for NOS enzymes. This is because their natural product is NO, and newly generated NO molecules coordinate to the ferric heme at the end of each catalytic cycle before leaving the enzyme (12–14) (see Fig. 1). In the three mammalian NOS, the rates of FeIIINO dissociation and reduction are set so that much of the NO can escape from the enzyme before the FeIIINO product complex is reduced to ferrous, which then dooms it to an oxidative reaction that forms a higher oxide of nitrogen in place of NO (15, 16). The FeIIINO dissociation rates of mammalian NOS enzymes are within a range that is typical for other ferric heme proteins, whereas in bsNOS this rate is near the lower end of the range (Table V). In contrast, the rates of ferric heme reduction in mammalian NOS enzymes range between 0.1 and 4 s$^{-1}$ at 10 °C, which are much slower than in other heme enzymes that contain attached flavin domains like cytochrome P450BM3 (23) or flavohemoglobin (24). We suspect that this circumstance evolved in the mammalian NOS to enable their NO release from the heme. As discussed previously (17, 18), the slower NO dissociation in bsNOS may predispose it to release less NO and thereby utilize NO in ways that may be distinct from its mammalian counterparts. Indeed, NO release from intact Bacillus cells has yet to be demonstrated.

Clearly, the amino acid residues that define heme pockets or active-site channels can impede NO release from most hemeproteins (Table V). Flash photolysis experiments have been done with the neuronal NOS and endothelial NOS FeIIINO product complexes to study NO rebinding kinetics within the heme pocket (25, 26). These studies report that most of the photolysed NO (>80%) undergoes very rapid recombination with the ferric heme (within picoseconds) with much of the remaining NO binding to the heme within nanoseconds. This amounts to a highly efficient geminate or
near gemicinate recombination within the pocket. Thus, binding NO within the heme pocket occurs much faster than the bimolecular process that takes place when the enzyme is exposed to extrinsic NO. The reported bimolecular association rate constants for NO are in the range of $10^3$–$10^7$ M$^{-1}$ s$^{-1}$ at 10 or 20 °C (22, 25, 26). The $k_{cat}$ values for mammalian NOS ferric-NOS complexes are reported to range between $2$ and $50$ s$^{-1}$ at 10 or 20 °C. The faster $k_{cat}$ values have typically been derived from kinetic analysis of observed $K_{on}$ values determined in either laser-flash or stopped-flow NO-binding experiments, as we have done here in Fig. 6. The slower $k_{cat}$ values have typically been derived from equilibrium NO titration experiments (25, 26, 26), in ligand displacement studies (16, 25, 26) or in single turnover catalytic studies (13, 14, 22, 25, 26) that directly follow NO dissociation from the ferric heme as we have done here in Figs. 3–5. Thus, a newly formed NO molecule is likely to undergo multiple ferric heme binding and dissociation events within the NOS heme pocket before it escapes from the enzyme. Under such circumstances, it is not surprising that residues like iNOSoxy Val-346 and bsNOS Ile-224, which help to define the size of the NOS heme pocket before it escapes from the enzyme. Indeed, crystal structure data predict that there is a very tight fit between NOHA and O$_2$ in the iNOSoxy heme pocket (30) and also suggest a steric interaction between bound O$_2$ and Ile-224 in bsNOS. In any case, the relative inability of the Ile/Val substitutions to measurably alter the spectral properties of the Fe$^{III}$O$_2$ intermediate or the Fe$^{III}$NO product complex suggests that their influence on $k_{cat}$ involves relatively subtle effects. This issue can be addressed in future studies.

Given the special constraint that Fe$^{III}$NO formation puts on NOS catalysis, why has the enzyme not evolved to support a faster NO dissociation? This would minimize the danger of ferric heme reduction becoming too fast and would even allow NOS to support a faster rate of NO synthesis in the steady state. Although many parameters are likely to determine NO dissociation from a hemeprotein, enlarging heme pocket access is certainly one way to speed NO dissociation. But therein lies a problem, because the heme pocket has multiple functions that must remain in harmony with one another. For example, any positive effect of widening the entrance regarding NO release might be counteracted by an increase in active-site solvation, a less optimal shielding of heme-oxo catalytic intermediates, or by issues related to substrate binding within the active site. Indeed, related heme-thiolate oxygenase enzymes like cytochrome P450 are thought to require a relatively shielded distal pocket to perform their oxygen activation chemistry and catalysis (31, 32). On the other hand, the available sequence data indicate that closing down the heme pocket (relative to mammalian NOS) must confer some selective advantage to the bacterial NOS-like enzymes. In fact, they contain another conserved residue switch (Ser to His-134 in bsNOS) that helps to further close down their heme pocket (18). But a problem associated with minimizing NO release in this way is that one must still accommodate entry of Arg and O$_2$ into the heme pocket. In fact, our data indicate that the Val to Ile substitution in iNOSoxy presents a kinetic barrier for NO and O$_2$ to access the heme, apparently mimicking what occurs for O$_2$ binding in bsNOS. This brings up related concerns about the biological O$_2$ tension under which each NOS has evolved to operate. There likely is a range of useful heme entryway sizes, and we suspect that those in the mammalian and bacterial NOSs are set according to their required functions and the environment under which they must operate. Beyond the structure-function insights, our mutants suggest a means to examine how changing the NO release rate of a given NOS might impact its biological function in the host organism.

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