Nitric oxide (NO) release from nitric oxide synthases (NOSs) is largely dependent on the dissociation of an enzyme ferric heme-NO product complex (FeIII NO). 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, V346I) and in bsNOS (I224V) and characterized the kinetics and extent of their NO synthesis from NOHA and their NO-binding kinetics. The mutations did not greatly alter binding of Arg, (6R)-tetrahydrobiopterin, or 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-dioxygen (FeIIO2) intermediate, FeIII NO, and an ending ferrous enzyme. The rate of each transition was increased relative to wild-type bsNOS, with FeIII NO dissociation being 3.6 times faster. In V346I iNOSoxy 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 FeIII NO 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, with FeIIINO dissociation being 3.6 times faster. In V346I iNOSoxy 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 FeIII NO 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.

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The atomic coordinates and structure factors (codes 1MTV, 1DXY, and 1F0P) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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enzyme to ferrous (futile cycle that releases a higher oxide of nitrogen. Reduction of ferric molecules engages in a productive cycle that releases free NO and in a Fe\(^{3+}\) made, an immediate product of catalysis is the ferric heme-NO complex Fe\(^{2+}\) generate a ferrous heme-NO complex (this difference.

phy by Pant et al compared with the bsNOS construct that was used in crystallography by Fattah et al to study NO synthases). We characterize 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 NOS.

**EXPERIMENTAL PROCEDURES**

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

**Mutagenesis—**Site-directed mutagenesis of mouse iNOSoxy DNA in the pCWori expression plasmid (coding for amino acids 66–498 plus a His\(_\alpha\) 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, Nael and Xhol for V346F and I224V mutations, respectively) was incorporated into the primers as follows: V346F, 5′-GCA CTC CGG GCC ATTA GCC AAC ATG CTA CTT-3′; V348R, 5′-CAG TAG CAT GGT ACC TAT GGC CCG CAG TGC-3′; I224V, 5′-GGG GTC GAA ATT GAT ATG ACC CCT GAG GTC GGG GG-3′; I224VR, 5′-CCC CCG ACC TCG AGC TTA TCA GAA ACA ATT GCC ACG CC-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 NOS enzymes were determined from the 444 nm absorbance of the ferrous-CO complex, using an extinction coefficient of 76 mM\(^{-1}\) cm\(^{-1}\) (21).

**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 \(\mu\)M 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 (\(K_a\)). Binding affinity of Arg was then measured in the same way except that substrate Arg was added gradually to enzyme solutions that contained H\(_2\)B and imidazole at either 1.6 mM (iNOSoxy enzymes) or 10 mM (bsNOS enzymes). The \(K_a\) value of Arg was then calculated using equation 1, \(K = K_{A,\text{free}}/1 + [\text{imidazole}]/K_{\text{A,imidazole}}\), in which \(K_{A,\text{imidazole}}\) 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 NOHA 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
Fig. 2. Comparison of structure surrounding the heme pockets of iNOSoxy and bsNOS. Left panel, cut-away view of the molecular surface (colored by electrostatic potential; red, negative; blue, positive) surrounding the immediate heme pocket in iNOSoxy (1DWX) and bsNOS (1MI7V). Viewer is looking down the substrate channel into the heme pocket. The increased projection of the Ile side chain (green) from the roof of the heme pocket in bsNOS compared with Val in iNOSoxy completely sequesters nitric oxide (NO) bound to the heme iron along side the substrate arginine (ARG). The NO position was modeled by superimposing the structure of bsNOS with eNOSoxy complexed with Arg and NO (1FOP). H4B resides outside of the Val/Ile substitution and thus its accessibility should not be affected by the residue change. Right panel, view is from the bound H4B looking out through the heme pocket toward the substrate channel. Figure rendered in SPock (41).

| TABLE 1 |
| --- |
| Structural Determinant of NO Release from NOS |
| UV-visible spectra were recorded at room temperature in the presence of saturating concentrations of the indicated additives or heme ligands. $K_s$ values were determined as explained under “Experimental Procedures.” The EC$_{50}$ value of H$_4$B was determined in an H$_2$O$_2$-driven NOHA oxidation assay. NA, not available. |
| | iNOSoxy | V346I iNOSoxy | bsNOS | 1224V bsNOS |
| --- | --- | --- | --- | --- |
| Ferric enzyme | 418 nm | 407 nm | 402 nm | 398 nm |
| + H$_4$B | 399 nm | 402 nm | 399 nm | 396 nm |
| + Arg | 397 nm | 399 nm | 398 nm | 396 nm |
| + Arg, H$_4$B | 396 nm | 399 nm | 398 nm | 396 nm |
| + Imidazole | 427 nm | 427 nm | 426 nm | 426 nm |
| + Dithiothreitol | 375, 459 nm | 378, 459 nm | 400 nm | 395 nm |
| Ferrous-CO | 444 nm | 444 nm | 445 nm | 444 nm |
| $K_s$ (imidazole) | 158 ± 6 µM | 179 ± 6 µM | 384 ± 10 µM | 506 ± 38 µM |
| $K_{obs}$ (Arg) | 175 ± 4 µM | 397 ± 27 µM | 129 ± 2 µM | 42 ± 3 µM |
| $K_{obs}$ (Arg) | 16.1 ± 0.7 µM | 40.9 ± 4.3 µM | 4.8 ± 0.1 µM | 2.0 ± 0.2 µM |
| EC$_{50}$ (H$_4$B) | 5.0 ± 0.4 µM | 1.6 ± 0.1 µM | NA | NA |

Results

Because the complementary Val and Ile mutations are located near the NOH pocket, 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 H$_4$B 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 H$_4$B, 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 1224V bsNOS displayed a lower affinity toward imidazole but an increased affinity toward Arg. In an H$_2$O$_2$-driven NOHA oxidation assay the H$_4$B concentration dependence of V346I iNOSoxy was somewhat enhanced. We can conclude that the mutations did not greatly alter NOS binding of Arg, H$_4$B, 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 respects despite their containing either a Val or Ile at the same position.

Next we 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 NOH oxidation by the mutants in a single turnover reaction. Solutions of ferrous enzymes containing NOH and H$_4$B were rapidly mixed with O$_2$-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 NOH 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 (Fe IIINO) 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, FeIII(NO) (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...
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. Stopped-flow analysis of heme transitions during NOHA oxidation by V346I iNOSoxy. Ferrous enzyme (6 μM) was mixed with air-saturated buffer in the presence of NOHA (0.2 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. B, shows one spectrum that was recorded at 0.361 s after initiating the NOHA reaction. C, shows the calculated concentrations of different species versus time of reaction.

To obtain an independent estimate for the dissociation rates of the Fe³⁺NO 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 Fe³⁺NO 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, which gave estimated dissociation rates of 0.34 ± 0.01, 0.60 ± 0.02, 2.09 ± 0.06, and 0.49 ± 0.02 s⁻¹, respectively, for bsNOS, I224V bsNOS, iNOSoxy, and V346I iNOSoxy. These values are of the same rank order and quan-
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 Fe^{III}-NO product complex in bsNOS, whereas the V346I mutation slows dissociation of this product complex in iNOSoxy.

We next studied binding kinetics of extrinsic NO to V346I iNOSoxy alone and in comparison with wild-type iNOSoxy. Anaerobic solutions that contained a ferric enzyme, NOHA, and H_4B were mixed in the stopped-flow spectrophotometer with anaerobic solutions that contained different concentrations of NO. Buildup of the Fe^{III}-NO product complex was monitored at 438 nm. The absorbance gain was monophasic and fit well to a single exponential function in all cases. Fig. 6 contains plots of the apparent rate constants that we observed at each NO concentration for wild-type iNOSoxy and V346I iNOSoxy. When fit to a linear function both plots gave a positive intercept consistent with NO binding being reversible. The estimated k_{on} and k_{off} values derived from the graphs are given in Table IV. The estimated k_{on} and k_{off} values that we obtained for wild-type iNOSoxy that contained NOHA plus H_4B are in general agreement with values we derived in identical experiments for ferric iNOSoxy that contained bound Arg and H_4B (22). The data in Table IV indicate that ferric V346I iNOSoxy has an ~8-fold slower k_{on} for extrinsic NO and an ~11-fold slower k_{off} for NO compared with wild-type iNOSoxy. The slower k_{off} for NO relative to wild-type as we estimated graphically in Fig. 6 qualitatively confirms our other measures of NO release rates by V346I iNOSoxy versus wild-type. The slower k_{on} toward extrinsic NO is also consistent with our observation that the rate of Fe^{IV}=O_2 formation in V346I iNOSoxy was slower than in wild-type iNOSoxy during the NOHA single turnover reactions (see Table III).

### DISCUSSION

Factors that control heme-NO dissociation are particularly important for NO 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 Fe^{III}NO dissociation and reduction are set so that much of the NO can escape from the enzyme before the Fe^{III}NO 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 Fe^{III}NO 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 heme proteins (Table V). Flash photolysis experiments have been done with the neuronal NOS and endothelial NOS Fe^{III}NO 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

### Table IV

| Protein | k_{on} | k_{off} |
|---------|--------|---------|
| iNOSoxy | 0.27 ± 0.03 | 20.7 ± 3.0 |
| V346I iNOSoxy | 0.033 ± 0.003 | 1.9 ± 0.3 |

### Table V

| Ferric model complex or protein | NO k_{off} | Temperature °C |
|-------------------------------|------------|----------------|
| Fe^{III}TPPS\_4-NO | 5 × 10^2 | 23 |
| Flavohemoglobin | 4 × 10^2 | 20 |
| Catalase | 1.7 × 10^2 | 25 |
| Prostaglandin H synthase | 60 | 21 |
| Myoglobin | 28 | 25 |
| Myeloperoxidase | 11 | 10 |
| Heme oxygenase-1 | 3 | 25 |
| Nitrophorins 1-4 | 0.006-2.6 | 25 |
| Cytochrome c | 4.4 × 10^{-2} | 25 |
| Neuronal NOS | 5 | 10 |
| Inducible NOS | 2.3 | 10 |
| bsNOS | 0.23 | 10 |

* TPFS, tetra(4-sulfonatophenyl)porphine.

b NO release from nitrophorin proteins is biphasic and pH-dependent.

c As measured in the NOHA single turnover reactions.
near guanidine 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 $10^3 - 10^7 \text{M}^{-1} \text{s}^{-1}$ at 10 or 20 °C (22, 25, 26). The $k_{cat}$ values for mammalian NOS ferric-NO 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 graphic 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, 40), 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 exit, would also influence the macroscopic association and dissociation rates of NO for the ferric heme.

Although the addition of an extra methyl group caused by the Val to Ile substitution removes only 10 Å$^2$ of the heme cavity, it extends the roof at the entrance of the heme pocket ~1.5 Å and into contact with the carboxylate side chain of heme pyrrole D ring, thereby completely sequestering heme ligands from solvent (Fig. 2). Structural superpositions of the conserved heme pockets of bsNOS (Protein Data Bank code: 1M7V), iNOSoxy (1DWX), and eNOSoxy (1FOP) bound to Arg and NO indicate that the terminal Ile methyl will generate a van der Waals interaction with heme-bound NO. Although protein dynamics allow access to the heme iron whether the Val or Ile is present, it is not surprising that even the conservative Val to Ile mutations had opposite affects on NO-binding rates among the iNOSoxy and bsNOS enzymes argue strongly that the described changes in side chain volume at this particular location help to regulate NO release from NOS. In this way, our results provide a structural basis that links heme pocket geometry to the 10–20-fold difference in NO release rate that is observed between the animal and bacterial NOS enzymes.

Our evidence suggests there was incomplete Fe$^{II}$NO formation in V346I iNOSoxy despite its catalyzing an efficient NOHA single turnover reaction (0.78 NO/heme). A simple explanation is that not all of the NO is captured by the ferric heme. This would be consistent with crystallographic data showing that the Ile-224 methyl group has a steric interaction with heme-bound NO in bsNOS. Perhaps the same steric interaction is magnified in the iNOSoxy V346I mutant to the point of its destabilizing NO binding in a subpopulation of the enzyme molecules. This phenomenon might enable a fraction of NO to quickly escape from the heme pocket even while escape of the heme-bound NO is retarded. We can now test this interesting possibility.

The Ile/Val mutations affected other aspects of catalysis in addition to altering NO binding and the Fe$^{II}$NO dissociation rates. These include changing the rates of the first two heme transitions in the NOHA reaction, namely the rate of Fe$^{II}$O$_2$ formation and $k_{cat}$. As was observed for Fe$^{II}$NO dissociation, the Ile substitution slowed these two kinetic parameters, whereas the Val substitution sped them up. At this point, we can assume that all of the kinetic effects are because of the mutations either increasing or decreasing heme pocket access. Thus, one can rationalize that Ile supports slower rates of Fe$^{II}$O$_2$ formation and extrinsic NO binding, because O$_2$ and NO access to the heme are similarly influenced by protein structural features in NOS (22, 27, 28) and in other hemeproteins (29). In contrast, the mutual effects on $k_{cat}$ were unexpected and cannot be so easily explained. The only documented way to slow $k_{cat}$ in iNOSoxy is to slow the H$_2$B reduction of the Fe$^{II}$O$_2$ intermediate during the Arg hydroxylation reaction (19), but it is not at all clear how the Val/Ile substitutions might influence that process. Perhaps the substitutions favor NO/haeme-oxo interactions that are either more or less optimal for catalysis. 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$^{II}$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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A Conserved Val to Ile Switch near the Heme Pocket of Animal and Bacterial
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