Nitric oxide (NO) is a signal molecule produced in animals by three different NO synthases. Of these, only NOS I (neuronal nitric-oxide synthase; nNOS) is expressed as catalytically active N-terminally truncated forms that are missing either an N-terminal leader sequence required for protein-protein interactions or are missing the leader sequence plus three core structural motifs that in other NOSs are required for dimer assembly and catalysis. To understand how the N-terminal elements impact nNOS structure-function, we generated, purified, and extensively characterized variants that were missing the N-terminal leader sequence (Δ296nNOS) or missing the leader sequence plus the three core motifs (Δ349nNOS). Eliminating the leader sequence had no impact on nNOS structure or catalysis. In contrast, additional removal of the core elements weakened but did not destroy the dimer interaction, slowed ferric heme reduction and reactivity of a heme-dioxy intermediate, and caused a 10-fold poorer affinity toward substrate L-arginine. This created an nNOS variant with slower and less coupled NO synthesis that is predisposed to generate reactive oxygen species along with NO. Our findings help justify the existence of nNOS N-terminal splice variants and identify specific catalytic changes that create functional differences among them.
D. radiodurans

the corresponding start sites of an inducible NOS N-terminal deletion may not be critical for enzyme assembly and catalysis. Our cells (27, 31), its leader sequence and N-terminal core motifs are the otherwise trypsin-resistant nNOS oxygenase domain dimer, particular deletion points represent trypsin-sensitive sites in NOS (33) or radiodurans subunit covalently links two oxygenase domains (17, 29, 32).

\((/H9004\) requirements of nNOS or why it is the only NOS isoform that

imply that underlying structure-function differences exist practically essential for dimer formation and associated NO parameters that combine to control its NO synthesis.

\((/H9252\) binding loop sequences indicated. The \(\text{Arrows}\) also indicate the initiation sites of the N-terminally truncated nNOS mutants used in this study (\(\Delta296\) and \(\Delta349\) nNOS) and the \(\text{NOS}\) start sites of an inducible NOS N-terminal deletion mutant (\(\Delta114\) mouse iNOS) and NOS-like proteins in genomes of D. radiodurans NOS and B. subtilis NOS.

B. subtilis NOS-like proteins and TnNOS splice variants in animals and to the bacterial NOS-like proteins differs substantially from those of NOS II and NOS III, which is consistent of nNOS splice variants and the bacterial NOS-like protein.

To explore these issues, we generated, purified, and extended our deletion mutants used in this study (\(\Delta296\) and \(\Delta349\) nNOS) (29, 32). These \(\text{Arrows}\) also indicate the initiation sites of the N-terminally truncated nNOS mutants used in this study (\(\Delta296\) and \(\Delta349\) NOS) and the corresponding start sites of an inducible NOS N-terminal deletion mutant (\(\Delta114\) mouse iNOS) and NOS-like proteins in genomes of D. radiodurans NOS and B. subtilis NOS.

face (29, 32), whereas \(\text{Zn}^{2+}\) binding to the CXXC motif in each subunit covalently links two oxygenase domains (17, 29, 32).

Because the N-terminal splice variants of nNOS have all been active to varying degrees when overexpressed in animal cells (27, 31), its leader sequence and N-terminal core motifs may not be critical for enzyme assembly and catalysis. Our recent work on bacterial NOS-like proteins from Deinococcus radiodurans NOS (33) or Bacillus subtilis NOS (34, 35) shows that they are also catalytically active despite their missing the three core motifs (Fig. 1). Surprisingly, this characteristic of nNOS splice variants and the bacterial NOS-like proteins differs substantially from those of NOS II and NOS III, because in these latter two NOSs the three core motifs are practically essential for dimer formation and associated NO synthesis activity (16, 29, 30). Together, the available data imply that underlying structure-function differences exist among the three NOSs despite their apparent structural congruence. However, little is known about the precise structural requirements of NOS or why it is the only NOS isoform that is naturally expressed in N-terminally truncated forms.

To explore these issues, we generated, purified, and extensively characterized two N-terminally deleted nNOS proteins, one missing the entire N-terminal leader sequence (\(\Delta296\)nNOS) and the other missing the leader sequence plus the three core sequence motifs (\(\Delta349\)nNOS) (29, 32). These particular deletion points represent trypsin-sensitive sites in the otherwise trypsin-resistant nNOS oxygenase domain dimer (nNOSoxy) (36). As shown in Fig. 1, the \(\Delta296\)nNOS is analogous to the naturally occurring splice variant nNOS\(_\text{S}\), whereas the \(\Delta349\)nNOS is analogous to the naturally occurring nNOS and TnNOS splice variants in animals and to the bacterial NOS-like proteins B. subtilis NOS and D. radiodurans NOS. Our results reveal in detail how the N-terminal structural elements impact nNOS dimer assembly, cofactor/substrate affinity, and several fundamental kinetic and thermodynamic parameters that combine to control its NO synthesis.

\(37123\)
followed by formation of the ferrous CO complex at 444 nm. Reactions were initiated by rapid mixing an anaerobic, buffered, CO-saturated solution containing 50 μM NADPH with an anaerobic, buffered, CO-saturated solution containing wild-type or mutant nNOS (2 μM), 40 mM EPPS buffer, pH 7.6, 10 μM H4B, 5 mM Arg, 0.3 mM DTT, 4 μM CaM, and 1 mM Ca2+. Flavin reduction was monitored under the same conditions at 485 nm. Signal/noise ratios were improved by averaging at least 10 individual mixing experiments. The time course of the absorbance change was fit to single or multiple exponential equations using a nonlinear least square method provided by the instrument manufacturer.

Ferrous Heme-NO Oxidation—An anaerobic buffered solution containing each full-length nNOS enzyme (4 μM) plus 5 mM Arg and 10 μM H4B was reduced with a minimum amount of dithionite solution, and then a saturated NO solution was added to give a NO concentration of ~0.1 mM and form the ferrous heme-NO enzyme complex. This solution was transferred to the stopped flow under anaerobic conditions and then rapidly mixed with air-saturated buffer solution at 10 °C. Rates of ferrous NO complex disappearance and ferric enzyme formation were determined from cross-sections of absorbance loss and gain at 436 and 393 nm, respectively.

Single Turnover Analysis of Arg or NOHA Oxidation—These reactions were performed as previously described (41). An anaerobic solution of nNOSxy enzyme (6 μM) containing 40 mM EPPS, pH 7.6, 1 mM DTT, and 2 mM NOHA or 5 mM Arg, with or without 20 μM H4B, was titrated with dithionite solution in an anaerobic cuvette until full reduction to ferrous nNOSxy was achieved. The solution was then transferred to the stopped flow instrument using a gas-tight syringe, where it was rapidly mixed in multiple shots with an equal volume of oxygen-saturated buffer (50 mM EPPS, pH 7.6) at 10 °C. Ninety-six spectral scans (350–700 nm) were obtained following each mixing. The diode array data were then fit to different reaction models by a Specfit program from Hi-Tech Ltd. to obtain the calculated number of species, their individual spectra, the concentration of each species versus time, and rate constants for each transition.

Measurement of Heme Midpoint Potential—The glass cell and electrodes used were as previously described (42). Spectroelectrochemical titration was performed at 25 °C using a Radiometer PGP201 potentiostat/galvanostat. The reference and auxiliary electrodes were made anaerobic overnight in a solution containing 0.5 mM methyl viologen, 0.1 mM potassium phosphate, pH 7. The reference electrode was calibrated using a 5 mM solution of ferricyanide/ferrocyanide in anaerobic 0.1 M potassium phosphate, pH 7, at 25 °C (E(ox) = +0.425 mV). The Δ296nNOSxy and Δ349nNOSxy were diluted to a final concentration of about 25 μM in a 0.1 M potassium phosphate buffer, pH 7, in the presence of 60 μM H4B, 0.1 mM EDTA, 5 mM Arg, 0.1 mM methyl viologen, 20 μM phenosafranine, and 20 μM neutral red. The solution was rendered anaerobic in the electrochemical cell by N2 refilling over 2 h. The titration was achieved using the internal source of the radiometer set on the galvanostat mode for current ranging between 5 and 60 μA and times ranging between 15 s and 5 min. After each adjustment, the potential was allowed to equilibrate (between 30 min and 1 h), the potential was measured (versus SHE), and a spectrum was recorded on a Hitachi U-3110 spectrophotometer. The one-electron midpoint potential was determined using the absorbance change at 400 and 645 nm, where change due to dye mediators was negligible. The midpoint potential was calculated using the Nernst equation.

RESULTS

Physical Characteristics of N-terminal Deletion Mutants—The full-length proteins Δ296nNOSFL and Δ349nNOSFL were generated by deletion mutagenesis, whereas corresponding nNOSxy proteins were generated by limited trypsin proteolysis of purified nNOSxy (43). Fig. 2 depicts SDS-PAGE and gel filtration profiles of the six proteins used in our study, namely wild-type nNOSxy (A), Δ296nNOSxy (B), Δ349nNOSxy (C), wild-type nNOSFL (D), Δ296nNOSFL (E), and Δ349nNOSFL (F). Each protein migrated at its expected molecular weight in SDS-PAGE. N-terminal sequencing confirmed that each protein had the correct N-terminal truncation (data not shown). Gel filtration patterns showed that N-terminal deletions of 296 or 349 amino acids did not greatly alter the dimer content of the nNOSxy proteins, given that even Δ349nNOSxy remained 83% dimeric (Fig. 2, panel 2, profile C). The full-length proteins, however, had generally less dimer content compared with their oxygenase counterparts, consistent with our earlier observations that attached reductase domains weaken the dimeric interaction between two oxygenase domains (44). Thus, nNOSFL, Δ296nNOSFL, and Δ349nNOSFL were estimated to be 88, 82, and 56% dimeric, respectively (Fig. 2, panel 3). Notably, the truncated nNOSFL proteins did not increase their dimer content after overnight incubation with 10 mM Arg and 20 μM H4B (data not shown) as otherwise normally occurs in wild type enzyme (13,43). Our results suggest that the N-terminal leader sequence and adjacent core elements need not be present for significant dimer formation in nNOS. This behavior makes nNOSxy somewhat similar to bacterial NOS-like proteins (33–35) and distinguishes them from the other two mammalian isoforms, iNOS or eNOS, where deletion of the same core elements renders them completely or predominantly monomeric (16, 29, 30).

Because a significant proportion of each N-terminal-deleted nNOS was dimeric, the nNOSs provided us an unprecedented opportunity to investigate how the N-terminal elements influence kinetic, thermodynamic, and catalytic properties of a nNOS dimer. These investigations are detailed below.

Role of N-terminal Elements in Stabilizing the Dimer—We compared dimer interaction strengths of the various full-length

FIG. 2. SDS-PAGE (panel 1) and gel filtration profiles (panels 2 and 3) of wild-type and N-terminally deleted nNOS proteins. 10–15 μg of each protein (A, wild-type nNOSxy; B, Δ296nNOSxy; C, Δ349nNOSxy; D, wild-type nNOSFL; E, Δ296nNOSFL; F, Δ349nNOSFL) were analyzed by gel electrophoresis after being boiled (panel 1) and run on a Superdex 200 HR column as described under “Materials and Methods.” Results are representative of three similar experiments. D, dimer; M, monomer.
proteins by examining the loss of a dimer-dependent and a dimer-independent catalytic activity as a function of increasing chaotropic stress due to urea (43). Fig. 3A shows that Δ349nNOSFL lost dimer-dependent activity (NO synthesis) at lower urea concentrations compared with wild type nNOSFL or Δ296nNOSFL. However, the three proteins were identical regarding stability of their dimer-independent activity (cytochrome c reduction; Fig. 3B). This indicates that the N-terminal leader sequence has no impact on nNOS dimer stability, whereas the three core structural elements do. However, significant dimer stability remains even in their absence.

Spectral Properties and Arg and H4B Affinity—Dithionite reduction of each nNOS mutant in the presence of CO formed a ferrous heme-CO complex that absorbed at 444 nm in all cases (not shown), indicating that they all have proper heme incorporation with stable cysteine thiolate axial ligation (44). Light absorbance spectra of the four ferric proteins are shown in Fig. 4. In the presence of 1 mM DTT, all proteins exhibited a split Soret absorbance peak at 380 and 460 nm, which indicated that DTT bound to ferric heme to form a bis-thiolate complex as occurs in wild-type nNOS (22). Arg and H4B were able to displace DTT and bind to all four mutants, as indicated by a characteristic shift in Soret absorbance toward a single peak near 400 nm (45, 46).

Arg binding affinities were determined by measuring displacement of imidazole bound to the ferric heme at different Arg concentrations (22, 39). The Δ349nNOSFL had almost 10-fold higher apparent K_d for Arg compared with wild type, whereas the apparent K_d of Δ296 nNOSFL was equivalent to wild type (Table I). Measures of the apparent K_m for Arg using the NO synthesis assay also showed a higher value for the Δ349nNOSFL (Table I). Measures of apparent K_m for H4B indicated a less than 2-fold increase for Δ349 nNOSFL compared with wild type (Table I). These data suggest that the N-terminal leader sequence has no impact on Arg or H4B binding affinity or K_m, whereas an additional deletion of the core elements lowers enzyme affinity toward Arg and H4B and increases their apparent K_m.

NOS Steady State Catalytic Activities—We compared catalytic activities of the three nNOS proteins by measuring their steady state rates of NO production and associated NADPH oxidation, H_2O_2-dependent nitrite formation from NOHA, and cytochrome c and ferricyanide reduction (38). Results are summarized in Tables II and III. After taking into account their differences in dimer content, NO synthesis activity of
Δ296nNOSFL was 93% of the wild type nNOSFL, whereas Δ349nNOSFL had 45% activity of wild type (Table III). Corresponding NADPH oxidation rates measured during NO synthesis showed that the ratio of NADPH oxidized per NO formed was similar in the Δ296nNOSFL and wild type enzymes (2.2 and 2.0, respectively) but was a bit greater in Δ349nNOSFL (2.6) (Table III). The rates of nitrite production by Δ296nNOS and Δ349nNOS in the H₂O₂-driven assay were 91 and 51% that of wild type, respectively, and were in consonance with their NO synthesis activities (Table II). All three enzymes had equivalent cytochrome c and ferricyanide reduce activities.

We also examined whether there was any change in the Ca²⁺-dependence of the Δ349nNOSFL as compared with the wild type. We found that both proteins showed a similar trend of Ca²⁺-dependence over a 0–50 μM concentration range (data not shown), indicating that the deletion of 349 amino acids from the N-terminal region did not affect Ca²⁺ response in nNOS.

Together, these data suggest that deleting the N-terminal leader sequence had no impact on catalysis, whereas removal of the three N-terminal core elements slowed NO synthesis by the nNOS dimer and caused some uncoupling of its NADPH oxidation. To better understand the basis for these catalytic effects, we performed kinetic and biophysical studies with the nNOS deletion mutants as described below.

**Kinetics of Heme and Flavin Reduction**—We determined the kinetics of NADPH-dependent flavin and heme reduction in CaM-bound nNOS proteins by stopped flow spectroscopy. The observed rate constants for reactions run at 10 °C are listed in Table IV, and the traces in Fig. 5 illustrate kinetics of absorbance change recorded at two diagnostic wavelengths for each nNOS protein (37).

Rates of NADPH-dependent flavin reduction were strikingly close in all three proteins (Fig. 5B), confirming that the N-terminal elements do not influence electron loading into the reductase domain. We determined the kinetics of heme reduction by following heme CO binding at 444 nm (47). The initial absorbance decrease present in each trace of Fig. 5B reflects an NADPH-dependent flavin reduction that occurs prior to electron transfer to the ferric heme (38) and was not considered in our rate calculations. Heme reduction was best described as monophasic in all three proteins (Fig. 5A). The Δ296nNOSFL and wild type enzymes were equivalent regarding their rates and extents of heme reduction. In contrast, heme reduction in Δ349nNOSFL was 27% slower and of smaller magnitude than wild type (Table IV). The smaller magnitude for Δ349nNOSFL heme reduction is likely to be due to the lower dimer content of this mutant. Control reactions showed that rates of CO binding by the prereduced ferrous forms of all three nNOS proteins were identical (data not shown), confirming that the differences we observed reflected their different ferric heme reduction rates.

Removal of the core N-terminal elements, therefore, caused a discernible slowing of ferric heme reduction in Δ349nNOSFL.

**Heme-NO Complex Formation and Oxidation**—We next compared rates of heme-NO complex buildup during the initial phase of NO synthesis by the three nNOSFL enzymes (47). Heme-NO complex formation was followed at 436 nm after initiating NO synthesis with NADPH addition in the stopped flow instrument. Complex buildup occurred within the first few seconds and followed a biphasic process in all cases, with the slow phase representing the majority of the total absorbance change (Table IV). The relative rates of heme-NO complex buildup were as follows: wild type = Δ296nNOSFL > Δ349nNOSFL. This matched the rank order of their ferric heme reduction rates and is consistent with ferric heme reduction being rate-limiting for heme-NO complex formation in the initial phase of NO synthesis.

During steady state NO synthesis, a significant proportion of nNOS builds up as a ferrous heme-NO complex, and oxidation of this complex to ferric enzyme is one of three kinetic parameters that together determine the release rate of NO (48). We therefore investigated whether the N-terminal deletions would affect the rate of ferrous heme-NO oxidation. All three nNOSoxy enzymes formed stable six-coordinate ferrous heme-NO complexes in the presence of Arg and H4B (data not shown). Their rates of oxidation to form ferric enzyme were similar (Table IV). This suggests that the N-terminal deletions do not alter the oxygen reactivity of the nNOS ferrous heme-NO complex.

**Single Turnover Reaction Kinetics**—To investigate whether the N-terminal deletions affect any of the defined biosynthetic steps that occur during a single catalytic turnover by nNOS.
enzymes with NADPH under anaerobic conditions in the stopped flow spectrophotometer. Heme reduction (\( \text{B} \)) was followed by CO binding at 10 \( ^\circ \)C and were initiated by rapidly mixing an oxygen-containing buffer with a prereduced ferrous solution. Values in parenthesis indicate the rate of ferric enzyme formation as determined at a separate wavelength in the same oxidation reactions. Details are described under "Materials and Methods." The values are representative of eight separate reactions for each experiment.

WT, wild-type.

**Fig. 5.** Kinetics of heme and flavin reduction in the nNOS enzymes at 10 \( ^\circ \)C. Reactions were initiated by mixing CaM-bound enzymes with NADPH under anaerobic conditions in the stopped flow spectrophotometer. Heme reduction (A) was followed by CO binding at 444 nm, whereas flavin reduction (B) was followed at 485 nm. Traces shown are the average of 7–10 individual scans.

Analysis of rapid scan data collected during the NOHA single turnover reactions discerned formation of the initial ferrous-dioxy intermediate, an initial ferric heme-NO product complex, and the ending ferri enzyme for all three nNOSoxy proteins (Fig. 6). Each spectral species had characteristic absorbance bands that have been previously observed for wild-type nNOSoxy (41). The three nNOSoxy proteins had similar rates for each of the three catalytic transitions that are observed during the NOHA reaction (Table V). This indicated that the N-terminal deletions do not impact the kinetics of NOHA oxidation or subsequent dissociation of NO from the ferric enzyme.

**Heme Midpoint Potential**—We also examined whether the N-terminal deletions changed the nNOS ferric heme midpoint potential. Fig. 7, *upper left panel*, shows light absorbance spectra collected during electrochemical titration of \( \Delta 296n\text{NOS} \). Absorbance change at 400 and 645 nm was used to calculate reduction potential, because at those wavelengths the spectral change due to the added mediator dyes is negligible. The *lower left panel* shows the Nernst plot for the absorbance data at 645 nm for \( \Delta 296n\text{NOS} \). The slope obtained was 0.046 (for 0.059), and the midpoint potential was calculated to be \(-260 \pm 5\) mV. The *upper right panel* of Fig. 7 contains spectra collected during titration of \( \Delta 349n\text{NOS} \), whereas the *lower right panel* shows the Nernst plot of the absorbance data collected at 650 nm. The calculated heme midpoint potential for \( \Delta 349n\text{NOS} \) was \(-273 \pm 5\) mV, with a slope of 0.062. These midpoint values are comparable with a wild type heme midpoint potential of \(-257 mV \pm 3 mV \).

**DISCUSSION**

The normal dimer content, urea resistance, and catalytic profile of our \( \Delta 296n\text{NOS} \) enzymes establish that the N-terminal leader sequence has practically no role in dimer stability or enzyme catalysis. Our results with \( \Delta 296n\text{NOS} \) confirm and extend previous work that had demonstrated that mouse nNOS lacking the PDZ domain (nNOS\( \delta \)), which is analogous to a \( \Delta 241n\text{NOSFL} \), retained about 80% of the wild-type nNOS activity when transfected into COS cells, with the activity being fully dependent on Ca\(^{2+}\)/CaM and displaying a normal \( K_m \) for Arg (27). It is now clear that except for providing binding sites for protein inhibitor or stimulator molecules like PIN (protein inhibitor of nNOS), the NMDA receptor, or CAPON (carboxyl terminal PDZ ligand of nNOS) (50–52), the nNOS leader sequence is not meant to discharge any structural or catalytic functions to the enzyme. Rather, its role is to direct nNOS toward various protein-protein interactions that help regulate NO production and signal transduction (53). In neuronal tissues, for example, nNOS enzymatic activity is dependent on calcium influx through NMDA receptors, and this may require
the nNOS to be attached to the NMDA receptor through the PDZ/GLGF domain located in the leader sequence (54). This localization might also be important for nitration of the NMDA receptor and its functional consequences (55). In skeletal muscles, nNOS is similarly localized through its PDZ/GLGF interaction with syntrophin, which in turn associates nNOS with the dystrophin complex that is anchored to the sarcolemma (27, 56). Variants like nNOS/H9252 or our/H9004296nNOS would thus lack proper localization for a precise regulation or delivery of NO in skeletal muscle tissues (27).

The N-terminal core elements of NOS consist of the N-terminal hook, a CXXXC Zn$^{2+}$ binding motif, and a segment of residues that interact with the dihydroxypropyl side chain of H4B (Fig. 1). Deleting the three elements in mouse iNOS led to a completely monomeric, thiol-ligated heme protein that was catalytically inactive and unable to bind Arg or H4B (16, 29, 30). A similar deletion in bovine eNOS (analogous to the first 115 amino acids in iNOS) generated an enzyme that was predominantly monomeric and retained little catalytic activity (16, 29, 30). In contrast, our/H9004349nNOSFL and /H9004349nNOSoxy proteins were 56 and 83% dimeric and maintained substantial catalytic activity. Thus, among the three mammalian NOS, only nNOS withstands such an extensive deletion.

Our previous work showed that dimer interaction strengths.

---

### TABLE V

**Kinetics of heme transitions during Arg and NOHA single turnover reactions**

Anaerobic ferrous nNOSoxy proteins that contained Arg or NOHA in the presence or absence of H4B were rapidly mixed at 10 °C in the stopped flow spectrophotometer with oxygen-saturated buffer to start the reactions. Heme transitions were examined by rapid scanning spectroscopy. Values are means ± S.D. for between 8 and 15 individual reactions per experiment. $k_1$, $k_2$, $k_2'$, and $k_3$ are the conversion rates for the transitions indicated in Reactions 1 and 2, the single turnover reactions of Arg and NOHA, respectively. WT, wild-type.

| Reaction | Heme-dioxygen buildup ($k_1$) | Heme-dioxygen disappearance ($k_2$, $k_2'$) | Ferric heme-NO decay ($k_3$) |
|----------|-------------------------------|---------------------------------------------|-------------------------------|
| WT nNOSoxy + Arg + H4B | 138 ± 8.2 | 18 ± 0.8 | 5 ± 0.6 |
| WT nNOSoxy + Arg | 121 ± 6.4 | 0.3 ± 0.05 | |
| WT nNOSoxy + NOHA + H4B | 129 ± 4.7 | 13 ± 0.6 | 26 ± 0.7 | 5 ± 0.3 |
| Δ296nNOSoxy + Arg + H4B | 118 ± 5.8 | 0.4 ± 0.02 | |
| Δ296nNOSoxy + Arg | 106 ± 8.3 | 8.2 ± 0.4 | 23 ± 0.4 | 4.4 ± 0.2 |
| Δ296nNOSoxy + NOHA + H4B | 146 ± 3.1 | 0.1 ± 0.02 | |
| Δ349nNOSoxy + Arg + H4B | 122 ± 5.5 | 7.2 ± 0.02 | |
| Δ349nNOSoxy + Arg | 93 ± 6.7 | 90 ± 7.2 | |
| Δ349nNOSoxy + NOHA + H4B | 3.1 ± 29 | 0.7 ± 5 | 0.3 |

**Fig. 6.** Light absorption spectra of four heme species identified during NOHA single turnover reactions (left panels) and their changes in concentration versus time (right panels). Anaerobic solutions of ferrous NOSoxy proteins (Δ296 and Δ349) containing NOHA (2 mM) and H4B (20 μM) were mixed with air-saturated buffer at 10 °C in the stopped flow instrument, and diode array spectra were collected. Traces in each panel are the averages from 8–10 reactions each.

---

The N-terminal Elements Control nNOS Structure and Catalysis
follow a rank order of eNOS > nNOS > iNOS (43). However, after deletion of the three core N-terminal elements, the rank order changes to nNOS > eNOS > iNOS. Thus, although the three core elements still help to stabilize the nNOS dimer (see Figs. 2 and 3), their contributions are less critical for nNOS than for iNOS or eNOS. This implies that nNOS depends more on interactions within the remaining dimer interface. The mechanisms by which N-terminal core elements may stabilize NOS dimers include exchange of N-terminal hooks between the two oxygenase domains in the dimer (29, 32) and Zn$^{2+}$ ligation of four cysteines located at the dimer interface (17, 32). Curiously, mutation of two conserved residues in the N-terminal hook of nNOS (Asp-314 and Thr-315) decreased dimer stability and catalysis to a greater extent than did our Δ349 deletion (57). Although surprising, this relationship is consistent with our finding that analogous point mutations in the iNOS N-terminal hook antagonize heterodimer formation to a greater extent than does complete deletion of the N-terminal core elements (16, 29, 30). Although Zn$^{2+}$ binding has been shown to help stabilize the nNOS dimer against dissociation in polyacrylamide gels (58), our results clearly indicate that an intact Zn$^{2+}$ (Cys)$_4$ complex is not required for formation of significant nNOS dimerization or for its catalytic function. This conclusion is consistent with point mutagenesis studies that showed an intact Zn$^{2+}$ (Cys)$_4$ complex is not required for the dimerization or activity of nNOS or iNOS (29, 59, 60).

Our current findings help justify the existence of biologically active nNOS splice variants like human TnNOS and the mouse nNOSγ (27, 31) and also justify how NOS-like proteins present in D. radiodurans NOS or B. subtilis NOS can be dimeric and catalytically active despite their missing all three N-terminal core elements (33-35). In fact, our Δ349nNOSoxy construct is exactly analogous to D. radiodurans NOS (Fig. 1). The D. radiodurans NOS and B. subtilis NOS enzymes were shown to produce either NO or nitrite when combined with a mammalian NOS subunit in a heterodimer, when supported by a free mammalian NOS reductase domain (33), or in a single turnover reaction using NOHA as substrate (34). Together, such evidence provides new perspective on the selective importance of the N-terminal core elements in NOS dimerization and function.

The NO synthesis activity of the Δ349nNOSFL dimer was only 45% compared with wild type enzyme. There are three key kinetic parameters that together determine the NO release rate from a given NOS during its steady state catalysis (48). These are the rate of ferric heme reduction, the rate of ferrous heme-NO complex dissociation, and the rate of ferrous heme-NO oxidation. We found that the rate of ferric heme reduction was slower in Δ349nNOSFL compared with wild type, whereas the two other key kinetic parameters were only slightly altered. A slowing of nNOS ferric heme reduction has been accomplished using CaM mutants (37, 61). In that circumstance, a slower rate of ferric heme reduction in nNOS was associated with a proportionally slower NO synthesis in the steady state. Computer simulation using a kinetic model that describes nNOS catalysis also predicted this same result (62). We therefore can conclude that the slower NO synthesis by the Δ349nNOSFL dimer is primarily due to its slower rate of ferric heme reduction, as measured directly in our stopped flow experiments.

How might the Δ349 deletion slow down heme reduction in nNOS? One mechanism could involve changing the thermodynamic driving force for heme reduction, as occurs in Trp$^{109}$ point mutants of nNOS (63, 64). However, we know this is not the case for Δ349nNOS, because its heme midpoint potential is similar to that of wild type. Alternatively, the N-terminal core elements may help to properly align or gate the FMN module in its contacting the oxygenase domain for ferric heme reduction. This role would be consistent with NOSoxy crystal structures that show the deleted N-terminal elements are adjacent to a region on the oxygenase domain surface that is predicted to help dock the FMN module for electron transfer to the heme (32, 35). Our previous work with iNOS N-terminal deletion mutants showed that their NO synthesis is also slower than wild type when tested in a heterodimer system (29). This possibly reflects a slower heme reduction, but further study will be required.

Why does the Δ349 deletion not affect rates of nNOS ferric heme-NO dissociation or ferrous heme-NO oxidation? In the former case, crystal structures show that the deleted N-terminal elements are located near the open end of the active site funnel (32) and so are probably too far away from the heme pocket to influence heme-NO dissociation. In the latter case, a lack of effect is consistent with this deletion not altering the...
nNOS heme midpoint potential, which otherwise affects the rate of ferrous heme-NO oxidation,\(^2\) and with its not causing any discernible change in the heme electronic environment.

Together, our analysis of the three key kinetic parameters predicts that Δ296nNOSFL and Δ349nNOSFL enzyme molecules will distribute themselves during steady state NO synthesis in much the same way as does wild type nNOS (i.e. the predominant enzyme species will be the ferrous heme-NO complex) (48). This means that both deletion mutants should preserve the high apparent \(K_{m\text{,Arg}}\) that is characteristic of wild type enzyme (\(K_{m\text{,Arg}}\) of \(\sim 0.4\) mM) (65). Thus, biological expression of such N-terminal splice variants is not likely to affect a nNOS with a modified physiologic oxygen response.

In addition to its poor dimerization and slower NO synthesis, two features that distinguish Δ349 nNOSFL are its less coupled NADPH oxidation and NO synthesis and a 10-fold poorer Arg binding affinity. The uncoupled NADPH oxidation may be biologically important, because the active enzyme will produce reduced oxygen species along with NO. Different factors may contribute to the greater uncoupling in Δ349 nNOSFL. For example, it could be due to problems with subdomain alignment as discussed above in the context of ferric heme reduction. Another causative factor may be the slower reaction of the ferrous-dioxy intermediate during Arg hydroxylation. The speed of this conversion is directly linked to the efficiency of Arg hydroxylation, and slowing this step is one way to uncouple NADPH oxidation and NO synthesis (66). Because the rate of ferrous-dioxy conversion is determined by the rate of H4B electron transfer in NO (66), our results imply that H4B radical formation might be slower in Δ349nNOSFL. Interestingly, conversion of the ferrous-dioxy species is also slower in \(D.\ radiodurans\) NOS and \(B.\ subtilis\) NOS (33, 34), which are also missing the N-terminal core elements. How the N-terminal core elements influence the rate of ferrous-dioxy conversion is an important structure-function question that will require further study.

Regarding the poorer Arg binding affinity of Δ349nNOS, the same effect was observed for N-terminal deletion mutants of eNOS (30) and for a NOS point mutant C331A, whose \(Zn^{2+}\) binding is selectively disrupted (67). However, poor Arg binding was not observed for iNOS point mutant C109A, whose \(Zn^{2+}\) binding is also selectively disrupted in the same way (29). Arg binding affinity is also normal in \(D.\ radiodurans\) NOS and \(B.\ subtilis\) NOS (33, 34) despite their missing the three N-terminal core elements. Thus, the influence of N-terminal core elements on Arg binding in NOS is isoform-specific. The poorer Arg affinity in Δ349nNOS is probably biologically significant, because it would increase by a factor of 10 the intracellular Arg concentration required to maintain saturation binding for coupled NO synthesis.

It is also noteworthy that Δ349nNOSFL displayed little change in its \(K_{m}\) for H4B. This same behavior was observed in the presence of Arg for an nNOS mutant with a disrupted \(Zn^{2+}\) binding site (67) and for N-terminal deletion mutants of eNOS (30). Similarly, point mutation of core N-terminal residues that interact with the dihydroxypropyl side chain of H4B had no significant effect on H4B affinity of iNOSoxy (29). Recently, \(B.\ subtilis\) NOS was also shown to have normal H4B affinity despite its missing the N-terminal core elements (34). On the other hand, an iNOSoxy whose \(Zn^{2+}\) center was disrupted by point mutations had about a 7-fold poorer affinity toward H4B (29), and differences observed in crystal structures of \(Zn^{2+}\)-free and \(Zn^{2+}\)-bound iNOSoxy dimer led to the conclusion that the \(Zn^{2+}\) center should strongly influence the energetics of H4B binding in iNOS (68). Taken together, these data argue that N-terminal core elements play relatively minor roles in regulating H4B binding to nNOS, eNOS, and possibly even iNOS. Indeed, key residues that surround the H4B ring and position it near the heme are conserved in all bacterial NOS-like proteins (35, 69) and remain present in all of the N-terminal deletion mutants discussed above. Individual mutations of these residues typically does lower NOS affinity toward H4B (29). A concept that does emerge from the NOS mutational studies is that maintaining a dimeric structure correlates well with maintaining a capacity to bind H4B. On the other hand, there is no such correlation between maintaining dimeric structure and a capacity to bind Arg (22, 39, 70). This may reflect that the H4B binding sites are integrated into the NOS dimer interface, whereas the Arg binding sites are not (15, 17).

Conclusions and Perspectives—Our study establishes that nNOS differs from other mammalian NOSs regarding its dependence on N-terminal structure elements, and this helps to explain why nNOS is the only isoform that naturally exists as active N-terminally truncated splice variants. The biochemical data are entirely consistent with the N-terminal leader sequence and a downstream trio of core elements (N-terminal hook, the \(Zn^{2+}\) binding motif, and residues binding the H4B dihydroxypropyl side chain) representing two distinct subdomains within nNOS (18, 71). Genetic deletion of the leader subdomain permits a distinct cellular localization of native nNOS activity, whereas an additional genetic deletion of the core element subdomain permits distinct localization of an nNOS with significantly altered structural and catalytic properties that predispose it to generate reduced oxygen species along with NO. These features, together with distinct patterns of tissue expression, may enable nNOS to impact a wider range of physiological and pathophysiological processes including neurotransmission, skeletal muscle contraction, sexual function, fluid homeostasis, addiction, atherosclerosis, and neurodegeneration (1–6). From an evolutionary point of view, it is intriguing that Δ349nNOSoxy (and presumably TnNOS and nNOS\(y\)) display striking similarities with the bacterial NOS-like proteins with respect to having a significant dimer content and catalytic activity. This suggests that among the three NOS isoforms, only nNOS has maintained these characteristics in common with primitive NOS-like proteins and probably uses them to distinct advantage in higher organisms.

REFERENCES

1. Dawson, V. L., and Dawson, T. M. (1998) Prog. Brain Res. 118, 215–229
2. Harrison, D. G. (1997) J. Clin. Invest. 100, 2153–2157
3. MacMicking, J., Xie, Q. W., and Nathan, C. (1997) Annu. Rev. Immunol. 15, 323–350
4. Huang, Z., Huang, P. L., Panahian, N., Balkara, T., Fishman, M. C., and Moskowitz, M. A. (1994) Science 265, 1883–1885
5. Gross, S. S., and Wolin, M. S. (1995) Annu. Rev. Physiol. 57, 737–769
6. Michel, T., and Piron, O. (1997) J. Clin. Invest. 100, 2146–2152
7. Griffith, O. W., and Stuehr, D. J. (1995) Annu. Rev. Physiol. 57, 707–736
8. Mayer, B., and Hemmens, B. (1997) Trends Biochem. Sci. 22, 471–478
9. Dinerman, J., Lowenstein, C. J., and Snyder, S. H. (1993) Circ. Res. 73, 212–222
10. Hemmens, B., and Mayer, B. (1988) Methods Mol. Biol. 100, 1–32
11. Stuehr, D. J. (1999) Biochem. Biophys. Acta 1411, 217–230
12. Baek, K. J., Thié, B. A., Lucas, S., and Stuehr, D. J. (1999) J. Biol. Chem. 268, 21210–21219
13. Klett, P., Pfeiffer, S., List, B. M., Lehner, D., Glatter, O., Bachinger, H. P., Werner, E. R., Schmidt, K., and Mayer, B. (1996) J. Biol. Chem. 271, 7326–7342
14. Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) J. Biol. Chem. 271, 11462–11467
15. 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
16. Ghosh, D. K., Wu, C., Pitters, E., Moloney, M., Werner, E. R., Mayer, B., and Stuehr, D. J. (1997) Biochemistry 36, 10609–10619
17. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998) Cell 95, 939–950
18. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Nurula, S. R., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
19. Bender, A. T., Nakatsuika, M., and Osawa, Y. (2000) J. Biol. Chem. 275,
