Control of Electron Transfer in Nitric-oxide Synthases

SWAPPING OF AUTOINHIBITORY ELEMENTS AMONG NITRIC-OXIDE SYNTHASE ISOFORMS

To clarify the role of the autoinhibitory insert in the endothelial (eNOS) and neuronal (nNOS) nitric-oxide synthases, the insert was excised from nNOS and chimeras with its reductase domain; the eNOS and nNOS inserts were swapped and put into the normally insertless inducible (iNOS) isoform and chimeras with the iNOS reductase domain; and an RRKKR sequence in the insert suggested by earlier peptide studies to be important (Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777) was mutated. Insertless nNOS required calmodulin (CaM) for normal NOS activity, but the Ca\(^{2+}\) requirement for this activity was relaxed. Furthermore, insert deletion enhanced CaM-free electron transfer within nNOS and chimeras with the nNOS reductase, emphasizing the involvement of the insert in modulating electron transfer. Swapping the nNOS and eNOS inserts gave proteins with normal NOS activities, and the nNOS insert acted normally in raising the Ca\(^{2+}\) dependence when placed in eNOS. Insertion of the nNOS insert into iNOS and chimeras with the iNOS reductase domain significantly lowered NOS activity, consistent with inhibition of electron transfer by the insert. Mutation of the nNOS RRKKR to an AAAAA sequence did not alter the eNOS Ca\(^{2+}\) dependence but marginally inhibited electron transfer. The salt dependence suggests that the insert modulates electron transfer within the reductase domain prior to the heme/reductase interface. The results clarify the role of the reductase insert in modulating the Ca\(^{2+}\) requirement, electron transfer rate, and overall activity of nNOS and eNOS.

The three major isoforms of nitric-oxide synthases differ biochemically and biologically in multiple ways (1–9). One of these differences, a dependence of activity on reversible calmodulin (CaM) binding, distinguishes the constitutive neuronal (nNOS) (10) and endothelial (eNOS) (11) isoforms from the inducible (iNOS) isoform (12). A second significant difference is provided by the level of catalytic activity, in that eNOS has a relatively low activity of ~30 nmol of NO/min/nmol of NOS, whereas nNOS and iNOS have higher activities of ~100 and 150 nmol of NO/min/nmol, respectively.

The differences in catalytic activity derive from the relative abilities of the reductase domains to transfer electrons to the heme, a process strictly controlled by CaM activation in the wild-type enzymes (13). A peptide insert of about 50 amino acids in nNOS and eNOS but not iNOS is evident upon alignment and comparison of the sequences of the three isoforms (14). We have shown in earlier work that this insert helps to lower the rates of electron transfer in eNOS in both the CaM-bound and -unbound states (15). Thus, removal of the insert from wild-type eNOS or from NOS chimeras in which the native flavin domain of nNOS has been replaced by the eNOS flavin domain leads to proteins that are hyperactive in both overall NO synthesizing activity and in electron transfer from the CaM-bound or -unbound protein to electron acceptors such as cytochrome c and ferricyanide.

Conflicting results have been obtained concerning the effect of the autoinhibitory domain on the CaM dependence of activity. In accord with our earlier results on eNOS, Guillemette and co-workers (16) found that nNOS mutants in which the peptide insert was deleted still required CaM for NOS activity. In contrast, Shimizu and co-workers (17) reported that removal of the nNOS insert yielded a protein that retained 25% of its activity in the absence of CaM, although this activity was <10% that of wild-type nNOS.

We sought to clarify the role of the nNOS insert by excising it from both wild-type nNOS and NOS chimeras in which the reductase domain of other NOS isoforms was replaced by the NOS reductase domain. The chimera mutants provide a larger sampling of data points and make it possible to determine the effects of the nNOS insert in various protein contexts. This diversity of NOS mutants was further increased by creating NOS proteins that varied solely in the nature of the insert in the reductase domain. In these latter variants, the eNOS and nNOS inserts in the parent proteins were exchanged, and the eNOS insert was added to insertless NOS proteins bearing the iNOS reductase domain, including both wild-type iNOS and a chimera in which the reductase domain of eNOS had been replaced by that from iNOS. Finally, we sought to determine whether a highly basic region in the insert was the primary molecular determinant of the autoinhibitory properties of the eNOS insert, as proposed by Masters and co-workers (14) based on peptide inhibition studies.

We have found that the nNOS insert acts similarly to the eNOS insert in raising the Ca\(^{2+}\) requirement for activity. When placed in the context of eNOS, the nNOS insert can substitute for the native one in this role as well as in maintaining the lower eNOS activity. The eNOS insert, however, does not exert the same full effects in nNOS as it does in its native context.

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The abbreviations used are: CaM, Ca\(^{2+}\)-dependent calmodulin; nNOS/eNOS/iNOS, neuronal/endothelial/inducible nitric-oxide synthases; E/NΔ, chimeras of the heme- and CaM-binding domains of eNOS with the nNOS reductase domain mutated to lack the autoinhibitory insert; E/I(E\(_{\text{insert}}\)), chimera E/I in which the autoinhibitory insert of eNOS was inserted; PCR, polymerase chain reaction.
Thus, autoinhibition of Ca\(^{2+}\) activation is decreased, thereby lowering the Ca\(^{2+}\) requirement; and activity levels are only partially lowered by the presence of the eNOS insert in nNOS. Finally, our results show that the five consecutive basic residues RRKRK implicated by the peptide studies as key features of the eNOS autoinhibitory insert are not actually required, as an insert with all these residues replaced by alanines is still able to fully autoinhibit CaM activation and to fully depress the activity level. The results are discussed in the context of possible structural requirements for the insert.

**EXPERIMENTAL PROCEDURES**

**Materials**

HEPES and agarose were from Fisher. DNA manipulations were done using enzymes, buffers, and reagents from New England Biolabs and purification kits from Qiagen, which also supplied the nickel-nitrotriacetic acid resin. Oligonucleotide primers were synthesized, and the DNA sequenced, by the Biomedical Resource Center, University of California, San Francisco. All other materials were from Sigma.

**General PCR Cycling**

A Progene thermocycler from Techne (Cambridge, UK) was employed. Mutagenesis was accomplished via standard overlap extension PCR techniques, utilizing as the template previously constructed NOS chimeras (18) that possessed engineered Nhel splice sites at amino acids 760–761, 538–539, and 527–528 in nNOS, iNOS, and eNOS, respectively. 5` end primers annealed at the Nhel splice site or at the Nhel splice site, whereas 3` end primers annealed after preexisting unique sites (AatII in nNOS, BsrGI in iNOS, and KpnI in eNOS).

Unless otherwise noted, the standard PCR cycling parameters were 25 cycles of 1 min each at temperature (94 °C melting, 60 °C annealing, and 72 °C extension) followed by a 10-min extension, using −0.2 ng/μl template, 0.5 μM primers, 400 μM dNTPs, and 0.02 units/μl Vent polymerase that possesses 3` to 5` proofreading activity. For extension of two overlapping PCR products, 5 cycles were performed without the appropriate end primers, followed by addition of primers and 20 cycles of amplification, and the final 10-min 72 °C extension. For syntheses of overlapping PCR products from megaprimers, a pair of primers (megaprimer and corresponding paired primer) were usually sufficient to give the desired amplification, except in the case of E/I/E insert, in which three primers were necessary (the megaprimer, its paired primer, and the same-strand primer which produced the megaprimer). The cycling parameters in this case were the same as for overlap extension reactions.

**Construction of nNOS Insert Deletion Mutants**

**pCWori/I/N**—The Nhel site and another unique site (AatII) downstream of the insert were used to subclone the insert-deleted gene fragment. Specifically, primers 1 and 2 (5`-TATATACTAG TCCATTAC- TAC TACTACCAGA and GGGTGCAGT ACTCTCCATTA AAGCAC- AGCC GAATTTCTCCC CG) and template pCWori/I/N were used to produce fragment A (25 standard cycles, except the first 5 cycles were at 55 °C annealing). Primers 3 and 4 (AGTACTGGCC CCGTGCACC CTTGAGA and TAAATCTGCA TTAAAGAGTC AAAAAT) were used to produce fragment B using identical cycling parameters. Equimolar amounts (1 nM) of fragments A and B, plus 500-fold excess primers 1 and 4 and the same cycling parameters produced product AB, which was subcloned via Nhel and AatII into chimera pCWori/I/N.Δ, yielding deletion chimera pCWori/I/N.Δ.

**pCWori/nNOSΔ**—Subcloned from pCWori/I/NΔ into pCWori/I/N.Δ, the Ndel/Nhel nNOS gene fragment corresponding to the heme- and CuA domains of nNOS replaced that of iNOS, yielding pCWori/ N/N.Δ, hereafter referred to as “nNOSΔ,” which differs from the wild-type insert deletion mutant by a T761S mutation resulting from introduction of the Nhel chimeric splice site. As serine, the native amino acid in eNOS, is highly homologous and represents a conservative mutation, we believe that this mutant is indistinguishable from the exact nNOSΔ insert deletion mutant.

**pCWori/E/NΔ**—This mutant was produced by subcloning of the Ndel-Nhel eNOS heme domain gene fragment from pCWori/E/N (18) into pCWori/I/N.Δ.

**Construction of Insert-swapped Mutants**—These mutants were constructed in the same way as the insert deletion mutants, except that the primers analogous to primers 2 and 3 above were “megaprimers” made via PCR amplification and were composed of the entire insert sequence plus small flanking regions corresponding to 6 and 3 amino acids at the 5` and 3` termini, respectively. The megaprimers, which were synthesized using primers 5 and 6 (GGTTGTTGACC AGACATTGTT GAGTAGCC) plus template cDNA nNOS or eNOS, anneal to sequences adjacent to the insert sequence that are virtually identical in nucleotide sequence (identical in protein sequence) between eNOS and nNOS isoforms.

**pCWori/nNOS(Einsert)—**Amplification of template pCWori/I/N using megaprimers (Einsert) and primer 7 (TATATACTAG TCCATTAC TAC TACTACCAGA) produced fragment C, whereas megaprimer and primer 8 (TAAATCTGCA TTAAAGAGTCC AAAAATCTG) produced fragment D. Overlap extension produced product CD, which was subcloned into pCWori/nNOS via the Nhel and AatII unique sites.

**pCWori/eNOS(Ninsert)—**Amplification of template pCWori/E using megaprimers (Ninsert) and primer 9 (TATATACTAG TCCATTAC TAC TACTACCAGA) produced fragment E, whereas megaprimer and primer 9 (CCCCGGAGGT CAGGAC) produced fragment F. Overlap extension gave product EF, which was subcloned into pCWori/E/EA (15) via the Nhel and KpnI unique sites.

**pCWori/E/I/E(Einsert)—**The sequence designated the eNOS insert sequence (55 amino acids 593MEMSG... ALGTL) was inserted into iNOS, replacing the analogous 10-amino acid sequence, 604FMLRELHFT. Megaprimer(eNOS) was synthesized from template pBlueScript/eNOS cDNA and primers 10 (GGCAGACTCT GAAGAAATCT CTGATGGAGA TTGGCGGCGG) and 11 (CAAGCGCAAA CAGCACTAT CGATGGGG CCGAGG) and 11 (CAAGCGCAAA CAGCACTAT CGATGGGG CCGAGG). The “outer” ends of the resulting megaprimer were identical to the iNOS sequences “outside” the insert, whereas the interior of the megaprimer was identical to the eNOS insert (Fig. 1).

Amplification of template pCWori/E/I using megaprimer(Einsert) and primers 11 and 12 (CCAGCAGCTT GGAAACTAG TCGCAGGCAG GGGCG) produced fragment G, while megaprimer and primers 10 and 12 (CGGACACCTT GGAAACTAG TCGCAGGCAG GGGCG) produced fragment H. Overlap extension gave product GH, which was subcloned into pCWori/E/I via the Nhel and BglII unique sites.

**pCWori/1/iNOS(Einsert)—**Replacement of the Ndel/Nhel eNOS heme domain gene fragment with that of iNOS resulted in pCWori/I/E(I/Einsert), hereafter referred to as “iNOS(Einsert),” which has a conservative T538S mutation due to the Nhel splice site.

**Construction of 5Ala Mutant eNOS**

RRKK extended in the bovine eNOS insert was mutated to five consecutive alanine residues. Mutagenesis was accomplished by standard inverse extension PCR mutagenesis using mutagenesis primers 13 (GGCGCCGACG GGGCGCAGAC CGCGACACA CAGACAC, forward) and 14 (CTGGGCGGCG TGCGGCGCGCAG GAGGAGCCA CCGG, reverse), end primers 12 and 9, and template pHis/E/E.

**Protein Expression and Purification**

Conditions were as reported previously (19, 20). Proteins were purified to >95% purity (as judged by SDS-polyacrylamide gel electrophoresis) via affinity chromatography on nickel-nitrotriacetic acid-agarose and 2,3′-ADP agarose.

**RESULTS**

To address the goals of this study, we constructed a series of mutant proteins by deleting or swapping the insert in the wild-type NOS isoforms as well as in chimeras derived from them. The constructs are shown schematically in Fig. 2.
nNOS Insert Deletion Proteins

Protein Expression and Purification—All the expressed and purified proteins exhibited similar stabilities and yields (∼2–4 mg/liter). I/N, like native iNOS, required coexpression with CaM to prevent significant proteolysis (19, 21, 22), presumably due to a folding or protection requirement for CaM. Therefore, I/NΔ and iNOS(Einsert) also were expressed in the presence of CaM. None of the other proteins required CaM for expression and purification.

Deletion of the eNOS autoinhibitory sequence was shown to affect the Ca2⁺ dependence of eNOS, decreasing the free Ca2⁺ concentration required for the expression of NOS activity in the presence of CaM (15, 23). The same eNOS deletion also resulted in enhanced NO production as well as enhanced electron transfer from both CaM-free and CaM-bound eNOS to external oxidants such as ferric cytochrome c and ferricyanide (15). The rate enhancements were seen not only upon deletion of the insert from wild-type eNOS, but also upon its deletion from the chimeric NOS proteins N/E and I/E, which were constructed by replacing the reductase domains of nNOS and iNOS, respectively, with that of eNOS.

Ca2⁺ Dependence of Activity—We likewise examined the effect of nNOS deletions on the Ca2⁺ requirement for NOS activity. In the presence of a 4-fold excess of CaM, the nNOS mutant lacking the insert, nNOSΔ, exhibited a lower Ca2⁺ requirement compared with parent nNOS (Fig. 3A, compare ○ to ●), as was seen for the eNOSΔ analog (Fig. 3A, compare □ to ■), and an approximate 10-fold lowering of the apparent EC50 for Ca2⁺ from 300 to 20 nM. Unexpectedly, E/N, a chimeric protein with eNOS oxygenase and CaM-binding domains but an nNOS reductase domain, similarly required less Ca2⁺ for NOS activity (EC50 ~70 nM) despite the fact that it possesses the autoinhibitory insert. The corresponding deletion E/NΔ did not exhibit a lower EC50 for Ca2⁺ (Fig. 3B, compare △ to ●).

The alternative approach is to prepare the I/N chimeras in which the insertless iNOS reductase is replaced by the insert-bearing nNOS reductase domain. We reported previously (18) that this replacement led to an altered Ca2⁺ dependence that was intermediate between that of the wild-type constitutive isoforms and iNOS, for which coexpression with CaM was required to obtain full-length, active protein. Approximately 50% activity was retained in 2.5 mM EGTA, compared with full activity for iNOS (Fig. 3B, compare ● to ×). We were interested in whether this disruption in the Ca2⁺ independence of iNOS could be explained by the introduction of the eNOS or nNOS insert which accompanied swapping of the reductase domains. Deletion of this insert produced I/NΔ whose Ca2⁺ dependence was nearly identical to that of I/N (Fig. 3B). Thus, the deletion did not produce the defining iNOS property of retaining NOS activity even at very low (<0.1 mM) free Ca2⁺ concentrations.

NO Synthesis and Cytochrome c Reduction—Deletion of the eNOS insert yielded an enzyme with enhanced NOS and reductase activities (15). Thus, the insert appears to be at least partially responsible for attenuating the reductase, and hence overall, activity of eNOS, both of which are intrinsically lower than those of nNOS and iNOS.

The nNOS reductase alone (24) and full-length nNOS (10, 25–31) typically exhibit 2–5 times higher activities than eNOS (31–42). Therefore, whereas the insert may help to lower activities in eNOS, it must not do so in nNOS. To confirm this, we compared the activities of proteins bearing the insert-deleted nNOS reductase, i.e. we compared the nNOSΔ, E/NΔ, and I/NΔ deletion mutants with wild-type nNOS and the E/N and I/N chimeras.

As reported by us and by others (15, 43, 44), the activity of both NOS and NADPH-cytochrome P450 reductase is significantly influenced by the ionic strength of the assay solution. For an increase in ionic strength from 25 to 125 mM, nNOS and eNOS show decreases in overall activity of 10 and 30%, respectively, whereas iNOS shows a decrease of 20% (see Fig. 4). Because activities vary with the NOS isoform, and for other reasons soon to become apparent, assays were done both as typically reported in the literature but also in the presence of 100 mM KCl. The salt enhancement is nearly maximal with this KCl content (43).

Overall Activity—Removing the insert from eNOS resulted in eNOSΔ, an enzyme that exhibited a roughly 2-fold increase...
in overall NO synthesis (15). The N/E and I/E chimeras also exhibited higher activities upon deletion of the insert to give the N/EΔ and I/EΔ insert-deleted chimeras.

Similar removal of the insert from nNOS gave nNOSΔ, which in the KCl-free assay had an activity approximately half that of wild-type nNOS (Fig. 4, 36 ± 2 versus 75 ± 2 min⁻¹), a decrease not unlike that reported by Shimizu and co-workers (17) (30%) and Guillemette and co-workers (16) (20%). However, we found virtually no difference in nNOS and nNOSΔ activities in the presence of 100 mM KCl. In fact, if anything, nNOSΔ exhibited a slightly higher activity (94 ± 2 versus 85 ± 5 min⁻¹). The same behavior was seen when the E/N and I/N chimeras bearing the nNOS reductase were compared with the corresponding insert-deleted proteins, although the “rescue” by KCl was incomplete in these cases. Nevertheless, 81 and 85% of the activity was retained with the E/NΔ and I/NΔ chimeras, respectively, in KCl.

**Cytochrome c Reduction**—For the previously constructed NOS chimeras, the overall activity reflected the activity of the reductase (18). We therefore examined the reductase activity to see if this characteristic was preserved in the deletion mutants of proteins with an nNOS reductase domain.

Whereas deletion of the insert did not significantly affect overall NOS activity in any of the three proteins with an nNOS reductase domain (Fig. 4), the CaM-bound cytochrome c reductase activity (Fig. 5) gave varied results. In the absence of KCl (Fig. 5A), nNOSΔ (72%) and E/NΔ (76%) were slightly lower than the corresponding nNOS and E/N parent proteins, whereas I/NΔ (139%) was slightly higher than I/N. When the assays were performed in the presence of KCl (Fig. 5B), the effect of insert deletion was abolished for nNOSΔ (91%) but was slightly enhanced for E/NΔ (54%) and I/NΔ (162%).

The cytochrome c reduction activity of CaM-free nNOSΔ was 4-fold greater than that of CaM-free nNOS (2300 ± 200 versus 590 ± 20 min⁻¹) in the absence of salt but was only 2.5-fold greater in 100 mM KCl (4400 ± 1600 versus 1620 ± 40 min⁻¹). When CaM was added to the assay, nNOSΔ and nNOS had similar activities, both of which were higher at higher ionic strength (Fig. 5; nNOS, 9800 ± 500 and 13,000 ± 360 min⁻¹ versus nNOSΔ, 7100 ± 1100 and 11,800 ± 600 min⁻¹). E/N and E/NΔ showed a similar trend in the CaM-free state; the deletion increased reductase activity relative to the parent (1030 ± 80 versus 360 ± 11 min⁻¹) but was increased by 36% in the CaM-free state observed with the deletion mutant at higher ionic strength.

True CaM-free rates (exogenous CaM-free assay results are shown in Fig. 5) for I/N and I/NΔ could not be measured because CaM coexpression is required to express active I/N and

![Fig. 4. Nitric-oxide synthase autoinhibitory element.](image-url)

**Fig. 4.** Nitric oxide synthesis by deletion mutant proteins lacking the neuronal isoform inserts. Rates were determined by the oxygen-hemoglobin method at 37 °C with or without the addition of 100 mM KCl and are presented as mol of product/min/mol of enzyme. Error bars are standard deviation for at least three measurements.

![Fig. 5. Cytochrome c reduction by deletion mutant proteins lacking nNOS inserts.](image-url)

**Fig. 5.** Cytochrome c reduction by deletion mutant proteins lacking nNOS inserts. Rates were measured at 37 °C with (black bars) or without (white bars) 1 μM exogenous CaM plus 100 μM CaCl₂ and with (A) or without (B) 100 mM KCl and are presented as mol of product/min/mol of enzyme. Error bars are standard deviations for at least three measurements. Gray bars indicate that the protein was coexpressed and copurified with CaM.

I/NΔ. Nevertheless, whereas removal of the insert from nNOS and the E/N chimera produced deletion mutants with lower activity than the parents proteins, removal of the insert from I/N gave I/NΔ with enhanced activity in both the presence (7900 ± 200 versus 4700 ± 100 min⁻¹) or absence (5290 ± 90 versus 3800 ± 200 min⁻¹) of KCl. This was true despite the fact that the insert-deleted proteins had a somewhat lower NO-synthesizing activity (Fig. 4).

Thus, removal of the nNOS insert from nNOSΔ and E/NΔ, the two proteins based on constitutive NOS isoforms (nNOS or E/N), resulted in a higher CaM-free rate relative to the CaM-bound rate regardless of KCl concentration, consistent with the results seen for the eNOS deletions.

**Insert-swapped Proteins**

Deletion analysis provides the opportunity to study the function of the nNOS autoinhibitory insert in the context of either native nNOS or eNOS. To extend this approach, we put the insert in the structural context of a non-native “host” NOS. By swapping the inserts of eNOS and nNOS, or by introducing the eNOS insert into iNOS or an iNOS reductase-bearing chimera, it should be possible to retain or eliminate potential “pairing” sites outside the insert and thereby implicate or rule out such external sites in the proper function of the autoinhibitory insert. This method refines our earlier approach of swapping the entire reductase domain and focuses attention on the specific interactions of the insert.

**Protein Expression and Purification**—The four “insert-swapped” proteins, eNOS(Ninsert), nNOS(Einsert), iNOS(Einsert), and E/I(Einsert), were expressed and purified identically as the parental native NOSs and chimera, producing similar yield and purity. iNOS(Einsert) was coexpressed with CaM.

**Ca²⁺ Dependence of Activity**—When the eNOS autoinhibitory insert was introduced into nNOS to give nNOS(Einsert), the
resulting protein exhibited a lower Ca\textsuperscript{2+} requirement (Fig. 6A). For this simple loop swap, EC$_{50}$(Ca\textsuperscript{2+}) dropped from 200 nM for nNOS to 50 nM. However, introducing the eNOS insert into the insertless iNOS had no significant effect. Similarly, the EC$_{50}$(Ca\textsuperscript{2+}) for the E/I(E insert) protein did not differ significantly from that of the insertless E/I. Thus, introducing the eNOS insert decreases that dependence. In fact, the insert swap exhibits an EC$_{50}$(Ca\textsuperscript{2+}) very similar to that of the eNOS from which the insert has been removed (40 versus 20 nM; compare $\triangle$ of Fig. 6A and gray $\bigcirc$ of Fig. 6A). Finally, eNOS(Ninsert), in which the eNOS insert was replaced by that of nNOS, exhibited Ca\textsuperscript{2+} requirements identical to those of nNOS. Thus, the nNOS loop thus appears to function in the context of the eNOS protein and evokes the Ca\textsuperscript{2+}-dependent behavior characteristic of eNOS and nNOS.

**Nitric Oxide Synthesis**—The insert-swapped proteins, nNOS(Einsert) and eNOS(Ninsert), had overall NOS activities similar to those of their parental protein (Fig. 7). Inclusion of 100 mM KCl increased the NOS activity by less than 40% for eNOS(Ninsert) but 70% for nNOS(Einsert). In the previous converse experiment, in which the insert was deleted from eNOS, the deletion enhanced the overall activity 2-fold (15). As shown here, inserting the eNOS loop into iNOS and the E/I chimera significantly lowers the overall activity (Fig. 8). The iNOS(Einsert) activity was 32 and 36% of iNOS activity, respectively, in the absence and presence of KCl, and the E/I(Einsert) activity was 40 and 25% of the E/I activity under the corresponding conditions.

**Cytochrome c Reduction**—In contrast to the similar NO synthesizing activities of nNOS and nNOS(Einsert) (Fig. 7), replacement of the nNOS with the eNOS insert decreases the rate of cytochrome c reduction by 50% in both the presence (Fig. 9A) and absence (Fig. 9B) of KCl. In contrast to insert deletion, the insert exchange did not alter the CaM-free cytochrome c reduction rate with respect to the CaM-bound rate (Fig. 9). Similarly, replacing the eNOS with the nNOS insert yielded eNOS(Ninsert) without significantly affecting the low eNOS rate, in agreement with the results for the NO synthesizing activity (Fig. 7).
Introducing the eNOS loop into iNOS and the E/I chimera gave the same trends for the CaM-bound cytochrome c reduction activity as observed for the overall NOS activity. The attenuating effect of the eNOS loop on cytochrome c reduction was similar for iNOS(Einsert) and E/I(Einsert) in the absence (21 and 25%, respectively) or presence (42 and 25%, respectively) of KCl (Fig. 10B). The effect on the CaM-free rates was similar.

We showed previously that the chimeric E/I and N/I proteins, both of which have the iNOS reductase domain, exhibit high reductase activity even in the absence of CaM, consistent with what was observed for the iNOS reductase expressed as a separate polypeptide without the oxygenase and CaM-binding domains (45). The CaM independence of cytochrome c reduction by E/I was retained in E/I(Einsert). Thus, whereas deletion of the eNOS insert from eNOS increased the reduction rate (Fig. 10), insertion of the eNOS loop into iNOS did not lower the high CaM-free rate. The CaM-free rate for E/I(Einsert) was identical (−KCl, Fig. 10A) or slightly higher (+KCl, Fig. 10B) than the CaM-bound rate, as observed for the E/I parent.

Finally, the effect of salt on the cytochrome c reduction rates depends on the identity of the reductase domain. The proteins with the iNOS reductase domain (iNOS, iNOS(Einsert), E/I, and E/I(Einsert)) in Fig. 10, and N/I in our previous study (18) exhibit substantially decreased activities in the presence of 100 mM KCl, whereas the proteins with eNOS and NOS(Einsert) domains (eNOS, eNOSΔ, nNOS, nNOSΔ, nNOS(Einsert), and eNOS(N-insert)), Figs. 5 and 9, and N/E and N/EΔ in our previous work (15) all show enhanced activity.

**RRKKK → AAAAA (5Ala) eNOS Mutant**

Early studies indicated that a pentapeptide based on a stretch of five basic residues within the eNOS autoinhibitory insert was an effective inhibitor of nNOS (14). However, as this eNOS-derived pentapeptide was a less effective inhibitor of eNOS itself, it was not clear whether the inhibition arose from a specific functional relationship between this basic region and CaM binding or was simply the result of a non-functionally relevant interaction. To clarify the role of the basic region, we converted the RRKKK amino acid sequence of the eNOS autoinhibitory insert into AAAAA. The Ca2+ dependence of nitric oxide synthesis by the mutant enzyme was unaffected by this gross mutation (Fig. 11). Although cytochrome c reduction was lower than for wild type (Fig. 10), the nitric oxide synthesizing activity was somewhat higher than that of wild-type eNOS (Fig. 8) but did not rise to the level seen upon removal of the entire insert.

**DISCUSSION**

The reductase domains of the nitric-oxide synthases employ their flavin prosthetic groups to provide electrons from NADPH to the P450-like heme domains. Maximal electron transfer to the heme groups depends on the binding of CaM (13), which occurs reversibly in the constitutive eNOS and nNOS isoforms. In the inducible isoform, CaM is essentially irreversibly bound, and NO synthesis occurs at negligible Ca2+ levels. Comparison of the primary sequences of the three isoforms reveals that eNOS and nNOS possess a 38–42-amino acid insert within their FMN-binding domain that is not present in either iNOS or NADPH-cytochrome P450 reductase. Thus, the presence or absence of the insert coincides, respectively, with either reversible (eNOS and nNOS) or irreversible (iNOS) CaM binding.

We demonstrated previously (15) that removal of the insert from eNOS lowered the Cu2+ requirement for the synthesis of nitric oxide from an EC50 of 150 to 20 nm. Furthermore, a decrease in the Cu2+ requirement for NO activity was also observed upon removal of the insert from chimeras in which the nNOS and iNOS heme domains were linked with the eNOS reductase domain. Somewhat to our surprise, removal of the insert also increased electron transfer to cytochrome c by more than 2-fold. Greatly increased electron transfer (>200-fold) to cytochrome c has recently been observed in C-terminal truncations of iNOS (46); however, the increased cytochrome c reducing ability was not reflected in a significantly increased ability to synthesize NO. The authors speculated that NO release may have become rate-limiting in these truncated iNOS proteins, but the fact that we and other laboratories have observed higher NO synthesis rates by the wild-type enzyme suggests that another explanation is likely. In any case, the enhanced electron transfer observed upon deletion of the insert from eNOS and chimeras bearing the eNOS reductase domain does translate into an increase in overall NO activity, leading us to conclude that the insert is at least partially responsible for the lower NO synthesizing activity of eNOS relative to iNOS and nNOS.

As a similar insert is also present in nNOS, which has a higher intrinsic NO synthesizing activity than eNOS, we examined the effect of removing the nNOS insert, both in terms of the Ca2+ requirement and its consequences for electron transfer. Two other laboratories have reported (16, 17) removal of
the insert from nNOS. In contrast to the report of Guillemette and co-workers (16), nNOS did not require coexpression with CaM and could be purified in the same way as wild-type nNOS. In addition to the discrepancy concerning the instability of the CaM-free protein, Guillemette and co-workers (16) also reported an activity for their ΔnNOS protein (8 nmol NO·min⁻¹·mmol⁻¹) that was considerably lower than that of our nNOSΔ protein (36 nmol NO·min⁻¹·mmol⁻¹). The deletion reported by Guillemette and co-workers (16) (ΔnNOS) differs from ours by only one amino acid, and it is surprising that this small difference has such a drastic effect on the stability of the CaM-free protein. We believe that the similar activity and similar stability of our CaM-free nNOSΔ and nNOS proteins reflect a preservation of the local tertiary structure near the mutation, making conclusions about the mode of action of the mutation valid.

All of our proteins required CaM for significant NO synthesis (>5% wild type), consistent with the eNOS deletions described by us previously. In contrast, Shimizu and co-workers (17) reported that deletion of 40 or 42 of the insert residues produced Δ40 and Δ42, respectively, that retained 30% of the maximum activity in the absence of Ca²⁺/CaM, the maximum activity being 22–30% that of the wild-type enzyme. Under similar salt-free conditions, Guillemette and co-workers (16) observed a decrease in the maximum activity to 20% that of the wild-type enzyme, but CaM was required for activity, and we observed a decrease to 50% of wild-type activity, again with CaM being required for NO synthesis (Fig. 4). However, the activity we observed was increased in 100 mM KCl to 111% that of the wild-type protein. Deletion of the insert from nNOS and chimeras with the nNOS reductase domain yields proteins that are more sensitive to salt than those obtained by deletion of the insert from eNOS or chimeras with the eNOS reductase domain (Fig. 4). For example, the activity of E/N without added KCl is 50% of the activity with KCl. In our previous paper, the authors pointed out, unlikely that CaM from the yeast expression system was carried through the purification procedure in the Shimizu experiments because the protein was purified in the presence of 1 mM EGTA and was also bound to CaM-Sepharose. The difference in CaM dependence between their experiments and ours is thus probably real. As we deleted Met⁸₂₈–Glu⁶₆₉ whereas Shimizu and co-workers (17) deleted Pro⁸₃¹–Ser⁸₇₀, the two mutant proteins differed by a total of only two amino acids. Nevertheless, it is possible that the structural difference inherent in these two additional amino acids enables slow leakage of electrons from the reductase to the heme in the latter protein. The much higher sensitivity of nNOSΔ than the other mutant constructs to ionic strength suggests, in fact, that nNOSΔ is structurally relatively unstable and is perhaps poised on a conformational knife edge. If so, a small difference in the amino acids that remain after the insert is deleted, or even in the purification and assay conditions, might be sufficient to trigger significant differences in the quaternary structures and properties of the mutant proteins.

Comparisons of the three wild-type isoforms and the eNOSΔ and nNOSΔ proteins without the reductase domain insert has been informative, but the comparison has been extended here to a more diverse pool of proteins to amplify and strengthen the attendant conclusions. This has been done by examining the effect of deleting the insert from chimeric NOS proteins, by swapping the inserts between the constitutive nNOS and eNOS proteins, and by introducing the eNOS insert into proteins with the insertless iNOS reductase domain.

This diversification has clarified the source of the differing salt effect on the NOS activities. In a study of salt effects on nNOS, Masters and co-workers (44) concluded that salts elicited dissociation of the autoinhibitory product NO, leading to the observed enhanced activity. In an earlier rigorous examination of the effects of many salts on all three NOS isoforms, Mayer and co-workers (43) observed a general correlation with the Hofmeister series and trends for the three wild-type isoforms similar to those found here for KCl, i.e. activation of eNOS and nNOS and inhibition of iNOS by increasing salt. Mayer and co-workers (43) attributed the salt effects to “aspecific changes in protein solvation” and ruled out specific binding, alteration in CaM binding, and dissociation of the NOS dimers. They concluded that “salts mainly affect the interdomain electron transfer, but we cannot rule out an additional effect on the preceding electron transfer steps.”

In the present study, we have shown that the nature of the salt dependence is directly linked to the identity of the reductase domain regardless of the heme- and CaM-binding domains. Thus, I/N, I/NΔ, E/N, E/NΔ, eNOS(Einsert), nNOS(I insert ), nNOS(E insert ), nNOS and nNOSΔ in this paper, and N/E, N/EΔ, I/E, I/EΔ, eNOS, and eNOSΔ in our previous paper (15), all exhibited enhanced activity with 100 mM KCl, whereas N/I, E/I, E/I(E insert ), iNOS, and iNOS(E insert ) were inhibited by 100 mM KCl. Thus, it is likely that the salts studied by Mayer and co-workers (43) alter electron transfer within the reductase domain, but irrespective of the insert, as the effects are independent of the nature of the interdomain region.

The Ca²⁺ requirement for activation of nNOS was lowered significantly by deletion of the insert (EC₅₀ = 300–20 nM). Surprisingly, the E/N chimera was activated by a lower Ca²⁺ concentration than eNOS itself (250 versus 70 nM) even though the nNOS reductase has a similar insert. Deletion of the insert from the chimera did not alter further the Ca²⁺ requirement. Since reductase structural elements are required to bring about the complete Ca²⁺ independence of iNOS (18, 47, 48), it is likely that reductase elements in addition to the autoinhibitory loop play a role in CaM binding and that the interactions between CaM and these elements have been perturbed in the E/N chimera. Thus, the interactions involved in normal CaM binding have been disrupted by the exchange of reductase domains in the chimera. This might also explain the finding that the slight loss of Ca²⁺ dependence is identical for I/N and I/NΔ, as this suggests that the exchange of reductase domains induces the alteration, independent of whether the autoinhibitory insert is present or not.

Our previous findings with chimeras incorporating the eNOS

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**Table I**

| Mutation | Expression system | CaM required? | EC₅₀(Ca²⁺) (nM) |
|----------|------------------|--------------|----------------|
| Ortiz de Montellane⁴ | E. coli | Yes | 300–20 |
| | E. coli | Yes | 242–175 |
| | S. cerevisiae | Partially | ND² |

⁴ Reported in this work.
⁵ Reported in Ref. 16.
⁶ Reported in Ref. 17.
⁷ Not determined.

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reductase with or without the insert might lead one to conclude that the effects of the insert on the Ca\(^{2+}\)/CaM dependence and electron transfer rates were directly related. Thus, deletion of the eNOS insert without exception enhanced both electron transfer and NO synthesis and lowered the Ca\(^{2+}\) requirement. However, the present results on the proteins with exchanged autoinhibitory inserts suggest that the Ca\(^{2+}\) dependence and electron transfer effects are discrete phenomena. For example, replacing the insert in nNOS with that from eNOS yields nNOS/E\(_{\text{nsert}}\) whose Ca\(^{2+}\) dependence is lower and yet whose activity is nearly as high (−KCl) or higher (+KCl) than that of nNOS. One possible explanation for this difference is a lack of binding requirements in the nNOS parent that is required by the eNOS insert. Regardless of whether this is the correct explanation, it is clear that one effect does not necessarily follow the other. Support for the idea that the Ca\(^{2+}\) dependence but not electron transfer effects of the insert require the participation of additional protein elements comes from the fact that the reductase and NO synthetic activities of both iNOS/E\(_{\text{nsert}}\) and E/I/E\(_{\text{nsert}}\) are decreased relative to those of the parent iNOS and E/I proteins, respectively. In iNOS/E\(_{\text{nsert}}\), no nNOS or eNOS structural features other than those within the insert are present, which suggests that the mere presence of the loop is sufficient to inhibit electron transfer. This could occur by a mechanism as simple as disruption of a key contact within the electron transport pathway. Since electron transfer to cytochrome c is decreased in addition to the NO synthesizing activity, inhibition occurs prior to the step in which the electron is transferred from the FMN to the heme donor or to cytochrome c. Inhibition therefore occurs upstream of the CaM-binding site. In E/I/E\(_{\text{nsert}}\), the heme and CaM-binding domains are complementary to the insert, yet the Ca\(^{2+}\) dependence is the same as for the E/I parent. This suggests that the effects of the insert on CaM binding depend on structural elements of the reductase in addition to those of the insert. It is unlikely that the structure of the reductase/heme electron transfer interface has been altered in the chimera, or that alterations have occurred in any required insert/heme domain interactions, as the catalytic activities of E/I are as high as those of iNOS. This finding is inconsistent with disruption of the quaternary structure. Further indication that the insert lowers electron transfer irrespective of CaM binding is seen in the rates of reduction of cytochrome c by E/I/E\(_{\text{nsert}}\) in the absence of CaM. The iNOS reductase has been shown to have CaM-Free reduction rates that are as high as the CaM-bound rates and as high as the rate supported by cytochrome P450 reductase (45). As a result, E/I and N/I have CaM-free cytochrome c reduction rates as high as those of iNOS itself. Moreover, that addition of the eNOS insert in E/I/E\(_{\text{nsert}}\) did not alter the Ca\(^{2+}\) dependence relative to that of E/I, the reductase and NO synthesizing activities were reduced regardless of whether CaM was bound or not. Thus, the inhibitory effect of the insert is complete within the reductase domain and is independent of CaM. Additional support for the view that the reductase domain provides binding elements that help to determine the Ca\(^{2+}\) dependence comes from the fact that the only proteins that show lowered Ca\(^{2+}\) requirements are those in which the eNOS or nNOS insert is present within its constitutive reductase domain. The converse, however, is not necessarily true; for example, eNOS/N\(_{\text{nsert}}\) and E/Na have parent-like Ca\(^{2+}\) requirements (Fig. 6). Nevertheless, eNOS\(_{\alpha}\), nNOS\(_{\alpha}\), N/E\(_{\Delta}\), and nNOS/E\(_{\text{nsert}}\) have lower EC\(_{50}\) (Ca\(^{2+}\)) values than the respective parent proteins, whereas iNOS(E\(_{\text{nsert}}\)) and E/I/E\(_{\text{nsert}}\) do not. Interestingly, in eNOS/N\(_{\text{nsert}}\), the nNOS insert appears to function normally when placed in eNOS, in that Ca\(^{2+}\) require-

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