Control of Nitric Oxide Synthase Dimer Assembly by a Heme−NO-Dependent Mechanism†

Yishi Chen, Koustubh Panda, and Dennis J. Stuehr*

Department of Immunology, The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195

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ABSTRACT: Homodimer formation is a key step that follows heme incorporation during assembly of an active inducible nitric oxide synthase (iNOS). In cells, heme incorporation into iNOS becomes limited due to interaction between self-generated NO and cellular heme [Albakri, Q., and Stuehr, D. J. (1996) *J. Biol. Chem. 271*, 5414−5421]. Here we investigated if NO can regulate at points downstream in the process by inhibiting dimerization of heme-containing iNOS monomer. Heme-containing monomers were generated by treating iNOS dimer or iNOS oxygenase domain dimer (iNOSoxy) with urea. Both monomers dimerized when incubated with Arg and 6*R*-tetrahydrobiopterin (H_4B), as shown previously [Abu-Soud, H. M., Loftus, M., and Stuehr, D. J. (1995) *Biochemistry 34*, 11167−11175]. The NO-releasing drug *S*-nitrosyl-*N*-acetyl-D,L-penicillamine (SNAP; 0−0.5 mM) inhibited dimerization of iNOS monomer in a dose- and time-dependent manner, without causing heme release. SNAP-pretreated monomer also did not dimerize in response to H_4B plus Arg. SNAP converted Arg- and H_4B-free iNOS dimer into monomer that could not redimerize, but had no effect on iNOS dimer preincubated with Arg and H_4B. Anaerobic spectral analysis showed that NO from SNAP bound to the ferric heme of iNOSoxy monomer or dimer. Adding imidazole as an alternative heme ligand prevented SNAP from inhibiting iNOS monomer dimerization. We conclude that NO and related species can block iNOS dimerization at points downstream from heme incorporation. The damage to heme-containing monomer results from a reaction with the protein and appears irreversible. Although dimeric structure alone does not protect, it does enable Arg and H_4B to bind and protect. Inhibition appears mediated by NO coordinating to the ferric heme iron of the monomer.

Nitric oxide (NO)[1] mediates numerous processes and serves as a physiological vasodilator, neurotransmitter, and cytotoxic agent in mammals (*1*−*3*). NO is synthesized from L-arginine (Arg) by the NO synthases (NOSs), which are distinct gene products including neuronal NOS (NOS-I or nNOS), endothelial NOS (NOS-III or eNOS), and inducible NOS (NOS-II or iNOS) (*4*). All NOSs are bi-domain proteins comprised of a C-terminal reductase domain and an N-terminal oxygenase domain linked by a central calmodulin binding sequence. The reductase domain contains binding sites for NADPH, FAD, and FMN and is related to other flavoproteins such as NADPH−cytochrome P450 reductase, bacterial sulfite reductase flavoprotein α-subunit, and cytochrome $P450_{BM3}$ reductase domain (*5*−*8*). In contrast, the NOS oxygenase domain contains binding sites for heme, H_4B, and Arg and does not share significant sequence or structural homology with any other proteins (*9*−*11*).

Dimerization is one of several posttranslational steps required to convert NOS polypeptides into active enzymes (Figure 1). Heme incorporation into the NOS monomer is essential and is the slow step for dimerization in cells expressing NOS (*12*−*15*). An extensive dimer interface is created between two oxygenase domains, and this helps activate NOS by sequestering the heme from bulk solvent, creating functional binding sites for Arg and H_4B, and allowing electron transfer between reductase and oxygenase domains located on adjacent subunits (*16*−*20*). In iNOS and nNOS, binding H_4B with or without Arg to the newly formed dimer favors additional structural changes, which although not apparent in existing crystal structures (*11*), are indicated by changes in proteolytic susceptibility (*21*), heme ligand binding (*22*−*25*), and increased resistance toward dimer dissociation by detergent (*26*−*28*). In this way, H_4B and Arg binding cause a transition from "loose" to "tight" NOS dimer. Zn^{2+} incorporation at the dimer interface can also occur and may provide additional stabilization (*29*).

Because dimerization is required to activate NOS, how cells control the process is of interest. Our studies of iNOS dimer assembly showed that cell-generated NO can block heme incorporation into an iNOS monomer (*13*). This special kind of NO feedback regulation explains why heme-free iNOS monomer accumulates with time in cytokine-activated cells that generate NO. Our related studies with iNOS heme-containing monomers (urea-generated) showed that H_4B, Arg, structural analogues of these molecules, or small

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* Correspondence should be addressed to this author at the Department of Immunology NB-3, The Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Phone: 1-216-445-6950. Fax: 1-216-444-9329. E-mail: stuehrd@ccf.org.

[1] Abbreviations: DETANONOate, (*Z*)-1-[2-aminoethyl-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; 2DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; H_4B, (6*R*)-2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; iNOS, inducible nitric oxide synthase; iNOSoxy, oxygenase domain of iNOS; NO, nitric monoxide; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine.

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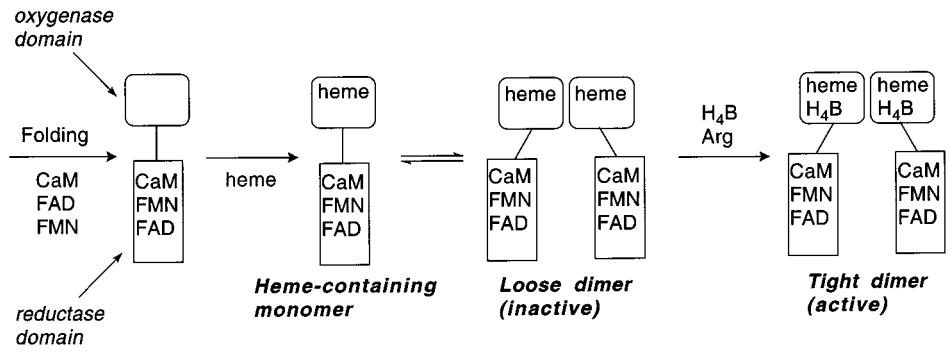

FIGURE 1: Stepwise model for active iNOS dimer assembly. After translation, the protein binds calmodulin (CaM), FMN, and FAD to create a heme-free monomer that contains a functional reductase domain. Heme binding enables two monomers to form a loose dimer. H_4B binding and Arg generate an active dimer. Adapted from ref *36*.

imidazoles all promote their conversion into dimer (*26, 30, 31*). In contrast, imidazoles that contain bulky side chains such as clotrimazole or miconazole antagonized dimerization through their binding to the NOS monomer heme (*32*). This recently served as a basis for pharmacologic discovery of selective iNOS inhibitors (*33*).

Although NO can inhibit heme insertion into iNOS monomer, nothing is known about how NO may influence downstream intermediates in the active dimer assembly pathway. We therefore investigated how the NO/NO^+ donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) would affect the stability and behavior of iNOS heme-containing monomer and loose dimer. These represent two intermediates along the pathway to active dimer (Figure 1) that normally do not accumulate in mammalian cells due to the presence of H_4B. The results expand our knowledge of how NO and related molecules can regulate NOS dimer assembly, and introduce new concepts regarding reversal of or protection against NO-based effects, and a role for heme−NO binding in mediating the effects.

MATERIALS AND METHODS

Materials. SNAP was purchased from Alexis Corp. (San Diego, CA). Ultrapure urea was obtained from Bio-Rad. Centricon microconcentrators were purchased from Amicon (Beverly, MA). All other regents and materials were obtained from Sigma Chemical Co. (St. Louis, MO) or from sources previously reported (*13, 18, 21, 26*).

Preparation of iNOS Dimer and Heme-Containing Monomer. Full-length iNOS and the iNOS oxygenase domain (iNOSoxy), containing a six-His tag attached to their N and C termini, respectively, were overexpressed in protease-deficient *E. coli* strain BL 21 DE cells and purified in the absence of Arg plus H_4B as previously described (*18, 21*). Dissociation of dimeric iNOS or iNOSoxy was carried out as described previously (*18, 21, 26*). Purified proteins (10−20 μM) were incubated at 30 °C for about 90 min in 40 mM EPPS buffer, pH 7.6, containing 0.5 mM DTT and either 2.5 M (iNOS) or 5 M (iNOSoxy) urea. Aliquots were removed and diluted 1:10 with 40 mM EPPS, pH 7.6, and 10% glycerol before use. Monomers were prepared fresh on the day of the experiments. The concentration of heme-containing iNOS monomer was determined based on the 444 nm absorbance of the dithionite-reduced, CO-bound form, using an extinction coefficient of 76 000 M^{-1} cm^{-1}.

Treatment of iNOS Proteins with SNAP. iNOS proteins were incubated at room temperature for 1 h or for the times indicated with different concentrations of SNAP in 40 mM EPPS buffer, pH 7.6, with 10% glycerol, 0.1 mM DTT, 5 mM Arg, and 10 μM H_4B. In some cases, Arg and H_4B were omitted. Residual SNAP was destroyed by adding 3 mM DTT to incubations prior to further experiments.[2]

Dimerization of iNOS Monomer. Urea-generated monomers (including those that were SNAP-pretreated) were incubated at concentrations of 1−2 μM at 25 °C for 1 h or for the indicated times in 150−300 μL of 40 mM EPPS, pH 7.6, containing 10% glycerol, 0.1 mM DTT, 10 μM H_4B, and 5 mM Arg. In some cases, H_4B and Arg were omitted, and 5 mM imidazole was added as noted in the text.

NO Synthesis Activity. The initial rate of NO synthesis by iNOS was measured at 37 °C using the oxyhemoglobin assay for NO. The iNOS (10−50 nM with respect to heme iron concentration) was added to a cuvette containing 40 mM EPPS, pH 7.6, supplemented with 10% glycerol, 0.3 mM DTT, 5 mM Arg, 4 μM each of FAD, FMN, and H_4B, 100 units/mL catalase, 10 units/mL superoxide dismutase, 0.1 mg/mL bovine serum albumin, and 5−10 μM oxyhemoglobin to give a final volume of 0.3 mL. Reactions were initiated by adding NADPH to give 0.1 mM. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an absorbance increase at 401 nm and quantitated using an extinction coefficient of 38 mM^{-1} cm^{-1}.

UV−Visible Spectroscopy. Spectra and absorbance measurements were recorded with a Hitachi U-2000 spectrophotometer. To monitor spectra of iNOSoxy monomer or dimer during SNAP treatment, cuvettes contained 3−5 μM protein in 40 mM EPPS buffer, pH 7.6, containing 10% glycerol, 5 mM Arg, 10 μM H_4B, 0.1 mM DTT, and 200 μM SNAP. In some cases, separate enzyme and SNAP solutions were made anaerobic by cycles of vacuum and purging with N_2, and spectra were collected after mixing them in an anaerobic cuvette.

RESULTS

Before studying the effects of the NO/NO^+ donor SNAP on dimerization, we first measured its NO release under our particular incubation conditions using the oxyhemoglobin spectrophotometric assay as described under Materials and Methods. Rates of NO release were measured at various times after adding SNAP to (iNOS-free) dimerization mixtures. Figure 2 shows that the rates varied in direct

[2] The oxyhemoglobin assay showed that adding 3 mM DTT caused rapid and complete release of NO from SNAP within 3 min.

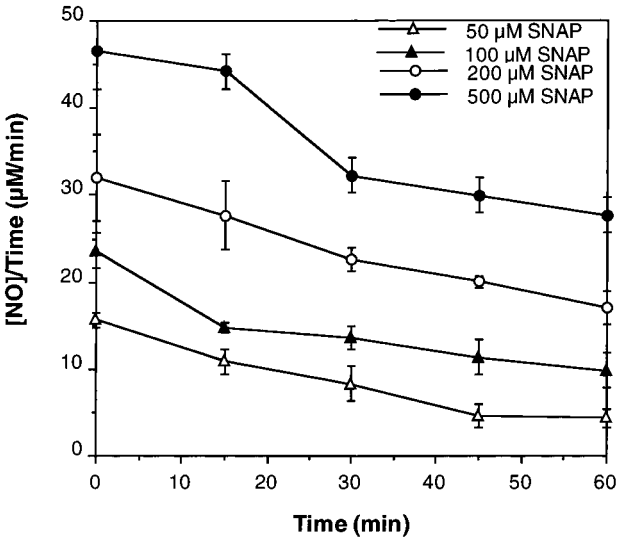

FIGURE 2: Rate of NO release by SNAP during a 1 h incubation. SNAP was added at the indicated concentrations to a buffered solution as described under Materials and Methods. Aliquots were removed at the indicated times and diluted in cuvettes to measure the rate of NO release using the oxyhemoglobin assay. Rates were then multiplied by the dilution factor to obtain those shown in the graph. Data are the mean and SD for three measurements.

proportion to the initial SNAP concentration, and decreased slowly with time, such that NO release was still occurring after 1 h of incubation in all cases. NO release from SNAP was also confirmed with an NO electrode (data not shown). We utilized these reaction conditions to treat iNOS as described below.

SNAP Inhibits Dimerization of iNOS Monomer. Dissociating dimeric iNOS with urea typically results in approximately a 80:20 mixture of heme-containing monomers and dimers (*21*, *26*). Assembly of such monomers into dimer is promoted by addition of H_4B and Arg. To determine how SNAP would influence dimerization, we incubated iNOS monomers with H_4B and Arg in the presence of different SNAP concentrations ranging from 0 to 0.5 mM. After 1 h, aliquots were removed to determine monomer−dimer content and NO synthesis activity. Figure 3 shows the gel filtration profiles of each iNOS dimerization reaction. In each chromatogram, the four peaks eluting at 7.5, 9, 11, and 14 mL represent iNOS aggregates, dimer, monomer, and contaminating iNOS reductase domain, respectively. The amount of dimer formed in the incubation ranged from 90% to 30% in the presence of 0−500 μM SNAP, respectively. As shown in Table 1, the dimer content correlated with a variation in NO synthesis specific activity that ranged from 68% to 14% between SNAP concentrations of 50 and 500 μM, respectively. Thus, SNAP antagonized dimerization of heme-containing monomers in response to H_4B and Arg.

We next investigated if SNAP acts on the heme-containing monomer itself. Heme-containing monomers were pretreated with different concentrations of SNAP ranging from 50 to 500 μM for 1 h, or with 100 μM SNAP for different time periods. Remaining SNAP was then destroyed, and the iNOS proteins were tested for dimerization in response to H_4B plus Arg. As shown in Figure 4 and Table 2, the 1 h SNAP pretreatment inhibited dimerization and recovery of NO synthesis activity in a concentration-dependent manner. We also preincubated heme-containing monomer with 500 μM

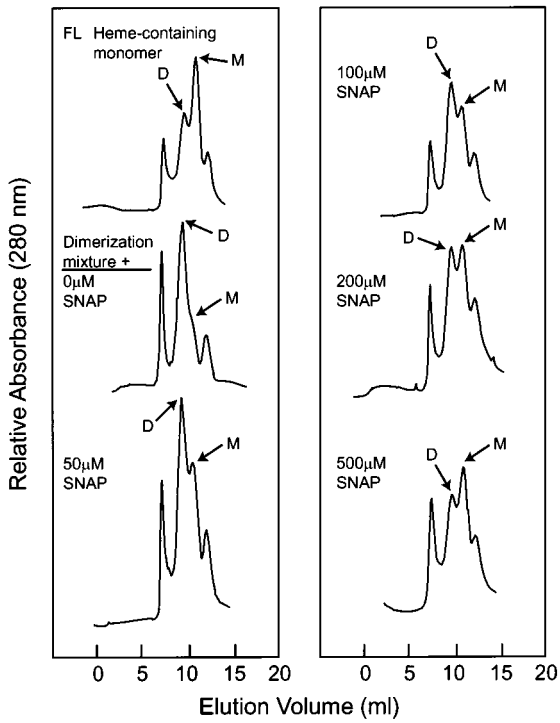

FIGURE 3: Effect of SNAP on iNOS dimerization in response to H_4B plus Arg. iNOS heme-containing monomer was coincubated with H_4B, Arg, and the indicated concentrations of SNAP for 1 h. The sample was then subjected to gel filtration chromatography. D and M indicate dimer and monomer peaks, respectively. Data are representative of at least three experiments.

Table 1: Effect of SNAP Coincubation on Ability of iNOS Monomer To Recover NO Synthesis Activity in Response to H_4B plus Arg[a]

dimerization incubation condition	[SNAP] (μM)	specific activity (nmol of NO min^{-1} mg^{-1})
$-H_4B$, −Arg	0	94 ± 3
$+H_4B$, +Arg	0	556 ± 34
same	50	403 ± 2
same	100	335 ± 29
same	200	182 ± 2
same	500	71 ± 10

[a] Specific activities were determined by assaying for NO synthesis activity using the oxyhemoglobin assay. Values are the mean and SD from two experiments, two measurements each.

DETANONOate in place of SNAP as an alternative NO donor. Subsequent iNOS dimerization in response to Arg and H_4B was inhibited approximately 85%, similar to what we observed for 500 μM SNAP (data not shown).

As shown in Figure 5 and Table 2, the kinetics of the pretreatment effect were such that 100 μM SNAP caused about 20% inhibition of dimerization and 24% inhibition of NO synthesis activity within 15 min. The effect reached a maximum after 45−60 min pretreatment, which produced about 50% inhibition of dimerization and 45% inhibition of NO synthesis activity. Spectra taken of SNAP-treated monomers showed that they did not lose their bound heme (data not shown). These results suggest that SNAP inhibits iNOS dimerization through an effect on the iNOS protein. To further test this, iNOS monomers were incubated 1 h under dimerization conditions (H_4B plus Arg) in the presence of 500 μM SNAP, and then the monomers that remained in the dimerization mixture were purified by gel filtration and

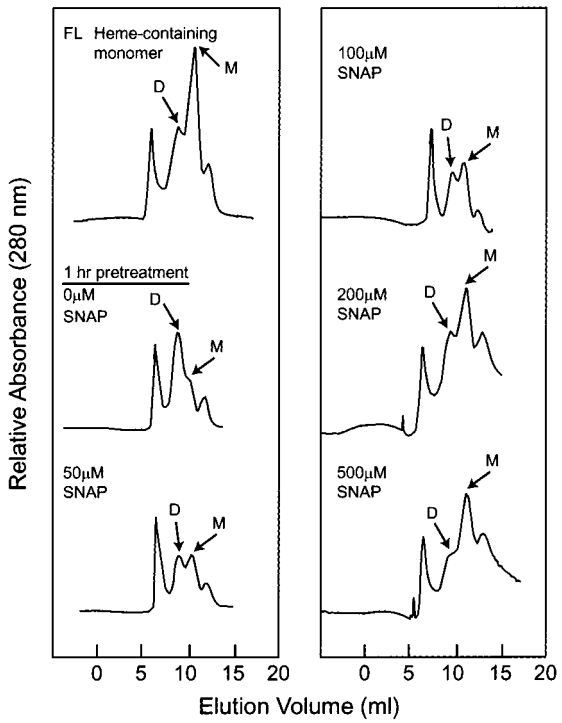

FIGURE 4: Effect of SNAP pretreatment on iNOS dimerization in response to H_4B plus Arg. iNOS heme-containing monomer was preincubated with the indicated concentrations of SNAP for 1 h. Excess SNAP was destroyed, and then the samples were incubated with H_4B plus Arg for 1 h and subjected to gel filtration chromatography. D and M indicate dimer and monomer peaks, respectively. Data are representative of at least two experiments.

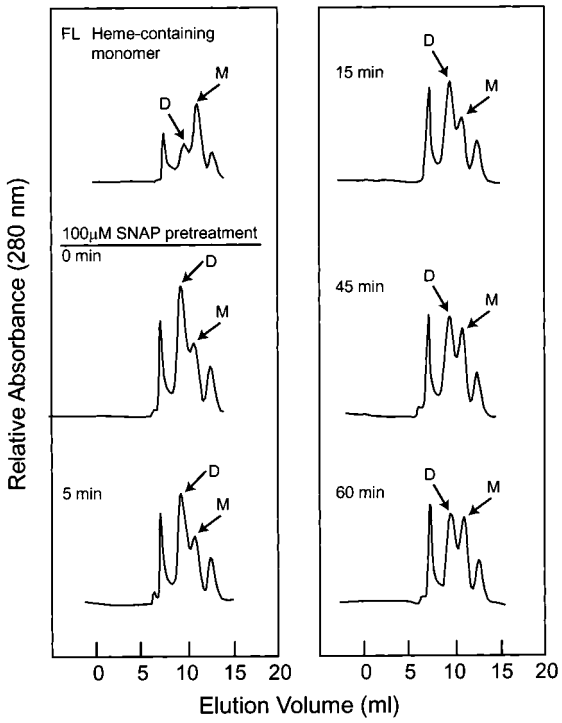

FIGURE 5: Effect of SNAP pretreatment on iNOS dimerization in response to H_4B plus Arg. iNOS heme-containing monomer was preincubated with SNAP for the indicated times. Excess SNAP was destroyed, and then the samples were incubated with H_4B plus Arg for 1 h and subjected to gel filtration chromatography. D and M indicate dimer and monomer peaks, respectively. Data are representative of at least two experiments.

Table 2: Effect of SNAP Pretreatment on Ability of iNOS Monomer To Recover NO Synthesis Activity in Response to H_4B plus Arg[a]

dimerization condition	[SNAP] (μM)	time with SNAP (min)	specific activity (nmol of NO min^{-1} mg^{-1}) expt 1	expt 2
$-H_4B$, −Arg	0	60	49	76
$+H_4B$, +Arg	0	60	615	847
same	50	60	295	366
same	100	60	167	303
same	200	60	159	298
same	500	60	89	ND
$-H_4B$, −Arg	0	60	78	59
$+H_4B$, +Arg	0	60	702	523
same	100	5	535	515
same	100	15	434	488
same	100	45	365	345
same	100	60	390	304

[a] iNOS heme-containing monomers were pretreated with SNAP and then incubated 60 min with Arg plus H_4B to promote dimerization. Either the SNAP concentration or the time of SNAP exposure was varied. After the dimerization incubation, NO synthesis activity was measured by the oxyhemoglobin assay. ND is not determined. Values are the mean from two measurements each.

tested regarding their ability to dimerize in response to H_4B plus Arg. They were unable to dimerize (data not shown), indicating that inhibition is irreversible under these conditions and can occur in the presence or absence of H_4B and Arg.

SNAP Can Dissociate Loose iNOS Dimer. We next investigated if SNAP could cause dissociation of H_4B- and Arg-free iNOS dimer (loose dimer) or an iNOS dimer saturated with H_4B plus Arg (tight dimer). Loose dimer was incubated 1 h alone or with two concentrations of SNAP (50 and 500 μM) in the absence or presence of Arg plus H_4B. As shown in Figure 6, incubation alone in the absence of SNAP or H_4B plus Arg did not cause dimer to dissociate. Treatment with 50 μM SNAP in the absence of Arg plus H_4B caused a portion of loose dimer (~60%) to become monomers, and also inhibited their NO synthesis activity (data not shown). Monomer so generated did not redimerize in response to H_4B plus Arg (data not shown). In contrast, dimer that had been given Arg plus H_4B just before adding SNAP was resistant to dissociation by 50 μM SNAP (Figure 6) or by 500 μM SNAP (data not shown), and maintained its NO synthesis activity (data not shown). Thus, SNAP caused dissociation of a loose iNOS dimer, but could not do so after it bound H_4B and Arg.

SNAP Influences iNOS Dimerization through NO Binding to Heme. NO can bind as a sixth ligand to the ferric iNOS heme (*22*). To investigate if heme−NO binding was important for SNAP inhibition of iNOS dimerization, we blocked its binding to the heme iron by adding 5 mM imidazole. Imidazole binds to the ferric heme iron of iNOS monomer or dimer with a K_d of approximately 30−40 μM (*34*, *35*). iNOS heme-containing monomers were incubated with 5 mM imidazole or with 5 mM Arg plus 10 μM H_4B immediately before being incubated with 500 μM SNAP at room temperature for 1 h. A spectrum taken of iNOS monomer in the presence of imidazole and SNAP confirmed that practically all of the ferric heme was imidazole-bound (data not shown). In the meantime, iNOS monomer was incubated with 500 μM SNAP alone, 5 mM imidazole alone, or 5 mM Arg plus 10 μM H_4B as controls. Then, all monomers preincubated in these ways were dialyzed in buffer containing Arg

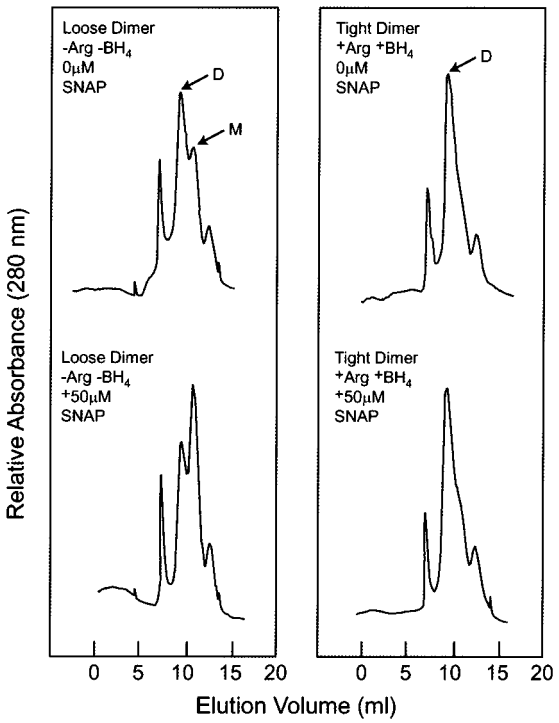

FIGURE 6: Effect of SNAP on the stability of loose or tight iNOS dimers. H_4B plus Arg was or was not added to iNOS dimer, and then 0 or 50 μM SNAP was immediately added. The samples were incubated for 1 h and then subjected to gel filtration chromatography. D and M indicate dimer and monomer peaks, respectively. Data are representative of at least two replicate experiments.

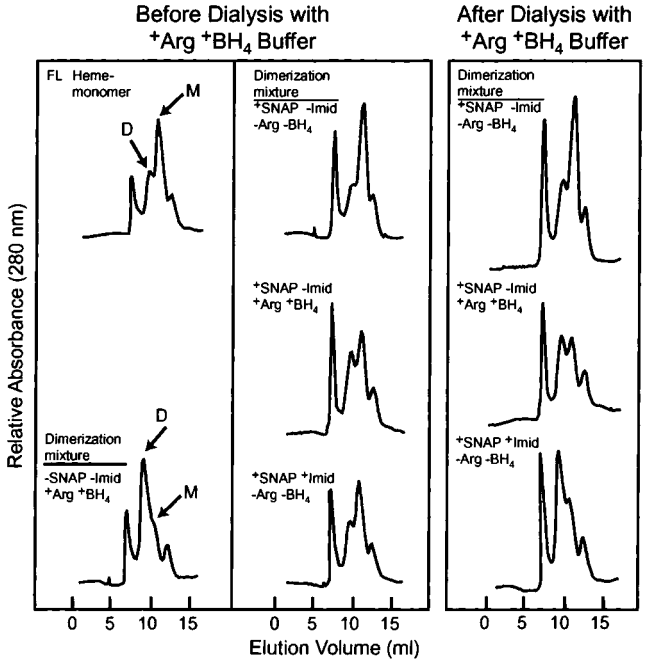

FIGURE 7: Effect of imidazole on SNAP inhibition of iNOS dimerization. iNOS monomer was incubated for 1 h with the indicated combinations of 0.5 mM SNAP, 5 mM imidazole, and H_4B plus Arg. Samples were then dialyzed overnight in buffer containing 5 mM Arg plus 10 μM H_4B. Samples that were removed before and after dialysis were subjected to gel filtration chromatography. D and M indicate dimer and monomer peaks, respectively. Data are representative of three experiments.

plus H_4B to test their ability to dimerize. Sample aliquots were removed before and after the dialysis step to analyze the dimer:monomer ratios and NO synthesis activities. Figure 7, left and center panels, shows that in samples prior to dialysis SNAP inhibited dimer formation in response to Arg plus H_4B, as we previously observed. SNAP also inhibited imidazole from promoting dimer formation that is normally observed in the absence of SNAP (*32*; Table 3). Figure 7, right panel, shows that subsequent dialysis of SNAP-treated samples in Arg plus H_4B containing buffer resulted in significant dimerization only in the imidazole-pretreated sample. The NO synthesis activities shown in Table 3 are in good agreement with the gel filtration results. Thus, imidazole prevented SNAP from inhibiting dimerization of iNOS heme-containing monomer. To test if the protective effect of imidazole was related to its heme biding, we repeated the experiment but replaced imidazole with 2-phenylimidazole, which has a poorer affinity toward iNOS ferric heme (K_d approximately 230 μM). Adding 2-phenylimidazole at 5 mM in the SNAP incubation failed to protect iNOS monomer against inhibition of dimer formation in response to Arg and H_4B (data not shown). Together, the results imply that SNAP inhibition requires NO binding to ferric heme in the iNOS monomer.

To investigate this further, we collected spectra during incubation of iNOSoxy dimer or heme-containing monomer with 200 μM SNAP to see if a ferric heme−NO complex could form under aerobic or anaerobic conditions. When the incubation was done under aerobic conditions, a ferric heme−NO complex did not build up to detectable levels (data not shown). In contrast, under anaerobic conditions, both monomer and dimer formed a ferric heme−NO complex whose intensity increased with time during the SNAP incubation (Figure 8A,B). The complexes showed characteristic absorbance at 440, 549, and 580 nm, although the 440 nm Soret peak was much more prominent in the iNOSoxy dimer than in the monomer.

Table 3: Effect of Imidazole on SNAP Inhibition of NO Synthesis Recovery[a]

preincubation condition			specific activity (nmol of NO min^{-1} mg^{-1}) before dialysis		after dialysis	
SNAP	imidazole	H_4B + Arg	expt 1	expt 2	expt 1	expt 2
−	−	−	108	103	NA	NA
−	−	+	723	698	725	788
−	+	−	524	455	596	479
+	−	−	174	ND	73	15
+	+	−	188	157	579	528
+	−	+	446	389	344	379

[a] iNOS monomer was preincubated for 60 min with the indicated combinations of 0.5 mM SNAP, 5 mM imidazole, and H_4B plus Arg. Samples were then dialyzed in buffer containing 5 mM Arg plus 10 μM H_4B. NO synthesis activity was measured for each sample before and after dialysis. Values are the mean of two measurements. NA, not applicable; ND, not determined.

DISCUSSION

We investigated how NO and related molecules influence the creation of an active iNOS dimer at points in the assembly process that are downstream from heme insertion. H_4B-free iNOS dimer and heme-containing monomer generated from it represent two intermediates along the pathway to active dimer (Figure 1) (*14*, *36*). Because these intermediates do not accumulate in mammalian cells, bacterial

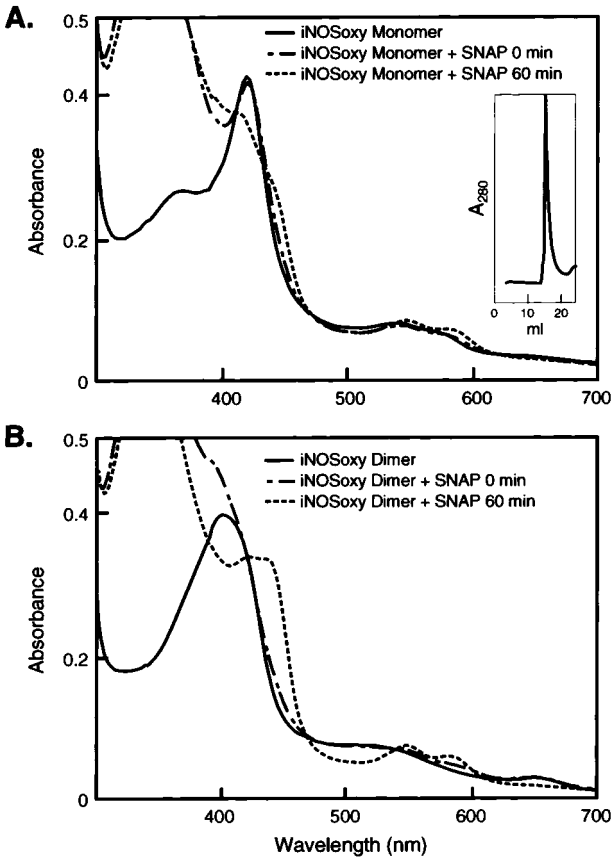

FIGURE 8: Light absorbance spectra of iNOSoxy monomer (A) or dimer (B) before and during incubation with 0.2 mM SNAP under anaerobic conditions. Incubations also contained 40 mM EPPS buffer, pH 7.6, 10% glycerol, 5 mM Arg, 10 μM H_4B, and 0.1 mM DTT. Spectra were recorded before, immediately after, and 60 min after SNAP addition. The inset shows a gel filtration profile of the iNOSoxy monomer used in the experiment. Data are representative of two similar experiments.

expression and urea dissociation provide a practical means to obtain them for experiments. Our previous work established that Arg plus H_4B cause heme-containing iNOS monomers to reassemble into dimer over a 30−60 min incubation. The reassembled dimer exhibited the same physical, spectral, and catalytic properties as native dimeric iNOS (*26*, *30*, *31*). Here we found that the NO/NO^+ donor SNAP inhibited dimerization when it was incubated with heme-containing monomer in the presence of Arg plus H_4B, or when it was preincubated with monomer alone. In the former circumstance, some inhibition we observed at high SNAP concentrations may have been due to NO or related species oxidizing H_4B. However, this explanation cannot hold for inhibition we observed following preincubation of iNOS monomers with SNAP, because in that case residual SNAP was destroyed before adding H_4B. SNAP-induced inhibition of dimerization was time- and concentration-dependent, in all cases was irreversible, and appeared to involve reaction of a SNAP-derived species with the monomer itself. Finally, we obtained similar inhibition upon preincubating heme-containing monomer with the alternative NO donor DETANONOate, indicating the inhibition was not specific to using SNAP as a donor.

Interestingly, SNAP treatment did not alter the structure or activity of an H_4B- and Arg-replete iNOS dimer. Although it is possible that bound Arg and H_4B molecules physically blocked reaction of a SNAP-derived species with key protein component(s), this is unlikely. Rather, the unique sensitivity of heme-containing monomer toward SNAP may stem from its adopting conformations that allow key protein component(s) to be chemically modified, and these modifications render monomer incapable of forming a stable dimer. This is consistent with monomer not containing functional H_4B or Arg binding sites, and its being unable to create these sites outside the context of a dimeric structure. SNAP also caused dissociation of an H_4B- and Arg-free (loose) iNOS dimer. Subunits within a loose dimer may be susceptible to the same modifications that occur in the monomer, and to their negative effect on dimer stability. However, in contrast to the monomer, adding H_4B plus Arg to the loose dimer instantly protected it from destabilization by SNAP. This is consistent with spectral and catalytic data that show loose iNOS dimer rapidly binds H_4B and Arg when they are given together (*21*, *31*). Our results support and extend the concept that H_4B and Arg binding stabilize iNOS in a conformation that otherwise is not well-populated in either the loose dimer or the heme-containing monomer forms (*36*).

How does SNAP prevent dimerization of heme-containing monomer? Although exact answers are still forthcoming, enough is known about the chemistry of SNAP and iNOS structure to discuss some possible mechanisms. SNAP has been used extensively as a NO donor in pharmacology (*37*, *38*), and its NO release here was demonstrated by the oxyhemoglobin reaction, formation of an iNOS ferric heme−NO species, and direct electrode measurement. When NO accumulates in aerobic solution, it generates reactive *N*-oxides such as N_2O_3 and NO_2 (*39*). N_2O_3 can transfer NO^+ to protein nucleophiles, while NO_2 can oxidize amino acids or metal centers. Because SNAP is an *S*-nitroso compound, it also can directly transfer NO^+ to protein sulfhydryls (*40*). Thus, besides ferric heme−NO complex formation, SNAP may cause cysteine nitrosation and methionine oxidation in iNOS, and may destroy the $Zn^{2+}(Cys)_4$ structure that can form at the iNOS dimer interface (*11*). We know from mutagenesis studies that the $Zn^{2+}(Cys)_4$ structure is not required for dimerization of heme-containing iNOS monomer in response to H_4B and Arg, or for subsequent catalytic function (*21*, *29*). There are several cysteine and methionine residues near the iNOS dimer interface and/or near structural elements that help form the interface or create binding sites for H_4B and Arg (*16*). Evidence suggests that S-nitrosation of proteins can regulate their structure or function (*41*, *42*). Because an Arg- and H_4B-replete iNOS dimer was insensitive to SNAP-induced dissociation, inhibition by SNAP as reported here clearly differs from how NO inhibits an active NOS dimer (*37*, *43*−*45*). An active NOS dimer may be stable enough to withstand damaging modifications brought on by incubation with SNAP, or alternatively may populate a conformation that protects key reactive groups from modification. Indeed, creating the dimer interface alters the solvent accessibility of many iNOS structural elements (*9*, *16*, *46*). Beyond that, there are other potentially important structural changes as indicated by comparing crystal structures of an imidazole-bound, heme-containing iNOSoxy monomer and an active iNOSoxy dimer (*9*, *16*). These include creation of a helical lariat to form the H_4B binding site and repositioning of helices in and near the distal heme pocket. Our results provide a starting point to explore NO/NO^+-induced protein

modifications in relation to structural changes that occur during assembly of active iNOS dimer.

Surprisingly, the iNOS heme may mediate SNAP-induced damage to the monomer. This concept is consistent with (a) imidazole protecting heme-containing iNOS monomer from becoming irreversibly damaged by SNAP, (b) NO exposure not causing irreversible inhibition of dimerization in a heme-free iNOS monomer,[3] and (c) our observing ferric heme−NO complex formation during anaerobic incubation of SNAP with iNOS monomer. Although SNAP blocked dimerization that normally occurs during incubation of heme-containing monomer with imidazole (ref *32* and Table 3), the resulting monomer was able to dimerize in response to H_4B plus Arg and regain full activity. Imidazole could protect simply by antagonizing NO binding to the heme, or by causing a protein conformational change after it binds to the heme. Regarding the former possibility, binding of NO or imidazole to ferric iNOS heme is rapidly reversible (*22*, *34*, *43*, *45*, *47*), and imidazole at 5 mM (16× its K_d) was surely sufficient to antagonize NO binding to the iNOS ferric heme during our SNAP incubations. This is consistent with 2-phenylimidazole being an ineffective antagonist at 5 mM and having 8-fold lower affinity toward iNOS ferric heme. Regarding the alternative possibility of an imidazole-induced conformational change, as discussed above there is crystallographic evidence showing that imidazole binding to the ferric heme of an iNOSoxy monomer does not promote the same conformational changes that are associated with dimer formation in response to H_4B plus Arg (*9*, *16*). Thus, we suspect that imidazole protects against irreversible damage by antagonizing heme−NO complex formation in iNOS monomer. This differs mechanistically from the protection obtained when H_4B and Arg bind to iNOS dimer, because H_4B and Arg do not bind directly to the ferric heme or antagonize its NO binding (*22*−*25*).

There are at least two ways that heme−NO binding could irreversibly disable dimerization of iNOS monomer. (A) It may irreversibly alter important properties of the heme−thiolate bond (to Cys194 in mouse iNOS). The heme−thiolate bond was shown to be essential for dimerization in a metal porphyrin substitution study with nNOS (*15*). Also, NO binding to ferrous NOS dimer caused a transition to cytochrome P420 NOS in the absence of Arg and H_4B (*22*, *48*). Whether this transition also occurred for ferric heme in our SNAP incubations was not tested, but our spectral data suggest that the ferric heme−NO complex of iNOS monomer does differ from that of the H_4B -and Arg-containing dimer (see Figure 8). (B) Heme−NO binding may deliver NO or related species to critical protein residues inside iNOS, as appears to occur between the heme group and cysteine residues within hemoglobin (*49*). In either case, it is remarkable that SNAP inhibition becomes inoperative or ineffective if H_4B plus Arg bind to the dimer, even though its heme still binds NO.

Summary. Our work adds to a developing picture of how NO and related species negatively regulate iNOS dimer assembly. NO and related species were previously shown to block heme insertion into iNOS monomer (*13*). We now propose that they can also act downstream in the assembly process to irreversibly inhibit heme-containing monomers from forming a stable loose dimer. Whether these principles extend to other NOS isoforms is unknown, but given that differences exist among their structural properties, a range of responses is certainly possible. Further work to identify key points of damage and how heme−NO binding facilitates the process is underway.

[3] Chen, Y., Albakri, Q., and Stuehr, D. J., unpublished results.

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