Activation of Peroxynitrite by Inducible Nitric-oxygen Synthase

A DIRECT SOURCE OF NITRATIVE STRESSa

Received for publication, September 29, 2006, and in revised form, March 16, 2007. Published, JBC Papers in Press, March 16, 2007, DOI 10.1074/jbc.M609237200

Amandine Maréchal†, Tony A. Mattioli†, Dennis J. Stuehr‡, and Jérôme Santolini††

From the †Laboratoire de Stress Oxydant et Détoxication, iBiTec-S, Commissariat à l’Énergie Atomique, Saclay, Gif-sur-Yvette 91191 Cedex, France and the ‡Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195

In mammals, nitric oxide (NO) is an essential biological mediator that is exclusively synthesized by nitric-oxygen synthases (NOSs). However, NOSs are also directly or indirectly responsible for the production of peroxynitrite, a well known cytotoxic agent involved in numerous pathophysiological processes. Peroxynitrite reactivity is extremely intricate and highly depends on activators such as hemoproteins. NOSs present, therefore, the unique ability to both produce and activate peroxynitrite, which confers upon them a major role in the control of peroxynitrite bioactivity. We report here the first kinetic analysis of the interaction between peroxynitrite and the oxygenase domain of inducible NO (iNOSoxy) (15–17). For the constitutive NOSs, Ca2+-dependent calmodulin binding enables NADPH-derived electrons to transfer from the flavins to the heme and to initiate NO synthesis (18). The chemistry catalyzed by all three isozymes appears also globally identical (19): NOSs catalyze a two-step oxidation of L-arginine with intermediate formation of Nω-hydroxy-L-arginine (20) (Scheme 1) by a mechanism analogous to that of monooxygenation by cytochromes P450 (21, 22). To date, all NOSs seem to share similar structure and mechanism, and the numerous structure-function investigations failed to elucidate the factors responsible for the observed different physiological specificities (i.e. signaling versus cytotoxicity) of constitutive and iNOSs.

Recently a new NOS catalytic model (Scheme 2) has been proposed by Stuehr and colleagues (23, 24). Before being released, NO actually binds to the heme within nanoseconds in a gammatic recombination process (25). Thus the actual NOS end-product should not be considered simply as NO but as a heme-NO complex, i.e. an FeIII-NO complex or an FeII-NO complex. This would lead to two different catalytic cycles (Scheme 2) with efficient release of NO from the FeIII-NO complex, or oxidation of the NO ligand of the FeIII-NO complex into other reactive nitrogen species (26). Although the identities of these reactive nitrogen species remain unknown, the nucleophilic attack of FeIII on oxygen should theoretically result in the transient build-up of peroxynitrite (PN), as suggested by several studies on FeIII oxidation in hemoglobins and myoglobin (27–30). However, NOSs can potentially produce peroxynitrite in other ways (31): (i) in uncoupling conditions, NOSs are prone to produce superoxide anion that will stoichiometrically react with NO to produce peroxynitrite (32), (ii) in H2B-free condi-
iNOS Enhances Peroxynitrite Oxidative and Nitrative Potency

Under these conditions the extent of oxygen activation remained minor compared to \( \text{Fe}^{4+} \text{O}_2 \) auto-oxidation and superoxide production.
iNOS Enhances Peroxynitrite Oxidative and Nitrative Potency

Analysis of Peroxynitrite Nitrative and Oxidative Properties Using 4-Hydroxyphenylacetic Acid (HPA)—The reaction of HPA with PN leads to the formation of oxidized and/or nitrated HPA metabolites that can be isolated, identified, and quantified by HPLC. In parallel, fluorescence assays gave an insight into the production of HPA dimers (di-HPA) resulting from the one-electron oxidation of HPA. 10 mM HPA and 20 μM iNOSoxy were added to 100 μl of a freshly degassed reaction buffer (KP, 0.1 M, pH 7.4) and 0.3 mM diethylenetriaminepentaacetic acid. Peroxynitrite (0.5, 1, 2, and 4 mM final concentrations) was added to the buffer, and the solution was immediately vigorously mixed. The solution was kept at room temperature for 10 min to ensure the completion of the reaction and was then diluted to 1 ml for analysis. Several control samples were measured under the same experimental conditions: (i) in the absence of both iNOSoxy and PN; (ii) in the absence of iNOSoxy, for all PN concentrations; (iii) with decomposed PN, with and without iNOSoxy; in this case, PN was first diluted at the desired concentration in the reaction buffer and kept at room temperature for at least 3 min to ensure full decomposition; and (iv) in the presence of 1 mM soluble CO₂. All samples were simultaneously analyzed by fluorescence spectroscopy and HPLC.

HPA-fluorescence Assays—The presence of di-HPA in the solution was determined by fluorescence spectroscopy on a Cary Eclipse fluorescence spectrometer (Varian Inc., Palo Alto, CA). Emission spectra were recorded with an excitation wavelength of 326 nm. Emission intensity was measured between 400 and 405 nm and plotted as a function of PN concentration. This series of experiments was repeated three times. Each plot was fitted to a linear function using Origin 6.0 software (OriginLab Corp.), and the slopes of all curves were averaged. We verified whether the presence of iNOSoxy could quench the fluorescence of di-HPA. HPA was allowed to react with PN in the absence of enzyme. Subsequent addition of 20 μM iNOSoxy led to negligible variations in the fluorescence emission intensity (<10%) indicating that iNOSoxy is not interfering with HPA fluorescence profile. Samples were frozen and kept at −20 °C until analysis by HPLC. We did not observe any modification of the fluorescence emission of the sample due to the freeze-thaw cycle, which indicates that the samples are not altered by freeze-thaw cycles.

HPA-HPLC Assays—iNOSoxy was removed from the solution by centrifugation (2300 × g at 8 °C) using Millipore (Bedford, MA) Ultrafree®-0.5 centrifugal filter device with a membrane filter (30-kDa cut-off). HPA derivatives were separated and analyzed by HPLC using a Dionex P680 instrument coupled to a Dionex UV/170U spectrophotometer (Dionex Corp., Sunnyvale, CA). The reverse-phase column was a Supelcosil LC-8, 150 × 4.6 mm protected by a Discovery C18, 20 × 4.0 mm pre-column (Sigma-Aldrich). The gradient was provided by changing the mixing ratio of the two eluents: water with 1% (v/v) acetic acid (A), and methanol containing 1% (v/v) acetic acid (B). The column was equilibrated with 5% B before injection of 20 μl of reaction solution. The gradient was then increased to 100% B for 20 min and held for 5 min to wash the column. The gradient returned to the initial condition within 10 min and held for 5 min for equilibration. Detection was...
achieved at 274, 280, 365, and 428 nm. Chromatograms were analyzed using Chromleap 6.40 software (Dionex Corp., Sunnyvale, CA). Retention times were identified at 280 and 365 nm using commercially available HPA (retention time = 9.25 min), 3,4-dihydroxyphenylacetic acid (HPA-OH, retention time = 8 min), and 4-hydroxy-3-nitrophenylacetic acid (HPA-NO₂, retention time = 11.55 min). Peak areas were calibrated using increasing concentrations of HPA, HPA-OH, and HPA-NO₂. Because HPA-NO₂ displays a specific peak at 365 nm, its production during HPA reaction with PN was quantified using both absorption peaks at 280 and 365 nm. At 280 nm, because of the crowding of the 11.3- to 11.8-min region, the chromatograms were converted into ASCII file and fitted to multi-Gaussianian functions using Origin 6.0 (OriginLab Corp.). Three different chromatogram peaks were identified with absorbance maxima occurring around 11.35, 11.55, and 11.75 min. The areas of the 11.55-min chromatogram peak measured at 365 nm were converted into HPA-NO₂ concentrations using calibration curves. HPA-NO₂ concentrations were plotted as a function of PN concentrations, and the data were fitted to linear functions. Experiments were repeated twice.

**DHR Fluorescence Assays**—DHR was used to assess the two-electron oxidative properties of peroxynitrite. The procedure was similar to the one described for HPA. DHR was diluted immediately prior to the assay in a degassed KP, (0.1 M and pH 7.4) buffer with 0.3 mM diethylenetriaminepentaacetic acid. The final concentration of DHR in the reaction buffer was set at 40 μM. Peroxynitrite at increasing concentrations (0, 10, 20, 30, 40, and 50 μM final) was vigorously mixed into the solution, in the presence or absence of iNOSoxy. Fluorescence emission spectra of the different samples were recorded with an excitation wavelength at 500 nm. Emission intensity was measured at 525 nm and plotted as a function of PN concentration. The curves were fitted to linear function, and the slopes of three series of experiments were averaged. Similar control experiments were performed, using the same protocol, with decomposed PN, in the absence of iNOSoxy or by adding iNOSoxy after the completion of the reaction between DHR and PN.

**Activity Assays**—iNOSoxy sample (1.5 μM) was rapid-mixed with successive additions of PN aliquots at 4 equivalents of iNOSoxy concentration. The number of additions ranged between 0 and 10. Bolus additions of 60 μM PN and of 60 μM decomposed PN were performed as controls. All samples were then washed by three successive cycles of concentration/dilution in a KP, (0.1 M and pH 7.4) buffer using a Millipore Ultrafree®-0.5 centrifugal filter device membrane filter concentrator (30-kDa cut-off). NOS activity of each protein sample was then measured using the Griess assay. Nitrite production activity was plotted as a function of the number of addition of PN. Experiments were repeated three times. Kinetics of PN decomposition in the presence of 10 μM iNOSoxy was measured for each sample with the protocol described here above. Absorbance decays at 302 nm were fitted to a mono-exponential function. Fitting to bi-exponential function did not lead to distinct and consistent rate constants. Mono-exponential apparent rate constants were plotted as a function of the number of PN aliquots added.

**RESULTS**

**iNOSoxy Activates Peroxynitrite Decomposition**—PN (100 μM final) and increasing concentrations of iNOSoxy were rapidly-mixed (final pH 7.4), and the kinetics of PN decay was monitored by stopped-flow coupled to a rapid-sampling diode array spectrophotometer. The inset of Fig. 1A shows time traces of absorbance changes at 302 nm that correspond to the kinetics of PN decay for increasing iNOSoxy concentrations. The kinetic traces obtained for each enzyme concentration were fitted to a mono-exponential function. The apparent PN decay rate constants (k_decay) were plotted as a function of iNOSoxy concentration (Fig. 1A, main). The PN decomposition rate was found to increase linearly with iNOSoxy concentration suggesting that iNOSoxy interacts with PN and accelerates its decomposition. This curve was fitted to a linear function: the y-intercept represents the spontaneous decomposition rate of PN under the experimental conditions (0.1 s⁻¹ at pH 7.4), and the slope corresponds to the second order rate constant of PN activation as defined in the introduction (see above) by iNOSoxy ((21 ± 2) × 10⁴ M⁻¹ s⁻¹). This experiment was repeated at pH 6.4 and 8.4 using the same protocol. At each pH value we observed an activation of PN decomposition by iNOSoxy (Fig. 1A). The rate of iNOSoxy-induced PN decomposition increases as the pH decreases (Table 1), indicating that PN activation is pH-dependent. Superposition of all the 750 UV-visible absorption spectra recorded during the kinetics measurement allows us to correlate PN decomposition with changes in the iNOSoxy heme absorption spectrum. The inset of Fig. 1B shows the time evolution between 12 and 324 ms of the absorption difference spectra with respect to the initial iNOSoxy absorption spec-
iNOS Enhances Peroxynitrite Oxidative and Nitrative Potency

TABLE 1
Peroxynitrite activation by iNOSoxy as a function of pH, substrates, and cofactor

| pH | Substrate/cofactor | PN decay, \(k_{\text{obs}}\) | iNOSoxy-induced, \(k_{\text{act}}\) |
|----|-------------------|-----------------|-----------------|
| 7.4| H_4B              | 4.8 ± 0.2       | 20 ± 1          |
| 7.4| Arg               | 0.15 ± 0.03     | 8.5 ± 1         |
| 7.4| Arg + citrulline  | 0.16 ± 0.01     | 7.0 ± 0.5       |
| 7.4| Arg + H_4B       | 0.12 ± 0.01     | 1.8 ± 0.3       |

*Corresponds to experiments performed in the absence of substrate and cofactor.

FIGURE 1. Kinetics of peroxynitrite decomposition activated by iNOSoxy. 100 μM peroxynitrite was added to a solution containing increasing concentrations of iNOSoxy. A, peroxynitrite decomposition kinetics. Inset: peroxynitrite decay was monitored at 302 nm for increasing concentrations of iNOSoxy (a, b; 0.5 μM, B, 1 μM, C, 2 μM, D, 4 μM). Kinetics was fitted to a mono-exponential function. The results were presented in Table 1. B, hemin spectral changes of iNOSoxy during peroxynitrite decomposition. Inset: superimposed difference spectra of iNOSoxy (with initial spectrum as reference) for time points between 12 and 324 ms. Main panel: kinetic traces of absorbance at 302 nm (peroxynitrite) and 420 nm and 445 nm (iNOSoxy Soret absorption band).

The decomposition of peroxynitrite (kinetic trace at 302 nm, Fig. 1B) coincides with a shift of the iNOSoxy hemin Soret maximum from 420 nm (corresponding to the resting ferric low spin six-coordinated species) to 445 nm (Fig. 1B). This transition is reversible: as PN becomes consumed, the iNOSoxy hemin Soret band shifts back to ~420 nm indicating that a ferric low spin form of iNOSoxy is regenerated (Fig. 1B). The weaker absorbance of the final iNOSoxy species at 420 nm suggests that initial native enzyme has been modified upon its reaction with PN (see “Discussion”). Because PN decay is kinetically coupled to the build-up of a new iNOSoxy intermediate with a modified heme group, this activation of PN is likely to involve the iNOS heme. These results suggest that the iNOSoxy heme binds PN and activates its decomposition in a pH-dependent manner.

Effects of Substrates and Cofactor on PN Activation by iNOSoxy—We repeated the previous experiment at pH 7.4 in the presence of various combinations of l-arginine (Arg), citrulline, and H_4B. For each combination, apparent PN decay rates were plotted as a function of enzyme concentration (Fig. 2A). No significant changes were observed for the rates of spontaneous decomposition of PN in the presence of substrates, products, or cofactor (Table 1). This suggests that the interaction between peroxynitrite and these compounds is kinetically negligible under our experimental conditions. The kinetics of peroxynitrite activation by iNOSoxy remain unchanged upon H_4B binding (\(k_{\text{act}} = (22 ± 2) \times 10^4 \text{M}^{-1}\text{s}^{-1}\), Table 1). In contrast, we observed dramatic changes upon Arg and citrulline binding for which the apparent activation rate constants were determined at (8.5 ± 10^4) M^{-1}s^{-1} and (7 ± 0.5) \times 10^4 M^{-1}s^{-1}, respectively. This effect was even amplified when both Arg and H_4B were bound to iNOSoxy (\(k_{\text{act}} = (1.8 ± 0.3) \times 10^4 \text{M}^{-1}\text{s}^{-1}\)). Thus, although H_4B binding seems unable to affect iNOSoxy capacity to activate PN decomposition, the binding of Arg and citrulline at the distal side of the heme pocket drastically reduces PN activation.

Effects of iNOSoxy on Peroxynitrite Decomposition in the Presence of CO_2—Under physiological conditions, PN reacts with CO_2 to form the nitrosoperoxycarbonate anion (NPC) (39, 56). To complete the previous experiments, done in CO_2-free degassed conditions, we investigated the reactivity of peroxynitrite with iNOSoxy in the presence of CO_2. CO_2 was included in iNOSoxy solutions via the addition of sodium bicarbonate (see “Experimental Procedures”). With 0.1 mM CO_2, kinetic traces of peroxynitrite decay monitored at 302 nm exhibit two distinct phases (spectra not shown), which could be fitted to a double-exponential function. As observed by Lymar and Hurst (57), two PN decay phases are observed when CO_2 concentrations are (sub)stoichiometric with respect to peroxynitrite. The fast phase corresponds to the reaction of PN with CO_2, whereas the slow phase, that appears when all CO_2 is consumed, corresponds to the decay of free peroxynitrite with only trace amounts of CO_2 slowly regenerated from carbonate. Apparent rate constants of both phases were plotted as a function of iNOSoxy concentration. The resulting curves were fitted to a linear function.
**TABLE 2**

Effects of substrate and cofactor on peroxynitrite activation by iNOSoxy in the presence of CO$_2$.

| Substrate/Cofactor | k$_{act}$ (Slow phase) | k$_{act}$ (Fast phase) |
|--------------------|------------------------|------------------------|
| +Arg               | 0.61 ± 0.04 s$^{-1}$   | 2.8 ± 0.2 s$^{-1}$    |
| +H$_4$B            | 0.57 ± 0.08 s$^{-1}$   | 2.6 ± 0.1 s$^{-1}$    |
| +Arg + H$_4$B      | 0.83 ± 0.04 s$^{-1}$   | 2.6 ± 0.2 s$^{-1}$    |
| +Arg + H$_4$B      | 0.33 ± 0.06 s$^{-1}$   | 1.5 ± 0.04 s$^{-1}$   |

For the fast phase (in the presence of CO$_2$), we observed that iNOSoxy still increases the rate of peroxynitrite decomposition (Fig. 2B, filled squares). This phase is characterized by a 30-fold faster spontaneous decay (2.8 s$^{-1}$), confirming the participation of CO$_2$ and an iNOSoxy-dependent activation rate (k$_{act} = (62 ± 8) \times 10^4$ M$^{-1}$ s$^{-1}$) three times greater than that observed in the absence of CO$_2$. The slow phase kinetics (after consumption of the initial pool of CO$_2$) were also dependent on iNOSoxy concentration (data not shown), but the iNOS-dependent activation rate was found to be similar to the one reported for peroxynitrite decay in the absence of CO$_2$ (k$_{act} = (22 ± 2) \times 10^4$ M$^{-1}$ s$^{-1}$).

We repeated these experiments in the presence of different combinations of Arg and H$_4$B using the same protocol. In these conditions both phases were still observed. The effects of Arg and H$_4$B on the kinetics of the fast phase (in the presence of CO$_2$) are displayed in Fig. 2B: we did not observe any major effect of Arg or H$_4$B binding alone on the iNOSoxy-induced peroxynitrite activation rate in the presence of CO$_2$. However, the addition of both Arg and H$_4$B substantially inhibited the effect of iNOSoxy on peroxynitrite decomposition rates in the presence of CO$_2$ (Table 2). The kinetic characteristics of the slow phase remained more similar to those determined in the absence of CO$_2$. Fig. 2C summarizes the values of PN activation rates by iNOSoxy obtained in the presence and in the absence of CO$_2$ as a function of the presence of substrate and cofactor (values from Table 2). These data suggest that iNOSoxy is able to activate peroxynitrite either in the absence and presence of CO$_2$.

**Two-electron Oxidative Properties of iNOS-activated Peroxynitrite**—Among the various chemical reactivities of peroxynitrite, we investigated those favored upon iNOSoxy activation. We first compared the two-electron oxidative properties of free and iNOS-activated peroxynitrite using the fluorescent probe DHR. DHR was mixed with increasing concentrations of PN (see “Experimental Procedures”). Reaction of PN with DHR leads to the two-electron oxidative formation of rhodamine, with characteristic fluorescence spectral properties ($\lambda_{ex} = 500$ nm with maximum emission around 525 nm; Fig. 3A, inset).
Rhodamine production was plotted as a function of PN concentration, and the resulting curve was fitted to a linear function (Fig. 3A, filled squares). We performed the same experiment in the presence of 20 µM iNOSoxy that were added to the DHR solution before PN addition (Fig. 3A, open squares). We observed in these conditions that the oxidation of DHR was diminished by 60%. This decrease was not linked to the quenching of rhodamine fluorescence by photon re-absorption due to iNOSoxy, because the addition of iNOSoxy after the reaction does not change the sample fluorescence properties, *i.e.* rhodamine fluorescence intensity (data not shown). Addition of decomposed PN, in the absence or presence of iNOSoxy, did not modify the observed sample fluorescence properties (data not shown), which confirms the absence of reaction between DHR and the PN decomposition products. We performed the same experiments in the presence of 1 mM CO₂ (Fig. 3A, filled circles). We observed a lowering of DHR oxidation by 70%, which indicates that CO₂, as expected, significantly decreased the two-electron oxidative power of peroxynitrite. Thus, iNOSoxy seems to exhibit the same capacity as CO₂ to suppress the two-electron oxidative properties of peroxynitrite.

The cumulative presence of both iNOSoxy and CO₂ did not provide an additional decrease in DHR oxidation by peroxynitrite (73% decrease of rhodamine production). This suggests that, although iNOSoxy remains able to activate peroxynitrite in the presence of CO₂, it might not be able to further change its reactivity.

**One-electron Oxidative Properties of iNOS-activated Peroxynitrite**—We analyzed the effects of iNOSoxy on the one-electron oxidative properties of peroxynitrite using HPA as a probe. One-electron oxidation of HPA by peroxynitrite leads to HPA dimerization into di-HPA, which is characterized by a specific fluorescence profile (λ<sub>ex</sub> = 326 nm with maximum emission ~405 nm; Fig. 3B, inset). HPA was mixed with increasing concentrations of peroxynitrite. Fluorescence intensities at 405 nm were plotted as a function of peroxynitrite concentration, and the resulting curve was fitted to a linear function (Fig. 3B, filled squares). We observed an increase in di-HPA production upon increase of peroxynitrite concentration. This effect was specific to peroxynitrite activity, because addition of decomposed peroxynitrite did not induce the production of di-HPA (data not shown). In the presence of iNOSoxy, the di-HPA production was enhanced by 40% (open squares). The absence of changes in fluorescence, when iNOSoxy was added after the reaction with peroxynitrite was completed, suggests that the enhancement of di-HPA production is related to the activation of PN by iNOSoxy.

The presence of CO₂ also induced an increase of di-HPA production (Fig. 3B, filled circles). The ratio between both slopes indicates that CO₂ enhances HPA dimerization by 68%. Once again, no additive effect was observed when both iNOSoxy and CO₂ were present in the milieu (filled squares). This series of experiments suggests that, like CO₂, iNOSoxy promotes peroxynitrite one-electron oxidation activity.

**Nitrative Properties of iNOSoxy-activated Peroxynitrite**—The reaction samples described in the preceding section were analyzed by reversed-phase HPLC to investigate the production of 4-hydroxy-3-nitrophenylacetic acid (HPA-NO₂). Injections of standard samples of HPA, HPA-NO₂, and HPA-OH allowed us to identify and quantify the production of HPA metabolites upon reaction with peroxynitrite (see "Experimental Procedures"). Fig. 4A exhibits a representative chromatogram of the elution of HPA metabolites when HPA was allowed to react with 2 mM PN in the presence of 20 µM iNOSoxy. In the absence of iNOSoxy, the reaction of HPA with PN did not lead to HPA-OH production. The main metabolite observed by HPLC was HPA-NO₂ (Fig. 4A). The production of HPA-NO₂ was assessed using its characteristic absorbance properties at 280 and 365 nm (see "Experimental Procedures") and plotted as a function of the concentration of added PN (Fig. 4B, open
squares). This production is specific to PN reactivity, because the chromatogram did not show any production of HPA-NO₂ or HPA-OH upon reaction of decomposed PN with HPA (data not shown). When HPA was allowed to react with PN in the presence of iNOSoxy, we observed a significant increase in HPA-NO₂ production (Fig. 4B, filled circles) with no observable production of HPA-OH. Because the same experiment performed with decomposed peroxynitrite did not lead to any HPA-NO₂ production (data not shown), this increase may have been due to an activation of peroxynitrite by iNOSoxy. The comparison of the slopes of HPA-NO₂ production in the presence or absence of iNOSoxy shows an enhancement of the production of HPA-NO₂ by 90% upon iNOSoxy addition. This series of experiments shows that iNOSoxy drastically modifies peroxynitrite reactivity favoring its nitrating and one-electron oxidizing activities.

**Effects of Activated Peroxynitrite on iNOSoxy Functioning**

iNOSoxy (1.5 mM) was mixed with successive aliquot additions of PN (PN concentration in each aliquot was 6 mM) in 100 mM KPO₄, pH 7.4, buffer (see “Experimental Procedures”). Fig. 5A shows the effect of these successive aliquot additions of PN on iNOSoxy ability to synthesize NO (histogram). We observed a significant inhibition of iNOSoxy activity with the increasing number of PN aliquots added. We also analyzed the effect of such successive additions of excess peroxynitrite on the ability of iNOSoxy to activate PN decomposition. iNOSoxy was mixed with successive aliquot additions of PN (final pH 7.4) using the same [iNOSoxy]/[PN] ratio. Kinetics of PN decay were monitored at 302 nm and fitted to a mono-exponential function. The apparent rate of peroxynitrite decomposition was plotted as a function of the number of peroxynitrite aliquot additions (stars). Bolus addition corresponds to a peroxynitrite concentration of 40-fold iNOSoxy concentration. Western blotting analysis of nitrotyrosine formation in iNOSoxy exposed to iterative additions of peroxynitrite. Conditions are described under “Experimental Procedures.” Starting from left, samples correspond to 0, 1, 3, 5, 8, and 10 iterative additions of 6 μM PN aliquots (final enzyme concentration was 1.5 μM), 60 μM PN (bolus addition), and 60 μM addition of decomposed PN. Each blot was quantified using ImageMaster TotalLab.
Arg and H$_4$B. This suggests that the loss of iNOSoxy function is directly correlated with iNOSoxy-activated peroxynitrite.

The PN-treated samples used for NOS activity assay were also analyzed by immunoblotting using anti-nitrotyrosine antibodies to look for potential nitration of tyrosine residues (see “Experimental Procedures”). Fig. 5B displays the results of the Western blot experiments on iNOSoxy treated with successive additions of peroxynitrite. As observed, the extent of tyrosine nitration increased with the number of PN aliquot additions. This signal vanished when the nitrosytrosine-containing samples were pretreated with sodium dithionite, which reduced nitrotyrosine into aminotyrosine (data not shown). In addition, no nitrotyrosine signal was observed upon peroxynitrite addition when the enzyme was incubated with Arg and H$_4$B. Because the binding of Arg also suppresses peroxynitrite activation by iNOSoxy and the enhancement of HPA nitration, it is likely that activation of peroxynitrite by iNOSoxy leads to an increased nitration of endogenous tyrosine that could result in iNOSoxy self-inhibition.

**DISCUSSION**

Hemoproteins have been shown to interact with peroxynitrite, to accelerate its decomposition, and to modify its chemical reactivity. Among them, NOSs have the unique ability to produce peroxynitrite. Because they can catalyze both the production and activation of peroxynitrite, NOSs could play a crucial role in the control of PN bioactivity. This report describes the first attempt to fully investigate the interaction between the iNOS oxygenase domain and peroxynitrite. We kinetically characterized the reaction between the iNOSoxy catalytic site and peroxynitrite and showed that iNOSoxy accelerates PN decomposition in a pH-dependent manner. We observed drastic changes in PN reactivity upon its activation by iNOSoxy. iNOSoxy-dependent activation of PN seems to lead to important structural modifications of the iNOSoxy catalytic site and to the inhibition of iNOS activity.

**Kinetic Analysis of PN Decay Activation**—We based the method of our investigation on the pioneering work of Groves and co-workers who first studied the kinetics and mechanism of interaction between PN and metalloporphyrins (58–60). This work was extended to metalloproteins by Herold and colleagues (43, 46), who showed that Mb and Hb catalyze the isomerization of PN in a way that is reminiscent of what had been observed for metalloporphyrins (61). In this report we used similar approaches to study the effects of iNOS oxygenase domain on PN decomposition. Our results clearly show that iNOSoxy, in its resting ferric state, increases the decay rate of peroxynitrite in a concentration-dependent manner, as has been reported for other hemoproteins, such as met-Mb and met-Hb (43), HRP (48, 62), chloroperoxidase (62), lactoperoxidase (48), myeloperoxidase (48), and cytochromes P450$_{BM3}$ (49), P450$_{NOR}$ (50, 62), and P450$_{Cam}$ (42, 47, 48, 62). The involvement of the heme in the activation of PN decay has been observed for all of these hemoproteins (42). For P450$_{BM3}$, P450$_{Cam}$, and myeloperoxidase, substrate addition (respectively, palmitate, camphor, and chloride) suppresses PN activation (47–49). We observed the same phenomenon for iNOSoxy: PN activation could be abolished as the heme accessibility is partially blocked by the presence of substrate or end-products, although Arg and citrulline effects on PN activation might also result from additional changes of heme pocket environment (due to monomer/dimer equilibrium, changes in the polarity of the distal side, changes in heme redox potential, etc.). Additionally we observed heme spectral changes upon PN addition that are kinetically coupled to PN decay: native iNOSoxy converts into an intermediate complex that progressively disappears as PN is consumed. This clearly confirms the involvement of iNOSoxy heme in PN decay activation.

We also found that the reaction of PN with iNOSoxy is pH-dependent with faster PN activation at low pH, which is similar to previous reports for globins (43) or P450$_{Cam}$ (47). Although this suggests that the reacting species is peroxynitrous acid (ONOOH), the mechanism proposed for these proteins still assumes the initial binding of peroxynitrite anion (ONOO$^-$) to the heme (42, 43). To explain this counterintuitive pH dependence, several hypotheses have been proposed: (i) the release of the water molecule, bound to the heme Fe$^{3+}$ atom as the sixth ligand, is required prior to PN binding. Because water binding is stronger at alkaline pH, a lower pH would facilitate water release and lead to a faster rate of PN binding and activation and (ii) PN activation by hemiolsiates such as the cytochrome P450 family of enzymes is believed to depend on the electron-donation properties of the proximal ligand; therefore, changes in pH could change the protonation state of the proximal cysteine and thus modify its ability to enhance PN decomposition. In parallel, one cannot exclude that a second and rate-limiting reaction might be taking place in large excess of PN, such as what has been described for metalloporphyrins (58). In our case, this additional reaction could involve ONOOH and would explain the observed pH dependence. All these hypotheses remain to be tested in the case of iNOSoxy.

**Analysis of PN Activation Products**—Our results indicate that an important flux of peroxynitrite is reacting with iNOSoxy. But unlike what has been reported for Mb and Hb (43), this flux of PN will not be scavenged and isomerized into nitrate. Instead, PN reactivity is enhanced by iNOSoxy action. Although its two-electron oxidation power is abolished, we observed a significant increase (up to 90%) in one-electron oxidation and in nitration activities. Thus, like what has been reported for P450 proteins (47, 50), iNOSoxy channels peroxynitrite toward one-electron chemistry processes and the nitration of exogenous phenol probes. This behavior is also reminiscent of the effect of CO$_2$ on PN reactivity (39, 63). Indeed, our results show the exact same effect of CO$_2$ and iNOSoxy; i.e. they both channel peroxynitrite toward a nitrative process and consequently diminish its two-electron oxidation power. The reactivity of iNOSoxy-activated PN therefore suggests a mechanism with electron or H* abstraction followed by recombination of one phenol radical with the NO$_2$ radical within the heme pocket, leading to the nitration of the phenolic probe. Because the production of NO$_2$ (upon PN homolytic cleavage) and of the phenol radical (upon oxidation by the resulting o xo-ferryl complex) does not occur concomitantly, each radical might escape the heme pocket before recombination can occur. In that case two phenol radicals could recombine and dimerize. Our results suggest that nitration is partic-
in iNOS.

**Mechanism of PN Activation**—The fact that iNOSoxy favors one-electron processes suggests that, like CO₂ and other heme-proteins, iNOSoxy may promote the homolytic cleavage of peroxynitrite. As has been described for hemiothiolate proteins, PN would bind to the Fe³⁺ atom of the heme, followed by the homolytic cleavage of the O-ONO bond, which would lead to the build-up of an oxo-ferryl complex and the production of an NO₂ molecule (50). Two important observations in our work support this mechanism: (i) we observed significant nitration of the iNOSoxy and of the external probes, as would be expected if NO₂ were generated; (ii) in our stopped-flow experiments we observed the steady-state build-up of an intermediate species that exhibits the characteristics of an oxo-ferryl complex. The Soret band of this species is red-shifted with a lower extinction coefficient (supplemental Fig. S1), which is compatible with the formation of a “Compound II-like” intermediate species, such as the one described for chloroperoxidase (64) or proposed for P450_{NOR} (50).

Our set of data leads to a simple mechanism that is depicted in Scheme 3. Peroxynitrite anion first binds to the ferric heme. Then PN undergoes an iron-catalyzed homolytic cleavage that leads to the build-up of an oxo-ferryl complex and the release of an NO₂ radical. This will lead to the one-electron oxidation or to the nitration of exogenous compounds within the heme pocket, or to the release of NO₂ in the medium. Ferric heme is then regenerated and available for another cycle of PN activation. In the presence of Arg, we did not observe any build-up of such an oxidative intermediate (data not shown). This coincides with the quasi-absence of PN activation, and, in these conditions, we did not observe any change in the nitration of iNOS.

As we mentioned above, the pH dependence of the observed PN activation suggests an interaction between the peroxynitrous acid and the heme that is in contradiction with the initial binding of a peroxynitrite anion. One explanation would reside in the fact that our experiments are performed in excess of PN. Under these conditions, we were unable to determine the nature of the rate-limiting step and we cannot exclude secondary reactions with free ONOOH. Although our results clearly indicate that the pathway described in Scheme 3 is the dominant one, our model has therefore to be incorporated into a more general scheme. We are currently analyzing PN decomposition kinetics for several conditions of PN/iNOSoxy ratio to investigate the existence of additional activation pathways and to precisely determine the rate-limiting step of each PN-activation cycle.

**PN Activation in the Presence of CO₂**—Peroxynitrite reactivity, in vivo, is directly linked to its reactivity toward CO₂. In the presence of CO₂, PN is rapidly converted into NPC anion. CO₂ acts as a Lewis acid and favors the homolytic cleavage of peroxynitrite into NO₂ and CO₂ radicals. In our kinetic experiments, (sub)stoichiometric concentrations of CO₂ were added to the enzyme solution. Upon rapid-mixing, PN will react with all available CO₂, until all the CO₂ is consumed. Remaining PN will then freely decompose or react with trace amounts of CO₂ slowly regenerated from carbonate. This leads to biphasic kinetics of PN decomposition. This biphasicity was observed in the presence of any concentration of iNOSoxy, and the rates of both phases were shown to increase with iNOSoxy concentration. This indicates that iNOS remains able to activate peroxynitrite decomposition in the presence of CO₂. Because the increase in PN decomposition rate observed in the presence of CO₂ is linked to the build-up of NPC, this suggests an interaction between iNOSoxy and NPC. This type of interaction has been already observed by Herold and colleagues who reported a faster rate of PN decay, in the presence of CO₂, probably linked to NPC build-up, and an activation of this rate by Hb and Mb (43).

In the presence of excess CO₂, peroxynitrite will be completely converted into NPC. Under these conditions, we did not observe any change in PN global reactivity, which means that iNOSoxy is unable to further modify the reactivity of NPC. This was expected: because CO₂ already acts as a Lewis acid, there cannot be any further O-ONO bond cleavage activation by iNOSoxy, leaving the PN apparent reactivity unchanged.

**Consequences on NOS Role and Functioning**—Unlike what has been reported for other hemoproteins (42, 50), the cumulative effects of PN activation via the addition of successive PN aliquots were found to directly lead to iNOSoxy inhibition. The inhibition of NOSs by the addition of a large bolus of PN has already been described, but the reports disagree on the extent, the characteristics, and the explanations for this inhibition (51, 65–68).

Herein we report evidence that this inhibition is linked to the specific nitration or one-electron oxidation of iNOSoxy and, moreover, that it is dependent on the enzyme itself. We observed that iNOSoxy NOS activity was inhibited after successive cycles of PN activation, which was not observed when peroxynitrite activation was suppressed upon Arg binding. This inhibition is concomitant with the loss of iNOSoxy ability to activate PN. The apparent pseudo-first-order profile of PN decay kinetics suggests that iNOS inhibition occurs in a second phase, after PN decomposition is completed. This self-inhibition did not correspond to the degradation of the protein or the loss of the heme. Resonance Raman and UV-visible absorption spectroscopies confirmed that, after several cycles of PN acti-

---

**Scheme 3. Model for peroxynitrite activation by iNOS oxygenase domain.** After water release from the native enzyme, peroxynitrite anion binds to the ferric heme. This intermediate undergoes a homolytic cleavage that leads to build-up of an oxo-ferryl complex and the release of a NO₂ radical. This will channel PN reactivity toward one-electron processes such as nitration or one-electron oxidation. NO₂ can also freely diffuse out of the heme pocket and react with other biomolecules.
vation the environment of the heme is modified but the porphyrin itself remains bound to iNOS and apparently unaltered. Nonetheless, this loss of activity seems directly correlated to functional modifications in the surroundings of the heme pocket. Our data suggest that this autoinhibition pattern corresponds to the self-nitration of tyrosine residues, a property that has already been reported for other hemothiolate proteins such as cytochromes $P_450_{BM3}$ and $P_450_{Cam}$ (42, 47, 49, 51).

Moreover, we showed that PN one-electron oxidation activity was also enhanced by iNOSoxy. The loss of activity could therefore result from the oxidation of several key residues such as the proximal cysteine. This is supported by the modification of the UV-visible absorption spectra of iNOSoxy after several PN activation cycles (10-nm shift of the Soret band, data not shown) that has been already reported for iNOS (67) and that accounts for the decrease in 420 nm absorption observed by stopped-flow (Fig. 1B). The oxidation could also target the zinc tetrathiolate moiety at the dimer interface and induce monomerization or irreversible dimerization of iNOSoxy as observed by Zou et al. (68, 69).

Modification of the monomer/dimer equilibrium is known to influence iNOSoxy activity (70). In our case, this monomer/dimer equilibrium did not seem to play a role in iNOSoxy ability to activate PN. We observed PN activation when iNOSoxy was in equilibrium between monomers ↔ loose dimers ↔ tight dimers (in the absence of substrate and cofactor) but also when iNOSoxy was purely a tight dimer, such as in the presence of $H_4B$. Besides, for NOS isoforms that remain dimeric in the absence of $H_4B$, the same PN activation was observed. Therefore, the quaternary structure of iNOSoxy does not seem to intervene in iNOSoxy activity to activate PN. Moreover, in the presence of Arg, in which case PN activation is repressed but the zinc tetrathiolate is not protected, we did not observe any inhibition of iNOSoxy. This suggests that oxidation of the zinc tetrathiolate complex or the formation of inter-dimer sulfide bonds is not responsible for the loss of activity of iNOSoxy. We are currently investigating the exact nature of the structural modifications (i.e., oxidation versus nitration) that are linked to iNOSoxy inhibition.

Physiological Relevance of PN Activation by iNOS—The characteristics of the interaction between PN and hemoproteins vary with the nature of the protein. The interaction of PN with $P_450_{BM3}$ is very fast (49) and leads to an increase in PN oxidative and nitrative power. This interaction can be rather slow as in the case of Mb and Hb (43), for which a PN-scavenging role of PN occurs.

REFERENCES

1. Lincoln, J., Hoyle, C. H. V., and Burnstock, G. (1997) Nitric Oxide in Health and Disease, Cambridge University Press, UK
2. Ignarro, L. J. (2000) Nitric Oxide: Biology and Pathobiology (Ignarro, L. J., ed) Academic Press, San Diego
3. Dawson, V. L., and Dawson, T. M. (1998) Prog. Brain Res. 118, 215–229
4. Mungrue, I. N., and Bredt, D. S. (2004) J. Cell Sci. 117, 2627–2629
5. Bredt, D. S. (2003) J. Cell Sci. 116, 9–15
6. Sessa, W. C. (2004) J. Cell Sci. 117, 2427–2429
7. MacMicking, J., Xie, Q.-W., and Nathan, C. (1997) Annu. Rev. Immunol. 15, 323–350
8. Lowenstein, C. J., and Padalino, E. (2004) J. Cell Sci. 117, 2865–2867
9. Bogdan, C. (2001) Nat. Immunol. 2, 907–916
10. Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001) Biochem. J. 353, 593–615
11. Ghosh, D. K., and Salerno, J. C. (2003) Front. Biosci. 8, d193–d209
12. Stuehr, D. J. (1999) Biochim. Biophys. Acta 1411, 217–230
13. Li, H., and Poulos, T. L. (2005) J. Inorg. Biochem. 99, 293–305
14. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. I., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
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. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998) Cell 95, 939–950
17. Garcin, E. D., Bruns, C. M., Lloyd, S. J., Hosfield, D. J., Tiso, M., Gachhui, R., Stuehr, D. J., Tainer, J. A., and Getzoff, E. D. (2004) J. Biol. Chem. 279, 37918–37927
18. Roman, L. J., Martasek, P., and Masters, B. S. (2002) Chem. Rev. 102, 1179–1190
19. Abu-Soud, H. M., Presta, A., Mayer, B., and Stuehr, D. J. (1997) Biochemistry 36, 10811–10816
20. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseeman, J. (1991) J. Biol. Chem. 266, 6259–6263
21. Meunier, B., de Visser, S. P., and Shaik, S. (2004) Chem. Rev. 104, 3947–3980
22. Denisov, I. G., Makris, T. M., Sligar, S. G., and Schlichting, I. (2005) Chem. Rev. 105, 2253–2277
23. Stuehr, D. J., Santolini, J., Wang, Z. Q., Wei, C. C., and Adak, S. (2004) J. Biol. Chem. 279, 36167–36170
24. Santolini, J., Adak, S., Curran, C. M., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 1233–1243
25. Negererie, M., Berka, V., Vos, M. H., Liebl, U., Lambry, J. C., Tsai, A. L., and Martin, J. L. (1999) J. Biol. Chem. 274, 24694–24702
26. Santolini, J., Meade, A. L., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 48887–48898
27. Arnold, E. V., and Bohle, D. S. (1996) Methods Enzymol. 269, 41–55
28. Herold, S., and Rock, G. (2005) Biochemistry 44, 6223–6231

A. Maréchal, T. A. Mattioli, D. J. Stuehr, and J. Santolini, manuscript in preparation.
iNOS Enhances Peroxynitrite Oxidative and Nitrative Potency

29. Andersen, H. J., and Skibsted, L. H. (1992) J. Agric. Food Chem. 40, 1741–1750
30. Herold, S. (1998) FEBS Lett. 439, 85–88
31. Stuehr, D., Pou, S., and Rosen, G. M. (2001) J. Biol. Chem. 276, 14533–14536
32. Rosen, G. M., Tsai, P., and Pou, S. (2002) Chem. Rev. 102, 1191–1200
33. Rusche, K. M., Spiering, M. M., and Marletta, M. A. (1998) Biochemistry 37, 15503–15512
34. Adak, S., Wang, Q., and Stuehr, D. J. (2000) J. Biol. Chem. 275, 33554–33561
35. Ishimura, Y., Gao, Y. T., Panda, S. P., Roman, L. J., Masters, B. S., and Weintraub, S. T. (2005) Biochem. Biophys. Res. Commun. 338, 543–549
36. Pfeiffer, S., Lass, A., Schmidt, K., and Mayer, B. (2001) FASEB J. 15, 2355–2364
37. Beckman, J. S., and Koppenol, W. H. (1996) Am. J. Physiol. 271, C1424–C1437
38. Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M., and Cayota, A. (2001) Free Radic. Biol. Med. 30, 463–488
39. Goldstein, S., Lind, J., and Mersony, G. (2005) Chem. Rev. 105, 2457–2470
40. Koppenol, W. H. (1998) Free Radic Biol. Med. 25, 385–391
41. Daiber, A., and Ullrich, V. (2002) Methods Enzymol. 359, 379–389
42. Herold, S., and Shivashankar, K. (2003) Biochemistry 42, 13460–13472
43. Herold, S., Matsui, T., and Watanabe, Y. (2001) J. Am. Chem. Soc. 123, 4085–4086
44. Herold, S., and Fago, A. (2005) Comp. Biochem. Physiol. A Mol. Integr. Physiol. 142, 124–129
45. Daiber, A., Schöneich, C., Schmidt, P., Jung, C., and Ullrich, V. (2000) J. Inorg. Biochem. 81, 213–220
46. Floris, R., Piersma, S. R., Yang, G., Jones, P., and Weaver, R. (1993) Eur. J. Biochem. 215, 767–775
47. Daiber, A., Herold, S., Schöneich, C., Namgaladze, D., Peterson, J. A., and Ullrich, V. (2000) Eur. J. Biochem. 267, 6729–6739
48. Mehl, M., Daiber, A., Herold, S., Shoun, H., and Ullrich, V. (1999) Nitric Oxide 3, 142–152
49. Mehl, M., Daiber, A., Herold, S., Shoun, H., and Ullrich, V. (2004) Biochim. Biophys. Res. Commun. 317, 873–881
50. Herold, S., Exner, M., and Boccini, F. (2003) Chem. Res. Toxicol. 16, 390–402
51. Ghosh, D. K., Crane, B. R., Ghosh, S., Wolan, D., Gachhui, R., Crooks, C., Presta, A., Tainer, J. A., Getzoff, E. D., and Stuehr, D. J. (1992) EMBO J. 11, 6260–6270
52. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550
53. Titheradge, M. A. (1998) Nitric Oxide Protocols: Methods in Molecular Biology pp. 83–91, Totowa, NJ
54. Meli, R., Nauser, T., Latal, P., and Koppenol, W. H. (2002) J. Biol. Inorg. Chem. 7, 31–36
55. Lymar, S. V., and Hurst, J. K. (1995) J. Am. Chem. Soc. 117, 8867–8868
56. Lee, J. B., Hunt, J. A., and Groves, J. T. (1998) J. Am. Chem. Soc. 120, 7493–7501
57. Lee, J. B., Hunt, J. A., and Groves, J. T. (1998) J. Am. Chem. Soc. 120, 6053–6061
58. Zou, M. H., Daiber, A., Peterson, J. A., Shoun, H., and Ullrich, V. (2000) Arch. Biochem. Biophys. 376, 149–155
59. Zou, M. H., Hou, X. Y., Shi, C. M., Nagata, D., Walsh, K., and Cohen, R. A. (2002) Arch. Biochem. Biophys. 376, 9749–9754
60. Nakajima, R., Yamazaki, L., and Griffin, B. W. (1985) Biochim. Biophys. Res. Commun. 128, 1–6
61. Pasquet, J. P., Zou, M. H., and Ullrich, V. (1996) Biochimie (Paris) 78, 785–791
62. Huhmer, A. F., Gerber, N. C., de Montellano, P. R., and Schöneich, C. (1996) Chem. Res. Toxicol. 9, 484–491
63. Huhmer, A. F., Nishida, C. R., Ortiz de Montellano, P. R., and Schöneich, C. (1997) Chem. Res. Toxicol. 10, 618–626
64. Zou, M. H., Hou, X. Y., Shi, C. M., Nagata, D., Walsh, K., and Cohen, R. A. (2002) J. Biol. Chem. 277, 32552–32557
65. Zou, M. H., Shi, C., and Cohen, R. A. (2002) J. Clin. Invest. 109, 817–826
66. Panda, K., Rosenfeld, R. J., Ghosh, S., Meade, A. L., Getzoff, E. D., and Stuehr, D. J. (2002) J. Biol. Chem. 277, 31020–31030