The nitric-oxide synthase (NOS) catalyzes the oxidation of L-arginine to L-citrulline and NO through consumption of oxygen bound to the heme. Because NO is produced close to the heme and may bind to it, its subsequent role in a regulatory mechanism should be scrutinized. We therefore examined the kinetics of NO rebinding after photodissociation in the heme pocket of human endothelial NOS by means of time-resolved absorption spectroscopy. We show that geminate recombination of NO indeed occurs and that this process is strongly modulated by L-Arg. This NO rebinding occurs in a multiphasic fashion and spans over 3 orders of magnitude. In both ferric and ferrous states of the heme, a fast nonexponential picosecond geminate rebinding first takes place followed by a slower nanosecond phase. The rates of both phases decreased, whereas their relative amplitudes are changed by the presence of L-Arg; the overall effect is a slow down of NO rebinding. For the isolated oxygenase domain, the picosecond rate is unchanged, but the relative amplitude of the nanosecond binding decreased. We assigned the nanosecond kinetic component to the rebinding of NO that is still located in the protein core but not in the heme pocket. The implications for a mechanism of regulation involving NO binding are discussed.

The enzyme nitric-oxide synthase (NOS) has been identified in mammals as three different isoforms, which have been cloned and expressed (1, 2). Although it is inducible in macrophages, the two other forms are constitutive in neurons and in endothelial cells. Its presence was also recently demonstrated in liver mitochondria (3). NOS isoforms catalyze the formation of highly diffusible NO and are involved in several physiological events (4–6). For instance, hypertension is associated with a deficiency of NO production, whereas excessive amounts of NO lead to hypotension. For blood pressure regulation, NO released from eNOS acts as an intermediate messenger in endothelial cells, targeting the enzyme guanylyl cyclase (7), which is activated and catalyzes the production of cGMP from GTP.

NOS catalyzes the oxidation of L-Arg to L-citrulline through O₂ consumption to produce NO in presence of NADPH, tetrahydrobiopterin, FAD, FMN, and Ca²⁺-calmodulin (8–10). The NOS structure is organized in two distinct domains, a reductase domain that binds FADN and FAD and an oxygenase domain that harbors the heme (iron-protoporphyrin IX) for O₂ ligation. The triggering role of calmodulin has been explained by a switch mechanism between the reductase and oxygenase domains mediating electron transfer from, respectively, NADPH to FAD, to FMN, and then to the heme (11, 12). In the resting state, iron is ferrie and five-coordinated with an axial ligand of Cys (13). The oxygenase domain can be expressed separately in an active form that binds cofactors (14). The formation of NO occurs in two steps and requires two molecular O₂ and 1.5 equivalent of NADPH (8, 15). N-Hydroxy-L-arginine is generated in the first stage of reaction and is subsequently further oxidized to the final products L-citrulline and NO. It has been shown by isotopic substitution that the nitrogen atom of NO comes from the NOH group of the intermediate N-hydroxy-L-arginine (8). The proposed reaction mechanisms involve the formation of a transient Fe²⁺ species before O₂ ligation and a Fe³⁺ one in the last step when L-citrulline and NO are formed (15, 16).

Several isoform-selective inhibitors of NOS are analogs of L-Arg with a modified guanidinium group such as propyl-L-Arg (17) or aminoguanidine (18), which may interfere with other L-Arg-utilizing enzymes. Because NOS possesses several cofactors and binds three substrates for NO synthesis, it may be regulated in different ways. This regulation is essential because NO is a very reactive species that cannot be stored. For therapeutics purposes, a detailed study of the regulation of the isoforms is of prime importance because inhibiting or enhancing the activity of one isoform without perturbing the others can be beneficial. A possible mechanism of regulation is through self-inhibition; although the NOH group from which NO is derived may not directly interact with the heme during oxidation of N-hydroxy-L-arginine, NO is released in close vicinity of the heme, and its subsequent binding to the heme must therefore be considered. Indeed, a 2-fold increase of the catalytic activity of the inducible murine NOS has been reported when oxy-hemoglobin, a NO scavenger, is present during the reaction cycle (19), but this effect was not observed for the constitutive murine neuronal NOS (20). It was shown that NO decreased nNOS activity (21) and that a ferric-nitrosyl complex formed during catalytic activity in the case of inducible NOS (19) as well as a ferrous-nitrosyl complex formed independently of exogenous NO concentration during activity of nNOS (20). Such observations are not yet reported for eNOS. Furthermore, the presence of NO scavengers such as oxymeloglobin or superoxide did not increase the activity of nNOS, nor...
did they preclude the formation of the nitrosyl complex. Because the use of NO scavengers allows investigation only of diffusion controlled mechanisms of inhibition, these observations suggest that two phases occur in self-inhibition of nNOS by NO, one of which involves a fast step in which NO binds to the heme right after synthesis, before diffusing out of the heme pocket. We addressed this issue at a molecular dynamics level by using time-resolved absorption spectroscopy, which powerfully relates structural properties and dynamical behavior. This is a particularly well suited technique because it allows simulation, by means of photodissociation, of the release of newly synthesized NO in the heme pocket. To follow the behavior of released NO and the parameters influencing the heme-NO interaction, we analyzed the rebinding of NO to eNOS after photolysis in both ferric and ferrous states of the heme and in the presence or absence of the substrate L-arginine.

**EXPERIMENTAL PROCEDURES**

L-Citrulline was from Alexis Biochemicals (San Diego, CA). L-[2,3,4,5-3H]Arginine was obtained from Amersham Pharmacia Biotech (specific activity, 77 Ci/mmol). [6R]-,5,6,7,8-Tetrahydro-L-biopterin (BH4) was obtained from Research Biochemical International. Gas was purchased from Alphagaz. All other chemicals used for purifications or spectroscopy were purchased from Sigma.

**Expression and Purification of eNOS and Oxygenase Domain of eNOS**—Recombinant human eNOS and oxygenase domain of eNOS were prepared using the baculovirus expression system. The BH4-depleted eNOS was expressed in *E. coli* (specific activity, 77 Ci/mmol). Expression and purification of recombinant proteins were essentially the same as described previously (14, 22). The purified enzyme preparations were stored at −80 °C.

**Biopterin Determination**—The BH4 content of purified eNOS and its oxygenase domain were measured as described previously (13) and quantitated from a standard curve of authentic BH4. The BH4 stoichiometry was determined to be 0.5–0.6 mol of BH4/mol of protein in eNOS and 0.3 mol of BH4/mol of oxygenase domain, respectively.

**Preparation of Samples for Spectroscopy**—After thawing, 100 µl of eNOS (60 µM) were put in a 1-mm optical path quartz cell sealed with a rubber stopper and immediately degassed with at least five cycles of vacuum and purging with argon (98.9999%). When indicated, 8 µl of 60 mM BH4 were added using a gas-tight syringe (Hamilton) previously rinsed with argon (final concentration, 5 mM; [NADPH]/[heme] = 1). The enzyme solution was prepared with Tris (pH 7.5, 0.15 M NaCl).

**RESULTS**

The equilibrium spectra of unliganded and NO-ligated species of eNOS are displayed together with the difference spectra in Fig. 1. When NO is unliganded, the Soret maximum is located at 398 nm for the oxidized form and at 412 nm for the NADPH-reduced form. Upon ligation of NO, the maximum is shifted to 441 and 434 nm for ferric and ferrous eNOS, respectively. The difference spectrum (Fig. 1A) for ferric eNOS discloses a characteristic minimum at 443 nm (shifted by 2 nm with respect to the maximum of NO-ligated eNOS Soret band). The Soret shift induced by NO binding is the opposite of that observed for histidine-coordinated iron proteins such as myoglobin or hemoglobin. When L-Arg is added to the ferric form (Fig. 1B), there is a slight shift from 398 to 395 nm, providing evidence for a modification of the heme electronic state.

The transient spectra (raw data) are shown in Fig. 2. Each of them corresponds to the calculated difference between a spectrum at the indicated time and the spectrum before photodissociation. For ferric eNOS alone, the transient spectra at all time points (Fig. 2, top panel) showed a minimum at the same position as the equilibrium spectrum (443 nm) corresponding to the photodissociation of NO. From 8 ps to 4 ns, the transient spectra have the same shape and exhibit a decreasing intensity because of rebinding of NO. The 2-ps spectrum has an isosbestic point around 423 nm slightly shifted by about 2 nm with respect to that of the longer time spectra. This is the only feature showing the presence of an intermediate before the rebinding of NO to the ferric ground state species. When L-Arg is bound to ferric eNOS, the transient spectrum at 2 ps displays absorption maxima centered at 415 and 465 nm. The isosbestic point moves from 428 nm at 2 ps to 423 nm at 4 ns, and the second isosbestic point, which was constant at 467 nm in the absence of L-Arg, is greatly displaced from 455 nm at 2 ps to 465 nm at 4 ns in the presence of L-Arg. These features are not observed in the case of ferrous eNOS alone. When NO is more pronounced in the 2-ps transient difference spectrum of L-Arg-bound and NADPH-reduced eNOS, indicating that the photodissociated excited state heme is different when the substrate L-Arg is bound. For ferrous eNOS, the amplitude of the transient difference spectra at 4 ns remains high, indicating a long-lived component. In all three conditions, the 8-ps to 4-ns spectra correspond to rebinding without contribution from transient excited species.

To disentangle the contributions from the different transient
species and their associated kinetics, we performed SVD analysis of the raw data. The 300-ps and the 4-ns time windows were recorded sequentially, and SVD analysis were performed separately. This resulted in two main spectral components displayed in Fig. 3 and compared with the equilibrium unliganded minus NO-liganded difference. Although a SVD spectral feature does not necessarily correspond to a particular chemical species, it does in this present case; for the three species, the spectral component having the highest singular value (SVD1) matches the deligandation difference equilibrium spectrum. The associated kinetics displayed in Fig. 4 thus represent the evolution of unliganded ground state eNOS and the subsequent rebinding of NO. In all cases this SVD component had the same spectrum in both time windows, which were therefore fit simultaneously to obtain kinetic parameters describing the overall spectral evolution. According to the physiological function of eNOS, the release of a certain amount of NO is expected to occur, and we thus needed a constant term for fitting the kinetics for the three conditions. The minimal model that we used for describing the kinetics of unliganded ground state and NO rebinding has up to three exponential decay components and a constant term. The decay components and constant term are associated with the same transient species, which is similar to the liganded-unliganded difference equilibrium spectrum (Fig. 3) having identical positions for minimum and isosbestic points. Therefore, these kinetic components represent different phases of NO rebinding for the three species. The parameters of the fits are listed in Table I.

In all cases a 5-ps rise was also present in the kinetics (not shown) associated with the SVD2 spectral component (Fig. 3) and is assigned to the formation of unliganded ground state species from the excited state. This component produced the change of isosbestic point near 423 nm seen in the raw spectra (Fig. 2) at 2 ps. The early time photophysics of eNOS heme will be discussed elsewhere.

**Ferric NOS**—The fit of kinetics associated with the SVD1 spectrum revealed three decay components and a constant (Fig. 4 and Table I) whose associated spectra are shown in Fig. 3A. The fast decay ($\tau_1 = 15$ ps) and the intermediate one ($\tau_2 = 200$ ps) are two phases of the recombination with NO still located in the heme pocket, as seen for other hemoproteins, and represent together 37% of the total rebinding. The long time component ($\tau_3 = 2$ ns) represents a rebinding phase of NO not necessarily located in the heme pocket. The constant term of 10% relative amplitude may be due either to a much longer time rebinding or to NO diffusing into the solution. About 34% of the photodissociated NO has rebound after 300 ps and 85% after 4 ns (Fig. 4).

**L-Arg-bound Ferric NOS**—When L-Arg is bound, the fit of kinetics also resulted in three decay terms. The SVD2 component has a spectrum quite different from that obtained in the absence of L-Arg (Fig. 3B), especially around 460 nm, and is the origin of the conspicuous shift of isosbestic point from 455 to 465 nm in the raw spectra (Fig. 2B). Compared with free eNOS, the presence of L-Arg increased the time constant of the fast NO-rebinding to 53 ps (Table I) and its relative amplitude 4-fold. Simultaneously, the amplitude $A_2$ of the intermediate rebinding phase was decreased 2-fold, whereas the long time constant increased dramatically to $\tau_3 = 17$ ns (it may also contain a constant term). Along with the increase of $A_1$, L-Arg binding resulted in the crossing of the normalized kinetics. The change of $\tau_1$ and $\tau_2$ is seen in the first time window (Fig. 4), whereas that of $\tau_3$ is evident in the second time window. About
36% of NO rebound after 300 ps and 50% after 4 ns. Thus the overall effect of L-Arg is a greatly reduced rate of recombination, whereas the phases are differently affected.

Importantly, there is no spectral SVD component (Fig. 3B) matching the difference spectrum of L-Arg deligation from eNOS (Fig. 1B); therefore we readily concluded that photodissociation of L-Arg did not occur and that rebinding of NO occurred when L-Arg remained bound. This also holds true for ferrous NOS.

NADPH-reduced NOS—For preparing ferrous eNOS we did not add calmodulin prior to adding NADPH. After reduction with NADPH the equilibrium spectrum discloses a Soret located at 412 nm, characteristic of unliganded reduced eNOS, similar to that obtained with sodium dithionite, which can reduce heme without calmodulin. Subsequent addition of NO shifted the Soret maximum of the NADPH-treated sample to 434 nm, again identical to that of eNOS reduced with sodium dithionite, and is clearly not the position for the NO complex of ferric eNOS (441 nm). A residual amount of calmodulin carried along the eNOS purification appears to sustain electron transfer from FMN to the heme (30). Because the reduction using NADPH may not lead to a full reduction of NOS (19), we expected that this sample contained an amount of unliganded

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**Fig. 2.** Transient spectra (raw data) for the NO rebinding for the three indicated species at four selected times after photodissociation of NO (spectra at 90 different time delays were recorded for each species).

**Fig. 3.** Spectra associated with the kinetic components from the SVD analysis for ferric eNOS (A), L-Arg-bound ferric eNOS (B), and L-Arg-bound NADPH-reduced eNOS (C). The SVD1 transient spectrum (solid line) corresponds to NO rebinding kinetics and is compared with the equilibrium difference spectrum (dotted line). SVD2 (dashed line) is associated with excited state decay. Each SVD1 component corresponds to one kinetic trace of Fig. 4A. In C, the rising edge at 400 nm in the equilibrium difference spectrum is due to NADPH depletion after addition of NO.
The transient spectra associated with these decays match those of unliganded NOS (solid line), ferric NOS (dotted line), and ferrous NOS with L-Arg (dashed line) and are compared with NO rebinding to ferric eNOS and to myoglobin. Note the difference in scale between B and C.

FIG. 4. A, normalized kinetics of NO rebinding associated with the first SVD spectral component in two time windows. In all cases, the transient spectra associated with these decays match those of unliganded minus NO-liganded eNOS equilibrium difference spectra. The parameters of fit are listed in Table I. The curves are, respectively, ferric NOS (solid line), L-Arg-bound ferric eNOS (dotted line), and L-Arg-bound NADPH-reduced eNOS (dashed line) and are compared with NO rebinding to myoglobin (dashed and dotted line); lines are the fit, and symbols are the results of the SVD analysis. B, distribution of rates for the three eNOS species from the MEM analysis of the same kinetic data. The curves have been normalized to the maximum values of \( a(k) \). C, comparison of the rate distribution for rebinding to ferric eNOS and to myoglobin. Note the difference in \( k \) scale between B and C.

oxidized heme, the absorption band of which is hidden by that of NADPH. The affinity for NO is higher for ferrous than for ferric eNOS (\( K_D = 65 \mu M \) and \(-\) mM for ferric and ferrous form, respectively\(^2\)), and we used a NO concentration of 0.5% in the gas phase; this gave an aqueous NO concentration of 0.5% in binding to ferric eNOS. Thus the fraction of the remaining ferric eNOS that is NO-ligated is negligible, and the NO rebinding kinetics is only due to ferrous eNOS. This is not true for the kinetics of excited states (SVD2 spectral component), which may be a mixture of contributions from both oxidized and reduced L-Arg-bound species.

As seen for the ferric eNOS, the NO rebinding proceeds with three exponential decays. The time constant of fast geminate rebinding decreased slightly for ferrous eNOS (\( \tau_1 = 36 \) ps) compared with ferric eNOS (\( \tau_1 = 53 \) ps). Interestingly, the relative amplitude of the picosecond components, associated with NO rebinding still located in heme pocket, decreased, whereas that of the long one increased (\( \tau_2 = 18 \) ns, \( A_2 = 0.63 \)). The multiple phases have time constants quite similar to those of L-Arg-bound ferric eNOS (Table I), but the general trend is a slightly slower rebinding than for ferric eNOS.

Because the combination of exponential values obtained from the fits are one of the most probable set, but not the unique solution, we analyzed the kinetics by another method: the MEM (28), which enables a better estimate of the uncertainty of the kinetic components. We obtained a rate distribution \( a(k) \) (Fig. 4B) for the three NO-ligated species. For the ferric NOS, three peaks are separated from each other by 1 order of magnitude (5 \( \times \) 10\(^{-2} \), 5 \( \times \) 10\(^{-3} \), and 5 \( \times \) 10\(^{-4} \) ps\(^{-1} \)) corresponding to the exponential lifetimes for ferric eNOS in Table I, and the amplitude \( a(k) \) tappers off at rates slower than 10\(^{-4} \) ps\(^{-1} \), indicating the completeness of rebinding in this case. The relative amplitudes \( a(k) \) of the rates match those of the components \( A_i \). For ferric eNOS with L-Arg, there is only one peak centered at 100 ps\(^{-1} \), the broadness of which reflects the uncertainty in determining the rates; indeed, if two rates lie too close to each other, depending on signal/noise ratio, MEM analysis may not separate them as distinct rates (31). By multieponential fitting, we obtained 53- and 300-ps lifetimes, whose ratio (5.7) represents the resolution limit of MEM with the signal/noise ratio of our data. Importantly, the distribution amplitude rises at low rates showing that rebinding is incomplete and that the slowest rate (\(<10^{-4} \) ps\(^{-1} \)) may include a constant term. For the third species, ferrous eNOS with L-Arg, two peaks are located at 2.5 \( \times \) 10\(^{-3} \) and 2 \( \times \) 10\(^{-2} \) ps\(^{-1} \) matching the picosecond exponential rebinding components and disclosing an inverted ratio of amplitudes when compared with ferric eNOS. As in the case of ferric eNOS with L-Arg, the amplitude rises at low rates, revealing the incompleteness of rebinding. Fig. 4C shows the comparison of rate distribution for ferrous eNOS and for myoglobin and revealed the overall faster rebinding to this latter hemoprotein, which was dominated by a bigger amplitude of the faster rate, whereas the slowest phase dominates that for the ferric eNOS.

BH\(_4\)-depleted Ferric eNOS—To estimate whether the BH\(_4\) content has an effect on geminate NO rebinding, we compared the kinetics of BH\(_4\)-depleted eNOS expressed in E. coli with that of eNOS containing 0.5 mol of BH\(_4\)/mol of protein, expressed with the baculovirus system (Fig. 5A). The kinetics are very similar, even on the nanosecond time scale. We therefore conclude that, in contrast to L-Arg, BH\(_4\) has no major effect on NO rebinding. This result is consistent with recent stopped flow measurements, which did not show an obvious effect of BH\(_4\) on NO binding\(^2\) and with the x-ray diffraction results (39), clearly indicating that the presence or absence of BH\(_4\) does not change the structure in the vicinity of the heme or the \( O_2 \) binding pocket of eNOS.

Ferric Oxygenase Domain—The rebinding of NO to the isolated oxygenase domain in the ferric state also proceeds through multi-exponential kinetics and is compared (Fig. 5B) with that of entire eNOS. The rates of kinetic components are similar for both species, but the relative amplitudes of the picosecond geminate rebinding (\( A_1 = 0.25 \)) decreased with respect to that of ferric eNOS possessing the reductase domain.

\(^2\) V. Berka and A.-L. Tsai, unpublished results.
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The model used for fitting consists of a sum of exponentials and a constant term. See Fig. 4.

**Table I**  
Parameters obtained by fitting the kinetics associated with the main SVD transient spectra, which correspond to the rebinding components.

| Species                        | $\tau_1$ (ps) | $A_1$ | $\tau_2$ | $A_2$ | $\tau_3$ | $A_3$ | $A_4$ | $A_5$ |
|-------------------------------|---------------|-------|-----------|-------|-----------|-------|-------|-------|
| Ferric NOS                     | 15            | 0.07  | 0.2       | 0.30  | 2         | 0.53  | cst   | 0.10  |
| L-Arg-bound ferric NOS         | 53            | 0.27  | 0.3       | 0.17  | 17        | 0.56  | $^c$  |
| Myoglobin (horse heart)        | 36            | 0.23  | 0.4       | 0.14  | 18        | 0.63  | $^c$  |

$a$ Relative amplitude: $\sum A_i = 1$ for rebinding components (decays of $\Delta t$), not including the amplitude of the transient due to the excited states.  
$b$ cst, constant component.  
$c$ Although $\tau_1$ may contain a constant term, the fit was not improved by adding it, or $A_1$ tended to vanish.

(A$_3 = 0.40$) with subsequent increase of the nanosecond recombination. The MEM analysis of the rate distribution (Fig. 5C) provides a striking contrast between the entire eNOS and its isolated oxygenase domain. The higher rate ($\sim 0.03$ ps$^{-1}$) is clearly the same for both species; as for the slower rate, it appears as a large peak for entire eNOS, in agreement with the higher amplitude of the 300 ps component with respect to the short one (Table I) and is followed by a nonzero amplitude. For the oxygenase domain this peak displays a much smaller relative amplitude and is replaced by an increased plateau matching the slow and constant terms in the time representation.

**DISCUSSION**

Nitrosyl complexes have been detected during the catalytic activity of the enzyme; there is a ferrous one in neuronal NOS (20), whereas for inducible NOS the ferric nitrosyl complex was observed (19). These complexes may be relevant to regulation of the enzyme activity. To probe the dynamics of NO released from N-hydroxy-L-arginine intermediate in eNOS heme pocket, we performed time-resolved absorption measurements. We emphasize that the photodissociation of NO previously liganded to the heme mimicks the release of NO newly formed in the heme pocket.

After photodissociation, rebinding of NO occurs in a multiphasic fashion. For all the NOS species probed here, the geminate rebinding decomposes in two phases, a nonmonoeaxponential picosecond binding (15–53 ps and 0.2–0.4 ns) and a longer one (≥2 ns). Similarly to wild type and mutants NO-liganded myoglobin, the picosecond phase is due to rebinding of NO that did not escape the heme pocket after photodissociation (32–35). This nonexponential character of the picosecond geminate recombination has been interpreted as a distribution of substates of the protein (36) and/or as a relaxation process of the heme pocket following photodissociation and developing simultaneously to the rebinding (32). Whatever model is considered, the structure of the distal side of heme pocket as well as the structural fluctuations influence the picosecond rebinding (33, 37). Because of the difference in nature of the proximal side chain covalently bound to the iron (Cys for eNOS, His for myoglobin) there should be a differential trans-effect between both proteins. However, this effect influences off rates, and our experiment probes on rates (more precisely a step of the process determining the on rate). The energy of photodissociation was in excess so that any difference in binding energy because of different proximal ligands does not affect the process. The overall rebinding of NO to eNOS is much slower than that measured for myoglobin (Fig. 4), in agreement with a lower affinity that is expected for an enzyme functionally releasing NO. The existence of a structure much more complex than that of myoglobin may account for the additional nanosecond phase of rebinding in eNOS and gives grounds to the comparative studies between the whole eNOS and its isolated oxygenase domain.

**The Isolated Oxygenase Domain**—In the isolated oxygenase domain, the rates of both phases of the geminate recombination of NO are similar to that of entire eNOS. The striking contrast of MEM rate distribution (Fig. 5C) shows that the higher rate, corresponding to the rebinding of NO still located in the heme pocket, is the same whether the oxygenase domain is isolated or not. Geminate recombination rates are very sensitive to small structural changes; a single mutation in the heme pocket such as V68A in myoglobin changed the rate of NO rebinding (33). Thus, the similarity of picosecond rates for eNOS and the isolated oxygenase domain shows that the surroundings of the heme pocket structure in the latter are unperturbed, consistent with its unchanged catalytic properties (14). However, the relative amplitudes of the picosecond and nanosecond phases changed. As seen in the MEM analysis, the slower rate distribution has a markedly decreased amplitude correlated with a simultaneous increase of a plateau because of the constant term. This is consistent with a smaller proportion of NO rebounding from protein core to the heme and more NO escaping to the solvent in the absence of the reductase domain. As shown by the crystal structure (38, 39), the heme is located on a side of the oxygenase domain and therefore possibly influenced by the reductase domain. Thus the isolation of this domain does not alter the structure of heme pocket nor the gating from it but instead changes the gating of a possible channel leading to it from the solution. We assign the nanosecond phase to the recombination of NO having left the heme pocket but still within the protein molecule. This may be due to a cavity such as that revealed in the crystal structure of the heme domain dimer (38, 39), which may be a channel for access of NO, L-Arg, and $O_2$.

**Effects of $L$-Arg on NO Rebinding**—Neither BH$_4$ nor L-Arg is required for dimerization of eNOS (39–42), and we ruled out the possibility that the effects observed in this study upon addition of L-Arg are due to dimerization. Two conspicuous effects are induced upon binding of L-Arg to its site: a change of the transient spectra at early time after photodissociation of NO from ferric heme and a change of the relative amplitudes of the three kinetic components. Dual effects of L-Arg are revealed by multieponential fitting and by MEM analysis (Fig. 4). Despite a slight increase of the picosecond rate, the overall effect is a dramatically slowed recombination. For the picosecond phase, the presence of L-Arg in its binding site results in an increase of the fast component relatively to the slower ones. Clearly, the binding of L-Arg changes the heme environment and the energy barrier for rebinding.

The conformation of the heme pocket in the distal side influences the rate distribution of the picosecond recombination. This was shown for NO geminate recombination in myoglobin by means of distal side mutations such as V68X (33, 37). Similarly, a different behavior of the picosecond and the nanosecond components was observed (34) when comparing mutants differing by the size of a residue (Leu$^{29}$) located in the distal side of the heme pocket. These results suggest that this side chain is located on the way out of the protein from the iron and acts as...
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Fig. 5. A, normalized nanosecond kinetics of BH₄-depleted eNOS expressed in E. coli (●) compared with that of eNOS containing 0.5 mol of BH₄/mol of protein expressed with a baculovirus system (△). B, normalized picosecond kinetics of geminate recombination for the isolated ferric oxygenase domain (●) and entire ferric eNOS (△). The relative amplitude for the picosecond rebinding (τ₁ = 21 ps and τ₂ = 300 ps) is 0.25 and 0.4 for the oxygenase domain and entire eNOS, respectively, and 0.75 and 0.6 for nanosecond rebinding (τ₁ = 2 ns) for the two species, respectively. C, comparison of the rate distribution from MEM analysis of the same kinetic data for entire eNOS and the isolated oxygenase domain. The curves have been scaled to match the larger k₂ components (−3 × 10⁻² ps⁻¹) to allow a direct comparison of the relative amplitudes in both species.

A barrier both for ligand escape and for rebinding from the exterior of the heme pocket. Furthermore, an inverse correlation was observed between the overall dissociation rate (kₒₘ) and the picosecond rate of the geminate recombination, suggesting that the same barrier influences both rates. As indicated by simulations for myoglobin (34, 43), variations of the rate of recombination are correlated with the size of a mutated residue (L29X) in the distal side that decreases the volume available for the ligand and consequently constrains the diffusion of NO in the pocket. Steric parameters thus affect the picosecond rebinding. L-Arg is H-bound to a Glu side chain (Glu³⁶¹ for human eNOS) (39, 44) in such a way that its guanidinium part is in close vicinity of O₂ liganded to the ferrous heme. The presence of L-Arg could therefore sterically hinder NO rebinding, slowing down the rate of NO exchange from and/or to the heme. This observation corroborates with recent stopped flow data of NO binding to the ferric eNOS. L-Arginging binding caused a 2–3 order decrease in the observed rates of NO binding.²

In addition to this direct steric effect, there may be structural effects. Indeed, mutations on the myoglobin molecular surface have been shown to influence picosecond rebinding (33), suggesting subtle changes of the heme pocket structure or of protein relaxation, and conformational changes after photodissociation have been revealed by time-resolved absorption (45) and by computation (46). Furthermore, the access of ligands to the heme iron of myoglobin is affected by structural fluctuations that depend strongly on the structural pattern (35). As revealed by EPR experiments, the binding of L-Arg to its site changes the spin-state of the heme in both eNOS (47) and nNOS (48, 49) and induces a shift of the Soret band in the unliganded ferric NOS spectrum (Fig. 1B and Refs. 22 and 50) without direct interaction with the heme iron. These equilibrium measurements underline a structural change in the heme pocket, and the dynamics effects shown by our measurements may be related to such a structural perturbation induced by the substrate, because all three phases of NO rebinding in eNOS are changed upon L-Arg binding, and the excited state has a different spectrum in the presence of L-Arg.

It is likely that L-Arg and NO access their respective sites through the same channel. Indeed, the structure of eNOS suggests that dimerization results in the formation of a channel leading to the heme pocket (39). Because the baculovirus-expressed eNOS is mostly in dimeric form, the presence of a channel leading to the heme may be at the origin of the nanosecond component of geminate recombination. Because L-Arg bound in the heme pocket changes the rate of this nanosecond NO-rebinding, it is likely that a structural perturbation occurs that changes the communication between the heme and the channel. Indeed, it was noticed (33) that in a myoglobin mutant with an altered rebinding rate, structural modifications are not seen on x-ray structure. Similar cases likely exist in the NOS isoforms.

L-Arg binding induced a 2–3-order decrease of both kₒₘ and kₑₐₙ rates of NO to ferric eNOS measured by stopped flow,² in agreement with the photodissociation data that reveal a decrease of the overall recombination rate upon L-Arg binding, which accounts for the change of kₒₘ. In contrast, L-Arg did not change the kₒₘ binding rate of NO for ferric inducible NOSs in the presence of BH₄ (25). The magnitude of the effect because of L-Arg on kₒₘ and on the geminate recombination rates is not necessarily the same, because geminate rebinding represents only a step of the process determining the on rate. Furthermore, initial conditions differ with stopped flow measurement initiated by mixing NOS and NO solution, whereas photodissociated NO was previously bound to the heme. The overall NO association rate from solution may also include competition with another distal ligand. Photodissociation measurement shows the effect of L-Arg binding free of this competition step, thus closely simulating the situation where NO binds to the heme right after synthesis.

Another effect of L-Arg binding on NO rebinding may be
through electrostatic interactions. The proximity of the guanidinium part of L-Arg to the heme-ligated NO is revealed by x-ray structure (38, 39), EPR spectroscopy (24) and also by Raman scattering (23). Without L-Arg, the ferrous NO complex is unstable and is changed from a hexa- to a penta-coordinated species with an unbound proximal Cys (24). Because L-Arg perturbs the electronic state of the ferrous heme-NO complex, as seen by the alteration of EPR spectrum, the electronic barrier for rebinding is likely to change, contributing to the modification of the rate distribution of the picosecond recombination.

In the presence of L-Arg, $k_{nm}$ for NO binding did not change for the ferrous eNOS, as measured with stopped flow, whereas it changed for ferric eNOS. We observed a slight difference in the rates of the picosecond components, the fastest one being increased for the reduced heme, which thus discloses a modified barrier. No change occurred for the nanosecond component, consistent with the influence of the heme redox state being limited to the heme pocket. During the catalytic activity of nNOS a ferrous-nitrosyl complex formed (20), whereas a ferric one was revealed in the case of inducible NOS (19). Because the proposed reaction mechanisms involve a Fe$^{3+}$ heme in the last step where NO is formed (15, 16), this difference of redox state of the complex may be due to different rates of NADPH reduction between both isoforms, as suggested earlier (25). In one case, rereduction may occur before NO leaves the heme pocket. This difference in redox state of the nitrosyl complexes is associated with different picosecond rebinding, and the isoform specificity may thus correlate with different requirements for catalytic regulation.

The formation of the ferrous nitrosyl nNOS complex was not decreased by NO scavengers or by enzyme dilution (20), and it was suggested that two phases could occur in self-inhibition of NOS by NO, involving a step in which NO binds to the heme right after synthesis, before leaving the heme pocket. The properties of geminate rebinding after NO release in heme pocket are consistent with such a process. Indeed the “gating” effect of L-Arg on the rebinding modifies the exchange pathway from the heme to the rest of the molecule and consequently changes the overall affinity for NO. The effect of L-Arg is outlined in Fig. 6A. On the potential energy curve, two energy barriers are modified upon L-Arg binding. The first one, from the heme pocket to the heme distal ligand position, decreased, whereas the second one, from the protein cage to the heme pocket, is raised.

Mechanistic Implications—X-ray structures and simulations have revealed that no direct pathway exists from the heme pocket of myoglobin and hemoglobin to exterior of the protein and that fluctuations of the structure are required for entry into and exit from diatomic ligands (51). For myoglobin the fast rebinding component dominates (Fig. 4) without a significant nanosecond component, we may conclude that in eNOS the rebinding component dominates (Fig. 4) without a significant nanosecond component.

Figure 6A. putative potential energy curve model for three main stages of NO rebinding to ferrous eNOS. The ordinate represents the Fe/NO distance. The dashed line is the potential energy curve in the presence of L-Arg (this minimal model does not take into account substates or other possible barriers). B, scheme for NO binding after synthesis and release. Synthesis occurs in step I (not detailed). The process II is that probed by the photodissociation and rebinding experiment, and this scheme does not prejudge of the iron redox state of the b species. Species b and c correspond to positions b and c in panel A, respectively.

A striking point is that the two rebinding phases are differently influenced by L-Arg. This leads to a subtle regulation of NO rebinding at different levels (directly in the heme pocket and in the exchange pathway with solution). Because NO is a very reactive species that cannot be stored after synthesis, a regulation of NOS by the formation of nitrosyl complexes should be better achieved through control of NO rebinding without leaving the heme pocket rather than through an equilibrium involving NO already in solution. We are currently undertaking further experiments to clarify these mechanisms.

Conclusion—The self-inhibition of NOS by ligation of NO newly synthesized to the heme prior to release from the protein core has been hypothesized (19, 20). We show that geminate recombination of NO does occur and that this process is modulated by the substrate. When NO is released in the heme pocket of eNOS, the rebinding to the iron is a multiphasic process with at least three kinetic components. We assigned the fastest picosecond phase to geminate rebinding of NO that is still located in the heme pocket. In contrast to the first one, the slowest phase, in nanosecond time scale, is influenced by the absence or the presence of the reductase domain. We thus assigned it to rebinding of NO located in the protein core but not in the heme pocket. The presence of only L-Arg on its binding site differentially modulates the rates and the relative amplitudes of both phases of recombination. L-Arg induces a structural perturbation of the heme pocket in the ferric state and directly influences the geminate binding of NO just released in the heme pocket, providing a possible mechanism of subtle and efficient regulation of NO synthesis and release by means of self-inhibition of eNOS. This may be achieved, once L-Arg is depleted, by a temporary storage of released NO.
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