Fast Dissociation of Nitric Oxide from Ferrous Pseudomonas aeruginosa cd₁ Nitrite Reductase
A NOVEL OUTLOOK ON THE CATALYTIC MECHANISM

Serena Rinaldo‡, Alessandro Arcovito§, Maurizio Brunori‡¶*, and Francesca Cutruzzola‡¶

From the ‡ Dipartimento di Scienze Biochimiche “A. Rossi Fanelli” and § Istituto di Biologia e Patologia Molecolari del Consiglio Nazionale delle Ricerche, Università di Roma “La Sapienza”, 00185 Rome and ¶ Istituto di Biochimica e Biochimica Clinica, Università Cattolica del Sacro Cuore, 00168 Rome, Italy

The heme-containing periplasmic nitrite reductase (cd₁ NIR) is responsible for the production of nitric oxide (NO) in denitrifying bacterial species, among which are several animal and plant pathogens. Heme NIRs are homodimers, each subunit containing one covalently bound c-heme and one d₁-heme. The reduction of nitrite to NO involves binding of nitrite to the reduced protein at the level of d₁-heme, followed by dehydration of nitrite to yield NO and release of the latter. The crucial rate-limiting step in the catalytic mechanism is thought to be the release of NO from the d₁-heme, which has been proposed, but never demonstrated experimentally, to occur when the iron is in the ferric form, given that the reduced NO-bound derivative was presumed to be very stable, as in other heme proteins. We have measured for the first time the kinetics of NO binding and release from fully reduced cd₁ NIR, using the enzyme from Pseudomonas aeruginosa and its site-directed mutant H369A. Quite unexpectedly, we found that NO dissociation from the reduced d₁-heme is very rapid, several orders of magnitude faster than that measured for b-type heme containing reduced hemeproteins. Because the rate of NO dissociation from reduced cd₁ NIR, measured in the present report, is faster than or comparable with the turnover number, contrary to expectations this event may well be on the catalytic cycle and not necessarily rate-limiting. This finding also provides a rationale for the presence in cd₁ NIR of the peculiar d₁-heme cofactor, which has probably evolved to ensure fast product dissociation.

Pseudomonas aeruginosa, a facultative anaerobe, can use denitrification as the anaerobic energy-producing pathway (1). This microorganism is an opportunistic pathogen, and it has been shown that in the host, the denitrification pathway not only works as a source of electrons (2) but may also be involved in nitric oxide (NO) scavenging, given that the classical flavohemoglobin-mediated detoxification pathway is not active (3). More recently, low concentrations of NO have also been shown to control the lifestyle of P. aeruginosa by inducing dispersal of the multicellular assemblies (biofilms) strictly related to chronic pulmonary infections (4). Therefore, pathogenesis, NO metabolism, and denitrification are strictly related.

The conversion of nitrite (NO₂⁻) to nitric oxide (NO) is catalyzed by the periplasmic nitrite reductases (NIR), which are either copper- or heme-containing enzymes (5). P. aeruginosa NIR belongs to the latter type, being a homodimer of two 60-kDa subunits, each containing one covalently bound c-heme and one d₁-heme. Extensive spectroscopic and functional studies have been carried out on cd₁ NIRs (5, 6); the c-heme domain is the entry site of the electrons, whereas catalysis occurs at the level of the d₁-heme.

The established physiological role of these enzymes is to catalyze the one-electron reduction of NO₂⁻ to NO. The reaction cycle involves binding of nitrite to the reduced protein at the level of the d₁-heme, followed by dehydration of nitrite to yield NO and release of the product (7–9). The rate-limiting step in the catalytic mechanism is thought to be the release of NO from the d₁-heme; the current working hypothesis postulates that release of NO is likely to occur when the d₁-heme iron atom is in the oxidized state, because the reduced NO-bound derivative of other hemeproteins is generally known to be very stable (10). According to this interpretation in the literature, the fully reduced NO-bound state of the protein has always considered a “dead-end” inhibited state of cd₁ NIRs (7–9, 11).

A puzzling observation concerning this hypothesis is that rapid kinetic studies failed to show kinetically competent NO dissociation from the oxidized d₁-heme, even at low reductive pressure (8, 12). A recent study on Paracoccus pantotrophus cd₁ NIR (12) has shown that in a site-directed mutant in which the protein has the c-heme oxidized and the d₁-heme reduced, reduction of nitrite can occur but the NO produced is unable to dissociate; thereby, the final species is the fully oxidized NO-bound enzyme.

We have measured for the first time the kinetics of NO binding and release from the fully reduced state of cd₁ NIR, using the enzyme from P. aeruginosa and its site-directed mutant.
H369A. We observe that the dissociation rate constant of NO from reduced d1-heme ($k_{\text{off}} \sim 6-35 \text{ s}^{-1}$) is much more rapid than previously assumed on the basis of extensive information available on b-type heme-containing ferrous proteins. This totally unexpected result allows us to re-discuss the reaction mechanism of nitrite reduction catalyzed by cd1 NIRs.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Purification—Wild type cd1 NIR was purified following Parr et al. (13). Mutagenesis of His-369 to Ala and purification of the mutant were already described in Ref. (9). In the Pseudomonas putida expression system, the H369A protein is synthesized with the c-heme, but no d1-heme; this semi-apo-NIR is then reconstituted in vitro with the d1-heme extracted from wild type cd1 NIR (14). Reconstitution was carried out by incubating the protein at 15 °C in 50 mM Bis-Tris buffer, pH 7.0, 20 °C. The d1-heme, followed by gel filtration.

The extinction coefficient used in this report for the oxidized cd1 NIR was $e_{412 \text{ nm}} = 141 \text{ mm}^{-1} \text{ cm}^{-1}$ (15). This corresponds to the active sites.

Amperometric NO Measurements—The NO production was measured anaerobically with a NO-sensitive Clark-type electrode (World Precision Instruments) at 20 °C in 50 mM Bis-Tris, pH 6.2 and 7.0, or 50 mM Tris/HCl, pH 7.6 and 8.0. The electrode was calibrated at each pH by addition of 1 μl of a NO stock solution prepared by equilibrating degassed buffer with a tonometer containing 1 atm NO (Air Liquide, Paris, France) ([NO] in solution = 2 mM at 20 °C).

The cd1 NIR (1 mM) was incubated in the reaction chamber with 10 mM sodium ascorbate and 0.1 mM $N,N,N',N'$-tetramethyl-$p$-phenylenediamine (Sigma-Aldrich) as electron donors, and the nitrite reductase activity was recorded after addition of sodium nitrite (0.3 mM; Sigma-Aldrich).

The deoxy myoglobin (Mb, from horse; Sigma-Aldrich) solution was prepared as follows. A ferric Mb solution was reduced by addition of solid sodium dithionite ($Na_2S_2O_4$), and the excess of reductant was removed by gel filtration chromatography on a Sephadex G25 column (GE Healthcare). The resulting oxyMb derivative was incubated in degassed 50 mM Bis-Tris buffer, pH 7.0, in the presence of 5 mM sodium ascorbate and 0.13 mg/ml of ascorbate oxidase (Sigma-Aldrich) to remove the oxygen ligands. The protein was added in the electrode chamber at the final concentration of 15 μM ($e_{560 \text{ nm}} = 13.8 \text{ mm}^{-1} \text{ cm}^{-1}$) (16).

Laser Photolysis Measurements—A 10-μM protein solution in 50 mM Bis-Tris, pH 7.0, at room temperature (both for the wild type and H369A cd1 NIR) was reduced anaerobically with 5 mM sodium ascorbate in a fully filled cuvette (1-cm path length) for fluorescence assays. Different amounts (30–90 μM) of a NO solution were added anaerobically to produce the reduced NO-bound derivative. The instrument used for photolysis experiments has been described elsewhere (17) and uses a Nd-YAG solid-state laser with a 5-ns pulse (Quanta System HIL 101, second harmonic $\lambda = 532$ nm, $E = 80$ mJ/pulse) that was focused onto the filled cuvette containing the desired solution. The experimental data thus collected are time courses at single wavelength; they are converted to absorbances by means of the IGORPRO package (Wavemetrics). Single wavelength time courses are fitted as a single exponential using the nonlinear least squares routines provided by the IGORPRO package (Wavemetrics). The observed recombination rate constants were plotted as a function of the final NO concentration to extrapolate the second order rate constant; all the data were corrected for the dilution of the sample upon each NO addition.

Stopped-flow Measurements—All the stopped-flow experiments described in this work were carried out anaerobically. The concentration of the samples in the different experiments is always given "before mixing"; a 1:1 dilution was always used in the symmetric mixing apparatus. All the experiments were performed with an Applied Photophysics stopped-flow apparatus (DX.17MV; Applied Photophysics, Leatherhead, UK). To calculate the rate of NO dissociation a monochromatic light source was used in the single wavelength acquisition mode; the use of the diode array acquisition mode was limited because photodissociation of NO is known to affect the rate, which is 2–4 times faster with this setup. The analysis of the kinetics was carried out with the IgorPro program (Wavemetrics).

The reduced cd1 NIR was prepared anaerobically by adding 5 mM sodium ascorbate to the oxidized protein in degassed buffer (either 50 mM Bis-Tris, pH 6.2, pH 7.0, or 50 mM Tris, pH 8.0) at 20 °C. The reduced NO-bound derivative was obtained after addition of a stoichiometric amount of either NO or NO2 to the reduced protein. All spectra were recorded in a JASCO V550 spectrophotometer.

The reduced NO-bound wild type cd1 NIR (8–18 μM) (cd1 NIR-NO) was rapidly mixed in the stopped-flow apparatus with a solution of human deoxy hemoglobin (deoxyHb) at different concentrations (18–80 μM in heme), calculated using $e_{555 \text{ nm}} = 12.5 \text{ mm}^{-1} \text{ cm}^{-1}$ (16) in 50 mM Bis-Tris, pH 7, at 20 °C. Under these conditions the following reaction occurs.

$$\text{cd}_1, \text{NIR-NO} + \text{deoxyHb} \rightarrow \text{cd}_1, \text{NIR} + \text{Hb-NO}$$

REACTION 1

The kinetics is rate-limited by NO dissociation from cd1 NIR. The deoxyHb was prepared by degassing a solution of oxy-hemoglobin in the presence of 5 mM sodium ascorbate and 20 μl of a 13-μg/ml solution of ascorbate oxidase (Sigma-Aldrich). To determine the pH dependence of the NO dissociation rate, the same experiment was carried out also at pH 6.2 (in 50 mM Bis-Tris buffer) and pH 8.0 (in 50 mM Tris buffer) by mixing a 15-μM solution of reduced NO wild type cd1 NIR with an 80-μM (in heme) solution of deoxyHb.

The NO dissociation rate constant from the reduced NO-bound H369A NIR was determined at pH 7.0 and 20 °C by mixing a 20-μM solution of the sample with a 64-μM (in heme) solution of deoxyHb in the stopped-flow apparatus, as reported for the wild type protein. To further investigate the dissociation rate constant of NO bound to the reduced d1-heme, the experiment with deoxyHb was also carried out for both the reduced wild type and H369A proteins (8 μM, pH 7.0, 20 °C) starting from the NO derivative prepared incubating anaerobically the reduced protein with a stoichiometric amount of sodium nitrite.
Fast NO Dissociation from Ferrous P. aeruginosa cd₁, NIR

**RESULTS**

**Production of NO under Steady-state Conditions**—The release of NO from P. aeruginosa cd₁ NIR under steady-state conditions can be measured directly using a NO-sensitive electrode. Fig. 1A shows the traces recorded at pH 6.2 and pH 7; the NO produced by the enzyme is rapidly scavenged if deoxymyoglobin is added to the reaction mixture, as expected. The release of NO is clearly pH-dependent, as previously suggested, with a turnover number of 11.2 (±1.2) s⁻¹ at pH 6.2 and 1.9 (±0.05) s⁻¹ at pH 8.0, in good agreement with literature data (15, 18) (Fig. 1B).

Preincubation (at pH 7.0) of the reaction mixture containing the reductants and nanomolar cd₁ NIR with micromolar NO before the addition of nitrite does not significantly inhibit the rate of production of NO (Fig. 1B, inset). This suggests that, in the presence of substrate, even a large excess of free NO is unable to inactivate the enzyme.

The NO Association Rate Constant—The NO association rate constant was measured by laser flash photolysis at pH 7.0 (Fig. 2). Time courses were followed at 460 nm with different ligand concentrations. The traces could be always fitted well with a single-exponential expression (Fig. 2, inset). The small absorbance change observed in the experiment is partly due to the laser setup, which enables us to obtain a very limited photolysis with NO. In the case of cd₁ NIR we cannot exclude that there may be a geminate effect, but it is also certain that the small amplitude observed is largely due to the lack of quanta.

Because it is known that at pH 7.0 some NO may bind to the ferrous c-heme of cd₁ NIR (15), the kinetics was also monitored at 630 nm (where no spectral contribution of the c-heme is present) and at pH 8.0 (where NO binding to the c-heme does not occur) (15) (data not shown). No difference was observed in the kinetics, suggesting that the association rate measured reflects NO binding to the reduced d₁-heme only.

The second-order association rate constant ($k_{\text{obs}}$), obtained from the linear dependence of the observed rate constant ($k_{\text{obs}}$) on ligand concentration (Fig. 2), is $3.9 \times 10^6$ M⁻¹ s⁻¹ (Table 1) at pH 7.0. NO significant pH dependence was observed between pH 6.2 and 8 (not shown).

The NO Dissociation Rate Constant—The dissociation of NO from the fully reduced NO-bound derivative of hemoproteins is usually very slow ($10^{-3} - 10^{-5}$ s⁻¹); the classical approach used to determine this value involves dilution of the reduced NO-bound hemoprotein into a solution containing a large excess of a NO scavenger, usually sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) or hemo-

(Sigma-Aldrich). Each sample was mixed in the stopped-flow apparatus with a 40-μM (in heme) solution of deoxyHb.

To determine the dissociation rate of NO from the reduced d₁-heme, the reduced NO-bound wild type cd₁ NIR (10.4 μM) was also rapidly mixed with a 5–20-mM potassium cyanide solution, and the formation of the reduced cyanide-bound derivative was followed at different wavelengths. Under these conditions, cyanide displaces NO by binding to the reduced d₁-heme; the kinetics is rate-limited by NO dissociation. As a reference, the fully reduced NO-bound protein (5.2 mM) was also incubated anaerobically with 20 mM cyanide, and the spectrum was collected in a JASCO V550 spectrophotometer.

**FIGURE 1. Steady-state NO production assay.** The reaction was carried out in the presence of 1 nM cd₁ NIR and reductants (10 mM sodium ascorbate and 0.1 mM N,N,N′,N′-tetramethyl-p-phenylenediamine). A, NO production at pH 6.2 (thin line) or pH 7.0 (bold line) at 20 °C; the first arrow indicates the addition of 0.3 mM NO₂⁻ and the following ones subsequent addition of 1.5 μM deoxymyoglobin as NO scavenger. At times longer than 100 s some chemical degradation of NO in the presence of the reductants and possibly some enzyme inhibition are likely to occur. B, turnover number of the nitrite reductase activity of cd₁ NIR as a function of pH, at 20 °C. The turnover number was calculated as the concentration of NO produced/s/1 nM catalytic center.

**FIGURE 2. Determination of the NO association rate constant by laser photolysis.** The experiment was carried out on reduced NO-bound wild type (black circles) and H369A (open circles) cd₁ NIRs. The reaction was followed at room temperature under anaerobic conditions in 50 mM Bis-Tris buffer, pH 7.0, containing different concentrations of NO (30–90 μM) in the cuvette. The dependence of the observed rate constant for NO recombination to reduced NIR, plotted as a function of [NO], allows us to calculate the $k_{\text{obs}}$ values (see Table 1) for the wild type and for H369A cd₁ NIRs as the slope of the linear fit of the data. The intercept was fixed in the fit equal to the value of the dissociation rate constant measured in this work (see Table 1) (open square and star for the wild type and the H369A cd₁, NIRs, respectively). Inset, time course of NO (90 μM) recombination to fully reduced wild type cd₁ NIR (10 μM). The reaction was initiated by photodissociation of NO with a laser pulse at 532 nm; the recombination was monitored at 460 nm. At this wavelength the small amplitude observed is largely due to the lack of quanta.
Fast NO Dissociation from Ferrous P. aeruginosa cd1, NIR

TABLE 1
Comparison of the kinetic parameters for NO binding and dissociation to reduced cd1, NIRs (determined in this work) and those of selected ferrous hemeproteins

| Protein              | pH    | $k_{on}$ | $k_{off}$ | Method            | Ref. |
|----------------------|-------|----------|-----------|-------------------|------|
| Wild type            | 6.2   |          |           |                   |      |
| c$^{2+}$d$^{2+}$NO   | 7.0   | 3.9(±0.3)×10$^8$ |           | deoxyHb          |      |
|                      | 7.0a  | 1.9(±0.2)×10$^9$ |           | deoxyHb          |      |
|                      | 7.0   | 1.7×10$^7$ | 10$^{-3}$ | Dithionite + CO   | 19   |
| H369A                | 7.0   | 0.4–1.0×10$^8$ | 4×10$^{-3}$ | Human deoxyHb     | 35   |
| c$^{2+}$d$^{2+}$NO   | 7.0   | 0.4–1.0×10$^8$ | 4×10$^{-3}$ | Human deoxyHb     | 35   |
| Mb (sw)              | 7.0   | 0.4–1.0×10$^8$ | 4×10$^{-3}$ | Human deoxyHb     | 35   |
| Hb (human)           | 7.0   | 0.4–1.0×10$^8$ | 4×10$^{-3}$ | Human deoxyHb     | 35   |
| Cytochrome c oxidase | 7.4   |          |           |                   |      |

$^a$ The reduced NO-bound cd1 NIR was obtained by addition of a stoichiometric amount of sodium nitrite to the fully reduced protein.

globin, and eventually a high affinity ligand such as carbon monoxide (CO). The reaction is followed spectrophotometrically over a long time range (hours), monitoring the appearance of the reduced CO-bound form; under these conditions the observed kinetics is rate-limited by NO dissociation (19).

In the case of cd1 NIR, however, the CO association rate for the reduced d$_1$-heme is fairly slow ($2×10^8$ M$^{-1}$ s$^{-1}$) (20, 21) as compared with other hemeproteins (0.5–5×10$^8$ M$^{-1}$ s$^{-1}$) (16, 22) and the amount of CO to compete effectively with NO is not achievable. Moreover, the products of dithionite oxidation are known to bind the reduced d$_1$-heme (23), which would further complicate analysis of the results. Thus, in the case of cd1 NIR the dithionite-CO method cannot be used to determine unequivocally the NO dissociation rates.

The alternative approach to measure NO dissociation rate constant is based on the high affinity of deoxyHb for NO ($K_a$~10$^{-11}$ M) (19); using an excess deoxyHb, the NO released by cd1 NIR is rapidly and effectively scavenged by Hb (see Reaction 1 under “Experimental Procedures”).

Upon mixing reduced NO-bound cd1 NIR with an excess deoxyHb in the stopped-flow (diode array), analysis of the spectra shows that a reaction has already occurred after 10 s (Fig. 3). The increase in absorbance at 460 nm, where the contribution of Hb is minimal, can only be interpreted with the formation of the reduced ligand-free cd1 NIR.

The NO dissociation was then time-resolved upon mixing reduced NO-bound cd1 NIR and excess deoxyHb in the stopped-flow and following the kinetics at single wavelength. Fig. 4 shows the time course at 460 nm recorded at three different pH values (6.2, 7.0, 8.0, panels A, B, and C, respectively). The observed kinetic amplitude corresponds to the expected absorbance change going from the reduced NO-bound to the reduced cd1 NIR (see also Fig. 3, inset). All traces can be fitted with a double-exponential expression, yielding the values of $k_1$ and $k_2$ listed in Table 1. The contribution of each exponential process was found to be ~50% of the total change in signal amplitude. The NO dissociation rate was found to be essentially independent of deoxyHb concentration in the range explored (1–4 molar excess of Hb with respect to cd1 NIR), which is consistent with the model assuming that NO dissociation is rate-limiting. A small (2-fold) decrease in the value for the two
rate constants has been observed at pH 8.0 (see Table 1). At pH 7.0 the two dissociation rate constants were determined to be \(27.5 \text{ s}^{-1}\) and \(3.8 \text{ s}^{-1}\). Very similar rates were obtained when the reduced NO-bound enzyme was prepared by addition of stoichiometric nitrite (Fig. 4D), indicating that the species from which NO is dissociating rapidly is the same in both cases.

Because this NO dissociation rate is much more rapid than values reported in the literature for many other ferrous NO-bound hemoproteins, we have confirmed this unexpected result by an independent experiment. It was shown previously (24, 25) that reduced cd\(_1\) NIR has an unusually high affinity for anionic ligands as cyanide (\(K_d = 9.5 \text{ mM}\)), and we have attributed the majority of this effect to the conserved His-369 on the distal side of the d\(_1\)-heme (25). Therefore we used cyanide in a displacement reaction, adding a concentrated KCN solution to reduced NO-bound cd\(_1\) NIR. The spectrum of the resulting mixture (containing 20 mM KCN) (Fig. 5A) shows that after \(2\) min the spectrum corresponds to that of the cyanide adduct, confirming that displacement of NO has already occurred. When the same experiment was repeated in the stopped-flow, the time course of formation of the cyanide derivative was easily followed (Fig. 5B), and the two rate constants are listed in Table 1. A small (2-fold) dependence of the measured NO dissociation rate constants upon cyanide concentration between 5–20 mM was observed (not shown). The values obtained using the cyanide displacement approach are in good agreement with those obtained from the deoxyHb experiments (see above and Table 1), confirming that fast NO dissociation occurs independently from the method used to monitor the reaction.

**NO Association and Dissociation Rate Constants for the H369A Mutant**—The site-directed mutant H369A was previously shown to be unable to catalyze effectively nitrite reduction to NO (9) and to bind anions with extremely low affinity (9, 25). To assess the role of the conserved distal His-369 residue in the reactivity with NO, we have measured both the association (Fig. 2, Table 1) and dissociation rate constants (Fig. 4, B).
Fast NO Dissociation from Ferrous P. aeruginosa cd1 NIR

The fast rates of NO dissociation were also observed starting from the NO complex obtained by allowing the enzyme to carry out one catalytic cycle by adding stoichiometric nitrite to the reduced protein (see Fig. 4D). This suggests that the species that dissociates NO rapidly is identical to the fully reduced NO-bound derivative obtained by direct binding of gaseous NO to ferrous cd1 NIR (Fig. 3, inset).

The nitrite reductase activity of cd1 NIRs was shown by us and other groups to yield NO as the reaction product, with turnover numbers ranging from 2 to 10 s⁻¹ (depending on the conditions used in the assay) (18, 30). Preincubation of nanomolar cd1 NIR with micromolar NO before the addition of nitrite does not significantly inhibit the production of NO from the enzyme (Fig. 1B, inset). This result clearly indicates that, in the presence of the substrate, a large excess of free NO is unable to irreversibly inactivate the enzyme.

These results cast some doubt on the belief that the fully reduced NO-bound derivative (c²⁺ d¹⁻²⁺ NO) can be considered a dead-end species, unable to dissociate NO. Indeed, present results demonstrate that NO dissociation from the reduced d₁-heme occurs much more rapidly than expected, suggesting that this species may likely be a genuine catalytic intermediate (Fig. 6, pathway 2). We thus propose that the fully reduced enzyme (c²⁺ d²⁺) may also originate along pathway 2. Given that the rate constant for dissociation of NO from reduced cd₁ NIR measured in the present report is somewhat faster than the turnover number, this event may well be on-pathway and not necessarily rate-limiting.

It must be mentioned that at present we have no information on the rate of NO dissociation from the c³⁺ d¹⁺ NO mixed valence species (see Fig. 6), which is unstable and difficult to populate. At this stage we cannot exclude that fast NO dissociation can also occur from this mixed valence species, before re-reduction of the c-heme moiety. However, the finding that NO release from the reduced d₁-heme occurs at a rate that is much faster than that of ferrous b-type heme-containing proteins stands out as a novelty.

Previous pre-steady-state studies failed to demonstrate that the oxidized d₁-heme NO complex is able to spontaneously dissociate NO (8); reduction of the enzyme by an external electron donor was already pointed out to be crucial to have product dissociation, because it is possible to observe steady-state turnover under conditions in which a large excess of reducing equivalents is available (9, 15, 30). The present rapid kinetics study is thus fully consistent with the idea that, under high reductive pressure, rapid NO dissociation may indeed occur.

The value of the association rate constant (3.9 × 10⁹ M⁻¹s⁻¹) measured for the P. aeruginosa cd₁ NIR lies in the range of values measured for other hemeproteins, which are ~10⁻⁸ - 10⁻¹⁰ M⁻¹s⁻¹ (10, 31). Thus, binding of NO to the reduced d₁-heme occurs rapidly; this is consistent with an unhindered d₁-heme site, which is known to be high spin-pentacoordinated (32).

Taking into account the values for the association and dissociation rate constants (Table 1) the affinity of P. aeruginosa cd₁ NIR for NO can be evaluated to be in the range Kₐ = 10⁻⁷ - 10⁻⁸ M. Most reduced hemeproteins display a much higher affinity, usually ~10⁻¹¹ M; the difference with cd₁ NIR is entirely due to the faster NO dissociation characteristic of the latter enzyme.

**DISCUSSION**

This work provides much new insight into the mechanism of *P. aeruginosa* cd₁ NIR by the unequivocal determination of the microscopic constants for NO binding to and release from the ferrous d₁-heme-NO complex. The reduction of nitrite to NO by a heme-containing enzyme such as cd₁ NIR poses a mechanistic problem because the product NO is known to bind with very high affinity to reduced hemes and thus must be quickly released in the bulk to avoid inhibition. Fig. 6 summarizes the current view of the catalytic cycle for the cd₁ NIR. Upon mixing nitrite with the reduced enzyme (c²⁺ d²⁺), the formation of the initial complex (c²⁺ d²⁺ NO⁻) is followed by production of NO involving bond breaking and loss of a water molecule; yet this is assumed to be reversible. The resulting mixed valence species (c³⁺ d³⁺ NO) may enter pathway 1 (where dissociation of NO and further reduction to the starting fully reduced NIR (c²⁺ d²⁺) occurs. This pathway, involving release of NO from the oxidized d₁-heme, was proposed to be the most likely one to account for the enzyme turnover (8, 9, 11).

The primary observation of the present work is that NO dissipates very rapidly from the ferrous d₁-heme, an unexpected and surprising result. The measured rate constants are 10⁴ - 10⁶-fold greater than those measured for any NO complex of ferrous b-type heme-containing proteins reported in the literature (10, 19, 26 and Table 1). The kinetics of NO release from the fully reduced NO-bound wild type cd₁ NIR is biphasic with values of 35 and 6 s⁻¹ at pH 7.0 (average from values listed in Table 1). Although the biphase is not understood at present, it might be related to the dimeric structure of the enzyme, which has been previously shown to display cooperative behavior with some ligands, such as CO (20) and cyanide (24). An intrinsic functional asymmetry of the enzyme is also evident in the reaction with macromolecular electron donors (such as azurin and cytochrome c₅₅₁) (27, 28) and in the intramolecular electron transfer step (29).
Fast NO Dissociation from Ferrous P. aeruginosa cd1, NIR

The other relevant outcome of this study is the observation that in the H369A mutant the dissociation rate for NO is essentially the same as for the wild type protein (only 2 times slower). We have previously shown that in this mutant the nitrite reductase activity is severely reduced (turnover number 0.08 s⁻¹, pH 6.2) (9). Because the mutant protein binds anions with a significantly lower affinity (25) but is able to dissociate NO equally well (this work), the major role of His-369 in P. aeruginosa cd1 NIR must be in controlling the binding of the anionic substrate NO₂⁻.

The unexpected finding reported here may also provide a rationale for the (to-date) puzzling presence in cd1 NIR of the peculiar d₁-heme, unique to this class of enzymes. The d₁-heme is a 3,8-dioxo-17-acrylate-porphyrindione; the presence of the electronegative oxo groups confers to the macrocycle distinct properties compared to the corresponding isobacteriochlorines (33). The d₁-heme structure might be a prerequisite for the fast rate of NO dissociation from the ferrous form, a property that cannot be achieved with a standard b-type heme.

Interestingly, other heme groups belonging to different hemoproteins, such as the heme d of cytochrome bd from Escherichia coli, have been recently shown to dissociate NO more rapidly (0.133 s⁻¹) than b-type-containing hemoproteins (34). In the latter case it has been suggested that expression of b-type oxidases, instead of heme copper ones, would promote pathogenicity of the microorganism by enhancing bacterial tolerance to nitrosative stress. Thus, the chemical structure of the heme controls the rate of NO dissociation even in proteins whose physiological role is not to produce NO; the need for cd1 NIR to have this peculiar cofactor can be now fully appreciated.

In summary, we have shown that P. aeruginosa cd1 NIR quickly dissociates NO from the ferrous d₁-heme iron. We have examined product formation under different experimental conditions and propose an alternative yet plausible mechanism of the catalytic cycle in which the fully reduced NO-bound derivative can be viewed as a genuine on-pathway intermediate.

Acknowledgments—We gratefully acknowledge A. Bellelli (Rome, I) for stimulating discussion on the laser experiments. I. Pecht and D. Goldfarb (Rehovot, Israel) are gratefully acknowledged for fruitful discussion of the results. M. Caruso (Rome, I) is acknowledged for skillful technical assistance in the fermentation of bacterial strains.

REFERENCES
1. Zumft, W. G. (1997) Microbiol. Mol. Biol. Rev. 61, 533–616
2. Hassett, D. J., Cuppoletti, J., Trapnell, B., Lymar, S. V., Rowe, J. J., Yoon, S. S., Hilliard, G. M., Parvatiyar, K., Kamani, M. C., Wozniak, D. J., Hwang, S. H., McDermott, T. R., and Ochsner, U. A. (2002) Adv. Drug. Deliv. Rev. 54, 1425–1443
3. Arai, H., Hayashi, M., Kuroi, A., Ishii, M., and Igarashi, Y. (2005) J. Bacteriol. 187, 3960–3968
4. Barraud, N., Hassett, D. J., Hwang, S. H., Rice, S. A., Kjelleberg, S., and Webb, J. S. (2006) J. Bacteriol. 188, 7344–7353
5. Rinaldo, S., and Cutruzzola, F. (2007) in Biology of the Nitrogen Cycle (Bothe, H., Ferguson, S. J., and Newton, W. E., eds) pp. 37–55, Elsevier, Amsterdam
6. Cutruzzola, F., Rinaldo, S., Centola, F., and Brunori, M. (2003) IUBMB Life 55, 617–621
7. Silvestrini, M. C., Tordi, M. G., Musci, G., and Brunori, M. (1990) J. Biol. Chem. 265, 11783–11787
8. George, S. J., Allen, J. W., Ferguson, S. J., and Thorneley, R. N. (2000) J. Biol. Chem. 275, 33231–33237
9. Cutruzzola, F., Brown, K., Wilson, E. K., Bellalli, A., Arese, M., Tegoni, M., Cambillau, C., and Brunori, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2232–2237
10. Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997) Biochemistry 36, 6814–6818
11. Averill, B. A. (1996) Chem. Rev. 96, 2951–2964
12. Zajicek, R. S., Cartron, M. L., and Ferguson, S. J. (2006) Biochemistry 45, 11208–11216
13. Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W., and Melling, I. (1976) Biochem. J. 157, 423–430
14. Silvestrini, M. C., Cutruzzola, F., D’Alessandro, R., Brunori, M., Fochesato, N., and Zennaro, E. (1992) Biochem. J. 285, 661–666
15. Silvestrini, M. C., Colosimo, A., Brunori, M., Walsh, T. A., Barber, D., and Greenwood, C. (1979) Biochem. J. 183, 701–709
16. Antonini, E., and Brunori, M. (1971) in Frontiers of Biology (Neuberger, A., and Tatum, E. L., eds) Vol. 21, p. 19, North-Holland Publishing Co., Amsterdam-London
17. Arcovito, A., Gianni, S., Brunori, M., Travaglini-Allocatelli, C., and Bellelli, A. (2001) J. Biol. Chem. 276, 41073–41078
18. Cutruzzola, F. (1999) Biochim. Biophys. Acta 1411, 231–249
19. Moore, E. G., and Gibson, Q. H. (1976) J. Biol. Chem. 251, 2788–2794
20. Parr, S. R., Wilson, M. T., and Greenwood, C. (1975) Biochem. J. 151, 51–59
21. Wilson, E. K., Bellalli, A., Liberti, S., Arese, M., Grasso, S., Cutruzzola, F., Brunori, M., and Brzezinski, P. (1999) Biochemistry. 38, 7556–7564
22. Springer, B. A., Slijper, S. G., Olson, J. S., and Phillips, G. N. Jr. (1994) Chem. Rev. 94, 699–714
23. Parr, S. R., Wilson, M. T., and Greenwood, C. (1974) Biochem. J. 139, 273–276
24. Barber, D., Parr, S. R., and Greenwood, C. (1978) Biochem. J. 175, 239–249
25. Sun, W., Arese, M., Brunori, M., Nurizzo, D., Brown, K., Cambillau, C., Tegoni, M., and Cutruzzola, F. (2002) Biochim. Biophys. Res. Commun. 291, 1–7
26. Azizi, F., Kielbasa, J. E., Adeyiga, A. M., Maree, R. D., Frazier, M., Yakubu, M., Shields, H., King, S. B., and Kim-Shapiro, D. B. (2005) Free. Radic. Biol. Med. 39, 145–151
27. Parr, S. R., Barber, D., Greenwood, C., and Brunori, M. (1977) Biochem. J. 167, 447–455
28. Silvestrini, M. C., Tordi, M. G., Colosimo, A., Antonini, E., and Brunori, M. (1982) Biochem. J. 203, 445–451
29. Blatt, Y., and Pecht, I. (1979) Biochemistry 18, 2917–2922
30. Richter, C. D., Allen, J. W., Higham, C. W., Koppenhoefer, A., Zajicek, R. S., Watmough, N. J., and Ferguson, S. J. (2002) J. Biol. Chem. 277, 3093–3100
31. Cassoly, R., and Gibson, Q. H. (1975) J. Mol. Biol. 91, 301–313
32. Walsh, T. A., Johnson, M. K., Greenwood, C., Barber, D., Springall, J. P., and Thomson, A. J. (1979) Biochem. J. 177, 29–39
33. Barkigia, K. M., Chang, C. K., Fajer, J., and Renner, M. W. (1992) J. Am. Chem. Soc. 114, 1701–1707
34. Borisov, V. B., Forte, E., Sarti, P., Brunori, M., Konstantinov, A. A., and Giuffré, A. (2007) Biochem. Biophys. Res. Commun. 355, 97–102
35. Sarti, P., Giuffré, A., Forte, E., Mastronuccia, D., Barone, M. C., and Brunori, M. (2000) Biochem. Biophys. Res. Commun. 274, 183–187