The catalytic mechanism of *Pseudomonas aeruginosa* cd1 nitrite reductase

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**Abstract**

The cd1 NiRs (nitrite reductases) are enzymes catalysing the reduction of nitrite to NO (nitric oxide) in the bacterial energy conversion denitrification process. These enzymes contain two distinct redox centres: one covalently bound c-haem, which is reduced by external electron donors, and another peculiar porphyrin, the d1-haem (3,8-dioxo-17-acyrate-porphyrindione), where nitrite is reduced to NO. In the present paper, we summarize the most recent results on the mechanism of nitrite reduction by the cd1 NiR from *Pseudomonas aeruginosa*. We discuss the essential catalytic features of this enzyme, with special attention to the allosteric regulation of the enzyme’s activity and to the mechanism employed to avoid product inhibition, i.e. trapping of the active-site reduced haem by the product NO. These results shed light on the reactivity of cd1 NiRs and assign a central role to the unique d1-haem, present only in this class of enzymes.

**Introduction**

*Pseudomonas aeruginosa* is a Gram-negative bacterium commonly found in soil and water, well known for its metabolic versatility; under anaerobic conditions it can use nitrate and nitrite to produce energy via the denitrification pathway. In natural environment, denitrification is the part of the biological nitrogen cycle in which nitrate is transformed into nitrogen gas; reduction of nitrate occurs in four stages each catalysed by a specific metalloenzyme [i.e. the nitrate, nitrite, NO (nitric oxide) and N2O reductases] [1], according to the following scheme: \( \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2 \rightarrow \text{N}_2\text{O} \).

The denitrification pathway is expressed under low oxygen tension in the presence of nitrogen oxides such as nitrate and nitrite [1].

It is well known that facultative aerobic bacteria are capable of adapting their respiratory capability and reorganizing their metabolism to cope with \( \text{O}_2 \) availability. A striking example is given by denitrifiers, including human pathogens such as *Ps. aeruginosa* and *Neisseria meningitidis*, which were shown to adapt to the variable supply of \( \text{O}_2 \) and nitrate/nitrite by regulating the expression and activity of the various reductases involved in respiratory metabolism.

In the denitrification pathway, NO is produced from nitrite by the enzyme NiR (nitrite reductase); two distinct classes of NiRs are found in bacteria both yielding NO as the main product. The two types of NiR contain either copper (CuNiR) or haem (cd1NiR) as cofactor, with the haem-containing enzymes occurring more frequently; in *Ps. aeruginosa*, nitrite reduction is carried out by cd1NiR. The membrane-bound enzyme NOR (nitric oxide reductase) is responsible for NO conversion into \( \text{N}_2\text{O} \).

*Ps. aeruginosa* is also a human pathogen, responsible for severe nosocomial infections, in particular in chronic respiratory diseases such as cystic fibrosis. In the airways of the patients, *Ps. aeruginosa* is capable of anaerobic growth by respiration using nitrate or nitrite as terminal electron acceptors [1]; thus pathogenesis, NO metabolism and anaerobic denitrification are strictly related [2,3]. Denitrification is important to detoxify NO under anaerobic conditions: the host’s defence systems are neutralized by the activity of NOR [4]. On the other hand, the denitrification machinery is a key signal for the expression of virulence factors; in a recent paper, van Alst et al. [5] demonstrated that production of NO by cd1NiR is required under aerobic conditions for *Ps. aeruginosa* virulence in a *Caenorhabditis elegans* model. Given that excess free NO may cause metabolic suicide of the pathogen [2], during anaerobic growth, *Ps. aeruginosa* controls the levels of NO by regulating its synthesis and degradation [6]. The key role of nitrite reduction is also highlighted by experiments showing that exposure to 15 mM \( \text{NO}_2^- \) at pH 6.5 kills a mucoid variant of *Ps. aeruginosa*, abundant in the airways of cystic fibrosis patients, in part because of the low NiR activity present in this mutant strain [7,8].

In the present paper, we describe the mechanism of nitrite reduction by cd1NiR from *Ps. aeruginosa* (Pa-cd1NiR) [9,10]. We discuss the essential catalytic features of this enzyme, with special attention to the recent experiments on the allosteric regulation of the activity and to the mechanism employed to avoid product inhibition, i.e. trapping of the active-site reduced haem by the product NO.

Pa-cd1NiR is a homodimer containing one c-haem and one d1-haem (3,8-dioxo-17-acyrate-porphyrindione) group in each subunit. Electrons are transferred from cytochrome C551 to the c-haem moiety of the enzyme [11], and thereby
Figure 1 | Chemical structure of the $d_1$-haem and other partially saturated porphyrins: (A) $d_1$-haem ($cd_1$NiR), (B) $b$-haem (myoglobin), (C) $d$-haem ($bd$ oxidase) and (D) sirohaem (sulfite and nitrite reductase).

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Internally to the active-site Fe(III) $d_1$-haem, to which the substrate NO$_2^-$ binds and is reduced to NO [1]. The $d_1$-haem (Figure 1A) is a partially saturated macrocycle with a set of oxo, methyl and acrylate substituents; other haems in biology in which the porphyrin ring is partially saturated are the $d$-haem in *Escherichia coli* and sirohaem of bacterial and plant sulfite and NiRs (Figures 1C and 1D) [12,13]. The $d_1$-haem is unique to the $cd_1$NiRs [1,14] and is synthesized by a specialized pathway present only in denitrifiers (strongly induced in *Pseudomonas aeruginosa* upon nitrite treatment).

**Nitrite reduction by cd$_1$NiR**

Nitrite reduction to NO is the physiologically relevant activity of $cd_1$NiR [15,16]; indeed the expression of $cd_1$NiR is induced by low O$_2$ tensions and presence of nitrogen oxides [1]. NO is produced efficiently by Pa-$cd_1$NiR (catalytic-centre activity $= 6 \text{ s}^{-1}$ at pH 7.0) [17] and the activity is pH dependent with an optimum between pH 5.8 and pH 6.5 [15,17]. The current knowledge on the individual steps in the catalytic cycle is summarized below.

**Substrate binding**

In $cd_1$NiR, nitrite binds to the reduced $d_1$-haem with high affinity ($K_m = 6 \mu$M) [18]; binding of nitrite to the Fe(II)-iron is expected in the reaction mechanism since this state of the iron has to supply the electron needed for the reduction process. The nitrite molecule is thought to bind via the nitrogen atom in Pa-$cd_1$NiR forming the so-called nitro complex; this is an important point to clarify since the chemical reactivity of the nitrite molecule may differ considerably depending on the mode of binding. The crystal structure of the reduced nitrite-bound derivative of Pa-$cd_1$NiR is not available, but in the *Paracoccus pantotrophus* $cd_1$NiR the nitrite molecule binds via the nitrogen atom [19]. This observation agrees with results on other haem proteins [20,21] and on synthetic iron porphyrin nitrite complexes in which, regardless of the iron oxidation state, the N-binding mode is observed [22–24]. The nitrite N-binding mode also agrees with the current mechanism of the reduction of nitrite by $cd_1$NiRs which is thought to occur via a double protonation of a terminal O atom of the nitrite molecule. Theoretical calculations have suggested that the O-binding mode is also possible for nitrite binding and reduction of $cd_1$NiR [25] and also for other haem proteins such as haemoglobin [26]. Although nitrite can bind through the O-binding mode (the so-called nitrito mode) to the haem of myoglobin and haemoglobin [27–29], in the case of the $d_1$-haem there is no experimental evidence that such O-binding mode may occur.

The high affinity for nitrite of the ferrous $d_1$-haem (see above) is a peculiar and physiologically relevant feature of all $cd_1$NiRs; indeed these proteins display unusually high affinity for several anionic molecules in the ferrous state, including the inhibitor cyanide [30,31]. This behaviour is remarkably different from that observed in the $b$-type haem-containing proteins in which the negatively charged molecules (nitrite and cyanide) usually bind much better to the Fe(III) iron. The
Figure 2 | Conformational changes seen after the reduction of Pa-cd₁NiR
Superimposition of the three-dimensional structure of the oxidized (in black) and the reduced form (in grey). Three relevant residues in the d₁-haem pocket are shown, i.e. His₃⁶⁹ and His₃²⁷ coming from monomer A (HIS₃⁶⁹A and HIS₃²⁷A in the Figure), whereas Tyr¹⁰ comes from the adjacent monomer B (TYR₁⁰B in the Figure). The reorganization of the 56–62 loop and the new hydrogen bond (broken line) formed between Thr⁵⁹ and Gln¹¹ in the reduced structure are clearly seen (THR₅⁹A and GLN₁¹B in the Figure respectively).

much higher affinity for nitrite of the d₁-haem can be partially explained with the presence of two electron withdrawing carbonyl groups on the d₁-haem ring (Figure 1A). The two conserved histidine residues (His₃²⁷ and His₃⁶⁹) in the active-site pocket [32] (see Figure 2) also contribute to the stabilization of the nitrite anion: upon mutagenesis of the latter histidine residues into alanine, the affinity for nitrite falls and the turnover rate is reduced 100-fold [18]. The high affinity for nitrite of reduced Pa-cd₁NiR is important for the catalytic mechanism as discussed below.

Electron transfer from the c-haem to the d₁-haem
As mentioned above, in the catalytic cycle the substrate (i.e. nitrite) binds to the ferrous enzyme and is then reduced to yield NO and oxidized d₁-haem. Reduction of the d₁-haem occurs (by intramolecular electron transfer) from the c-haem, which in turn is reduced by external electron donors (see above). Early kinetic studies of cd₁NiRs isolated from Ps. aeruginosa [33], P. pantotrophus [34] and Pseudomonas stutzeri [35] yielded considerably different rate constants for this step: 3, 1400 and 23 s⁻¹ respectively. Interestingly, the internal electron transfer rate from the c-haem to the d₁-haem is tightly regulated by an allosteric mechanism. In more detail, the c-haem to d₁-haem electron transfer rate constant was found to decrease (by more than one order of magnitude) as the number of electrons introduced in the enzyme is increased [36]. This decrease in rate may, in principle, arise from a decrease in the electronic coupling between donor and acceptor sites due to conformational changes between reduced and oxidized Pa-cd₁NiR [37]. These conformational changes include the relocation of the side chain of Tyr¹⁰ located nearby the d₁-haem, the coupled dissociation of the OH⁻ ligand of the Fe(III) iron and the disruption of the hydrogen bonds at the interface between the two haem domains (Figure 2). In agreement with this structural interpretation, the intramolecular electron transfer is faster (approx. 10-fold) when the conserved His₃⁶⁹ is replaced by alanine [36]. The positive charge density on the distal side of the d₁-haem is decreased in this mutant and thus the hydroxide ligand is destabilized, lowering the energy barrier for electron transfer and thus increasing the rate.

In summary, we propose that, in Pa-cd₁NiR, allosteric communication between identical monomers may depend on a perturbation of the domain–domain interface within each monomer and a coupled large relocation of the
c-haem domain [38] on reduction of the enzyme. These conformational changes disrupt the above-mentioned hydrogen bonds, decreasing the electronic coupling between the c-haem and the d1-haem. Thus the control of the electron transfer rates in Pa-cd1-NiR involves contributions from local changes, affecting the driving force of the intramolecular electron transfer, and more global changes, which may affect the electronic coupling between sites (within and between monomers), further rising the energy barrier for electron transfer as the enzyme undergoes reduction.

The complex allosteric behaviour observed for the homodimeric Pa-cd1-NiR indirectly confirms in our opinion the relevance of this step in the control of the reactivity of the enzyme.

**Catalysis and product release**

In the catalytic cycle, the formation of a complex between NO and the reduced d1-haem might slow down product release; indeed, the reduced d1-haem–NO complex may be considered an inhibited species [39] given that NO binds tightly to ferrous haem proteins [40,41]. However, we have clearly shown that the rate constant of NO dissociation from reduced Pa-cd1-NiR is high (up to 70 s\(^{-1}\)) [17], several orders of magnitude greater than that of haem b-containing proteins. Consequently, the affinity of Pa-cd1-NiR for NO is lowered and the ferrous enzyme is not firmly inhibited by NO [17,42]. It is noteworthy that nitrite reduction can still be monitored after pre-incubation of reduced Pa-cd1-NiR with NO [17].

These conclusions on Pa-cd1-NiR are also supported by the experiments carried out on the enzyme from *P. pantotrophus* suggesting that intramolecular c-haem to d1-haem electron transfer triggers product release [43]; indeed it has been shown that *P. pantotrophus* d1-Fe(III)–NO complex is a very long-lived species in the absence of excess reductant [44,45]. Therefore also *P. pantotrophus* cd1-NiR works efficiently only in the presence of substrate and electron donor proteins [16,44,46], i.e. it cannot release NO in the absence of reducing equivalents.

We have also shown that nitrite can displace the NO bound to the ferrous enzyme [42], allowing the enzyme to enter a new catalytic cycle; therefore the high affinity of the active-site ferrous d1-haem for nitrite (see above) actively contributes to NO dissociation during the catalytic cycle. In agreement with this observation, if the affinity of Pa-cd1-NiR for nitrite is decreased (as in the H369A mutant) the fully reduced NO-bound derivative accumulates [18,42].

The observation that NO and nitrite can compete for binding may suggest that the formation of N\(_2\)O\(_3\) could in principle occur, for example, in a reaction similar to that proposed for Hb [47]. This event is, however, highly unlikely, mainly because during catalysis the d1-haem is maintained in the reduced state by internal electron transfer from the c-haem. Moreover, ferric d1-haem has low affinity for nitrite [48], a feature that probably limits further reaction with free NO to produce N\(_2\)O\(_3\).

In summary, the high affinity for nitrite, the allosteric control of the internal electron transfer rate and the exceptionally high NO dissociation rate ascertain that, when nitrite is available, the NiR is active and can actively produce and release NO.

**Concluding remarks**

In facultative aerobic bacteria, such as *Ps. aeruginosa*, nitrite is used as an electron acceptor and converted into NO by the specialized class of haem-containing enzymes called cd1-NiRs [1]. The mechanism employed by cd1-NiRs is now becoming clear, highlighting the essential role of the peculiar d1-haem, whose presence in these enzymes was largely unexplained. As mentioned above, the high affinity of reduced d1-haem for nitrite [18] and the exceptionally fast NO dissociation [17] are trademarks of the d1-haem, and are tuned by two conserved histidine residues present in the active site of Pa-cd1-NiR.

We previously proposed [49] that ancient haems, such as the d1-haem of cd1-NiR or the sirohaem of bacterial and plant nitrite and sulfite reductases, have been evolutionarily conserved because their role is strategic to the organism where they are found today. The peculiar low affinity for NO of the reduced d1-haem of *Ps. aeruginosa* cd1-NiR is a component of this strategy, which could not be achieved by the more common b-type haem. It would be very interesting to measure and compare the NO releasing properties of the various porphyrins shown in Figure 1; such experiments, together with a computational study of the electronic structure of the same porphyrins may allow us to correlate the functional properties of these partially saturated macrocycles with NO with their structure.

Finally, the recent evidence that nitrite can also be reduced to NO under hypoxic conditions in eukaryotes, suggests that anaerobic/microaerobic nitrite reduction in higher organisms is a vestigial function originating from the denitrifying metabolism and operative long before the advent of aerobic respiration [50].

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**References**


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