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Nitrosylation of c heme in cd_1-nitrite reductase is enhanced during catalysis

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ABSTRACT

The reduction of nitrite into nitric oxide (NO) in denitrifying bacteria is catalyzed by nitrite reductase. In several species, this enzyme is a heme-containing protein with one c heme and one d_1 heme per monomer (cd_1NiR), encoded by the *nirS* gene.

For many years, the evidence of a link between NO and this hemeprotein represented a paradox, given that NO was known to tightly bind and, possibly, inhibit hemeproteins, including cd_1NiRs.

It is now established that, during catalysis, cd_1NiRs diverge from "canonical" hemeproteins, since the product NO rapidly dissociates from the ferrous d_1 heme, which, in turn, displays a peculiar "low" affinity for NO (K_D = 0.11 μM at pH 7.0).

It has been also previously shown that the c heme reacts with NO at acidic pH but c heme nitrosylation was not extensively investigated, given that in cd_1NiR it was considered a side reaction, rather than a genuine process controlling catalysis.

The spectroscopic study of the reaction of cd_1NiR and its semi-apo derivative (containing the sole c heme) with NO reported here shows that c heme nitrosylation is enhanced during catalysis; this evidence has been discussed in order to assess the potential of c heme nitrosylation as a regulatory process, as observed for cytochrome c nitrosylation in mammalian mitochondria.

1. Introduction

Nitric oxide (NO) is a well-established signalling molecule among both eukaryotes and bacteria [1–4]. In bacteria, dedicated redox regulators perceive NO to promote N-oxides scavenging, nitrogen assimilation or anaerobic respiration metabolisms [5–9]; NO, in turn, originates from both chemical and enzymatic synthesis *via* oxidation of L-arginine, or assimilative nitrate/nitrite reduction (as a by-product) or dissimilative nitrite reduction (as an intermediate of denitrification) [2,10–12].

In denitrifying bacteria, the reduction of nitrite into NO is catalyzed by dedicated nitrite reductase. In several species, this enzyme is a heme-containing protein with one c heme and one d_1 heme per monomer (cd_1NiR), encoded by the *nirS* gene [13]. In human pathogens such as *Pseudomonas aeruginosa* expression of *nirS* gene is crucial for growing as biofilm in chronic infections sites including the airways of cystic fibrosis (CF) patients [4].

The reaction mechanism of cd_1NiR represented a biochemical conundrum, given that NO, the product of NiR enzymes, binds to the d_1 heme and, to a lower extent, the c heme [14]; generally speaking, NO tightly binds ferrous hemeproteins, thus acting as a powerful inhibitor [15–17]. In the last few years, kinetic studies on cd_1NiR from *P. aeruginosa* elucidated the mechanistic details controlling the NO binding and release events from the active site (i.e. d_1 heme) [12,18,19]. Briefly, ferrous d_1 heme active site displays an unusual low affinity for NO (K_D = 0.11 μM at pH 7.0), due to the rapid dissociation rate of this ligand from ferrous heme iron [12]; moreover, ferrous d_1 heme binds with high affinity anions such as the substrate nitrite, which replaces NO during catalysis, allowing productive NO release [18]. This unusual reactivity of the d_1 heme with both NO and anions is a general property of this cofactor [20]. The reaction of NO with the ferrous active site of cd_1NiR, which generally represents a dangerous event for heme-containing enzymes, does not inhibit the enzyme, which may enter a new catalytic cycle in the presence of excess reductants and nitrite [18,20]. A decrease in the concentration of both substrates leads to progressive product inhibition and finally

Abbreviations: NO, nitric oxide; cd_1NiR, cd_1 nitrite reductase.

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populates the ferrous-NO bound adduct of the d_1 heme, since affinity for NO, although lower than that of other hemeproteins, becomes significant under substrate(s) depletion; the reduced-NO bound adduct represents a "resting" state which can enter catalysis when substrates become again available [12,18]. NO may also react with c heme under certain conditions (i.e. acidic pH, or high levels of NO or nitrite and reductants), even though the role of this reaction in controlling catalysis has not been proposed or elucidated [14,21]. Interestingly, when the concentration of reductants (i.e. electrons) is $\leqslant$ than that of nitrite during turnover, c heme nitrosylation occurs to a lower extent (~50% lower) [21]. Since electrons and nitrite availability controls NO dissociation from ferrous d_1 heme and productive turnover, it is not excluded that c heme nitrosylation might be a probe of a fully functional nitrite reductase enzyme, and thus, indirectly, of the availability of nitrite and electrons.

It is clear that c heme nitrosylation represents a strategy in eukaryotes to control the release of cytochrome c in the cytoplasm during early apoptotic events [22,23]; it would be exciting if also in prokaryotes a role for c heme nitrosylation as a signal with a regulatory role could be demonstrated.

Here a detailed spectroscopic analysis of the reactivity of *P. aeruginosa* cd_1NiR and its semi-apo derivative (containing the sole c heme) with NO is reported; we found that the c heme reacts with NO mainly during nitrite reductase turnover. The susceptibility of c heme to NO is here discussed, together with the re-evaluation of literature data, also in light of the new knowledge gained in the last years on this class of enzymes.

2. Materials and methods

2.1. Protein purification

Wild type NiR and the semi-Apo NiR were purified as previously published [12,24]. Protein concentration was determined spectroscopically according to [14] and to [24] for the oxidized holo and as-purified semi-Apo NiR, respectively. Protein concentrations reported here refer to the concentration of the monomer.

2.2. Absorbance spectra of semi-Apo NiR

5.4 μM reduced semi-Apo NiR solution (c^{2+}-NiR as purified, see [24]) was equilibrated anaerobically under nitrogen atmosphere either at pH 7.0 or pH 6.2 (in 50 mM Bis–Tris) in a gas tight tonometer and the corresponding absorbance spectra were collected.

Oxidized semi-apo NiR (c^{3+}-NiR) was prepared by adding 2 equivalents of ferricyanide (SIGMA) to 400 μl of 13.5 μM c^{2+}-NiR; excess of ferricyanide was removed by gel filtration on Sephadex G-25 resin (GE-Healthcare) in 50 mM Bis–Tris pH 6.2 and the eluted protein was equilibrated anaerobically under nitrogen atmosphere.

100 μM NO was added anaerobically to each of the protein solutions with a gas tight syringe from a saturated solution of NO (2 mM at 20 °C) and the reaction was followed spectroscopically. All spectra were recorded in a JASCO V550 spectrophotometer.

2.3. Absorbance spectra of holo NiR

5.6 μM oxidized holo NiR ($c^{3+}d_1^{3+}$-NiR) or 6.1 μM fully reduced ($c^{2+}d_1^{2+}$-NiR) holo NiR were equilibrated under nitrogen atmosphere at pH 7.0 in 50 mM Bis–Tris in a gas tight tonometer; $c^{2+}d_1^{2+}$-NiR was obtained after addition of 10 mM sodium ascorbate under anaerobic conditions. The mixed valence ($c^{2+}d_1^{3+}$-NiR) holo NiR solution (13.2 μM) was prepared anaerobically in 50 mM Bis–Tris pH 7.0 and complete c heme reduction was obtained after the addition of a slight excess of sodium ascorbate (20–25 μM). The corresponding NO-bound derivatives were obtained as described above. Re-oxidation of $c^{2+}d_1^{3+}$-NiR was carried out by adding 3 equivalents of ferricyanide in the gas-tight tonometer.

Spectra recorded under steady-state conditions were collected starting from $c^{2+}d_1^{2+}$-NiR (5 μM) handled as described above at pH 6.2, 7.0 or 8.0 (the latter in 50 mM Tris buffer); the reaction was started by the addition of 1 mM sodium nitrite (SIGMA) under anaerobic conditions.

All spectra were recorded in a JASCO V550 spectrophotometer.

3. Results and discussion

3.1. Reactivity of semiApo cd_1-Nir with nitric oxide

In order to assess the involvement of c heme nitrosylation in controlling cd_1NiR activity, the reactivity of c heme with NO was probed, for the first time, using the semi-Apo NiR (c^{2+}-NiR), the recombinant form of cd_1NiR lacking the d_1 heme. This approach allowed us to evaluate the intrinsic reactivity of the c heme, devoid of possible effects due to the presence of d_1 heme.

As depicted in Fig. 1A, binding of NO (100 μM; pH 6.2) to ferrous c heme (c^{2+}) occurs strikingly slowly at $4.3 \pm 0.2 \times 10^{-3}$ s^{-1}; UV–Vis spectrum of the final species presents two peaks at 529 and 562 nm (Fig. 1A, inset, bold line), as expected for the c^{2+}-NO^+ species rather than for the c^{2+}-NO species of ferrocytochrome, where the peaks are at 534–540 and 567–568 nm [14,25]. At this stage we cannot rule out that the observed absorbance spectrum accounts for the presence of several species including unreacted c^{2+}-NiR and the c^{2+}-NO species.

Reaction of NO (under the same experimental conditions) with c^{3+}-NiR triggers the reduction of the c heme within the first hour (occurring at $4.0 \pm 0.8 \times 10^{-4}$ s^{-1}, Fig. 1B), as confirmed by the appearance of the two peaks at 550 and 522 nm typical of ferrocytochrome (Fig. 1B, inset, grey line); only the addition of further NO (600 μM) allows the c heme to be (partly) nitrosylated (this second process occurring at $2.1 \pm 0.4 \times 10^{-4}$ s^{-1}) (Fig. 1B, inset, bold line). Interestingly, under these experimental conditions, a c^{2+}-NO species is populated together with the unreacted c^{2+} protein (peaks at 532, 550 and 569), in agreement with literature data [14].

Reaction of c^{2+}-NiR with NO at pH 7.0 occurs even more slowly, involving a little fraction of c heme (~30%, at $4.6 \pm 0.8 \times 10^{-4}$ s^{-1}, data not shown), indicating that the reaction of the c heme with NO is favoured at acidic pH, in agreement with previous results on holo cd_1NiR [14].

3.2. Reactivity of holo cd_1-NiR with nitric oxide

Previous data demonstrated that, holo cd_1-NiR (hereinafter cd_1-NiR) reacts with NO both at the level of the d_1 heme and of the c heme (either in the ferric and in the ferrous state) at acidic pH, in the presence of saturating NO atmosphere.

In order to understand whether c heme nitrosylation is a mechanism to "sense" the turnover conditions, the experiments reported below were carried out at pH 7.0, where the intrinsic reactivity of the sole c heme with NO is minimal, but the turnover is still significant [12]. We performed this analysis in order to evaluate the role, if any, of the d_1 heme to enhance the reactivity of the c heme with NO, possibly by inducing conformational changes which destabilize coordinating protein residues bound to the c heme iron, as found in other cytochromes c [23,26].

Reaction of fully reduced cd_1-NiR ($c^{2+}d_1^{2+}$-NiR) with NO leads to the nitrosylation of the sole d_1^{2+} heme (Fig. 1S) and it does not affect the reactivity of the c^{2+} heme with NO, where nitrosylation does not occur.

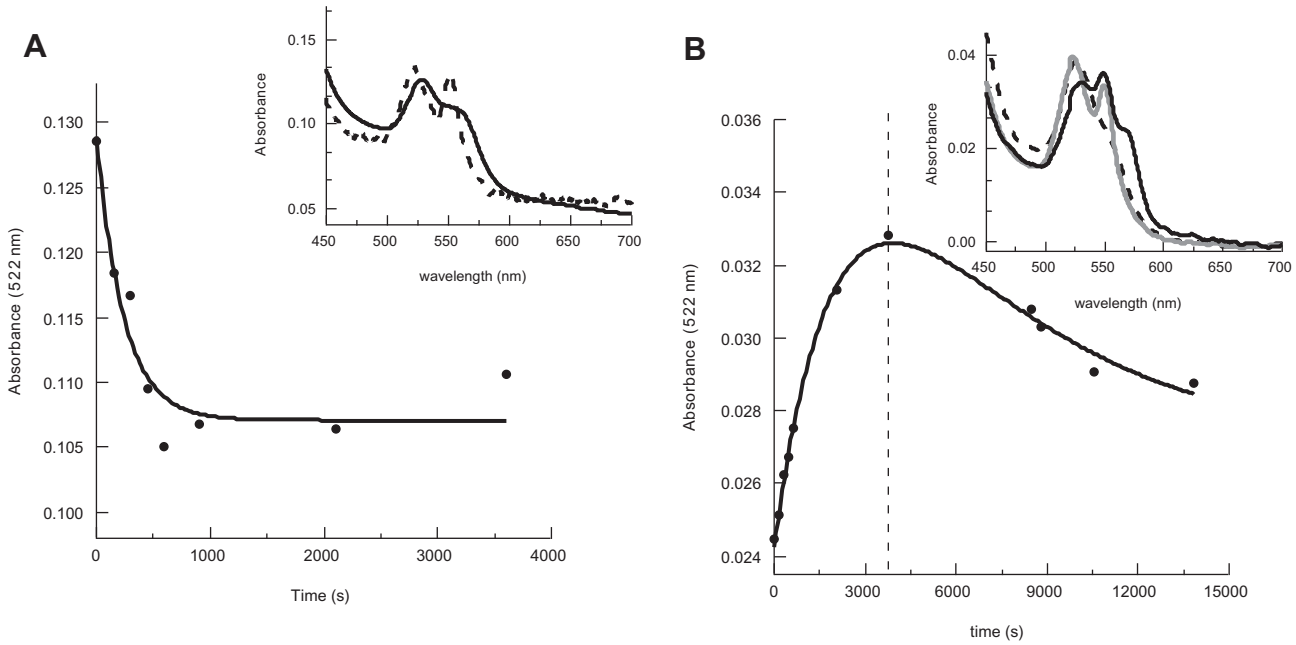

Fig. 1. Reaction of semi Apo-NiR with NO. (A) Timecourse of NO (100 μM) binding to c^{2+}-NiR (5.4 μM, pH 6.2) at 522 nm (black circles) corresponding to one of the typical peaks of the ferrous c heme, which shifts to 529–532 nm upon nitrosylation, has been fitted with a single-exponential (continuous line). Inset: spectra of the ferrous semiApo NiR (dashed line) and of the nitrosylated adduct (continuous line), 1 h after NO addition. (B) The timecourse of NO binding (initially 100 μM and then 600 μM; the time of the second addition is indicated by the dashed line) to ferric semiApo-NiR (2.5 μM) at 522 nm (black circles) has been fitted with a double-exponential (continuous line). Inset: spectra of the ferric semi-Apo NiR (dashed line) and of the intermediates after NO addition (1 and 3.5 h, grey and black lines, respectively).

On the other hand, the reactivity of ferric cd_1NiR ($c^{3+}d_1^{3+}$-NiR, Fig. 2A, bold line) with NO was found to be more complex. Reaction with NO leads to spectroscopic changes which occur simultaneously at both redox centres ($1.7 \pm 0.1 \times 10^{-3}$ s^{-1}, Fig. 2B): d_1^{3+} heme reacts with NO to populate an intermediate with a peak at 638 nm, meanwhile ~50% of the c heme becomes reduced (Fig. 2A, grey thin line). Taking into account that (i) this process proceeds with the same rate on both redox centres and that (ii) the NO-mediated c heme reduction occurring in the semi-apo NiR is about 4-fold slower (see above) than in the holo NiR, it is likely that c heme reduction occurs *via* internal electron transfer from the d_1 heme, which in turn takes the electron from the bound NO radical.

The observed spectroscopic changes could represent the conversion between the fully ferric enzyme ($c^{3+}d_1^{3+}$) into $c^{2+}d_1^{3+}NO^+$ *via* a transient $c^{3+}d_1^{3+}$-NO ⇔ $c^{3+}d_1^{2+}$-NO species, which does not accumulate. Interestingly, the formation of $c^{2+}d_1^{3+}NO^+$ intermediate is followed by a further process occurring at $8.1 \pm 0.9 \times 10^{-4}$ s^{-1} (Fig. 2B), which again involves both redox centres. It seems that the electron comes back from the c heme to the d_1 one to populate a species with a peak at 636 nm (Fig. 2A, dashed line). This adduct could correspond to the $d_1^{2+}NO^+ \Leftrightarrow d_1^{3+}NO$ derivative(s) with a (partly) oxidized c heme. Addition of excess of sodium ascorbate slowly fully reduces the $c^{3+}d_1^{2+}$-NO^+ adduct (confirmed by the appearance of a pronounced shoulder above 650 nm, Fig. 2C, grey line) but no nitrosylation of the c^{2+} heme is observed. A scheme summarizing all the reaction discussed is reported in the Supplemental data (Fig. 2S), together with the peaks of the d_1 heme for each intermediate.

Taken together our data indicate that, in the holo NiR, NO reacts solely with the ferric d_1 heme.

Binding of NO was also assayed starting from the mixed valence holo protein ($c^{2+}d_1^{3+}$) (Fig. 2D, bold line), which populates a species with a peak at 636 nm ($c^{2+}d_1^{3+}NO \Leftrightarrow c^{2+}d_1^{2+}NO^+$, dashed line) and no changes were again observed on the c heme. Oxidation of this species triggers the simultaneous formation of a species with a peak at 638 nm ($d_1^{3+}NO^+$) and of c^{3+} heme (disappearance of the peaks at 522 and 551 nm, Fig. 2D, grey thin line), which does not further react.

In conclusion, binding of NO to holo NiR at pH 7.0 occurs only at the level of the d_1 heme and none of the possible redox state(s) of the c or the d_1 heme promotes/enhances the c heme nitrosylation.

3.3. C heme nitrosylation during cd_1-NiR catalysis

Cd_1NiR catalyzes the reduction of nitrite into NO at the level of the d_1 heme iron; it is likely that also the c heme has evolved a strategy to cope with the NO radical. We investigated the c heme nitrosylation during the reduction of nitrite (i.e. NO production) at pH 7.0: according to the data reported above, no significant c heme nitrosylation should be observed under these experimental conditions, within the timeframe of the catalysis.

$C^{2+}d_1^{2+}$-NiR protein was incubated in the presence of excess nitrite and reductants anaerobically at pH 7.0 and the spectra were collected during the reaction time (Fig. 3A). Contrary to expectations, complete c heme nitrosylation is observed, occurring rapidly at a rate of $1.8 \pm 0.2 \times 10^{-3}$ s^{-1} (Fig. 3B, open circles), very similar to the nitrite reduction rate ($8.0 \pm 0.1 \times 10^{-3}$ s^{-1}, Fig. 3B, black circles), thus indicating that the unexpected reaction of the c heme with NO occurs mainly during the turnover.

Accordingly, kinetics of the c heme nitrosylation is expected to be pH dependent as observed for catalysis of nitrite reduction. At acidic pH (pH 6.2), i.e. the optimal pH for nitrite reductase activity [18] and for c heme nitrosylation (see above), the latter reaction occurs at $1.2 \pm 0.3 \times 10^{-2}$ s^{-1} and it is completed when the enzyme is still working (Fig. 3C); on the other hand, at basic pH (pH 8.0), where the turnover strongly decreases [18], nitrosylation occurs at $2.0 \pm 0.2 \times 10^{-4}$ s^{-1}, 10-fold slower than nitrite reduction (not shown). Moreover, at pH 8.0 only ~30% of c heme nitrosylation is observed, as compared to pH 7 counterpart. The pH dependence of the c heme nitrosylation is likely due to both a different rate of

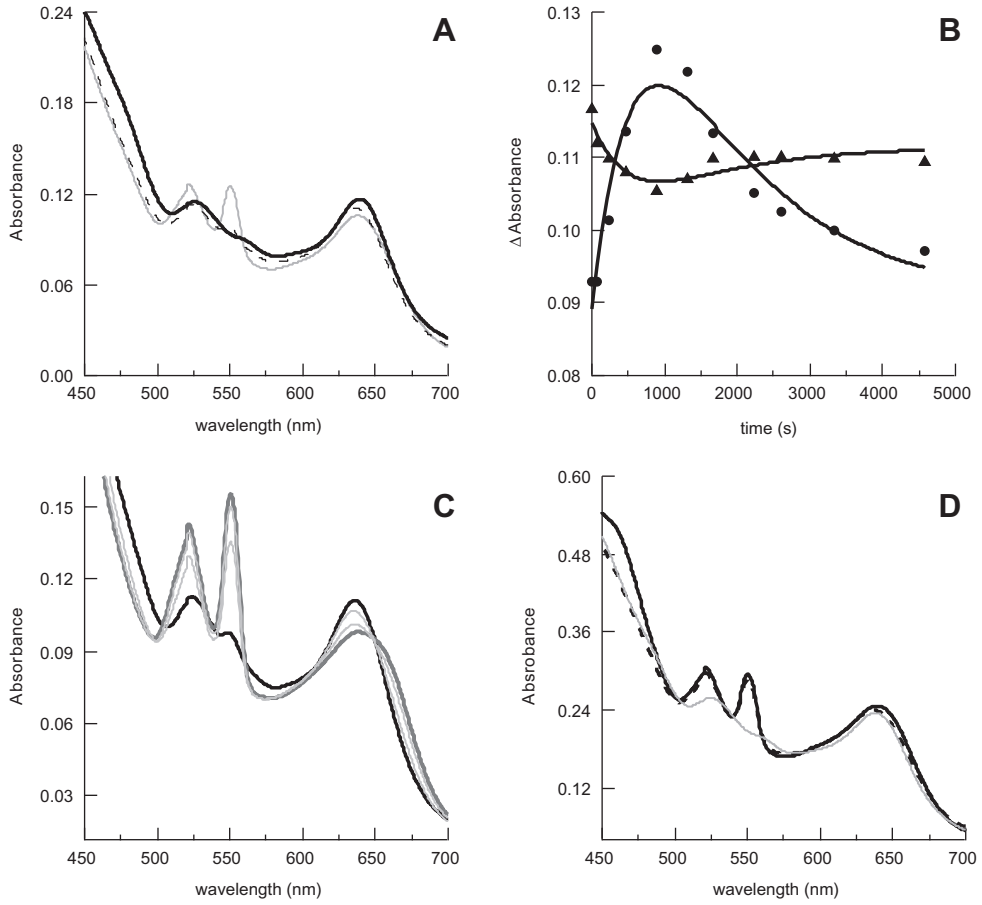

Fig. 2. Reaction of holo-NiR with NO. (A) Spectra of the ferric holo-NiR (5.6 μM, pH 7.0, continuous line) and of the first intermediate populated after the addition of NO showing a peak at 638 nm (grey thin line), which is then converted into a second species with a peak at 636 nm (dashed line). (B) Timecourse of the reaction described in (A) followed at 551 nm (black circles) and 640 nm (black triangles), which are indicative of the c heme and of the d_1 heme, respectively. (C) The second intermediate populated in (B) (black line) was incubated with sodium ascorbate as a reductant and the spectra were recorded (grey lines) until the c heme was fully reduced (grey bold line). (D) Reaction of the mixed valence NiR (13.5 μM, pH 7.0, black line) with NO. A species showing a peak at 636 nm (dashed line) is populated which, in turn, is converted into a ferric species showing a peak at 638 nm upon addition of ferricyanide (grey thin line).

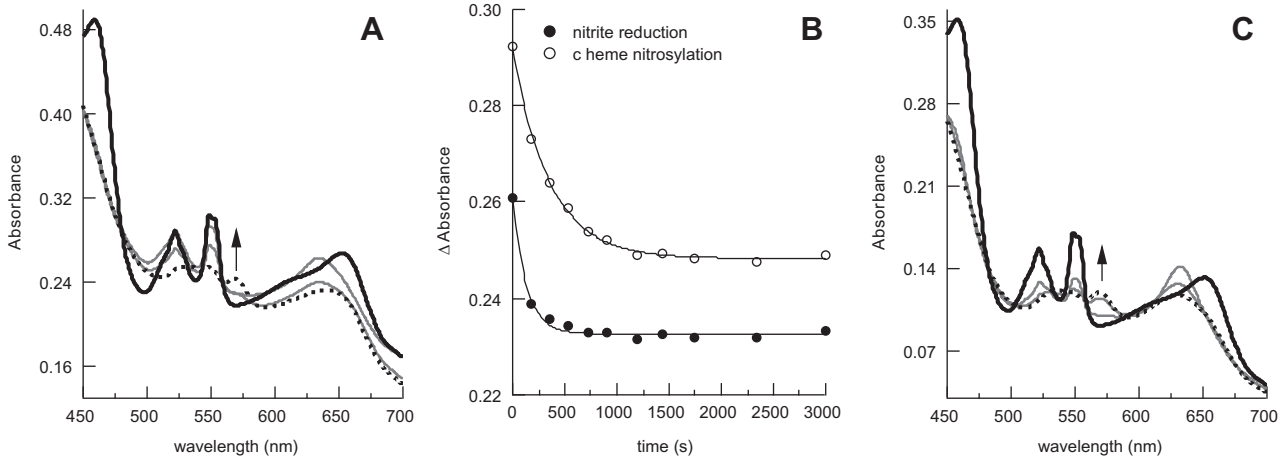

Fig. 3. Reaction of holo-NiR with nitrite. (A) Representative spectra of the reaction of the fully reduced enzyme (5 μM, pH 7.0, black line) with 1 mM sodium nitrite at 0.5, 2 and 15 min (thin grey lines and dotted line, respectively); the arrow indicates the direction of the c heme nitrosylation. (B) Timecourse of the reaction described in (A) followed at 551 nm (open circles) and 640 nm (black circles), which are indicative of the c heme and of the d_1 heme, respectively; data were fitted with a single exponential (continuos line). (C) Representative spectra of the reaction of the fully reduced enzyme (5 μM, pH 6.2, black line) with 1 mM sodium nitrite at 0.5, 2 and 15 min (grey lines and dotted line, respectively); the arrow indicates the direction of the c heme nitrosylation.

NO production at given pH and increased reactivity of the c heme at acidic pH (as observed with the semi-Apo protein).

Nevertheless, the unexpected reactivity at pH 7.0 is clearly due to the peculiar reactivity of the c heme during catalysis, when the

enzyme is fully active (i.e. under excess of nitrite and electrons availability).

We know that these conditions are mandatory (i) to allow productive turnover (nitrite binds very rapidly only the ferrous d_1 heme, which in turn is productively reduced by the c one only under reductive pressure) and (ii) to minimize product inhibition (nitrite replaces NO only from the reduced d_1^{2+}NO adduct) [12,18]. Here we show that these conditions promotes c heme nitrosylation, which can ultimately be considered a probe of the "wealth" of cd_1NiR.

This interpretation gives a rationale to previous evidence showing that substrate imbalance (excess of nitrite vs reductants) dramatically lowers c heme nitrosylation (20%, even at pH 6) as compared to the same reaction carried out with balanced excess of substrates (70% of c heme nitrosylation). The first set up populates mainly the d_1^{3+}NO species (70%), which does not react with further nitrite being in the ferric state, while the second set up leads to the d_1^{2+}NO species, competent to react with nitrite [21], in agreement to our aforementioned hypothesis.

As shown here, catalysis positively affects the reactivity of the c heme with NO, thus suggesting that the coordination bond with the sixth ligand (i.e. Met88) of the c heme may be weaken, in order to allow NO to bind; it is likely that destabilization of iron-methionine coordination involves conformational change(s) occurring mainly during turnover.

It is known that cd_1-NiRs conformation largely depends on the redox state of the protein; major changes in the structural organization were found to occur upon reduction in both *P. aeruginosa* and, more dramatically, in *P. panthotrophus* cd_1-NiRs, in which reduction triggers heme iron ligand exchange and c heme domain rearrangement [11,27,28].

In *P. aeruginosa* cd_1-NiR, the rearrangement occurring upon reduction requires the sole reduction of the c heme, which in turn triggers a cascade of movements (i.e. rotation of distal d_1 heme residue and a loop displacement). We proposed that this rearrangement may represent the "tip of iceberg" of the possible conformations of the c heme domain, which may swing to populate an "open" conformation of the enzyme, possibly relevant as a catalytic intermediate [29,30].

The fact that c heme reduction dictates protein conformation as well c heme nitrosylation, the latter if nitrite is also available, suggests that the c domain rearrangement (structurally characterized, [29]) likely controls the reactivity of the c heme with NO.

This study allowed us to unveil a peculiar reactivity of the c heme with NO during nitrite reduction catalysis, the latter occurring at the d_1 heme. Given that the enzyme works in the presence of balanced excess of both substrates (i.e. nitrite and electrons), when c heme nitrosylation is favoured, it is likely that the latter event could have a physiological role; c heme nitrosylation may report on the status of nitrite reductase activity and represents a signal in bacteria, similarly to cytochrome c in eukaryotes [22,23].

The molecular mechanism underlying this process needs to be clarified; it is likely that the swing and the relocation of the c domain, previously characterized by crystallographic studies and proposed to be an intermediate conformation experienced by the enzyme during catalysis, could also control nitrosylation of the c heme, only observed during turnover.

Further experiments and, possibly, structures are needed to demonstrate whether the c heme nitrosylation "locks" the c heme-containing domain in the proper conformation to support catalysis, until electrons and nitrite are available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.020.

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