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.

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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

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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 ⩽ than that of nitrite during turnover, c heme nitrosylation occurs to a lower extent ($\sim$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 ($\sim$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.