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Functional interactions between nitrite reductase and nitric oxide reductase from *Paracoccus denitrificans*

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Denitrification is a microbial pathway that constitutes an important part of the nitrogen cycle on earth. Denitrifying organisms use nitrate as a terminal electron acceptor and reduce it stepwise to nitrogen gas, a process that produces the toxic nitric oxide (NO) molecule as an intermediate. In this work, we have investigated the possible functional interaction between the enzyme that produces NO; the *cd₁* nitrite reductase (*cd₁NiR*) and the enzyme that reduces NO; the c-type nitric oxide reductase (*cNOR*), from the model soil bacterium *P. denitrificans*. Such an interaction was observed previously between purified components from *P. aeruginosa* and could help channeling the NO (directly from the site of formation to the side of reduction), in order to protect the cell from this toxic intermediate. We find that electron donation to *cNOR* is inhibited in the presence of *cd₁NiR*, presumably because *cd₁NiR* binds *cNOR* at the same location as the electron donor. We further find that the presence of *cNOR* influences the dimerization of *cd₁NiR*. Overall, although we find no evidence for a high-affinity, constant interaction between the two enzymes, our data supports transient interactions between *cd₁NiR* and *cNOR* that influence enzymatic properties of *cNOR* and oligomerization properties of *cd₁NiR*. We speculate that this could be of particular importance *in vivo* during metabolic switches between aerobic and denitrifying conditions.

Denitrification is an anaerobic process in which nitrate (NO₃⁻) is reduced stepwise to nitrogen gas (N₂) via the intermediates nitrite, nitric oxide and nitrous oxide. There is widespread interest in denitrification because it limits the amount of nitrogen available to crops by decreasing the amount of nitrate and nitrite in the soil and because incomplete denitrification yields nitrous oxide which is a potent green-house gas. In *Paracoccus* (*P.*) *denitrificans*, a model organism for both aerobic respiration and denitrification, the enzymes that catalyze these reactions are: nitrate reductase (NAR), reducing nitrate to nitrite, nitrite reductase (NiR) which reduces nitrite to nitric oxide, nitric oxide reductase (NOR), reducing nitric oxide to nitrous oxide and finally nitrous oxide reductase (N₂OR), which reduces nitrous oxide to nitrogen gas (for a review on denitrification enzymes, see¹).

The stepwise reduction of nitrate requires the product of one enzyme to be the substrate for the next enzyme in the pathway and as a consequence the expression of all four enzymes should be coordinated and regulated in such way that the concentrations of nitrite and nitric oxide are kept at concentrations that are not toxic to the cell². Lethal nitric oxide concentrations have been shown to vary between organisms, with some bacteria such as *Agrobacterium tumefaciens* accumulating μM NO concentrations during rapid switches between oxic and anoxic conditions, but in *P. denitrificans* nitric oxide is kept at (or below) ~30 nM³.

The enzyme catalyzing the reduction of nitrite to nitric oxide (NO₂⁻ + e⁻ + 2 H⁺ → NO + H₂O) in *P. denitrificans* is cytochrome *cd₁* nitrite reductase (*cd₁NiR*), a soluble protein located in the periplasm (for a recent review on *cd₁NiR* and nitrite, see⁴). The almost identical (97% sequence identity) and well characterized *cd₁NiR* from *Paracoccus pantotrophus* is purified⁵ and crystallized as a dimer⁶. Each *cd₁NiR* monomer consists of one small

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heme *c* domain and one large *d*₁ domain, where NO_2^- reduction takes place. The heme *c* domain receives electrons from one of two soluble donors; either cytochrome *c*⁵⁵⁰ or the copper protein pseudoazurin⁷. The heme *d*₁ in the catalytic domain has an unusual ability (as compared to other hemes) to rapidly release NO, thereby lowering the degree to which the *cd*₁NiR enzyme activity is inhibited by its product NO⁸.

The well-characterized *cd*₁NiRs from *P. pantotrophus* and *Pseudomonas (Ps) aeruginosa* (see e.g.⁹ and⁴) have many properties in common including a similar overall fold especially in the larger, catalytic *d*₁ domain. They also use similar electron donors; a soluble *c* cytochrome or a blue copper protein. However, there are also striking differences, such as the 'domain swapping' that occurs only in the *Ps. aeruginosa* *cd*₁NiR dimer, where the N-terminal arm (in the cyt. *c* domain) of one monomer crosses over to interact with the *d*₁ domain of the second monomer.

Nitric oxide, produced from *cd*₁NiR, is further reduced to nitrous oxide ($2\text{NO} + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$), by nitric oxide reductase (NOR). NORs are members of the heme-copper oxidase (HCuO) superfamily. This superfamily (comprising the cytochrome *c* oxidase in mitochondria) is large and diverse and some of its members are capable of NO-reduction^{10–12}, and all that have been investigated also show that the physiological O_2 -reduction reaction is inhibited by NO (reviewed in^{13,14}, see also¹⁵), an effect which is linked to the use of NO as a signaling molecule in mammals¹⁶.

The NOR from *P. denitrificans* is a cytochrome *c*-dependent NOR (cNOR) that, as purified, is composed of two subunits; NorB and NorC. The NorB is an integral membrane protein and harbors a low-spin heme *b* and the active site, composed of a high-spin heme *b*₃ and a non-heme iron, Fe_B. NorC is membrane-anchored and contains a periplasmic heme *c*, which receives electrons from soluble donors such as cytochrome *c* or pseudoazurin (the same as for *cd*₁NiR). The enzyme uses protons and electrons from the same side of the membrane (periplasmic, see^{17,18}) and is thus non-electrogenic^{19,20}, which differs from the O_2 -reducing HCuOs. The crystal structure of the cNOR from *P. aeruginosa* supports this as putative proton transfer pathways are only found leading from the periplasm into the active site²¹.

Respiratory chain complexes in mitochondria commonly form higher-order complexes, so-called supercomplexes. Such supercomplexes have also been found in bacteria (see e.g.^{22,23}), but the functional advantage of them is not always fully understood. Recently, the crystal structure of a complex between separately purified *cd*₁NiR and cNOR from *Ps. aeruginosa* was presented²⁴. The complex has a 2:2 stoichiometry (dimer of *cd*₁NiR with two monomers of cNOR), and the interaction was suggested to be present also under native conditions, but then in a 2:1 stoichiometry since the membrane-location of cNOR is not compatible with the 2:2 complex observed. Such a *cd*₁NiR-cNOR complex could confer advantages *in vivo* as the toxic NO molecule would, instead of being released into the periplasmic solution, rather be 'channeled' into the membrane in which it is more soluble. From the membrane, NO could directly enter the gas channel suggested for cNOR^{21,25}, see Fig. 1.

The aim of this work was to determine whether the *P. denitrificans* *cd*₁NiR and cNOR form a molecular complex *in vivo* and/or *in vitro* and to study potential functional interactions *in vitro*. To this end we investigated the localization of *cd*₁NiR in *P. denitrificans*, and we also used the cNOR catalyzed reaction as an *in vitro* 'handle' to report on a possible complex with *cd*₁NiR. We also used fluorescence spectroscopy to investigate *cd*₁NiR dimerization and the interactions of *cd*₁NiR with artificial and native membranes as well as with cNOR. Our data implies interference from *cd*₁NiR binding on electron donation to cNOR, consistent with an overlapping interaction surface. This effect of *cd*₁NiR on cNOR activity shows a titration profile consistent with an interaction primarily with a single *cd*₁NiR monomer. Our fluorescence data is consistent with this dimerization occurring in the relevant concentration range (20–40 nM *cd*₁NiR). However, we could not observe any clear long-lived high-affinity binding between *cd*₁NiR and cNOR going beyond the rather high affinity *cd*₁NiR showed to artificial membranes, nor could we observe a large fraction of the *cd*₁NiR associated with the membrane-bound cNOR in *P. denitrificans* membranes. Potential *in vivo* consequences of our results are discussed.

Results

The influence of *cd*₁NiR on catalytic activity of cNOR. If cNOR and *cd*₁NiR interact with each other, they could influence each other's catalytic parameters, therefore we measured the influence of the presence of *cd*₁NiR on NO-reduction by cNOR (which is straightforward to measure). Surprisingly, we observed clear inhibition of cNOR-catalyzed NO-reduction in the presence of *cd*₁NiR, see Fig. 2a. NO-reduction by the *P. denitrificans* cNOR exhibits a sigmoidal curve, due to substrate inhibition^{26,27} at $\text{NO} > 10 \mu\text{M}$. The value we report for cNOR activity is the maximum activity (k_{max}) note that this k_{max} is not a k_{cat} since there is substrate inhibition at higher $[\text{NO}]$ observed at $\sim 5 \mu\text{M}$ NO. In the presence of *cd*₁NiR (Fig. 2a), two effects are observed; the maximum activity is lowered and the substrate inhibition pattern changes, see below.

We investigated the inhibitory effect as a function of *cd*₁NiR concentration added, the raw data is shown in Supporting Fig. 1, and a plot of the maximum rate as a function of added *cd*₁NiR is shown in Fig. 2b (and Supporting Fig. 2). The maximum activity of cNOR decreases gradually the more *cd*₁NiR is added and the effect reaches a maximum level of inhibition ($\sim 50\%$) at $\sim 30 \text{ nM}$ *cd*₁NiR (approximately equimolar to cNOR). Surprisingly, at higher concentrations of *cd*₁NiR, the inhibition is released (Fig. 2b), the possible reasons for this are discussed further below (see Fluorescence section). For the investigations of the influence of the electron donor described in the next section, we used the *cd*₁NiR concentration (and cNOR/*cd*₁NiR ratio) giving the maximum inhibition.

Electron donation to cNOR in the presence of *cd*₁NiR. In the co-crystal structure of the complex between the *cd*₁NiR and cNOR from *P. aeruginosa*²⁴, the interaction surface (see Fig. 1) could possibly overlap with interaction of the electron donor to cNOR. Thus, one reason for the inhibition observed with *cd*₁NiR could be that it interferes with electron donation, and we therefore studied the titration behavior of electron donors for cNOR catalysis in the absence and presence of *cd*₁NiR.

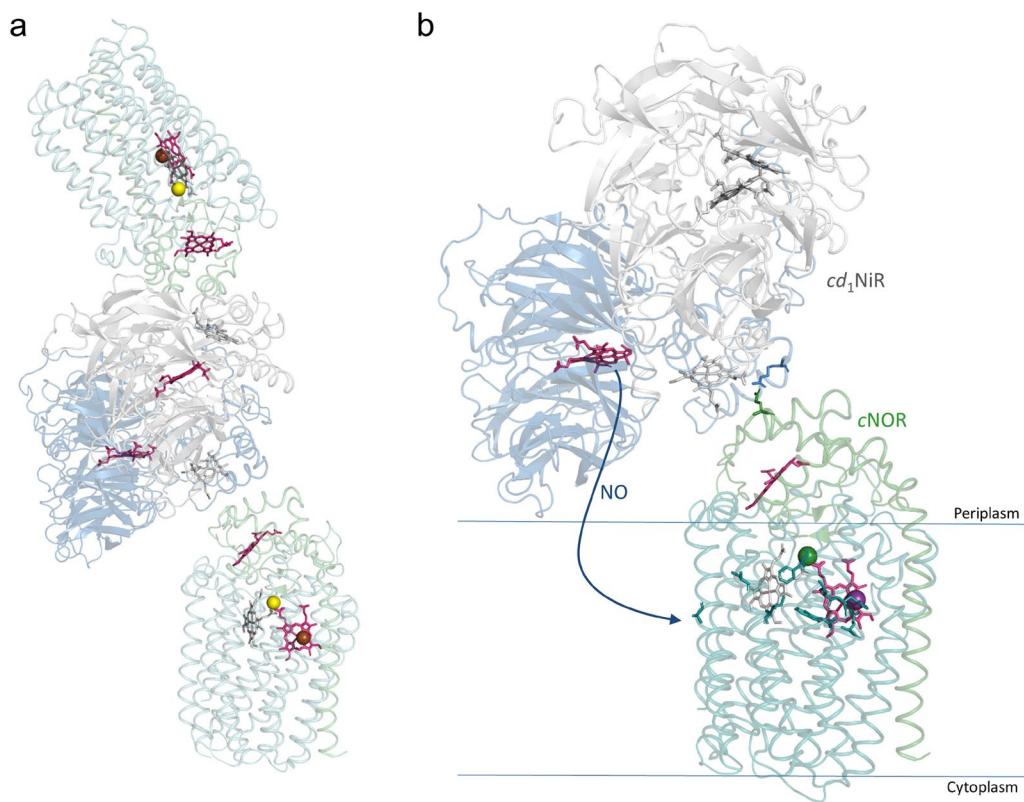


Figure 1. Structure of the co-complex of the *P. aeruginosa* cNOR and the cd_1 NiR dimer (PDB ID: 5GUW²⁴). (a) The full 2:2 structure with the two cNOR molecules in light green (NorC) and teal (NorB) and the cd_1 NiR dimer in blue/gray. (b) Enlargement of the co-complex interaction area for cd_1 NiR and a single cNOR, with the interaction between Arg-71 (cd_1 NiR, blue stick) and Glu-119 (cNOR, green stick) shown. Also shown are schematic outlines of the cytoplasmic membrane in which cNOR sits and the path for NO from the release from the d_1 heme of cd_1 NiR (pink) into the membrane from which it would travel through the suggested gas channel (indicated by green sticks) to the active site heme b_3 (pink) in cNOR²⁵. Also highlighted is the initial electron-accepting heme c in NorC (pink), other heme groups in grey.

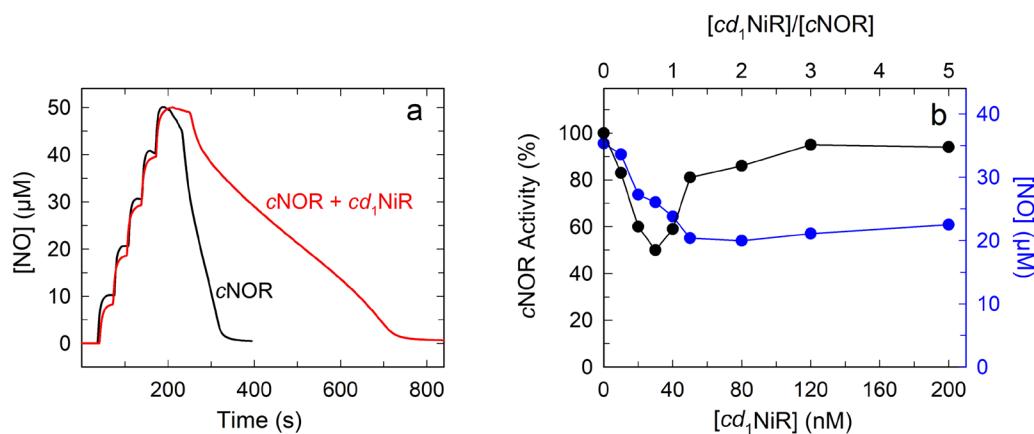


Figure 2. The inhibitory effect of cd_1 NiR on cNOR catalysis. (a) NO reduction profile of *P. denitrificans* cNOR in the absence (black line) and presence (red line) of cd_1 NiR. Experimental conditions: 50 mM HEPES pH 7.0, 50 mM KCl, 0.05% DDM, 30 mM glucose, 1 U/ml glucose oxidase, 20 U/ml catalase. Once the chamber was anaerobic, cyt. c (15 μM), TMPD (0.5 mM), and 5 times 10 μM NO (from NO-saturated water) was added. At t ~250 s, ascorbate (3 mM) and cNOR (80 nM) were added. For the trace with cd_1 NiR (80 nM), it was added before the addition of NO. (b) Titration of the inhibitory effect of cd_1 NiR on cNOR catalysis. Experimental conditions as in A, except the cNOR concentration was 40 nM, and the cd_1 NiR concentration varied between 0–200 nM. cNOR activity (black circles) refers to the k_{max} at ~5 μM NO, with the k_{max} in the absence of cd_1 NiR set at 100%. Also shown is the effect of adding cd_1 NiR on substrate inhibition (blue circles) for NO-reduction by cNOR. The right y-axis refers to the [NO] where $k_{max}/2$ is reached (termed K_i^{app} in the text, note that this is higher than k_{max}).

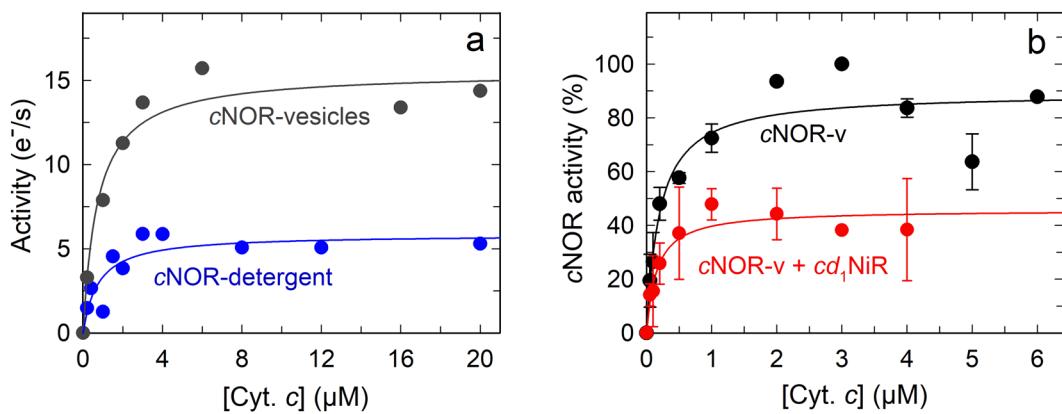


Figure 3. Determinations of the K_m for cytochrome *c* for NO reduction by cNOR. **(a)** Comparison between cNOR in detergent (blue) and reconstituted in liposomes (grey). Experimental conditions as in Fig. 2, except for with liposomes, DDM was omitted. cNOR activity refers to the k_{max} at $\sim 5 \mu\text{M}$ NO with the k_{max} obtained without cyt. *c* subtracted. The lines shown are fits giving $k_{\text{cat}} = 6 \pm 1 \text{ (e}^{-}/(\text{s} \cdot \text{cNOR}))$, $K_m = 0.8 \pm 0.3 \mu\text{M}$ cyt. *c* (blue, detergent) and $k_{\text{cat}} = 15 \pm 1 \text{ (e}^{-}/(\text{s} \cdot \text{cNOR}))$, $K_m = 0.8 \pm 0.2 \mu\text{M}$ cyt. *c* (dark grey, liposomes). **(b)** Comparison between liposome-reconstituted cNOR in the absence (black) and presence (red) of *cd*₁NiR. cNOR activity refers to the k_{max} at $\sim 5 \mu\text{M}$ NO, with the k_{max} at $3 \mu\text{M}$ cyt. *c* in the absence of *cd*₁NiR set to 100%. The curves were fitted as in a, giving $K_m = 0.20 \pm 0.05 \mu\text{M}$ cyt. *c* (black, cNOR only) and $k_{\text{cat}} = 46 \pm 4\%$, $K_m = 0.15 \pm 0.05 \mu\text{M}$ cyt. *c* (red, + *cd*₁NiR). Experimental conditions as in (a).

As a pre-requisite for the investigation of possible interference of electron donation caused by *cd*₁NiR binding to cNOR, we determined the K_m for cytochrome *c* (horse heart) during NO reduction by cNOR. For these titrations, we always used the maximum activity, k_{max} at $\sim 5 \mu\text{M}$ NO. The results are shown in Fig. 3a and can be fitted with a $k_{\text{max}} = 6 \pm 1 \text{ e}^{-} \text{ s}^{-1}$ (electrons/(s·cNOR)) and $K_m = 0.8 \pm 0.3 \mu\text{M}$. As seen in this graph, the data is scattered and the standard deviation in the K_m quite large. We therefore instead measured the activity with cNOR reconstituted into liposomes. The aim of this was two-fold, first the activity of *P. denitrificans* cNOR is higher in liposomes^{17,28}, giving us a larger total change in activity during titration and hence smaller relative errors. Secondly, the presence of a membrane might influence a putative cNOR-*cd*₁NiR interaction, as suggested for the *P. aeruginosa* complex²⁴. In liposome-reconstituted cNOR, we determined the k_{max} to $15 \pm 1 \text{ e}^{-} \text{ s}^{-1}$ and the K_m for cyt. *c* to $0.8 \pm 0.2 \mu\text{M}$ (Fig. 3a), i.e. no change in K_m was observed.

Side by side experiments were conducted to determine the K_m for cyt. *c* of liposome-reconstituted cNOR in the absence or presence of $\sim 30 \text{ nM}$ *cd*₁NiR (Fig. 3b). This is the *cd*₁NiR concentration which maximally inhibits detergent-solubilized cNOR (Fig. 2b; see also corresponding data for liposome-reconstituted cNOR in Supporting Fig. 2). Surprisingly the observed K_m was unchanged in the presence of *cd*₁NiR ($K_m = 0.15 \pm 0.05 \mu\text{M}$) compared to the control ($K_m = 0.20 \pm 0.05 \mu\text{M}$) as shown in Fig. 3b. However, the relative k_{max} in the presence of *cd*₁NiR was $\sim 50\%$ of the control. Thus, only the k_{max} and not the K_m value is affected, indicating that the *cd*₁NiR and cyt. *c* do not bind at the same place to cNOR.

We note that the K_m value determined (in the absence of *cd*₁NiR) in this experiment is different from that determined in the previous experiment (Fig. 3a). This is probably due to the K_m values being low and therefore the data obtained possibly not represented well by a simple Michaelis-Menten fit. Also, the concentration of *cd*₁NiR is about equimolar to cNOR and small differences in the relative concentrations between experiments might affect the data. These considerations are the reasons for doing comparative experiments 'side-by-side'.

Since the K_m for cyt. *c* does not change significantly in the presence of *cd*₁NiR, we scrutinized the raw data used for Fig. 3b, and re-plotted it without subtracting the background rate (with Ascorbate (Asc)/tetramethyl-p-phenylenediamine (TMPD)) (see Supporting Fig. 3A). This shows that there is inhibition of the basal activity by *cd*₁NiR with only Asc/TMPD to provide electrons that does not change significantly when cyt. *c* is added. This observation suggests that *cd*₁NiR inhibits the electron donation from TMPD rather than that from cyt. *c*. To verify this, we studied the effect of titrating *cd*₁NiR on cNOR catalysis in the absence of TMPD (with only cyt. *c* and Asc), see Supporting Fig. 3B which shows that in the absence of TMPD, there is no inhibition.

We then studied the cNOR activity as a function of the TMPD concentration (with ascorbate, but in the absence of cyt. *c*), both in the absence and presence of *cd*₁NiR, see Fig. 4. Here there is a clear inhibition by *cd*₁NiR. The data indicates that there might be more than one interaction with TMPD, but assuming a single binding site, the obtained constants are; in the absence of *cd*₁NiR: $k_{\text{max}} = 31 \pm 2 \text{ e}^{-} \text{ s}^{-1}$ and $K_m = 1.2 \pm 0.2 \text{ mM}$, and in the presence of *cd*₁NiR: $k_{\text{max}} = 12 \pm 2 \text{ e}^{-} \text{ s}^{-1}$ and $K_m = 0.7 \pm 0.2 \text{ mM}$. In this scenario, both the k_{max} and K_m are affected (so-called mixed inhibition). Our data does not allow for any unambiguous fit to a more complex behaviour.

We also observe inhibition by *cd*₁NiR when PMS is used as an electron mediator instead of TMPD (see Supporting Fig. 4). Thus, there is an inhibition of cNOR activity by the presence of *cd*₁NiR with both TMPD and PMS, indicating that the interaction surface (or part of it, cf. the data with TMPD) on cNOR is similar for TMPD and PMS, and that this surface overlaps with *cd*₁NiR binding.

As controls for the measurements described above, we studied the possibility that TMPD directly affects auto-reduction of NO, as well as the possibility that small amounts of nitrite formed (from NO) in the buffer

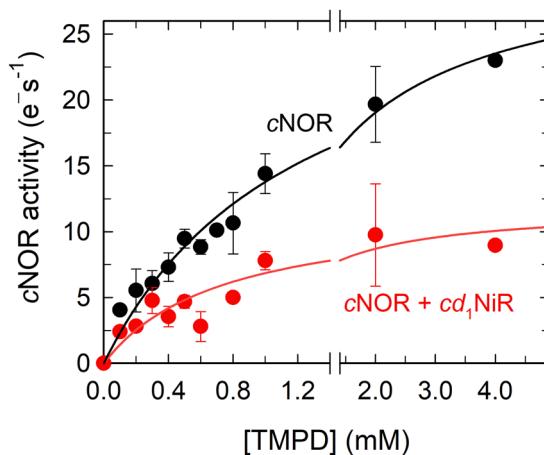


Figure 4. Determination of the K_m for TMPD for NO reduction by liposome-reconstituted cNOR in the absence (black) and presence (red) of cd_1 NiR. Experimental conditions, and data treated as in Fig. 3. The black line is a single-hyperbolic fit to the cNOR data giving $k_{cat} = 31 \pm 3$ ($e^-/(s \cdot cNOR)$), $K_m = 1.2 \pm 0.2$ mM TMPD. The red line is the same fit for the cNOR + cd_1 NiR data, giving $k_{cat} = 10 \pm 2$ ($e^-/(s \cdot cNOR)$), $K_m = 0.6 \pm 0.2$ mM TMPD.

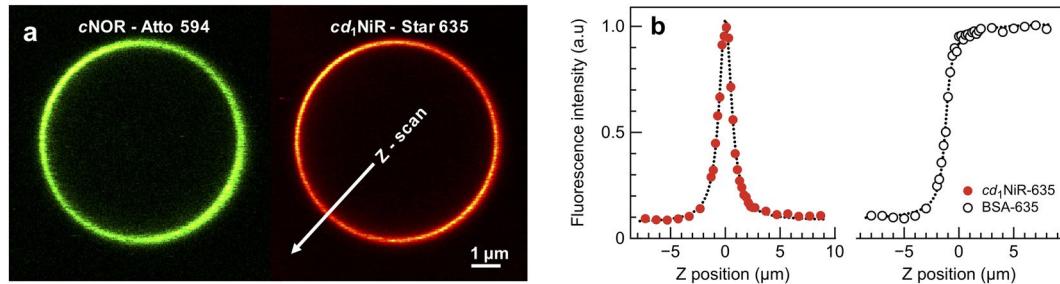


Figure 5. Fluorescently labeled cNOR (ATTO 594) and cd_1 NiR (STAR 635) visualized with a laser scanning confocal microscope. (a) cNOR was reconstituted in giant unilamellar vesicles (GUVs) and cd_1 NiR was added to the GUV solution. cNOR was detected in the membrane (green GUV) and cd_1 NiR was found to be highly associated with the membrane (red GUV). (b) Fluorescence intensity scan across the membrane (Z-plane), from the inside (−) to the outside (+) of a GUV after adding cd_1 NiR or BSA, labeled with STAR 635. cd_1 NiR (in contrast to BSA) is highly enriched at the membrane surface. For experimental conditions, see Material and Methods.

could have effects interfering with our results. However, we found no effects that were significant enough to influence the data presented. For nitrite, we see that it can inhibit cNOR activity, but only at high (mM) concentrations, consistent with previous studies²⁹.

Substrate inhibition in cNOR in the presence of cd_1 NiR. As described above, adding cd_1 NiR during NO-reduction by cNOR has two effects; both reducing the maximum activity investigated above, and in changing the pattern of substrate inhibition, see Fig. 2 and Fig. S1. Thus, a plot of the NO concentration where $k_{max}/2$ is reached as a function of cd_1 NiR added is shown in Fig. 2b (together with the corresponding effects on the k_{max}). Note that this refers to the NO concentration at *higher* NO (than that which gives k_{max}) where $k_{max}/2$ is reached, and therefore refers to an apparent K_i (rather than an apparent K_m). We note that the decrease in maximum rate at low cd_1 NiR correlates well to the decrease in the K_i^{app} for NO (that is a higher apparent affinity for NO at an inhibitory site), whereas the K_i^{app} for NO then roughly saturates at ~40 nM cd_1 NiR. It is thus clear that even though the inhibition on the maximum rate is released at higher cd_1 NiR, there is still an influence also at higher cd_1 NiR concentrations, indicating an interaction between cNOR and cd_1 NiR that persists (see Discussion).

SDS page analysis for localization of cd_1 NiR in *P. denitrificans*. To investigate the localization of cd_1 NiR in *P. denitrificans* cells grown under denitrifying conditions, cells were fractionated, and the presence of cd_1 NiR analyzed using Western blot with a specific antibody for cd_1 NiR. The results, shown in Supporting Fig. 5, demonstrate that although cd_1 NiR is present mainly in the periplasm, it is also found in the membrane fraction. We investigated many different conditions for this analysis including different detergents and ionic strength, but although using a milder detergent (digitonin) for solubilisation of the membrane fraction gave a somewhat larger fraction of cd_1 NiR bound to it, this fraction is still small, see Discussion.

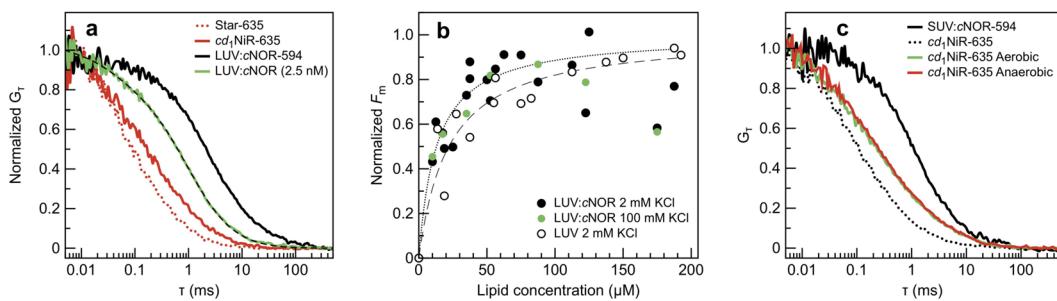


Figure 6. Interactions between cd_1 NiR and liposomes, cNOR-liposomes and native membranes. (a) Fluorescence autocorrelation curves measured on a sample containing 5 nM cd_1 NiR-STAR 635 (red), and after addition of LUVs containing 2.5 nM cNOR (green). The dashed (black) line is a fit of the data using a model with two diffusion times. As references, measurements of a sample containing free dye STAR 635 (red dotted line) and a sample with LUVs containing cNOR labelled with ATTO 594 are also shown. (b) Titration of 5 nM cd_1 NiR-STAR 635 with increasing concentrations of LUVs containing unlabeled cNOR in buffer containing 2 mM KCl (black) or 100 mM KCl (green), and titration with the same amount of LUVs without protein (white). The plot shows the amplitude of the slow component where F_{max} has been set to 1. The data for LUVs with and without protein were fitted with a simple binding model (see text for details). (c) FCS curves from titration experiments with native membranes from *P. denitrificans* grown under aerobic (green) or anaerobic denitrifying (red) conditions. Sonicated membranes were added to a solution of 50 nM cd_1 NiR-STAR 635 (black dotted line). A sample with DOPC-liposomes produced in the same way containing cNOR labelled with ATTO 594 is shown as reference (black line).

Interactions of cd_1 NiR and cNOR investigated by fluorescence spectroscopy. Purified cd_1 NiR and cNOR proteins were fluorescently labeled with ATTO 594 and STAR 635, respectively. cNOR was successfully reconstituted in giant unilamellar vesicles (GUVs), anchored to a biotin-covered glass surface imaged using a confocal laser scanning microscope (see Fig. 5a, green GUVs). cd_1 NiR was then added in equimolar concentration (to cNOR) to the GUV solution. The cd_1 NiR was also added to ‘empty’ GUVs. The cd_1 NiR was found to be highly associated with the membrane (Fig. 5a, red GUV). A scan across the membrane (Z-plane, Fig. 5b) showed an increase in fluorescence intensity in the membrane plane ($Z = 0$), i.e. the concentration of cd_1 NiR is higher at the membrane surface than in the surrounding solution. A control with STAR 635-labeled BSA protein (Fig. 5b) showed no such increase in the membrane plane.

The possible interaction between cd_1 NiR and cNOR was then assayed using fluorescence correlation spectroscopy (FCS, see Methods) in combination with the confocal setup. First, we measured FCS on the reconstituted cNOR-ATTO 594 and cd_1 NiR-STAR 635 simultaneously on the membrane surface and looked for interaction by using two-color FCS and cross-correlation analysis (FCCS). However, no significant interaction could be distinguished by this approach (Supporting Fig. 6). In agreement with this, there was no significant difference in the degree of cd_1 NiR binding between the GUVs with or without cNOR reconstituted.

To further probe potential interactions, cNOR was reconstituted in DOPC liposomes (LUVs) and the interaction with cd_1 NiR was assayed by monitoring changes in diffusion of labelled cd_1 NiR upon binding. The diffusion time of the liposomes containing cNOR labelled with ATTO 594 was 2.6 ms determined with FCS (Fig. 6b, black trace). This would correspond to a liposome size of approximately 90 nm (corresponding well to the 100 nm expected from the LUV-forming protocol). Liposomes containing unlabeled cNOR were added in increasing concentrations to a solution containing 5 nM cd_1 NiR-STAR 635. The diffusion of cd_1 NiR-STAR 635 alone was 0.34 ms, corresponding to a hydrodynamic radius of ~ 100 Å. The addition of liposomes changed the apparent diffusion time of cd_1 NiR indicating that cd_1 NiR binds to the LUVs containing cNOR (Fig. 6). The fraction of bound cd_1 NiR was determined by fitting the FCS data with a two-component diffusion model (Eq. 1), where the amplitude of the component with a long (2.6 ms) diffusion time was taken to represent liposome-bound cd_1 NiR. The amplitudes of the 2.6 ms component (Fig. 6b) were fitted with a simple ligand-binding model (Eq. 2). The same experiment was repeated with ‘empty’ LUVs, and the binding constant for liposome binding to cd_1 NiR, compared on the basis of lipid concentration, was $\sim 13 \pm 1$ μ M with and $\sim 22 \pm 2$ μ M (see Fig. 6b) without cNOR present in the membrane. This difference is likely within the experimental uncertainties and cd_1 NiR has a similar, rather high, affinity to the liposomes independently of the presence or absence of cNOR.

We also increased the ionic strength in the buffer from 2 to 100 mM (KCl) in order to shield purely electrostatic interactions between cd_1 NiR and the membrane. However, no decrease in the fraction bound cd_1 NiR was observed (green circles in Fig. 6b) which indicates that the association of cd_1 NiR to the DOPC membrane is not purely electrostatic in nature (see Discussion).

Although diffusion of both cd_1 NiR and cNOR was detected when measuring FCS on the GUV membrane surface, there was no interaction observed using cross-correlation analysis. Thus, neither the titration experiment using small liposomes, nor the FCCS, measured directly on the membrane surface, could detect an interaction between cd_1 NiR and cNOR going beyond the interaction between cd_1 NiR and the membrane under these experimental conditions. However, it should be noted that the rather high-affinity interaction between cd_1 NiR and the DOPC liposomes themselves might ‘hide’ a relatively weak interaction between cNOR and cd_1 NiR, see Discussion.

Since cd_1 NiR showed such significant interaction with the pure DOPC liposomes, we also wanted to investigate whether an interaction between cd_1 NiR and the membrane (with or without cNOR expressed) could be observed using native *P. denitrificans* membranes. Small membrane vesicles were made from cells grown under either aerobic or anaerobic denitrifying conditions and mixed with a solution containing 50 nM cd_1 NiR-STAR 635. Although cNOR expresses only during denitrifying conditions no differences were observed. The diffusion time of cd_1 NiR-STAR 635 (Fig. 6c) was partly slowed down in both cases with a fraction matching the diffusion time (~1.5 ms) of sonicated DOPC liposomes containing cNOR-ATTO 594. In both cases the slow fraction was maximum ~ 25% of the total cd_1 NiR-STAR 635 population, in comparison to up to 85% when using pure DOPC liposomes. Although these fractions do not necessarily correspond directly to the fraction bound cd_1 NiR, we can conclude that cd_1 NiR interacts much more strongly with artificial ‘lipid-only’ liposomes than it does with native membranes, see Discussion.

Dimerization of cd_1 NiR. From the functional cNOR-catalyzed NO-reduction data presented above, the observed effect of adding increasing amounts of cd_1 NiR (see Fig. 2b) made us consider that this dependence could be linked to dimerization of cd_1 NiR. cd_1 NiR is a dimer in the X-ray crystal structures from both *P. aeruginosa*^{9,24} and *P. denitrificans*⁶ and also reported to be a dimer in solution^{5,30}, but to our knowledge, there is no reported value for the dimerization constant. To further investigate if there is such a cd_1 NiR dimer dissociation/association in the concentration range used, we analyzed the fluorescence intensity from labeled cd_1 NiR as a function of its concentration. The fluorescence intensity as well as the particle number of cd_1 NiR-STAR 635 obtained by FCS (parameter N in Eq. 1) was used to determine the photon count-rate per molecule (CPM). Figure 7 shows that the CPM increases with increasing concentrations when adding cd_1 NiR-STAR 635 alone. Assuming that the majority of cd_1 NiR-STAR 635 is present as a monomer at very low concentrations (<1 nM) an increase in CPM at higher concentrations indicates dimerization. In comparison, the CPM of cNOR labelled with the same fluorophore (cNOR-STAR 635) showed only a small increase, indicating that there is no change in its oligomeric state in this region.

This cd_1 NiR titration was done both in the presence and absence of cNOR. Interestingly, both the maximum CPM for cd_1 NiR-STAR 635 and its concentration dependence changed when 40 nM cNOR (unlabeled) was present in the solution. The CPM data could be fitted using the ligand-binding model (Eq. 2) allowing for a simple comparison; the apparent binding constants for the suggested dimerization of cd_1 NiR-STAR 635 was 3.5 ± 0.1 nM without and 5.3 ± 0.2 nM with cNOR present. We observed a slight decrease in CPM at cd_1 NiR-STAR 635 concentrations above 15 nM, but only in the case when cNOR was not present and these data points were not included in the fit (dashed line). The maximum CPM reached was lower in the presence of cNOR, indicating either a quenching effect, or that even when dimerized, the cd_1 NiR is influenced by/binds cNOR, or that there is a fraction of cd_1 NiR that cannot dimerize in the presence of cNOR.

Discussion

The denitrification process is tightly controlled in *P. denitrificans*, in order to avoid release and accumulation of toxic intermediates; nitric oxide and (to a lesser degree) nitrite. This control occurs on the level of transcription, by a tight coupling of the expression of the enzymes involved (see e.g.^{31,32}). It has also been suggested that *in vivo*, kinetic parameters for cNOR are significantly different from those obtained *in vitro*, with e.g. a very high NO affinity thereby helping to keep the steady-state NO levels low³. A different way to minimize toxic intermediates would be to control the enzymes themselves, by e.g. forming a functional complex between cd_1 NiR and cNOR that shuttles the NO produced from cd_1 NiR directly to cNOR without release into the bulk phase. Support for this hypothesis was recently presented in the form of a co-complex structure of the cd_1 NiR and cNOR from *P. aeruginosa* obtained from separately purified components²⁴, see Fig. 1. It should be noted that in aerobic respiration in both eukaryotes and prokaryotes, supercomplexes of individual enzyme components involved are frequently found (see e.g.^{22,23,33} and references therein).

In this work, we investigated the possibility of a cNOR/ cd_1 NiR complex for *P. denitrificans*, a denitrification model bacterium. The two enzymes cd_1 NiR and cNOR from *P. denitrificans* share 48% (cNOR) and 61% (cd_1 NiR) overall sequence identity with their counterparts from *P. aeruginosa*. We purified the cd_1 NiR from *Paracoccus pantotrophus* and not *denitrificans*, but these two enzymes are 97% identical.

The addition of cd_1 NiR during NO-reduction by cNOR shows some intriguing effects. First, both the substrate inhibition pattern and maximum rate of NO reduction is affected by cd_1 NiR (Fig. 2). Since both these parameters are presumed to be linked to the effective electron donation (see ref.³⁴ for a discussion on substrate inhibition), it seems plausible that a complex of cd_1 NiR and cNOR forms and that the complex interface interferes with the access of the electron donor to cNOR. This conclusion is supported by the co-crystallised *Ps. aeruginosa* cd_1 NiR/cNOR complex²⁴, which shows that the cd_1 NiR interacts with the NorC subunit (see Fig. 1) that harbors the initial electron acceptor (a heme c) of cNOR. As is clear from Fig. 3b (and Supporting Fig. 3) however, the observed K_m for cyt. c does not change in the presence of cd_1 NiR but direct electron donation by TMPD is clearly affected (see Fig. 4). Although we have not used the presumed physiological *c*⁵⁵⁰ cytochrome^{7,35}, but the readily available horse heart (hh) cyt. c, the structures align very well and hh cyt. c works well as electron donor to cNOR. The small TMPD molecule (MW: 164 g/mol) presumably has a less defined or multiple interaction surfaces on cNOR, as indicated by our titration data (Fig. 4), and these (or some of them) presumably overlap with the interaction surface for cd_1 NiR. We also observe inhibition by the presence of cd_1 NiR with the electron mediator PMS (instead of TMPD, see Supporting Fig. 4).

An interesting parallel is that the antibody used for crystallisation of the *Ps. aeruginosa* cNOR (only)²¹, was shown to interfere with electron donation from cytochrome c, but not from PMS²¹. The binding site for this antibody has some, albeit small, overlap with the binding of cd_1 NiR in the co-crystal complex²⁴.

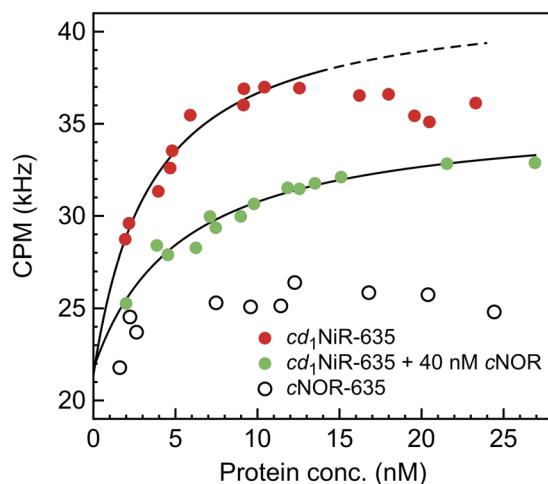


Figure 7. Fluorescence intensity (count per molecule (CPM)) when adding increasing amounts of *cd₁NIR-STAR* 635 to a solution with (green circles) or without (red circles) 40 nM cNOR. The fluorescence intensity measured with increasing concentrations of cNOR-STAR 635 alone is also shown (white circles). The black lines are ligand-binding fits for *cd₁NiR* dimerization, giving $K_D = 3.5 \pm 0.1$ nM (without) and 5.3 ± 0.2 nM (with cNOR) respectively.

The inhibition of cNOR activity observed upon addition of *cd₁NiR* shows a clear correlation in extent to the concentration of added *cd₁NiR* up until approximately equimolar amounts to cNOR (20–40 nM), but at higher concentrations of *cd₁NiR*, the inhibition is relieved. This is a surprising but highly reproducible observation which we suggest could be due to an effect of dimerization of *cd₁NiR*, which is purified and crystallized in the dimeric form both in *P. pantotrophus*⁶ and *P. aeruginosa*⁹. Our fluorescence intensity measurements with labeled *cd₁NiR* showed a fluorescence ‘count-rate per particle’ (CPM) increase (Fig. 7), consistent with dimerization with an apparent K_D of ~ 3.5 nM. To our knowledge, an apparent dimerization constant for *cd₁NiR* has not previously been determined. In the *P. aeruginosa* *cd₁NiR* dimer there is domain ‘swapping’ between the monomers, leading to a presumably obligatory dimer, whereas no such swapping occurs in the *P. pantotrophus* (and hence *denitrificans*) *cd₁NiR*. This difference is likely to affect the stability of the dimer and also the propensity to interact with cNOR. Also consistent with our functional data is that this K_D is affected (increases) in the presence of cNOR, from 3.5 nM to ~ 5 nM, supporting an interaction between cNOR and *cd₁NiR* in the same concentration range as used in the functional assay. The total CPM for *cd₁NiR* is also affected by cNOR, and the 40 nM cNOR used in this experiment might not be enough to saturate the effects, such that the influence of cNOR for *cd₁NiR* dimerization might be somewhat underestimated.

The *P. aeruginosa* co-complex structure, where each *cd₁NiR* monomer binds a cNOR on opposite ‘ends’ (Fig. 1) is a structure that cannot be formed *in vivo* because of the restrictions imposed by the cytoplasmic membrane. This is thus consistent with a cNOR/*cd₁NiR* interaction that is stronger when both proteins are in their monomeric forms. In this context, we do see differences in the inhibition patterns when adding *cd₁NiR* to cNOR in detergent versus in liposomes, but qualitatively, the results are similar (see Supporting Fig. 2).

Although the effect *cd₁NiR* has on the maximum rate of NO-reduction was interpreted above in terms of only occurring for the monomer of *cd₁NiR*, even at higher *cd₁NiR* concentrations (i.e. when *cd₁NiR* is predominantly a dimer) it still influences cNOR catalysis as seen in the plots of the K_i^{app} (Fig. 2b). A possible interpretation for this is that *cd₁NiR* still interacts with cNOR even in its dimeric form, but that the interaction surface changes. It’s also possible that the effect on the cNOR substrate inhibition pattern originates from structural changes occurring in *cd₁NiR* itself as a response to changes in [NO] or in reduction levels (as seen in³⁶, see below).

Even though there are clear influences on the function of cNOR by the presence of *cd₁NiR*, we could not find evidence for a high-affinity, constant complex between the *P. denitrificans* *cd₁NiR* and cNOR, as indicated e.g. by the Western Blot results (Supporting Fig. 5) and the lack of clear differences between the interactions of (fluorescently labeled) *cd₁NiR* with either the native aerobic or anaerobic (denitrifying) *P. denitrificans* membranes shown in Fig. 6c. Interpreting this data is complicated by the observation that there is a rather high affinity of the *cd₁NiR* for lipid membranes (see Figs. 5 and 6), as reported also previously^{37,38}. This interaction is not purely electrostatic in nature, whereas only electrostatic interactions between the *d₁* domain and the membrane were discussed for the *P. aeruginosa* *cd₁NiR*-cNOR co-complex simulations²⁴. An interaction between *cd₁NiR* and the cytoplasmic membrane would enable the NO produced to directly dissolve into the membrane bilayer from which it can migrate to the gas channel in cNOR (see Fig. 1) without equilibrating with the bulk water phase even with no direct contact between the two enzymes.

Since a co-complex structure of *cd₁NiR*-cNOR exists only for the *P. aeruginosa* proteins, we overlayed the potential interaction between the two homologous proteins from *P. denitrificans*. For the *P. denitrificans* cNOR, we constructed a model based on the *P. aeruginosa* structure (to which it has 54% (NorB) and 47% (NorC) sequence identity), as shown in Supporting Fig. 8A, and the Glu-119 of *P. aeruginosa* cNOR that forms the main interaction with the *P. aeruginosa* *cd₁NiR* overlays well with the corresponding Asp-123 in *P. denitrificans* cNOR.

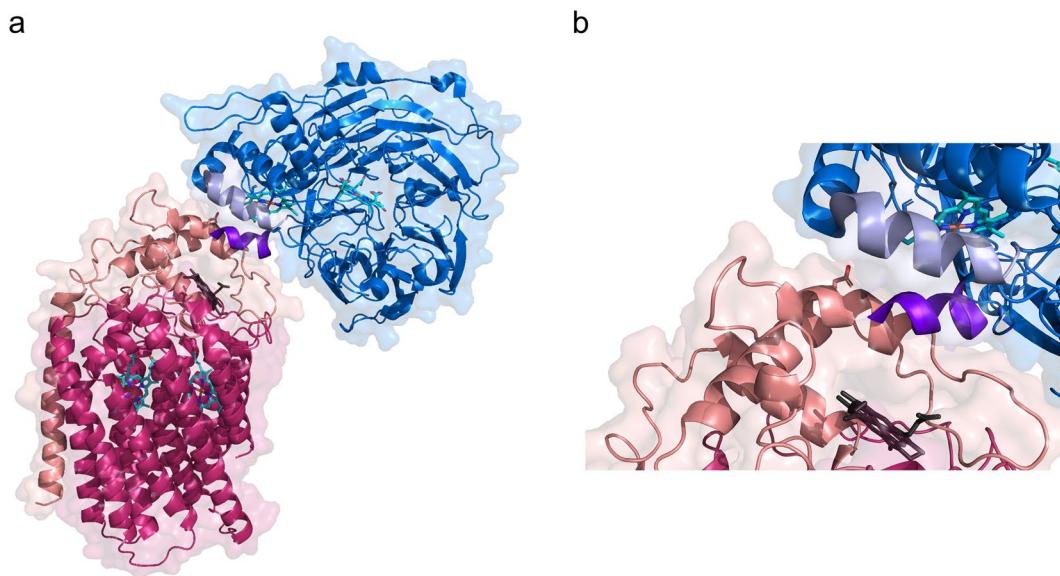


Figure 8. (a) The model of the *P. denitrificans* cNOR (NorB in magenta, NorC in salmon) and the *P. pantotrophus* cd_1 NiR monomer structure (blue, PDB ID: 1QKS⁶) overlapped on the cd_1 NiR/cNOR co-complex structure from *P. aeruginosa* (PDB ID: 5GUW²⁴ (not shown)). The cd_1 NiR helix that interacts with NorC (in the co-complex) in light blue and the ‘extra’ N-terminal helix in purple. Note that this ‘extra’ helix would clash into the NOR/NiR interface. Shown is also the D123 (stick) of NorC (equivalent to the E-119 in *Ps. aeruginosa*) and the heme groups of the proteins. (b) Zoom-in of interface. The picture outlines also the surface (transparent) of the proteins except for the ‘extra’ helix.

However, the *P. pantotrophus* cd_1 NiR structure (sequence identity 97% to *P. denitrificans* cd_1 NiR) shows significant differences to the cd_1 NiR from *P. aeruginosa*, and there is no Arg equivalent to the R-96 (numbering from our alignment (Supporting Fig. 7), corresponds to the R-71 in the alignment from Terasaka *et al.*²⁴) that interacts with the E-119 on cNOR (in *P. denitrificans* and *P. pantotrophus* cd_1 NiR, the corresponding residue is a Leu). The structural overlay of the *P. pantotrophus* and *P. aeruginosa* cd_1 NiRs (see Supporting Fig. 8B) further shows that the cyt. *c* domain is more different than the d_1 domain and specifically the region on cd_1 NiR that is interacting with cNOR in the *Ps. aeruginosa* co-crystal structure is markedly different in *P. pantotrophus* cd_1 NiR, there is a small N-terminal helix that would ‘clash’ with the cNOR, as shown in Fig. 8, whereas in *Ps. aeruginosa* cd_1 NiR, the N-terminal is involved in ‘domain swapping’ and forms part of the d_1 domain (see Supporting Fig. 8C).

However, it is also known that the *P. pantotrophus* cd_1 NiR *c* domain structure is significantly different in the reduced state³⁶, and thus suggested to undergo large-scale conformational changes upon reduction (Supporting Fig. 9). Such changes could affect both the cNOR interaction and the dimerization constant, since the *c* domain of cd_1 NiR ‘swings’ out of dimer contact in the reduced state. It is difficult to predict what would happen to a putative cd_1 NiR/cNOR complex when cd_1 NiR is reduced since the N-terminal ‘clashing’ helix (Fig. 8), is not even resolved in the reduced cd_1 NiR structure³⁶, and there is hardly any overlap between the oxidised and reduced *c* domain structures (Supporting Fig. 9). Presumably the interaction between *P. denitrificans* cNOR and cd_1 NiR, if it occurs using a similar interaction surface as in *Ps. aeruginosa*, would affect this cd_1 NiR conformational change and hence could be involved in controlling cd_1 NiR activity. We also note that in our functional cNOR assays, cd_1 NiR is presumably predominantly in the reduced state (depending on if it has turned over, see³⁹) since there is an excess reductant and no nitrite added.

So, are there physiological consequences of having an interaction between the cd_1 NiR monomer and cNOR that becomes much less pronounced once the cd_1 NiR dimerizes? It is possible that such regulation on the enzyme level (on top of the major transcriptional regulation) is there to fine tune flux through denitrification in response to rapidly fluctuating environmental conditions and is especially important when expression levels are low.

Materials and Methods

cd_1 NiR; growth of bacteria and purification. *Paracoccus pantotrophus* (G6) was grown anaerobically and cd_1 NiR purified essentially as in⁵. Briefly, bacteria were grown until $OD_{600} \sim 0.8$ in a medium containing nitrate as electron acceptor and acetate as carbon source, supplemented with 50 μ g/ml kanamycin. To obtain the periplasmic fraction, the cell pellet was resuspended in 200 mL buffer containing 0.5 M sucrose, 3 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 400 mg of lysozyme was added. The solution was then incubated with constant stirring at 30 °C for 40 minutes. The cell solution was then centrifuged at 25000 g for 10 min. The supernatant (containing the periplasm) was applied to a DEAE anionic-exchange column (GE Healthcare), from which bound fractions were eluted with a 0–300 mM NaCl gradient in 100 mM Tris/HCl pH 8.0. The brown-colored fractions were pooled, solid ammonium sulfate was added to 40% (w/v) and the precipitated protein removed by centrifugation at 30000 g for 30 min.

The solution was applied to a phenyl-sepharose column (GE Healthcare), and a 40-0% ammonium sulfate gradient was applied. The fractions that contained pure *cd*₁NiR ($A^{406}/A^{280} \sim 1.25$)⁸ were pooled and concentrated. The concentration of *cd*₁NiR was determined by using $\epsilon^{418} = 268 \text{ mM}^{-1} \text{ cm}^{-1}$. For antibody generation the enzyme was further purified using size exclusion chromatography in 100 mM Tris/HCl pH 7.0 on a Superose 10/300 column (GE-Healthcare).

cNOR; growth of bacteria, protein purification and model building. Purification of cNOR (*P. denitrificans* overexpressed in *E. coli*) was performed as described in¹⁸, based on the original protocol from⁴⁰. Briefly, the plasmid pNOREX was transformed into a JM109 strain which contained the pEC86 vector⁴⁰. cNOR expression was induced by IPTG. The membranes were solubilized in 100 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA and 1% n-dodecyl-β-D-maltoside (DDM). The membrane solution was incubated with constant stirring for 1 hour at 4 °C. The unsolubilized membranes were removed by centrifugation, and the supernatant was applied to a Q-Sepharose high performance (GE-HealthCare) column, which was equilibrated in 20 mM Tris/HCl pH 7.6, 0.04% DDM and 5 mM NaCl. The column was washed with the same buffer but containing 250 mM NaCl and cNOR was eluted with a 250 mM-500 mM NaCl gradient in 20 mM Tris/HCl pH 7.6, 0.04% DDM.

The pure fractions of cNOR were pooled, diluted 3 times in 100 mM Tris/HCl, 50 mM NaCl, and the concentration of NaCl was lowered to below 50 mM by repeated dilution and reconcentration in concentrating vials (Millipore Merck, Ltd). Aliquots were flash frozen in liquid nitrogen and stored in -80 °C.

The structural model of *P. denitrificans* cNOR was constructed with SWISS-MODEL (<https://swissmodel.expasy.org>) using the default parameters and refinement procedure. The crystal structure of *Pseudomonas aeruginosa* cNOR (PDB ID: 3o0r²¹, sequence identity 54% for NorB and 47% for NorC) was used as the structural template. The *P. denitrificans* cNOR could also be modelled on the *Roseobacter denitrificans* cNOR (sequence identity 75% for NorB and 69% for NorC) structure⁴¹, but since this cNOR, unlike *P. denitrificans* cNOR, was found to bind a Cu⁺ ion in the NorC subunit, which could potentially influence the region around the presumed interaction with *cd*₁NiR, we chose to use the *P. aeruginosa* cNOR-derived model.

Detection of *cd*₁NiR in anaerobically grown (on nitrate) *P. denitrificans* cells. *P. denitrificans* (Pd1222) cells were grown anaerobically on nitrate (32 mM) as electron acceptor at 37 °C, the cells were harvested and the periplasm was obtained by osmotic shock as described above. The pellet was sonicated, and the membranes were extracted by high-speed centrifugation (100 000 g). The different cell components (whole cell, periplasm (PL) and membrane (M) fractions) were subjected to SDS-PAGE (Invitrogen, 4–12%) analysis followed by Western blot using a PDVF membrane and an antibody against *cd*₁NiR, obtained from Biogenes GmbH (Germany).

Protein reconstitution in vesicles. For the generation of small unilamellar vesicles (SUVs), a solution of 40 mg/ml soybean lipids in 50 mM Tris/HCl pH 7.0, 50 mM KCl was sonicated until it became clear. 2–4 μM cNOR was added to the liposomes in the presence of 0.6% Na-cholate and the mixture was incubated for 1 hour at 22 °C. The detergent was then removed on a PD-10 column (GE-Healthcare). For generation of large unilamellar vesicles (LUVs), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) lipids dissolved in CHCl₃ were dried and then rehydrated to 2 mM in a 10 mM phosphate buffer (pH 7.4) with 2 mM KCl. Unilammellar liposomes were made by passing the lipid solution through a filter with a 100 nm pore size 21 times. cNOR was reconstituted into the liposomes by gently solubilizing the vesicles with 0.6% Na-Cholate before adding the protein at a 10:1 molar ratio (protein:liposome), giving a protein to lipid ratio of ca. 1:3500 in the outer monolayer. The detergent was then slowly removed by dialysis at 4 °C over night.

For generation of giant unilamellar vesicles (GUVs), a 1 mM stock solution of DOPC supplemented with 1% DPPE-biotinyl (2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl) (Avanti Polar Lipids) was used according to the procedure described in⁴². cNOR labelled with ATTO 594 (see below) was reconstituted into the GUVs using a mild detergent treatment with DDM; the protein solution containing 1 mM DDM was mixed with 20 μl GUV-solution to a final concentration of 0.05–0.25 μM protein and 0.05 mM DDM and incubated at room temperature for 30 min. The proteo-GUVs were then diluted 20 times in a 100 mM buffered glucose solution (10 mM phosphate buffer pH 7.4, 2 mM KCl) and transferred to a LabTek microscope chamber coated with streptavidin and further incubated at room temperature for 2 h. The dilution gave a final detergent concentration of 2.5 μM DDM in the sample.

Steady-state activity measurements. The interaction between cNOR and *cd*₁NiR was investigated by studying the multiple turnover activity of cNOR, either in detergent (0.05% DDM) or incorporated in vesicles, using a Clark-type electrode (World Precision Instruments, WPi) as in¹⁸. Briefly, the activity was measured in 50 mM HEPES at pH 7.0 with 50 mM KCl at room temperature. The buffer in the reaction chamber (total volume = 1 ml) was made anaerobic by adding the glucose (30 mM)/glucose oxidase (1 U/ml)/catalase (20 U/ml) system. Substrates were added with a syringe in the following order, horse heart (*hh*) cyt. *c* (varying concentrations), TMPD at varying concentrations, 5 equal additions of 10 μM NO (from NO-saturated water), and 3 mM sodium ascorbate. cNOR was added at various concentrations (20–80 nM) either prior to (cNOR in vesicles) or after all substrate additions (detergent solubilized). *cd*₁NiR was added prior to the addition of NO, when specified. The data was recorded with the LabScribe2 software (WPi), and the maximum NO-reduction rate was calculated (at ~5 μM NO).

Fluorescence labelling. *cd*₁NiR and cNOR were fluorescently labelled using amino-reactive dyes. The protein concentration was set to 3 mg/ml and a 1/20 volume of NaHCO₃ (pH 9.0) was added. *cd*₁NiR was labelled with a 5-fold molar excess of Abberior STAR 635 (Abberior GmbH) and cNOR was labelled with a 3-fold molar excess of ATTO 594 (ATTO Tec GmbH) by incubating at room temperature while gently shaking for 1.5 h. Unbound dye was removed using a PD-10 column (GE Healthcare), equilibrated with a 10 mM phosphate buffer (pH 7.4) supplemented with 100 mM sucrose, 2 mM KCl and 1 mM (~0.05%) DDM.

Fluorescence correlation spectroscopy (FCS) measurements and analyses. FCS measurements were performed on an instrument from Abberior Instruments (Göttingen, Germany), built on a stand from Olympus (IX83), and modified for two-color imaging (see⁴² for a detailed description of the experimental set up). Two fiber-coupled, pulsed (20 MHz) diode lasers emitting at 637 nm (PicoQuant AG, Berlin) and 594 nm (Abberior Instruments) were used for excitation, with the excitation pulses of the two lasers out of phase, to minimize cross-talk and enable fluorescence cross correlation of *cd*₁NiR-STAR 635 on the membrane surface of the GUVs containing cNOR-ATTO 595. For details on the correlation and cross correlation analysis, see^{42,43}.

The diffusion time of *cd*₁NiR-STAR 635 (5 nM) was determined with FCS in the presence of increasing concentration of LUVs, with or without reconstituted cNOR. Normalized autocorrelation curves of the recorded fluorescence intensity fluctuations, $G(\tau)$, were calculated using a MatLab script, and the recorded $G(\tau)$ curves were then fitted using a model for 3D-diffusion, including two diffusional components and a population of a non-fluorescent triplet state (T) with a relaxation time τ_T :

$$G(\tau) = \frac{1}{N(1 - T)} \times a \left[1 + \frac{\tau}{\tau_{D1}} \right]^{-1} \left[1 + \frac{\tau}{\beta^2 \tau_{D1}} \right]^{-1/2} + b \left[1 + \frac{\tau}{\tau_{D2}} \right]^{-1} \left[1 + \frac{\tau}{\beta^2 \tau_{D2}} \right]^{-1/2} \times [1 - T + Te^{-\frac{\tau}{\tau_T}}] \quad (1)$$

Here, τ_{D1} is the diffusion time of free *cd*₁NiR-STAR 635 and τ_{D2} is the diffusion time of LUV-bound *cd*₁NiR-STAR 635. $\beta = \omega_2/\omega_1$, where ω_2 and ω_1 denote the $1/e^2$ extension of the FCS detection volume in along and perpendicular to the excitation beam direction, respectively. N is the average number of fluorescent molecules in the detection volume, and a and b the fractions of fluorescent molecules belonging to each of the two different diffusion components (with $a + b = 1$). The amplitudes of the component with diffusion time τ_{D2} were fitted with the binding model:

$$F_m = \frac{F_m^{\max} \times c_{\text{sol}}}{K_D + c_{\text{sol}}} \quad (2)$$

where F_m represents fraction of liposomes bound to *cd*₁NiR-STAR 635, c_{sol} represents non-bound liposomes (plotted as number of free lipids), and K_D is the binding constant defined by the concentration at which half of the liposomes are bound ($F_m = 0.5$). The value of F_m^{\max} was set to unity.

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Author contributions

Designed study: I.A., J.S., J.W., P.Ä., Performed experimental work: I.A., J.S., J.t.B. Analysed data: I.A., J.S., J.t.B., J.W., N.W., P.Ä. Wrote the manuscript: I.A., J.S., P.Ä. All authors reviewed and revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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