FEBS Letters 565 (2004) 48–52 FEBS 28314

Paracoccus pantotrophus NapC can reductively activate cytochrome cd_1 nitrite reductase

Richard S. Zajicek[a], James W.A. Allen[a], Michaël L. Cartron[a], David J. Richardson[b], Stuart J. Ferguson[a,*]

[a] *Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[b] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*

Received 9 February 2004; revised 23 March 2004; accepted 25 March 2004

First published online 7 April 2004

Edited by Peter Brzezinski

Abstract The oxidized "as isolated" form of *Paracoccus pantotrophus* cytochrome cd_1 nitrite reductase has a *bis*-histidinyl coordinated *c* heme and a histidine/tyrosine coordinated d_1 heme. This form of the enzyme has previously been shown to be kinetically incompetent. Upon reduction, the coordination of both hemes changes and the enzyme is kinetically activated. Here, we show that *P. pantotrophus* NapC, a tetraheme *c*-type cytochrome belonging to a large family of such proteins, is capable of reducing, and hence activating, "as isolated" cytochrome cd_1. NapC is the first protein from *P. pantotrophus* identified as being capable of this activation step and, given the periplasmic co-location and co-expression of the two proteins, is a strong candidate to be a physiological activation partner.

Keywords: NapC; Nitrite reductase; Enzyme activation; Heme ligand exchange

1. Introduction

Cytochrome cd_1 nitrite reductase is an enzyme of the bacterial periplasm; it plays a key role in denitrification, the respiratory reduction of nitrate, via nitrite, nitric oxide and nitrous oxide, to nitrogen gas (N_2). The cytochrome *c* domain of the homodimeric enzyme, carrying a heme that is attached to the protein via two thioether bonds, receives electrons from donor proteins [1–4]. The active site on each subunit contains a specialized d_1 heme at which the substrate, nitrite, binds and is reduced to NO. The d_1 heme is in turn re-reduced by electrons supplied by the *c* heme of the same subunit.

Crystal structures of *Paracoccus pantotrophus* cytochrome cd_1 have produced a number of surprises. Among these is the unusual ligation of the two types of heme in the oxidized, "as isolated", form of the enzyme. The heme of the *c*-type cytochrome centre is *bis*-histidinyl coordinated, while the d_1-type centre has a proximal histidine ligand but a distal tyrosine, residue 25 [5]. In two crystal structures of the reduced *P. pantotrophus* enzyme, histidine 17 was replaced as a ligand of the *c*-type heme iron by methionine 106, and tyrosine 25 dissociated from the active site d_1 heme leaving a five coordinate iron atom [6,7]. In solution, spectroscopic analysis is consistent with ligation of tyrosine to the d_1 heme iron of the oxidized as prepared *P. pantotrophus* enzyme and has demonstrated *bis*-histidinyl coordination of the *c*-type heme, as seen in the crystal [8].

Studies using physiological electron donor proteins to *P. pantotrophus* cytochrome cd_1 (pseudoazurin and cytochrome c_{550}) with various electron acceptors (nitrite, oxygen or hydroxylamine) have shown that the oxidized "as isolated" conformer of the enzyme is far from catalytically competent. However, a catalytically much more active form of the protein is obtained if the enzyme is first reduced and then reoxidized by the non-physiological substrate hydroxylamine [9]. This very active (initially) oxidized form has His/Met coordination at the *c* heme [10], while the visible absorption and EPR spectra indicate that tyrosine 25 is not bound to the d_1 heme. Pre-reduction of the enzyme has also resulted in much higher values of k_{cat} than could be obtained with "as isolated" enzyme for any combination of the known electron donors and acceptors [3,9].

There appears to be a very tight coupling between both the oxidation states and coordination at the *c* and d_1 heme iron atoms that is most clearly illustrated by a complex, highly cooperative (thermodynamically and kinetically), hysteretic, redox titration [11]. The potential required for reduction of the oxidized "as isolated" state of the enzyme is ca. 60 mV, while oxidation of the reduced form occurs at ca. +250 mV [11]. It has been shown that in the very closely related organism *Paracoccus denitrificans*, both pseudoazurin (E_m ca. +230 mV) and cytochrome c_{550} (E_m ca. +260 mV) can act as steady-state electron donors to cytochrome cd_1 in denitrifying conditions in vivo (i.e., for turnover of nitrite) [12]. Turnover was also observed in vitro for these electron donors with various electron acceptors [3]. Additionally, Pearson et al. [12] showed that no other electron donor was capable of sustaining reduction of nitrite by cytochrome cd_1 in vivo. However, despite the latter observation, in vitro studies have shown that neither pseudoazurin nor cytochrome c_{550} are capable of reducing (and hence initially activating for catalysis) "as isolated" *P. pantotrophus* cytochrome cd_1 [3,9]. The physiological role of this conformer of the enzyme (*c*-heme His/His coordinated, d_1 His/Tyr) remains unclear; it has been proposed that it is related to insertion or retention of the biosynthetically expensive d_1 heme, or alternatively protects the d_1 heme against reactive oxygen species when *P. pantotrophus* encounters aerobic conditions [13]. The enzyme clearly exhibits a very strong tendency to

* Corresponding author. Fax: +44-1865-275259.
E-mail address: stuart.ferguson@bioch.ox.ac.uk (S.J. Ferguson).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.03.072

return to the "as isolated" conformer when oxidized [9,11,14]. However, presently, there is no evidence as to how the catalytically inert "as isolated" form of the cytochrome cd_1 might be reduced/activated in the cell.

NapC is a *c*-type cytochrome with four *bis*-histidinyl coordinated hemes in a periplasmic soluble domain, anchored to the cytoplasmic membrane by a single transmembrane helix [15]. The protein forms part of a family of multi-heme quinol dehydrogenases, including TorC and CymA, that transfer electrons from quinol pools in the cytoplasmic membranes of Gram-negative bacteria to various periplasmic oxidoreductases [16]. This process provides a cytochrome bc_1 complex independent mechanism for electron transfer from quinols to periplasmic enzymes. NapC donates electrons to the diheme *c*-type cytochrome NapB and those electrons are in turn transferred to the molybdoenzyme NapA, which reduces nitrate to nitrite. In *P. pantotrophus*, the Nap system has been proposed to be a mechanism of dissipation of excess redox energy during oxidative metabolism of reduced carbon substrates [17]. However, Nap is constitutively expressed to some extent [17] and is thus also present in anaerobic conditions when the organism is respiring by denitrification. A form of *P. pantotrophus* NapC (hereafter called $NapC^{sol}$) has been constructed in which the membrane anchor has been removed to leave a soluble protein containing the hemes [16]. In such protein, the four hemes have midpoint reduction potentials ranging from −235 to −56 mV [16]. Therefore, both thermodynamically and biologically, NapC is a protein that has the potential to catalytically activate the "as isolated" conformation of *P. pantotrophus* cytochrome cd_1 by reduction. In this paper, we report an investigation of this hypothesis.

2. Materials and methods

Paracoccus pantotrophus cytochrome cd_1 was purified according to the method of Moir et al. [18] from the periplasms of cells grown anaerobically, as previously described [18], with nitrate as terminal electron acceptor. $NapC^{sol}$ was purified from the periplasm of aerobically grown *Escherichia coli*, according to Cartron et al. [15]. The *E. coli* cells were co-transformed with the $NapC^{sol}$ expression vector and pEC86 [19], a plasmid containing the *ccm* genes coding for the *E. coli* cytochrome *c* maturation accessory proteins.

The concentration of cytochrome cd_1 was determined using the oxidized extinction coefficient 285 mM^{-1} cm^{-1} at 406 nm [20]. $NapC^{sol}$ concentrations were determined using the oxidized extinction coefficient of 434 mM^{-1} cm^{-1} at 406.5 nm [16] or the reduced value of 93 mM^{-1} cm^{-1} at 551 nm. All protein solutions were made up in 50 mM potassium phosphate buffer, pH 7.0, and experiments were conducted at 25 °C. Cytochrome cd_1 has an inherent oxidase activity [3,21] and reduced $NapC^{sol}$ has proved highly susceptible to oxidation on exposure to air (MLC and SJF unpublished observation); therefore, all experiments were carried out in an anaerobic cabinet (Belle Technology) (>5 ppm O_2). $NapC^{sol}$ was reduced with excess dithionite within the anaerobic cabinet and the excess reductant removed by passing the solution down a desalting column containing P6-DG resin (Bio-Rad) [9,10,22].

A Hi-Tech SX-61 stopped-flow apparatus was used to mix oxidized cytochrome cd_1 and reduced $NapC^{sol}$ (in the absence of excess reductant) over varying timescales and to record the resulting absorption spectra. Kinetic data analysis was carried out using TableCurve 2D (Jandel Scientific) and Origin (Microcal).

3. Results

Oxidized "as isolated" *P. pantotrophus* cytochrome cd_1 was first mixed with 50 mM potassium phosphate buffer in a stopped-flow apparatus. The resultant spectrum was, as expected, identical to that of the oxidized "as isolated" protein. The cytochrome cd_1 used in our experiments was, therefore, deemed to initially be oxidized and to have His/His coordination at the *c* heme and His/Tyr coordination at the d_1 heme [5,8] (hence in the kinetically incompetent state) [3,9]. The concentration of dimeric cytochrome cd_1 in the flow cell was ca. 5 μM (i.e., 20 μM in total heme).

Fig. 1 shows the rich absorption spectra of *P. pantotrophus* cytochrome cd_1. In the oxidized enzyme the broad 500–600 nm peak is assigned to the *c* heme and peaks at 420 (shoulder), 640 and 700 nm are assigned to the His/Tyr coordinated oxidized d_1 heme [8]. Upon reduction of the enzyme an unusual and characteristic doublet (split) α band, arising from the *c* heme, is observed at 548 and 554 nm. The d_1 heme component of the spectrum also changes significantly, with a resolved peak being apparent at 460 nm and a now single peak, also with characteristic shape, centred on 650 nm (Fig. 1). All the significant bands in the spectrum of cytochrome cd_1 with a wavelength above 600 nm may be solely attributed to the d_1 heme [23]. NapC has the spectrum of a typical *c*-type cytochrome [16]; the Soret band is red shifted upon reduction from 406.5 to 416 nm and a sharp (singlet) α band appears at 551 nm in the reduced, but not the oxidized, spectrum.

When oxidized "as isolated" cytochrome cd_1 was mixed with 10 μM reduced *P. pantotrophus* $NapC^{sol}$ (i.e., 40 μM in total heme) and left for ca. 4 min, the spectrum shown in Fig. 1 (solid line) was observed. This mixture clearly contained a component with the spectrum of reduced cytochrome cd_1.

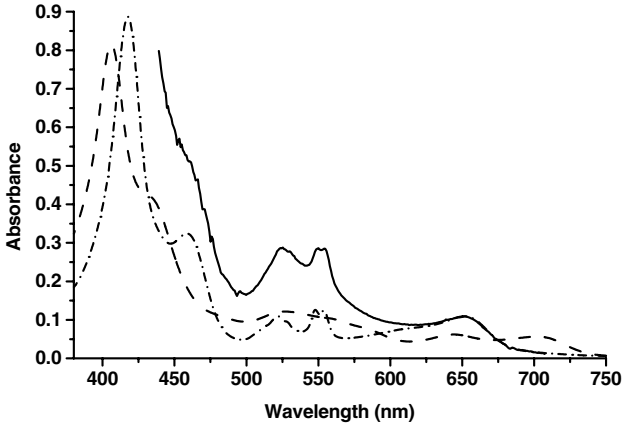

Fig. 1. Absorption spectra of various states of *P. pantotrophus* cytochrome cd_1. Oxidized "as isolated" (– – – –) and reduced (–·–·–) cytochrome cd_1; the reduced enzyme spectrum was obtained from the same sample as the oxidized after a minimal addition of disodium dithionite. (——) A mixture of initially oxidized "as isolated" cytochrome cd_1 and reduced *P. pantotrophus* NapC, 150 s after mixing. 5 μM oxidized "as isolated" cd_1 (20 μM in total heme) was mixed with an equal volume of 10 μM reduced NapC (40 μM total heme) in a stopped-flow apparatus and left for ca. 4 min. The spectrum from the mixing experiment was normalized relative to that of the pure enzyme using the extinction coefficient of reduced cd_1 at 650 nm, given that NapC does not absorb at that wavelength. All proteins were in 50 mM phosphate buffer, pH 7.0, and at 25 °C. Note that the spectra of oxidized and reduced cd_1 were recorded in a standard spectrophotometer, whereas the spectrum of the NapC-cd_1 mixture was recorded using a diode array detector attached to the stopped-flow instrument. The diode array saturated in the Soret band region of the spectrum (380–440 nm) and so that spectrum is necessarily truncated at 440 nm.

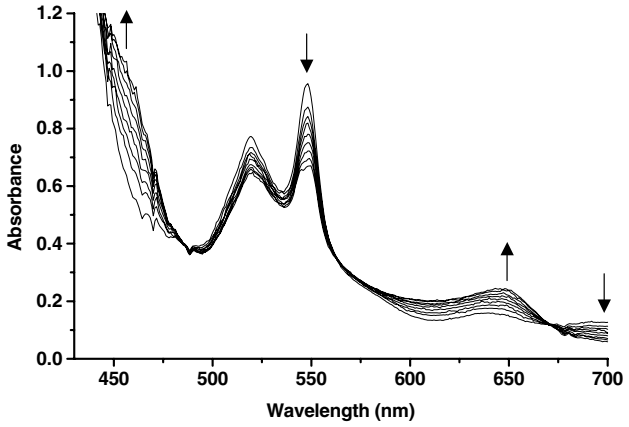

Fig. 2. Spectral change over 150 s on mixing oxidized "as isolated" *P. pantotrophus* cytochrome cd_1 with reduced *P. pantotrophus* NapC. Arrows indicate the direction of change at key wavelengths (see text). Experimental conditions were as in Fig. 1. Absorbances in the Soret band region of the spectrum (380–440 nm) saturated the diode array detector and thus could not be resolved.

Notably, there was a single peak above 600 nm, at 650 nm, and clear splitting of the *c* heme α band. There was also an increase of absorbance at 460 nm and a decrease at 700 nm, the latter a wavelength where oxidized His/Tyr coordinated d_1 heme absorbs [8]. These observations are consistent with a change of oxidation state at both hemes of cytochrome cd_1 to produce the reduced form of the enzyme.

Stopped-flow experiments were then performed to monitor the extent and rate of these changes. Fig. 2 illustrates the spectral changes over 150 s, indicating clearly reoxidation of $NapC^{sol}$ and reduction of cd_1. Fig. 3 shows single wavelength time-courses (obtained from similar stop-flow data) at four characteristic wavelengths: 460 nm (reduced d_1 heme), 550 nm (reduced NapC), 650 nm (reduced d_1 heme) and 700 nm (oxidized His/Tyr coordinated d_1 heme). It is apparent that the form of the curve at each wavelength, and the associated rates, were identical. This, combined with the clear isosbestic points in the spectra (Fig. 2), indicates a two state system in which $NapC^{sol}$ fully reduced cd_1 with no accumulation of intermediates. Reduction of the *c* and d_1 hemes of "as isolated" *P. pantotrophus* cytochrome cd_1 by NADH (with mediators) or dithionite is highly cooperative (i.e., both hemes must be reduced synchronously) [11]. The *c* heme of cd_1 is the redox centre of the protein that has been both modelled and observed to receive electrons from partner proteins [1,2,4]. As $NapC^{sol}$ can act as a multi-electron reductant, we therefore argue that the *c* heme of cd_1 (for which there is no "signature" wavelength where either NapC or d_1 heme does not also absorb) was reduced at the same rate as the d_1 heme (and by implication from Fig. 3 also the same rate as $NapC^{sol}$ was oxidized). Note that

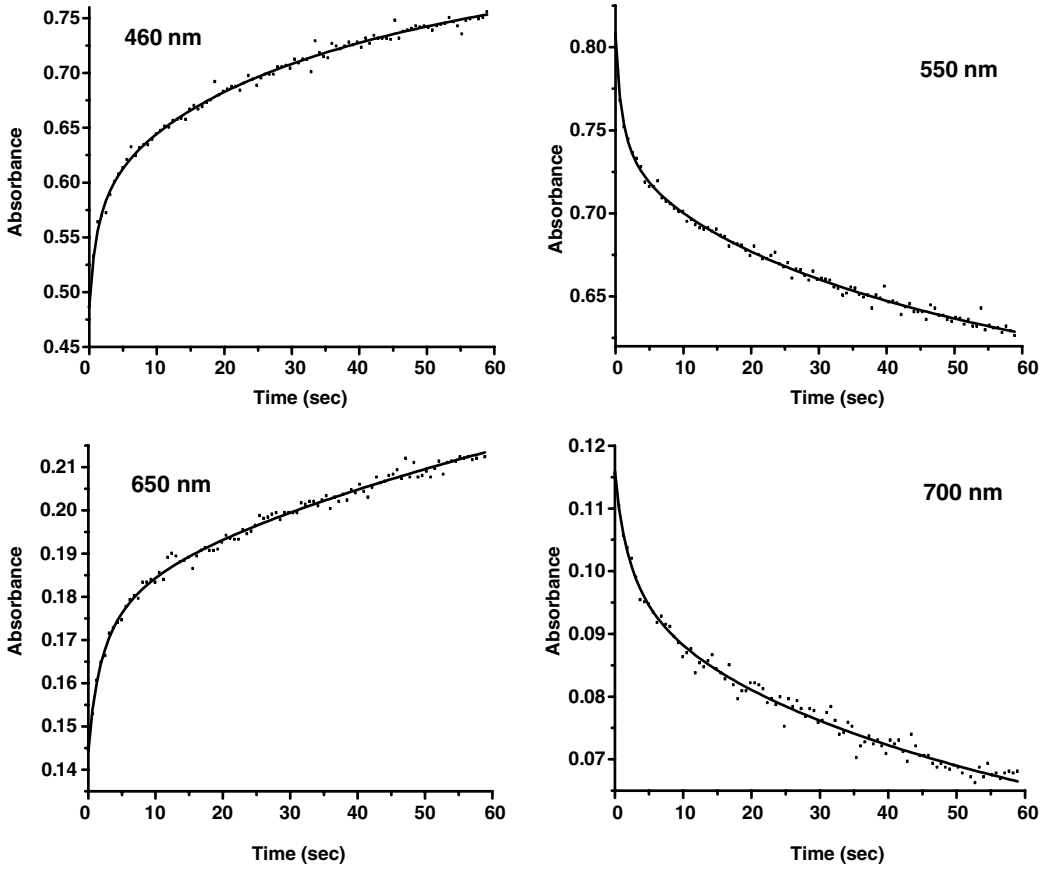

Fig. 3. Absorbance changes at wavelengths characteristic of *c*-type (550 nm) or d_1-type (460, 650 and 700 nm) heme on mixing oxidized "as isolated" cytochrome cd_1 with reduced NapC. Absorbance changes were monitored after mixing at 25 °C 5 μM oxidized "as isolated" cd_1 with 10 μM reduced NapC, both in 50 mM potassium phosphate buffer, pH 7.0. Data were obtained from spectra as shown in Fig. 2. The dots are the measured data points; the solid lines are best fits to the data, shown for clarity.

previous work has shown that the reduced *c* heme to oxidized d_1 heme electron transfer rate in *P. pantotrophus* cytochrome cd_1 is several orders of magnitude faster than the kinetics reported here [20,22] and thus is extremely unlikely to be rate limiting in the present experiments. Our time-courses (Fig. 3) indicated that the reaction of reduced NapC^{sol} with oxidized cd_1 was biphasic, consisting of a fast phase followed by a much slower phase. The origin and significance of these kinetics is presently unclear. More rapid kinetic experiments (on a timescale of 1 s) show that no very fast phases were overlooked in our longer timebase scans. Using the difference extinction coefficient between oxidized "as isolated" and reduced cytochrome cd_1 [24], the observed spectral change after 150 s at 650 nm was 98% of that expected for complete reduction of the cytochrome cd_1. This confirms near total reduction of cytochrome cd_1 by reduced NapC^{sol}.

4. Discussion

The heme ligations of oxidized "as isolated" *P. pantotrophus* cytochrome cd_1 have been puzzling since the surprising finding in the X-ray crystal structure that the *c*-heme had *bis*-histidinyl coordination while the d_1 heme had His/Tyr coordination and was blocked substrate access [5]. This was compounded by the remarkable observation of a change in coordination at both hemes on reduction [7]. In vitro, it has been shown that a pre-reduction step is required to achieve high rates of substrate turnover [3], but once activated the enzyme remains catalytically competent in turnover conditions, with His/Met ligation at the *c* heme [3,9,10,25]. However, the physiological electron donors to cd_1 (pseudoazurin and cytochrome c_{550}) [12] are both incapable of reducing and/or kinetically activating the "as isolated" enzyme in vitro [3,9]. In this work we clearly show, for the first time, that a protein found in the *P. pantotrophus* periplasm, NapC, is capable of reducing (and hence activating) oxidized "as isolated" cytochrome cd_1.

The presumed principal role of NapC in *P. pantotrophus* is the delivery of electrons from the ubiquinol pool to NapB from where they are transferred to NapA for the two-electron reduction of nitrate to nitrite. The fact that NapC is a tetraheme protein that donates two electrons to a periplasmic diheme protein (NapB) also makes it an ideal candidate to reduce oxidized "as isolated" cytochrome cd_1, which also requires two electrons per monomer in a cooperative process [11]. The apparent midpoint potential for reduction of "as isolated" *P. pantotrophus* cd_1 is ca. +60 mV, but the potential for oxidation of the reduced protein is +250 mV [11]. The latter will be the effective potential reached by electrons arriving at cd_1 from reduced NapC^{sol}, whose hemes have potentials between −56 and −235 mV [16]. Thus, there is 300–500 mV potential difference between the redox centres of NapC and cd_1; this is fully consistent with the complete reduction of cd_1 by NapC that we observed.

The timescale of cytochrome cd_1 reduction by the soluble form of NapC reported here is not as fast as may have been predicted for a physiological reductant, although our data (Fig. 3) indicate that it is the rate-limiting step in these experiments. However, rates in the periplasm will be affected by various factors such as relative protein concentrations, ionic strength, etc. In vivo, NapC can be expected to be maintained in a substantially reduced state. In contrast, in our experimental conditions the NapC^{sol} was increasingly oxidized as the reaction progressed, raising its effective reduction potential, decreasing the concentration of reduced NapC available to reduce the cd_1 and increasing the possibility of competitive inhibition by the already oxidized NapC. More significantly, in vivo, in the presence of nitrite, the reductive activation of cd_1 *need only occur once*, following which the enzyme maintains a catalytically competent state in turnover [3,9,10]. In that sense, there may be no need for the activation to be particularly fast. Indeed, the rate may be tuned in the cell for a particular purpose. If, for example, the role of the "as isolated" conformer of cd_1 is to protect the biosynthetically demanding d_1 heme in the presence of oxygen (and hence reactive oxygen intermediates) [13], it would not be productive for the enzyme to be rapidly re-reduced by NapC. In such conditions, the rate of reversion to the "as isolated" (hence protected) state may be favoured relative to the rate of activation (deprotection) by NapC. In contrast, cd_1 activated by NapC in the presence of nitrite (hence in anaerobic conditions) would rapidly be kinetically trapped in turnover by nitrite binding to the d_1 heme, which is known to be fast [22,26]. Mutants of *Paracoccus denitrificans* have been used to demonstrate unequivocally that the only effective steady-state electron donors to cd_1 in denitrifying conditions are pseudoazurin and cytochrome c_{550} [12], and thus there is no question that NapC is a physiological electron donor that can sustain turnover by cytochrome cd_1; this in turn suggests that the in vivo rate of reduction of cd_1 by NapC is slow.

It presently seems likely that cytochrome cd_1 is synthesized in an at least partially reduced state in the cell. Many *c*-type cytochromes are observed to be synthesized reduced, for instance when overproduced in the periplasm of aerobically grown *E. coli*. In these conditions, natural reductants of *c*-type cytochromes are thought to be absent and the expression system therefore probes the oxidation state of the end product of the cytochrome *c* biogenesis apparatus. Both the *c* heme binding domain of *P. pantotrophus* cytochrome cd_1 [27] and the semi-apo form of cd_1 (which contains no d_1 heme) (RSZ and SJF, unpublished data) are obtained in the reduced state when produced in this way. Similarly, our recent work has shown that in vitro the characteristic covalent attachment of heme to *c*-type cytochromes requires reducing conditions [28,29]. If *P. pantotrophus* cytochrome cd_1 is made in a fully or semi-reduced state, it will be in the catalytically active form and immediately primed to reduce nitrite. Synthesis of cd_1 requires anaerobic denitrifying conditions, implying the ready availability of nitrite. However, should the cells then encounter aerobic growth conditions in natural environments, or use another oxidant, cd_1 will probably revert to the "as isolated" form. We thus envisage the most likely role for *P. pantotrophus* NapC as a cd_1 reductant to be a "rescue" function, whereby populations of cd_1 that have somehow relaxed to the catalytically incompetent "as isolated" state can be reactivated.

Cytochrome cd_1 expression in *P. pantotrophus* is controlled by the transcriptional regulator NNR and as such requires NO for transcription of the structural gene and the d_1 heme biosynthesis genes. This situation implies that there must be a system outside of the NNR control that is able to reduce nitrite to NO in order to instigate large scale nitrite reduction by cytochrome cd_1. It has been shown that in *P. denitrificans* and *Rhodobacter sphaeroides*, a constitutive basal level of nitrite

reductase transcription is apparent [30,31]. If such basal levels of cytochrome cd_1 expression occur in *P. pantotrophus*, the enzyme may largely be in the "as isolated" conformation due to periods of extended aerobiosis. This cytochrome cd_1 may well require activation when needed to produce NO.

NirT was the first member of the NapC/NirT family of multi-heme quinol dehydrogenases to be identified. It was found in the *Pseudomonas stutzeri nirSTBM* gene cluster, *nirS* being the structural gene for cytochrome cd_1. *nirT* was knocked out in order to examine its role in cytochrome cd_1 function [32]. The deletion strain was unable to reduce nitrite in vivo, but active cd_1 was obtained from the cells. There is presently no information concerning whether *P. stutzeri* cytochrome cd_1 requires a kinetic activation step analogous to that for the *P. pantotrophus* enzyme. The former has His/Met *c*-heme coordination in its oxidized "as isolated" state [8] and thus cannot undergo heme ligand exchange reactions directly analogous to those in the latter, although the ligand to the d_1 heme of "as isolated" *P. stutzeri* cd_1 has not yet been identified. However, the reported specific activity of *P. stutzeri* cytochrome cd_1 is very similar to that of the unactivated state of the *P. pantotrophus* enzyme [3,33]. If nitrite reduction is to generate optimal proton translocation stoichiometry, electron transfer from the quinol pool to cytochrome cd_1 must proceed via the cytochrome bc_1 complex, rather than via NapC/NirT. It has been shown that electron transfer to *P. stutzeri* nitrite reductase is sensitive to the cytochrome bc_1 inhibitors antimycin and mucidin [34]. Considering these factors together, it may be that *P. stutzeri* NirT is a required activating partner for nitrite reductase in that organism, while electrons for steady-state turnover of nitrite are provided via the energetically preferred cytochrome bc_1 complex. However, the postulated interaction/activation effect of NirT would appear to be required continuously (or repeatedly) in *P. stutzeri* [32], which is often grown so as to denitrify microaerobically [35], in contrast to the limited "rescue" function we propose (see above) for *P. pantotrophus* NapC reducing-reactivating cytochrome cd_1 in the latter organism.

In summary, the present work shows that *P. pantotrophus* $NapC^{sol}$ is capable of reducing, and hence kinetically activating, the otherwise catalytically incompetent "as isolated" form of cytochrome cd_1. NapC is the first protein from *P. pantotrophus* identified as being capable of this activation step and, given the periplasmic co-location and co-expression of the two proteins, is a strong candidate to be a physiological activation partner.

Acknowledgements: This work was funded by Grants C13443, C19430 and P15877 from the BBSRC to S.J.F. and D.J.R. R.S.Z. is supported by a studentship from the BBSRC and M.L.C. by a studentship from St Edmund Hall, Oxford and the University of Oxford. We are particularly grateful to Professor Fraser Armstrong for his generosity in allowing us to use his laboratory facilities, and to Dr. Kylie Vincent for assistance with the stop-flow instrument.

References

[1] Allen, J.W.A., Ferguson, S.J. and Fülöp, V. (2001) in: Handbook of Metalloproteins (Messerschmidt, A., Huber, R., Poulos, T. and Wieghardt, K., Eds.), Wiley, Chichester, pp. 424–439.
[2] Fülöp, V., Watmough, N.J. and Ferguson, S.J. (2001) Adv. Inorg. Chem. 163–204.
[3] Richter, C.D., Allen, J.W.A., Higham, C.W., Koppenhöfer, A., Zajicek, R.S., Watmough, N.J. and Ferguson, S.J. (2002) J. Biol. Chem. 277, 3093–3100.
[4] Williams, P.A., Fülöp, V., Leung, Y.C., Chan, C., Moir, J.W.B., Howlett, G., Ferguson, S.J., Radford, S.E. and Hajdu, J. (1995) Nat. Struct. Biol. 2, 975–982.
[5] Fülöp, V., Moir, J.W.B., Ferguson, S.J. and Hajdu, J. (1995) Cell 81, 369–377.
[6] Sjögren, T. and Hajdu, J. (2001) J. Biol. Chem. 276, 29450–29455.
[7] Williams, P.A., Fülöp, V., Garman, E.F., Saunders, N.F.W., Ferguson, S.J. and Hajdu, J. (1997) Nature 389, 406–412.
[8] Cheesman, M.R., Ferguson, S.J., Moir, J.W.B., Richardson, D.J., Zumft, W.G. and Thomson, A.J. (1997) Biochemistry 36, 16267–16276.
[9] Allen, J.W.A., Watmough, N.J. and Ferguson, S.J. (2000) Nat. Struct. Biol. 7, 885–888.
[10] Allen, J.W.A., Cheesman, M.R., Higham, C.W., Ferguson, S.J. and Watmough, N.J. (2000) Biochem. Biophys. Res. Commun. 279, 674–677.
[11] Koppenhöfer, A., Turner, K.L., Allen, J.W.A., Chapman, S.K. and Ferguson, S.J. (2000) Biochemistry 39, 4243–4249.
[12] Pearson, I.V., Page, M.D., van Spanning, R.J.M. and Ferguson, S.J. (2003) J. Bacteriol. 185, 6308–6315.
[13] Gordon, E.H.J., Sjögren, T., Löfqvist, M., Richter, C.D., Allen, J.W.A., Higham, C.W., Hajdu, J., Fülöp, V. and Ferguson, S.J. (2003) J. Biol. Chem. 278, 11773–11781.
[14] Jafferji, A., Allen, J.W.A., Ferguson, S.J. and Fülöp, V. (2000) J. Biol. Chem. 275, 25089–25094.
[15] Cartron, M.L., Roldan, M.D., Ferguson, S.J., Berks, B.C. and Richardson, D.J. (2002) Biochem. J. 368, 425–432.
[16] Roldan, M.D., Sears, H.J., Cheesman, M.R., Ferguson, S.J., Thomson, A.J., Berks, B.C. and Richardson, D.J. (1998) J. Biol. Chem. 273, 28785–28790.
[17] Ellington, M.J.K., Bhakoo, K.K., Sawers, G., Richardson, D.J. and Ferguson, S.J. (2002) J. Bacteriol. 184, 4767–4774.
[18] Moir, J.W.B., Baratta, D., Richardson, D.J. and Ferguson, S.J. (1993) Eur. J. Biochem. 212, 377–385.
[19] Arslan, E., Schulz, H., Zufferey, R., Kunzler, P. and Thöny-Meyer, L. (1998) Biochem. Biophys. Res. Commun. 251, 744–747.
[20] Kobayashi, K., Koppenhöfer, A., Ferguson, S.J. and Tagawa, S. (1997) Biochemistry 36, 13611–13616.
[21] Timkovich, R. and Robinson, M.K. (1979) Biochem. Biophys. Res. Commun. 88, 649–655.
[22] George, S.J., Allen, J.W.A., Ferguson, S.J. and Thorneley, R.N.F. (2000) J. Biol. Chem. 275, 33231–33237.
[23] Koppenhöfer, A., Little, R.H., Lowe, D.J., Ferguson, S.J. and Watmough, N.J. (2000) Biochemistry 39, 4028–4036.
[24] Koppenhöfer, A. (1998) Functional studies on the cytochrome-type nitrite reductase from *Thiosphaera pantotropha*, DPhil Thesis, University of Oxford.
[25] Allen, J.W.A., Higham, C.W., Zajicek, R.S., Watmough, N.J. and Ferguson, S.J. (2002) Biochem. J. 366, 883–888.
[26] Silvestrini, M.C., Tordi, M.G., Musci, G. and Brunori, M. (1990) J. Biol. Chem. 265, 11783–11787.
[27] Gordon, E.H.J., Steensma, E. and Ferguson, S.J. (2001) Biochem. Biophys. Res. Commun. 281, 788–794.
[28] Daltrop, O., Allen, J.W.A., Willis, A.C. and Ferguson, S.J. (2002) Proc. Natl. Acad. Sci. USA 99, 7872–7876.
[29] Daltrop, O. and Ferguson, S.J. (2003) J. Biol. Chem. 278, 4404–4409.
[30] Van Spanning, R.J.M., Houben, E., Reijnders, W.N., Spiro, S., Westerhoff, H.V. and Saunders, N.F.W. (1999) J. Bacteriol. 181, 4129–4132.
[31] Kwiatkowski, A.V. and Shapleigh, J.P. (1996) J. Biol. Chem. 271, 24382–24388.
[32] Jungst, A., Wakabayashi, S., Matsubara, H. and Zumft, W.G. (1991) FEBS Lett. 279, 205–209.
[33] Zumft, W.G. (1997) Micro. Mol. Biol. Rev. 61, 533–616.
[34] Kučera, I., Hedbávný, R. and Dudák, V. (1988) Biochem. J. 252, 905–908.
[35] Arese, M., Zumft, W.G. and Cutruzzolà, F. (2003) Protein Expr. Purif. 27, 42–48.