Cytochrome cd₁ is fundamental to denitrification in Paracoccus pantotrophus as it catalyses the one-electron reduction of nitrite to nitric oxide. This is the second step in the denitrification process which comprises the sequential reductions of nitrate, nitrite, nitric oxide and nitrous oxide. In addition, it is the first committed step of denitrification, producing a toxic gaseous product, nitric oxide.

Cytochrome cd₁ is a soluble periplasmic homodimer with a subunit mass of approx. 65 kDa (365 amino acids). Each monomer is made up of two domains; a predominantly α-helical N-terminal domain (1-134) that covalently binds a c haem and an 8-bladed β-propeller C-terminal domain (135–565) that contains a non-covalently bound d₁ haem cofactor [1]. The d₁ haem cofactor is a dioxygenobacteriochlorin and is only found in cytochrome cd₁ nitrite reductases. As isolated, the enzyme from Paracoccus pantotrophus is oxidized; the c haem is bis-histidinyl co-ordinated (His-17 and His-69) and the d₁ haem is Tyr-25/His-200 co-ordinated [1]. The d₁ haem cofactor is a dioxygenobacteriochlorin and is only found in cytochrome cd₁ nitrite reductases. As isolated, the enzyme from Paracoccus pantotrophus is oxidized; the c haem is bis-histidinyl co-ordinated (His-17 and His-69) and the d₁ haem is Tyr-25/His-200 co-ordinated [1].

The d₁ haem Tyr-25 ligand extends from the N-terminal domain on a polypeptide loop. On reduction, the c haem becomes His-69/Met-106 co-ordinated and the Tyr-25 ligand departs from the active site and leaves the d₁ haem pentacoordinate and available for substrate binding [2]. On exposure to oxidizing conditions, reduced P. pantotrophus cytochrome cd₁ shows a strong tendency to revert to the oxidized ‘as isolated’ conformation [3,4].

Steady-state kinetic analysis of P. pantotrophus cytochrome cd₁ in vitro has shown that only the reduced form of the enzyme is able to reduce nitrite at a catalytically competent rate when using physiological electron donors [5]. This evidence leads to the conclusion that the oxidized ‘as isolated’ form of cytochrome cd₁ is inactive owing to the N-terminal Tyr-25 bound to the d₁ haem that blocks nitrite binding. Therefore it is thought that the enzyme in the cell must be first activated by reduction for substrate turnover. The bis-histidinyl co-ordinated c haem of cytochrome cd₁ has a reduction potential of approx. +60 mV, whereas the His/Met co-ordinated reduced c haem of cytochrome cd₁ has a reduction potential of approx. 200 mV higher [3]. Consequently, the physiological electron donors cytochrome c₅₅₂ and pseudoazurin [6] would not be able to reduce the bis-histidinyl co-ordinated oxidized enzyme because they themselves have reduction potentials of +230 and +260 mV respectively. However, they can sustain steady-state turnover of nitrite by activated cytochrome cd₁ [5], implying that the oxidized ‘as isolated’ form of the enzyme plays no part in catalysis. Cytochrome cd₁ from Pseudomonas aeruginosa carries no conserved His-17 or Tyr-25 residues and as such has a His/Met co-ordinated oxidized c haem [7] and no reported inactive oxidized state. In addition, no other reported cytochrome cd₁ has been shown to require this method of reductive activation.

Recently, various forms of oxidized Paracoccus pantotrophus cytochrome cd₁ have been produced in an active state. Kinetic and structural experiments were performed on a Tyr-25 to Ser variant of Paracoccus pantotrophus cytochrome cd₁ [8]. The crystal structure of the oxidized protein showed that the c haem still carried bis-histidinyl co-ordination, whereas the d₁ haem bound a sulphate molecule and not the -OH group of the Ser-25. In addition, steady-state kinetic experiments showed this enzyme to be fully active in both the oxidized and reduced states when using physiological electron donors [8]; the need for activation by pre-reduction was abolished. These results demonstrated that, even with an initially bis-histidinyl co-ordinated c haem, nitrite could bind to the d₁ haem and turnover was initiated. This can be explained using the idea of electron tunnelling in which an electron transfer chain can go via a thermodynamically unfavourable step, e.g. electron transfer from pseudoazurin to the bis-histidinyl co-ordinated c haem of Paracoccus pantotrophus cytochrome cd₁, if the overall reaction is favourable [9]. Although these experiments do not pertain to the wild-type enzyme, they do highlight that the binding of Tyr-25 to the d₁ haem is vital for the inactivation of the wild-type enzyme. In addition, they also demonstrate that Tyr-25 is not required during the catalytic cycle.

Key words: cytochrome cd₁, NapC, nitrite reductase, Paracoccus pantotrophus, periplasm, reductive activation.

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In addition to the Y25S mutation, an active oxidized form of the enzyme was produced by adding the non-physiological substrate hydroxylamine to the fully reduced wild-type enzyme [4]. This can be further stabilized by the addition of nitrite [10]. This form of the enzyme is not only active but carries His/Met co-ordination that would allow thermodynamically favourable electron transfer from the physiological electron donor, either reducing the protein before or after substrate binding. Probably, this form of the enzyme represents something similar to an oxidized intermediate of the catalytic cycle without Tyr-25 bound to the $d_1$ haem, the interaction that could inactivate the enzyme.

There is a great deal of evidence that $c$-type cytochromes are produced in a reduced form within the periplasm ([11] and R.S. Zajicek and S.J. Ferguson, unpublished work). If this is the case, after expression in a nitrite-rich anaerobic environment, P. pantotrophus cytochrome $d_1$ would remain in an active form until aerobiosis. However, during periods of aerobic growth, any cytochrome $d_1$ produced by the cell would be inactivated rendering the biosynthetically demanding cytochrome $d_1$ potentially useless for further nitrite reduction. Recently, it has been shown that the tetra-haem periplasmic nitrate reductase from P. pantotrophus, NapC, is able to reductively activate ‘as isolated’ oxidized cytochrome $d_1$ in vitro [12]. We propose NapC could be used as an activator of cytochrome $d_1$ in vivo after exposure to an aerobic environment. It has been shown that, for the production and cofactor insertion of cytochrome $d_1$, nitric oxide is required to activate the NNR transcription factor that initiates cytochrome $d_1$ expression [13]. Upon returning to anaerobiosis after periods of aerobic growth, active cytochrome $d_1$ would be required initially to reduce nitrite to nitric oxide to induce large-scale cytochrome $d_1$ expression. In P. pantotrophus, NapC is maximally transcribed during periods of aerobic growth [14]. This observation agrees well with the need for continual activation of cytochrome $d_1$ following aerobic growth and/or when the organism encounters an aerobic/anaerobic interface.

In conclusion, there is now a great deal known about the activation properties of cytochrome $d_1$ from P. pantotrophus. Is this property of the enzyme purely an artifact or does it have a significant purpose? Speculation includes this enigma reflecting a method of effective $d_1$ haem retention during periods of aerobiosis [8]. It has also been proposed that this conformation of the enzyme may protect the $d_1$ haem from reactive oxygen species, preventing unwanted radical chemistry from taking place [15].

References

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