The role of tryptophan 272 in the Paracoccus denitrificans cytochrome c oxidase

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Abstract The mechanism of electron coupled proton transfer in cytochrome c oxidase (C\textsubscript{c}O) is still poorly understood. The P\textsubscript{M} intermediate of the catalytic cycle is an oxoferryl state whose generation requires one additional electron, which cannot be provided by the two metal centres. The missing electron has been suggested to be donated to this binuclear site by a tyrosine residue. A tyrosine radical species has been detected in the P\textsubscript{M} and F\textsuperscript{'} intermediates (formed by addition of H\textsubscript{2}O\textsubscript{2}) of the Paracoccus denitrificans C\textsubscript{c}O using electron paramagnetic resonance (EPR) spectroscopy. From the study of conserved variants its origin was determined to be Y167 which is surprising as this residue is not part of the active site. Upon inspection of the active site it becomes evident that W272 could be the actual donor of the missing electron, which can then be replenished from Y167 or from the Y280-H276 cross link in the natural cycle. To address the question, whether such a direct electron transfer pathway to the binuclear centre exists two tryptophan 272 variants in subunit I have been generated. These variants are characterised by their turnover rates as well as using EPR and optical spectroscopy. From these experiments it is concluded, that W272 is an essential component of the active site.

Keywords: Cytochrome c oxidase; Tyrosine radical; Variants; Electron paramagnetic resonance spectroscopy; Bioenergetics

1. Introduction

Cytochrome c oxidase (C\textsubscript{c}O) is the terminal enzyme of the respiratory chain (for recent reviews, see [1–3]). Using cytochrome c as electron donor it catalyses the reduction of dioxygen to water and additionally pumps protons across the mitochondrial or bacterial membrane. This reaction contributes to the generation of an electrochemical proton gradient across the membrane that is used by the ATP-synthase for the formation of ATP. The net reaction can be written as:

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4\text{cyt}^2^+ + \text{O}_2 + 8\text{H}_2^+ \rightarrow 4\text{cyt}^3^+ + 2\text{H}_2\text{O} + 4\text{H}_2^+ \quad (1)
\]

with H\textsubscript{2}\textsuperscript{+} denoting protons taken up from the inner phase (the bacterial cytoplasm or mitochondrial matrix) and H\textsubscript{2}\textsuperscript{+} referring to protons released into the outer phase (the periplasm or mitochondrial intermembrane space).

Although the structure [4–6] and function of C\textsubscript{c}O has been studied for many years, it is still a matter of debate how electron and proton transfer are coupled, the precise mechanism of proton pumping has remained elusive. During one turnover electrons are transferred from cytochrome c initially to Cu\textsubscript{A} then further to heme a and from there into the binuclear centre made of heme a\textsubscript{3} and Cu\textsubscript{B} where dioxygen reduction takes place. During one turnover of C\textsubscript{c}O several intermediates are formed that represent different redox states of these metal centres.

After the binding of dioxygen to the doubly reduced binuclear center an intermediate compound A is formed. By reductive cleavage of the dioxygen bond the oxoferryl intermediate P is formed. But only three of the four electrons that are required for this process can be provided by the metal centres. The “missing” electron has been suggested to be donated by an amino acid residue close to the binuclear center [7–9]. The oxoferryl intermediates P\textsubscript{M}, F\textsuperscript{'} and F of C\textsubscript{c}O can be generated artificially by addition of hydrogen peroxide to the fully oxidized enzyme [10–12]. In P\textsubscript{M} and F\textsuperscript{'} intermediates a tyrosine radical has been identified by steady state EPR-spectroscopy [13]. This radical has been assigned to the residue tyrosine 167 by a site-directed mutagenesis study of several tyrosine variants close to the binuclear center [14] and by analysis of the dihedral angles arising from the β-methylene protons [13–15]. But tyrosine 167 may not be involved in the direct donation of the “missing” electron, because the function of the enzyme in not severely impaired by the mutation of this residue [14], and the residue tyrosine 167 is not part of the active site.

By investigation of the reaction of reduced C\textsubscript{c}O from P. denitrificans and E. coli with molecular oxygen using a microsecond freeze hyperquenching (MHQ) technique a mixture of two radicals has been observed in both these enzymes [16]. A tryptophan cation radical has been tentatively assigned based on the observed spectral properties of the EPR lineshape while for the second species it was less clear and a tyrosine radical could not be ruled out. The authors suggested that in the P\textsubscript{M}-state these two radicals may exist in rapid electronic equilibrium.

The observed tyrosine radical species may either be tyrosine 280 or tyrosine 167. A strong candidate for the tryptophan
cation radical is tryptophan 272. This residue is positioned at a hydrogen-bonding distance of 3 Å from tyrosine 167 and lies directly inbetween Y167 and the binuclear centre (see Fig. 1).

Here we present evidence for an involvement of tryptophan 272 in process of the radical formation at tyrosine 167 in the steady state after reaction of Cr(O) with hydrogen peroxide. Further the characterisation of two conservative variants W272M and W272F reveals an almost complete loss of catalytic activity. Oxoferryl states and the radical at tyrosine 167 cannot be generated by these variants. The possible importance of these findings for the mechanism of the oxygen bond cleavage is discussed.

2. Materials and methods

Site-directed mutagenesis in subunit I of Cr(O) (cytochrome aa₃) from P. denitrificans was performed as described previously [14]. Phenylalanine was chosen as a variant for tryptophan thus maintaining an uncharged large hydrophobic group, which is also planar and is thus the most conservative change for such a large aromatic residue. In addition the methionine variant was also made for comparison. Wild type and mutant cells were grown in succinate medium as described [17], except that the manganese content was decreased in the medium. The enzyme was pulsed with stoichiometric amounts of sodium dithionite, reoxidised and buffered in (50 mM KPi, pH 8.0, 0.05% LM). The enzyme was pulsed with stoichiometric amounts of hydrogen peroxide to Cr(O) (buffered in 1 mM Tris–HCl, pH 9 (8.6), 50 mM KCl, 0.05% LM) was used. In pH shift experiments the L of 1 M Mes-KOH, pH 6.0 were mixed with large excess of hydrogen peroxide had no additional effect. The turnover rate of variant W272M is also very low and again using of a large excess of hydrogen peroxide (up to 100-fold). The difference spectrum at pH 9 shows maxima at 604 and at 443 nm. These maxima are not identical with the characteristic wavelengths of 610 and 438 nm of the P₄₅₀-intermediate that are detected in the P. denitrificans wild type enzyme as well as in variant Y167F. In addition, when hydrogen peroxide is added after a pH jump to pH 6 the reactivity of variant W272M is also very low and again using of a large excess of hydrogen peroxide had no additional effect. The optical difference spectrum shows maxima at 604, 561, 444 and 424 nm, whereas the characteristic maxima for the F-O-/F-intermediate, observed with wild type enzyme and variant Y167F are found at 575–580 and at 436 nm (see Fig. 2 C and D).

From these experiments with variant W272M it can be concluded that neither P₄₅₀ nor F- or F-intermediates are formed by the variant enzyme using this fivefold molar excess of hydrogen peroxide at these low protein concentrations, see however the discussion in [14].

The tyrosine radical (shown as trace A in Fig. 3), assigned to tyrosine 167 [14], observed with EPR spectroscopy after addition of stoichiometric amounts of hydrogen peroxide to Cr(O) is also not detected in variant W272M (see Fig. 3). In the optical absorption spectrum of oxidised variant W272M the Soret maximum is also significantly different from that of the wild type. In this variant enzyme this maximum is shifted from 425 to 414 nm and is broader than in the wild type enzyme (data not shown). Cyanide binding using the variant W272M showed a significantly different cyanide binding spectrum as compared to the wild type. This result indicates that the cyanide binding ability of heme a₃ is also strongly affected by the mutation.

3. Results

3.1. Characterisation of variant W272M

The turnover rate of variant W272M is drastically reduced compared to the wild type enzyme. In the reaction with reduced cytochrome c the variant enzyme displays only 1% of the wild type activity. Further, the reactivity of W272M with a fivefold excess of hydrogen peroxide is also extremely low. At pH 9 only very small changes in the difference spectrum were observed (see Fig. 2A and B). This reactivity was not altered by using larger molar excesses of hydrogen peroxide (up to 100-fold). The difference spectrum at pH 9 shows maxima at 604 and at 443 nm. These maxima are not identical with the characteristic wavelengths of 610 and 438 nm of the P₄₅₀-intermediate that are detected in the P. denitrificans wild type enzyme as well as in variant Y167F. In addition, when hydrogen peroxide is added after a pH jump to pH 6 the reactivity of variant W272M is also very low and again using of a large excess of hydrogen peroxide had no additional effect. The optical difference spectrum shows maxima at 604, 561, 444 and 424 nm, whereas the characteristic maxima for the F-O-/F-intermediate, observed with wild type enzyme and variant Y167F are found at 575–580 and at 436 nm (see Fig. 2 C and D).

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3.2. Characterisation of variant W272F

As observed for the variant W272M the turnover rate of variant W272F was almost abolished compared to the wild type enzyme. The reaction of the W272F variant with a fivefold (or higher) excess of hydrogen peroxide increased the auto reduction rate at high and low pH, so that typical redox shifts were observed in the difference spectra (see Fig. 2). The difference spectrum at high pH again revealed maxima at 604 and 445 nm similar to those seen for the W272M variant but more
intense, whereas the wild type and the Y167F variant have characteristic maxima at 610 and 438 nm specifying PM states (see Fig. 2A and B). At low pH the same 604 and 443 nm maxima were observed in the W272F variant difference spectrum but the maxima at 561 and 424 nm of the W272M variant were not detectable. The characteristic maxima for the F-/F-intermediate (575–580 and 436 nm) of the wild type enzyme and the Y167F variant were not observed (see Fig. 2C and D). For both the W272M and W272F variants it can be concluded that neither the PM- nor the F-/F-intermediates are induced after hydrogen peroxide treatment. As with the W272M variant, the tyrosine 167 radical was not observed by EPR spectroscopy after addition of stoichiometric amounts of hydrogen peroxide to the variant W272F (see Fig. 3). In the optical absorption spectrum of oxidised variant W272F the Soret maximum is shifted from the wild type enzyme absorption at 425 to 420 nm and the maximum is broadened in the same way compared to the variant W272M (data not shown). Similar optical properties occurred in the spectrum of the oxidised W272F enzyme like for the W272M variant; the small differences seen are assumed to be redox potential shifts of the heme a and the binuclear centre due to the additional methionine sulfur atom. Cyanide binding to heme a was also not observed in the W272F variant.

3.3. Characterisation of variant R473N

The turnover rate of this variant is also diminished and shows only 6.5% of wild type activity. Its oxidised absorption spectrum reveals a broad Soret maximum at 422 nm and a shoulder at 414 nm (data not shown). At pH 9, no PM can be observed after reaction with hydrogen peroxide. The difference spectrum shows maxima at 436 and 582 nm and a shoulder at 601 nm which may be indicative of the F-state. The
treatment with H$_2$O$_2$. Experimental conditions: Frequency: 9.43 GHz, shows the magnetic field region around the tyrosine radical ($g = 2$) in: (A) Wild type, (B) R473N, (C) W272M, and (D) W272F. After treatment with H$_2$O$_2$. Experimental conditions: Frequency: 9.43 GHz, microwave power, 0.1 mW; field modulation, 100 kHz; amplitude ± 0.4 mT peak to peak, $T = 20$ K. The inset shows the magnetic field region around the tyrosine radical ($g = 2$) in: (A) Wild type, (B) R473N, (C) W272M, and (D) W272F. After treatment with H$_2$O$_2$. Experimental conditions: Frequency: 9.43 GHz, microwave power, 0.1 mW; field modulation, 100 kHz; amplitude ± 0.4 mT peak to peak, $T = 20$ K.

Fig. 3. EPR spectra of cytochrome c oxidase after reaction with H$_2$O$_2$. The main spectrum is from wild type CcO which contains a radical EPR spectrum which is identical to that published previously [13,14] but which is recorded here at a lower temperature (20 K). The inset overall reactivity with hydrogen peroxide is very low and only results in formation of about 4% of the F-state. At pH 6 again no F is observed. The difference spectrum shows maxima at 436 and 580 nm and a shoulder at 600 nm, however, there is a much higher reactivity with hydrogen peroxide resulting in about 45% yield of the F-state. When the reaction of stoichiometric amounts of hydrogen peroxide is performed at much higher proteins concentrations an EPR signal is indeed observed with characteristics typical of the tyrosine radical seen in wild type CcO (Fig. 3), as has been observed previously for other variants [14].

4. Discussion

The origin of the radical that can be observed in the reaction of CcO with hydrogen peroxide has been previously assigned to tyrosine 167 [14], however it was also demonstrated that this residue is not essential for the function of the enzyme, because the variant Y167F is still able to form an oxoferryl state and maintains a relatively high turnover rate while retaining full proton pumping activity.

It has been proposed that residue tyrosine 167 is not the direct donor of the “missing” electron that is required for the reductive cleavage of the dioxygen bond in CcO but that the radical species may be formed in a secondary process in the steady state of the reaction with hydrogen peroxide. Thus the original donor of the “missing” electron is still unknown.

Recently Wiertz et al. [16] suggested that in pre-steady state reaction kinetics of CcO from P. denitrificans two configurations of the P$_{554}$-state exist in rapid electronic equilibrium. One species was assigned to a tryptophan cation radical while for the second species a tyrosine radical could not be ruled out. The authors postulated that tryptophan 272 or tryptophan 164 might be the source of this radical (see Fig. 1). In addition Sivistunenko et al. [15] discussed the possible involvement of a tryptophan residue in the oxygen bond cleavage. They suggested that a tryptophan residue might form an intermediate radical state between the original donor of the electron which is supposed to be the cross linked tyrosine 280 and the observed tyrosine radical at residue 167. At variance, Siegbahn and Blomberg [20] have discussed on the basis of quantum chemical calculations recently that the actual donor of the missing electron might be tryptophan 272.

The experiments performed here using the W272M and W272F variants reveal two important pieces of information. (1) Mutation of this tryptophan residue drastically affects the catalytic function of the enzyme. (2) These variants completely lose the ability to form the F$_{554}$, F$^\cdot$ and F-intermediates in the reaction with hydrogen peroxide, indicating that also in the catalytic cycle the cleavage of the oxygen bond might be not possible in both W272 variants.

Further no EPR signal from a radical species was observed with W272 variants. From this it is concluded that tryptophan 272 plays an important role in the formation of a radical species at tyrosine 167 and also possibly in the catalytic mechanism of the enzyme itself. Tryptophan 272 is located in subunit I of CcO at a distance of 8.5 Å from the heme $a_1$ iron and 5 Å to CUB and is also directly hydrogen bonded to tyrosine 167 (see Fig. 1). A close relationship between these two residues and the possibility of electron transfer between them thus seems to be obvious.

As at present no other radical EPR signal has been observed in the W272 variants, one may speculate if this tryptophan really is the original donor of the “missing” electron required for oxygen bond cleavage. However the involvement of another amino acid residue, e.g., tyrosine 280 cannot be excluded because the lifetime of this radical species may be very short. In this case tryptophan 272 could form an intermediate radical state between the original electron donor and tyrosine 167.

Another reason why the radical at Y167 may not be formed in variants W272M and W272F could be the close position of this mutated residue to the binuclear center. This may affect the functionality of the active site and especially the reaction with hydrogen peroxide may be impaired as is indicated by the change of the spectral properties of these variants. However, this argument has to be compared to the fact that in variant Y280H oxoferryl intermediates could also not be detected by optical spectroscopy under experimental conditions where low protein and hydrogen peroxide concentrations were used (see discussion in [14]). But due to the different reactivity of CcO with hydrogen peroxide under conditions where high protein concentrations were used like for EPR-measurements the radical at tyrosine 167 was detected in the variant Y280H [14].

In the variant R473N an EPR-signal from Y167 is still observed. This residue is also very close to Y167: indeed in W272 variants reveal two important pieces of information. (1) Mutation of this tryptophan residue drastically affects the catalytic function of the enzyme. (2) These variants completely lose the ability to form the F$_{554}$, F$^\cdot$ and F-intermediates in the reaction with hydrogen peroxide, indicating that also in the catalytic cycle the cleavage of the oxygen bond might be not possible in both W272 variants.

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In the variant R473N an EPR-signal from Y167 is still observed. This residue is also very close to Y167: indeed in MD-simulations it has been suggested to be transiently coordinated to Y167 via hydrogen bonding [21]. Its turnover rate is also very low and the changes in the oxidised spectrum indicate structural perturbations of the hemes. In the reaction with hydrogen peroxide no P$_{554}$ to F transition can be observed in variant R473N (but maybe F-state especially at low pH). But in spite of all these similarities a radical at residue tyrosine 167 can still be detected in variant R473N but not in variants W272M and W272F. This observation supports the special importance of W272 for the formation of the radical at tyrosine 167.

The scenario that W272 is the original donor of the missing electron which is then replenished by a nearby tyrosine residue,
either tyrosine 167 or the cross linked tyrosine 280, also provides a likely explanation why in the PM state created by hydrogen peroxide a radical caused by tyrosine 167 is observed whereas not in PM states created by other means: hydrogen peroxide carries two hydrogen atoms into the active site, for the reductive cleavage of the O=O double bond therefore no extra protons are required for the formation of an oxoferryl state, whereas in the native cycle a proton is also required. The hydroxyl group of tyrosine 280 can deliver a proton plus an electron, whereas tyrosine 167 appears to be too far away for a proton delivery to the active site. Therefore it is feasible that in the native cycle tyrosine 280 is the secondary donor of the missing electron whereas upon treatment with hydrogen peroxide it can also be tyrosine 167.

The proposal that tryptophan 272 is the primary donor of the missing electron makes good sense also with respect to the conservation of this residue. Tryptophan 272 appears to be absolutely conserved within the superfamily of heme-copper containing terminal oxidases, whereas tyrosine 280, commonly believed to be the donor of the missing electron at present, is absent in the most distant members of this superfamily, namely the cbb3 type cytochrome c oxidases (see e.g., Ref. [22]). A recent study of the cbb3 oxidase from Vibrio cholerae has suggested that a tyrosine residue, structurally homologous to Y280, is however present in the cbb3 family of oxidases but is found on a different transmembrane helix within the protein [23].

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