

## Minireview

## Exploring the molecular nature of alternative oxidase regulation and catalysis

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**Abstract** Plant mitochondria contain a non-protonmotive alternative oxidase (AOX) that couples the oxidation of ubiquinol to the complete reduction of oxygen to water. In this paper we review theoretical and experimental studies that have contributed to our current structural and mechanistic understanding of the oxidase and to the clarification of the molecular nature of post-translational regulatory phenomena. Furthermore, we suggest a catalytic cycle for AOX that involves at least one transient protein-derived radical. The model is based on the reviewed information and on recent insights into the mechanisms of cytochrome *c* oxidase and the hydroxylase component of methane monooxygenase. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Alternative oxidase; Ubiquinol-oxygen oxidoreductase; Post-translational regulation; Active site structure; Catalytic mechanism; Tyrosine radical

## 1. Introduction to ‘a waste of energy’

Plants exhibit respiration that is partly resistant to cyanide, a characteristic that has been long recognised [1] and is accounted for by the presence of a single mitochondrial respiratory enzyme, the alternative oxidase [2,3]. The occurrence of this oxidase is widespread, as it has been identified in all higher plants studied to date as well as in many algae, fungi, yeasts and protozoa. The enzyme is non-protonmotive [4,5] and its activity does therefore not contribute to the conservation of energy, in contrast to the proton-pumping activity of cytochrome *c* oxidase [6]. The heat that is dissipated during alternative oxidase activity is put to good use in thermogenic plants which exploit it to volatilise primary amines to attract pollinating insects [7]. However, the ubiquitous presence of the enzyme throughout the plant kingdom, including all non-thermogenic species studied to date, would suggest that the oxidase has a more general physiological role. Although all currently available evidence is circumstantial, it appears that the alternative oxidase is important under conditions where cytochrome pathway activity is in some way impaired

[8]. Based on this notion, two main hypotheses that are not mutually exclusive have been proposed respectively predicting that the alternative oxidase allows Krebs-cycle turnover when the energy charge of the cell is high and that the enzyme protects against oxidative stress (see [8,9] for reviews).

Extensive studies have been performed (and most recently reviewed in the cited references) in an attempt to reveal the in organello kinetic behaviour of the alternative oxidase [10,11] and the mechanisms that account for the genetic [9] and post-translational [12] regulation of the enzyme. Furthermore, several structural models have been developed in which the active site of the oxidase is considered to comprise a non-haem di-iron centre [13]. Recent site-directed mutagenesis studies have provided experimental data that allow evaluation of these structural hypotheses, something that has traditionally been difficult, since the lack of a stable fully purified enzyme has severely hampered spectroscopic studies. In this paper we review theoretical and experimental research in which key alternative oxidase features are specifically related to the molecular nature of the enzyme. We evaluate previous considerations as to the reaction mechanism of the oxidase, leading to the development of a new catalytic model.

## 2. Post-translational regulation of alternative oxidase activity

In vitro alternative oxidase activity can be modulated in several ways. Activity of the oxidase in plant mitochondria is for example considerably increased upon reduction of an intersubunit disulphide bridge, yielding a non-covalently linked homodimeric protein [14,15]. The reduced enzyme is further activated by  $\alpha$ -keto acids, pyruvate in particular [16], which have been shown to react with a protein-derived sulphhydryl moiety [17], most likely to form a thiohemiacetal [17]. In contrast, the fungal alternative oxidase is not stimulated by pyruvate [18,19], nor is it possible to cross-link the protein covalently [18] suggesting that the fungal enzyme is exclusively monomeric (see however [20]). The fungal alternative oxidase is stimulated by purine nucleotides including ADP, AMP and GMP (cf. [12]).

It is evident that the stimulatory phenomena observed in plant systems involve at least one cysteine residue, two of which (C122 and C172, Fig. 1)<sup>1</sup> are highly, but not universally (cf. [12]), conserved throughout the plant alternative oxidase

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**Abbreviations:** MMOH, hydroxylase component of methane monooxygenase; Q, ubiquinone

<sup>1</sup> *Sauromatum guttatum* numbering is used throughout this paper.

sequences. Site-directed mutagenesis studies that were independently performed by Rhoads et al. [21] and Vanlerberghe and co-workers [22] have conclusively revealed that C122 is the site where the aforementioned regulatory events occur. Importantly, it was demonstrated that mutation of C122 to glutamate yields an enzyme that, although slightly less active than the pyruvate-stimulated wild-type oxidase, is constitutively in an activated state [21]. Since the carboxylate moiety of the glutamic acid resembles that of a pyruvate-induced thiohemiacetal, such an observation is in good agreement with the suggested mechanism of activation [17]. In subsequent studies, Djajanegara et al. [23] showed that mutation of C122 to serine results in an enzyme that is no longer activated by pyruvate but, intriguingly, specifically by succinate. Mutation of C122 to alanine yields an alternative oxidase protein that is also insensitive to pyruvate [21–23] and, based on unpublished observations (as stated in [12,23]), it is known that this mutant too is stimulated by succinate. In contrast,

succinate does not significantly affect the wild-type enzyme [23].

The constitutively high activity of the C122E mutant would suggest that it is the presence of an additional carboxyl moiety in this particular area of the protein that accounts for increased alternative oxidase activity. That activity of the enzyme is stimulated by carboxylic acids such as pyruvate and succinate supports this notion. To date, the mechanism that accounts for activation of the mutant alternative oxidase proteins by succinate is unclear, although a covalent interaction with the enzyme, as appears to be the case for pyruvate and the wild-type oxidase, is unlikely. In this respect, it remains puzzling how a site-specific substitution of a sulphhydryl with a hydroxyl group could result in such a drastic alteration of the oxidase's sensitivity to succinate.

It has been pointed out that the fungal alternative oxidase does not contain the primary domain of approximately 40 amino acids that surrounds and includes the 'regulatory' cys-

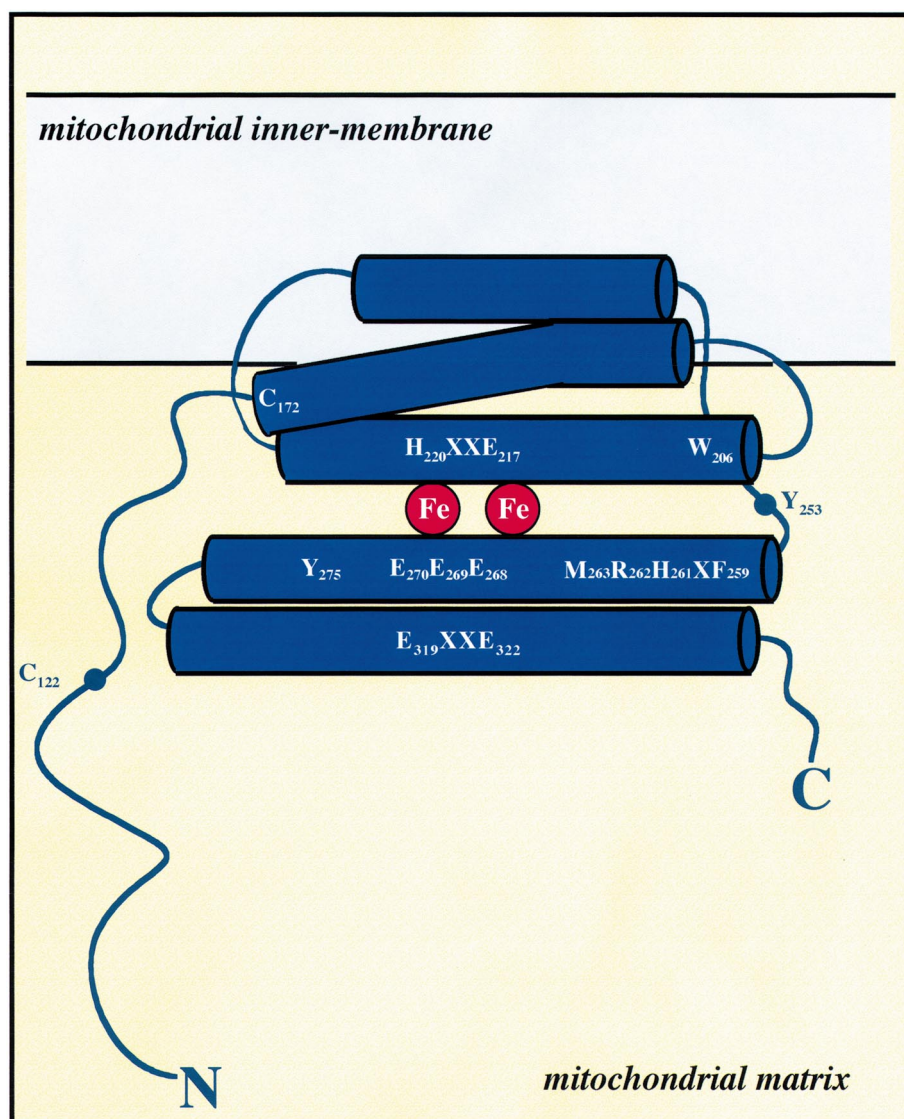


Fig. 1. Schematic structural representation of the alternative oxidase. The helical region harbouring the binuclear iron centre (shown as red spheres) is based on the structure proposed by Andersson and Nordlund [29]. Highlighted amino acid residues (*Sauromatum guttatum* numbering) are discussed in the text. Note added in proof: as indicated in the text, residue E322 should be H322.

teine (C122) in most plant sequences [18]. As mentioned before, the fungal enzyme is not affected by pyruvate which correlates with this notion [18,19]. Umbach and Siedow [12,18] have suggested that it is this structural domain that allows dimerisation of the plant enzyme, its absence in fungi explaining why most fungal alternative oxidases studied to date have been found to be monomeric proteins. The enzyme identified in the phytopathogenic fungus *Gaeumannomyces graminis*, however, appears to be an exceptional case [20]. When mitochondrial membranes isolated from this species are incubated with monoclonal antibodies raised against the *S. guttatum* alternative oxidase [24], two proteins cross-react with apparent molecular weights of approximately 30 and 66 kDa, respectively [20]. By analogy to the plant oxidase, the latter species was considered to be a dimeric form of the enzyme [20]. Based on this observation, Joseph-Horne et al. [19] suggested that the functional fungal protein is a homodimer with its subunits held together exclusively through electrostatic interactions. Clearly, such a model is difficult to reconcile with the work of Umbach and Siedow [18]. In this respect, it is unfortunate that the extent to which the *G. graminis* protein can be covalently connected with chemical cross-linking agents, and whether the apparent dimeric species disappears when gel electrophoresis is performed under fully denaturing conditions, has not been reported. Similarly, it is a pity that the published partial *G. graminis* alternative oxidase sequence [19] lacks the part that is of most interest in this context, namely the region that harbours the proposed [18] plant dimerisation domain.

### 3. Structure of the alternative oxidase

#### 3.1. Distinction between two structural hypotheses

The structure of the alternative oxidase has been subject of much speculation (see [13] for a recent review). Although neither crystallographic nor spectroscopic data are as yet available to provide any direct evidence, the currently adopted view is that the active site of the oxidase comprises a non-haem di-iron centre (Fig. 1). This idea was first postulated by Siedow and colleagues [25,26] who identified, in the relatively few alternative oxidase sequences that were available at the time, two conserved glutamate-histidine motifs (E270-X-X-H273 and E319-X-X-H322) which in structurally relatively well characterised carboxylate di-iron proteins such as the hydroxylase component of methane monooxygenase (MMOH; see [27] for a recent review) have been shown to be pivotal in the co-ordination of the iron atoms. This led to the development of a structural hypothesis with two distinct features: (a) the di-iron centre is buried in a four-helix bundle that forms an independent matrix-located domain and (b) the protein is integrally associated with the mitochondrial inner membrane via two membrane-spanning helices (predicted from early hydropathy analysis [28]) that are connected by a helix located in the inter-membrane space [26].

Subsequently, it was pointed out by Andersson and Nordlund [29] that neither the ligand spacing in the above model, the order and direction of the unusually short helices harbouring the active site, nor the proposed overall fold of the alternative oxidase protein agree with the available structural information on well-established di-iron enzymes. These weaknesses, together with the notion that one of the iron-co-ordinating histidines (H273) is not fully conserved

throughout the by then numerous newly emerged alternative oxidase sequences, provoked these authors to revise the model proposed by Siedow and colleagues [29]. Using an until recently [30] ignored but indeed fully conserved glutamate-histidine motif (E217-X-X-H220), a structure was proposed in which there is a greater degree of resemblance to other di-iron enzymes. Arguably the most distinct feature of the revised model is that the alternative oxidase protein is no longer predicted to be trans-membranal, but rather to be peripherally associated with the matrix side of the inner membrane (Fig. 1 [29]).

Very recently, Albury et al. [31] showed that E217 is an essential amino acid residue in terms of alternative oxidase activity. Such an observation has ramifications as to the likelihood of the respective structural hypotheses described above. According to the model of Siedow et al., E217 resides in the inter-membrane space [25,26], a location that most likely rules out a catalytic function for this residue. In contrast, Andersson and Nordlund [29] assign an essential role to this glutamate residue which, in their model, is predicted to bridge the di-iron centre. Therefore, the complete lack of activity of an alternative oxidase protein in which E217 was mutated to alanine [31] supports the interfacial structure postulated by these authors [29].

#### 3.2. Is the primary ligation sphere of the active site universally conserved?

It has recently been demonstrated that the *IMMUTANS* gene of *Arabidopsis* encodes a protein that is distantly related to the alternative oxidase [32,33]. This plastid terminal oxidase, as its name reflects, is associated with the thylakoid membranes of these plants and since it couples oxidation of plastoquinol to the reduction of oxygen to water [34], has been implicated in chlororespiration (e.g. [34]). Alignment of the primary structures of the plastid and other alternative oxidases reveals limited yet significant homology between the chloroplastic and mitochondrial enzymes [13]. The amino acid residues that co-ordinate the di-iron centre in the model of Andersson and Nordlund are with one exception (E269) all conserved in the sequence of the chloroplast oxidase [13]. In all mitochondrial alternative oxidase sequences, E269 forms part of a sequential quintet of conserved amino acids that also includes E270 and E268 (Fig. 1). Since the latter is the only carboxylate residue that is present in the plastid oxidase, Berthold et al. [13] proposed that E268 rather than E269 is iron-co-ordinating. The obvious strength of such a choice is that the active sites of several enzymes with similar reactivity are explained by a single model. However, some experimental evidence exists to suggest that the primary co-ordination sphere of the alternative oxidase's di-iron centre may not be universally conserved [35,36].

Site-directed mutagenesis studies have revealed that E270 is a key residue with respect to activity of both the plant [35] and trypanosomal [36] alternative oxidase. Mutation of this glutamate to asparagine renders a plant enzyme that is almost completely inactive [35], whilst activity of the trypanosomal enzyme is fully abolished upon mutation to either alanine, leucine, asparagine or glutamine [36]. The oxidase was detected immunologically in both systems, demonstrating that the lack of activity was not because of impaired protein expression [35,36]. It was furthermore shown that the mutated *S. guttatum* alternative oxidase was correctly targeted to and

indeed exclusively incorporated in the mitochondrial inner membrane [35]. Such a result strongly suggests that the E270N mutation does not prevent the correct folding of the oxidase, as it is most unlikely that an unfolded protein would integrate successfully in its target membrane. Interestingly, upon mutation of E270 to aspartate or histidine, the trypanosomal alternative oxidase retains approximately 20% of its original activity [36]. It is tempting to interpret such observations in favour of an important role for E270, based on the rationale that aspartate and histidine are both residues that are theoretically capable of ligating iron, thereby being able to at least to some extent take over the function of the glutamate. Coincidence perhaps, but it is noteworthy that position 270 in the sequence of the chloroplast oxidase is occupied by a histidine residue [32]. Of course, we realise that it is difficult to ascertain that mutation of E270, due to its proximity, does not merely indirectly disturb the stability of the di-iron centre. Nevertheless, until further data have been gathered to conclusively clarify this matter, we are reluctant to exclude the possibility of species-specific nuances as to the precise ligation sphere of the active site of the various alternative oxidases.

### 3.3. Emerging clues regarding quinone binding

The structure of the quinone (Q)-binding site(s) of the alternative oxidase was probed by Berthold [37] who expressed a library of randomly mutated proteins in a haem-deficient *Escherichia coli* strain (cf. [38]) to screen for cells with an increased resistance to salicyl hydroxamic acid. Four single amino acid mutations were recovered (F259L, M263I, M263V and G347E) that are considered to reflect residues that are potentially involved in Q binding [37], based on the rationale that hydroxamic acids inhibit the alternative oxidase by interfering with this process [39]. More recently, Albury et al. [31] mutated Y253 (Fig. 1), a completely conserved residue previously implicated in Q binding [26], to phenylalanine and found that the mutant enzyme retains full activity. Preliminary results suggest that the Y253F mutant is marginally more sensitive to phenolic antagonists such as octyl-gallate and salicyl hydroxamic acid than the wild-type enzyme; the in situ kinetic behaviour of the respective enzymes with respect to the Q-redox poise, however, is indistinguishable (Crichton, P.G., Affourtit, C., Albury, M.S. and Moore, A.L., unpublished observations).

It may be evident from the previous paragraph that further experiments, preferably at a higher time resolution, are required to clarify the exact roles the mentioned residues play in Q binding. Interestingly, however, Y253 and particularly F259 and M263 are all relatively close to an almost fully conserved histidine-arginine pair (H261-R262, Fig. 1) identified by Fisher and Rich [40] who pointed out that such HR pairs form part of the two Q-binding sites of the bacterial reaction centre. In this respect, these authors furthermore discovered a very weakly conserved motif in the more amino-terminal region of the alternative oxidase, roughly between residues 135 and 150 [40]. This motif has a triad composition that may be generalised as 'aliphatic-(X)<sub>3</sub>-H-(X)<sub>2/3</sub>-(L/T/S)' and appears to be present in a variety of other enzymes that react with quinones [40]. Although no serious attempts have been made as yet to gain insight into the three-dimensional fold of the amino-terminal part of the alternative oxidase, it is not inconceivable that the protein folds in such a fashion that the triad motif residues arrive at a position that is proximate

to the region harbouring the mentioned HR pair and indeed to the putative di-iron centre.

## 4. Mechanism of the alternative oxidase

Experimental data that could provide direct insights into the catalytic mechanism of the alternative oxidase are scarce. Purely based on in organello steady-state kinetic measurements, two closely related mechanistic models were initially developed [41,42] with as main prediction that the oxidase is fully reduced by the sequential oxidation of two ubiquinol molecules before it reacts with oxygen. Given the reasonable probability that the alternative oxidase is a di-iron protein, such an order of events is unlikely, as it is hard to envisage how four electrons would be accommodated simultaneously by a single binuclear centre (cf. [25]). The steadily increasing relative wealth of information on the structure of reaction intermediates of other di-iron enzymes (see e.g. [27]) led to the proposal of two alternative catalytic models that both address this 'overload' aspect [13,30]. Although similar in nature, these models differ as to the exact sequence in which oxygen and quinol bind to the enzyme. Furthermore, the reaction mechanism suggested by Affourtit et al. [30] involves a high-valent diferryl intermediate, whereas Berthold and colleagues [13] questioned the catalytic necessity of a compound with such strong oxidising potential and hence did not include it in their model.

Over the last few years it has become apparent that many di-iron proteins, including stearoyl-ACP  $\Delta^9$ -desaturase [43], MMOH [44] and rubrerythrin [45], are capable of fully reducing oxygen to water as a side reaction to their main respective catalytic activities. Measurements of oxygen kinetic isotope effects have recently revealed that the initial dioxygen activation steps for both the oxygenase and oxidase reactions of MMOH are the same [46]. It was suggested, based on the observed competition between these reactions, that oxidase activity is the consequence of reduction of the diferryl MMOH intermediate 'Q' [46]. Such a mechanism provides a catalytic precedent for alternative oxidase activity as is illustrated in Fig. 2 (cycle I). The reaction cycle is in essence similar to that proposed previously [30], but is less assuming as to the nature of iron-ligating amino acids. Oxidation of one quinol molecule results in the reduction of the resting diferric iron centre. The resultant diferrous centre interacts with oxygen to initially establish an enzyme-dioxygen Michaelis complex, which subsequently leads to formation of a peroxide intermediate. Analogous to the suggested oxidation reaction of MMOH [46], reduction of oxygen occurs in two steps that are separated by a detectable diferryl compound. This high-valent species is reduced upon oxidation of a second quinol molecule, which completes the reaction cycle (Fig. 2, cycle I).

Recent work concerning the reaction of cytochrome *c* oxidase has highlighted that in general both haem and non-haem oxygen-metabolising enzymes rapidly reduce oxygen in one single step [47]. The generated oxidising equivalents are transferred to the enzyme for further processing [47]. Reaction cycles II–IV, shown in Fig. 2, represent potential alternative oxidase mechanisms that involve such a one-step oxygen reduction. In all suggested cases, an enzyme-dioxygen Michaelis complex is formed as described before. However, in contrast to mechanism I (Fig. 2), further reaction with dioxygen does not result in a peroxide intermediate, but rather in an enzyme



species where fully reduced, yet incompletely protonated oxygen atoms are bound to the di-iron site. The valency of the binuclear metal centre of this species depends on the source of the four electrons required for the oxygen reduction. All four electrons may be obtained either exclusively from the two ferrous iron atoms, yielding a diferryl centre (Fig. 2, cycle II), or in part from amino acid residues that are in close vicinity of the active site. In the latter case, one or two transient protein-derived radicals are formed together with, respectively, a divalent ferric-ferryl or a diferric iron site (Fig. 2, cycles III and IV, respectively). Following oxidation of a second quinol molecule, protonation of the oxygen atoms is completed, whilst the amino acid radical(s) and/or the iron atom(s) are reduced to their resting state.

Recent work by Albury and colleagues [31] has revealed that mutation of a fully conserved tyrosine (Y275, Fig. 1) to phenylalanine results in total loss of alternative oxidase activity, demonstrating that this residue is of mechanistic importance. The proximity of Y275 to the di-iron centre (it forms a putative hydrogen bond with a proposed iron-co-ordinating carboxylate [13]), would render this residue a site where an electron may be abstracted during enzyme turnover. The observation that enzyme activity is fully abolished upon removal of the hydroxyl moiety (i.e. Y275F) that is required for this process is in accordance with this suggestion. Involvement of a redox-active tyrosine in oxygen reduction is not without precedent, since it has been conclusively shown that a radical tyrosine residue forms an integral part of the structure of the catalytic cytochrome *c* oxidase intermediate 'P' [47]. It has furthermore been pointed out that a tyrosine residue is present at an analogous locus in the primary structure of rubrerythrin, a di-iron enzyme that also exhibits oxidising activity

[45]. The data obtained by Albury et al. [31] would therefore favour a mechanism that involves at least one protein-derived radical as illustrated by either cycle III or IV shown in Fig. 2. The consequence of a single protein-donated electron would be the occurrence of an EPR-detectable divalent ferric-ferryl species (Fig. 2, cycle III). The di-iron site of one of the reaction intermediates of the R2 subunit of ribonucleotide reductase has this valency [48]. If two electrons are protein-derived, an additional appropriate amino acid donor would be required (Fig. 2, cycle IV). Scrutiny of all currently available alternative oxidase sequences reveals that a universally conserved tryptophan residue (W206, Fig. 1) could be a potential candidate for such a role. Future site-directed mutagenesis of W206 should reveal whether or not this hypothesis is reasonable.

Finally, it is of interest to consider how pyruvate and indeed succinate would affect the mechanism of the alternative oxidase to increase its activity (cf. Section 2). Experiments to address this matter have yielded ambiguous results. Umbach and Siedow [15] demonstrated that, in isolated mitochondria, the kinetic behaviour of the alternative oxidase with respect to the Q-reduction level is altered by pyruvate such that activity is increased predominantly at relatively low reduction levels. Such data would suggest that pyruvate increases the apparent affinity of the oxidase for ubiquinol relative to its affinity for ubiquinone [15]. Using quinol analogues as artificial reducing substrate, however, Hoefnagel et al. [49] showed that pyruvate exclusively affects the  $V_{\max}$  of the oxidase. Based on this observation, pyruvate was considered to increase the proportion of active enzyme in the sample and hence was deemed a prerequisite for alternative oxidase activity [49]. To explain the apparent discrepancy between the single enzyme kinetic data

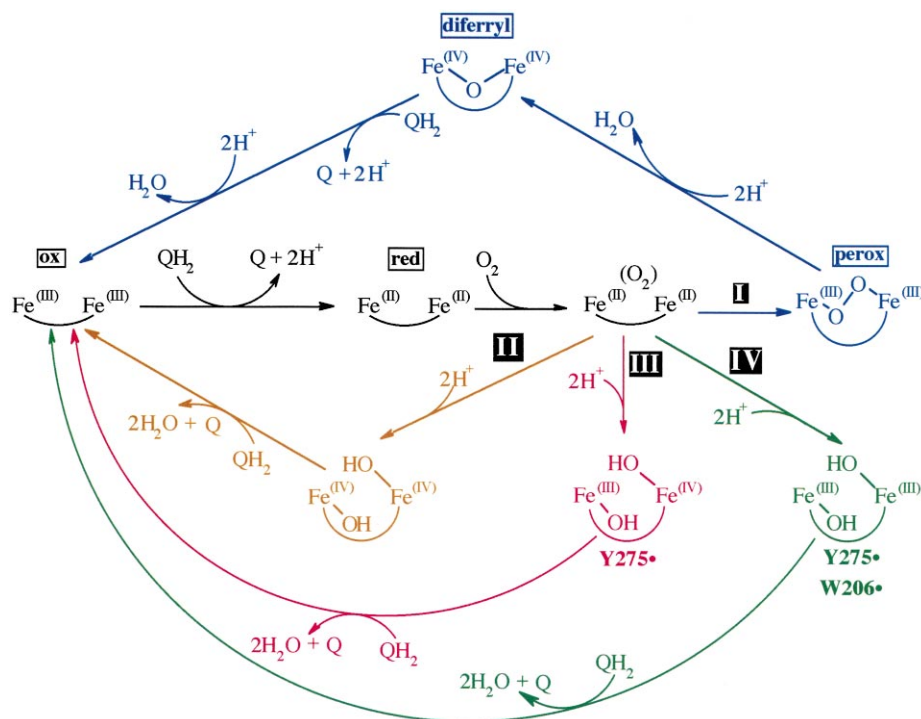


Fig. 2. Potential catalytic mechanisms of the alternative oxidase. The proposed reaction intermediates that are common to cycles I–IV are given in black. Mechanism I (blue) is based on that proposed for the oxidising activity of MMOH [46], whereas mechanisms II–IV (brown, red and green, respectively) are inspired by recent knowledge on cytochrome *c* oxidase that was reported in [47]. 'Ox', 'red', 'perox' and 'diferryl' respectively indicate oxidised, reduced, peroxide and diferryl reaction intermediates. See text for a detailed explanation.

[49] and those determined *in situ* [15], Hoefnagel et al. [49] suggested that pyruvate could affect the redox potential of the alternative oxidase or could reduce binding of oxidised product (Q), in both cases allowing higher enzyme activities at relatively low Q-reduction levels. It may be evident that it is indeed the *relative* affinity of the oxidase for quinol/quinone that would be affected in either case. It is the authors' current opinion that pyruvate has a dual effect on the alternative oxidase, respectively increasing  $V_{\max}$  and decreasing the apparent  $K_m$  of the enzyme for the Q-reduction level. In this respect it is interesting to note that the triad motif that is possibly involved in Q binding [40] is found in a region of the alternative oxidase's primary structure that is reasonably close to the site where activation occurs (cf. Sections 2 and 3.3).

## 5. Concluding remarks

In conclusion, it may be evident from the above discussion that despite difficulties in obtaining a stable purified protein, considerable progress has been made in the last decade as to the understanding of the molecular nature of alternative oxidase regulation and catalysis. The range of available site-directed mutant proteins in combination with the current development of more efficient bacterial over-expression systems should pave the way to thoroughly test and improve the current structural and mechanistic hypotheses.

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