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Review

Active site intermediates in the reduction of O_2 by cytochrome oxidase, and their derivatives☆,☆☆

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ABSTRACT

The mechanism of dioxygen activation and reduction in cell respiration, as catalysed by cytochrome *c* oxidase, has a long history. The work by Otto Warburg, David Keilin and Britton Chance defined the dioxygen-binding heme iron centre, viz. *das Atmungsferment*, or cytochrome a_3. Chance brought the field further in the mid-1970's by ingenious low-temperature studies that for the first time identified the primary enzyme-substrate (ES) Michaelis complex of cell respiration, the dioxygen adduct of heme a_3, which he termed Compound A. Further work using optical, resonance Raman, EPR, and other sophisticated spectroscopic techniques, some of which with microsecond time resolution, has brought us to the situation today, where major principles of how O_2 reduction occurs in respiration are well understood. Nonetheless, some questions have remained open, for example concerning the precise structures, catalytic roles, and spectroscopic properties of the breakdown products of Compound A that have been called P, F (for peroxy and ferryl), and O (oxidised). This nomenclature has been known to be inadequate for some time already, and an alternative will be suggested here. In addition, the multiple forms of P, F and O states have been confusing, a situation that we endeavour to help clarifying. The P and F states formed artificially by reacting cytochrome oxidase with hydrogen peroxide are especially scrutinised, and some novel interpretations will be given that may account for previously unexplained observations. This article is part of a Special Issue entitled: Respiratory Oxidases.

Work during the last 20 years has revealed that cytochrome *c* oxidase is actually member of a huge and quite diverse superfamily of enzymes, the respiratory heme–copper oxidases [1,2], the activity of which is responsible for more than 90% of the O_2 consumed by biological reactions on Earth. These enzymes are both structurally and functionally very different from the respiratory cytochrome *bd*-type oxidases [3], or the non-heme iron alternative oxidase of plants [4], which do not belong to the heme–copper superfamily. The heme–copper oxidases are related to one another not only by the theme of a heme–copper active site, but also by a low-spin heme next to it. Functionally, they are related by their property of conserving part of the free energy of the O_2 reduction reaction as a protonmotive force across the mitochondrial or bacterial membrane [5]. Our title refers to the classical "respiratory enzyme", i.e. Warburg's *Atmungsferment* [6], or Keilin's cytochrome oxidase [7], the latter a name originally given by Malcolm Dixon in 1929 [8] for the cyanide-sensitive enzyme that catalyses the oxidation of cytochrome (i.e. cytochrome *c*). Both heme groups in the mitochondrial enzyme are of type A (aa_3), which is distinguished from heme B (protoheme) by a formyl substituent instead of methyl in position 8 (in pyrrol ring A), and a long hydroxyethyl farnesyl chain instead of a vinyl group at position 2 (in pyrrol ring B). Even though there is a strictly conserved theme of a low spin heme *plus* a high spin heme/copper centre, the heme structures themselves vary among heme–copper oxidases from different prokaryotes. Thus heme B, or the more recently discovered heme O, which differs from heme A by having a methyl group in place of the formyl [9,10], may be represented instead of, or in addition to, heme A. Two other features of the binuclear site are fully conserved (for details, see below), namely the copper ion (called Cu_B) on the distal side of heme a_3, and with three histidine ligands plus a fourth hydroxy or aquo ligand in some states, and a tyrosine residue covalently bonded to one of the histidine ligands.

Here, our current view of the mechanism of O_2 reduction by cytochrome *c* oxidase (COX), will be summarised with special emphasis on the structural, functional and spectroscopic properties of the intermediates of this reaction called P, F and O, which have been experimentally produced in a number of different ways, both natural and artificial.

1. The catalytic cycle

The main concepts of the catalytic cycle of COX (Fig. 1 shows our current view) started to emerge in the middle 1970's after

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☆☆ If not mentioned otherwise, the numbering of amino acids is based on the sequence of subunit I of cytochrome *c* oxidase from bovine heart mitochondria.

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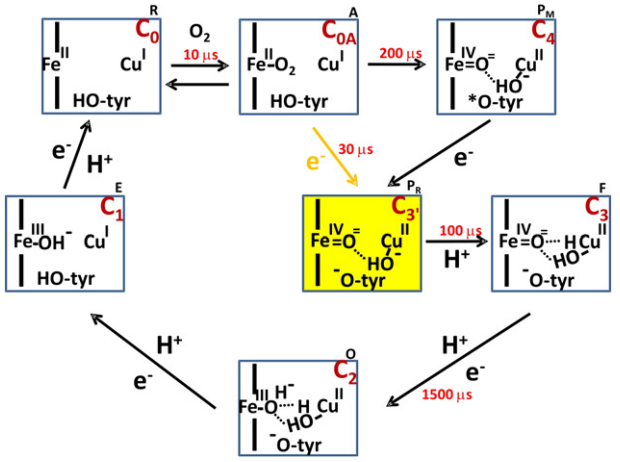

Fig. 1. Catalytic cycle of cytochrome *c* oxidase. The rectangle represents the binuclear site, which includes heme a_3 (Fe), Cu_B, and the cross-linked tyrosine (HO-tyr). The three histidine ligands of Cu_B and the proximal histidine of heme a_3 are omitted for clarity. Note that bonds are not shown between oxygen and hydrogen in water, hydroxide and phenolic OH, and that dotted lines indicate hydrogen bonds. A new nomenclature (see text) is introduced in the upper right corner, the old nomenclature is outside the rectangle. Approximate time constants (1/k; room temperature) are given in red for the reaction of COX with ~1 mM O_2; these vary somewhat depending on the source of COX. The fully reduced enzyme (Cu_A and heme *a* reduced) takes the path indicated by the yellow arrow, where an electron from heme *a* is transferred to the binuclear site in conjunction with scission of the O–O bond, yielding state **$C_{3'}$** (P_R) marked in yellow. If only the binuclear site is reduced (mixed valence enzyme), the reaction with O_2 goes instead to state **C_4** (P_M), where the fourth electron has been taken from the tyrosine. Note that the protons indicated at reaction arrows are "substrate" protons to be consumed in formation of water from reduced dioxygen. The steps where water leaves the site are not known exactly, but the scheme implies the steps **$C_2 \rightarrow C_1$** and **$C_1 \rightarrow C_0$**, albeit not shown explicitly. Proton pumping is not depicted. Hydrogen bonding from a hydroxide (**C_4**, **$C_{3'}$**) or water ligand (**C_3**, **C_2**) of Cu_B to the oxo or hydroxy ligand of the heme is depicted schematically (dotted). Note also that the strong hydrogen bonding to the hydroxide-ligated heme in **C_2** keeps the ferric iron in a high spin state, slightly proximally displaced from the porphyrin plane.

demonstration of the primary dioxygen adduct (Compound A) at cryogenic temperatures by Chance et al. [11], as confirmed at room temperature both by Raman [12–14] and optical spectroscopy [15]. The structure of the subsequent P state(s) was less clear. Wikström [16] had proposed it to be a ferric–cupric-**p**eroxy state (hence the P nomenclature) of the heme–copper site, because it could be formed by a *two-electron oxidation* of the ferric–cupric enzyme (plus water), i.e. by reversal of part of the cycle driven by ATP hydrolysis in mitochondria, *via* generation of protonmotive force and reversal of the proton-pumping function of COX. Moreover, the P (P_M) state is spectroscopically identical to Chance's Compound C [11], that was formed at low temperatures in the reaction between (two-electron reduced) "mixed-valence" enzyme and O_2, although Chance et al. did not consider it a catalytic intermediate at the time. Also, at that time the reaction of the fully reduced COX with O_2 had not yet revealed a P intermediate (P_R, see below). The fact that P could also be formed by reacting the ferric/cupric enzyme with hydrogen peroxide at high pH ([17], and see below), or by a controlled reaction of the "mixed-valence" enzyme (with only the binuclear site reduced; state **C_0** (R) in Fig. 1) with O_2 at room temperature [18], seemed both to support the "peroxy" assignment. Only after the important lead by Weng and Baker [19] in 1991, suggesting that the O–O bond is already broken in the P state and that the heme may be in the ferryl form, was this indeed proven to be the case by the elegant resonance Raman experiments of Kitagawa and Ogura and their coworkers [20,21], by Babcock et al. [22], and by an ingenious dioxygen labelling experiment by Fabian et al. [23]. A ferryl heme structure for the P (P_M) state could thus be definitely established (**C_4** in Fig. 1). This was an important achievement, because it indicated that the bound dioxygen is reduced to the level of water in a single reaction step, without experimentally observable intermediates. The entire oxidising power of O_2 is transferred to the enzyme's active site in this apparently single reaction. However, this finding also created a problem, namely how to account for "the fourth electron equivalent" required in O_2 reduction to water. In Fig. 1 it is accounted for by formation of a neutral tyrosine radical, for which there is ample evidence, as we shall see.

The state that we now call **C_3** (or F) was probably first observed by Erecinska et al. [24] in ferricyanide-oxidised pigeon-heart mitochondria treated with ATP. It was described as a high-energy form of COX in which heme a_3 had undergone a high to low spin transition. This species was later found to be the product of energy-linked *single electron oxidation* of the binuclear ferric–cupric site (plus water) and was assigned as a ferryl–cupric state of the binuclear centre [16] (state **C_3**, Fig. 1). An equivalent state could also be produced by treatment of COX with an excess of hydrogen peroxide (see [18,19], and below). The Raman data by Babcock, Kitagawa and Rousseau, and their collaborators [20,21,25–27], supported the notion that this state has indeed also a ferryl heme a_3 structure, but with a Raman Fe=O stretching frequency ($\nu_{Fe=O}$) at ~785 cm^{-1} rather than at ~804 cm^{-1}, as found for the P state. Incidentally, it may be worth commenting here that the commonly used term oxo-ferryl or ferryl-oxo, or their equivalent, for the heme structure Fe[IV]=O is a tautology, because the term "ferryl" was originally coined by Philip George in 1951 precisely for that structure (see [28]).

The ferric/cupric **C_2** state (state O) that results from uptake of the next electron (and a proton) into state **C_3** is at the same reduction level as cytochrome *c* oxidase as isolated. However, there is a lot of evidence to suggest that the isolated enzyme is probably never in the same state as the **C_2** redox state during catalysis, but that in the latter the binuclear centre attains a metastable high-energy configuration, the nature of which is still enigmatic (see Section 6 and [5,29,30] for recent reviews on this topic). Using resonance Raman spectroscopy, Rousseau et al. [25,26] were the first to show that the ferryl state **C_3** (F) is followed by a state in the reaction of reduced COX with O_2, where ferric heme a_3 has a hydroxide ligand. This is an interesting constellation, because it is one of very few cases of a high-spin ferric heme–hydroxide complex, which is made possible only by considerable weakening of the Fe–OH bond (as seen by the low Raman frequency) by strong H-bonding to the –OH group from an adjacent residue [26,31], which is here proposed to be the aquo ligand of Cu_B (Fig. 1). The structure of **C_2** in Figs. 1 and 2, or its close relative, is proposed to be the metastable and catalytically active form of the binuclear site at this redox level. There is good evidence from electron injection experiments (see [30] for a review) that it is Cu_B that is reduced in the subsequent reaction step that yields the **C_1** state (Fig. 1).

Since none of the intermediates of type P (see below for distinction between P_M and P_R) actually has a peroxide ligand in the active site, it may be warranted to revise the current nomenclature. Wikström and Verkhovsky [29] tentatively proposed a "C state" nomenclature, by analogy to the "S state" nomenclature of the water-splitting $CaMn_4$ centre of photosystem II [32], an idea originally proposed by Todd P. Silverstein (see also [33]). In this nomenclature (Fig. 1), the subscript describes the number of oxidising equivalents in the binuclear heme–copper site. Chance's Compound A (**C_{0A}** in Fig. 1) is an exception in two ways. First, in this case the number of oxidising equivalents due to the O_2 bound to the site is not counted. Secondly, in this oxygen adduct there is considerable charge transfer from heme iron to the dioxygen ligand, so that a ferric-superoxide structure (**C_{1A}**) may be a more appropriate description. However, we prefer Chance's original annotation for historical reasons, as well as to stress the effective reversibility of O_2 binding to the binuclear site [11,34]. Another possible nomenclature has been proposed by Konstantinov [35], who pointed out the homology of states **C_4** (P_M)

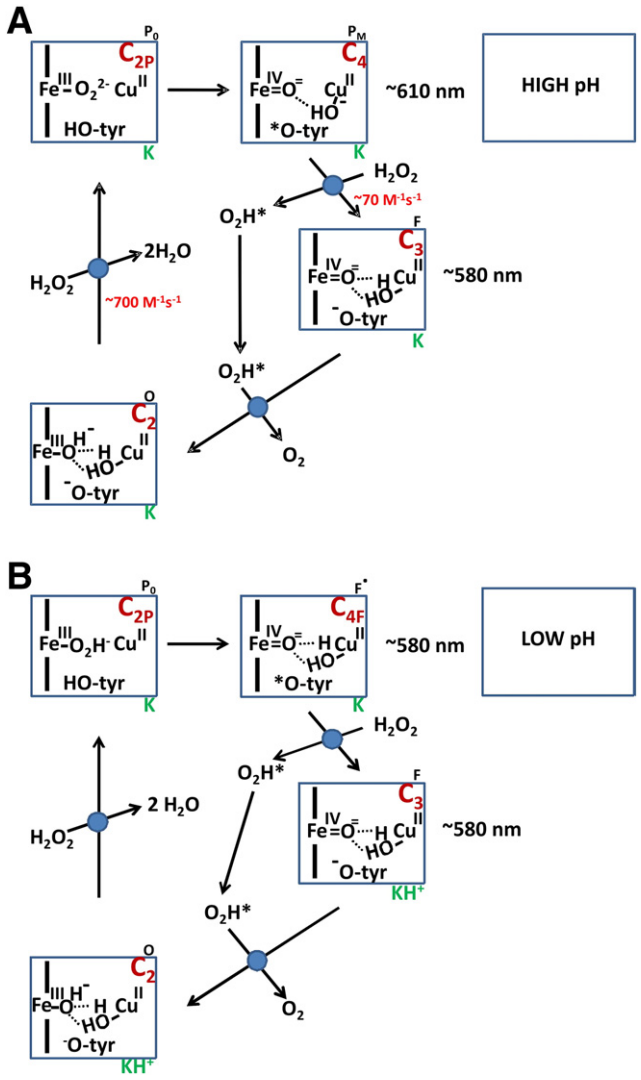

Fig. 2. The hydrogen peroxide cycle. Tentative scheme of how hydrogen peroxide reacts with ferric/cupric (state $\mathbf{C_2}$) cytochrome *c* oxidase at high (A) and at low (B) pH. The rectangle represents the binuclear site (see Fig. 1). The green K below the lower right corner represents the lysine that has given the proton-transferring K-channel its name (lysine 319 in subunit I of the bovine heart enzyme). Panel A includes approximate second order rate constants (in red) from the literature cited (see text).

and $\mathbf{C_3}$ (F) (Fig. 1) to the classical Compounds I and II of peroxidases and catalases, as had Weng and Baker [19].

2. States P_M and P_R

Some confusion has arisen from the fact that a state with an optical spectrum identical to the $\mathbf{C_4}$ state (P_M) described above is also formed when *fully reduced* COX reacts with O_2, viz. as the intermediate following the initial state $\mathbf{C_{0A}}$ (A) [36,37]. Differently from above, in this case the O–O bond splitting reaction is accompanied by transfer of the electron in heme *a* to the binuclear site (Fig. 1, yellow arrow; state $\mathbf{C_{3'}}$ or P_R), which thus becomes isoelectronic with intermediate $\mathbf{C_3}$ (F). It is indeed remarkable that states $\mathbf{C_4}$ (P_M) and $\mathbf{C_{3'}}$ (P_R, from fully **r**educed enzyme) have identical optical spectra, considering the additional electron in the binuclear site in the latter state. Moreover, the resonance Raman Fe═O signature of $\mathbf{C_{3'}}$ (P_R) is the same as for $\mathbf{C_4}$ (P_M) [20–22], proving the ferryl structure of the heme in both. As already mentioned, it is generally thought that the fourth oxidising equivalent of $\mathbf{C_4}$ resides in the fully conserved tyrosine residue in the active site that is covalently bonded to one of the three histidine ligands of Cu_B forming a neutral radical [30,38,39] (see below).

$\mathbf{C_4}$ (P_M) *minus* $\mathbf{C_2}$ (O) difference FTIR spectra of isotopically labelled COX from *P. denitrificans* [40] showed a band at 1519 cm^{-1} present in $\mathbf{C_4}$, but not in $\mathbf{C_3}$ or $\mathbf{C_2}$, and was assigned as due to the neutral tyrosine radical, whereas a trough near 1311 cm^{-1} was interpreted as due to a tyrosinate anion present in the $\mathbf{C_2}$ state but absent in $\mathbf{C_4}$. Later FTIR work [41,42] showed that a similar component absorbing at 1308 cm^{-1} was formed on the transition from $\mathbf{C_0}$ (R) to $\mathbf{C_{3'}}$ (P_R), and was retained in state $\mathbf{C_3}$ (F), and partially in $\mathbf{C_2}$ (O). This provided evidence for deprotonation of the tyrosine during splitting of the O–O bond, and since the 1308–1311 cm^{-1} band is lacking in the $\mathbf{C_4}$ state, this data indirectly supports the notion of a neutral tyrosine radical in this state. Cu_B is EPR-silent (as is the putative tyrosine radical) in state $\mathbf{C_4}$ presumably due to magnetic interactions, but is definitely cupric in $\mathbf{C_{3'}}$ (P_R), as shown despite magnetic coupling to the ferryl iron by a fortuitous and unusual EPR signal in this state [43]. Altogether, we may thus conclude that the identical optical spectra for states $\mathbf{C_4}$ and $\mathbf{C_{3'}}$ find a quite natural explanation, as the extra electron in $\mathbf{C_{3'}}$ has no effect on the heme macrocycle.

It might seem unlikely that cytochrome oxidase would ever become fully reduced in physiological aerobic steady states, and that inclusion of state $\mathbf{C_{3'}}$ (P_R) in the catalytic cycle may therefore seem mistaken. However, we have included $\mathbf{C_{3'}}$ in Fig. 1 (yellow) for two reasons. First, even in normoxic tissues the local O_2 concentration may be two orders of magnitude lower than in air-saturated media [44], and even lower concentrations may be envisaged in hypoxia, especially for the cells furthermost from the nearest capillary. Transient full reduction of cytochrome oxidase may well occur under such conditions. Secondly, it is quite possible, even during normoxic turnover, that $\mathbf{C_{3'}}$ is a true transient intermediate between $\mathbf{C_4}$ (P_M) and $\mathbf{C_3}$, in a sequence $\mathbf{C_0 \rightarrow C_{0A} \rightarrow C_4 \rightarrow C_{3'} \rightarrow C_3}$ (Fig. 1, black arrows), even though this has not been demonstrated explicitly. At any rate, state $\mathbf{C_{3'}}$ (P_R) is of considerable interest as such, because it is the only relatively stable state of the bimetallic site where the charge of the extra electron has not apparently been neutralised. $\mathbf{C_{3'}}$ thus appears to be one equivalent more negative than all other states depicted in Fig. 1, which is of particular interest with respect to the mechanism of proton pumping [30,43], and because it seemingly violates the electroneutrality principle of Peter Rich [45]. As discussed elsewhere [30], no such violation actually occurs, but the extra negative charge of the binuclear centre drives a proton into the so-called proton-loading site, before completion of the proton-pumping mechanism.

3. On the nature and origin of the 580 and 607 nm absorption bands

As much as it may have seemed odd that the optical spectra of states $\mathbf{C_4}$ and $\mathbf{C_{3'}}$ are the same (but see above), it may seem especially strange that the optical spectra of $\mathbf{C_{3'}}$ (P_R) and $\mathbf{C_3}$ (F) are very different, considering that the binuclear centre has the same overall electronic composition and that the heme (which is by far the major contributor to the optical spectrum) is in the ferryl state in both species. It is important to note, however, that the large spectral difference is limited to the α- (or Q_O) band, whereas the γ- (Soret or B) band is virtually the same [18,19]. States $\mathbf{C_{3'}}$ (P_R) and $\mathbf{C_4}$ (P_M) are characterised by a sharp Q_O band at ~607–610 nm and a Q_V (β) band at ~570 nm in the difference spectrum, whereas the corresponding bands of $\mathbf{C_3}$ (F) have maxima at ~580 and ~535 nm, with the Q_O band much less intense and broader than for the P states [16,18,46,47].

The intensity of the Q_O band is especially sensitive to the axial iron ligands and other structural alterations that affect the axial symmetry of the heme [48,49]. On excitation, the two π to π* transition dipoles (a_{1u} to e_g and a_{2u} to e_g) add to produce the high intensity Soret band, but subtract for the α-band. If the heme were perfectly symmetric

around the axis through the Fe perpendicular to the porphyrin plane, the intensity of the α-band would vanish but the Soret band would remain strong. Any deviation from such axial symmetry, such as e.g. binding of O_2 or CO to heme iron, will therefore make the α-band sharper and more intense without appreciable effect on the Q_V (β) band. This effect, in which the Q_O and Q_V bands separate into two from having been overlapping in the unliganded case, is especially pronounced in single crystal spectra of, for example, deoxy- and oxy-myoglobin, but is also evident in the respective solution spectra [48].

In state **C_3** the binuclear site has one more proton than in **$C_{3'}$** (see e.g. [30,35,38,50]). The additional proton cannot reside on the tyrosine (see FTIR data, Section 2), and has been suggested to cause protonation of the oxygenous Cu_B ligand to yield water from OH^- [30,50]. Molecular modelling outgoing from the available crystal structures (e.g.[51–53]) suggest that the fourth oxygenous Cu_B ligand, identified by both EXAFS and ENDOR spectroscopy [54], can easily interact with the distal oxo ligand of the ferryl heme, and may therefore perturb the heme's axial symmetry and hence the optical spectrum [18,43], as discussed above. A hydroxo ligand on Cu_B[II] may donate a hydrogen bond to the oxo ligand of the ferryl heme causing such asymmetry, and a sharp and intense Q_O band. Resonance Raman work has indeed provided evidence for H-bonding to the heme oxo group in the **C_4** state [55,56]. By contrast, an aquo ligand of Cu_B in the **C_3** state (and in state F·, see below) can obviously provide more axially symmetric H-bonding to the ferryl oxo group, thus resulting in a less intense Q_O band at ~580 nm. The asymmetric H-bonding to the oxo group in the **C_4** and **$C_{3'}$** states could furthermore distort the Fe═O axis not to be perpendicular to the heme plane, whereas the copper-bonded water molecule in **C_3** may be expected to keep the Fe═O axis more closely perpendicular, resulting therefore in a more axially symmetric structure and a less intense α-band.

Another explanation relates to especially the a_{2u} to e_g excitation being associated with displacement of electrons in the heme plane towards the perifery (see e.g. [49]). The strong H-bonding to the distal oxo group in **C_3**, relative to **C_4** and **$C_{3'}$**, brings more positive charge to the centre of the porphyrin ring, and is thus expected to raise the energy of the excitation with shift of the absorption maximum from ~610 to ~580 nm. A further explanation for the much lower molar absorptivity of the 580 nm peak could be due to it being very broad [22]. Thus the distal heme pocket in **C_3** might be a mixture of several slightly dissimilar structures, whilst **C_4** and **$C_{3'}$** have a more homogeneous structure.

4. Artificial P and F states

As we have already seen, the ferryl/cupric binuclear site can attain a number of different states, some of which have been observed during catalysis (see above), whereas others have been described under artificial conditions. In the higher oxidation state it is thought that the cross-linked tyrosine in the site forms a neutral radical, the **C_4** (P_M) state being the prototype (Fig. 1) with λ_{max} near 610 nm in the difference spectrum. However, adding stoichiometric amounts of hydrogen peroxide to oxidised COX at low pH creates the so-called F· (F dot, or F', or "fast F") state [18,47,57,58] with an optical spectrum reminiscent of **C_3** (F) (λ_{max} near 580 nm) even though it is isoelectronic with **C_4** (λ_{max} near 610 nm). **C_4** is formed by hydrogen peroxide treatment of oxidised COX under otherwise the same conditions, but at high pH [17,18,45,57,58]. The different effects of hydrogen peroxide at different pH could thus again be interpreted as being due to a fourth aquo (**C_3**) versus hydroxo (**C_4**) ligand of Cu_B[II], as discussed above. Conversely, the lower oxidation state with the **C_3** (F) state as the prototype, and where the tyrosine is in the anionic form based on FTIR data (see above), has the other variant, **$C_{3'}$** (P_R), with a different absorption spectrum. As already discussed above, these two may again differ by the protonation state of the oxygenous ligand of Cu_B[II] (Fig. 1).

Fabian and Palmer [18] prepared the P and F intermediates by careful oxidation of mixed valence COX with O_2, and by H_2O_2 treatment, respectively. Surprisingly, the enzyme that was in the P state as judged from the optical spectrum (λ_{max} ~610 nm) nevertheless titrated ~1.3 equivalents more oxidised than state **C_2** (O), whereas enzyme in state F titrated ~1 equivalent more oxidised, as expected. As discussed by these authors, the **C_4** (P_M) state with its typical 610 nm band most likely dissipates to **C_3** within a few minutes *without a change in the optical absorption spectrum*, probably due to migration of the radical at the cross-linked tyrosine, and if so, with formation of state **$C_{3'}$** (or P_R; Fig. 1). As already discussed, the latter state is, of course, also a well-known intermediate in the reaction of fully reduced COX with O_2, but in that case the radical at the cross-linked tyrosine presumably never forms. Instead, the fourth electron required for O – O bond splitting is rapidly transferred from the low-spin heme *a* during the ~30 μs process in which the electron tunnelling between the hemes (~1 ns) is not rate-limiting ([36,37]; Fig. 1).

4.1. The problem with the pH-effect on peroxide reactivity…

From the above properties it thus seems clear that low pH, or explicit proton uptake, causes a transition from the 610 nm species to the 580 nm species in the above cases, independently of oxidation state. However, the situation is more complicated because it has been shown that production of F· by adding hydrogen peroxide to enzyme in the **C_2** (O) state at low pH is not associated with uptake (or release) of protons [59,60]. Formation of the **C_4** (P) state from **C_2** (O) with hydrogen peroxide at high pH is also not accompanied by proton uptake, whereas formation of P from O using carbon monoxide as reductant and O_2 bubbling is linked to uptake of two protons [61]. For this reason it was deduced that the additional proton in F· is present already in the O state, prior to the reaction with H_2O_2. Thus state **C_2** (O) might exist in a protonated and a deprotonated form, distinguished by the protonation state of a residue with an apparent pK_a of 6.5–7 [45,47,57].

Pecoraro et al. [58] made the important observation that the effect of pH in generating either the ~610 nm P (at high pH) or the ~580 nm F· state (at low pH), when enzyme in state **C_2** (O) reacts with H_2O_2, is abolished in a structural variant of COX where the key lysine residue of the proton-conducting K-pathway is mutated to a methionine. The K pathway leads from the negatively charged *N*-side of the membrane to the binuclear site at the position of the cross-linked tyrosine [51–53], and has been envisaged as the path for uptake of two of the four substrate protons required for reduction of O_2 to water at the active site [62], i.e. the two protons taken up coupled to reduction of the binuclear site in **$C_2 \rightarrow C_0$**. In pioneering work, Vygodina and Konstantinov [57] had indeed already shown that the pH-dependence of the reaction of COX with peroxide is exerted from the *N*-side of the membrane. In the lysine/methionine variant, hydrogen peroxide always produced a P state (λ_{max} ~607 nm), i.e. *irrespective of pH*, suggesting in agreement with the above, that the proton required to form F· is taken up from the *N*-side of the membrane via the K-channel [58]. However, it was also noted that even if the lysine/methionine variant was incubated at low pH for 5 min, the phenotype still remained the same, still forming the P state, which seemed to exclude the possibility that the reaction pathway would depend on the protonation state of **C_2** (i.e. the state *before* H_2O_2 addition), as had been anticipated [58]. The authors therefore proposed that uptake of the proton via the K-pathway to form state F· would have to occur during or after the binding of H_2O_2 [58], but this disagrees with the direct measurements which, as already mentioned, definitely showed lack of such proton uptake [59,60]. Obviously, there is a serious dilemma in understanding the basis for the pH-dependence.

The reason why even a 5 min incubation at low pH failed to produce the 580 nm species on hydrogen peroxide addition to the lysine/methionine variant [58] could stem from a change in the pK_a of some critical groups caused by the mutation. Electrostatic calculations have indeed shown that a significant fraction of the K-channel lysine may be protonated at pH 7 [63]. However, removing this positive charge by mutating the lysine to methionine would a priori be expected to *increase* the pK_a of neighbouring protolytic residues, thus favouring their *protonated* forms, not vice versa.

4.2. …and its solution

The solution to this seemingly difficult problem may be quite simple, and it is possibly unique. Enzyme in state **C_2** may indeed contain an extra proton on the average at low pH, and *that proton may reside on the lysine in the K-pathway*. As shown by electrostatic calculations, the pH-dependence of the protonated state of the lysine is quite broad due to interactions with other residues, mainly a neighbouring glutamic acid [63]. As depicted schematically in Fig. 2A,B, the proton on this lysine will participate in the reactions of the binuclear site with hydrogen peroxide, in agreement with the known proton transfer function of the K-channel, which leads from the *N*-side (and the lysine) to the cross-linked tyrosine [51–53]. Thus, it is suggested that the primary product of the reaction with hydrogen peroxide will be the peroxide dianion at high pH (Fig. 2A), but the hydroperoxide anion at low pH (Fig. 2B). After the subsequent O – O bond splitting reaction, the aquo or hydroxy ligand of Cu_B will determine whether the 580 nm (F·) or the 607 nm species (P) is formed, as already discussed.

Of course, a different sequence of events is possible at low pH than that shown in Fig. 2B. The proton on the lysine may be transferred later, i.e. to preformed Cu[II] – OH^-. However, from a structural viewpoint it seems more likely that the peroxide dianion is formed initially also at low pH (not shown in Fig. 2B), followed by its protonation by lysine via the tyrosine. The protonated lysine may be thought to lower the pK_a of the tyrosine by electrostatic interaction.

Due to the broad pH-dependence of the occupancy of the protonated lysine [63], both spectral forms will be present over a wide pH range, as is well known, even though F· dominates at low pH and P at high pH. Thus, in reality, the situation is always a mixture of the extremes as shown in Fig. 2A and B. Finally, in this explanation it is obvious why mutation of the lysine to methionine leads to formation of the P state by hydrogen peroxide also at low pH.

4.3. The hydrogen peroxide cycle

Fig. 2 (A,B) summarises our current view of the major reaction sequence when hydrogen peroxide reacts with cytochrome *c* oxidase at high and low pH. As suggested in early work (e.g. [47,50,57,58,60]) the primary reaction yields a peroxy adduct that in our scheme has either bound peroxide dianion (Fig. 2A) or hydroperoxide anion (Fig. 2B) depending on the pH-dependent initial state. This primary peroxy adduct is hypothetical insofar as it has not been experimentally observed, possibly because its formation is much slower than its decomposition (kinetic effect), or because it lies at a high energy level (thermodynamic effect). The next step is cleavage of the O – O bond, which is much like it is known to occur in the catalytic cycle (Fig. 1), yielding ferryl heme and a neutral tyrosinyl radical, as discussed above. Depending on the initial pH, the fourth Cu_B ligand is either a hydroxyl ion or water, and this determines the optical spectrum of the heme in the Q band, as already discussed (Section 3). So far, the added hydrogen peroxide has acted as an oxidant being itself reduced to two molecules of water. As also proposed originally (see above), the next step is a reaction with a second molecule of hydrogen peroxide, which now acts as a reductant. The product is the **C_3** (F) state in which the cross-linked tyrosine is deprotonated based on FTIR data [40–42]. The **C_4** to **C_3** transition of the catalytic cycle is known to be linked to uptake of a proton. When, at low pH, the oxygenous Cu_B ligand is already protonated in state **C_4**, the proton is bound to the K-channel lysine (Fig. 2B). The novel suggestion here is that hydrogen peroxide donates both an electron and a proton in this reaction, forming a local neutral perhydroxyl radical, which then serves as reductant in the subsequent reaction step **$C_3 \rightarrow C_2$**. In this way, the overall reaction scheme (Fig. 2) predicts catalase activity, which is indeed a property of cytochrome *c* oxidase [64,65]. Konstantinov et al. [60] reported superoxide generation from hydrogen peroxide as catalysed by COX. In view of the scheme in Fig. 2, it is quite possible that a fraction of the produced hydroperoxyl radical (i.e. the protonated superoxide; $pK_a \sim 4.9$) escapes into the surrounding medium, where it would readily dissociate to superoxide and a proton (see also below). It is also possible that superoxide rather than hydroperoxyl radical is formed in the **C_4** to **C_3** step with release of two protons. After that, one proton is taken up into the lysine via the K-pathway in **C_3**, and the other is taken up on reduction of **C_3** to **C_2** by the previously formed superoxide, again in agreement with the observation of no net proton uptake or release during the reaction [59,60]. As a matter of fact, we do not have enough information to know exactly at what point the lysine becomes reprotonated.

4.4. Properties of hydrogen peroxide-induced states

Recently, von der Hocht et al. [65] described yet another P state (λ_{max} near 610 nm) that replaced the F state (λ_{max} near 580 nm) at high pH when 20 mM ammonia was added in the presence of excess hydrogen peroxide. This state (called P_N) was interpreted to have ammonia as the fourth ligand of Cu_B[II], replacing OH^- (or water). The finding was presented as a contradiction to the order of events in the natural catalytic cycle (Fig. 1): "The formation of this previously undescribed P-like state from the F state appears to be not compatible with the usual forward direction of the catalytic cycle" [65].

We emphasise that adding an excess of hydrogen peroxide to the oxidised enzyme, as in [65], creates a cyclic steady state situation, as described in Fig. 2A,B, in which the **C_3** (F) state normally dominates [18,19,35,47,57–60]. In such conditions catalase activity is observed, i.e. dismutation of hydrogen peroxide to water and O_2 [64,65], and formation of superoxide has been detected [60]. In their truly informative paper, Weng and Baker [19] showed that both the formation of **C_4** (P_M) from **C_2** (O) and the decay of **C_4** to **C_3** (F) are bimolecular reactions with hydrogen peroxide as the second reactant. In the reaction schemes proposed so far, also the final conversion of **C_3** back to **C_2** has been considered to be a bimolecular reaction with hydrogen peroxide (see e.g. [18,19,35,47,57,58]). Whilst we cannot exclude this possibility, we suggested the simpler alternative shown in Fig. 2A,B, but both routes might in fact well exist in parallel. The dominance of the **C_3** state (580 nm) in the presence of an excess of H_2O_2 is a kinetic property of the cycle, and thus, for example, any perturbance that will sufficiently slow down the $\mathbf{C_4} + H_2O_2 \rightarrow \mathbf{C_3} + O_2H^*$ reaction step will be observed as an apparent "conversion of F to P", especially at high pH (see above), even though no such direct conversion has actually occurred.

Ammonia might promote the 580 to 610 nm transition by replacing the oxygenous ligand of Cu_B, as proposed [65]. According to our notion of the origin of the 580/610 nm spectral difference (see Section 3), hydrogen-bonding from the copper-bound ammonia to the heme oxo group would then exert a distorsion from axial symmetry that is significantly larger than for a bound water ligand. As this seems unlikely to us, a perhaps more likely explanation is that ammonia at the high concentrations used (~20 mM) blocks access of hydrogen peroxide into the active site by occupying the ligand-conduction channel that has previously been identified in the structure [66,67]. If this were the case, the local effective hydrogen peroxide concentration at the binuclear site would be lowered,

whereby the 610 nm species would be favoured over the 580 nm compound in the steady state. At any rate, the observed spectral conversion caused by ammonia at high pH in the presence of hydrogen peroxide is in our view fully compatible with the usual forward direction of the catalytic cycle.

5. Free radicals

The proposal of a neutral radical at the cross-linked tyrosine in the **C_4** state (P_M) was to our knowledge first suggested in [68] and in more detail in [22], and was supported experimentally by Proshlyakov et al. [69], who were able to label it with radioactive iodide. The cross-linked his-tyr fragment was the only protein material labelled, and such protein labelling was absent in the **C_3** (F) and **C_2** (O) states. We find this to be quite compelling evidence for a radical at the cross-linked tyrosine in **C_4**, despite the small observed labelling yield, considering the multitude of reasons for low yield in such an experiment. However, the **C_4** intermediate, as prepared with O_2 from mixed-valence enzyme, does not exhibit an EPR-detectable tyrosine radical, which has been ascribed to magnetic interactions with the nearby ferryl iron and cupric copper paramagnets. In contrast, generation of **C_4** by treatment with hydrogen peroxide has been found to be associated with production of free radicals, suggested to derive from either tyrosine or tryptophan residues (see [70] for a recent comprehensive review). Recent EPR work has identified the two observed radicals formed with H_2O_2 treatment as the cross-linked tyrosine [70] and tyrosine-129* [70–73]. Wiertz et al. [74] studied the reaction between fully reduced *Paracoccus* enzyme and O_2 by a rapid freeze-quench technique, and described a radical attributed to tryptophan at the **C_4** stage, but in their subsequent work [72, 75] the tryptophan radical (ascribed to W-236*, or W-272 in the *Paracoccus* enzyme) was reported to occur during a later stage of the reaction, roughly at the same time as the **C_3** (F) intermediate. By contrast, the recent work by Yu et al. [70], studying the reaction of fully reduced bovine COX with O_2, and also employing ultrafast quenching, only recorded an ascorbyl or a sulphur dioxide anion radical ($SO_2\cdot^-$), depending on whether the enzyme was pre-reduced by ascorbate or dithionite, but no protein-based radicals. In fact, based on our scheme in Fig. 1, free radicals with any significant occupancy would not be expected in the reaction of fully reduced COX with O_2, which proceeds directly from **C_{0A}** to **C_3**, and **C_3**.

Tryptophan 236* is completely conserved among all heme–copper oxidases, and lies parallel and π-stacked to one of the three histidine ligands of Cu_B. MacMillan et al. [76] demonstrated that mutations of this tryptophan prevented formation of the **C_4** and **C_3** states with hydrogen peroxide, and also prevented formation of the associated EPR-detectable radical from tyrosine 129, which accepts a hydrogen bond from the tryptophan. Consequently, tryptophan 236 has been proposed to be involved in a radical transfer process, either being itself the primary radical site in **C_4** [76], or being an intermediate in a radical transfer chain initiated at the cross-linked tyrosine [70]. MacMillan et al. [76] discussed why the tyrosine 129 radical is observed after hydrogen peroxide treatment, but not in the **C_4** state created by reacting the mixed valence enzyme with O_2, and ascribed the difference to the two protons carried into the active site with H_2O_2. However, relative to the **C_2** state reacting with H_2O_2, its prior reduction to state **C_o**, the species that reacts with O_2, is also associated with uptake of two protons [61]. Taken together, recent FTIR data [40–42] provided evidence for the notion that the cross-linked tyrosine donates the proton required in the reaction **$C_{oA} \rightarrow C_4$**. If trp 236 would provide the primary electron in this reaction, the structure would end up with a positive charge at the tryptophan and a negative charge at the tyrosine, i.e. an extensive dipole that should be easily visible by time-resolved electrometry. The reaction of mixed-valence enzyme with O_2 is not associated with such charge displacement [77]. Thus, while we agree that the trp 236 may well be intermediate in radical transfer to tyr 129, we think the evidence favours the cross-linked tyrosine as the primary radical site in the natural reaction mechanism (Fig. 1).

The question remains as to why radicals at the **C_4** stage of the reaction are observed (albeit at fairly low occupancy) when **C_4** is produced by hydrogen peroxide, but not "naturally". Referring to the schemes in Fig. 2A,B, this might be due to local production of the perhydroxyl radical when the site at state **C_4** reacts with a second molecule of H_2O_2. A proportion of the HO_2^* thus produced may escape from the suggested cyclic mechanism to produce the observed protein radicals.

6. "Fully oxidised" cytochrome oxidase

In the binuclear site of COX, as isolated, heme a_3 is high spin ferric and Cu_B is cupric, as evidenced from a large body of spectroscopic and magnetochemical data [78,79]. "*Fully* oxidised" is another misnomer insofar as this is a **C_2** state, although not necessarily the structure for **C_2** shown in Fig. 1. Several **C_2** states have indeed been described almost throughout COX history, starting from the "resting" and "pulsed" forms of Antonini and Brunori et al. [80,81], and the "fast" and "slow" forms of the enzyme reviewed by Moody et al. [82]. Briefly, these forms differ dramatically in their reactivity with ligands such as cyanide, and by the apparent rate by which electrons are transferred from heme *a* to the binuclear centre. More recently, X-ray data with COX from bovine heart [83] and *P. denitrificans* [84] has been interpreted to show a peroxide molecule in the active site of the oxidised (ferric/cupric) enzyme. Crystallographic data with the *Rh. sphaeroides* COX [85] appear very similar, but the authors' interpretation is more cautious, suggesting water and hydroxide ligands to the two metals (as in Fig. 1). Early on, Yoshikawa et al. [86] reported that full reduction of the oxidised enzyme with dithionite required six reducing equivalents, as would indeed be required if peroxide is a ligand of the ferric/cupric binuclear site. It is noteworthy, however, that the same authors found that only four oxidising equivalents were required to fully oxidise the reduced enzyme with O_2, albeit then arriving at a different oxidised state. Also, when the reductive titrations were performed in the presence of catalytic amounts of phenazine methosulphate, only ~4 reducing equivalents were required [86].

Kim et al. [87] criticised the peroxide suggestion based on its structural properties and suggested that the bound ligand might even be O_2. Recent quantum-chemical calculations support this proposal, and suggest that bound O_2 might be reduced to superoxide in the X-ray beam [88]. Very recently Liu et al. [89] reported optical spectra of single crystals of COX from *Rh. sphaeroides* at 100 K that clearly showed enzyme reduction by the X-ray beam. Most interestingly, development of the ferrous band of heme *a* at ~610 nm was accompanied by an absorption increase at ~590 nm, which was absent from crystals previously reduced by dithionite and frozen, and which disappeared on transient warming of the sample. It is suggested here that the 590 nm species may represent the dioxygen adduct, Compound A [11] or state **C_{0A}** (Fig. 1). If so, it would strongly support the notion that the crystallographically observed bridging ligand in the ferric/cupric oxidised enzyme is not peroxide, but O_2 or superoxide.

Finally, a stable peroxide bridge between the ferric iron and cupric copper in the binuclear centre is incompatible with the scenario discussed above regarding the reactivity of COX with added hydrogen peroxide. According to that picture, which is well founded by current knowledge, peroxide bound to the ferric/cupric site will be readily decomposed to the equivalent of two water molecules with acceptance of two electrons, one from heme iron forming the ferryl state, and the other from the cross-linked tyrosine forming a tyrosyl radical (**C_4** or **C_{4F}**) (Figs. 1 and 2).

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