



## Review

# Analysis of structure–function relationships in cytochrome c oxidase and its biomimetic analogs via resonance Raman and surface enhanced resonance Raman spectroscopies<sup>☆</sup>



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## ABSTRACT

Cytochrome c oxidase (CcO) catalyzes the four electron reduction of molecular oxygen to water while avoiding the formation of toxic peroxide; a quality that is of high relevance for the development of oxygen-reducing catalysts. Resonance Raman spectroscopy has been used since many years as a technique to identify electron transfer pathways in cytochrome c oxidase and to identify the key intermediates in the catalytic cycle. This information can be compared to artificial systems such as modified heme–copper enzymes, molecular heme–copper catalysts or CcO/electrode complexes in order to shed light into the reaction mechanism of these non-natural systems. Understanding the structural commonalities and differences of CcO with its non-natural analogs is of great value for designing efficient oxygen-reducing catalysts. In this review therefore Raman spectroscopic measurements on artificial heme–copper enzymes and model complexes are summarized and compared to the natural enzyme cytochrome c oxidase. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.

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## 1. Introduction

The ability of cytochrome c oxidase (CcO) and other heme–copper oxidases to reduce oxygen to water gives them a unique place in the field of bioenergetics. The selective reduction of oxygen to water requires the uptake of 4 electrons and 4 protons, whereas reduction to hydrogen peroxide is achieved with only 2 electrons and 2 protons; thus enzymatic activity and selectivity in heme–copper oxidases are tightly coupled to efficient electron and proton transfer reactions. The energy gained from this highly exothermic reaction is used in nature to pump protons from the N- to the P-side of the membrane, but could in principle also be transferred into electrical energy as is done in biofuel cells. For the latter application the natural enzyme has some serious drawbacks as electrical accessibility of the catalytic center is difficult to achieve in oxidase/electrode systems. CcO bioinspired molecular catalysts, however, could overcome this disadvantage but they lack the efficiency and specificity of the natural enzyme. In order to improve the design of these molecular catalysts, a profound understanding of the structure–function relationship in CcO as well as its biomimetic analogs is necessary. Such information can be provided by vibrational

spectroscopy, which has been applied intensely to CcO and also in recent years more and more to CcO functional model complexes.

The key molecular unit in CcO and many other O<sub>2</sub>-reducing catalysts is a heme that exhibits a strong Soret band absorption maximum around 400 nm. This strong absorbance allows studying very selectively the structure of the heme, even when bound in a very complex protein matrix, via resonance Raman (RR) spectroscopy. As a result heme proteins and heme model compounds have been studied extensively by RR spectroscopy since the 1970s [1,2]. From these works it is known that the vibrational band pattern in the marker band region between 1300 and 1700 cm<sup>−1</sup> is dominated by the enhanced porphyrin ring vibrations ( $\nu_4$ ,  $\nu_3$ ,  $\nu_2$ ) and depends sensitively on the core radius of the porphyrin ring [3,4]. By analyzing the positions of the marker bands it is possible to gain information about the redox and spin states of the central iron atom. In the biological system at least one of the heme axial ligands is occupied by an amino acid residue namely a histidine (cytochrome c oxidase and most peroxidases), a cysteine (cytochrome P450) or a tyrosine (catalase). The sixth coordination site may be used for adduct binding such as CO, NO or O<sub>2</sub> with each adduct exhibiting a specific vibrational fingerprint [5,6].

CcO exhibits two heme centers; a six coordinated heme *a* and a five coordinated heme *a*<sub>3</sub>, which is part of the catalytic binuclear Fe–Cu center. Using RR spectroscopy at Soret band excitation the redox and spin states of the two hemes could be identified [7–9]. By analyzing RR difference spectra of oxygenated CcO using isotopically labeled oxygen

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species the oxo intermediates in the catalytic cycle could be identified [10–12] and recently RR H<sub>2</sub>O/D<sub>2</sub>O difference measurements made it possible to assign the CH<sub>2</sub> twisting modes of both heme propionates [13] and to shed light on the proton pathway via heme *a* [14].

The detailed information that has been accumulated from CcO RR measurements over the last decades can be used to compare the reaction mechanism of the natural enzyme with either modified heme–copper biomolecules or CcO bioinspired molecular catalysts. Raman spectroscopic studies of CcO and CcO functional model systems that correlate structural information of the catalytic center with their oxygen reduction ability will therefore be the focus of this review. Furthermore, the electrocatalytic properties of these systems when bound to electrodes, gained from surface enhanced resonance Raman (SERR) spectroscopic measurements, will be reviewed.

## 2. Tuning oxidase activity: role of the distal heme environment

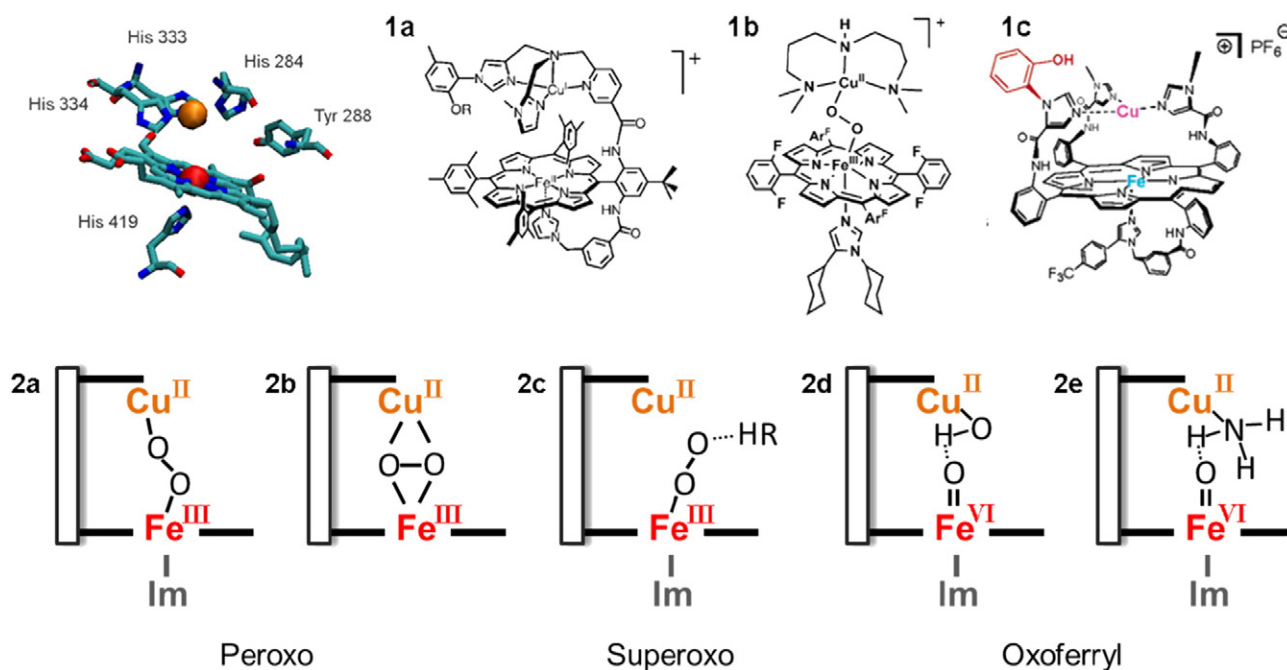
It has always been remarkable that the different catalytic reactions in catalase, peroxidases, monooxygenases and oxidases are performed by the same heme cofactor. Distinct differences are, however, found in the composition of the heme environment in the respective enzymes that promote efficiency for a selective reaction. To identify the responsible parameters that tune reaction selectivity is of great importance not only to understand the effectiveness of enzymatic catalysis but also for the development of heme-based molecular catalysts. One key element is the presence and nature of the proximal ligand that modulates the electron density at the Fe center [15]. Further reaction selectivity is introduced by the arrangement of amino acid residues and additional metal centers in the distal ligand environment [16,17]. Both parameters have to be considered carefully for the design of bioinspired molecular catalysts.

The high efficiency of CcO in respect to oxygen reduction is to a great amount introduced by the second metal Cu<sub>B</sub> in close vicinity of the heme *a*<sub>3</sub> iron that provides a docking site for the second oxygen atom and thus promotes fast splitting of molecular oxygen. Therefore, artificial addition of Cu to the catalytic center of a heme enzyme or heme model compound might increase its oxidase activity while blocking

the Cu<sub>B</sub> docking site in CcO should have the opposite effect. Further key elements for efficient oxygen to water reduction that have been identified are a histidine proximal ligand that increases the oxygen binding affinity of the Fe atom and a tyrosine located in the distal heme environment that works as an additional electron donor and therefore promotes the 4e<sup>−</sup>/4H<sup>+</sup> reduction pathway. These and other considerations have led to the design of several CcO inspired oxygen-reducing catalysts that are depicted in Fig. 1 1a–c.

RR measurements of the oxygenated catalytic center can be very useful to identify the key intermediates in the catalytic cycle which in turn sheds light on the reaction mechanism in the natural enzyme as well as in the molecular catalysts. Several oxo intermediates can be identified by measuring <sup>16</sup>O<sub>2</sub>–<sup>18</sup>O<sub>2</sub> RR difference spectra. Most prominently the  $\nu(\text{Fe}=\text{O})$  and  $\nu(\text{O}=\text{O})$  stretching vibration of a peroxo/superoxo species and the  $\nu(\text{Fe(IV)}=\text{O})$  stretching vibration of an oxoferryl species are detected with this method. The exact position of the  $\nu(\text{Fe(IV)}=\text{O})$  vibration is sensitive to changes of the proximal ligand and the hydrogen bonding of the Fe bound oxygen. It has to be noted that the  $\nu(\text{Fe}=\text{O})$  and  $\nu(\text{O}=\text{O})$  vibrations occur at similar frequencies, impairing an easy assignment; thus additional measurements with single substituted molecular oxygen (<sup>16</sup>O–<sup>18</sup>O) have to be performed for an unambiguous assignment. Different oxo intermediates discussed in the literature are presented schematically in Fig. 1 2a–e.

In CcO time resolved RR <sup>16</sup>O<sub>2</sub>–<sup>18</sup>O<sub>2</sub> difference spectra were able to identify the  $\nu(\text{Fe}=\text{O}_2)$  stretching vibration of the initial O<sub>2</sub> adduct at 568 cm<sup>−1</sup> [18] as well as the two oxoferryl intermediates P and F with their respective  $\nu(\text{Fe(IV)}=\text{O})$  stretching vibration at 804 and 785 (790) cm<sup>−1</sup> [11,12]. It has to be noted that no RR bands of the  $\nu(\text{O}=\text{O})$  stretching vibration of a peroxide or superoxide during catalytic turnover could be identified so far. Initially a band at 358 cm<sup>−1</sup> was assigned to the  $\nu(\text{Fe}=\text{O}_2^-)$  vibration of such a peroxide intermediate [12], however, since then the origin of this vibrational band has been discussed controversially. From different RR measurements other assignments of this band, i.e. to the His–Fe(IV)=O bending vibration, have been favored [19,20]. Recently, however, for the resting state of CcO a band at 755 cm<sup>−1</sup> has been detected that was assigned to the  $\nu(\text{O}=\text{O})$  stretching vibration of a bridging peroxide [21].

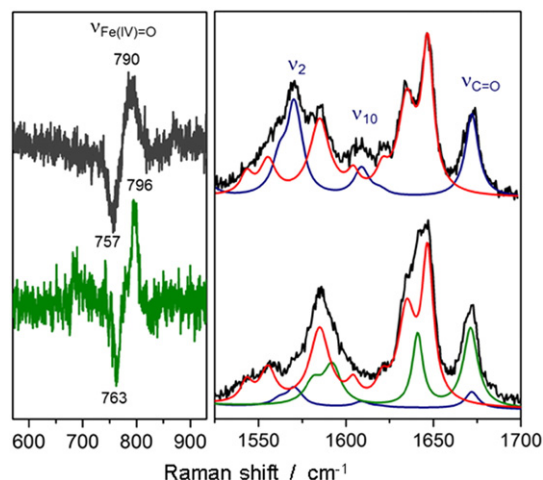


**Fig. 1.** Top: Binuclear heme *a*<sub>3</sub>-Cu<sub>B</sub> center of CcO from *Rhodobacter spaeroides* (PDB ID: 1M56) and the Fe–Cu model complexes taken from Refs. [41,44,47]. Bottom: Schematic drawing of possible intermediate states of CcO or Fe–Cu model compounds (2a) Fe–μ-1,2 peroxo-Cu, (2b) Fe–μ-1,2 peroxo-Cu (2c) Fe–superoxo-(2d) Fe=O and (2e) Fe=O NH<sub>3</sub>-Cu.

### 3. Modifications of natural enzymes

One way to gain information on the parameters that tune oxygen-reduction efficiency in enzymatic catalysis is the modification of the catalytic center in natural enzymes. Here introducing Cu to monometallic heme enzymes has been used to study its role as a promoter of oxygen reduction efficiency [16,22]. RR spectroscopy was used to study how distal heme adducts are affected by addition of Cu [23]. Sperm whale myoglobin was mutated in a way that a Cu atom is placed in the distal heme pocket via 3 histidine residues. To check whether this compound is a suitable model system for CcO the RR vibrations of a Cu<sub>B</sub>Mb-CO complex were analyzed. Without Cu the  $\nu(\text{Fe}-\text{CO})$  and  $\nu(\text{C}-\text{O})$  stretching vibrations were seen at 520 and 1947  $\text{cm}^{-1}$ , respectively. In the Cu containing mutants these vibrations were shifted to 513 and 1967  $\text{cm}^{-1}$ .  $\nu(\text{Fe}-\text{CO})$  vs.  $\nu(\text{C}-\text{O})$  inverse correlation plots give information on the polarity of the distal environment of the heme [24]. The values for the  $\nu(\text{Fe}-\text{CO})$  vs.  $\nu(\text{C}-\text{O})$  vibrations for the system without Cu matched the line observed in general for His coordinated monometallic heme proteins while the values for the system with Cu were on the same line as CcO. Hence the authors conclude that Cu<sub>B</sub>Mb can work as a good model system for CcO. Flash photolysis measurements of the Cu<sub>B</sub>Mb-CO complex showed that the presence of Cu modulated the rebinding kinetics of CO to the catalytic center. Thus it was concluded that Cu, similar as in CcO, regulates ligand entry and exit by providing a temporary ligand docking side. However, the introduction of Cu alone – albeit into a functional enzyme – does not facilitate automatically oxidase activity. The authors observe for this enzymatic CcO model no ferryl intermediate but a degradation of the heme to verdoheme and attribute this to the lack of protons available in the catalytic center. This underlines the strong influence of proton dynamics on the functionality of oxygen reducing enzymes, which has to be considered when designing inorganic molecular catalysts.

It is known that CcO exhibits catalase activity [25], which has been used in the past to create artificial oxo intermediates of CcO via addition of  $\text{H}_2\text{O}_2$  [26,27]. Under  $\text{H}_2\text{O}_2$  exposure the RR spectra of CcO in the marker band region show a shift of the  $\nu_4$ ,  $\nu_2$  and  $\nu_{10}$  vibrational modes to higher frequencies (Fig. 2) in agreement with a high spin to low spin transition of heme  $a_3$  upon formation of an oxoferryl species. RR  $\text{H}_2^{16}\text{O}_2$ - $\text{H}_2^{18}\text{O}_2$  difference spectra of the  $\text{H}_2\text{O}_2$  generated oxo intermediates show a  $\nu(\text{Fe(IV)=O})$  stretching vibration at 785/790 and 804  $\text{cm}^{-1}$  depending on the  $\text{H}_2\text{O}_2$  excess ratio [28,29]. These band positions



**Fig. 2.** Left: RR  $\text{H}_2^{16}\text{O}_2$ - $\text{H}_2^{18}\text{O}_2$  difference spectra of the  $\text{H}_2\text{O}_2$  induced intermediate of CcO with (bottom) and without (top) addition of ammonia. Right: RR spectra of CcO and CcO- $\text{H}_2\text{O}_2$  in the high frequency region. The spectra are fitted using the component spectra of heme  $a$  (red), high spin heme  $a_3$  (blue) and low spin heme  $a_3$  (green). Adapted from Ref. [35] with permission from the American Chemical Society.

correspond to the ones observed for the F and P intermediates, respectively, during turnover in the oxygen reduction cycle [12,30]. The origin for the position of the oxoferryl stretching vibration has been discussed controversially. As a reason either different hydrogen bonding of the oxoferryl oxygen or a change in the trans ligand strength of the proximal histidine was given [28,31,32]. Furthermore, recent nonlinear mechanics calculations of the  $\nu(\text{Fe(IV)=O})$  vibration in CcO from *Paracoccus denitrificans* demonstrated an influence of the apparent band positions on the protonation/deprotonation state of residues in the heme  $a_3$  environment [33]. The calculations showed that different protonation states of the propionic acid chain A and the close by amino acid Asp399 modulate the relative intensities of the  $\nu(\text{Fe(IV)=O})$  stretching vibration bands leading to shifted  $^{16}\text{O}$ - $^{18}\text{O}$  difference bands in the region between 750 and 850  $\text{cm}^{-1}$ . The authors state that this phenomenon might also account for the different characteristic vibrational pattern observed for the  $\nu(\text{Fe(IV)=O})$  vibration in the P and F intermediates of CcO.

Recently it has been observed that addition of ammonia to the  $\text{H}_2\text{O}_2$  generated F state leads to a more than twofold catalase activity of CcO from *P. denitrificans* [34] while at the same time the oxidase activity is decreased. The authors concluded that this is a direct consequence of ammonia binding to Cu<sub>B</sub> in the binuclear center. RR  $\text{H}_2^{16}\text{O}_2$ - $\text{H}_2^{18}\text{O}_2$  difference spectra show a shift in the  $\nu(\text{Fe(IV)=O})$  vibration upon addition of ammonia from the F state from 790 to 796  $\text{cm}^{-1}$  [35] (Fig. 2). This observation confirms a direct interaction of ammonia with the Fe-bound oxygen in the catalytic center and makes a scenario where ammonia occupies the Cu<sub>B</sub> docking site (Fig. 1 2e) very probable. It is interesting to note that this binding of ammonia to Cu<sub>B</sub> in the resting state of CcO is not possible most likely due to the presence of a  $\mu$ -peroxo ligand [36], which underlines the assumption that the presence of a Fe-Cu bridging ligand might serve as protection against spontaneous reactions of the metals with other molecules. In respect to the design of molecular catalysts and their long term stability this protection mechanism is of high importance and should be considered carefully.

### 4. Functional CcO inspired metal complexes

The information gained by spectroscopy on the reaction mechanism of CcO can be used for the design of CcO bioinspired oxygen-reducing metal complexes. Those metal complexes should adopt the quality of CcO to reduce oxygen very fast and selectively via the  $4e^-/4\text{H}^+$  reduction pathway to water and not the  $2e^-/2\text{H}^+$  pathway to  $\text{H}_2\text{O}_2$ . The latter quality is important not only to avoid formation of reactive peroxide species but also to increase the cell potential in fuel cell applications [37]. Several functional models of the active side of CcO have been developed and characterized by RR spectroscopy in the past [38–44]. A selection of these complexes is shown in Fig. 1.

A tri(2-pyridylmethyl)amineCu linked iron meso-tetraphenylporphyrin (tpaCu-TPPFe) complex was proposed as a good functional model system for CcO [42]. The compound contains Fe and Cu as the bimetallic catalytic center but lacks the histidine axial ligand and the tyrosine residue present in CcO. A  $\nu(\text{O}-\text{O})$  stretching vibration at 803  $\text{cm}^{-1}$  could be identified in this model compound which was assigned to a Fe- $\mu$ -peroxo-Cu intermediate. Moreover, it was proposed that  $\text{O}_2$  binds first to Cu(I), because Fe lacking a nitrogen axial ligand has a low affinity for oxygen. A few years later an improved model compound (( $L^{\text{N}4\text{-OH}}$ )Cu)/FeII(TMPIm)) was presented that includes the axial imidazole and a cross-linked Tyr residue (Fig. 1 1a) [41]. UV-vis and RR spectroscopies, performed at  $-70^\circ\text{C}$ , revealed a slow transition from one intermediate (A) to another one (B) on a minute time scale. From RR difference spectra with  $^{16}\text{O}$  and  $^{18}\text{O}$  two vibrational bands at 611 and 787 (803)  $\text{cm}^{-1}$  in intermediate A were assigned to the  $\nu(\text{Fe}-\text{O}_2^-)$  and  $\nu(\text{O}-\text{O})$  stretching vibrations of a Fe- $\text{O}_2^-$ -Cu-bridged peroxo intermediate (Fig. 1 2a). In the subsequently formed intermediate state B only a band at 574  $\text{cm}^{-1}$  was observed that was assigned to the  $\nu(\text{Fe}-\text{O}_2^-)$  vibration of a superoxo species (Fig. 1 2c). This heme- $\mu$ -peroxo-Cu(II) to heme-superoxo-Cu(I) conversion is rather unique for functional Fe-Cu

model compounds and its occurrence was attributed to the hydrogen bonding stabilization properties of the phenolic hydroxyl group of the added Tyr residue. The timescale, on which either a peroxo or a superoxo intermediate is formed, might be highly important for tuning the reaction to either the  $2e^-/2H^+$  or the  $4e^-/4H^+$  reduction pathway. Albeit not discussed for biomimetic CcO complexes such a scenario was proposed for similar build-up Pacman complexes: These oxygen reducing catalysts consist of two cofacial bisporphyrins held together in a finite distance by a flexible spacer and do show a high selectivity for the  $4e^-/4H^+$  reduction pathway to water [45]. In the proposed reaction mechanism for Co-Pacman complexes the formation of a superoxo intermediate is postulated that can either be protonated or transferred into a peroxo species. The formation of the peroxo species is considered to favor selective reduction of oxygen to hydrogen peroxide whereas fast protonation of the superoxo species leads to water as the final product.

An alternative Fe–Cu model complex ( $(F_8)Fe(L^{Me2N})Cu$ ) was presented by Kim et al. [40]. In this work two vibrational RR  $^{16}O$ – $^{18}O$  difference bands at 752 and 767  $cm^{-1}$  were observed under low temperature steady state conditions, which were both assigned to the  $\nu(O-O)$  vibration of a Fe- $\mu$ -peroxo-Cu species. It was, however, not unambiguously clear whether this peroxo species was present in a  $\mu$  (end-on as in Fig. 1 2a) or  $\mu-\eta^2:\eta^2$  (side-on 2b) configuration. This aspect was further investigated in an improved model compound where an axial imidazole (DCHIm) was added (Fig. 1 1b) [44]. Addition of the imidazole-containing compound leads to the occurrence of a band at 789 nm in the UV–vis spectrum. RR spectroscopy at 775 nm showed three  $^{16}O$ – $^{18}O$  isotope sensitive bands at 586, 796 and 394  $cm^{-1}$  which were assigned to the  $\nu(Fe-O)$  stretching, the  $\nu(O-O)$  stretching and  $\delta(N-Fe-O)$  bending mode of a peroxide intermediate, respectively. The frequency of the  $\nu(O-O)$  vibration is 40  $cm^{-1}$  higher than measured for the complex without the axial ligand [46]. The authors therefore conclude that the axial coordination of the imidazole causes the peroxo bridge to change from a side-on  $\mu-\eta^2:\eta^2$  to an end-on  $\mu-1,2$  conformation. The strong enhancement seen for the  $\nu(Fe-O)$  vibration was attributed to a peroxo-Fe charge transfer band seen at 789 nm in the UV–vis spectrum. Based on their results it is suggested that the 755  $cm^{-1}$  band observed in the resting state of CcO [21] is rather due to a side-on  $\mu-\eta^2:\eta^2$  than to an end-on  $\mu-1,2$  conformation.

As a third CcO mimic, a model complex featuring the heme  $a_3$ -Cu<sub>B</sub> motif (FeCu) was introduced by Collman et al. [43]. On this complex for the first time a vibrational band at 570  $cm^{-1}$  was detected via RR spectroscopy that could be assigned to a superoxide intermediate. Some years later results were published on a modified complex that included a histidine cross linked Tyrosine (FeCu-Tyr Fig. 1 1c) This complex was able to reduce oxygen selectively to water when immobilized on functionalized Au electrodes under conditions where fast electron transfer from the electrode to the catalyst could be achieved [39]. RR measurements of this complex at  $-60^\circ C$  yielded a 575/549  $cm^{-1}$   $^{16}O_2$ / $^{18}O_2$  difference band that was also assigned to a superoxo species [47]. At higher temperatures the authors propose a transformation into an oxoferryl state. This transformation, however, was not shown by RR spectroscopic measurements in this paper.

A similar model complex (FeFe) was presented a few years later where the Cu atom was replaced by a second Fe atom [48].  $^{16}O_2$ – $^{18}O_2$  RR difference spectra were recorded at  $-80^\circ C$  and  $-20^\circ C$  for the complex in solution. Depending on the temperature two different oxo-intermediates could be trapped. At  $-80^\circ C$  two bands at 822  $cm^{-1}$  and 583  $cm^{-1}$  were assigned to the  $\nu(O-O)$  and  $\nu(Fe-O)$  stretching vibrations of an end-on  $\mu$ -peroxo species. At  $-20^\circ C$  two other difference bands at 808  $cm^{-1}$  and 756  $cm^{-1}$  were observed which were assigned to the  $\nu(Fe(IV)=O)$  stretching vibration of the heme and non-heme ferryl species, respectively.

The positions of the  $\nu(Fe-O)$ ,  $\nu(O-O)$  and  $\nu(Fe(IV)=O)$  stretching vibrations observed for the different intermediates of CcO and their corresponding model complexes are summarized in Table 1.

These molecular catalysts have shown that by mimicking the environment of the CcO catalytic center one is able to improve the efficiency and selectivity of oxygen reducing catalysts. However, there are still differences in the reaction mechanism observed for the biological and the biomimetic systems. This includes the  $\mu$ -peroxo bridge only observed in the molecular catalyst during turnover conditions, which might work against rapid transformation into the active oxoferryl state. These discrepancies show that some important parameters such as fast supply of multiple electrons and protons as well as prevention of unwanted binding processes have not yet been considered conclusively.

## 5. Electrocatalytic oxygen reduction: CcO vs. molecular catalysts

Although CcO does show superior properties regarding oxygen activation, it does not play a significant role as an electrocatalyst. This is especially surprising as biocatalysts for efficient and selective oxygen reduction to water are strongly needed as cathode material in biofuel cells. The reasons for the lack of CcO and other heme–copper oxidases in this field are manifold.

First, it is highly challenging to establish a fast electron transfer pathway between the electrode and the catalytic center of CcO. In contrast to most functionalized electrode surfaces, the natural electron donor Cyt c has the right size and surface properties to bind to subunit II of CcO in such a way that the distance between heme c and Cu<sub>A</sub> becomes small and a fast electron transfer is possible via the propionic acid chains of heme c [49]. Such a type of interface is difficult to mimic by an electrode surface. Therefore, sufficient fast electrical communication between cytochrome c/ubiquinol oxidase and the electrode could so far only be achieved by using the respective natural redox partners cytochrome c [50,51] or ubiquinol [52] as redox mediators. But even in these cases the electrochemical induced oxygen reduction occurred at comparably high overpotentials, which makes CcO unattractive for biofuel cell applications. Other oxygen reductases such as Laccase work at much lower overpotentials and are therefore more common in biotechnical applications [53].

Secondly, it is not known how the immobilization of CcO on conducting electrodes will affect its structure and functionality. A unique possibility to study the structure of the heme centers of immobilized CcO is surface enhanced resonance Raman spectroscopy (SERRS) that makes use of the plasmonic field enhancement at the metal/dielectric interface of nanostructured noble metals. The enhanced light intensity at the metal surface leads to a Raman signal enhancement of molecules in its close vicinity that is roughly in the order of  $10^4$ – $10^8$  [54]. Thus it is possible to selectively probe the molecules that are attached to the metal surface and to analyze their structure during an electrocatalytic reaction. In order to combine surface enhancement with resonance Raman measurements at Soret band excitation of CcO, silver (Ag) has to be used as support material. Early SERR measurements had shown severe denaturation effects of proteins when bound directly to silver electrodes [55]. However, this drawback could be overcome by coating the electrode with  $\omega$ -functionalized alkanethiols or polymers. SERR measurements of heme proteins attached to such coated Ag interfaces showed that it is possible to preserve the native structural state of the heme environment also in the immobilized state [56–58].

## 6. Surface enhanced resonance Raman investigations of CcO on electrodes

SERR measurements not only decrease the amount of protein needed for sufficient signal to noise ratio, but – when combined with electrochemical measurements – allow correlating the redox and catalytic activity with structural changes of the catalytic center [59,60]. Structure–function relationships of immobilized heme proteins have been widely investigated by SERRS. Therefore it is surprising that only very few SERR measurements have been published so far on heme–copper oxidases. In the works of Friedrich et al. [61] and Hrabakova

**Table 1**

Positions of the vibrational bands of the peroxo/superoxo and oxoferryl intermediates in CcO and CcO functional models.

Compound-ligand	Peroxo		Oxoferryl	Superoxo	Ref.
	$\nu(\text{Fe}-\text{O}_2^{2-})$	$\nu(\text{O}-\text{O})^{2-}$			
CcO					
CcO-O <sub>2</sub>			786		[18]
CcO-O <sub>2</sub> Bovine			790		[12]
CcO rest. state Bovine		755			[21]
CcO-H <sub>2</sub> O <sub>2</sub> Bovine			785 <sup>a</sup>		[19]
CcO-H <sub>2</sub> O <sub>2</sub> <i>Paracoccus den.</i>			790 <sup>a</sup>		[28]
CcO-NH <sub>3</sub> -H <sub>2</sub> O <sub>2</sub> <i>Paracoccus den.</i>			796 <sup>a</sup>		[35]
<i>Fe-Cu model complexes</i>					
tpaCu-TPPFe-O <sub>2</sub>		803			[42]
(L <sup>N4+OH</sup> )Cu/Fe	611	787		574	[41]
(TMPIIm)-O <sub>2</sub> (1a)					
(F <sub>8</sub> )Fe[(L <sup>Me2N</sup> )Cu]-O <sub>2</sub>		767/752			[40]
(F <sub>8</sub> )Fe[(L <sup>Me2N</sup> )Cu]-	586	796			[44]
DCHIm-O <sub>2</sub> (1b)					
FeCu-O <sub>2</sub>				570	[43]
FeCu-Tyr-O <sub>2</sub> (1c)				575	[47]
<i>Other heme based CcO model complexes</i>					
FeFe-O <sub>2</sub>	583	822	808		[48]
FeEs4-O <sub>2</sub>	631	830	782		[66]

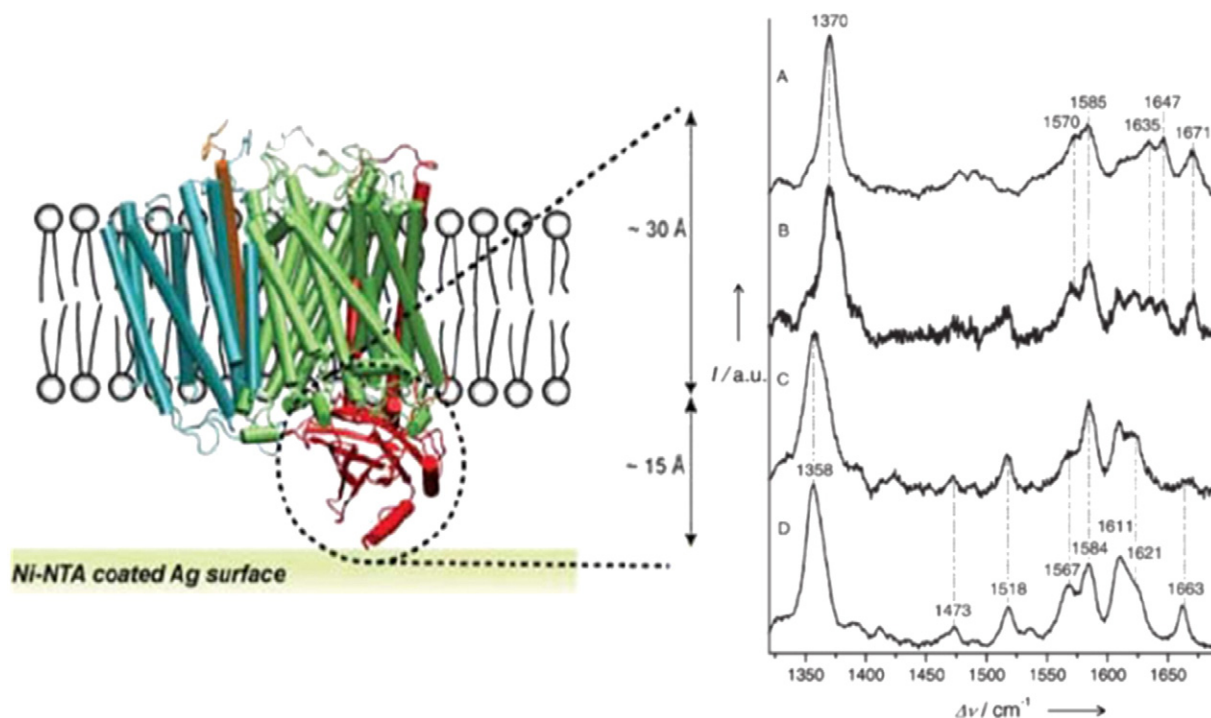
<sup>a</sup> High H<sub>2</sub>O<sub>2</sub> excess ratio.

et al. [62], CcO from *Rhodobacter spaeroides* was tethered to an Ag electrode via a His-Tag, which is located at the C-terminus of subunit I or II, and embedded in a phospholipid bilayer. Although this immobilization method leads to a long distance between the heme centers and the electrode surface, high SERR spectra of the immobilized enzyme were observed (Fig. 3). The SERR spectra recorded with 413 nm excitation line

resemble the RR spectra in solution demonstrating that at least the heme environment stays intact upon immobilization. Analysis of the porphyrin vibrational modes in the frequency region between 1300 cm<sup>-1</sup> and 1700 cm<sup>-1</sup> yielded the component spectra of reduced and oxidized heme *a* and heme *a*<sub>3</sub> respectively and thus the relative contribution of each species could be determined as a function of electrode potential. The authors showed that, upon decreasing the electrode's potential, heme *a* could be reduced whereas heme *a*<sub>3</sub> remained oxidized even when very high overpotentials were applied. This observation was rationalized by a drastically slowed down intramolecular electron transfer between heme *a* and *a*<sub>3</sub> in the presence of high electric fields in combination with a remaining presence of oxygen in the current experimental setup. This interpretation is in line with the observation by Takahashi et al. [63], using time resolved RR spectroscopy, that the oxygenated heme *a*<sub>3</sub> species of CcO in intact mitochondria is stabilized possibly due to the presence of a transmembrane potential.

A different behavior, however, was observed by Todorovic et al. [64] for the immobilized quinol oxidase from *Acidianus ambivalens* (QA). Here heme *a* and heme *a*<sub>3</sub> could be treated as independent redox-couples with almost ideal Nernstian behavior. Both hemes could be fully reduced with the respective midpoint potentials of E<sup>0</sup> = 0.32 V for heme *a* and E<sup>0</sup> = 0.39 V (vs. NHE) for heme *a*<sub>3</sub>. This is in contrast to CcO where the midpoint potential of heme *a*<sub>3</sub>, in the absence of oxygen, was determined to be more negative than E<sup>0</sup> of heme *a* [65]. It was concluded that the *inverted* midpoint potentials in QA allow a downhill electron transfer from heme *a* to heme *a*<sub>3</sub>, whereas the electron transfer cascade from heme *a* to heme *a*<sub>3</sub> in CcO has to undergo an *uphill* electron transfer that is facilitated by a sophisticated network of cooperativities.

These SERRS experiments do shed light into the intrinsic problems of CcO as an electrocatalyst. As the intramolecular electron transfer pathway in CcO is not accomplished by a cascade of redox potentials but involves cooperativity effects and is closely related to proton translocation, already small deviations from the natural environment can lead to a loss in functionality. Understanding the charge transfer dynamics of



**Fig. 3.** Left: CcO immobilized on a Ni-NTA coated Ag surface. Right: RR spectra of CcO in the oxidized (A) and reduced (D) states. SERR spectra of CcO at open circuit (B) and -0.65 V (vs. Ag/AgCl) (C).

Adapted from Ref. [61] with permission from the Royal Society of Chemistry.

CcO on electrodes can thus help enormously to improve the applicability of this enzyme as cathode material in biofuel cells.

## 7. SERRS of biomimetic complexes for oxygen reduction

In contrast to the natural enzyme, fast electron transfer might be easier achieved in CcO biomimetic complexes as the distance between the catalytic center and the electrode is in general much shorter. Furthermore, due to the much smaller size of the molecular catalysts, a higher surface coverage can be achieved on the electrodes. These advantages might make up for the lesser efficiency compared to CcO and thus oxygen reducing functional CcO models are of high interest for electrocatalytic applications. Especially the electron transfer properties of oxygen reducing compounds are very important as it could be shown that the electron transfer rate directly influences whether the reduction occurs via the  $4e^-/4H^+$  or the  $2e^-/2H^+$  route with either  $H_2O$  or  $H_2O_2$  as the final product [39].

Again surprisingly only a few SERR measurements on heme based catalysts for oxygen reduction have been published so far [66–69]. Very recently Sengupta et al. [66] showed SERR spectroscopic investigations of iron porphyrin catalysts during  $O_2$  reduction turn-over conditions. Two different catalysts were investigated: Iron Tetrphenylporphyrin (FeTPP) and iron  $\alpha_4$ -tetra-2-(4-carboxymethyl-1,2,3-triazolyl) phenylporphyrin (FeEs<sub>4</sub>). The latter exhibits a hydrogen bonding distal superstructure that allows stabilization of Fe– $O_2$  [70]. For FeEs<sub>4</sub> a gradually rising catalytic current was observed for potentials below its midpoint potential of  $-0.1$  V (vs. Ag/AgCl). This observation was accompanied by a gradual increase of the  $\nu_4$  and  $\nu_2$  vibrational bands characteristic for a Fe(III) and a Fe(IV) low spin species in the presence of oxygen that was missing in the measurements under anaerobic conditions. Using  $^{16}O_2$ – $^{18}O_2$  SERR difference spectra the  $\nu(Fe(IV)=O)$ , and  $\nu(Fe-OOH)$  species were identified at an electrode potential of  $-0.5$  V. The difference band at  $782\text{ cm}^{-1}$  was assigned to the  $\nu(Fe(IV)=O)$  stretching vibration, while the  $830\text{ cm}^{-1}$  and  $631\text{ cm}^{-1}$  bands were assigned to the respective  $\nu(O-O)$  and  $\nu(Fe-O)$  stretching vibrations of an Fe–OOH intermediate.

## 8. Conclusions

RR and SERR spectroscopies of heme–copper oxidases and their biomimetic analogs allow identifying key intermediates in the catalytic oxygen reduction cycle and can furthermore gain insight into the electron transfer pathways of CcO when bound to functional electrodes.

Comparing the nature of oxo intermediates in CcO and CcO functional models, the most noticeable difference is the detection of a bridging Fe– $\mu$ -peroxo–Cu species in the functional Fe–Cu model compounds under steady state conditions while in CcO this oxo intermediate has been so far only observed in the resting state. For some model systems a superoxide intermediate could be observed, which was explained by the presence of a His-linked tyrosine. This conclusion, however, cannot be conclusively drawn as also for a Fe–Cu complex that lacks the tyrosine such a superoxide intermediate was detected. This remains a delicate point as the formation of a superoxide was proposed to be crucial for generating a reduction pathway to water in other oxygen reducing molecular catalysts.

Time resolved RR spectroscopy is important for identification of short lived intermediates as the kinetics of electron transfer and the transiently formed oxo intermediates largely determines the catalytic efficiency. Although there has been a lot of work published in this respect on CcO, there is still a lack of time resolved RR measurements for the biomimetic compounds. This, however, is necessary to conclusively compare the reaction pathways in CcO and its functional models.

Finally to get more insights into the electrocatalytic properties of CcO and its biomimetic analogs, more measurements of these molecules on electrodes have to be performed. Here, SERRS gives a great opportunity to learn more about the role of electron transfer kinetics in oxygen-

reducing electrocatalysis. Previous SERR experiments hint already that the intramolecular electron transfer between heme  $a$  and heme  $a_3$  is altered in CcO when attached to metal electrodes but further experiments have to be done to understand the role of external electron injection and high electric fields in these systems.

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