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Original Paper

Electrochemical determination of hydrogen peroxide using *Rhodobacter capsulatus* cytochrome *c* peroxidase at a gold electrode

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Abstract. We describe the redox behaviour of horse heart cytochrome *c* (HHC) and *Rhodobacter capsulatus* cytochrome *c* peroxidase (RcCCP) at a gold electrode modified with 4,4′-bipyridyl. RcCCP shows no additional oxidation or reduction peaks compared to the electrochemistry of only HHC, which indicates that it most likely binds to HHC and results in a potential downshift of the voltammetric signals of the latter. Furthermore, the electrochemical determination of hydrogen peroxide at a RcCCP/HHC modified gold electrode is shown. The results demonstrate that HHC can substitute for cytochrome c_2, the physiological electron donor. The buffer 2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid (HEPES) and tris(hydroxymethyl) methylamine (Tris) electrochemically are not as inert as previously believed. They can react with oxygen (radicals) during electrochemical measurements, and the products formed can give rise to additional redox peaks. We therefore also have conducted a voltammetric study on theses buffers.

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Thanks to the pioneering work of different research groups [1–6], voltammetric methods are now routinely used in the determination of formal potentials of redox systems in proteins. Using appropriate electrodes, a reversible electrochemical behaviour is observed for redox compounds such as cytochromes, azurines and ferredoxines, even in the absence of a mediator. More specifically, voltammetric analysis of the electrode modified with enzymes is an important tool in the study of oxidation and reduction reactions. The activity of the enzyme can be determined by measuring the current corresponding to the reduction or oxidation of the target molecule. An electrochemical study of the redox process under study can lead to important information concerning the kinetics of the reaction.

In this paper, we report on the electrochemistry of *Rhodobacter capsulatus* cytochrome *c* peroxidase (RcCCP) and horse heart cytochrome *c* (HHC). RcCCP is a bacterial enzyme located in the periplasm. It binds two heme *c* groups covalently and uses them

as platforms to catalyse the two-electron reduction of hydrogen peroxide to water by means of the one-electron donor ferrocytochrome *c*.

$$2\text{cyt c}(Fe^{2+}) + H_2O_2 + 2H^+ \rightarrow 2\text{cyt c}(Fe^{3+}) + 2H_2O \quad (1)$$

As other bacterial CCPs, RcCCP is only active in the mixed valence state in which the high-potential, electron transferring heme is in the reduced state and the peroxidatic low-potential heme is in the Fe(III) state [7–10]. Upon the two-electron reduction of hydrogen peroxide, one electron is initially abstracted from the reduced high-potential heme group, which becomes reoxidized; this requires intramolecular electron transfer from the high- to the low-potential heme. The second electron is abstracted from the peroxidatic low-potential heme group with the formation of an oxy-ferryl center. The high-potential heme, with a midpoint potential of +270 mV, therefore functions as the electron transferring cofactor, while the low-potential heme has a midpoint potential for Fe(III)/Fe(II) between −190 and −310 mV [10] and constitutes the peroxidatic center.

In the past, the electrochemical behaviour of other CCPs was investigated by different research groups. A mutant of yeast CCP [11], CCP from *Nitrosomonas europaea* [12] or *Paracoccus panthotrophus* [13] and a redox polymer containing osmium ions and horseradish peroxidase [14] were examined using the technique of catalytic protein film voltammetry.

Here, we focus on the interaction between RcCCP, HHC and a modified gold electrode. The compound 4,4′-bipyridyl acts as an electron transfer promoter between the electrode surface and the enzyme, as reported in the literature [3]. This research shows that RcCCP can form a complex with HHC and that this complex is a mediator for the electrocatalytic reduction, and thus the determination, of hydrogen peroxide.

As the buffer component, we have used 2-[4-(2-hydroxyethyl)piperazinyl] ethanesulfonic acid (HEPES) and tris(hydroxymethyl)methylamine (Tris). They belong to the N-substituted buffers, first introduced by Good, which are widely used in biological and biochemical research due to their high solubility and convenient pK_a values between 6 and 8 [15]. However, some studies indicate that Good's buffers are not as inert as originally believed [16–18]. Therefore, this paper begins with an electrochemical study of HEPES and Tris.

Experimental

HHC, Tris–HCl, HEPES, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma-Aldrich (www.sigmaaldrich.com). The Tris buffer solution of 10×10^{-3} mol L^{-1} was set to pH 7.0 with 0.15 mol L^{-1} NaOH. The same was done with the 10×10^{-3} mol L^{-1} HEPES buffer solution. The enzyme RcCCP was heterologously expressed in *E. coli* and was purified to homogeneity using a four-step procedure [10]. Briefly, the periplasmic fraction was loaded onto a Q-Sepharose column and eluted with a step gradient of 0–500 × 10^{-3} mol L^{-1} NaCl. The fractions between 0.2 and 0.3 mol L^{-1} salt were pooled and concentrated using ammonium sulphate precipitation. The RcCCP-containing fractions were separated on an octyl-Sepharose hydrophobic interaction column. As a polishing step, the RcCCP was subjected to anion-exchange chromatography.

The basic electrochemical setup consisted of a three-electrode cell using a saturated calomel reference electrode (SCE, E = 0.244 mV vs NHE at 298 K, Radiometer, Copenhagen, www.radiometer.com) and a platinum counter electrode. The gold working electrodes of 1.6 mm diameter (BASi, West Lafayette, U.S.A., www.bioanalytical.com) were pretreated by mechanical and electrochemical polishing. Prior to its first use, the electrode surface was scoured briefly on SiC-emery paper 1200 grit to obtain an active surface. To smoothen this relatively rough surface it was further subjected to sequential polishing using a cloth covered with alumina powder (Buehler, Illinois, U.S.A., www.buehler.com) of 1, 0.3 and 0.05 μm particle size for respectively 5, 10 and 20 min. To remove any adherent Al_2O_3 particles, the electrode surface was rinsed thoroughly with doubly deionised water and cleaned in an ultrasonic bath (Branson 3210) for 2 min. Voltammetry measurements were performed by placing a 20 μL droplet containing protein and buffer between the tip of the reference electrode and the horizontal working electrode disk (modified from Hagen [19]). A PGSTAT20 potentiostat (ECO Chemie, The Netherlands, www.ecochemie.nl), controlled by the GPES 4.9005 software package (ECO Chemie, The Netherlands, www.ecochemie.nl) running on a Pentium II computer, was used to record the voltammetric curves.

Results and discussion

Electrochemical behaviour of a Tris, HEPES and phosphate buffer solution at a gold electrode

Curve 1 in Fig. 1 shows the electrochemical response of a 10×10^{-3} mol L^{-1} Tris buffer solution pH 7.0 at a gold electrode in nitrogen atmosphere. Only the redox processes of gold (oxidation of gold with formation of gold oxides and their subsequent reduction) are observed in the cyclic voltammogram around 0.4 V vs SCE [20–23]. Curves 2–6 show that the current potential behaviour becomes more complex when oxygen is present in the solution. Next to the redox processes of gold, a new oxidation peak, at about 0.16 V vs SCE (B_a), and two new reduction waves, at about −0.15 (A) and 0.11 (B_c) V vs SCE, occur. Process A can be explained as the reduction of oxygen present in the solution. The current corresponding to both the anodic and cathodic reactions B_a and B_c increases as a function of scan number and is not ob-

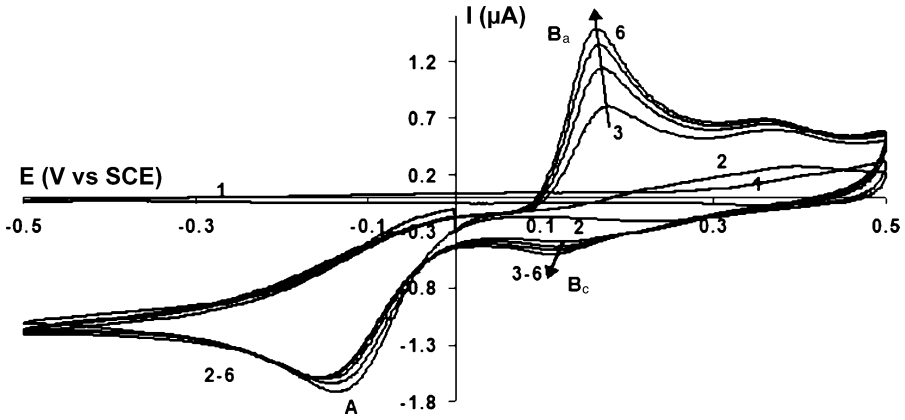

Fig. 1. Current-potential curves recorded at a gold electrode in a 10×10^{-3} mol L^{-1} Tris buffer solution pH 7.0 in the absence (*1*) and presence of oxygen (*2–6*) at a scan rate of 50 mV sec^{-1} and a temperature of 298 K. The graphs *2–6* represent successive voltammetric scans

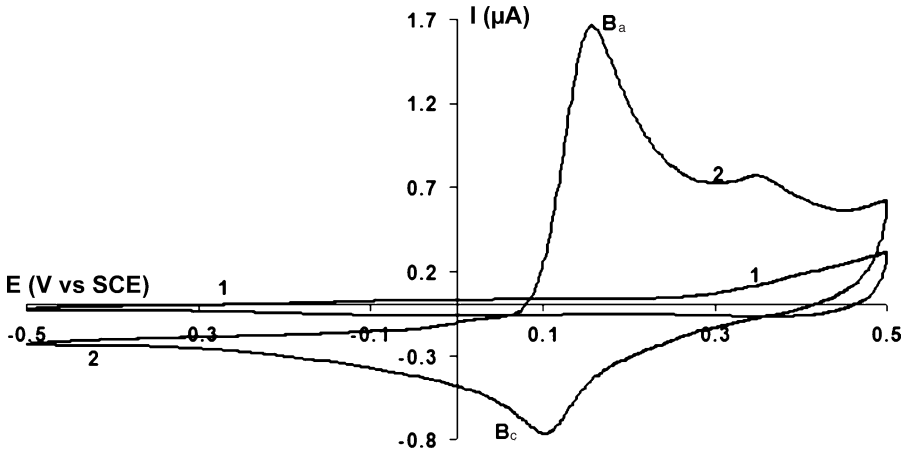

Fig. 2. Current-potential curves recorded at a gold electrode in a 10×10^{-3} mol L^{-1} Tris buffer solution pH 7.0 in the absence of oxygen (*1*) at a scan rate of 50 mV sec^{-1} and a temperature of 298 K. *2* was recorded after *6* (Fig. 1) and 20 min after flashing with nitrogen

served in the first scan. When oxygen is removed by flushing the headspace above the droplet with nitrogen right after the recording of curve 6, both redox processes are still observed (curve 2, Fig. 2). Curve 1 in Fig. 2 is identical to the one in Fig. 1 and represents the current potential behaviour of a nitrogen saturated Tris buffer solution at a gold electrode. The presence of peak B_a and B_c in curve 2 means that the compounds responsible for them are formed after contact between the buffer solution and oxygen. This effect is mentioned below as the 'oxygen effect'. A scan rate study is performed to investigate the nature of the process. The slope of the relationship between I_p and ν equals ca. 1, corresponding to a reaction of an adsorbed species; while a slope of 0.5 rather indicates a rate determining diffusion of a species towards the electrode surface. Analoguous results were obtained for a 10×10^{-3} mol L^{-1} HEPES buffer solution, for

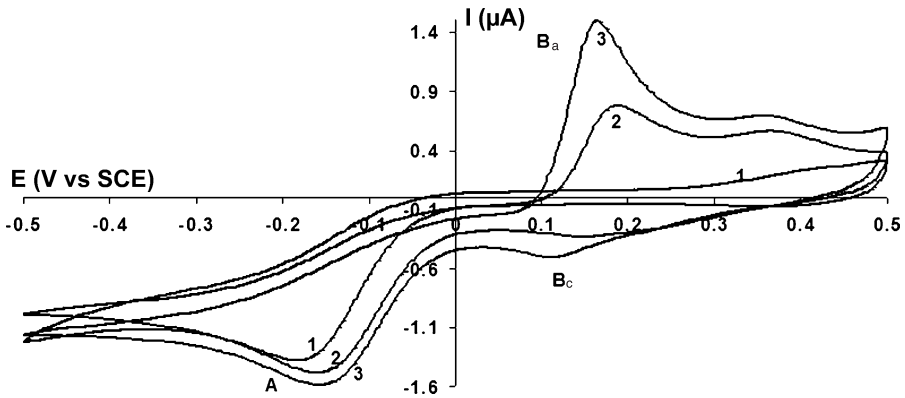

Fig. 3. Current-potential curves recorded at a gold electrode in a phosphate (*1*), 10×10^{-3} mol L^{-1} HEPES pH 7.0 (*2*) and 10×10^{-3} mol L^{-1} Tris pH 7.0 (*3*) buffer solution in the presence of oxygen at a scan rate of 50 mV sec^{-1} and a temperature of 298 K

which the oxidation peak B_a as well as the corresponding reduction peak B_c were observed (Fig. 3).

The oxygen effect can be explained by the fact that Good's buffers, such as Tris and HEPES, are not inert as was previously believed. They are able to generate N-oxides in the presence of molecular oxygen and Fe(II) and Fe(III)-polymers [16–18]. We presume that these compounds can react with oxygen radicals (superoxides) formed at the electrode, resulting in their N-oxide form. Oxygen radicals are formed during the reduction of oxygen at the end of scan 1, which is the reason why the processes B_a and B_c are not seen during the first scan. To confirm this hypothesis, a similar experiment was performed in a phosphate buffer solution (Na_2HPO_4/NaH_2PO_4, pH 7.0). The current potential behaviour of a 10×10^{-3} mol L^{-1} phosphate buffer solution at a gold electrode in the presence of oxygen is shown in Fig. 3 as curve 1. As this buffer solution can not form N-oxides, no additional peaks are seen in the cyclic voltammogram. Based on all these results, it can be concluded that oxygen should be kept out of the buffer solution when recording cyclic voltammograms in order to avoid the formation of radicals and oxidized buffer compounds, as the latter give rise to interfering adsorption redox processes. For compatibility with the optical cuvet activity assay [10], we choose to use a HEPES pH 7 buffer solution in a nitrogen atmosphere for all further experiments.

Electrochemical behaviour of HHC and RcCCP at a gold electrode

The voltammetric behaviour of a gold electrode modified with 4,4′-bipyridyl (bipy) in a 10×10^{-3} mol L^{-1} HEPES buffer solution pH 7.0 in the absence of oxygen is shown in Fig. 4 (curve 1). No oxidation or reduction process is observed. As expected [24], when HHC is added, a reversible redox behaviour appears. Curve 2 represents the redox processes of 12.2×10^{-6} mol L^{-1} HHC. The midpoint potential is 0.046 V vs SCE, corresponding to the oxidation and reduction of the heme group in the protein [24]. Interestingly, when 11.9×10^{-6} mol L^{-1} RcCCP is added to the latter (curve 3 in Fig. 4), the midpoint potential of the HCC redox process shifts downwards by 20 mV to 0.026 V vs SCE, which suggests the formation of a complex between HHC and RcCCP. RcCCP shows no additional oxidation or reduction peaks but makes HHC to become a more powerfull reductant. We recently demonstrated, using a bacterial two hybrid system, that soluble cytochrome c_2 from *Rhodobacter capsulatus* forms a complex with RcCCP [25], and hence can function as the electron donor for the latter as described before [26]. After fusing both proteins to two non-functional but complementing β-galactosidase truncations, the level of complemented β-galactosidase activity indicated that both cytochromes were interacting. Our voltammetric results therefore confirm the *in vitro* data that, although HHC is not the physiological electron-donor for RcCCP, it can substitute for *Rhodobacter capsulatus* cytochrome c_2 [10].

Determination of hydrogen peroxide at a RcCCP/HHC modified gold electrode

As shown in Fig. 5, HHC and RcCCP at a bipy modified gold electrode (curve 1) and in the presence of hydrogen peroxide (curve 2) yield catalytic electrochemical signals observed as reversible, sigmoidal

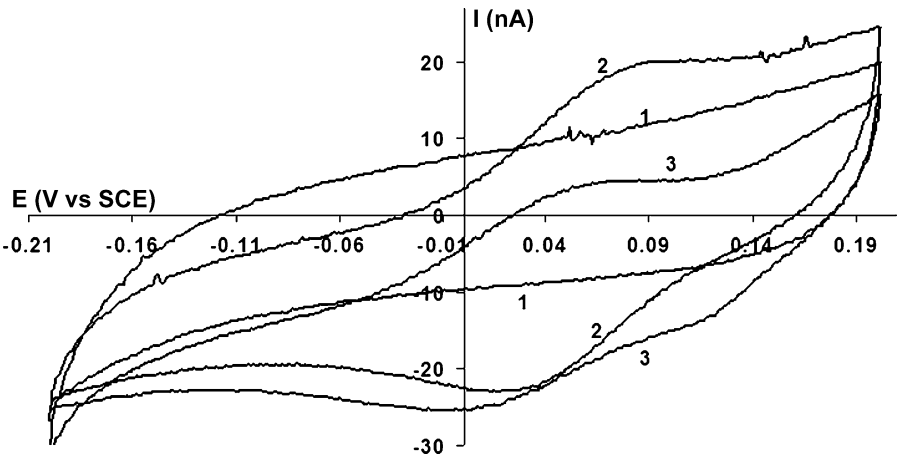

Fig. 4. Current-potential curves recorded at a gold electrode modified with 4,4′-bipyridyl in a 10×10^{-3} mol L^{-1} HEPES buffer solution pH 7.0 (*1*) containing 12.2×10^{-6} mol L^{-1} HHC (*2*) and 11.9×10^{-6} mol L^{-1} RcCCP added on top (*3*) at a scan rate of 50 mV sec^{-1} and a temperature of 298 K

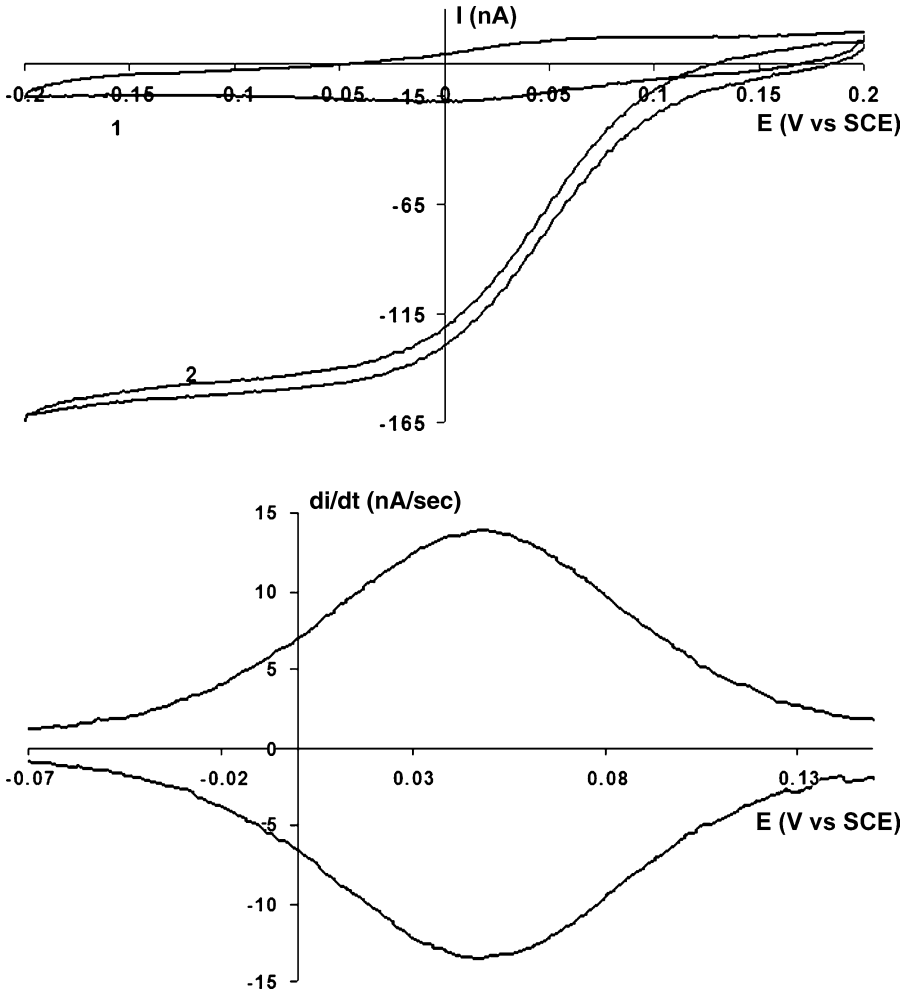

Fig. 5. Current-potential curves recorded at a gold electrode modified with 4,4′-bipyridyl in a 10×10^{-3} mol L^{-1} HEPES buffer solution pH 7.0 containing 12.2×10^{-6} mol L^{-1} HHC and 11.9×10^{-6} mol L^{-1} RcCCP (*1*) and 0.45×10^{-3} mol L^{-1} H_2O_2, added last, (*2*) at a scan rate of 50 mV sec^{-1} and a temperature of 298 K. Inset: derivate of the current as a function of the potential

waves. Such waves display a limiting current that corresponds to an enzymatic velocity, and are centered at a potential E_{cat}. As given in the inset of Fig. 5 the peaks are centered at an E_{cat} of 0.049 V vs SCE and the full widths at half-height are 90 mV, indicative for a one-electron rate-limiting step during catalysis. The E_{cat} is close to the midpoint potential of the high-potential heme of RcCCP, and also close to that of free HHC (Fig. 4, curve 2). However, and as said above, the potential of HHC is lowered by interaction with RcCCP (Fig. 4, curve 3), which suggests that E_{cat} represents a rate-limiting one-electron transfer to the high-potential heme in RcCCP. Therefore, this heme group functions as the electron relay site between HHC and the low-potential peroxidatic heme. The latter shows no direct electrochemical activity in the probed potential window.

The necessity of the presence of both HHC and RcCCP for the electrocatalytic reduction of hydrogen peroxide on a modified gold electrode was proven by the fact that no catalytic wave was observed when one of the compounds was deleted. In both cases, curve 1 of Fig. 5 is obtained. Only when HHC and RcCCP are both present, an electrocatalytic behaviour as shown in Fig. 5 (curve 2) is obtained.

The HCC/RcCCP system may offer potentials for selective peroxide determination because of the low overpotentials required, the decreased activity of the gold electrode by modification, and the intrinsic substrate specificity of the enzyme. It was also investigated whether hydrogen peroxide has any effect on the stability of any of the reaction partners. Figure 6 shows that upon recording subsequent voltammograms, the limiting current keeps on decreasing. The first and seventh voltammetric scan are presented by curves 2 and 3 respectively. The decrease in limiting current indicates that one of the compounds is chemically attacked by hydrogen peroxide. When a small amount of the substrate is now added to the droplet, curve 4 is obtained resulting from a recovery of the

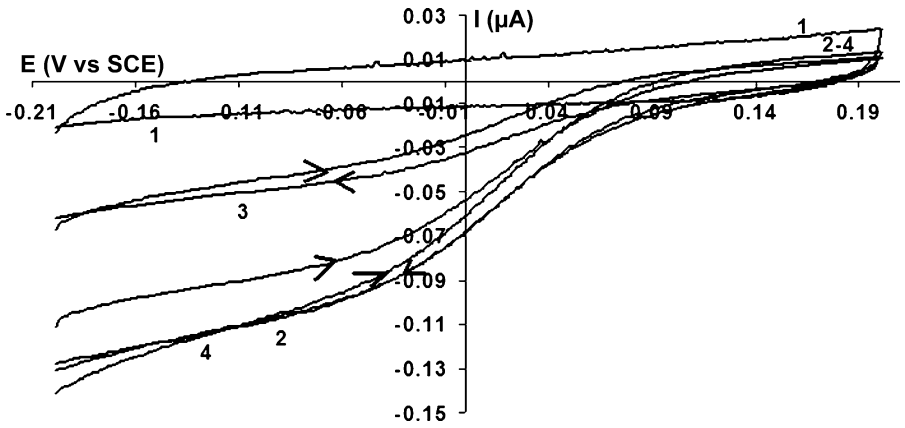

Fig. 6. Subsequent current-potential curves recorded under the same conditions as Fig. 5 but with H_2O_2 added after HHC and before RcCCP. *2* is the first scan, *3* the seventh. *4* is recorded after curve *3* and addition of 0.21×10^{-3} mol L^{-1} H_2O_2

electrocatalytic behaviour and thus of the limiting current. Analoguous curves as in Fig. 6 were obtained when HHC was added after the decrease in limiting current. These features indicate that it is HHC, and not the RcCCP enzyme, which is attacked by hydrogen peroxide. This is corroborated by the reported similar effect of hydrogen peroxide on yeast cytochrome *c* [27]. The smaller limiting current and small potential shift of the sigmoidal wave, compared to Fig. 5, can be explained by the fact that hydrogen peroxide was added in between the addition of HHC and RcCCP, and thus had the time to attack some of the HHC, whereas hydrogen peroxide was added to the solution after the addition of HHC and RcCCP in the case of Fig. 5. For the complex being able to effectively transfer electrons, the exposed edge of the heme of HHC is most likely at or near the docking interface thus shielding it from hydrogen peroxide. This would be analoguous to what is the case for the yeast cytochrome *c*/cytochrome *c* peroxidase complex as deduced from the crystal structure [24].

Conclusion

This article shows that oxygen should be kept out of the N-substituted buffer solution when performing cyclic voltammetry in order to avoid the formation of radicals and oxidized buffer compounds. The latter give rise to interfering redox processes.

A hypothesis could be formulated regarding the complexation between HHC and RcCCP. The midpoint potential of HHC is 0.046 V vs SCE, corresponding to the oxidation and reduction of the heme group in the protein. No additional oxidation or reduction peaks, but a downshift of the midpoint potential was observed when adding RcCCP.

Hydrogen peroxide could be detected at RcCCP/HHC modified gold electrodes (with 4,4′-bipyridyl, mercapto hexanol or mercapto propionic acid as linker). The necessity of the presence of HHC as well as RcCCP for the determination of hydrogen peroxide was proven. When one of the compounds is absent, no catalytic wave was observed.

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