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Evidence for Distinct Electron Transfer Processes in Terminal Oxidases from Different Origin by Means of Protein Film Voltammetry

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Supporting Information

ABSTRACT: Cytochrome aa_3 from *Paracoccus denitrificans* and cytochrome ba_3 from *Thermus thermophilus*, two distinct members of the heme−copper oxidase superfamily, were immobilized on electrodes modified with gold nanoparticles. This procedure allowed us to achieve direct electron transfer between the enzyme and the gold nanoparticles and to obtain evidence for different electrocatalytic properties of the two enzymes. The pH dependence and thermostability reveal that the enzymes are highly adapted to their native environments. These results suggest that evolution resulted in different solutions to the common problem of electron transfer to oxygen.

Terminal oxidases are the final complexes of the respiratory chain and catalyze the reduction of oxygen to water. Most oxidases are members of the heme−copper family because of their heterodinuclear active sites, which consist of a heme moiety and a copper center (Cu_B). The electrons for the reaction are supplied either by a quinol in the case of quinol oxidases or by a *c*-type cytochrome for cytochrome *c* oxidases (CcOs). One common feature of this family of proteins is the coupling between the redox reaction and the translocation of protons across the membrane.[1] The charge separation induced by the proton pumping and the reduction of oxygen leads to a transmembrane electrochemical gradient used by ATP synthase to phosphorylate ADP into ATP.

Heme−copper oxidases can be classified into three families (A, B, and C) on the basis of their amino acid sequence homologies. Among the distinctions between enzymes in these families are the nature and number of proton pathways and their oxygen affinities.[2] They exhibit different properties depending on the environmental conditions such as temperature and pH.[3] In contrast to mesophilic organisms such as *Paracoccus denitrificans*, organisms like *Thermus thermophilus* have to adapt to extreme temperatures. This adaptation not only concerns increased protein thermostability but also oxygen solubility, which is inversely proportional to temperature. Haltia et al.[4] reported that cytochrome aa_3 from *P. denitrificans* denatures at 67 °C, as shown by IR spectroscopy and calorimetry. In contrast, it was found that the Cu_A domain of cytochrome ba_3 oxidase from *T. thermophilus* is stable up to 100 °C, even at pH 4.[5] In addition, the mixed-valence state of cytochrome ba_3 has been shown to be different from the more classical cytochrome aa_3.[12] Differences in ligand binding properties have also been reported, including the high oxygen affinity of Cu_B found for the thermophilic organism.[6] Unfortunately, there are no structural studies available concerning the thermostability of the whole cytochrome ba_3.

Whether the CcOs all share a common catalytic mechanism is commonly discussed. Crucial differences in structure, proton pathways, and number of protons pumped have been identified.[7−11] Within these lines, differences could be expected when comparing the electrochemical properties of the ba_3 CcO from *T. thermophilus*, a member of the B family, with those of the aa_3 CcO from *P. denitrificans*, a member of the A family that includes all canonical residues as determined for mitochondrial oxidases.[2]

The complex cooperative behavior of the equilibrium potential of the hemes in mitochondrial CcOs has been described before in detail (see ref 12 for a review). Equilibrium measurements revealed two redox transitions, each including contributions from both hemes as a result of their cooperativity. When one heme changes its redox state, the second heme changes as well.[13,14] Upon addition of cyanide, the heme potentials are uncoupled, allowing the high-potential transition to be assigned to the heme *a*. Interestingly, the order of the potentials in the ba_3-type oxidases was found to be inverted compared with that in the aa_3-type oxidases: at equilibrium heme *a* from cytochrome aa_3 exhibits a higher potential than heme a_3,[15] whereas heme *b* from cytochrome ba_3 exhibits a lower potential than heme a_3.[16] Also, the pH dependence was found to be inverted.[17,18] However, all of these values were obtained at equilibrium.

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In order to study the importance for the electrocatalytic activity, it is interesting to perform protein film voltammetry experiments. In this method, the catalytic activity can be determined from the current observed upon variation of the potential in the presence of substrate.[19−22] The immobilization of membrane proteins such as CcO, however, is known to be an intriguing challenge because of their hydrophobic and amphiphilic nature.[7,8] Moreover, because the cofactors are buried in the polypeptide backbone, it becomes difficult to immobilize these proteins in such a way that they can perform direct electron transfer with satisfactory electron transfer rates.[23] Hence, only a few studies have been reported.[24−26] Because of their exceptional properties, gold nanoparticles are widely used to study soluble proteins.[23,27−30] In a preliminary study, we showed that these properties could also be exploited to perform a direct electron transfer between a terminal oxidase, cytochrome bo_3, and gold nanoparticles.[31] Herein we show that protein film voltammetry on gold nanoparticles can be used to discriminate catalytic mechanisms from terminal oxidases of different origins. In this paper, we compare the electrocatalytic properties of cytochrome aa_3 from *P. denitrificans* with those of cytochrome ba_3 from *T. thermophilus* at different pHs and temperatures. In conjunction with IR spectroscopy, we correlate the temperature behavior with structural modification occurring in the proteins.

Protein film voltammetry. Figure 1 shows a comparison of voltammograms of cytochrome aa_3 from *P. denitrificans* and cytochrome ba_3 from *T. thermophilus* immobilized on modified gold nanoparticle electrodes under turnover conditions (aerobic conditions) at different pH values. Measurements in the absence of O_2 are shown in the Supporting Information (SI). Gold nanoparticles with a diameter of 15 nm were drop-cast on the electrode surface to increase the surface area from 0.1 to 15 cm^2. Such a large specific surface area allows the immobilization of a large amount of protein on the electrode. As terminal oxidases are mostly hydrophobic, a functionalization composed of mercaptohexanol and hexanethiol was favored. Decreasing the amount of detergent in the protein samples resulted in a quite stable film of cytochrome aa_3 and cytochrome ba_3 on the surface. For further details on the experimental conditions, see the SI.

The sigmoidal shape of the curve reflects the electrocatalytic reduction of oxygen, where the intensity of the current includes information on the turnover.[32−34] As described by Léger et al.,[35] the slope of the limiting current gives insight into the distribution of orientations of the proteins at the electrode surface. Moderate slopes are observed for both proteins at pH 7, which suggests that the proteins have a rather homogeneous orientation distribution. The catalytic potentials at pH 7 for cytochrome aa_3 and cytochrome ba_3 were observed at −100 and +220 mV respectively, and the turnover frequencies for the immobilized proteins are in the range of 1 to 10 s^{-1} (details on these measurements are provided in the SI). The large difference in the reduction potentials should be considered in light of the fact that both proteins are multi-redox-site proteins. The potential measured for cytochrome aa_3 on the electrode surface is downshifted by approximately 350 mV compared with the value obtained in solution. This phenomenon was already observed by Friedrich et al.[36] The discrepancy was explained by a transition between a nonactivated state and an activated state corresponding to the protein working under turnover conditions.[37] Importantly, the large downshift in the potential was not observed for cytochrome ba_3 until pH 7.5. This major change may originate from the differences in the equilibrium potentials of each heme. For the aa_3 oxidase, the potential of heme *a* changes after the first electron transfer to allow for the second electron transfer. In contrast, the corresponding cofactor in the ba_3 oxidase, heme *b*, already exhibits a potential that allows the electrons to be transferred to the active site.[38] This inversion of potentials probably hampers the electron transfer to the active site and thus could be responsible for the overpotential observed in cytochrome aa_3.

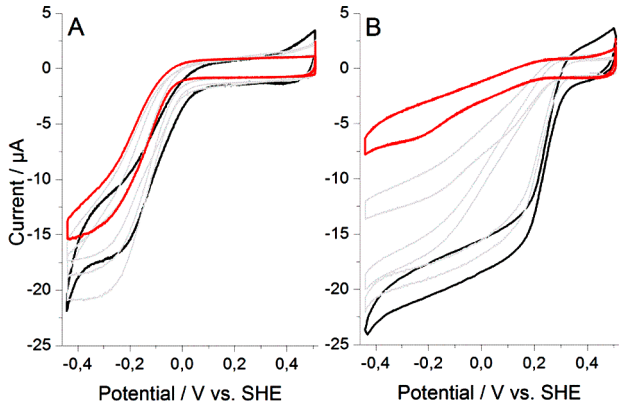

Figure 1. Voltammograms of (A) cytochrome aa_3 and (B) cytochrome ba_3 from pH 6.5 (black) to pH 8.5 (red) (ν = 0.02 V/s, 20 °C).

The electrocatalytic properties of cytochrome aa_3 (Figure 1A) exhibit a pH dependence of about 25 mV/pH unit. Thus, a large overpotential is expected over the entire pH range. In contrast, for cytochrome ba_3 the electrocatalytic oxygen reduction is highly pH-dependent (Figure 1B). The catalytic potential is observed at 220 mV at pH 6.5 and at 60 mV at pH 7.5. The catalytic current also dramatically decreases when the pH is increased. This observation can be correlated to the decrease and inversion of the difference in potentials between heme *b* and heme a_3 at different pH values.[17] Thus, the electron transfer between heme *b* and heme a_3 is impeded when the pH is increased, and as a consequence, the oxygen reduction efficiency is lowered. These results suggest that the relative order of the potentials of the binuclear center and the last electron donor plays a key role in the rate of oxygen reduction.[39,40] Our results are also consistent with the observation reported by von Ballmoos et al.[11] of a decrease in the formation rate of a key catalytic intermediate (O state) when the pH is increased. In summary, the study of the electrocatalytic reaction suggests that distinct catalytic mechanisms occur in the two proteins.

To further determine whether some of the differences observed may originate from the extreme environment where *T. thermophilus* thrives, we determined the electrocatalytic current for oxygen reduction as a function of temperature. Figure 2 shows the voltammograms of cytochrome aa_3 oxidase and cytochrome ba_3 oxidase at different temperatures in the presence of oxygen at pH 7. The temperature was varied between 10 and 70 °C, and the measurements were performed at 1000 rpm with a thermal equilibration time of 10 min. From 20 °C up to 40 °C, the electrocatalytic current of the cytochrome aa_3-modified electrode increased before decreasing at higher temperatures, whereas in the case of cytochrome ba_3 the current increased as the temperature increased from 25 to 45 °C and was stable between 45 and 70 °C. These results confirm that the thermophilic oxidase is significantly more stable and active at higher temperatures than the mesophilic counterpart. The increased turnover of the oxygen reduction at high temperatures indicates an evolution-adapted structure of the CcO.

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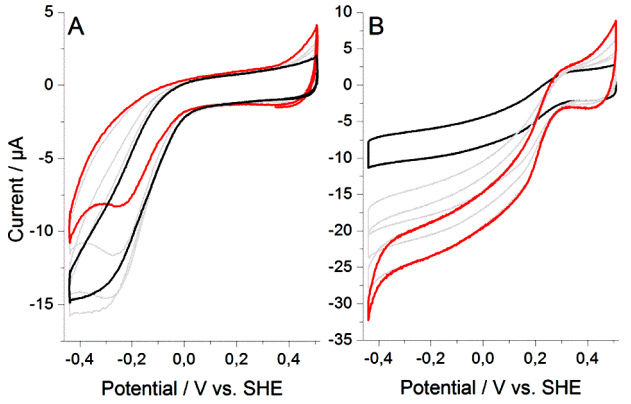

Figure 2. Voltammograms of (A) cytochrome aa_3 and (B) cytochrome ba_3 from 10 °C (black) to 70 °C (red) at pH 7 (ν = 20 mV/s).

Temperature-induced denaturation monitored by IR spectroscopy. The stability of the protein was confirmed by studies of the temperature dependence of the amide I band between 1700 and 1600 cm^{-1}, which includes the coordinates of the ν(C═O) vibration that are specific to the protein's secondary structure.[41] Figure 3 shows the evolution of the amide I bands of cytochrome aa_3 and cytochrome ba_3 oxidases obtained by monitoring the IR spectra from 15 to 75 °C. The measurements were performed in D_2O buffer because H_2O also contributes in this spectral region. Upon being heated, the mesophilic protein denatures at about 65 °C. At this temperature, a shoulder at approximately 1620 cm^{-1} appears and the signal at 1658 cm^{-1} is broader, indicating the presence of nonorganized secondary structure. The amount of α-helices decreases from 50% to 30% between 55 and 65 °C, indicating an increase in the amounts of disordered structure and β-sheets. This is in line with the transition temperature reported before.[4] Interestingly, the denaturation temperature obtained is about 65 °C, whereas the decrease in activity starts at 40 °C in protein film voltammetry. The loss of activity can be induced by subtle changes in tertiary structures or in the oxygen channel, which cannot be detected by IR spectroscopy.

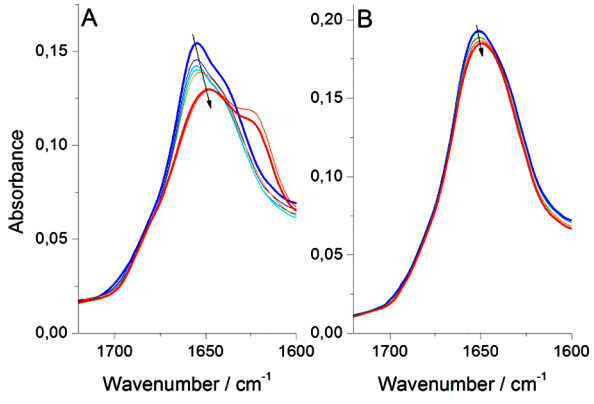

Figure 3. Evolution of the amide I bands of (A) cytochrome aa_3 and (B) cytochrome ba_3 from 15 to 75 °C in D_2O buffer at pD 7.

In contrast to cytochrome aa_3, the shape of the amide I band of cytochrome ba_3 remains unchanged over the entire temperature range, confirming that the cytochrome ba_3 remains stable even at high temperature. The thermostability of a protein is induced by several modifications, including a more rigid structure.[42] Interestingly, the oxygen channel of cytochrome ba_3 is known to be more hydrophobic than the channel in A-type oxidases.[9,43] Proteins showing predominantly hydrophobic interactions typically denature at higher temperatures than those showing more electrostatic interactions.[44]

Conclusion. In this study, we have for the first time used protein film voltammetry to gain new insight into the mechanism of terminal oxidases from different origins. Clear differences in the electrocatalytic properties, their pH dependence, and their thermostability were determined. Importantly, it can be concluded that the electron transfer between the two hemes is a crucial point for the catalytic reaction of oxygen. Within these lines it may be discussed that the oxygen reaction is optimized in the thermophilic enzyme, leading to the possibility of circumventing the low quantity of oxygen present in its native environment.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, voltammograms under noncatalytic conditions, Koutecky–Levich plots, characterization of the surface by IR spectroscopy, and deconvolution of the amide I band at 15 and 75 °C for each enzyme. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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