2D-SEIRA spectroscopy to highlight conformational changes of the cytochrome c oxidase induced by direct electron transfer†‡

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Potentiometric titrations of the cytochrome c oxidase (CcO) immobilized in a biomimetic membrane system were followed by two-dimensional surface-enhanced IR absorption spectroscopy (2D SEIRAS) in the ATR-mode. Direct electron transfer was employed to vary the redox state of the enzyme. The CcO was shown to undergo a conformational transition from a non-activated to an activated state after it was allowed to turnover in the presence of oxygen. Differences between the non-activated and activated state were revealed by 2D SEIRA spectra recorded as a function of potential. The activated state was characterized by a higher number of correlated transitions as well as a higher number of amino acids associated with electron transfer.

Introduction

The structure of large membrane proteins such as cytochrome c oxidase (CcO) is now well established by X-ray crystallography even in the reduced and oxidized states.5–4 These static images are valuable sources of information. However, structure-function relationships of such proteins require methods to monitor structural changes as a function of a perturbation. Time-resolved X-ray crystallography has recently been introduced but its applicability is still very limited.5 Conformational changes of proteins can be conveniently accessed by FTIR-spectroscopy as function of different kinds of perturbation such as time, pH, temperature etc.6,7 Particularly useful in this context is the possibility to manipulate even large complex multi-center redox proteins by electrochemical methods and to measure FTIR spectra as a function of potential.8 For example conformational transitions of the cytochrome c oxidase were revealed by potentiometric titrations followed simultaneously by UV/VIS and FTIR-spectroscopy.9–15 Functional groups characteristic of amino acids and secondary structures could be identified and a large number of vibrational modes could thus be assigned to particular redox transitions. In order to obtain this information, however, soluble redox compounds, so-called mediators had to be used to equilibrate the redox center with the electrode.10 Particularly interesting in this context seemed a surface architecture designed to facilitate direct electron transfer (ET) from the redox centers of the CcO to a gold electrode. FTIR spectra could then be measured at different redox states without the use of mediators. We had developed such a surface architecture for use with the CcO. The protein was bound via an NTA linker to a silver surface followed by reconstitution into a protein-tethered bilayer lipid membrane (ptBLM).16,17 Preliminary experiments employing surface-enhanced resonance Raman spectroscopy (SERRS) had shown that CcO could be reduced by electronic wiring provided the protein is immobilized in the orientation with CuA, the first electron acceptor, directed toward the electrode.18 However, attempts to measure time-resolved SERRS at a similar system resulted in an only extremely slow electron transfer, which was even unspecific with regard to the orientation.19 Later attempts to apply surface-enhanced IR absorption spectroscopy (SEIRAS) to a similar system did not even result in any electron transfer.20 This might have been due to an unsuitable packing density of the protein layer immobilized on the surface. A systematic study of the mixing ratio of the linker with a dilution molecule revealed a critical dependence of electron transfer kinetics from the packing density of the protein/lipid layer on top of the linker. Preparation

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conditions were thus optimized to exemplify the effective electronic wiring of the CcO from R. Sphaeroides with the his-tag attached to subunit II. Rate constants were determined by fast scan voltammetry. Modelling studies of the data revealed a four electron sequential pathway along the four redox centers CuA, heme a1, heme a3, and CuB. This was considered a closer mimic of the ET initiated by the natural substrate of the CcO, cytochrome c, than the one exemplified by mediators. This assumption was supported by SEIRA spectra recorded in the ATR-mode as a function of potential for activated (A) and non-activated (B) CcO immobilized via His-tag on subunit II. Potential applied was 900 mV vs. SHE for the fully oxidized state and varied from 500 mV to –700 mV in 100 mV steps for reduced states (bottom to top). No mediators added. Insets show spectra plotted on an expanded scale between 1300 and 1570 cm⁻¹.

Fig. 1 Difference spectra reduced–fully oxidized as a function of potential for activated (A) and non-activated (B) CcO immobilized via His-tag on subunit II. Potential applied was 900 mV vs. SHE for the fully oxidized state and varied from 500 mV to –700 mV in 100 mV steps for reduced states (bottom to top). No mediators added. Insets show spectra plotted on an expanded scale between 1300 and 1570 cm⁻¹.

Materials and methods

Materials and methods were used as described before, briefly a two layer gold surface was prepared on top of the ATR crystal of a (VERTEX 70 FTIR spectrometer, from Bruker, Karlsruhe) as well as the spectrometer. Materials and methods were used as described before, briefly a two layer gold surface was prepared on top of the ATR crystal of a (VERTEX 70 FTIR spectrometer, from Bruker, Karlsruhe) as well as the spectrometer.
and purified according to Ferguson-Miller et al.\textsuperscript{31} CcO was immobilized via his-tag technology on the two-layer gold surface functionalized with dithiobis (N-succinimidyl propionate) (DTSP) coupled to N-(5-amino-1-carboxypentyl) iminodiacetic acid and reconstituted by \textit{in situ} dialysis into a protein-tethered bilayer lipid membrane (ptBLM).\textsuperscript{22} Measurements under anaerobic conditions were performed in a buffer solution flushed with Ar containing K\textsubscript{2}HPO\textsubscript{4} 0.1 M, KCl 0.05 M, pH = 8 and the oxygen trap consisting of glucose (0.3% w/w), glucose oxidase (75 µg ml\textsuperscript{-1}) and catalase (12.5 µg ml\textsuperscript{-1}).

Spectra were analyzed using the software package OPUS 6 (Bruker Optics GmbH; Ettlingen). A linear baseline correction was performed between 2500 cm\textsuperscript{-1} and 1300 cm\textsuperscript{-1}. Spectra of the protein were corrected with respect to the substrate in buffer solution at the appropriate potential. In addition, the band at 1643 cm\textsuperscript{-1} due to water incompensation was subtracted from the respective spectrum of the protein. In order to be able to do the correction effectively, it was useful to keep the temperature of the spectro-electrochemical cell constant at 27 °C using a thermostat.

Two-dimensional infrared autocorrelation maps were constructed from a series of difference spectra as a function of potential using the software package 2Dshige\textsuperscript{6} Shigeaki Morita, Kwansei-Gakuin University, 2004–2005. Peak positions thus obtained were used to deconvolute the overlapping bands of the broad band as shown in Fig. 3A for the activated and 3B for the deactivated CcO.

Carbonylcyanid-\textit{m}-chlorophenylhydrazon (CCCP) was from Sigma.

\section*{Results and discussion}

Reduced-minus-oxidized and oxidized-minus-reduced SEIRA difference spectra of the CcO in the conventional 1D mode had been presented in ref. 22. Midpoint potentials derived from sigmoid functions of the absorbance vs. potential were in the range 200 to 500 mV corresponding to known midpoint potentials, but only from measurements performed in a strictly anaerobic solution, when the enzyme was considered to be in the non-activated state. In the presence of oxygen the enzyme was shown to be catalytically active as indicated by two peaks in the cyclic voltammogram at −137 mV and −600 mV (Fig. 2). These peaks have previously been attributed to repeated electron and proton transfer, characterized by the amplified current density. This statement was challenged by a control experiment in the presence of CCCP. The electron transfer peak shifted to even more negative potentials which was explained in terms of high-affinity binding sites for CCCP found in the CcO considered to modify electron transfer pathways (Fig. 2).\textsuperscript{13} Upon returning to the experiment without CCCP, however, under anaerobic conditions, the electron transfer peak persisted whereas the proton transfer peak disappeared as expected.\textsuperscript{22} Surprisingly, however, the electron transfer peak was shifted by more than 400 mV in the negative direction vs. known midpoint potentials. We deduced from these results that the enzyme undergoes a gradual transition from a non-activated to an activated conformational state when the enzyme, under aerobic conditions, passes through a number of redox cycles. This is equivalent to the transition from the resting to the pulsed state when the CcO reconstituted in liposomes is subjected to oxygen pulses. This was explained in terms of a conformational transition of the CcO, consistent with a change in the environment of the heme and Cu centers, in the course of which we are able to capture the “fast” or “pulsed” state of the enzyme known from independent studies. In order to characterize all of the redox transitions, a broad potential range was covered in the potentiometric titrations.

Spectra recorded in the reductive direction are presented in Fig. 1A and B in the activated and non-activated state, respectively. In both cases, spectra are dominated by broad positive bands in the amide I region. Smaller bands are found in the fingerprint region. Tentative band assignments were performed in ref. 22 on the basis of potentiometric titrations of the CcO in solution, which had been performed independently by several groups in the presence of mediators followed simultaneously by UV-VIS and FTIR spectroscopy. Specific changes in the UV-VIS spectra of the chromophores as a function of the redox state were used to assign vibrational modes in the IR to particular redox centers, even in the amide I region. Other criteria were the sigmoid potential dependence of the absorbance of these bands characteristic of a redox transition. Unspecific changes merely due to the electric field do not yield sigmoid dependencies. An attempt was made to separate such unspecific contributions.\textsuperscript{22}

Synchronous and asynchronous 2D-IR correlation maps (Fig. 3 and 4) were constructed from the SEIRA spectra shown in Fig. 1, separately for the amide I 1550–1750 cm\textsuperscript{-1} (Fig. 3A and B, Fig. 4A and B) and the fingerprint region 1300–1550 cm\textsuperscript{-1} (Fig. 3C and D, Fig. 4C and D), of the CcO in the activated (Fig. 3) and non-activated state (Fig. 4), respectively. They were used to deconvolute the 1D SEIRA spectra into single bands. Tentative band assignments are collected in Table 1. They were done similarly to ref. 22 mostly on the basis potentiometric tetrations of the CcO followed simultaneously by UV/VIS and FTIR spectroscopy.\textsuperscript{9,13,25} Data regarding single amino acids and secondary structures were also taken into account.\textsuperscript{33–35} However, due to the better resolution a higher number of bands could be considered.

The amide I region shows the characteristic band pattern of secondary structures, which can also be found in proteins...
without any redox function, particularly using 2D IR.\textsuperscript{33,34} For example the band at 1654–56 cm\textsuperscript{-1}/C\textsubscript{0} indicates the α-helical structure whereas the assembly of bands at 1618–1623, 1638–1641 and 1681–1684 cm\textsuperscript{-1}/C\textsubscript{0} is a characteristic feature of β-increased sheets. The same bands had been associated before\textsuperscript{9–12,25} with redox transitions of heme a\textsubscript{3} and Cu\textsubscript{A} of CcO. Sigmoid functions could be constructed from 1D spectra for the bands at 1623 and 1655 cm\textsuperscript{-1}/C\textsubscript{0}.\textsuperscript{22} 2D spectra should not be used for the same purpose because quantitative analysis should not be performed on deconvoluted spectra.\textsuperscript{36} The band at 1600–1607 cm\textsuperscript{-1}/C\textsubscript{0} has usually not been associated with the β-sheet structure. However, on the basis of potentiometric titrations of the CcO this band was attributed to the redox transition of Cu\textsubscript{A}, located in the middle of the β-sheet structure within the aqueous domain of the protein. Moreover, sigmoidic functions could be constructed from this band under conditions of direct and mediated ET.\textsuperscript{22}

Fig. 3 2D correlations maps of CcO activated, synchronous (A, C) and asynchronous (B, D) in the amide I (A, B) and fingerprint region (C, D) from SEIRA difference spectra reduced–fully oxidized for CcO immobilized via His-tag on subunit II (the lower panel shows correlations maps of a series of spectra as a function of potential). Potential applied was 900 mV vs. SHE for the fully oxidized state and varied from 500 mV to −700 mV in 100 mV steps for reduced states. No mediators added. The upper panel shows one of the 1D-SEIRA difference spectra (taken at −700 mV) as an example for the deconvolution.

The above mentioned band pattern was analyzed using the procedures developed by Noda\textsuperscript{26,27,37} in order to understand the difference between the activated state (AS, Fig. 3) and non-activated state (NAS, Fig. 4) of the CcO. For example the autocorrelation peaks or diagonal peaks of the synchronous spectra (Fig. 3A and 4A) at 1618–1623, 1638–1641, 1654–56 and 1681–1684 cm\textsuperscript{-1}/C\textsubscript{0} indicate strong correlations between amide I vibrations characteristic for α-helices (1654–1656 cm\textsuperscript{-1}/C\textsubscript{0}) and β-sheets (1600–1607, 1618–1623, 1638–1641 and 1681–1684 cm\textsuperscript{-1}).\textsuperscript{33,34} Hence, in both states of CcO, there are substantial changes of structural elements characteristic for the protein backbone in the IR spectra upon oxidation/reduction. Importantly, the spectroscopic changes are different for the AS and NAS of CcO. For example, in the AS, the spectroscopic changes are larger for the band due to α-helical structure (1654–1656 cm\textsuperscript{-1}/C\textsubscript{0}) relative to the changes in the bands due to β-sheet structure. The less prominent band of the α-helices in the NAS indicates a larger distance from the surface (Fig. 4A). From this it is concluded that upon activation the β-sheet structure is drawn closer to the hydrophobic domain so that the distance to the α-helical domain becomes smaller. The deconvolution of the amide I band also revealed

Fig. 4 2D correlations maps of CcO non activated, synchronous (A, C) and asynchronous (B, D) in the amide I (A, B) and fingerprint region (C, D) from SEIRA difference spectra reduced–fully oxidized (reductive titration) for CcO immobilized via His-tag on subunit II. (the lower panel shows correlations maps of a series of spectra as a function of potential). Potential applied was 900 mV vs. SHE for the fully oxidized state and varied from 500 mV to −700 mV in 100 mV steps for reduced states. No mediators added. The upper panel shows one of the 1D-SEIRA difference spectra (taken at −700 mV) as an example for the deconvolution.
Table 1  Tentative band assignments of the spectra after deconvolution by 2D correlation mapping

<table>
<thead>
<tr>
<th>Literature</th>
<th>Act.</th>
<th>Non-act.</th>
<th>Redox center</th>
<th>Redox state</th>
<th>Tentative assignment</th>
<th>Reference</th>
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<tr>
<td>1746–1747</td>
<td>1742</td>
<td>—</td>
<td>Heme a₃</td>
<td>Ox</td>
<td>Glu-COOH; ν(C=O); E286</td>
<td>9 and 10</td>
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<td>1700</td>
<td>1700</td>
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<td>1682</td>
<td>1681</td>
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<td>1656</td>
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<td>Ox</td>
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<td>1614–1642</td>
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<td>Formyl, ν(C=O), amide I β-sheet, ν(C=O)</td>
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<td>1620</td>
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<td>1600</td>
<td>1607</td>
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<td>1586</td>
<td>—</td>
<td>Cu₄</td>
<td>Ox</td>
<td>HisH; ν(C–C); H260, H214; Arg-H₂⁺; νₛ(CN₂H₅⁺); R481; Asp-COO⁻; νₛ(COO⁻); D214</td>
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<td>Heme</td>
<td>10</td>
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<tr>
<td>1561</td>
<td>1565</td>
<td>—</td>
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<td>Ox</td>
<td>Trp-NH; ν(CC), δ(CH); W172, propionate</td>
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<td>Heme ν₃ₜₜ;</td>
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<td>1515</td>
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<td>1517</td>
<td>?</td>
<td>?</td>
<td>Y</td>
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<td>—</td>
<td>1510</td>
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<td>Red</td>
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<td>1412</td>
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<td>1389</td>
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<td>9 and 10</td>
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</table>

that the band widths of the bands at 1681–1682, 1654–1656, 1620–1623 and 1600 cm⁻¹ are the same as requested for a number of overlapped bands, but only for the AS (Fig. 3a). This is different from the NAS, where the bands at 1607, 1620, 1681 cm⁻¹ assigned to the β-sheet structure are shown to adopt a larger bandwidth (Fig. 4a). A large bandwidth generally indicates exposure to water and an increased hydrophilic environment, in this case of the β-sheet structure. Considering the hydrophobic and hydrophilic surfaces in the X-ray structure of the CcO from R. Sphaeroides38 (Fig. 5), the change to a more hydrophobic environment of the β-sheets is consistent with the conclusion mentioned above of the β-sheets being folded back or drawn closer to the α-helices during transformation into the AS.

Bands indicating α-helices (1654–1660 cm⁻¹) present in FTIR spectra of the CcO were attributed to redox transitions of Cu₄ and heme a₃ (1651 and 1655 cm⁻¹), respectively, whereas bands characteristic of β-sheets (1600–1601, 1618–1623, 1682 cm⁻¹) were attributed mostly to Cu₄ (e.g. 1603, 1684 cm⁻¹) and heme a₃ (1618 cm⁻¹).9 Bands indicating heme a are very close (e.g. 1546, 1661 cm⁻¹) to those attributed to Cu₄ and heme a₃. So the discrimination between these three redox centers on the basis of IR spectra is problematic.

Fig. 5 Chimera image of the hydrophobic (red) and hydrophilic (blue) surfaces of the X-ray structure of CcO from R. Sphaeroides.

Nevertheless, these prominent bands are strongly correlated with smaller bands from single amino acids, particularly in the AS. For example the bands at 1586 (1592 in ref. 9) representing the amino acids H260, H217, R481, D214 are associated with Cu₄ whereas the band at 1565 cm⁻¹ (1561 in ref. 9) representing W172 and the heme propionate9,10 are associated with heme a₃.
The histidines that coordinate CuB were thought to play a role in proton translocation by the histidine cycle. Heme a₃ propionates were considered to share a proton with aspartate D407 and thus play a role of and in the regulation of proton pumping. Multi-Conformation Continuum Electrostatics (MCCE) calculations, however, show that their pKa₃s are well below pH = 7 so that they do not bind protons on heme a₃ reduction. The negative band at 1742 cm⁻¹, which was assigned in ref. 13 to the protonated form of glutamic acid (E286) and/or an aspartate, was associated with the reduction of heme a 15,42 E286 as the terminal amino acid of the D-proton-channel is considered to play a central role in proton pumping, although MCCE calculations indicate that it also does not act as proton acceptor. Nevertheless, the pumped protons of all the redox transitions in the oxidative part of the enzyme cycle are transferred via E286 43,44 (Fig. 3A and 4A).

The band at 1700 cm⁻¹ was assigned to an amide I β-turn associated with heme a₃. For infrared absorption bands of amino acid side chains in general see also. Amino acids detected by SEIRAS in the amide I region of the AS and NAS, respectively, are depicted in Fig. 6A and B, for comparison purposes.

Different information is obtained if we examine the peaks off the diagonal line, the so-called cross peaks. According to Noda 27,37 the presence of cross peaks suggests the possibility of the existence of inter- and intramolecular interactions among functional groups. Synchronous cross peaks are generated when two dipole-transition moments associated with molecular vibrations of different functional groups are reoriented simultaneously. Synchronous correlation squares can be constructed between two or more of such bands. For example the AS exhibits correlation squares between 6 bands at 1742 cm⁻¹ (due to E286), 1565 (α-helices, heme a₃), 1620, 1600 (β-sheets, Cuₐ), 1586, 1565 cm⁻¹ (H260, H217, D214 and W172, and the heme propionates). They are all assigned to redox reactions of the hemes, whereas an independent square exists between 1704, 1682 cm⁻¹ (Fig. 3A), the latter one is assigned to the amide I vibration of α-helices and β-sheets in conjunction with Cuₐ. In the NAS separate correlation squares can be constructed between 1681 and 1639 cm⁻¹, between 1639 and 1607 cm⁻¹ (all of them β-sheets, Cuₐ), and between the bands at 1586 and 1565 cm⁻¹ (W172 and heme propionates) (Fig. 4A), whereas the band at 1742 (E286) is not included in the NAS and the band at 1586 cm⁻¹ (H260, H217, D214) is shifted to 1574 cm⁻¹. The reduced number of correlation squares underlines the reduced cooperativity in the NAS as compared to the AS.

Another type of information obtained from the cross peaks of synchronous 2D IR correlation spectra is the knowledge of the relative reorientation direction of electric dipole-transition moments. Cross peaks in the AS at 1600 and 1742 cm⁻¹ in the synchronous 2D IR spectra are positive while those at 1565, 1586, 1623 cm⁻¹ are negative indicating an identical or opposite reorientation direction of the respective groups (Fig. 3A). In the NAS, on the other hand, the groups at 1607, 1620 and 1639 cm⁻¹ reorient in the same direction, whereas the groups at 1565 cm⁻¹ (H260, H217, D214, W172 and the heme propionates) orient in the opposite direction (Fig. 4A). This is clearly visualized particularly in a 3D presentation of the 2D SEIRA spectra of the amide I region (Fig. 7A and B). The groups H260, H217, D214, W172 and the heme propionates were discussed above to play a possible role in proton translocation. Hence their electric dipole-transition moments not in line with those of the secondary structures could be an indication of the inactive state (Fig. 8).

Finally, the direction of these cross peaks in the asynchronous reveal information about the sequential order of the conformational transitions as a function of potential of different amino acids and secondary structures of peptide groups. Cross peaks in the asymmetric spectra of every two bands in Fig. 3B and 4B were analyzed according to the rules of Noda 47 and compared with the direction of cross peaks in the synchronous spectra (Fig. 3A and 4A). This way...
Moreover, in the AS protonations and deprotonations of amino acids always happen before the secondary structures, for example propionate, W172, and D 214. Tryptophan W280 (W272 in CcO from P. denitrificans) was discussed as a possible source for a radical species of tyrosin (Y288). Additional protonations and deprotonations are also found at R 481, H 217, H 260, R 481. These are arginine and the histidines coordinated with CuA, which are not present in the spectra of the NAS, see also amino acids recognized by SEIRAS in the amide I region of the AS and NAS are depicted in Fig. 6A and B, respectively.

Considering the fingerprint region, the overall pattern is the same, although more sequential events can be discriminated with respect to single amino acids rather than secondary structures, see Table 2 in the ESI; as well as Fig. 3C and D, Fig. 4C and D. A much larger number of autocorrelation peaks as well as more interdependent synchronous correlation squares in the synchronous spectra of the AS (Fig. 3C) than in the NAS (Fig. 4C). They indicate a considerable higher number of strongly correlated transitions of single peptide groups of the AS. This is supported by a higher number of cross peaks in different directions in the AS (Fig. 3C and D) compared to the NAS (Fig. 4C and D), indicating dipole moments in different directions. This indicates substantial reorientations not only in the region of secondary structures but also with respect to single peptide groups. This is reflected particularly also in the 3D representation of the 2D SEIRA spectra in the fingerprint region. (Fig. 7C and D). Tentative band assignments were again done on the basis of equilibrium titrations, see Table 1. Specifically, a number of amino acids associated with reduction of CuB are found, particularly in the AS with bands at 1327–30, 1450, 1481–83, 1495 cm\(^{-1}\), where the band at 1450 and 1495 cm\(^{-1}\) is missing in the NAS. Specifically, they represent W280, D407, T352, T48, H334, H333, H284. H284 was considered crosslinked to Y288, particularly under turnover conditions, where it is supposed to play a role in O–O bond splitting. Interestingly both amino acids are observed here even under anaerobic conditions. These bands are strongly correlated with practically all of the bands in the spectrum of the AS (see the cross correlation peaks in Fig. 3C and D), for example with the other histidines H260, H 217, represented by the band at 1424 cm\(^{-1}\). In both states there is a change in W280 mentioned above as shown by the band at 1327–30 cm\(^{-1}\). This tryptophan (W272 in P. denitrificans) was discussed as an electron donor under Fig. 9 Amino acids in the fingerprint region recognized by 2D SEIRAS in the amide I region of the AS and NAS are depicted as in Fig. 6A and B, respectively.

![Fig. 7](image1)

**Fig. 7** 3D representation of 2D SEIRA spectra (synchronous) in the amide I region (A) in the AS (B) in the NAS of the CcO, in the fingerprint region (C) in the AS (D) in the NAS of the CcO.

![Fig. 8](image2)

**Fig. 8** Amino acids indicated according to 2D SEIRAS by the band at 1565 cm\(^{-1}\), whose electric dipole-transition moments in the AS are in line with those of the secondary structures, whereas in the NAS they are in the opposite orientation.

the sequential order of a large number of correlated peaks was isolated and collected in Table 1 and 2 in the ESI; Tentative band assignments of these peaks were done as described above. For example the cross peak between 1565 and 1654 in the spectrum of the AS (Fig. 3B) is positive in the upper left corner of the spectrum. Hence the amide I band of \(\alpha\)-helices 1654 would change at a less negative potential than the 1565 band of the amino acids W172 and propionate (rule No. 1). However, since the respective cross peak in the synchronous spectrum (Fig. 3A) is negative, the sequence is reversed. Hence in the AS the amino acids W164 and propionate change at a less negative potential than the amide I band of \(\alpha\)-helices (rule No. 2). By contrast in the NAS no cross peak can be seen at 1558 cm\(^{-1}\) in the asynchronous spectrum. Hence no sequential order can be attributed to other bands.

Other differences between AS and NAS can be deduced from Table 1 and 2 of the ESI; Generally in the AS, there is a much larger number of correlated bands occurring simultaneously at the same potential compared to the NAS. Sequential events are also more frequent. These findings indicate a more concerted action of groups when the enzyme is in the AS.
fingerprint region of the spectrum of the AS compared to the NAS (Fig. 9A and B, respectively) is also clearly seen in the 3D representation (Fig. 7C and D).

**Conclusion**

Direct ET had allowed us to demonstrate the CcO to be catalytically active under our experimental conditions. Current densities in the order of magnitude of 100 μA cm⁻² were measured in the presence of oxygen. Proton pumping could also be detected. Electrochemistry combined with SEIRA spectroscopy had indicated that the enzyme undergoes a gradual transition from a non-activated to an activated state when the enzyme, under aerobic conditions, passes through a number of redox cycles. This is equivalent to the transition from the resting to the pulsed state when the CcO reconstituted in liposomes is subjected to oxygen pulses. The two states were assumed to represent two different conformations of the enzyme. However, conventional 1D SEIRA spectra of the two states were not markedly different. With the present investigation we could demonstrate unequivocally that two different conformations can be allocated to the two states. Differences can be clearly recognized by 2D-SEIRAS, particularly considering reduced-minus oxidized difference spectra changing as a function of potential.

Importantly, the activated state is characterized by a higher number of strongly correlated conformational transitions. This suggests that the enzyme under turnover conditions undergoes a more global conformational transition than hitherto expected. This is consistent with the considerable conformational change in response to reduction found by X-ray crystallography, which was proposed to be of functional significance.⁴ In this context it should be mentioned that the activated state of the CcO is also generated under reducing conditions.

The result of these conformational transitions seems to be a more condensed form of the CcO, thus optimizing tunnel pathways for electrons and protons. The assumption of a more condensed form of the AS is consistent with several observations in 2D SEIRAS such as the narrower bandwidth and higher amplitude of bands characteristic for α-helices vs. those of the β-sheets. But above all, the global conformational change is consistent with the shift in the midpoint potentials of the CcO in the AS revealed by both cyclic voltammetry and potentiometric titration.⁷⁷ Midpoint potentials were shown to depend strongly on the environment of the heme structure in the case of P450, which is characterized by an enzyme cycle very similar to the CcO.⁵¹,⁵² Midpoint potentials in the same range were observed by cyclic voltammetry of P450. Eₘₒₚ of hemes spanning several hundred millivolts were calculated depending on the environment of the molecule.⁵³

The large number of correlations in 2D IR spectra also suggest a high degree of cooperativity between single transitions, particularly in the AS. Correlations were found in the case of H bridges, hydrophobic interactions, dipole interactions, which are well known to exhibit cooperative behaviour. This is in line with the hysteresis found in the sigmoid dependences of the band amplitudes from potential.²² Hysteresis is another expression of cooperativity, which has long been considered to play a role in membrane transport.⁵⁴

Another significant finding concerns the number of amino acids recognized by 2D-IR, which is considerable higher in the case of the activated CcO. Conformational changes of the following amino acids are found in the AS but not the NAS: E 286, D214, H217, H260, R481, H333, H334, H284, W143. E286 in particular was considered essential for proton pumping. However, the measurements presented here were conducted under strictly anaerobic conditions when proton pumping is totally excluded. This could be demonstrated before by electrochemical measurements.²² Amino acids from the D and K channel were not indicated under these conditions, see Table 1. They become apparent, however, when the enzyme works under oxygenated conditions.
(result not shown). Another interesting finding concerns the amino acids Y288 and W143 found in both the AS and NAS. Y288 was considered as part of the H284-Y288 crosslinked pair responsible for -O=O- bond splitting. However, it is recognized here also under conditions where higher oxidized states must be absent. The tryptophan W143, on the other hand was considered involved in electron transfer from Cyt c to CuA. It could play a similar role in the direct ET from the electrode to CuA.

2D IR has recently attracted considerable attention revealing information about structure-function relationships of small molecules. It seems still more promising in the case of large complex proteins such as the CoO.

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Notes and references