Similarities and dissimilarities in the structure-function relation between the cytochrome *c* oxidase from bovine heart and from *Paracoccus denitrificans* as revealed by FT-IR difference spectroscopy

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Abstract The redox dependent changes in the cytochrome *c* oxidase from bovine heart were studied with a combined electrochemical and FT-IR spectroscopic approach. A direct comparison to the electrochemically induced FT-IR difference spectra of the cytochrome *c* oxidase from *Paracoccus denitrificans* reveals differences in the structure and intensity of vibrational modes. These differences are partially attributed to interactions of subunits influencing the heme and protein modes. In the spectral regions characteristic for ν(C=O) and ν(COO$^-$)$^{s/as}$ modes of protonated and deprotonated Asp and Glu residues, additional signals at 1736, 1602 and 1588 cm^{-1} are observed. On this basis, the possible involvement of Asp-51, a residue specifically conserved in mammalian oxidase and previously proposed to show redox depended conformational changes in the respective X-ray structures, is critically discussed.

Key words: Bovine heart; Cytochrome *c* oxidase; UV-VIS spectroscopy; FT-IR spectroscopy; Protein electrochemistry; *Paracoccus denitrificans*

1. Introduction

Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain and catalyzes the stepwise reduction of oxygen to water. In the course of this process, electron and proton transfer are efficiently coupled to contribute to the formation of an electrochemical proton gradient which drives ATP synthesis. (For recent reviews, see [1,2]). Four redox-active cofactors are involved in electron transfer: Cu_A, Cu_B, hemes *a* and a_3. Two putative pathways for pumped and consumed protons, the K- and D-pathway, have been discussed. A third pathway, the so called H-pathway, was recently suggested [3]. The crucial residue in this proposed pathway, Asp-51, is only conserved in cytochrome *c* oxidase from animals.

Cytochrome *c* oxidases consist of two essential subunits (I and II) which host the redox-active sites and catalyze electron and proton transfer. A varying number of additional subunits can be found in mitochondrial (up to 11) and bacterial (up to two) oxidases. The structure of the oxidized beef heart cytochrome *c* oxidase (with 13 subunits) [4,5] as well as of the reduced form [3] has been reported. In the case of the cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans*, which consists of four subunits, the structure of the four subunit enzyme [6], as well as that of the two subunit complex [7], was determined.

A surprisingly high degree of structural similarity of the subunits I, II and III between the bacterial and the mitochondrial enzymes has been observed. Biochemical and biophysical studies indicate analogous catalytic function and proton transfer mechanisms for the bacterial and mammalian oxidases (see [8]). Nevertheless, significant differences have been proposed on the basis of recent structural data of the reduced and oxidized form of the enzyme [3].

In order to elucidate these differences, a combined electrochemical and FT-infrared (IR) spectroscopy approach is used here to study the redox dependent alterations in the cytochrome *c* oxidase from bovine heart. A comparison to the electrochemically induced FT-IR difference spectra of cytochrome *c* oxidase from *P. denitrificans* is presented on the basis of a number of previous approaches [9–13]. The entity of difference signals in the electrochemically induced FT-IR difference spectra represents the total of molecular changes concomitant with the redox reactions, compromising conformational changes of the backbone, from amino acid side chains, protonation processes and charge redistribution in the cofactor sites. These signals are presented and discussed in this manuscript to assess differences between both oxidases.

2. Materials and methods

2.1. Sample preparation

Bovine heart oxidase was prepared as previously described by Soulimane and Buse [14].

For electrochemistry, the protein samples were diluted in 200 mM phosphate buffer (pH 7) containing 100 mM KCl and 3.2 mM *n*-decyl-β-D-maltopyranoside and concentrated to approximately 0.3 mM using Microcon ultrafiltration cells (Millipore). Exchange of H_2O against D_2O was performed by repeatedly concentrating the enzyme and re-diluting it in a D_2O buffer. H/D exchange of amide protons was found to be better than 65% as judged from the shift of the amide II mode at 1550 cm^{-1} in the FT-IR absorbance spectra (data not shown).

2.2. Electrochemistry and FT-IR spectroscopy

The ultra-thin layer spectroelectrochemical cell for the UV-VIS and IR was used as previously described [15]. The gold grid working electrode was chemically modified with a 2 mM cysteamine solution. In order to accelerate the redox reaction, 16 different mediators were added to a final concentration of 45 μM each. For experimental details, see [10]. Potentials quoted with the data refer to the Ag/AgCl/3 M KCl reference electrode; add +208 mV for SHE′ (pH 7) potentials. Electrochemically induced difference spectra were recorded and processed as previously described in [13].

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3. Results and discussion

3.1. Electrochemically induced FT-IR difference spectra in H_2O and D_2O

Fig. 1 shows the oxidized minus reduced FT-IR difference spectra of the cytochrome *c* oxidase from bovine heart for the potential step from the fully reduced (−0.5 V) to the fully oxidized state (0.5 V) equilibrated in H_2O (solid line) and D_2O (dotted line) buffer.

In the amide I range (1680–1620 cm^{-1}), strong signals at 1684, 1662, 1646 and 1620 cm^{-1} indicate absorbance changes arising from alterations of C=O groups from the polypeptide backbone. These difference signals can be related to subtle perturbations in the structure upon the redox process. In addition, contributions from the formyl group of heme *a* and a_3, the heme propionates and from individual amino acid side chains (Asn, Gln and Arg) are expected in this spectral region. In the spectral region from 1560 to 1520 cm^{-1} (the amide II range), contributions from coupled CN stretching and NH bending modes are expected. An assignment for all these signals to amide II modes is not very realistic, since the majority of the bands do not show the expected strong shifts upon H/D exchange. In addition to amide II signals, vibrational modes from aromatic amino acid side chains and heme C=C groups also contribute here, as well as antisymmetric COO^- modes caused by protonation/deprotonation or perturbation of COO^- groups (Asp, Glu and heme propionate modes). At 1748 and 1736 cm^{-1}, two positive signals, correlating with the oxidation of the enzyme, can be seen. In the spectral region above 1710 cm^{-1}, exclusively contributions from protonated Asp and Glu side chains are expected. A down shift of 8–10 cm^{-1}, characteristic for COOH groups upon H/D exchange, is depicted in the inset in Fig. 1. We note a change in intensity of the signals upon H/D exchange, which may indicate a difference in the local pK_A value in D_2O.

Tentative assignments in the IR spectra of the bovine heart oxidase can be derived for protein modes from the comparison with the characteristic modes of secondary structure elements (e.g. [18]) or isolated amino acids [17]. Further tentative attributions may be suggested on the basis of the comparison with resonance Raman data, like for example for the heme formyl substituent [16]. While for the electrochemically induced FT-IR difference spectra of the cytochrome *c* oxidase from *P. denitrificans* band assignments could be substantiated on the basis of specific ^{13}C labelling at the COOH group of the heme propionates [11] and of site-directed mutations [10], the assignments for the bovine enzyme remain tentative. Table 1 lists these assignments together with the data previously published for the cytochrome *c* oxidase from *P. denitrificans* in [10,13].

3.2. Comparison to the electrochemically induced FT-IR difference spectra of cytochrome c oxidase from P. denitrificans

Fig. 2 shows the direct comparison of the electrochemically induced FT-IR difference spectra of the cytochrome *c* oxidase

Table 1

Bovine heart	Redox state	*P. denitrificans*	Redox state	Tentative assignments
1748	Ox	1746	Ox	ν(C=O) Glu-278 (pox)/Glu-242 (box)
1736	Ox	1734	Red	ν(C=O) Glu-278 (pox)/Glu-242 (box)
				Asp-51
				Asp-399 (pox)/Asp-264 (box)?
1702	Ox	1708	Ox	ν(C=O) Asp/Glu
1694	Red	1694	Red	ν(C=O) Asp/Glu
				Amide I (β-sheet)
1684	Ox	1688	Ox	Amide I (β-sheet)
		1684	Red	Amide I (β-sheet, loops)
1672?	Ox	1676	Ox	ν(C=O) CHO heme a_3
				ν(C=O) heme propionates
				ν(C=O) Asn/Gln
				$\nu(CN_3H_5)^{as}$ Arg
1662	Red	1662	Red	ν(C=O) CHO heme a_3
				Amide I (α-helical)
				$\nu(CN_3H_5)^{as}$ Arg
		1656	Ox	Amide I (α-helical)
1646	Ox	1644	Ox	ν(C=O) CHO heme *a*
1630	Red	1632	Red	$\delta(NH_2)$ Asn/Gln
				$\nu(CN_3H_5)^{s}$ Arg
				Amide I (β-sheet)
1602	Ox			$\nu(COO^-)^{as}$ Asp/Glu
1608?	Red	1606	Red	ν(C=O) CHO heme *a*
				ν_{37} heme *a*
1588	Red			$\nu(COO^-)^{as}$ Asp-51
1584	Ox	1588	Ox	ν_{37} heme a_3
1566	Ox	1564	Ox	ν_{38x} heme a_3
				$\nu(COO^-)^{as}$ heme propionate
				$\nu(COO^-)^{as}$ Asp/Glu
1572	Ox	1568	Ox	ν_{38x} heme *a*
				$\nu(COO^-)^{as}$ heme propionate
				$\nu(COO^-)^{as}$ Asp/Glu
1546	Red	1548	Red	ν_{38y} heme *a*
		1538	Ox	?
1530	Red	1528	Red	ν_{38y} heme a_3
1522				$\nu(COO^-)^{as}$ heme a_3 propionate

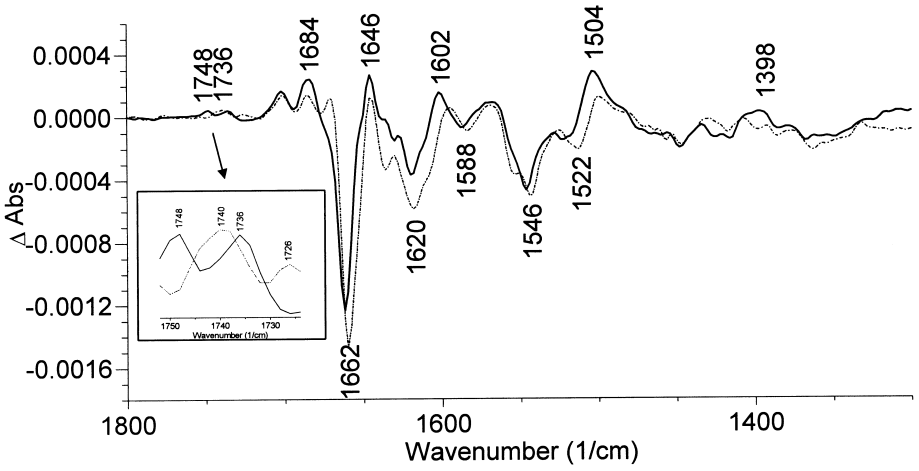

Fig. 1. Oxidized minus reduced FT-IR difference spectra (−0.5 V→0.5 V) of the cytochrome *c* oxidase from bovine heart equilibrated in H_2O buffer (solid line) and in D_2O buffer (dotted line). The inset depicts the spectral range from 1750 to 1725 cm^{-1}.

from *P. denitrificans* (previously published in [9–11]) and from bovine heart. We note clear differences between the spectra, reflecting subtle differences in the arrangement of amino acid side chains and cofactor sites in spite of the high degree of structural similarity between both enzymes. Some of the signals vary in intensity and point to similar processes. Additional or absent signals reflect stronger deviations.

Both enzymes host the same heme centers, heme *a* and a_3, with far reaching similarities regarding their environment [5,7]. On this basis, the same redox dependent changes of the porphyrin ring, the formyl and vinyl substituent and the heme propionates are expected to manifest in the spectra. The modes, tentatively assigned and listed in Table 1, do not differ by more than 4 cm^{-1}. As previously discussed in the comparison of the electrochemically induced FT-IR difference spectra of the two and four subunit oxidase, protein-protein interactions, induced by additional subunits, influence the absorption and the intensity of signals [13]. Heibel et al. [16] compare in a resonance Raman spectroscopic study the oxidized and the reduced form of the two and four subunit cytochrome *c* oxidase from *P. denitrificans* with the 13 subunit oxidase from bovine heart. They report quantitatively similar heme vibrations, however, showing differences in intensity and absorptions depending on the number of additional subunits. The IR data presented here are in line with these observations from resonance Raman spectroscopy.

The most interesting changes occur in the spectral region between 1760 and 1710 cm^{-1}. A positive difference signal at 1734 cm^{-1} can be observed in the oxidized minus reduced FT-IR difference spectra of the bovine heart oxidase (solid line). In the spectra for the cytochrome *c* oxidase from *P. denitrificans* (dotted line), a negative mode can be seen at the same position. The difference signals at 1746/1734 cm^{-1} could previously be assigned to Glu-278 (numbering for *P. denitrificans*) in a combined IR spectroscopic and mutagenesis study [10]. The obvious change of the peak sign in the characteristic spectral region of protonated Asp and Glu side chains for the spectra of both enzymes may be explained in two different ways: an additional protonated Asp and Glu amino acid side could contribute and overlap the negative mode or a change in extinction coefficient of the contributing groups may occur.

Although a contribution from an Asp or Glu residue located in the additional subunits of bovine heart oxidase could in principle be possible, we consider this a highly unlikely possibility. The comparison of the two and four subunit ox-

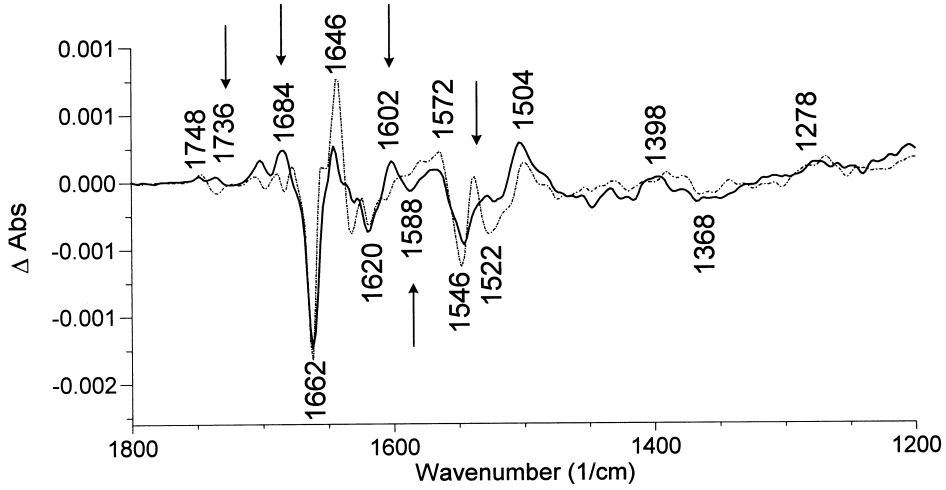

Fig. 2. Oxidized minus reduced FT-IR difference spectra (−0.5 V→0.5 V) of the cytochrome *c* oxidase from *P. denitrificans* (dashed line) and from bovine heart (solid line).

idase from *P. denitrificans* has already shown that contributions from other subunits than I and II can be excluded [12]. However, it could well be that a contribution of protonated Asp or Glu, which shows a very small extinction coefficient in the spectra of the cytochrome *c* oxidase from *P. denitrificans* at pH 7, may be significantly more prominent in the spectra of the bovine heart oxidase. One potential candidate is Asp-399 (Asp-364 in bovine heart oxidase), not observable at pH 7 between 1760 and 1730 cm^{-1} in the electrochemically induced FT-IR difference spectra of the cytochrome *c* oxidase from *P. denitrificans*, as demonstrated for the Asp-399-Asn mutant enzyme [10]. The perturbation of the mutated residue (Asn-399), however, was observed at 1668 cm^{-1}, indicating the involvement of this residue. A different extinction coefficient of the absorption of the $\nu(C=O)$ mode of the COOH group in the bovine heart oxidase, namely Asp-364, cannot be excluded.

Yoshikawa et al. [3] compared the X-ray structure of the reduced and oxidized form of the cytochrome *c* oxidase from bovine heart. Among the residues investigated for possible structural changes upon the redox transition, Asp-51 is proposed to show redox-coupled conformational changes. On the basis of the structural data, the group is suggested to be deprotonated in the reduced state. Protonation of the group in the oxidized state is proposed to be coupled to tautomerism with the polypeptide backbone. On this basis, the positive signal at 1736 cm^{-1} can be tentatively attributed to the protonation of Asp-51. The $\nu(COO^-)^{as}$ mode from deprotonated aspartates contribute at approximately 1590–1570 cm^{-1}, and the $\nu(COO^-)^{s}$ mode at approximately 1420–1390 cm^{-1}, depending on the local protein environment. A negative signal is observed at 1588 cm^{-1}, not observable in the spectra of the *P. denitrificans* oxidase, that may be related to the $\nu(COO^-)^{as}$ vibrational mode of Asp-51 and thus potentially reflects the deprotonation of Asp-51 with the reduction of the enzyme. This would be in line with the structural data [3]. An alternative assignment for the signal at 1588 cm^{-1} is in terms of an environmental change of a deprotonated acid group.

Asp-51 is only conserved in mammalian oxidases. Its redox dependent conformational change was proposed to be coupled to the heme *a* redox transition and to make part of a unique proton path, exclusive for mammalian oxidases [3]. This is not in line with the current understanding of the cytochrome *c* oxidases [8]. Alternatively, the conformational change of Asp-51 may be coupled to the Cu_A redox transition. Asp-51 is $\sim$6 Å away from it and may stabilize the charged Cu_A center. However, the IR difference signals discussed for Asp-51 here may arise from other protonated/deprotonated Asp or Glu residues, like Asp-364, Asp-51 or Glu-198 (the latter being a ligand of Cu_A), as pointed out above, an unequivocal assignment cannot be made for cytochrome *c* oxidase from bovine heart.

Further differences between the electrochemically induced FT-IR difference spectra of cytochrome *c* oxidase from bovine heart and *P. denitrificans* are evident at 1684 cm^{-1}, where the $\nu(C=O)$ mode from protonated heme propionates, from Asn and Gln, or from β-sheet secondary structure elements can be expected. At 1656 cm^{-1}, a signal contributes in the characteristic spectral region for α-helical secondary structure elements in the spectra of the cytochrome *c* oxidase from *P. denitrificans*, which is not present in the spectra of the bovine heart oxidase. Further discrepancies can be seen between 1540 and 1200 cm^{-1}.

In spite of the high degree of structural similarity for the oxidized form [3–7], clear differences can be seen in the electrochemically induced FT-IR difference spectra, especially for the structurally well-characterized oxidized form. We mainly attribute them to the influence of the additional subunits and additionally to possible contributions from groups like Asp-51, exclusively conserved in mammalian oxidases. Another point concerns the difference in calcium binding. Whereas the cytochrome *c* oxidase from *P. denitrificans* binds stoichiometrically one calcium ion, the bovine heart oxidase lacks calcium [19,20]. The interactions with the heme propionates discussed in [21] may also explain some of the variations in the IR spectra presented here and can be studied by combining electrochemically induced FT-IR difference spectra with site-directed mutagenesis studies in the cytochrome *c* oxidase from *P. denitrificans*.

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