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MALDI-TOF MS Lipid Profiles of Cytochrome *c* Oxidases: Cardiolipin Is Not an Essential Component of the *Paracoccus denitrificans* Oxidase

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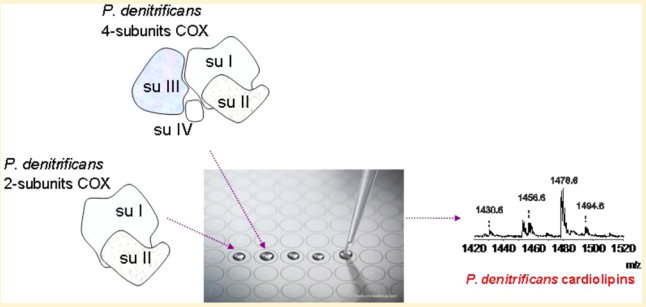
Supporting Information

ABSTRACT: Lipids of cytochrome *c* oxidase (COX) of *Paracoccus denitrificans* have been identified by MALDI-TOF MS direct analyses of isolated protein complexes, avoiding steps of lipid extraction or chromatographic separation. Two different COX preparations have been considered in this study: the enzyme core consisting of subunits I and II (COX 2-SU) and the complete complex comprising all four subunits (COX 4-SU). In addition, MALDI-TOF MS lipid profiles of bacterial COX are also compared with those of the isolated mitochondrial COX and bacterial bc_1 complex. We show that the main lipids associated with bacterial COX 4-SU are phosphatidylglycerol (PG) and phosphatidylcholine (PC), and minor amounts of cardiolipin (CL). PG and PC are absent in the COX 2-SU preparation lacking subunits III and IV, whereas CL is still present. Quantitative analyses indicate that at variance from mitochondrial COX, cardiolipin is present in substoichiometric amounts in bacterial COX, at a CL:COX molar ratio of ∼1:10. We conclude that bacterial COX does not require CL for structure or its activity.



Paracoccus denitrificans is a Gram-negative soil bacterium well-known for its close evolutionary relationship to present-day mitochondria. The cytoplasmic membrane of *P. denitrificans* contains a respiratory chain structurally and functionally similar to the electron transport chain of mammalian mitochondria, in particular with respect to its cytochrome composition.[1,2] *P. denitrificans* also shares other biochemical features with mitochondria, with respect to the lipid composition of membranes containing the electron transport systems, which contain phosphatidylcholine (PC) as a major phospholipid and oleic acid as the predominant fatty acid.[3]

Both the cytochrome *c* oxidase and the cytochrome bc_1 (electron transfer complexes IV and III, respectively) of *P. denitrificans* have been characterized in terms of structure and function. The heme aa_3-type oxidase is a monomer consisting of four different subunits (SU I–IV). Subunits I and II contain all the redox-active metals, i.e., the Cu_A center, heme *a*, and heme a_3–Cu_B center (binuclear center), thus representing the functional core of the enzyme; the function of subunits III and IV of the enzyme, on the other hand, is still unknown.[4] The enzyme has been crystallized in both its 2-SU and 4-SU forms,[4,5] and the kinetics of both forms have been characterized.[6]

Complex III mediates the transfer of an electron from ubiquinol to cytochrome *c* and like oxidase contributes to the generation of an electrochemical gradient used for ATP synthesis. The complex of *P. denitrificans* has recently been crystallized and the X-ray structure determined at 2.7 Å resolution.[7]

A specific lipid requirement is necessary for the shape, structure, and function of each cellular organelle;[8] this is especially true for mitochondria, for which the continuous supply and exchange of lipids are required for membrane integrity and overall cellular function.[9] A number of reports in the literature have pointed out that lipids are required to sustain the electron transport reactions catalyzed by mitochondria as well as isolated respiratory complexes. The dimeric phospholipid cardiolipin, for example, is essential for the optimal activity of inner mitochondrial membrane proteins, including respiratory complexes I, III, and IV, ATP synthase, and the ATP/ADP translocase,[10,11] as well as for the stability of respiratory chain supercomplexes.[12,13]

Little is known about the lipid composition of the isolated complexes of *P. denitrificans*. At variance from mitochondria, where an absolute requirement for cardiolipin in cytochrome oxidase has been reported, Shinzawa-Itoh et al. reported that

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two different types of PG are strictly associated with the cytochrome *c* oxidase of *P. denitrificans*, but no other anionic phospholipids were identified in their study.[14]

In this study, we describe the results of analysis of the lipid composition of complexes III and IV, i.e., the bc_1 complex and cytochrome *c* oxidase, of the respiratory chain of *P. denitrificans*, obtained by applying a recently developed MALDI-TOF MS method for the analysis of intact mitochondria.[15] Because of the sensitivity of our analytical approach, novel information about lipids tightly bound to this bacterial COX have been obtained; quantifying its cardiolipin content, we conclude that unlike the mitochondrial situation, the bacterial COX shows no preference for CL.

MATERIALS AND METHODS

Materials. 9-Aminoacridine hemihydrate (9-AA) was purchased from Acros Organics (Morris Plains, NJ). The following commercial glycerophospholipids were used as standards: 1,1′,2,2′-tetra(9*Z*-octadecenoyl) cardiolipin, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphate, 1,2-ditetradecanoyl-*sn*-glycero-3-phospho(1′-*rac*-glycerol), 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-L-serine, 1,1′,2,2′-tetramyristoyl cardiolipin (purchased from Avanti Polar Lipids, Inc., Alabaster, AL). All organic solvents used in the MS analyses were commercially distilled, of the highest available purity, and purchased from Sigma-Aldrich, J. T. Baker, or Carlo Erba.

Cell Growth and Membrane Preparation. *P. denitrificans* (ATCC 13543) was grown aerobically on methylamine as the major carbon source, and a crude membrane fraction was obtained, as described previously.[16,17]

Purification of Cytochrome *c* Oxidase Samples and the bc_1 Complex. Purification of the cytochrome *c* oxidase samples (2-SU and 4-SU forms) was performed as previously described.[18,19] Purification of the bc_1 complex was performed as described by Schröter et al.[20]

Lipid Extraction. Lipids were extracted from isolated and purified COX as briefly described here. A 7 mg/mL solution of COX 2-SU (250 μL) purified with Triton X-100 detergent was extracted by the method of Bligh and Dyer.[21] The extract was dried under N_2 and resuspended with chloroform to a final concentration of 10 mg/mL. The residual denatured COX 2-SU protein pellet after the first lipid extraction was again mixed with organic solvent to obtain the lipid re-extract.

Preparation of Isolated Protein Complexes for Direct MALDI-TOF MS Lipid Analysis. The procedure for sample preparation for intact analysis is analogous to the method recently developed for the analysis of isolated intact mitochondria.[15] The purified, detergent-solubilized protein complexes were diluted 7-fold with water to a final concentration of 1 μg of protein/μL; then 1 μL of the solution was spotted onto the MALDI target. After water evaporation, a thin layer (0.3 μL) of the matrix solution [9-AA, 40 mg/mL in a 60:40 (v/v) 2-propanol/acetonitrile mixture] was applied to the dried sample and evaporated. The samples were then directly analyzed by MALDI-TOF MS.

Preparation of Lipid Extracts and Lipid Standards in Solution for MALDI-TOF MS. Samples were prepared as previously described.[22] Briefly, the total lipid extracts (10 mg/mL) were diluted from 20 to 200 μL with a 60:40 (v/v) 2-propanol/acetonitrile mixture. Next, 10 μL of a diluted sample was mixed with 10 μL of 9-aminoacridine (10 mg/mL) dissolved in a 60:40 (v/v) 2-propanol/acetonitrile mixture. Then 0.3 μL of the mixture was spotted on the MALDI target (Micro Scout Plate, MSP 96 ground steel target).

MALDI-TOF MS. MALDI-TOF mass spectra were generated on a Bruker Microflex LRF mass spectrometer (Bruker Daltonics, Bremen, Germany). The system utilizes a pulsed nitrogen laser emitting at 337 nm and uses an extraction voltage of 20 kV. Gated matrix suppression was applied to prevent detector saturation; 999 single laser shots (sum of 3 × 333) were averaged for each mass spectrum. The laser fluence was kept ∼5% above threshold to have a good signal-to-noise ratio. All spectra were acquired in reflector mode using the delayed pulsed extraction, and spectra were acquired in both positive and negative ion mode. A mix containing 1,1′,2,2′-tetratetradecanoyl cardiolipin, 1,1′,2,2′-tetra(9*Z*-octadecenoyl) cardiolipin, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphate, 1,2-ditetradecanoyl-*sn*-glycero-3-phospho(1′-*rac*-glycerol), and 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-L-serine was always spotted next to the sample as an external standard. An external calibration was performed before each measurement. The mass range of the authentic standards is 590−1450 atomic mass units (amu). Peak areas, spectral mass resolutions, and signal-to-noise ratios were determined using the software of the instrument, Flex Analysis 3.3.65 (Bruker Daltonics).

The laser intensity was kept at 55−60% for the analyses of the lipid extracts and 65−70% for the analysis of intact complexes. To gain information about the nature of lipid chains, post source decay (PSD) spectra were also recorded using the method of Fuchs.[23]

Lipid Quantitative Analyses of COX 2-SU. To quantify CL in COX 2-SU of *P. denitrificans*, two different approaches were taken.

(a) Two aliquots, each containing 77 pmol of the cytochrome *c* oxidase (COX 2-SU) in detergent micelles, were incubated by being gently shaken overnight with or without 76 pmol of tetramyristoyl CL as an internal standard. After incubation, the samples were diluted 1:7 with water, to a final concentration of 1 μg/μL, and a 1 μL volume was directly loaded on the target; after being dried, the samples were covered with a layer of 0.3 μL of 30 mg/mL 9-AA, dried again, and analyzed.

The standard curve for quantifying cardiolipin was constructed as follows: 26.6, 13.3, and 2.66 nmol of CL 56:0 (as internal standard) in a chloroform solution were pipetted in glass microvials and dried under N_2; 50 μL of detergent micelles containing buffer was added to each vial and left overnight while being gently shaken. After incubation, the micellar system containing CL was diluted 1:7 with water as described above, and 0.3 μL containing 22.8, 11.4, and 2.28 pmol was loaded on the target. After being dried, the sample was covered with a layer of 0.3 μL of 30 mg/mL 9-AA, dried again, and analyzed. Triplicates of each sample were run.

(b) In parallel, we have quantified endogenous cardiolipin in the total lipid extract of COX 2-SU, as previously described.[22] The standard curve for quantifying cardiolipin, was obtained by preparing three different CL $(14:0)_4$ concentrated solutions of cardiolipin: 760, 380, and 76 μM CL.

Five microliters of each was added to 45 μL of the matrix solution (10 mg/mL 9-AA); 0.3 μL of this mixture, containing 22.8, 11.4, and 2.28 pmol of internal standard, was loaded on the target.

The total lipid extract from the COX 2-SU preparation was diluted from 1 to 10 μL with the matrix solution [30 mg/mL 9-AA in a 60:40 (v/v) 2-propanol/acetonitrile mixture], and 0.5

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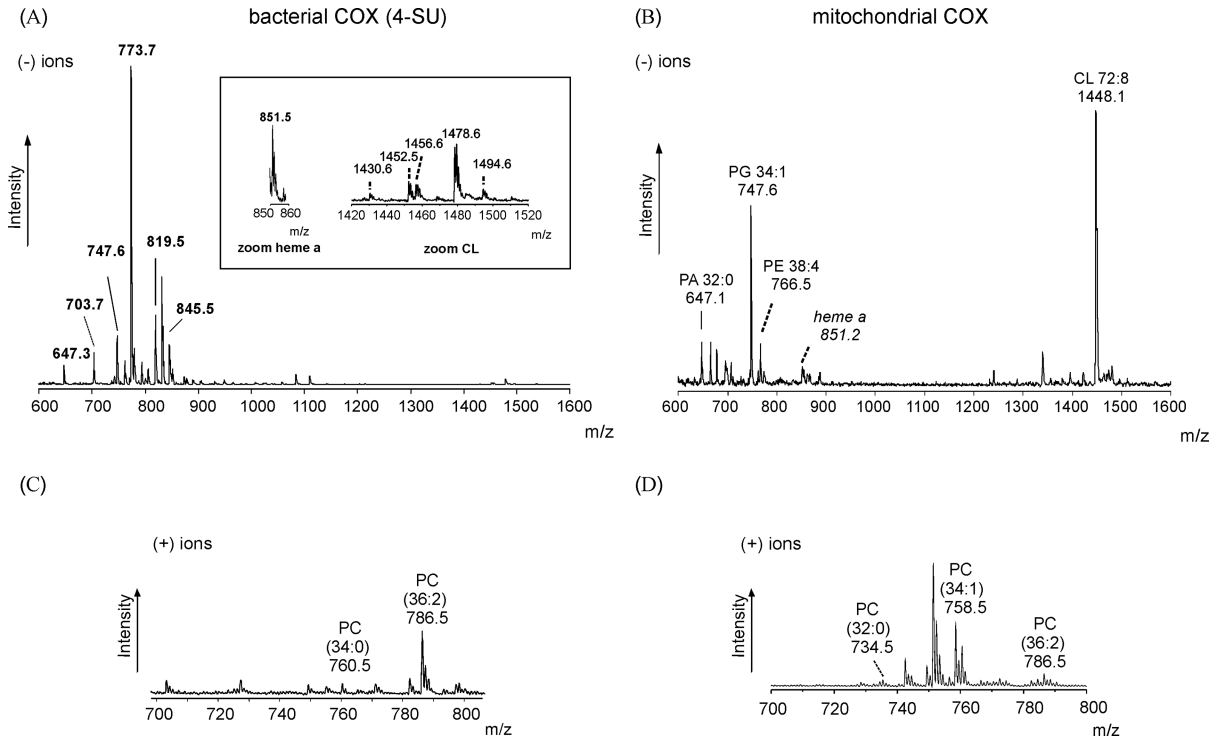

Figure 1. Lipid profiles of intact bacterial and mitochondrial cytochrome *c* oxidase enzymes. MALDI-TOF MS lipid analyses (negative ion mode) of complex IV (cytochrome *c* oxidase, four subunits) of (A) *P. denitrificans* and (B) bovine heart mitochondria. In the inset, close-ups of the heme and cardiolipin *m*/*z* ranges of the bacterial enzyme are shown. Clusters of phosphatidylcholine peaks obtained by positive ion mode analyses of (C) *P. denitrificans* COX 4-SU and (D) bovine heart mitochondria COX.

μL of 400 μM internal standard tetramyristoyl-CL was added to the solution; then 0.3 μL of the final solution was loaded on the target.

RESULTS

Specific Lipids Associated with Respiratory Complex IV of *P. denitrificans*. The MALDI-TOF MS lipid profile obtained by direct analysis (negative ion mode) of isolated COX of *P. denitrificans* is reported in Figure 1A. In the phospholipid range, minor peaks at *m*/*z* 647.3 and 703.7 can be attributed to PA 32:0 and PA 36:0, respectively; the peaks at *m*/*z* 747.6 and 773.7 correspond to PG 34:1 and PG 36:2, respectively. In the range of *m*/*z* 1400−1500 (reported in the inset of Figure 1A), the peak at *m*/*z* 1430.6 can be attributed to cardiolipin (CL) 70:3, likely consisting of three oleic acyl chains and one palmitate, while the peak at *m*/*z* 1452.5 to its sodiated form; the peak at *m*/*z* 1456.6 can be attributed to CL 72:4, consisting of four oleic acyl chains, and the peaks at *m*/*z* 1478.6 and 1494.6 to its sodium and potassium adducts, respectively.

Interestingly, the method shows in the same mass spectrum not only phospholipids, including CL, but also the peak that can be attributed to the heme prosthetic groups containing ionizable propionic acid residues. The peak at *m*/*z* 851.5 (see the inset in Figure 1A) can be attributed to the two hemes *a*. The assignment is also indirectly supported by the absence of this peak for the bc_1 complex (which does not contain heme *a*), analyzed under the same condition used for the COX complex (Figure 2).

Minor signals in the range of *m*/*z* 800−900 in the lipid profile of Figure 1A suggest the presence of sulfoglycolipids belonging to the family of SQDG (sulfoquinovosyl diacylglycerol). Peaks at *m*/*z* 793.6, 819.5, and 845.5 could be attributed to SQDG 32:0, SQDG 34:1, and SQDG 36:2, respectively. Details about the chemical structure of peaks attributed to SQDG have been obtained by post source decay (PSD) analyses; as an example, PSD spectra of SQDG peaks at *m*/*z* 793.6 and 819.5 are shown in Figure 2S of the Supporting Information. The presence of a sulfate group in the SQDG lipids has also been confirmed by Azure A staining after thin layer chromatography (TLC) of the lipid extract (not shown).

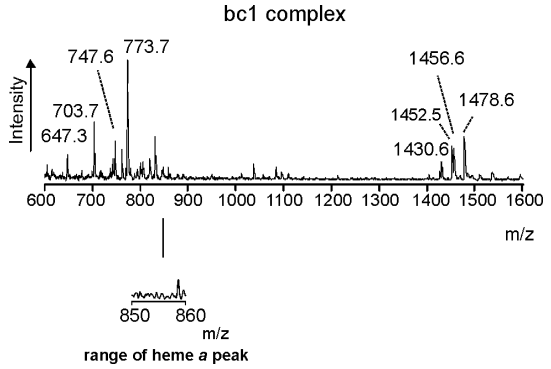

Figure 2. Lipid profile of the intact bacterial bc_1 complex. MALDI-TOF MS analysis (negative ion mode) of the lipids associated with complex III (bc_1 complex) of *P. denitrificans*. The close-up in the range of *m*/*z* 850−860 shows that the peak at *m*/*z* 851.5, attributed to heme (present in the mass spectra of bacterial COX), is absent. The lipid profile refers to 1 μg of total proteins. Major peaks at *m*/*z* 647.3 and 703.7 can be attributed to PA 32:0 and PA 36:0, respectively; the peaks at *m*/*z* 747.6 and 773.7 correspond to PG 34:1 and PG 36:2, respectively. In the range of *m*/*z* 1300−1500, the peaks at *m*/*z* 1430.6, 1456.6, 1478.6, and 1494.6 can be attributed to cardiolipin 70:3 and 72:4 and the sodium and potassium adducts of CL 72:4, respectively.

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The presence of SQDG has not been described previously and may well be reminiscent of the photosynthetic capacity of *P. denitrificans*, lost by this bacterium during evolution (for details of peak assignments, see Table 1). The COX lipid profile has been compared with that of the bacterial membranes used as starting material for the enzyme isolation and purification (shown in Figure 1S of the Supporting Information). At variance from CL, PG appears to be enriched in the COX enzyme fraction, compared to the starting membranes.

Table 1. Phospholipid Assignments of Peaks Identified in the Mass Spectrum of Complex III and/or IV of *P. denitrificans*

lipid class	*m/z* value	assignment $[M - H]^-$
phospholipids	647.3	PA 32:0
	673.5	PA 34:1
	699.5	PA 36:2
	703.7	PA 36:0
	716.6	PE 34:2
	719.5	PG 32:1
	721.6	PG 32:0
	742.6	PE 36:2
	747.6	PG 34:1
	773.7	PG 36:2
	789.6	PG 37:0
sulfoglycolipids	793.6	SQDG 32:0
	819.5	SQDG 34:1
	821.6	SQDG 34:0
	845.5	SQDG 36:2
	847.9	SQDG 36:1
	849.6	SQDG 36:0
cardiolipins	1378.2	CL 66:1
	1404.2	CL 68:2
	1430.6	CL 70:3
	1452.5	CL 70:3 + Na^+
	1456.6	CL 72:4
	1468.2	CL 70:3 + K^+
	1472.2	CL 73:2
	1478.6	CL 72:4 + Na^+
	1494.6	CL 72:4 + K^+
lipid class	*m/z* value	assignment $[M + H]^+$
phospholipids	734.6	PC 32:0
	758.5	PC 34:1
	760.5	PC 34:0
	786.5	PC 36:2

Finally, the lipid profile of the COX 4-SU preparation of *P. denitrificans* resulting from the direct lipid analyses of the intact enzyme was compared with that of COX isolated and purified from bovine heart (see the spectrum reported in Figure 1B). PA, PG, and CL peaks can be recognized in the spectrum of bovine COX reported in Figure 1B, together with the heme peak. PC was detected in the lipid analyses performed in the positive mode (see the range of *m/z* 700−800 in the close-up). The ratio of peak areas for cardiolipin and heme *a* is much higher in bovine heart COX than in bacterial COX.

Lipids of intact bovine COX detected in this study match perfectly with those previously reported by Shinzawa-Itoh et al. in COX crystals.[14] Remarkably, PG appears to be highly enriched in the lipid profile of isolated and purified mitochondrial COX, compared to the lipid profile of intact organelles, where PG is barely detectable.

Lipid profiles of bacterial COX (4-SU) and mitochondrial COX have been also analyzed in positive ion mode. Ranges of *m/z* 700−800 of both spectra are reported in panels C and D of Figure 1, respectively. In the spectrum of bacterial COX 4-SU, peaks at *m/z* 760.5 and 786.5 can be attributed to PC 34:0 and 36:2, respectively; in the spectrum of mitochondrial COX, peaks at *m/z* 734.5, 758.5, and 786.5 correspond to PC 32:0, PC 34:1, and PC 36:2, respectively.

MALDI-TOF MS Lipid Profile of Complex III of *P. denitrificans*. Lipids of the intact bc_1 complex have also been analyzed (Figure 2). Lipids specifically associated with the purified bc_1 complex are PA, PG, and CL. The peaks at *m/z* 647.3 and 703.7 can be attributed to PA 32:0 and 36:0, respectively; the peaks at *m/z* 747.6 and 773.7 correspond to PG 34:1 and PG 36:2, respectively. In the range of *m/z* 1300−1500, the peak at *m/z* 1430.6 can be attributed to cardiolipin 70:3 while the peak at *m/z* 1452.5 to its sodiated form; the peak at *m/z* 1456.6 can be attributed to CL 72:4 and the peak at *m/z* 1478.6 to its sodium adducts.

In the lipid profile of the bc_1 complex, the area peaks of PG and CL are similar; the situation is therefore different from that of COX, where PG is the prominent phospholipid.

Specific Lipids Associated with the COX 2-SU Preparation. We have also analyzed the residual lipids associated with the isolated cytochrome *c* oxidase two-subunit complex, i.e., after removal of subunits III and IV resulting from extensive treatment with Triton X-100 as a detergent. It is well-known that the complex consisting of subunits I and II represents the core of the functional enzyme.[4] As a consequence, lipids present in this highly purified preparation are specifically associated with the functional core of the enzyme. As described above, the spectrum has been acquired in negative ion mode using 9-AA as the matrix, by analyses of the intact membrane enzyme in detergent micelles (Figure 3A). In the phospholipid range, the peak at *m/z* 673.5 can be attributed to PA with 34:1 acyl chains; the minor peak at *m/z* 773.5 can be assigned to PG 36:2. In the CL range (*m/z* 1350−1500), the CL 70:3 peak at *m/z* 1430.4 and the CL 72:4 peak at *m/z* 1456.2 are present; in addition, the sodium and potassium adducts of CL 72:4 at *m/z* 1478.2 and 1494.2 are clearly visible.

As expected, the peak of the heme molecule at *m/z* 851.4 is also present. Results appear to indicate that the presence of PG is not required for the enzyme to function. Positive ion analyses did not indicate the presence of PC in this COX preparation (not shown).

To estimate the amount of cardiolipin associated with COX 2-SU, we have performed quantitative analyses. As it has been demonstrated that the ionization and/or desorption efficiency of phospholipids in MALDI-TOF MS analyses is not significantly influenced by the presence of different chain lengths and degrees of unsaturation, CL 14:0 has been used as an internal standard. An aliquot of the enzyme sample (in detergent buffer) has been added to a thin film of the dried internal standard (I.S.) and equilibrated while being gently shaken overnight, to ensure complete and homogeneous dispersion of the I.S. in the micelles. The standard curve of CL 14:0 used to quantitate cardiolipin has been obtained by using a micellar solution of CL 14:0 solubilized in buffer containing Triton X-100 micelles (in the absence of the enzyme) (Figure 3D). Results indicate that the cardiolipin:-COX molar ratio is 0.06 ± 0.02 (average value from duplicate experiments).

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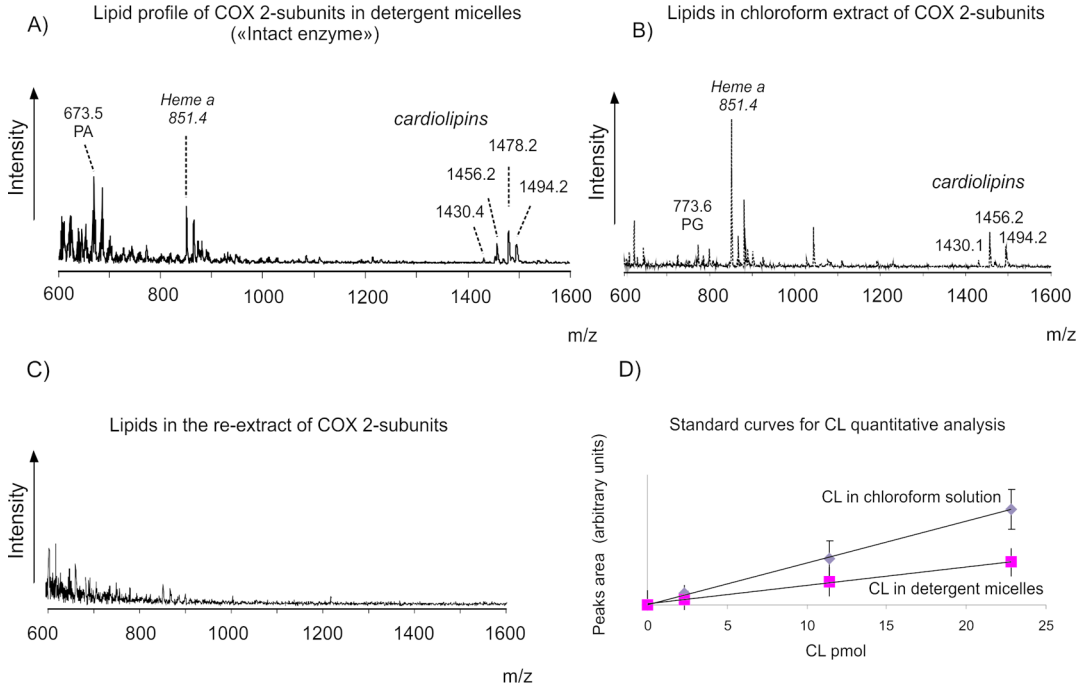

Figure 3. Lipid profiles of bacterial cytochrome *c* oxidase (COX 2-SU) consisting of subunits I and II only. MALDI-TOF MS analyses of the lipid profile of COX 2-SU, performed on (A) the intact enzyme and (B) the lipid extract. (C) Lipids in the re-extract of COX 2-SU. (D) Standard curves of tetra-myristoyl cardiolipin (CL 14:0)$_4$ for quantitative MALDI-TOF MS analyses. CL (2.28, 11.4, and 22.8 pmol) was in a chloroform solution (diamonds) or equilibrated in COX 2-SU solubilization buffer (squares), i.e., in detergent micelles. Other experimental details as in Materials and Methods.

To validate the results described above, we have also estimated the endogenous cardiolipin content in COX 2-SU by another analytical approach. A small-scale extract of COX 2-SU has been prepared (see Materials and Methods) and analyzed by MALDI-TOF MS in the presence and/or absence of the internal standard. In this case, the standard curve has been constructed by using C14:0 CL solubilized in chloroform (Figure 3D). Qualitatively, lipids in the extract (Figure 3B) are the same ones present in Figure 3A; it can be seen that the main peaks of the lipid extract of 2SU-COX are those of heme *a* and cardiolipin. PG is only a very minor component. PA was not detectable. As expected, the ionization mode of lipid components in the extract is different from the ionization mode of lipids in intact membranes. Results confirm that PG is not associated with the enzyme core. Quantitative analyses of CL in the lipid extract indicate that the CL:COX molar ratio is 0.12 ± 0.05 (average value from duplicate experiments), i.e., in substoichiometric amounts.

In addition, to verify the full recovery of cardiolipin content in the lipid extract, a re-extraction has been performed starting from the protein pellet left after the first lipid extraction. MALDI-TOF MS analysis of the re-extract (Figure 3C) clearly shows the absence of signals of cardiolipin, suggesting that the full recovery of cardiolipin has already been achieved in the first lipid extraction.

DISCUSSION

Our group recently developed a new MALDI-TOF MS method for the direct analysis of the membrane lipids of mitochondria, which was also applied to the study of the membrane lipid composition of *P. denitrificans*.[15] We demonstrated that the main lipid components of the *P. denitrificans* membranes, phospholipids PA, PE, PC, PG, and CL, can be identified in a reliable and reproducible way, by directly analyzing membranes, thus avoiding lipid extraction and any further separation steps.

Rather little is known about the specific lipids associated with the respiratory chain complexes of *P. denitrificans*,[14] while no previous studies report quantitative analyses of lipids specifically associated with isolated and purified bacterial respiratory complexes. In this study, we have analyzed the lipid composition of two respiratory complexes isolated from *P. denitrificans*, by means of the method previously used for the bacterial membranes.[15] We addressed complexes III and IV of the respiratory chains, and in particular two different forms of complex IV (COX 4-SU and 2-SU), of interest because they represent the entire enzyme and the functional core of the enzyme, respectively.

In the case of the COX 4-SU complex (Figure 1), results indicate that PG and CL are present; in addition, the peak of heme *a* is identified as well in the spectrum of COX, whereas it is absent as expected in that of the bc_1 complex (Figure 2). Different species of PC are also present.

Unexpectedly, also residual sulfoglycolipid molecules (SQDG) appear to be associated with the purified enzymes. The presence of these lipids typically present in Gram-negative photosynthetic bacteria has not been reported previously in membranes or in isolated and purified complexes of the respiratory chain of *P. denitrificans*. The presence of significant amounts of sulfoglycolipids in membranes used to isolate complexes analyzed in this study might be related to the specific growth conditions and/or the composition of the growth medium. These results add novel information with respect to previous literature reports; in the study of Shinzawa-Itoh et al.,[14] no sulfoglycolipids were detected and cardiolipin peaks were also not observed in the ESI-MS lipid profiles of the COX 4-SU enzyme. Furthermore, via analysis of the lipid

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profile of the 2-SU complex in intact mode (Figure 3A), it is evident that PG almost disappeared after the removal of subunits III and IV, whereas cardiolipin was still detectable together with heme *a*. The fact that PG is completely removed in parallel with the removal of subunits 3 and 4 suggests that PG might have a role in the assembly of subunits III and IV with the enzyme core whereas it is not required for the functioning of the core enzyme.

The lipid profile of COX 2-SU obtained analyzing the lipid extract of the enzyme (Figure 3B) shows the same lipid composition as that of COX 4-SU (Figure 3A). Differences in the relative intensities of lipid peaks simply depend on the different modalities of ionization in the two different analyses.

We also tried to quantify the cardiolipin content of the cytochrome *c* oxidase of *P. denitrificans*, so we performed a quantitative analysis of the 2-SU enzyme using two different systems. In the first, we quantified the cardiolipin content adding the internal standard (I.S.) to the purified enzyme in Triton X-100 micelles. In the second, the quantification was conducted with the lipid extract of the enzyme. Therefore, for quantification, two different standard curves of tetramyristoyl cardiolipin were constructed (Figure 3D). Both experimental approaches indicated that the cardiolipin:COX 2-SU molar ratio was ∼1:10 or lower. Cardiolipin quantitative analyses have been performed only on samples of COX 2-SU; on the basis of cardiolipin:heme peak area ratios, we can conclude that cardiolipin is only a minor lipid component in COX 4-SU samples.

As previous reports[6,19] have indicated that the core enzyme (i.e., COX 2-SU studied here) is fully functional in terms of its kinetic and energy transduction properties despite the negligible amount of cardiolipin, we conclude that CL is not required for the activity of the enzyme. Therefore, the situation is quite different from that of the isolated heart mitochondrial COX, which contains at least one or two CL molecules per enzyme molecule. It has been demonstrated that COX produced in markedly CL-depleted *Rhodobacter sphaeroides* cells still maintains its wild-type function and characteristics despite the nearly complete depletion of cardiolipin.[24] Therefore, our results describing lipids associated with complex IV of *P. denitrificans* are in agreement with those obtained for the photosynthetic bacterium *R. sphaeroides*.

Structural analyses suggest that cytochrome *c* oxidase of bovine heart mitochondria is in the dimeric state,[14] whereas the cytochrome *c* oxidase of *P. denitrificans* is a monomer.[25] Four phospholipids (CL, two PEs, and PG) bridge monomers to stabilize the dimer state; some information about the specific location of cardiolipin in the dimer and about its role in stabilizing the interactions between subunits of mitochondrial COX is available.[14] Lower levels of cardiolipin in the cytochrome *c* oxidase of *P. denitrificans* are therefore consistent with the structure of the bacterial cytochrome *c* oxidase, which does not contain the nuclear-encoded subunits of bovine heart cytochrome *c* oxidase and is in the monomeric state.

Whereas the functional role of cardiolipin in the mitochondrial counterpart is widely accepted,[26,27] studies of the catalytic function of cardiolipin in the bacterial COX complex are not yet available. In the past, it was recognized that optimal enzymatic activity could be obtained only after the addition of exogenous lipids.[25] Further studies are required to elucidate the role of CL (and possibly other phospholipids) on the operation modes of COX. Bacterial systems might offer the opportunity to clarify at least some aspects of the intriguing functional role of CL in respiratory chain complexes.

■ ASSOCIATED CONTENT

Supporting Information

MALDI-TOF MS lipid profile of *P. denitrificans* membranes (Figure 1S) and the fragmentation pattern of SQDG 32:0 (m/z 793.6) and the PSD spectrum of the ion having a monoisotopic mass weight (i.e., m/z ratio) of 819.6 (Figure 2S). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

COX 2-SU, cytochrome *c* oxidase two subunits; COX 4-SU, cytochrome *c* oxidase four subunits; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; TLC, thin layer chromatography; I.S., internal standard; 9-AA, 9-aminoacridine; CL, cardiolipin; PG, phosphatidylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyl diacylglycerol.

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