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Assembly of Respiratory Complexes I, III, and IV into NADH Oxidase Supercomplex Stabilizes Complex I in *Paracoccus denitrificans**

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Stable supercomplexes of bacterial respiratory chain complexes III (ubiquinol:cytochrome *c* oxidoreductase) and IV (cytochrome *c* oxidase) have been isolated as early as 1985 (Berry, E. A., and Trumpower, B. L. (1985) *J. Biol. Chem.* 260, 2458–2467). However, these assemblies did not comprise complex I (NADH:ubiquinone oxidoreductase). Using the mild detergent digitonin for solubilization of *Paracoccus denitrificans* membranes we could isolate NADH oxidase, assembled from complexes I, III, and IV in a 1:4:4 stoichiometry. This is the first chromatographic isolation of a complete "respirasome." Inactivation of the gene for tightly bound cytochrome c_{552} did not prevent formation of this supercomplex, indicating that this electron carrier protein is not essential for structurally linking complexes III and IV. Complex I activity was also found in the membranes of mutant strains lacking complexes III or IV. However, no assembled complex I but only dissociated subunits were observed following the same protocols used for electrophoretic separation or chromatographic isolation of the supercomplex from the wild-type strain. This indicates that the *P. denitrificans* complex I is stabilized by assembly into the NADH oxidase supercomplex. In addition to substrate channeling, structural stabilization of a membrane protein complex thus appears as one of the major functions of respiratory chain supercomplexes.

With mitochondria, eukaryotic cells possess specialized organelles dedicated mainly to oxidative phosphorylation. Inner mitochondrial membranes of higher eukaryotes like mammalia are highly enriched for the four respiratory chain complexes, NADH:ubiquinone oxidoreductase (complex I), succinate: ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV), which generate an electrochemical potential across this membrane. F_1F_O-ATP synthase (complex V) uses this electrochemical proton gradient to synthesize ATP (1).

Bacterial respiratory complexes are located in the cytoplasmic membrane. *Paracoccus denitrificans*, a Gram-negative soil bacterium, uses an electron chain for aerobic growth that comprises a full complement of mitochondrial respiratory complexes I–IV plus ATP synthase. In context of the endosymbiotic theory (2), proteobacteria like *P. denitrificans* are therefore regarded as likely ancestors of present day mitochondria (3, 4). In addition to its "canonical" respiratory chain, respiration in *P. denitrificans* is characterized by many branching points. Alternative oxidases (ba_3 quinol oxidase and cbb_3 cytochrome oxidase) allow growth under strongly varying oxygen concentrations. Additionally, *Paracoccus* may use a variety of electron donors (*e.g.* hydrogen) or nitrate as terminal electron acceptor, which leads to high environmental flexibility (5).

Bacterial complexes usually comprise a substantially lower number of subunits, *e.g.* 14, 3, and 4 subunits for *P. denitrificans* complexes I, III, and IV (6–9), respectively, compared with 46, 11, and 13 subunits for the corresponding bovine complexes (10–13). Association of respiratory chain complexes to supercomplexes was observed in mitochondrial (14, 15) and bacterial respiratory chains (16–21). Although stable interactions of bacterial complexes III and IV to form quinol oxidases have been reported, participation of complex I in supercomplex formation escaped detection so far. This may largely be due to a pronounced detergent sensitivity of complex I from various bacteria. So far, complex I could only be isolated from four bacteria: *Escherichia coli* (22), *Rhodothermus marinus* (23), *Klebsiella pneumoniae* (24), and *Aquifex aeolicus* (25).

We found that digitonin can retain a supramolecular assembly of NADH oxidase from *P. denitrificans* and isolated for the first time chromatographically a complete "respirasome" comprising complexes I, III, and IV in a 1:4:4 stoichiometry that is suitable for detailed structural and functional analyses.

EXPERIMENTAL PROCEDURES

P. denitrificans Strains—The parental strain used in this study was Pd1222 (DSM413 derivative, Ri^r, Sp^r, enhanced conjugation frequencies, m^-; Ref. 26). In strain MK6 (Pd1222, Δ*fbc*::Km^r; Refs. 27 and 28), the *fbc* operon (coding for the three-subunit complex III) was replaced by a kanamycin resistance cassette introduced via homologous recombination. Both alleles of cytochrome *c* oxidase subunit I are replaced by kanamycin and tetracyclin resistance genes in strain MR31 (Pd1222, Δ*cta*DI::Km^r; Δ*cta*DII::Tc^r; Ref. 29). In strain AT110 (Pd1235, *cyc*M::Km^r; Refs. 30 and 31), the *cyc*M gene coding for cytochrome c_{552} is inactivated by insertion of the kanamycin resistance gene. All strains were grown on succinate or methylamine medium (32, 33).

Isolation of Membranes—The cells were suspended in 100 mM potassium phosphate, pH 8.0, 1 mM EDTA, 100 μM Pefabloc SC, and broken using a Manton-Gaulin Press (400 bar, 2 × 10 min). After centrifugation (12,000 × *g*, overnight), the reddish brown membrane portion of the

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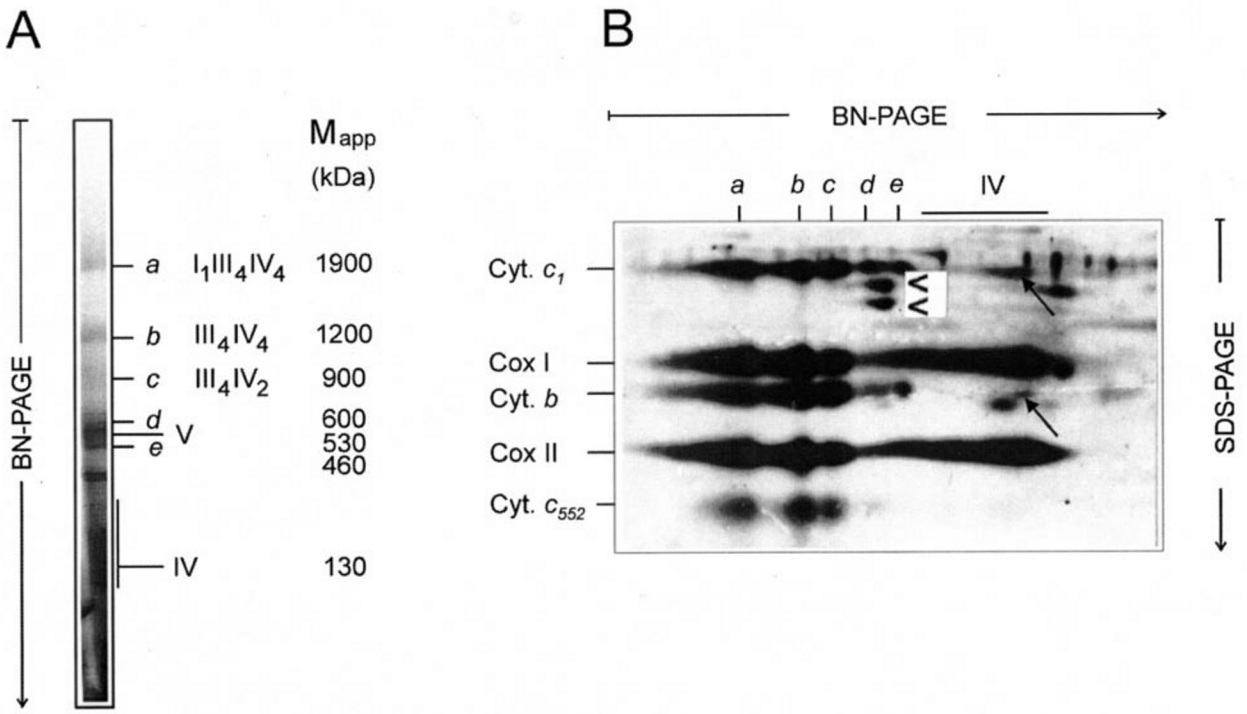

FIG. 1. **Separation of respiratory chain complexes and supercomplexes by BN-PAGE.** *A*, *P. denitrificans* membranes were solubilized using a digitonin/protein ratio of 2 (g/g), and the native membrane protein complexes were separated using a 4–13% acrylamide gradient gel. *Band a* was identified as NADH oxidase supercomplex ($I_1III_4IV_4$) containing complexes I, III, and IV in a 1:4:4 stoichiometry (*cf.* "Results"). *Bands b* and *c* also contained a core structure of tetrameric complex III associated with four or two copies of complex IV but missed complex I. *Bands d* and *e* correspond to complete tetrameric complex III and a subcomplex missing the Rieske iron sulfur protein and cytochrome c_{552}, respectively. *V*, complex V or ATP-synthase; *IV*, individual complex IV. Oxidative phosphorylation complexes I-V from bovine heart mitochondria were used for molecular mass calibration (not shown; Ref. 34). *B*, Western blot after two-dimensional resolution by Tricine-SDS-PAGE using a 10% acrylamide gel. A mixture of antibodies against subunits of complex III (*cyt.* c_1, cytochrome c_1; *cyt. b*, cytochrome *b*), of complex IV (*Cox I*, subunit 1; *Cox II*, subunit 2), and against cytochrome c_{552} (*cyt.* c_{552}) were used to localize individual respiratory chain complexes and supercomplexes. *Black arrows* mark subunits cytochromes *b* and c_1 of the minor amounts of dimeric complex III. Nonspecific interaction of the antibody mixture with the α and β subunits of ATP synthase is indicated by *arrowheads* on a *white background*. For immunodetection of complex I in *band a* see Fig. 5A.

sediment was resuspended in 20 mM potassium phosphate, 1 mM EDTA, pH 8, and centrifuged again (140,000 × *g*, 1 h). The sediment was resuspended in a small volume of 20 mM potassium phosphate, 1 mM EDTA, pH 8, to a final protein concentration of approximately 30 mg/ml.

Isolation of P. denitrificans NADH Oxidase—The membranes (200 mg of protein) were suspended in 150 mM NaCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 150 mM imidazole/HCl, pH 7.0 (8 ml), and solubilized by adding 3 g of digitonin/g protein (6 ml from a 10% digitonin solution). Following 30 min of ultracentrifugation at 100,000 × *g*, 150 mM sodium phosphate, pH 7.2, was added to the supernatant before application to a hydroxylapatite column (40 ml; equilibrated with 20 mM sodium phosphate, pH 7.2; run at room temperature). Following washing with 2 column volumes of 0.1% digitonin, 150 mM sodium phosphate, pH 7.2, individual respiratory chain complexes and supercomplexes were eluted by 0.05% Triton X-100, 150 mM sodium phosphate, pH 7.2. The eluate was concentrated approximately 5-fold to obtain a volume of 2.5 ml (Vivaspin 100,000 MWCO PES) and applied to a Sepharose Cl-6B column (80 ml; length, 1 meter; equilibrated with 0.05% digitonin, 100 mM NaCl, 5 mM 6-aminohexanoic acid, 1 mM EDTA, 20 mM Na-MOPS, pH 7.3; run at 4 °C). The digitonin buffer was stored at least 24 h before use at 4 °C to precipitate insoluble material (approximately 30% of the added digitonin).

Electrophoretic and Spectrometric Techniques—Blue native (BN)[1] PAGE, two-dimensional SDS-PAGE, and densitometric quantification were performed as described recently (34, 35). Fluorometric flavin mononucleotide determination followed the protocols of Koziol (36) and Hatefi and Stempel (37). Heme contents were determined by pyridine hemochrome analysis (38). For determination of cytochrome contents of native proteins, redox difference spectra (dithionite-reduced minus ferricyanide-oxidized) were recorded using the following absorption coefficients: *c*-type cytochromes (cytochrome c_1 of complex III and cytochrome c_{552}), $\epsilon_{550-540\ nm}$ = 19.1 $mM^{-1}\ cm^{-1}$; cytochrome *b* of complex III (containing hemes b_L and b_H), $\epsilon_{562-575\ nm}$ = 57 $mM^{-1}\ cm^{-1}$; cytochrome aa_3, $\epsilon_{605-630\ nm}$ = 23.4 $mM^{-1}\ cm^{-1}$.

Enzymatic Analyses—All of the enzymatic assays were performed at 30 °C. Decylquinazolinamine-sensitive NADH:decylbenzoquinone (DBQ) oxidoreductase activity of complex I was measured in 50 mM Tris/HCl, pH 7.4, 200 μM NADH, 60 μM decylbenzoquinone, 2 mM KCN ($\epsilon_{NADH,\ 340-400\ nm}$ = 6.22 $mM^{-1}\ cm^{-1}$). Deamino-NADH:hexamino-ruthenium oxidoreductase was determined in 250 mM sucrose, 0.2 mM EDTA, 2 mM NaN_3, 20 mM Na-HEPES, pH 8.0, 200 μM deamino-NADH, 2 mM hexamino-ruthenium, measuring the oxidation of deamino-NADH. Thenoyltrifluoroacetone-sensitive succinate:dichloroindophenol (DCIP) oxidoreductase activity of complex II was measured in 50 mM sodium phosphate, 1 mM EDTA, 2 mM NaN_3, 20 mM sodium succinate, 0.002% DCIP, pH 7.0 ($\epsilon_{DCIP,\ 610-750\ nm}$ = 20.5 $mM^{-1}\ cm^{-1}$). Horse heart cytochrome *c* and a buffer containing 150 mM NaCl, 75 mM imidazole/HCl, pH 7.0, was used for the following assays. DBH:cytochrome *c* oxidoreductase activity of complex III was measured in buffer supplemented with 2 mM KCN, 75 μM DBH, 50 μM cytochrome *c*, by the antimycin-sensitive reduction of cytochrome *c* ($\epsilon_{Cyt.\ c,\ 540-550\ nm}$ = 19.1 $mM^{-1}\ cm^{-1}$). Cytochrome *c* oxidase activity of complex IV was measured in buffer supplemented with 50 μM reduced cytochrome *c* by the cyanide-sensitive oxidation of cytochrome *c*. NADH:cytochrome *c* oxidoreductase, the coupled activities of complexes I and III, was measured in buffer supplemented with 2 mM KCN, 200 μM NADH, 50 μM cytochrome *c*, by the antimycin- or decylquinazolinamine-sensitive reduction of cytochrome *c*. DBQ oxidase activity, the coupled activities of complexes III and IV, was determined in buffer supplemented with 50 μM cytochrome *c* and 70 μM DBH by the cyanide-sensitive oxidation of DBH ($\epsilon_{DBH,\ 280-290\ nm}$ = 4.2 $mM^{-1}\ cm^{-1}$). NADH oxidase activity (complexes I, III, and IV) was measured by the cyanide-sensitive oxidation of 200 μM NADH.

Protein, Phospholipid, and Ubiquinone Determination—Protein determination was performed using a modified Lowry protocol (39), ac-

[1] The abbreviations used are: BN, blue native; DBQ, decylbenzoquinone; DBH, decylbenzoquinol; DCIP, dichloroindophenol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

TABLE I

Composition of P. denitrificans respiratory chain supercomplexes

M_{app}, apparent masses of bands *a–e* after BN-PAGE. The cytochrome c_{552} copy number/complex is not known and not considered for calculation of molecular masses (M_{calc}).

Band	Composition	M_{app}	M_{calc}	Remarks
		kDa	*kDa*	
a	$I_1III_4IV_4$	1900	1548	NADH oxidase supercomplex contains about two copies of cytochrome c_{552}
b	III_4IV_4	1200	984	
c	III_4IV_2	900	722	
d	III_4	600	460	Tetrameric complex III containing Rieske iron sulfur protein and traces of cytochrome c_{552}
e	III_4	460	379	Tetrameric complex III lacking Rieske iron sulfur protein and cytochrome c_{552}

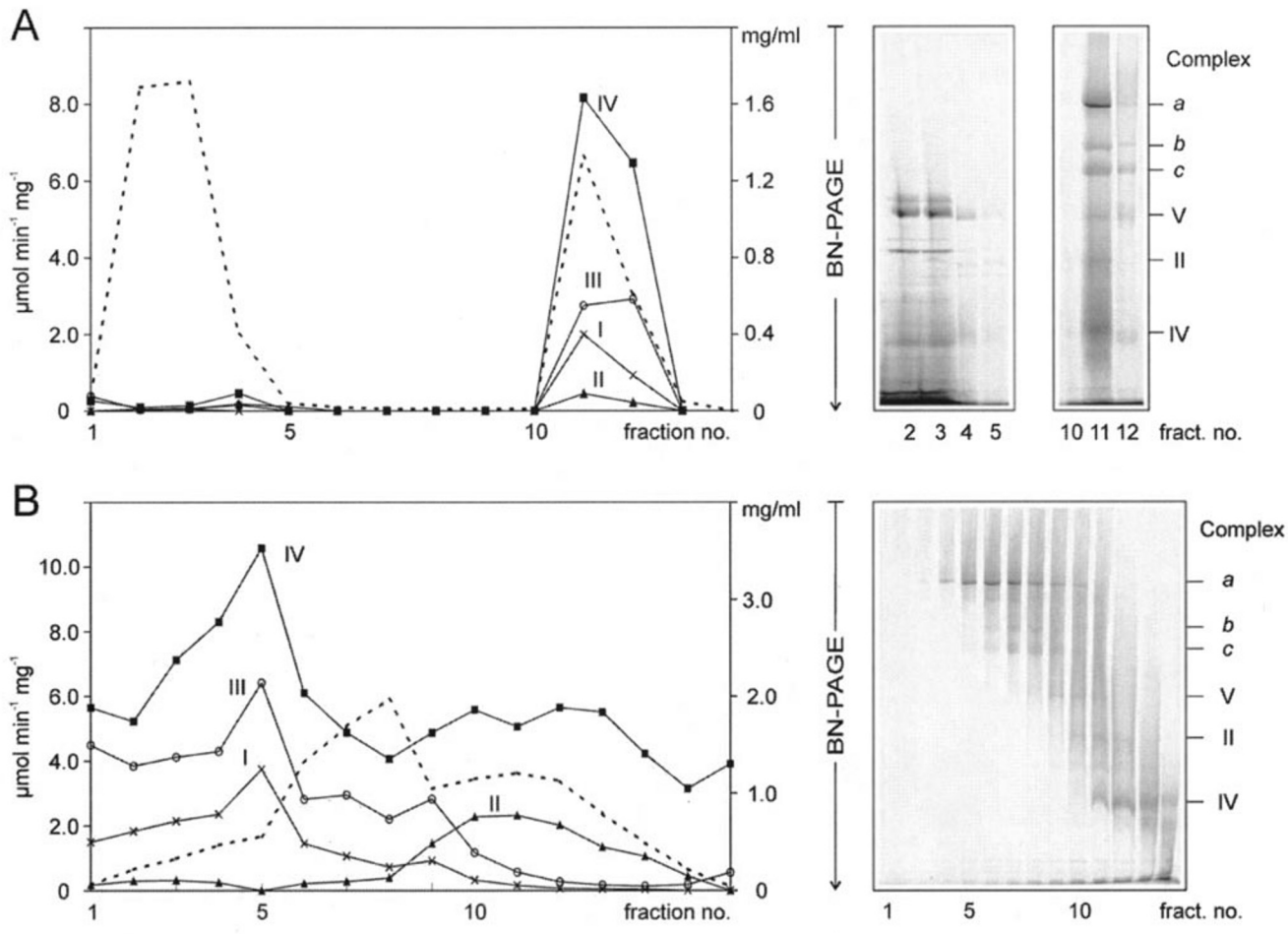

FIG. 2. **Typical elution profiles for the hydroxylapatite chromatography and gel filtration steps in NADH oxidase supercomplex isolation.** *A*, *left panel*, hydroxylapatite chromatography. More than 70% of total protein was found in the flow-through fractions 1–5. Almost all respiratory chain activity was restricted to fractions 11 and 12. *A*, *right panel*, BN-PAGE of the corresponding fractions indicated that ATP synthase (complex V) was removed with the pass through, and elution fractions contained apparently undisturbed assemblies of respiratory chain complexes. *B*, *left panel*, gel filtration using Sepharose CL-6B largely separated complex II activity from complex I and III activities. *B*, *right panel*, BN-PAGE indicated that the first fractions essentially contained holo NADH oxidase (*supercomplex a*) with little contamination by complex I-free *supercomplexes b* and *c*. *Dotted line*, protein (mg/ml); ×, NADH:DBQ oxidoreductase (complex I); ▲, succinate:DCIP oxidoreductase (complex II); ○, DBH:cytochrome *c* oxidoreductase (complex III); ■, cytochrome *c* oxidase (complex IV).

cording to Bradford (40), and by the Biuret (41) and BCA methods (42). Phospholipid and ubiquinone were determined according to Chen *et al.* (43) and Dröse *et al.* (44).

RESULTS AND DISCUSSION

Identification and Purification of Respiratory Chain Supercomplexes—P. denitrificans membranes were solubilized by digitonin and separated by BN-PAGE. A major band representing monomeric complex V (M_{app} = 530 kDa) and several additional bands with apparent masses in the range 460–1900 kDa were detected (Fig. 1*A*, *bands a–e*). Assignment of these bands was possible after SDS-PAGE in a second dimension and immunological detection (Fig. 1*B*). Band *d* contained cytochromes c_1 and *b* and the Rieske iron sulfur protein (separate identification not shown), suggesting that complex III was fully assembled. Some cytochrome c_{552} was bound in addition, indicating that cytochrome c_{552} can bind not only to complex IV (described below) but also to complex III. *Band e* was identified as a subcomplex of complex III missing the Rieske iron sulfur protein and cytochrome c_{552}. The apparent masses of *bands d* and *e* from BN-PAGE suggest that fully assembled complex III and also the subcomplex are tetrameric in *P. denitrificans*. Minor amounts of dimeric com-

TABLE II
Purification of NADH oxidase supercomplex from P. denitrificans

	Membranes	Supernatant	Hydroxylapatite	Sepharose fractions 4–6
Protein mg (%)	202 (100)	84 (42)	23 (11)	4 (2)
NADH:DBQ oxidoreductase				
μmol min^{-1} (%)	83 (100)	87 (106)	38 (47)	8.7 (10)
μmol min^{-1} mg^{-1}	0.41	1.04	1.66	2.18
DBH:cytochrome *c* oxidoreductase				
μmol min^{-1} (%)	101 (100)	76 (76)	64 (64)	16 (16)
μmol min^{-1} mg^{-1}	0.50	0.91	2.80	3.99
nmol cytochrome *b* (%)	25.1 (100)	20.5 (82)	12.9 (51)	2.8 (11)
μmol cytochrome *b*/g	0.12	0.24	0.56	0.71
Cytochrome *c* oxidase				
μmol min^{-1} (%)	349 (100)	272 (78)	176 (50)	30.5 (9)
μmol min^{-1} mg^{-1}	1.73	3.24	7.63	7.62
nmol cytochrome aa_3 (%)	34.8 (100)	27.8 (80)	20.9 (60)	3.14 (9)
μmol cytochrome aa_3/g	0.17	0.33	0.90	0.80
Succinate:DCIP oxidoreductase				
μmol min^{-1} (%)	30.7 (100)	31.1 (101)	8.7 (28)	0 (0)
μmol min^{-1} mg^{-1}	0.15	0.37	0.38	0

plex III, which is the minimal structural unit of cytochrome bc_1 complexes (45–48), were identified by immunological detection (Fig. 1*B*, *arrows*).

Assignment of *bands a–c* was facilitated by the analysis of chromatographically isolated NADH oxidase supercomplex that was found to correspond to *band a*. It contained monomeric complex I, four copies of complex III, four copies of complex IV, and approximately two molecules of cytochrome c_{552} (described below). The smaller complexes *b* and *c* were devoid of complex I (see Fig. 5*A*). They contained tetrameric complex III and four and two copies of complex IV, respectively, as deduced from the apparent mass in BN-PAGE (Table I) and densitometric analyses of Coomassie-stained two-dimensional gels (not shown). Because other species containing one or three copies of complex IV were not detected, this suggested that dimeric complex IV is bound to the supercomplexes, although both the structural and functional units have been described as monomers (49).

Evidence for similar supercomplexes was obtained in comparable experiments using Triton X-100 and dodecylmaltoside. However, a stable NADH oxidase supercomplex could only be purified from digitonin-solubilized membranes.

Chromatographic purification, as described under "Experimental Procedures," involved three major steps: solubilization/centrifugation, hydroxylapatite chromatography, and gel filtration. The elution profile from the hydroxylapatite column and corresponding BN-PAGE is shown in Fig. 2*A*. More than 70% of the applied protein including ATP synthase (complex V) passed the column unbound. Yet all of the respiratory chain complexes were almost quantitatively bound and eluted together. The enzymatic activity elution profile of a subsequent gel filtration and the corresponding BN-PAGE (Fig. 2*B*) indicated that the NADH oxidase associate of complexes I, III, and IV eluted first, followed by assemblies of complexes III and IV and individual complexes V, II, and IV. Table II summarizes the purification of this NADH oxidase supercomplex. Complex II clearly was not associated with NADH oxidase. Only fractions 4–6 from gel filtration were used for further spectrometric and enzymatic analyses.

NADH oxidase supercomplex might comprise some protein constituents in addition to components of complexes I, III, and IV (molecular mass = 1.55 MDa) that have not been identified so far. In this case this potential extra mass should total approximately 350 kDa, because the apparent mass of the holocomplex in BN-PAGE was approximately 1.9 MDa. The cytochrome *b* and cytochrome aa_3 contents of the novel NADH oxidase supercomplex preparation were 0.71 and 0.80 μmol/g, respectively. This is far below the theoretical values of 2.6 or 2.1 μmol/g based on a stoichiometry of 4 mol/mol supercomplex for both cytochromes and the calculated mass (1.55 MDa) or the apparent mass from BN-PAGE (1.9 MDa), respectively. This substantial deviation can only be partly explained by protein impurities, because the subunit composition of NADH oxidase isolated chromatographically or by BN-PAGE was very similar, except for some additional bands present in the chromatographic preparation (Fig. 3). However, it seems more likely that the protein elution profile of Fig. 2*B* reflected an unknown non-protein contaminant most prominent in fractions 5–9, which interfered with the Lowry protein determination method. Digitonin and lipids can be excluded as this unknown non-protein contaminant, because digitonin and extracted *Paracoccus* lipids were tested not to interfere with the Lowry method. However, less than 1% digitonin in the sample does considerably interfere with the Biuret and Bradford methods, and more than 1% digitonin also interferes with the BCA method.

Ratio of Complexes in NADH Oxidase Supercomplex—The ratios of complexes were determined by fluorometric quantification of flavin mononucleotide as a marker for complex I and from pyridine hemochrome spectra (Table III). Calculation of the ratio is based on ratios of one flavin mononucleotide/complex I, two hemes *a*/complex IV, two hemes *b*, and one *c*-type heme (cytochrome c_1)/complex III (50). Cytochrome c_{552} concentration was calculated from the total heme *c* content (cytochromes c_1 plus c_{552}) considering that cytochrome c_1 concentration should equal the cytochrome *b* concentration, which is half the heme *b* concentration. Complexes I, III, and IV were found to be present in a 1:4:4 ratio. Cytochrome c_{552} was substoichiometric to complex IV (approximately 1.6 ± 0.8/4). Assuming an initial 1:1 ratio, this would indicate that 40–80% of total cytochrome c_{552} was lost during isolation.

Enzymatic Activities of Isolated NADH Oxidase Supercomplex—Turnover number of cytochrome *c* oxidase was 160 ± 15 s^{-1} (n = 3) in isolated NADH oxidase, compared with 168 ± 15 s^{-1} with digitonin-solubilized membranes assuming that all spectral absorption at 603 nm in the membranes used was due to cytochrome aa_3. These matching data seemed to indicate that alternative enzymes like ba_3 quinol oxidase and cbb_3 oxidase were not present in considerable amounts in the aerobically grown cells used. Turnover number of DBH:cytochrome *c* oxidoreductase (complex III) was 95 ± 14 s^{-1} (n = 3) in isolated NADH oxidase complex. Spectral absorption of complex III at approximately 560 nm in membranes may be superimposed by several other *b*-type cytochromes, *e.g.* of succinate dehydrogenase (51). Therefore, complex III activity in membranes cannot be quantified reliably.

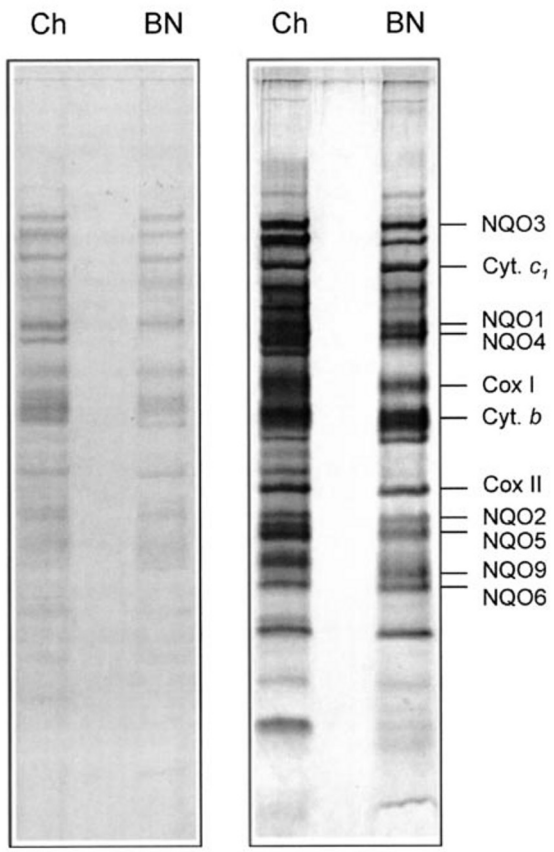

FIG. 3. **Subunit composition of NADH oxidase supercomplex.** NADH oxidase was prepared chromatographically (*Ch*) and by BN-PAGE (*BN*). The subunits were resolved by Tricine-SDS-PAGE using a 10% acrylamide gel. *Left panel*, Coomassie stain. *Right panel*, silver stain. Some subunits of complex I (*NQO 1–6* and *9*), complex III (*cyt. c_1* and *cyt. b*), and complex IV (*Cox I* and *Cox II*) were identified immunologically (experiments not shown). Subunits Cox III, cytochrome c_{552}, and the Rieske iron sulfur protein colocalized to the area of NQO 9 and NQO 6 bands and are not indicated.

NADH:DBQ oxidoreductase (complex I) turnover number in digitonin-solubilized membranes could not be calculated on the basis of the extractable flavin mononucleotide in membranes (0.096 μmol flavin/g; $n = 6$), because extractable flavin apparently originated also from proteins other than complex I, and calculated numbers were approximately 70 s^{-1} compared with 186 $\pm$ 26 s^{-1} for the isolated NADH oxidase complex. However, by comparing deamino-NADH:DBQ oxidoreductase activity and deamino-NADH:hexamino-ruthenium oxidoreductase activity, a stable catalytic activity of the hydrophilic part of complex I that is not altered by a potential dissociation of the hydrophobic part, it was possible to test whether the complex I turnover number was altered or unchanged during isolation of NADH oxidase (52). Deamino-NADH was used instead of NADH to exclude potential alternative dehydrogenases, although alternative dehydrogenases do not seem to exist in *P. denitrificans* (53). The deamino-NADH:hexamino-ruthenium/deamino-NADH:DBQ ratio was 3.9 $\pm$ 0.2 in membranes and 4.6 $\pm$ 0.3 in isolated NADH oxidase supercomplex, indicating that no considerable changes occurred during isolation.

NADH:ubiquinone oxidoreductase (complex I) activity of isolated NADH oxidase supercomplex was 2.24 $\pm$ 0.08 μmol min^{-1} mg^{-1}. This is the maximal activity theoretically obtainable for coupled activities of complexes I, III, and IV, because activities of complexes III and IV were significantly higher because of the 4-fold excess of the corresponding enzymes to complex I (see turnover numbers above and Table III). NADH:cytochrome *c* oxidoreductase (complexes I and III) activity of the same protein sample (1.51 $\pm$ 0.01 μmol min^{-1} mg^{-1}) indicated almost optimum electron transfer from complex I to complex III, in agreement with high endogenous phospholipid and quinone contents required for electron transfer. Based on the Lowry protein determination, the phospholipid content decreased from 980 $\pm$ 80 nmol/mg in membranes to 290 $\pm$ 10 nmol/mg in isolated NADH oxidase, whereas the ubiquinone content increased from 2.7 $\pm$ 0.3 nmol/mg in membranes to 6.7 $\pm$ 0.8 nmol/mg in isolated NADH oxidase. The 8-fold increase of the quinone/phospholipid ratio seems to indicate specific enrichment of ubiquinone in the NADH oxidase supercomplex. A ubiquinone/supercomplex ratio of 10 mol/mol was calculated based on the Lowry protein determination. Considering a potential interference of a non-protein contaminant with the Lowry method as described above, the ratio can approach a value of 30 mol/mol.

However, specific NADH oxidase activity (complexes I, III, and IV) was reduced by 74% to 0.53 $\pm$ 0.07 μmol min^{-1} mg^{-1}. Similarly, DBH oxidase (complexes III and IV) was reduced by 70% to 1.19 $\pm$ 0.01 μmol min^{-1} mg^{-1} compared with 4.07 $\pm$ 0.12 μmol min^{-1} mg^{-1} for DBH:cytochrome *c* oxidoreductase (complex III). This seems to indicate that the transfer of electrons between complexes III and IV was impeded, presumably by partial loss of cytochrome c_{552}, as described above.

Assembly into NADH Oxidase Supercomplex Stabilizes Complex I—Digitonin is one of the mildest detergents known. However, it was not clear whether retention of the complex I integrity in isolated NADH oxidase was due to the particular detergent properties or to an additional stabilizing role of supercomplex formation. To address this question, we compared respiratory chain activities in digitonin-solubilized membranes and the stability of complex I from wild-type and mutant strains during chromatography and BN-PAGE.

Complex III activity was not adversely affected by the loss of cytochrome c_{552} or complex IV in the corresponding mutant strains (Fig. 4). However, the loss of complex III in the complex III mutant strain led to considerable reduction of complex I and IV activities, indicating that complex III is required for assembly/stability of both complexes. A moderate reduction of complexes I and IV was also observed in the mutant strain lacking cytochrome c_{552}, indicating that also cytochrome c_{552} favors assembly/stability of the two complexes.

Complex I activity was substantially lower in all mutant strains compared with wild type, which might be taken as a hint for impeded assembly or stability of complex I in these membranes. Although all complex I activities were stable for 60 min after solubilization using low digitonin/protein ratios, reduced stability of complex I in the mutant strains lacking complexes III or IV became apparent from the subsequent analyses.

Attempts to isolate complex I from mutant strains lacking complexes III or IV led to an almost complete loss of NADH:DBQ oxidoreductase activity when the same isolation protocol as for parental strain was applied (results not shown). Even omission of the hydroxylapatite step that requires use of low Triton X-100 concentrations for protein elution and direct gel filtration using digitonin was not successful. These findings seem to indicate that complex I from these mutant strains is sufficiently stable at the low digitonin/lipid ratios used for solubilization, but stability is reduced under the conditions of chromatography. Because the presence of only one of the two complexes in the corresponding mutant strains could not protect complex I from inactivation during chromatography, we

TABLE III

Determination of redox factor contents (nmol / mg) and respiratory complex stoichiometries in isolated NADH oxidase supercomplex

Cytochrom c_{552} content was calculated from the total heme *c* content considering that cytochrome c_1 concentration equals the cytochrome *b* (complex III) concentration.

	FMN	Heme *a*	Heme *b*	Heme *c*	
Sample 1	0.16	1.18	1.20	0.90	
Sample 2	0.20	1.42	1.62	0.89	
Sample 3	0.13	1.04	1.00	0.90	
Average	0.16 ± 0.03	1.21 ± 0.16	1.27 ± 0.26	0.9 ± 0.01	
	Complex I	Complex IV	Complex III	Cytochrome c_1	Cytochrome c_{552}
Average	0.16 ± 0.03	0.61 ± 0.08	0.64 ± 0.13	0.64 ± 0.13	0.26 ± 0.13
Ratio	1.00	3.8 ± 0.5	4.0 ± 0.8	4.0 ± 0.8	1.6 ± 0.8

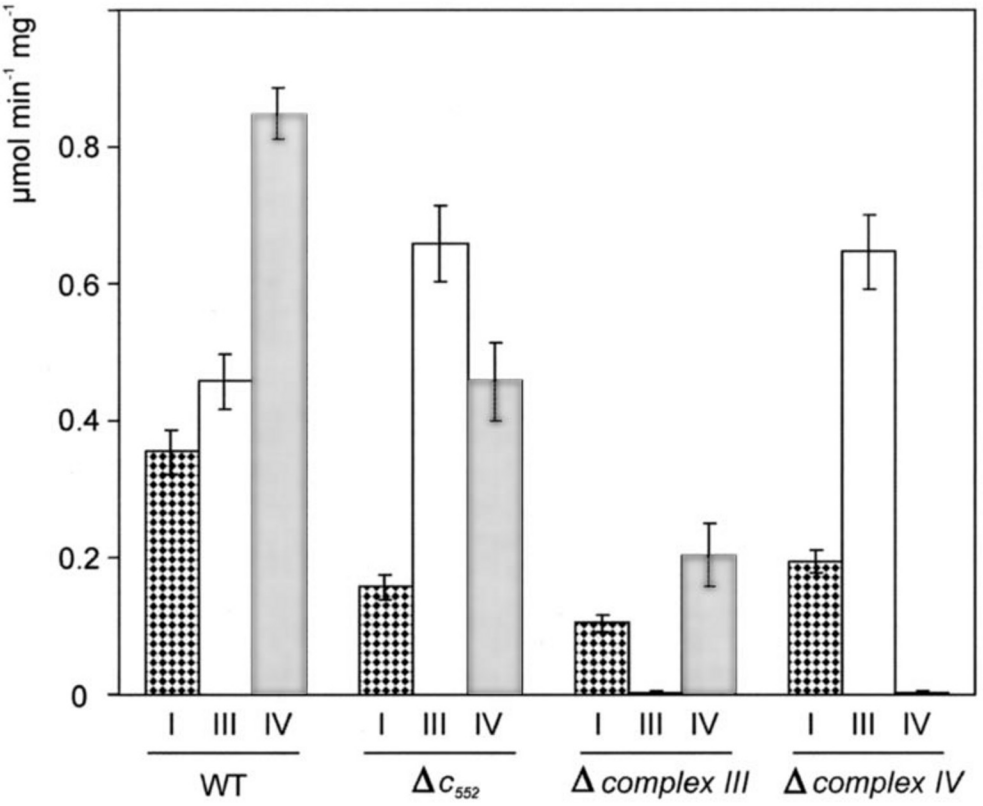

FIG. 4. **Catalytic activities of digitonin-solubilized respiratory chain complexes from *P. denitrificans* parental and mutant strains.** *I*, NADH:DBQ oxidoreductase activity of complex I; *III*, DBH:cytochrome *c* oxidoreductase activity of complex III; *IV*, cytochrome *c* oxidase activity of complex IV. *WT*, parental strain; Δc_{552}, Δ*complexIII*, and Δ*complexIV*, mutant strains lacking cytochrome c_{552}, complex III, and complex IV, respectively.

conclude that complex I requires assembly into a complete NADH oxidase complex for optimal stability.

Reduced stability of complex I in these mutant strains was also apparent from the complete dissociation of complex I under the conditions of BN-PAGE. Following two-dimensional SDS-PAGE and electroblotting, a mixture of specific antibodies was used to identify the location of assembled complexes and dissociated subunits. Using parental strain Pd1222 (Fig. 5*A*), the antibodies identified supercomplexes *a*, *b*, and *c* and individual complexes III and IV, but intact individual complex I was not present. Dissociated complex I subunits NQO 3 and 1 were found at the running front of BN-PAGE, *i.e.* at the right-hand side of the two-dimensional gel. At present we cannot discriminate between two possibilities. The dissociated subunits might either originate from individual complex I if larger fractions of it were in equilibrium with supercomplexes *b* and *c*. On the other hand, almost all complex I might initially assemble into NADH oxidase supercomplex, which partially disintegrates during BN-PAGE.

Using the complex III deletion strain (Fig. 5*C*), individual complex IV was detected but no assembled complex I. This seems to indicate that all complex I that was functional after solubilization by digitonin dissociated under the conditions of BN-PAGE. Analysis of the complex IV deletion strain (Fig. 5*D*) led to a similar result, except that stable tetrameric complex III was identified.

The proteins involved in the interaction of complexes III and IV are not known. Tightly bound cytochrome c_{552} that possesses a transmembrane anchor (31) initially was regarded as a candidate linker protein, because it binds preferentially to complex IV, and minor amounts were also found associated with complex III. Analyzing the strain carrying an inactivated cycM gene coding for cytochrome c_{552} (Fig. 5*B*) indicated that this electron carrier was not essential for

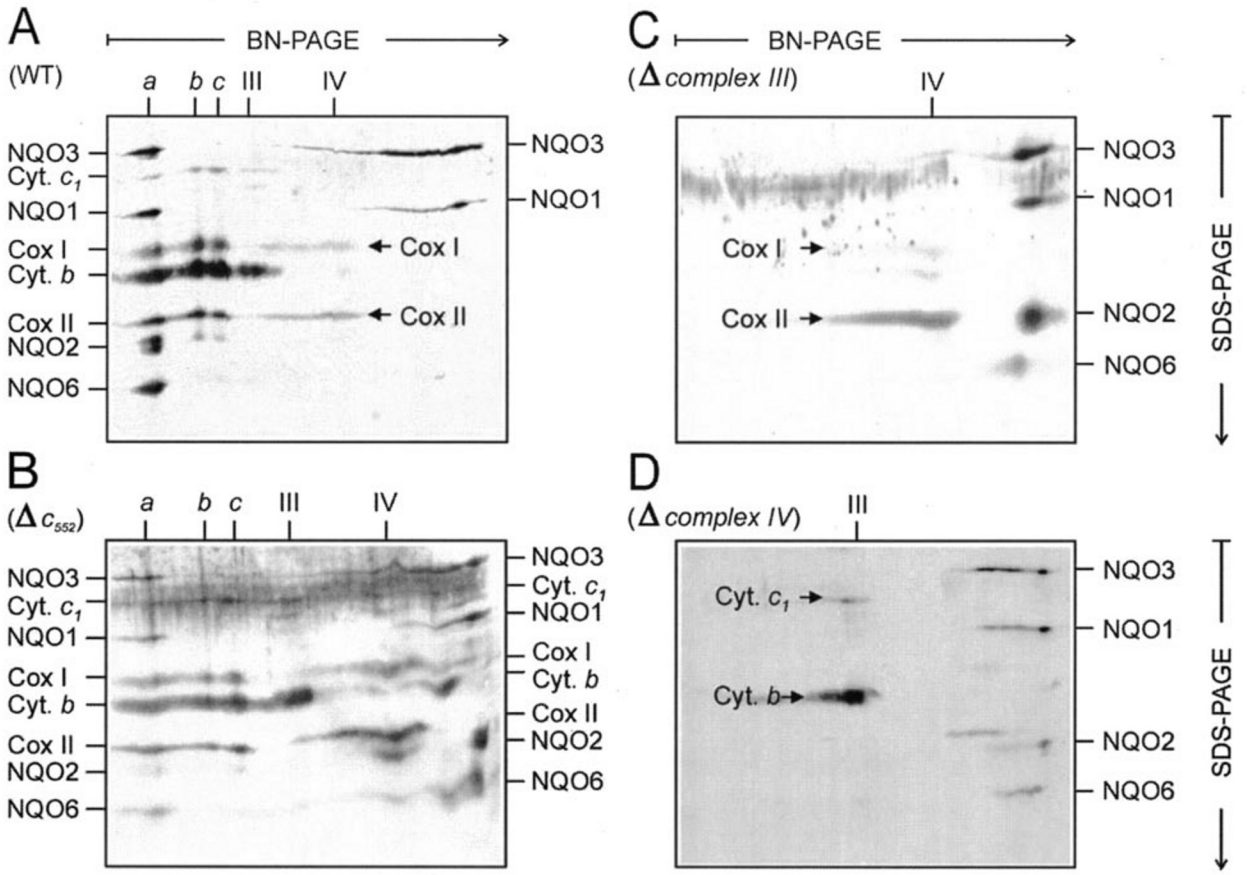

FIG. 5. **Identification of respiratory chain complexes and subunits in *P. denitrificans* parental and mutant strains.** Parental and mutant strains were solubilized using digitonin (3 g/g protein), separated by BN-PAGE followed by two-dimensional SDS-PAGE, and blotted onto polyvinylidene difluoride membranes. A mixture of antibodies directed against subunits of complexes I, III, and IV was used for immunodetection. *A*, *P. denitrificans* parental strain Pd1222 (*WT*). Antibodies identified *supercomplexes a*, *b*, and *c* and small amounts of individual complexes III and IV but no intact individual complex I. Dissociated complex I subunits NQO 3 and 1 were found at the running front of BN-PAGE, *i.e.* at the right side of the two-dimensional gel. *B*, AT110 strain with insertional inactivation of the cycM gene coding for cytochrome c_{552} (Δc_{552}). Cytochrome c_{552} was found not to be essential for formation of *supercomplexes a*, *b*, and *c*. However, amounts and stability of supercomplexes seemed to be reduced. *C*, MK6 strain with deletion of the *fbc* operon coding for complex III (Δ*complex III*). Assembled complex IV and dissociated subunits of complex I were identified. *D*, MR31 strain with inactivation of both subunit I gene copies of complex IV (Δ*complex IV*). No supercomplex but stable tetrameric complex III and dissociated subunits of complex I were identified.

formation of supercomplexes *a*, *b,* and *c*. However, the amounts of supercomplexes were reduced, and the amounts of dissociated subunits of complex I were increased, indicating that cytochrome c_{552} favors supercomplex formation and indirectly stabilizes complex I.

Summarizing, we conclude that detergent-labile complex I from *P. denitrificans* is protected by supercomplex formation. Deletion of complexes III and IV causes decreased complex I contents in membranes, suggesting altered assembly/stability of complex I that is not assembled into a complete NADH oxidase supercomplex also in membranes. However, detergent-stable NADH dehydrogenases, like *E. coli* complex I, do not require supercomplex formation. In fact, *E. coli* does not possess complex III. Similarly, complexes I from the yeast *Yarrowia lipolytica*, and the hyperthermophilic eubacterium *A. aeolicus* are rather stable (25, 54), and no respiratory chain supercomplexes could be detected. Structural stabilization of a labile membrane protein complex seems to be a major function of supercomplex formation, in addition to substrate channeling, which is easily envisaged for the interaction of complexes III and IV in *P. denitrificans* via cytochrome c_{552} but also seems to play a role for the interaction of complexes I and III via ubiquinone, as recently reported for the bovine respirasome (55).

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REFERENCES

1. Hatefi, Y. (1985) *Annu. Rev. Biochem.* **54,** 1015–1069
2. Margulis, L. (1970) *Origin of Eukaryote Cells*, Yale University Press, New Haven, CT
3. John, P., and Whatley, F. R. (1975) *Nature* **254,** 495–498
4. Stouthamer, A. H. (1992) *Antonie Leeuwenhoek* **61,** 1–33
5. Baker, S., Ferguson, S. J., Ludwig, B., Page, M. D., Richter, O.-M. H., and van Spanning, R. J. M. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 1046–1078
6. Xu, X., Matsuno-Yagi, A., and Yagi, T. (1993) *Biochemistry* **32,** 968–981
7. Yang, X., and Trumpower, B. L. (1986) *J. Biol. Chem.* **261,** 12282–12289
8. Kurowski, B., and Ludwig, B. (1987) *J. Biol. Chem.* **262,** 13805–13811
9. Raitio, M., Jalli, T., and Saraste, M. (1987) *EMBO J.* **6,** 2825–2833
10. Carroll, J., Fearnley, I. M., Shannon, R. J., Hirst, J., and Walker J. E. (2003) *Mol. Cell Proteomics* **2,** 117–126
11. Schägger, H., Brandt, U., Gencic, S., and von Jagow, G. (1995) *Methods Enzymol.* **260,** 82–96
12. Kadenbach, B., Jarausch, J., Hartmann, R., and Merle, P. (1983) *Anal. Biochem.* **129,** 517–521
13. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) *Science* **280,** 1723–1729
14. Schägger, H., and Pfeiffer, K. (2000) *EMBO J.* **19,** 1777–1783
15. Cruciat, C. M., Brunner, S., Baumann, F., Neupert, W., and Stuart, R. A. (2000) *J. Biol. Chem.* **275,** 18093–18096
16. Berry, E. A., and Trumpower, B. L. (1985) *J. Biol. Chem.* **260,** 2458–2467
17. Sone, N., Sekimachi, M., and Kutoh, E. (1987) *J. Biol. Chem.* **262,**

15386–15391
18. Iwasaki, T., Matsuura, K., and Oshima, T. (1995) *J. Biol. Chem.* **270,** 30881–30892
19. Iwasaki, T., Wakagi, T., Isogai, Y., Iizuka, T., and Oshima, T. (1995) *J. Biol. Chem.* **270,** 30893–30901
20. Komorowski, L., Verheyen, W., and Schäfer, G. (2002) *Biol. Chem.* **383,** 1791–1799
21. Niebisch, A., and Bott, M. (2003) *J. Biol. Chem.* **278,** 4339–4346
22. Leif, H., Sled, V. D., Ohnishi, T., Weiss, H., and Friedrich, T. (1995) *Eur. J. Biochem.* **230,** 538–548
23. Fernandes, A. S., Pereira, M. M., and Teixeira, M. (2002) *J. Bioenerg. Biomembr.* **34,** 413–421
24. Gemperli, A. C., Dimroth, P., and Steuber, J. (2002) *J. Biol. Chem.* **277,** 33811–33817
25. Peng, G., Fritzsch, G., Zickermann, V., Schägger, H., Mentele, R., Lottspeich, F., Bostina, M., Radermacher, M., Huber, R., Stetter, K. O., and Michel, H. (2003) *Biochemistry* **42,** 3032–3039
26. DeVries, G. E., Harms, N., Hoogendijk, J., and Stouthamer, A. H. (1989) *Arch. Microbiol.* **152,** 52–57
27. Korn, M. (1994) *Doppeldeletion der Cytochrom c-Oxidase und -Reduktase (cta und fbc-Operon) in Paracoccus denitrificans.* Diploma thesis, Johann Wolfgang Goethe-Universität Frankfurt/Main, Germany
28. Schröter, T., Hatzfeld, O. M., Gemeinhard, S., Korn, M., Friedrich, T., Ludwig, B., and Link, T. A. (1998) *Eur. J. Biochem.* **255,** 100–106
29. Raitio, M., and Wikström, M. (1994) *Biochim. Biophys. Acta* **1186,** 100–106
30. Turba, A. (1993) *Molekularbiologische und biochemische Charakterisierung des membrangebundenen Cytochrom c_{552} aus Paracoccus denitrificans.* Ph. D. thesis, Johann Wolfgang Goethe-Universität Frankfurt/Main, Germany
31. Turba, A., Jetzek, M., and Ludwig, B. (1995) *Eur. J. Biochem.* **231,** 259–265
32. Ludwig, B. (1986) *Methods Enzymol.* **126,** 153–159
33. Van Spanning, R. J., Wansell, C., Harms, N., Oltmann, L. F., and Stouthamer, A. H. (1990) *J. Bacteriol.* **172,** 986–996
34. Schägger, H., and von Jagow, G. (1994) *Anal. Biochem.* **217,** 220–230
35. Schägger, H. (1995) *Electrophoresis* **16,** 763–770
36. Koziol, J. (1971) *Methods Enzymol.* **18,** 253–285
37. Hatefi, Y, and Stempel, K. E. (1969) *J. Biol. Chem.* **244,** 2350–2357
38. Berry, E. A., and Trumpower, B. L. (1987) *Anal. Biochem.* **161,** 1–15
39. Helenius, A., and Simons, K. (1972) *J. Biol. Chem.* **247,** 3656–3661
40. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
41. Itzhaki, R. F., and Gill, D. M. (1964) *Anal. Biochem.* **9,** 401–410
42. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., OLson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
43. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem.* **28,** 1756–1758
44. Dröse, S., Zwicker, K., and Brandt, U. (2002) *Biochim. Biophys. Acta* **1556,** 65–72
45. Xia, D., Yu, C. A., Kim, H., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997) *Science* **277,** 60–66
46. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y., Kim, K. K., Hung, L. W., Crofts, A. R., Berry, E. A., and Kim, S. H. (1998) *Nature* **392,** 677–684
47. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Iwata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) *Science* **281,** 64–71
48. Hunte, C., Koepke, J., Lange, C., Roβmanith, T., and Michel, H. (2000) *Structure* **8,** 669–684
49. Iwata, S., Ostermeier, C., Ludwig, B., and Michel H. (1995) *Nature* **376,** 660–669
50. Gabellini, N., Bowyer, J. R., Hurt, E., Melandri, B. A., and Hauska, G. (1982) *Eur. J. Biochem.* **126,** 105–111
51. Pennoyer, J. D., Ohnishi, T., and Trumpower, B. L. (1998) *Biochim. Biophys. Acta* **935,** 195–207
52. Zickermann, V., Barquera, B., Wikström, M., and Finel, M. (1998) *Biochemistry* **37,** 11792–11796
53. Finel, M. (1996) *FEBS Lett.* **393,** 81–85
54. Kashani-Poor, N., Kerscher, S., Zickermann, V., and Brandt, U. (2001) *Biochim. Biophys. Acta* **1504,** 186–193
55. Genova, A. L., Bianchi, C., and Lenaz, G. (2003) *Ital. J. Biochem.* **52,** 58–61