Research paper

Supramolecular organizations in the aerobic respiratory chain of *Escherichia coli*

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ABSTRACT

The organization of respiratory chain complexes in supercomplexes has been shown in the mitochondria of several eukaryotes and in the cell membranes of some bacteria. These supercomplexes are suggested to be important for oxidative phosphorylation efficiency and to prevent the formation of reactive oxygen species.

Here we describe, for the first time, the identification of supramolecular organizations in the aerobic respiratory chain of *Escherichia coli*, including a trimer of succinate dehydrogenase. Furthermore, two heterooligomerizations have been shown: one resulting from the association of the NADH:quinone oxidoreductases NDH-1 and NDH-2, and another composed by the cytochrome bo_3 quinol:oxygen reductase, cytochrome bd quinol:oxygen reductase and formate dehydrogenase (*fdo*). These results are supported by blue native-electrophoresis, mass spectrometry and kinetic data of wild type and mutant *E. coli* strains.

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1. Introduction

The respiratory chain of eukaryotic cells is located in the inner mitochondrial membrane, where a set of membrane proteins (complexes I–IV) and small electron carriers (ubiquinone and cytochrome *c*) mediate electron transfer from reducing substrates like NADH and succinate to oxygen. Coupled to the redox reactions, complexes I (type I NADH:ubiquinone oxidoreductase or NDH-1), III (ubiquinol:cytochrome *c* oxidoreductase) and IV (cytochrome *c*:oxygen oxidoreductase) translocate protons from the matrix to the intermembrane space, generating a proton motive force that will enable ATP synthase to synthesize ATP, along with the reflux of protons to the matrix. In contrast to complexes I, III and IV, complex II (succinate:ubiquinone oxidoreductase, SDH) does not translocate protons [1].

Similarly, bacterial aerobic respiratory chains are assembled in the cytoplasmic membrane, through which proton translocation occurs from the cytoplasm to the periplasmic space. Unlike mitochondria, bacteria may have type I, II and III NADH:quinone oxidoreductases [2], together with different types of oxygen reductases, such as cytochrome *bd*-like oxygen reductases and heme–copper oxygen reductases, the latter being either cytochrome *c*/high potential iron–sulfur protein (HiPIP) or quinol:oxygen reductases [3].

The aerobic respiratory chain of *Escherichia coli* comprises type I and II NADH:quinone oxidoreductases, succinate:quinone oxido-reductase, and at least two quinol:oxygen oxidoreductases, cyto-chromes *bd* and *bo₃*, all enzymes being differentially expressed in response to the oxygen tension of the culture medium and growth phase [4,5].

Supramolecular organization of respiratory chains has been recently extensively reported for all life domains, challenging the random diffusion model [6] and providing new evidence in strong support of the "solid state" model proposed by Chance and Williams [7]. In eukaryotes, supercomplexes formed by complexes I, III and IV, the so-called respirasomes, have been observed in

mitochondria of bovine heart [8–10], mouse liver [11], potato tuber [12,13], *Neurospora crassa* [14] and *Yarrowia lipolytica* [15]. Associations of complexes III and IV have also been described in these organisms, as well as in *Saccharomyces cerevisiae*, that lacks complex I, and for which a mitochondrial dehydrogenase supercomplex has been proposed [16].

Respiratory chain supercomplexes have also been described in archaea and bacteria. It was reported that *Sulfolobus* sp. strain 7 has a terminal oxygen reductase supercomplex resulting from the functional fusion of complexes III and IV, containing cytochromes of the *b* and *a* types, and a Rieske-type iron–sulfur protein [17,18]. A mitochondrial-like respirasome was identified in *Paraccocus denitrificans* [19] and supramolecular associations of complexes III and IV were detected in *P. denitrificans* [19,20], *Corynebacterium glutamicum* [21], and *Bacillus* PS3 [22,23].

Although little is known regarding a possible interaction between ATP synthase and respiratory proton translocating complexes, the interaction between the caa_3 oxygen reductase and the ATP synthase of *Bacillus pseudofirmus* has been suggested [24]. Furthermore, oligomers of ATP synthase have been reported in bovine heart mitochondria and seem to shape the inner membrane cristae [25]. In *S. cerevisiae*, dimeric ATP synthase complexes were characterized and proposed to influence the assembly of the complex III–IV supercomplex, providing further evidence for a close relation between ATP synthase and the electron transport complexes [26].

In spite of the fact that the functional relevance of such organizations requires further clarification, there is clear consensus regarding the benefits it may bring to oxidative phosphorylation in the channeling of electrons, sequestration of reactive oxygen species, induction of mitochondrial cristae shape and structural stabilization of individual complexes [15,19,27–29].

To date, evidence to support the existence of supercomplexes in the aerobic respiratory chain of *E. coli* is lacking. Indeed, it has been suggested that since it contains a detergent stable complex I and is devoid of complex III, such supramolecular organization is not necessary [19]. Nevertheless, co-localization of oxidative phosphorylation complexes has been suggested [30].

Here, we present evidence for the presence of two supercomplexes, one formed by complex I and the type II NADH:quinone oxidoreductase (NDH-2), and another comprised of cytochromes bo_3 and bd quinol:oxygen reductases and a protein with NADH:NBT oxidoreductase activity, that has been identified by mass spectrometry to be the aerobic formate dehydrogenase. Moreover, the trimer of succinate dehydrogenase was observed for the first time in solubilized membranes of this bacterium.

2. Materials and methods

2.1. Solubilized membrane preparation

E. coli K-12 (ATCC 23716) and selected respiratory chain mutants were grown manually in Luria–Bertani medium adjusted to pH 7, at 37 °C, under vigorous agitation, the volume of cultures corresponding to one fifth of the total volume of the flasks, and harvested at early stationary phase. Upon suspension in MES 50 mM pH 6.0 [31] and disruption in a French press (6000 psi), cells were submitted to low speed centrifugation ($14000 \times g$, 15 min) to remove intact cells and cell debris and the supernatant was ultra-centrifuged ($138000 \times g$, 2 h) to separate the soluble from the membrane fraction. The isolated membrane fraction was aliquoted, frozen in liquid nitrogen and stored at −80 °C.

Membrane protein concentration was determined by the BCA assay [32]. Membrane solubilization trials were performed using several detergents (Triton X-100, DDM and digitonin) with variable detergent/protein ratios and analyzed by BN-PAGE (SM1). Solubilization was performed in a buffer containing NaCl 50 mM, aminocaproic acid 5 mM, EDTA 1 mM, PMSF 2 mM and imidazole/HCl 50 mM pH 6, on ice for 15 min and vortexing each 3 min, followed by centrifugation ($14000 \times g$, 30 min). A ratio of 6 g of digitonin per g of protein was found to be the best solubilization condition that preserved protein–protein interactions within the respiratory chain complexes and therefore used for the subsequent work herein described.

2.2. Electrophoretic techniques

Proteins from the solubilized membranes (150 μg per lane) were resolved by BN-PAGE [33–35].

The activities of NADH:NBT and succinate:NBT oxidoreductase were detected *in gel* [36] as well as *b*-type hemes [37]. Solubilized membranes (12.5 mg) were also applied on top of continuous sucrose gradients (0.3–1.5 M and 1–1.5 M) in a buffer containing 15 mM Tris/HCl pH 7, 20 mM KCl and 0.2% digitonin, resolved by ultra-centrifugation at 4 °C (20 h, $150000 \times g$) [38] and 1 mL fractions were collected, frozen in liquid nitrogen and stored at −80 °C.

2.3. Spectroscopic techniques

Spectroscopic characterization of intact or solubilized wild type membranes was performed by UV–visible absorption spectroscopy. Spectra were recorded with the as-isolated membranes or gradient fractions (oxidized state) and upon reduction by the addition of trace quantities of sodium dithionite powder. To obtain the CO-reduced spectrum, dithionite-reduced samples were incubated with CO gas for 2–3 min before spectral acquisition. The pyridine–hemochrome method [39] was used to quantify the type *b* hemes present in the above mentioned samples.

2.4. Catalytic activities

NADH:potassium ferricyanide and succinate:DCPIP oxidoreductase activities were spectrophotometrically measured in solubilized membranes and in sucrose gradient fractions. NADH:ferricyanide oxidoreductase activity was determined following oxidation of NADH at 340 nm ($\varepsilon_{NADH} = 6.22$ mM^{-1} cm^{-1}) in a reaction buffer containing 100 mM MOPS pH 7.2, 250 μM NADH and 250 μM K$_3$[Fe(CN)$_6$] [40]. Succinate:DCPIP oxidoreductase activity was monitored by following the PMS-coupled reduction of DCPIP at 578 nm at 37 °C ($\varepsilon_{DCPIP} = 20.5$ mM^{-1} cm^{-1}). The reaction mixture contained 100 mM MOPS pH 7.2, 0.05 mM PMS, 0.05 mM DCPIP and 20 μM succinate [41].

Oxygen consumption rates due to NADH, succinate and quinol oxidation were polarographically determined in a Rank Broths oxygen electrode (Hansatech) at 37 °C, in intact membranes, sucrose gradient fractions and NADH:NBT oxidoreductase positive bands. NADH and succinate oxidation were measured in a buffer containing 50 mM MOPS pH 7.2 and 250 μM NADH or 20 mM succinate, respectively. Quinol:oxygen oxidoreductase activity was monitored in a reaction mixture containing 100 mM MOPS pH 7.2, 50 mM KCl, 0.5 mM EDTA, 5.7 mM dithiothreitol, and 80 μM coenzyme Q$_1$. Specific inhibitors of the respiratory chain complexes such as rotenone (200 μM), piericidin A (2 μM), malonate (15 mM), and KCN (0.5 mM or 2.5 mM) were used. All reactions were initiated by addition of membranes (0.1 mg) or gradient fractions (40 μL).

2.5. Mass spectrometry

Proteins from digitonin-solubilized membranes were resolved by BN-PAGE and bands revealing NADH:NBT oxidoreductase activity were excised for digestion and MS analysis [42]. Tryptic peptides were deposited on a 192-well MALDI plate with 5 mg/mL α-CHCA (LaserBiolabs, 1:1) in 0.1% trifluoroacetic acid/60% acetonitrile (v/v) and MS analysis was performed on a MALDI-TOF/TOF MS (ABI 4700 Proteomics Analyzer, Applied Biosystems). Internal calibration, data acquisition, processing and interpretation and database search were performed according to standard criteria [43].

In addition, *in-gel* digestion was performed and peptide extracts were resuspended in 0.1% trifluoroacetic acid and analyzed by reversed-phase liquid chromatography (Ultimate 3000, Dionex Corporation, Sunnyvale, CA) coupled online to a linear ion trap MS (LTQ-XL, ThermoFisher Scientific, San Jose, CA). Separations were performed using a 75 μm i.d. × 360 o.d. × 20 cm long fused silica capillary columns (Polymicro Technologies, Phoenix, AZ) slurry packed in-house with 5 μm, 300 Å pore size C-18 silica-bonded stationary phase (Jupiter, Phenomenex, Torrance, CA). Following sample injection onto a C-18 precolumn (Dionex), the column was washed for 3 min with mobile phase A (2% acetonitrile, 0.1% formic acid) at a flow rate of 30 μL/min. Peptides were eluted using a linear gradient of 1% mobile phase B (0.1% formic acid in acetonitrile)/minute for 40 min, then to 95% B in an additional 10 min, all at a constant flow rate of 200 nL/min. Column washing was performed at 95% B for 15 min for all analyses, after which the column was re-equilibrated with mobile phase A prior to subsequent injections.

The MS was operated in data-dependent MS/MS mode in which each full MS scan was followed by seven MS/MS scans performed in the linear ion trap (LIT) where the seven most abundant peptide molecular ions were selected for collision-induced dissociation (CID), using a normalized collision energy of 35%. Data were collected over a broad precursor ion selection scan range of m/z 375–1800 and dynamic exclusion was enabled to minimize redundant selection of peptides previously selected for CID. Tandem mass spectra were searched against the UniProt-derived-*E. coli* protein database (6/10 release) from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8), using SEQUEST (ThermoFisher Scientific). Additionally, peptides were searched for dynamic methionine oxidation (15.995 amu). Peptides were considered legitimately identified if they met specific charge state and proteolytic cleavage-dependent cross correlation scores of 1.9 for $[M + H]^{1+}$, 2.2 for $[M+2H]^{2+}$ and 3.5 for $[M+3H]^{3+}$ and a minimum delta correlation of 0.08.

3. Results and discussion

3.1. Characterization of the E. coli aerobic respiratory chain activities

Supramolecular organizations involving the NADH:quinone oxidoreductase activity were investigated in the *E. coli* K-12 aerobic respiratory chain, which was thoroughly characterized prior to supercomplex detection.

UV−visible absorption spectra of the *E. coli* solubilized membranes were recorded in the oxidized and dithionite-reduced states and upon incubation with CO. The difference spectrum (spectrum of the dithionite-reduced state minus that of the oxidized state) indicates the presence of hemes *b* (maxima at 416 and 560 nm) and hemes *d* (shoulder at 441 nm, maxima at 631 nm and trough at 651 nm) (Fig. 1A). Furthermore, the difference spectrum of the dithionite-reduced membranes incubated with CO minus the spectrum of the dithionite-reduced membranes revealed the presence of hemes *o* (maxima at 416 and 567 nm and trough at 430 nm) and *d* (shoulder at 420 and maxima at 642 nm and trough at 430, 442, 560 and 622 nm) (Fig. 1B), indicating that both cytochromes bo_3 and bd oxygen reductases were expressed [4,44]. The study of the aerobic respiratory chain activities, performed polarographically with an oxygen electrode, resulted in the detection of NADH:oxygen oxidoreductase activity [2,45]. NADH oxidation was only 70% inhibited by two complex I inhibitors (rotenone and piericidin A), suggesting the presence of another NADH dehydrogenase (NDH-2) as has been previously described for the *E. coli* electron transfer chain [46]. A malonate-sensitive succinate:oxygen oxidoreductase activity was also measured, suggesting the presence of SDH [47]. Both NADH and succinate oxidoreductase activities were nearly completely inhibited by KCN (Table 1), which is known to inhibit heme−copper oxygen reductases [3], like cytochrome bo_3. In addition, a high rate of KCN sensitive quinol:oxygen oxidoreductase activity was observed (Table 1).

3.2. Identification of supramolecular organizations in the aerobic respiratory chain of E. coli

3.2.1. Detection of supercomplexes by electrophoretic techniques

The solubilized membrane fraction was resolved by BN-PAGE and, after staining for NADH:NBT and succinate:NBT oxidoreductase artificial activities, 4 bands with NADH:NBT oxidoreductase activity (bands 1–4), and 2 bands with activity of succinate:NBT oxidoreductase (bands 5 and 6) were apparent (Fig. 2). In addition,
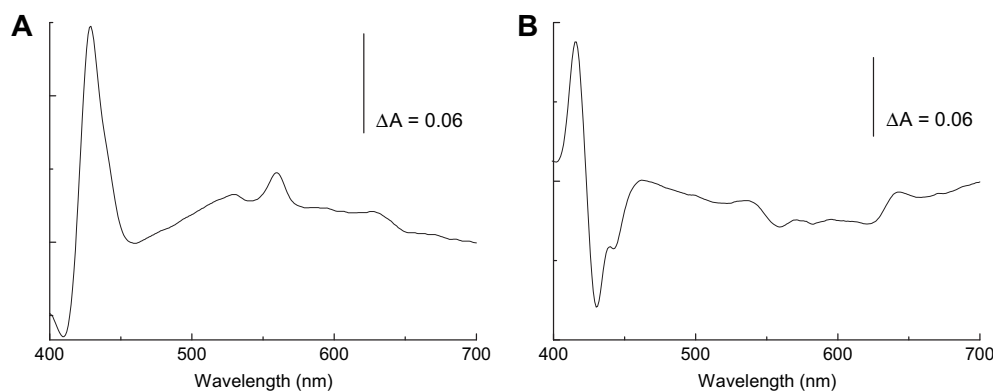
Fig. 1. UV−visible absorption spectra of triton X-100 *E. coli* membranes. Spectral acquisition was performed to the as-isolated membranes (oxidized state) and after the addition of sodium dithionite (reduced state). The dithionite-reduced samples were incubated with CO to obtain the CO spectrum. Shown are difference spectra representing dithionite-reduced minus oxidized (A) and CO-reduced minus dithionite-reduced (B).

heme staining allowed the detection of another band (band 7). The apparent molecular mass of bands 1–7 was estimated from the BN-PAGE in reference to molecular markers extracted from bovine heart mitochondria [48] and the molecular mass of the individual respiratory chain complexes was deduced from their primary structures (Table 2).

Bands 5 and 6, with apparent molecular masses of 305 ± 14 and 128 ± 2 kDa, respectively, most likely correspond to the trimeric and monomeric forms of SDH (Fig. 2B). It is noteworthy that the *E. coli* enzyme has been reported to crystallize as a trimer and this oligomerization of the protein was suggested to have physiological relevance [49]. Band 7, detected by heme staining (Fig. 2C), may be attributed to the dimer of cytochrome bo_3 oxygen reductase, previously observed in *E. coli* intact cells resolved by BN-PAGE [50], due to the proximity of the estimated masses (320 kDa vs. 316 ± 12). This is the first time that it is identified in solubilized membranes, indicating that the solubilization conditions used in this investigation serve to preserve these inter-complex interactions.

The assignment of bands 1–4 (Fig. 2A) is more complex, since none of the estimated molecular masses fall within those as deduced from the amino acid sequence of complex I, suggesting that at least some of these bands may correspond to respiratory chain supercomplexes.

The quinol:oxygen oxidoreductase activity of bands 1–4 was determined by means of an oxygen electrode. The activity of band 7, already assigned to cytochrome bo_3, was used as a positive control. In contrast with the low quinol oxidation activity exhibited by bands 1–3, bands 4 and 7 exhibited high rates due to oxygen consumption, which were inhibited by addition of KCN, indicating the presence of oxygen reductases in these bands (Fig. 3).
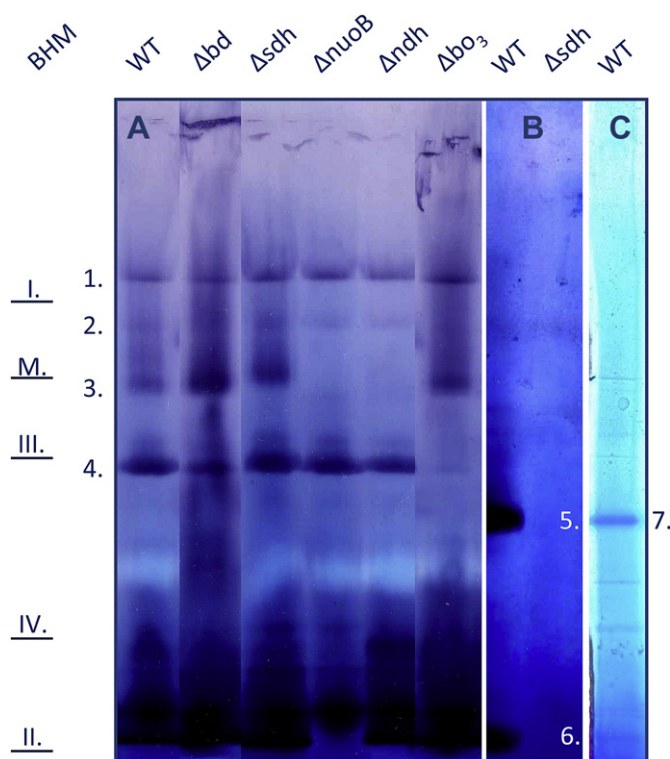
To assign bands 1–4 (Fig. 2A) to *E. coli* respiratory chain components and enumerate the composition of these super-complexes, the activity NADH:NBT oxidoreductase was detected in the solubilized membrane fraction of *E. coli* strains wherein cytochrome bo_3 (ML20S2), cytochrome *bd* (ML15A), succinate:quinone oxidoreductase (DW35), complex I (ann021), and NDH-2 (ann001) were deleted.

The activities of bands 1 and 2 were not affected by any of the referred gene deletions, suggesting that the proteins responsible for the NADH:NBT oxidoreductase activity in these bands do not arise from the aerobic electron transfer chain of this bacterium. Band 1 was analyzed by MALDI-TOF/TOF and identified as pyruvate dehydrogenase (data not shown), further corroborating this suggestion. In contrast, the activity of band 3 is absent in those strains mutant in complex I and NDH-2, and the activity of band 4 is lacking in the membranes prepared from the cytochrome bo_3 mutant strain. These results indicate the presence of a supramolecular organization involving the NADH:quinone oxidoreductases

Fig. 2. *In-gel* analyses of *E. coli* supercomplexes. Membrane fractions of *E. coli* and bovine heart mitochondria were solubilized with digitonin and resolved by BN-PAGE. A. Detection of NADH:NBT oxidoreductase activity in wild type and respiratory chain mutant strains. B. The activity of succinate:NBT oxidoreductase as detected in the wild type and SDH deletion strains. This activity was tested in the other respiratory chain mutants, bands 5 and 6 were also detected (data not shown). C. Heme staining of wild type membranes. Band 7 was observed in membranes of all mutants (data not shown). BHM – bovine heart mitochondria, I, II, III, IV and M stand for the mitochondrial complexes I–IV, and the ATP synthase, respectively, with molecular weights of 1000, 123, 482, 205 and 597 kDa.

of *E. coli* electron transport chain in band 3. Moreover, the apparent mass of band 3 (606 ± 5 kDa) agrees well with the sum of the individual masses of NDH-1 and NDH-2 (585 kDa). A physical association of both enzymes is supported by the fact that in the absence of NDH-2, a 48 kDa-polypeptide, band 3 is not detected. The presence of complex I in band 3 was further corroborated by MS. In a preliminary approach, MALDI-TOF/TOF MS allowed the

Table 1

Substrate:oxygen oxidoreductase activities of the *E. coli* aerobic respiratory chain, at 37 °C. The results are an average of three assays with –80 °C frozen/thawed membranes.

|	Activity nmol O_2 min^{-1} mg^{-1}	Inhibition (%)
NADH	1284 ± 24	
KCN	40 ± 9	97
Succinate	283 ± 4	
KCN	$6 \pm 0,6$	98
Quinol	4248 ± 67	
KCN	0	100

Table 2

Primary sequence deduced and BN-PAGE estimated molecular masses in kDa of respiratory chain components. The estimated molecular masses are the average of at least 3 BN-PAGE experiments.

	DMM[a]	NADH:NBT[b]	Succinate:NBT[b]	Heme staining
NDH-1	537			
NDH-2	48			
SDH	121			
Cytochrome bo_3	142			
Cytochrome *bd*	99			
Fdo	205			
Band 1		991 ± 17		
Band 2		797 ± 11		
Band 3		606 ± 5		
Band 4		432 ± 7		
Band 5			305 ± 14	
Band 6			128 ± 2	
Band 7				316 ± 12

[a] DMM – deduced molecular mass.
[b] oxidoreductase activity.
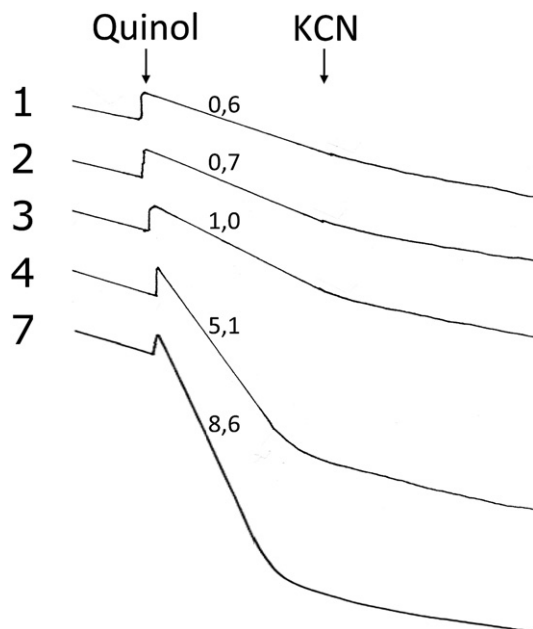
Fig. 3. Quinol:oxygen oxidoreductase activity of NADH:NBT oxidoreductase stained bands. Sets of 6 bands 1, 2, 3, 4 and 7 were excised from the BN-PAGE, placed in the oxygen electrode chamber and assayed for quinol:oxygen oxidoreductase activity. The rates of quinol oxidation activity are presented in nmol O_2 min^{-1} $band^{-1}$ after subtraction of the rates due to oxygen consumption before substrate addition. All activities are fully inhibited by the heme−copper oxidase inhibitor KCN.
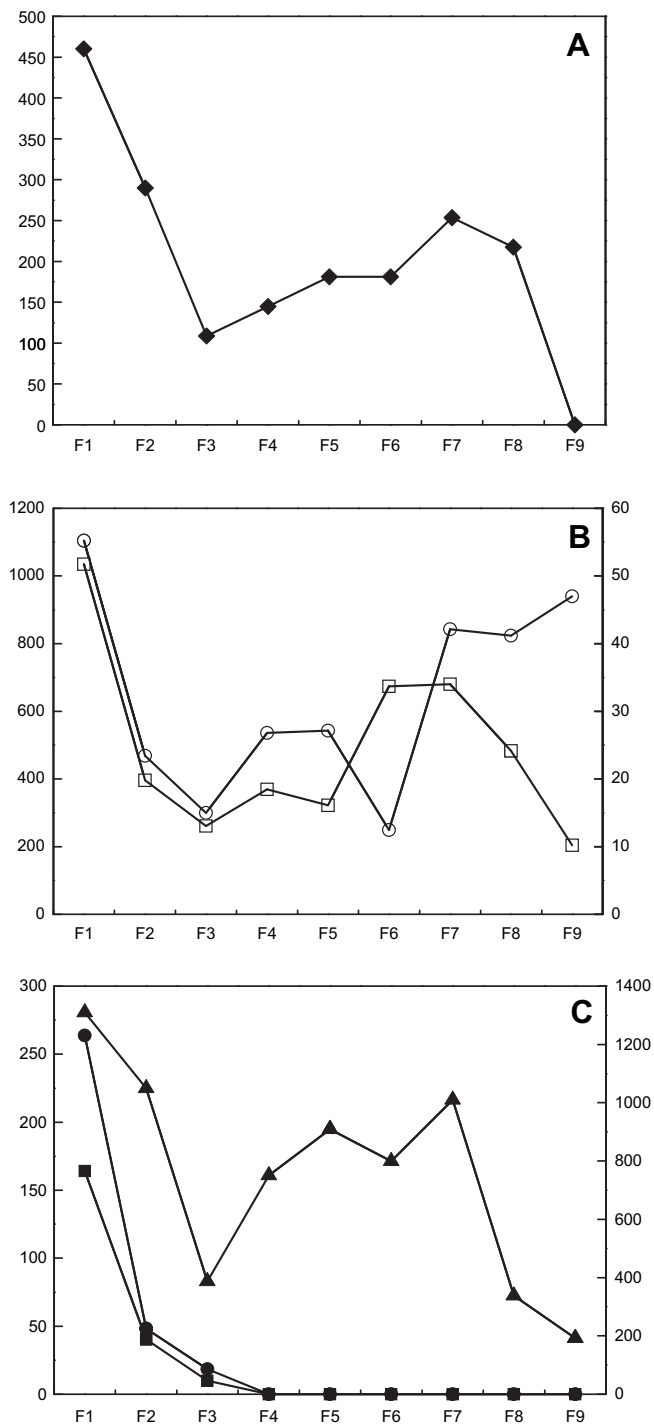
identification of 2 peptides of NuoB [sequences R.QADLM VVAGTCFTKMAPVIQR.L + oxidation of M (residues 92−112) and R.IAVTNLR.T (residues 209−215)]. To refine and improve this identification, the band was also analyzed by LC-MS/MS, which identified several peptides of subunits Nuo A, B, CD, F, G, H, I and L (Table 3, SM Table 1).

Band 4 is absent from the cells of the strain deleted in the bo_3 oxygen reductase, thus indicating the presence of a NADH oxidase supercomplex. To identify the partner of cytochrome bo_3 in this supercomplex, band 4 was also analyzed by LC-MS/MS. As expected, peptides originating from subunits I and II of bo_3 oxygen reductase were identified, along with peptides from subunit I of cytochrome bd. This result correlates with the observation that the intensity of band 4 is considerably reduced in the strain devoid of cytochrome bd and this decrease in intensity is reproducible. In addition, several peptides from the three components of the aerobic membrane-bound formate dehydrogenase, FdoG, H and I, were also identified (Table 3, SM Table 1). This enzyme catalyses the reversible oxidation of formate coupled to the reduction of NAD^+.

Table 3
LC-MS/MS of bands 3 and 4 in Fig. 2.

Band	Enzyme	Subunit	Gene	UniProt Accession	Peptides identified
3	NADH-quinone oxidoreductase	NuoCD	nuoCD	P33599	19
		NuoG	nuoG	P33602	15
		NuoI	nuoI	P0AFD6	8
		NuoB	nuoB	P0AFC7	7
		NuoH	nuoH	P0AFD4	5
		NuoF	nuoF	P31979	5
		NuoA	nuoA	P0AFC3	3
		NuoL	nuoL	P33607	3
4	Formate dehydrogenase-O	FdoG	fdoG	P32176	61
		FdoH	fdoH	P0AAJ5	7
		FdoI	fdoI	P0AEL0	4
	bo_3 oxygen reductase	Subunit I	cyoB	P0ABI8	3
		Subunit II	cyoA	P0ABJ1	3
	bd oxygen reductase	Subunit I	cydA	P0ABJ9	11

Fig. 4. Characterization of 0.3−1.5 M sucrose gradient fractions. The sucrose gradient fractions were investigated polarographically with respect to their substrate:oxygen oxidoreductase activity, and their heme b content was spectrophotometrically assessed measuring the pyridine-hemochrome formation. The artificial activities NADH:K_3[Fe(CN)$_6$] and succinate:DCPIP oxidoreductase were also measured. A. Pyridine−hemochrome content (nM). B. NADH: K_3[Fe(CN)$_6$], open balls, (nmol$_{NADH}$ mL^{-1} min^{-1}) and succinate:DCPIP oxidoreductase activities, open squares, (nmol$_{DCPIP}$ mL^{-1} min^{-1}). C. NADH: (filled balls, primary axis), succinate: (filled squares, primary axis) and quinol: (filled triangles, secondary axis) oxygen oxidoreductase activities (nmol O_2 min^{-1} mL^{-1}).

The aerobic formate dehydrogenase of *E. coli* oxidizes formate from the cytoplasm [51] and has been suggested to be able to use O_2 as a final electron acceptor [52]. The co-localization of the 3 enzymes in band 4 and the absence or reduction of the NADH:NBT oxidoreductase activity in the membranes of the *E. coli* strains devoid of cytochrome bo_3 or bd, respectively, suggests the assembly of a formate oxidase supercomplex, with a stoichiometry of 1:1:1, which is further supported by the estimated mass of band 4, 432 ± 7 kDa, in agreement with the sum of the 3 enzymatic components of the supercomplex hypothesized herein, 446 kDa. It is tempting to speculate that in the cytochrome bd mutant strain, band 4 may account for a formate oxidase supercomplex where the cytochrome bd unit was replaced by a second molecule of cytochrome bo_3, in a 1:2 stoichiometry, a supramolecular organization with a molecular mass in close proximity to that of the corresponding wild type supercomplex. The proposed respiratory chain supercomplex could provide an alternative pathway to generate the proton motive force to drive ATP synthesis or secondary transport in aerobic conditions.

3.2.2. Sucrose gradient analysis of E. coli membranes

An alternative procedure to resolve the *E. coli* respiratory chain components according to their molecular mass was also performed using a sucrose gradient (0.3–1.5 M) centrifugation of the solubilized membranes. The resolved gradient was divided in 1 mL fractions (F1–F9 from highest to lowest molecular mass, respectively) and the respiratory chain activities with artificial electron acceptors, the oxygen consumption rates and heme b content were determined. NADH:K_3[Fe(CN)$_6$] oxidoreductase activity was present throughout the gradient fractions being maximal in fraction 1. Such distribution may be explained by the presence of type I and II NADH:quinone oxidoreductases as monomers, homodimers or combined with other respiratory chain complexes in supercomplexes. Recently, Muster et al. [53] suggested that there may be a population of respiratory chain complexes that is not assembled in supercomplexes, based on *in vivo* data obtained from living HeLa cells. Succinate:DCPIP oxidoreductase activity was also maximal in fraction 1 but also significant in fractions 6 and 8, probably accounting for the trimeric and monomeric forms of the SDH complex, respectively (Fig. 4B). The amount of b-type hemes is maximal in fraction 1, followed by fractions 6–8, although present in the other fractions (Fig. 4A). Considering that *E. coli* bo_3, bd quinol:oxygen reductases and SDH are heme b-containing proteins, the distribution of hemes b in several gradient fractions may also result from the different oligomerization states of these enzymes, as well as from their supramolecular association with other complexes.

Oxygen consumption due to NADH or succinate oxidation was only present in fractions 1 and 2, being significantly higher in fraction 1 (Fig. 4C). These activities were more than 90% inhibited
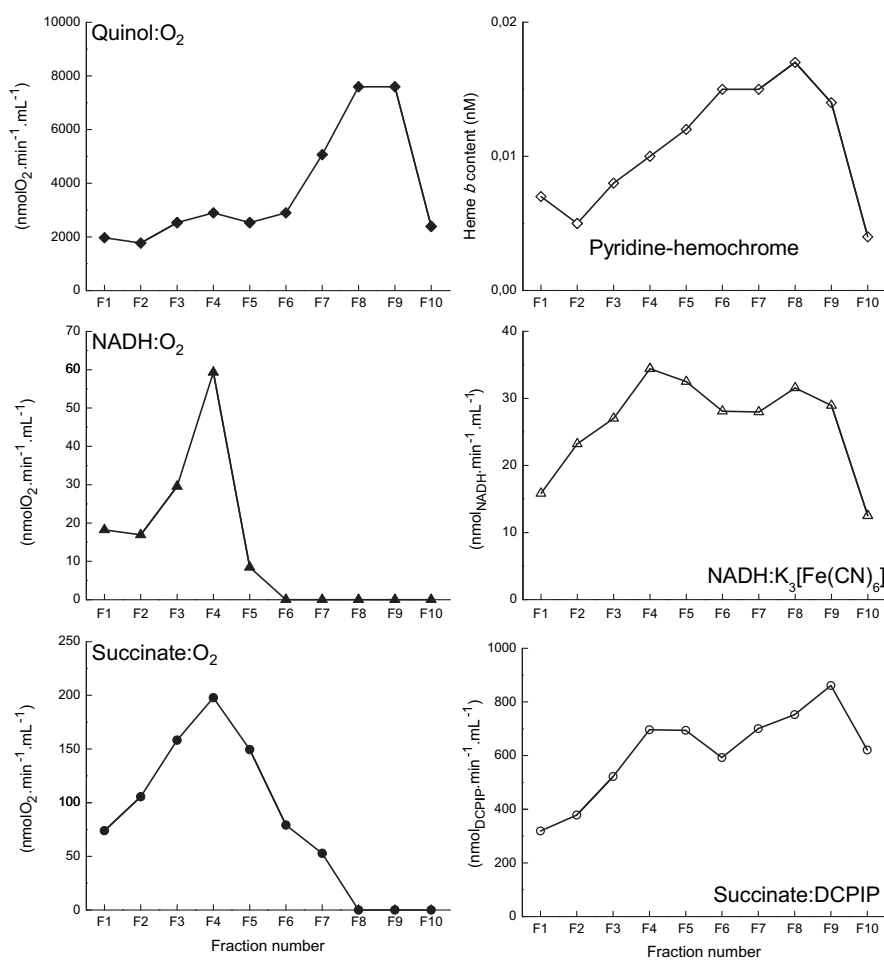
Fig. 5. Resolution of digitonin-solubilized *E. coli* membranes by sucrose gradient centrifugation. The gradient fractions were analyzed as in Fig. 4. The highest NADH: and succinate:oxygen oxidoreductase activities were observed in fraction 4, where quinol:oxygen oxidoreductase activity was also present (left side). Fraction 4 also contains type b hemes, presumably from cytochrome bo_3 oxygen reductase and succinate:ubiquinone oxidoreductase, and NADH:K_3[Fe(CN)$_6$] and succinate:DCPIP oxidoreductase activities (right side).

by KCN, indicating that a complete respiratory chain is operative. Quinol:oxygen oxidoreductase activity was also evident in the sucrose gradient fractions. In this case, the activity was distributed throughout the gradient, the activity of which was also maximal in fraction 1, and completely inhibited by KCN (Fig. 4C). The results from the analysis of the sucrose gradient fractions further support the notion that the *E. coli* aerobic respiratory chain is organized in a supramolecular fashion.

A refined sucrose gradient (1–1.5 M) fractionation was performed showing that NADH and succinate:oxygen oxidoreductase activities were maximal in fraction 4, thus excluding the possibility of the results obtained in the wider range-gradient being explained by protein aggregation (Fig. 5). The latter results were corroborated by the observation that NADH and succinate:oxygen oxidoreductase activities of *E. coli* membranes and fraction 1 of the 0.3–1.5 M sucrose gradient were eliminated in the presence of 0.025% Triton X-100, while the activities of the individual enzymes were retained, as assessed by the spectrophotometric measurement of NADH:ferricyanide and succinate:DCPIP oxidoreductase activities. This observation indicates that the presence of intact respiratory chain modules, upon digitonin solubilization, does not result from protein aggregation but from inter-complex interactions, which, although preserved upon digitonin solubilization, are disrupted by the subsequent addition of the harsher detergent Triton X-100.

Here is provided for the first time evidence for the presence of supercomplexes in the aerobic respiratory chain of *E. coli*, namely a supercomplex formed by NDH-1 and NDH-2, and a second representing the assembly of formate dehydrogenase with the oxygen reductases bo_3 and bd. In the context of prokaryotes, the supramolecular associations of the aerobic respiratory chain reported to date involved only cytochrome *c*:oxygen oxidoreductases, similarly to those present in mitochondria, namely the aa_3 cytochrome *c*:oxygen oxidoreductase in the *P. denitrificans* respirasome and the caa_3 cytochrome *c*:oxygen oxidoreductase in *Bacillus* PS3. It is, thus, noteworthy that this is the first report of supercomplexes involving enzymes such as type II NADH:quinone oxidoreductase, the bd and bo_3 quinol:oxygen reductases, and the aerobic formate dehydrogenase, thereby providing further evidence that supramolecular organization of the respiratory chain complexes is a widespread strategy to optimize oxidative phosphorylation.

Acknowledgements

Appendix. Supplementary material

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