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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.

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 oxidoreductase, and at least two quinol:oxygen oxidoreductases, cytochromes *bd* and bo_3, 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

Abbreviations: BCA, bicinchoninic acid; BN-PAGE, Blue Native Polyacrylamide Gel Electrophoresis; DCPIP, 2,6-dichlorophenolindophenol; DDM, dodecyl-β-d-maltoside; EDTA, Ethylenediamine tetraacetic acid; Fdo, Formate dehydrogenase, aerobic; LC-MS/MS, Liquid Chromatography coupled to tandem Mass Spectrometry; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; MALDI-TOF/TOF, Matrix-assisted laser desorption/ionization tandem time of flight; MS, mass spectrometry; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene fluoride; SDS, sodium dodecyl sulfate.

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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 × *g*, 15 min) to remove intact cells and cell debris and the supernatant was ultracentrifuged (138000 × *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 × *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 × *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 (ε_{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 (ε_{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).