

# Are *Escherichia coli* OXPHOS complexes concentrated in specialized zones within the plasma membrane?

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## Abstract

Most organisms are able to synthesize ATP by OXPHOS (oxidative phosphorylation). Mitochondria in eukaryotes perform OXPHOS in the inner mitochondrial membrane, whereas the plasma membrane is used by prokaryotes. However, whereas OXPHOS is a well-understood process at the biochemical level, relatively little is known about its operation at the level of the whole-organism/cell. We observed that a fluorescently labelled terminal oxidase, the cytochrome *bd* complex, is heterogeneously distributed in the *Escherichia coli* plasma membrane. This observation forms the basis of a working hypothesis that patches of the *E. coli* plasma membrane ('respirazones') are dedicated to respiratory function by the high concentration of OXPHOS components in these zones relative to the adjacent membrane. The formulation and physiological significance of this hypothesis are discussed in this paper.

## Introduction

Nearly all living organisms are capable of producing ATP by OXPHOS (oxidative phosphorylation), in which oxidation of electron donors by membrane-bound enzymes is coupled, by a series of stepwise redox reactions, with the generation of a p.m.f. (protonmotive force), which powers the ubiquitous  $F_1F_o$ -ATPase. The best-understood OXPHOS system is that of mammalian mitochondria, which serves as a general model for OXPHOS [1]. The mitochondrial system is located in the convoluted inner mitochondrial membrane and is composed of five enzyme complexes [Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (the cytochrome *bc*<sub>1</sub> complex), Complex IV (cytochrome *c* oxidase) and Complex V (the  $F_1F_o$ -ATPase)], mobile electron carriers and protons. The mobile electron carriers, ubiquinol and cytochrome *c*, functionally link complexes I–IV, which make up the ETC (electron transport chain). This in turn is linked to Complex V by the p.m.f. that is generated by the coupling of proton translocation with the electron transfer reactions in the ETC, as diffusion of protons from the intermembrane space into the mitochondrial matrix powers the rotational mechanism of the ATPase.

Mitochondria and present-day bacteria are considered to have descended from a common ancestor, but bacterial and mitochondrial OXPHOS systems differ in a few significant ways. While bacterial OXPHOS complexes share homology with mitochondrial complexes, the mitochondrial complexes have acquired additional subunits. Complex I [2] and

Complex V [3] are particularly augmented with additional subunits that seem to be involved in supramolecular organization [4]. In addition to homologues of the five mitochondrial complexes, most bacteria possess alternative proton-translocating oxidoreductases and are therefore capable of OXPHOS using a much wider variety of substrates than mammalian mitochondria. Bacterial ETCs are therefore considered branched. For instance, the model bacterium *Escherichia coli* has a Complex I homologue consisting of just the 14 'core' subunits in 13 polypeptides (as opposed to a maximum of 46 subunits in mitochondria) [2], unusually, does not possess a homologue of Complex III (and therefore terminal oxidases are hydroquinone oxidases), and is capable of anaerobic OXPHOS using nitrate and fumarate in the place of molecular oxygen as terminal electron acceptors [5].

Discussions on the arrangement of respiratory complexes have focused on mitochondrial systems and on the steric relationships between the complexes. Thus the solid versus fluid-state arguments in the literature (reviewed in [6]) are a debate focused on the biochemical consequences of OXPHOS complexes forming rigid protein superstructures in the membrane. The question of how OXPHOS complexes are distributed across the entire bioenergetic membrane, regardless of whether they form supercomplexes or not, is one that has not been addressed in much detail, and yet the answer to this question could give valuable insights into how mitochondria and bacteria function at the organellar and cellular levels respectively. At the organellar/cellular level, bioenergetic membranes perform functions in addition to OXPHOS. The inner mitochondrial membrane also transports numerous solutes and proteins and is involved in other metabolic pathways; bacterial plasma membranes, in addition to transport functions, also perform cell wall synthesis and chemotaxis.

**Key words:** cytochrome *bd*, *Escherichia coli*, membrane, oxidative phosphorylation (OXPHOS), respirazone, total internal reflection fluorescence (TIRF).

**Abbreviations used:** ETC, electron transport chain; GFP, green fluorescent protein; OXPHOS, oxidative phosphorylation; p.m.f., protonmotive force; TIRF, total internal reflection fluorescence.

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All these functions necessarily exist alongside OXPHOS in bioenergetic membranes, but the question of how they can do so (is the membrane compartmentalized?) is rarely raised.

The current general model for biological membranes is that of a partitioned fluid or fluid patchwork [7,8], replacing the 1972 Singer–Nicholson fluid mosaic model [9]. The current scheme predicts OXPHOS domains, particularly in multifunctional membranes, such as the bacterial plasma membrane, rather than the free mixing of all membrane components. We recently observed that GFP (green fluorescent protein)-tagged cytochrome *bd-I*, a terminal oxidase in *E. coli*, is localized to mobile clusters within the plasma membrane (T. Lenn, M.C. Leake and C.W. Mullineaux, unpublished work). This observation forms the basis of a hypothesis that describes the organization of OXPHOS at the whole-cell level.

### Supramolecular organization of OXPHOS complexes on the nanometre scale

The organization of OXPHOS complexes has been studied at the protein level by using a variety of *in vitro* techniques, which have provided increasingly convincing evidence that OXPHOS complexes do form stable inter- and intra-complex associations in bioenergetic membranes. Supramolecular assemblies of various OXPHOS complexes have been identified from mammalian, plant and yeast mitochondria and from *Paracoccus denitrificans* by blue-native PAGE [11]. Supercomplexes have also been identified in thermophilic bacterium PS3 [12], *Sulpholobus* sp. strain 7 [13] and *Corynebacterium glutamicum* [14]. Recently, dimeric *E. coli* cytochrome *bo* was reported [15]. The structures of some OXPHOS supercomplexes have been investigated by single-particle electron microscopy [16,17]. The compositions and structures of these supercomplexes have recently been reviewed by Boekema and Braun in [4], which also presents a model whereby supercomplexes coexist with monomeric complexes.

This model harmonizes apparently contradictory observations which equally support both fluid and solid-state hypotheses, but although it powerfully explains biochemical observations, its prediction of dynamic ordering is on a very small, protein-level (nanometre) scale and makes no comment on membrane organization on an organellar/cellular (micrometre) scale.

### Imaging the distribution of protein complexes in intact bioenergetic membranes

Electron microscopy techniques have been used to investigate protein organization in bioenergetic membranes. Early freeze-fracture electron micrographs showed a random distribution of transmembrane particles in inner mitochondrial membrane vesicles [18]; however, immunogold labelling of mitochondrial membrane proteins in intact mitochondria

suggests subcompartmentalization of the inner membrane [19]. Electron cryo-tomographs of intact mitochondria have revealed ATPase dimer ribbons along mitochondrial cristae [20], and freeze-etched *E. coli* cells show a heterogeneous distribution of proteins in the *E. coli* plasma membrane [21].

Electron microscopes are preferred to optical microscopes for resolving ultrastructural details because of their superior resolution (0.2–5 nm). However, the technique requires that samples are fixed or frozen in preparation. It therefore remains an *in vitro* technique, in which high spatial resolution is traded off for dynamic information.

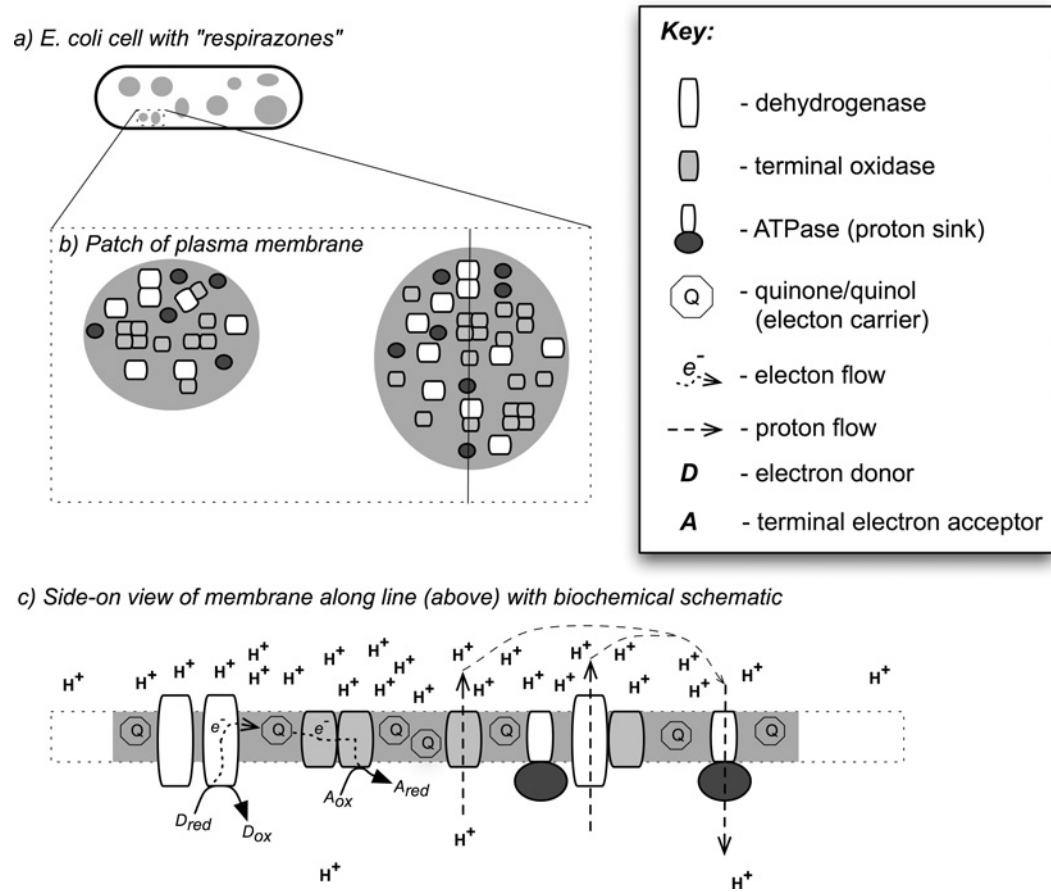
A whole suite of fluorescence imaging techniques are now available, some with resolution better than the traditional diffraction limit [22]. The resolution always remains lower than that of electron microscopy, but the advantage of fluorescence imaging is that dynamic information can be gathered from living cells. Also, specific proteins of interest can be labelled with 100% fidelity using GFP fusions. When designing experiments, it is important to consider the functionality of the GFP-tagged proteins, and their expression levels. Overexpression could lead to aggregation and artefactual distribution of proteins. Also, the temporal resolution of fluorescence imaging techniques is limited by the speed of image acquisition, and in general, the higher-resolution techniques [NSOM (near-field scanning optical microscopy) and STED (stimulated emission depletion)] require longer image acquisition times than lower resolution confocal, epifluorescence and TIRF (total internal reflection fluorescence) techniques.

Fluorescence imaging has been used to probe the organization of OXPHOS at the membrane level. Busch et al. [23] fused mammalian cells containing mitochondrial complex I labelled with different tags. Confocal imaging of the fused cells suggested the presence of fused ‘patchwork’ mitochondria in which Complex I populations remained spatially segregated, implying that Complex I is immobile or tightly associated into large supramolecular assemblages. However, the extent to which the mitochondria had fused is uncertain. Johnson et al. [24] combined YFP (yellow fluorescent protein) and CFP (cyan fluorescent protein) tagging with confocal microscopy to probe the localization and mobility of Complex II and ATPase in the *Bacillus subtilis* plasma membrane *in vivo*. They reported a dynamic, heterogeneous distribution of these complexes. However, expression of the complexes was under the control of heterologous promoters, raising the possibility of artefacts due to inappropriate timing and levels of expression. Also the time resolution of the confocal measurements was low (1 s exposures at 1 min intervals).

We have been using fluorescence microscopy to study the distribution of respiratory complexes in the *E. coli* plasma membrane. A custom-built epifluorescence/TIRF microscope with an Andor™ EM-CCD (electron multiplying charge-coupled-device) sensor allows video-rate imaging of the cells, with a frame rate of 25 Hz (40 ms frames) – a marked improvement in temporal resolution. TIRF microscopy allows specific imaging of the plasma membrane at one side of the cell close to the coverslip, with minimal background signal from the membrane at the other side of the

**Figure 1 | Illustration of the respirazone hypothesis**

Respirazones are specialized patches of membrane (grey patches) (a) where respiratory components are highly concentrated relative to the adjacent membrane (b), resulting in local enhancement of proton and electric circuits (c).



cell and autofluorescence from the cytoplasm. We replaced the genomic allele for *cydB* with a *cydB-gfp* chimaeric gene, and thus expressed GFP-tagged cytochrome *bd*-I oxidase under the control of native promoters (T. Lenn, M.C. Leake and C.W. Mullineaux, unpublished work). The tag appeared not to interfere with the function of the enzyme as no phenotypic differences were found between the *cydB-gfp* mutant and wild-type cells. In this strain, we observed mobile spots of fluorescence of heterogeneous intensity. As the expression of CydB-GFP was under the control of native promoters, the protein was not overexpressed and the cells produced a relatively low fluorescence signal. The spots are not clearly resolved by traditional epifluorescence or confocal microscopy as these techniques require long (1 s) exposures or frame averaging to produce a good signal-to-noise ratio and the mobile structures are blurred.

These spots were determined to be clusters of the cytochrome *bd*-I oxidase in the *E. coli* plasma membrane. By observing stepwise photobleaching of tracked clusters [25], we were able to count the number of GFPs per spot and found a broad distribution of GFPs per spot. The mean number of GFPs counted per spot was  $\sim 80$ , over a broad range of

approx. 10–150. There also appeared to be a periodicity of 4 in the probability distribution of GFPs per spot, suggesting that the clusters contain cytochrome *bd*-I tetramers.

The apparent spot width also did not increase with spot intensity, indicating that the actual diameters of the clusters are not determined solely by the number of cytochrome *bd* complexes they contain. It therefore appears that mobile patches, approx. 100 nm in diameter, exist in the *E. coli* plasma membrane, which contains variable numbers of cytochrome *bd*-I tetramers and other as yet unknown components.

### The respirazone hypothesis

Much remains to be determined concerning the physical character and physiological role of the cytochrome *bd* spots discussed above. However, given that (i) respiratory complexes in *B. subtilis* are also localized to mobile membrane domains [24], (ii) electron micrographs of freeze-etched *E. coli* [21] and fluorescently labelled lipids [26] also show a 'patchy' membrane, (iii) *E. coli* complex I, Complex II and Complex V may also be heterogeneously distributed in the *E. coli* membrane (T. Friedrich, personal communication),

(iv) respirasomes and OXPHOS supercomplexes have been isolated from other bacteria [12–15], and (v) the observed patch size is unlikely to be fully occupied by cytochrome *bd*, we propose the hypothesis that other respiratory components are also located within these patches and we refer to these patches as ‘respirazones’.

We hypothesize that respirazones are specialized compartments within bioenergetic membranes, of the order of 100 nm in diameter, that are dedicated to respiratory function. Within the respirazones, OXPHOS complexes and electron carriers are highly concentrated relative to the adjacent membrane (Figure 1).

The concentration of OXPHOS complexes into respirazones could serve to enhance the efficiency of the electron and proton circuits. If the quinone/hydroquinone pool were confined within respirazones, non-specific redox interactions would be effectively minimized, increasing energetic efficiency and minimizing damage to other membrane components. At the same time, the quinone/hydroquinone pool would be sufficiently delocalized to signal to redox sensors and allow the operation of multiple ETCs. If ATPases and proton symporters and antiporters are also associated with respirazones, the efficiency of the proton circuit could also be enhanced by the close proximity of proton pumps and sinks, as proton diffusion at the membrane surface leads to decay of the p.m.f. with increasing distance from the proton pump [27].

Thus respirazones could improve the energetic efficiency of OXPHOS and other p.m.f.-dependent processes. Conceivably, such processes could include flagellar rotation. A focus for future research will be to test the co-localization of OXPHOS enzymes with membrane transporters and flagellar motors in the plasma membrane. If this does occur, there would be localized power supplies for different p.m.f.-dependent processes in the membrane, raising the possibility that respiratory electron flow is tuned to provide power for different processes according to the needs of the cell.

The preferential association of OXPHOS complexes with specific membrane lipids is an attractive mechanism for generating these structures as it is known that lipids are heterogeneously distributed in bacterial membranes [26]. In particular, diphosphatidylglycerol, which is implicated in the formation of respiratory supercomplexes [28], is heterogeneously distributed in the *E. coli* plasma membrane [29].

In summary, the emerging picture of OXPHOS is that the OXPHOS system is organized and controlled. We propose that this organization extends to the cellular level in prokaryotes, and the observation of cytochrome *bd*-I clusters in *E. coli* and similar patterns observed in *B. subtilis* is evidence of this and is in keeping with the current membrane paradigm. As depicted in Figure 1, we hypothesize that the cytochrome *bd*-I-rich patches of membrane observed contain other respiratory complexes and constitute respiratory zones within the *E. coli* plasma membrane, based on (i) the observation of OXPHOS supercomplexes in representative prokaryotes and eukaryotic mitochondria, (ii) the current opinion that mitochondrial OXPHOS supercomplexes exist in dynamic

equilibrium with single complexes, and (iii) knowledge of the fact that lipids are heterogeneously distributed in the *E. coli* membrane. This hypothesis makes functional and structural sense to us, but further experiments are required to confirm the truth. Whatever that may be, we assert that these speculations are useful for guiding future work.

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