Supramolecular Organization of Respiratory Complexes

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Abstract

Since the discovery of the existence of superassemblies between mitochondrial respiratory complexes, such superassemblies have been the object of a passionate debate. It is accepted that respiratory supercomplexes are structures that occur in vivo, although which superstructures are naturally occurring and what could be their functional role remain open questions. The main difficulty is to make compatible the existence of superassemblies with the corpus of data that drove the field to abandon the early understanding of the physical arrangement of the mitochondrial respiratory chain as a compact physical entity (the solid model). This review provides a nonexhaustive overview of the evolution of our understanding of the structural organization of the electron transport chain from the original idea of a compact organization to a view of freely moving complexes connected by electron carriers. Today supercomplexes are viewed not as a revival of the old solid model but rather as a refined revision of the fluid model, which incorporates a new layer of structural and functional complexity.

INTRODUCTION

The mitochondrial oxidative phosphorylation (OXPHOS) system is one of the best-understood cellular processes at the genetic, molecular, structural, and biochemical levels (**Figure 1*a***). The immense progress in the description of OXPHOS components, their biogenesis, and the mechanism of action of the respiratory chain has been paralleled by a remarkable set of unexpected findings. These findings include the encoding of critical OXPHOS proteins by mitochondrial DNA (mtDNA) (1, 2); the transduction of electrochemical energy into mechanical energy in the chemiosmotic theory (3); the role of core OXPHOS system components in cellular processes unrelated to electron transport and ATP synthesis, such as the critical role of cytochrome *c* (cyt *c*) in apoptosis (4); and the participation of ectopically located ATP synthase components at the plasma membrane in hepatic HDL (high-density lipoprotein) endocytosis (5).

The vast amount of information about the OXPHOS system is not sufficient to explain the phenotypic consequences of its dysfunction. We still have a very poor understanding of why, where, how, and when disease will manifest if the OXPHOS system is impaired. Moreover, the investigation of specific diseases has revealed significant inadequacies in the standard models of the organization of the mitochondrial electron transport chain (mtETC) (6, 7). A prominent example is the unexpected discovery that some complex III (CIII) deficiencies disrupt the activity not only of CIII, but also that of complex I (CI) (8). This observation was even more puzzling when it was revealed that the physical ablation of CIII destabilizes CI (**Figure 1*b***) by making CI susceptible to active degradation within mitochondria (9). This observation was later independently confirmed (10) and extended to cyt *c* and CI and complex IV (CIV) (11, 12). Therefore, even though we have a complete description of all the genes encoding the OXPHOS system components, the mitochondrial protein synthesis apparatus, and the mitoribosomal proteins, rRNAs, and tRNAs, we cannot explain why the same mutation can cause different disease phenotypes.

This problem is exemplified by the 3243 mutation in the $tRNA^{LeuUUR}$ mtDNA gene, which is linked to the MELAS syndrome (marked by mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) in some patients (13) but to diabetes and deafness in others (14). Equally, we cannot explain why the same mutation can produce a fatal disease in some patients and result in no symptoms in others (15), even though the molecular defect is the same in both patient groups (16). By the same token, there is an almost complete lack of treatment alternatives for diseases affecting the OXPHOS system, with the exception of CoQ deficiencies. Thus, what human OXPHOS diseases teach us is that our basic knowledge of the system is far from complete and that a description of the component elements, even if exhaustive, does not provide a functional understanding of the whole system.

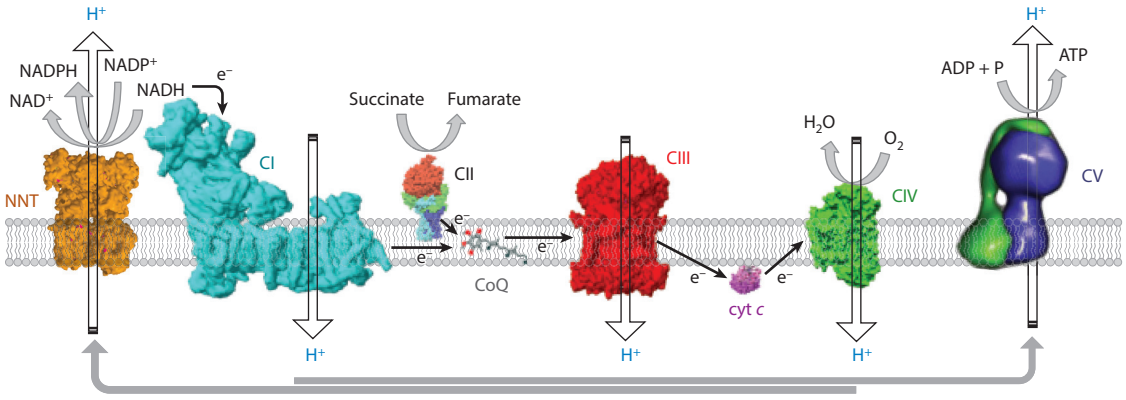
ORGANIZATION OF THE OXPHOS COMPONENTS: THE DISPUTE

The OXPHOS system is composed of respiratory complexes, electron carriers, and the H^+-ATP synthase. For historical reasons, the four respiratory complexes are named CI (NADH:ubiquinone

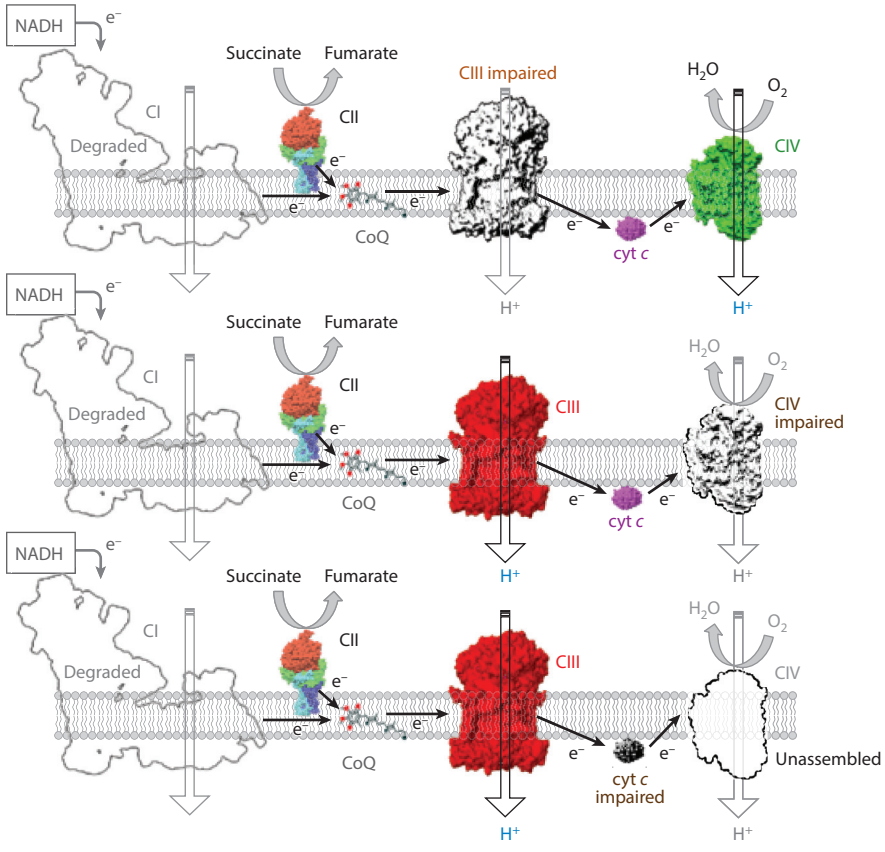
Figure 1

(*a*) Oxidative phosphorylation components and the flux of electrons and proton pumping. (*b*) The dependence of CI stability on other components of the respiratory chain. The lack of CIII and CIV promotes degradation of CI immediately after its assembly. The lack of cyt *c* prevents the assembly of CIV and promotes the degradation of CI. The respiratory chain components are modeled as opaque molecular surfaces of the bovine CI (18), CIII (168), CIV (26), and CV (from J. Walker) and the porcine CII (25), and the volume of the NNT is from *Thermus thermophilus* (169). Abbreviations: CI, NADH:ubiquinone reductase; CII, succinate:coenzyme Q reductase; CIII, coenzyme Q:cytochrome *c* oxidoreductase; CIV, cytochrome *c* oxidase; CV, H^+-ATP synthase; CoQ, coenzyme Q; cyt *c*, cytochrome *c*; NNT, NAD(P) transhydrogenase.

a The OXPHOS system



b Loss of CIII, CIV, or cyt *c* induces the degradation of CI



oxidoreductase), complex II (CII) (succinate:ubiquinone oxidoreductase), CIII (ubiquinol:cyt *c* reductase), and CIV (cyt *c* oxidase). The two electron carrier molecules are ubiquinone [also known as coenzyme Q (CoQ) or ubiquinol when in the fully reduced state ($CoQH_2$)] and cyt *c*. The H^+-ATP synthase is also known as complex V (CV). All structural protein components of the mammalian OXPHOS system are believed to be known. CI is composed of 44 different protein subunits (17, 18); NDUFAB1 is present twice (17, 18), and thus there are a total of 45 proteins in CI. CII has 4 protein subunits, CIII and CV have 11 each, and CIV has 14 (17). In addition to the structural components, assembly factors continue to be described for all the complexes, and research into the assembly pathways remains a very active field (19).

Research into cell respiration goes back more than 100 years, years that have witnessed spectacular peaks of tantalizing advances. These advances include the isolation of the NADH and FAD nucleotides and their identification as electron donors (20), the characterization of the fundamental roles of CoQ (21) and cyt *c* (22) in the mtETC, the discovery of ATP (23), and the determination of the crystal structure of the OXPHOS system components (24–29). A historically persistent controversy emerged about the physical and functional organization of the mETC complexes, provoking strong debate. During the period in which the order of the mtETC was established, in 1947 Keilin & Hartree discussed that the most plausible structure of the OXPHOS system would be one in which the "respiratory components were more or less rigidly held together in a framework that ensures their mutual accessibility and a consequent high catalytic activity" (6). This model won wide acceptance, and in 1963 Chance et al. introduced the concept of the oxysome, defined as "a functional unit for electron transfer and oxidative phosphorylation" (30). At the time, the generally accepted view was that ATP synthesis occurs through a chemical mechanism resembling substrate-level phosphorylation (31). The mtETC as envisioned by Keilin, Chance, and their respective colleagues was termed the solid model (**Figure 2*a***). The first crack in the Briton-Chance oxysome model was Mitchell's chemiosmotic proposal (in 1961), later confirmed (3).

The chemiosmotic model was so radical that it was some time before it gained acceptance. Because the model proposes a diffuse driving force for ATP synthesis, there is apparently no requirement for physical interaction between the mtETC and the ATP synthase. In addition, the mitochondrial inner membrane stores energy in charge separation (a battery) that can be used where needed, whereas strict biochemical coupling requires the added complexity of regulation of stoichiometries at each step. However, the chemiosmotic mechanism imposes very severe structural constraints and adds unexpected functional constraints because it connects membrane transport to ATP synthesis. In the model, factors like proton permeability, membrane conductance, and membrane surface, shape, and organization are critical to the function of the OXPHOS system, and the local building of asymmetry (heterogeneity) is important for the proper function of the system. An important achievement that contributed to the questioning of the solid model was the purification and reconstitution of the four respiratory complexes from mitochondria by Hatefi et al. (32) in 1962; this work demonstrated that purified respiratory complexes were functional. These findings led to the proposal from Green & Tzagoloff (33), in 1966, that the mtETC combines intracomplex electron transfer between redox components in the solid state and intercomplex electron transfer by rapid diffusion of mobile components acting as cosubstrates, i.e., CoQ and cyt *c*. The poorly understood aspect of this model is how charged species, electrons and protons, can be handed off to acceptors across water or lipid-filled gaps between proteins without significant loss. In 1977, with the increased knowledge of the properties of the inner mitochondrial membrane (IMM), Hackenbrock (34) proposed a change of perspective with the first suggestion of the fluid model. He presented a number of interesting experiments that showed the high degree of motive freedom of particulate (then unidentified) components of the IMM (34).

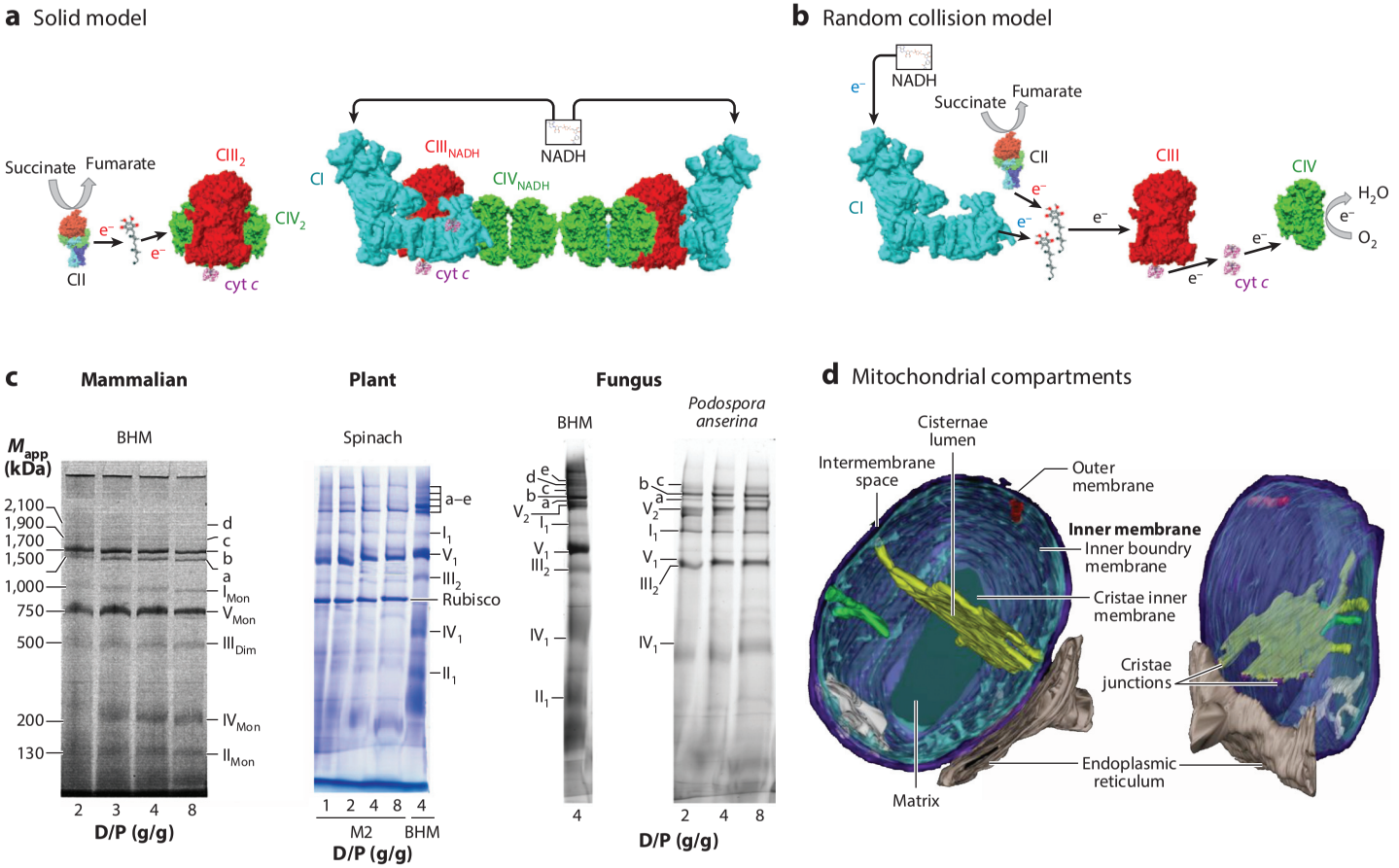


Figure 2

(*a*,*b*) The solid model (*a*) and the fluid model (*b*) of the organization of the mitochondrial electron transport chain. (*a*) All components of the respiratory chain are arranged in stable superassemblies containing CI, CIII, and CIV in different proportions. The scheme presents a free interpretation of one version of the solid model that postulated superassembly in respiratory strings, made by two hypothetical respirasomes connected by CIV-CIV interactions (46). (*b*) The respiratory chain is organized as large, multiprotein complexes that move freely in the inner mitochondrial membrane and that are connected by freely diffusible electron carriers: CoQ to connect CI and CII to CIII and cyt *c* to connect CIII to CIV. (*c*) Blue native electrophoresis analysis of mitochondrial preparations from representative organisms of three taxonomic kingdoms, prepared with a range of digitonin/protein (D/P) concentrations. The putative respiratory supercomplexes observed are remarkably similar, regardless of the origin of the mitochondria. Other abbreviations: BHM, bovine heart mitochondria; Dim, dimer; Mon, monomer; M2, mitochondria-rich fraction. Panel *c* reproduced from References 35 (*left panel*), 39 (*middle panel*), and 170 (*right panel*) with permission. (*d*) Three-dimensional reconstruction of mitochondria by electron tomography, illustrating the heterogeneity in structural domains defined by the different inner membrane folds. Images in panel *d* taken by T.G. Frey, San Diego State University, and G.A. Perkins, University of California, San Diego, and reproduced with permission.

Approximately a decade later, he published the full description of his paradigm-changing model, termed the random collision model and also known as the fluid model (**Figure 2*b***). In his own words (7):

> The mitochondrial inner membrane is a fluid-state rather than a solid-state membrane and. . .all membrane proteins and redox components which catalyze electron transport and ATP synthesis are in constant and independent diffusional motion.... We present five fundamental postulates upon which the random collision model of mitochondrial electron transport is founded: (*1*) All redox components are independent lateral diffusants; (*2*) cytochrome *c* diffuses primarily in three dimensions; (*3*) electron transport is a diffusion-coupled kinetic process; (*4*) electron transport is a multicollisional, obstructed, long-range diffusional process; (*5*) the rates of diffusion of the redox components have a direct influence on the overall kinetic process of electron transport and can be rate limiting, as in diffusion control.

The random collision model subsequently gained general acceptance and became the textbook model for the mtETC until 2000, when Schägger & Pfeiffer (35) published the first analysis of digitonin-solubilized yeast and bovine mitochondrial preparations by blue native gel electrophoresis (BNGE). In addition to reporting individual intact respiratory complexes, these authors reported discrete gel bands containing multiple comigrating respiratory complexes. Although these complexes were reminiscent of Chance's oxysome model, they were fundamentally different because they lacked the ATP synthase. Schägger & Pfeifer named them respiratory supercomplexes (SCs) and called for a complete overhaul of the Hackenbrock random collision model:

> Around 30–40 years after the first isolation of the five complexes of oxidative phosphorylation from mammalian mitochondria, we present data that fundamentally change the paradigm of how the yeast and mammalian system of oxidative phosphorylation is organized. The complexes are not randomly distributed within the inner mitochondrial membrane, but assemble into supramolecular structures.

Reading the original words used to present these conflicting models vividly illustrates the level of passion generated in the debate about the nature of the mtETC. After the publication of the Schägger & Pfeiffer (35) paper, two almost irreconcilable schools of thought—the defenders of the fluid model and the defenders of the solid model—were, scientifically speaking, at arms. Adherents to each faction argued their case largely by highlighting the experimental observations that could not be explained by the opposing model and by interpreting observations inconsistent with their own preferred view as wrong, false, or inaccurate.

The dispute can be summarized as follows. Defenders of the fluid model considered the supercomplexes observed by BNGE to be a technical artifact due to the use of detergents (and the detergent-like dissociation of proteins by Coomassie Brilliant Blue) and considered the free respiratory complexes observed on blue native gels to be the true representation of respiratory complexes in vivo. Those in the opposing camp believed the respirasome (the supercomplex containing CI+CIII+CIV) to be the true in vivo state of the mitochondrial respiratory chain and the observed free respiratory complexes as artifactual breakdown products of the true respirasome.

ORGANIZATION OF THE RESPIRATORY CHAIN: ALL FLUID OR ALL SOLID?

As discussed above, the description of SCs in 2000 by Schägger & Pfeiffer (35) automatically led to the revival of the Keilin-Chance solid model (6, 30). In principle, the observation of superassembled respiratory complexes need not necessarily contradict the fluid model. These superassemblies

could be envisioned as an efficient and organized way to store the respiratory complexes in an inactive stage from which they could be released when needed. In this view, defenders of the fluid model need not resist accepting supercomplexes as real physical entities.

However, although obtaining experimental support would have taken a few more years, supercomplexes were immediately proposed as the only true respiratory entities, a proposal that directly challenges the generally accepted understanding of the mtETC (35). Thus, the concept of the supercomplexes as the respirasome was proposed:

> The data presented in this work on the interactions of complexes within bovine heart mitochondria were used to depict a model for a network of respiratory chain complexes that may be called a respirasome.... This model assumes two copies of a $I_1III_2IV_4$ building block and one copy of a III_2IV_4 building block to fit the overall 1:3:6 stoichiometries of complexes I:III:IV, determined by Hatefi (1985). (35)

However, the data provided did not fully agree with the respirasome concept, because the III_2IV_4 SC predicted by the solid model was not observed, whereas unexpected superassemblies (I_1III_2, III_2IV_1, and I_1IV) and free complexes were detected (35). The absence of predicted SCs and the presence of unexpected SCs and free complexes were explained as a consequence of the membrane solubilization procedure, which was proposed to fragment the respirasome. These findings were not considered to be evidence that potentially challenged the validity of the solid model: "A complete III_2IV_4 complex was not detected, most likely because CIV-CIV interactions are easily dissociated by detergents in the presence of the anionic Coomassie dye" (35).

Schägger & Pfeiffer's (35) observations were of great interest and agitated the field, but further investigations did not confirm the conclusion that the SCs were equivalent to the physical structures proposed in the Keilin-Chance solid model. The Schägger & Pfeiffer proposal, however, provoked strong criticism that questioned not only the interpretation of the results, but the results themselves, with the observed superassemblies being dismissed by some as solubilization artifacts. In extreme cases, critics even questioned the validity of the BNGE technique.

Respiratory Supercomplexes: Real and Universally Observed Physical Entities

From the beginning, proponents of supercomplexes assumed that comigration of respiratory complexes on BNGE implies superassembly (35), even though this is not necessarily true. Comigration is necessary but not sufficient to demonstrate interaction. In addition, it was believed that supercomplexes were observable only upon digitonin solubilization of mitochondrial membranes. Therefore, the Schägger & Pfeiffer (35) proposal was received with significant skepticism, and researchers argued that the putative supercomplexes could be interpreted as a detergent-induced electrophoretic artifact. Nevertheless, further observations confirmed that comigration reveals true supercomplexes.

First, mitochondria from different sources differ significantly in protein sequence and composition and in the lipid content and physical properties of their membranes. Nonetheless, laboratories working with mitochondria from diverse sources [yeast and other fungi (35–37), plants (38–40), vertebrates (35, 41–44), and invertebrates (45)] found supercomplexes by BNGE. In all cases, the associations were remarkably similar, defining a limited number of combinations (**Figure 2*c***). Second, the same supercomplexes were observed in samples prepared with a variety of detergents (44, 46). Moreover, purification of respiratory supercomplexes with amphipol demonstrated that they are stable in the absence of detergent (47). Detection of the putative supercomplexes is prevented only in the presence of dodecyl maltoside (DDM) (35). The specific ability of DDM to disrupt respiratory complex superassemblies likely makes it suitable for purifying respiratory complexes

for crystallization. However, with some complexes, its action also removes peripheral subunits, which are therefore missing from the crystal structures (17, 48).

That comigration on BNGE or sucrose gradients reveals true physical interactions was supported by the observation that ablation of one component of a putative SC specifically affects the migration of its partners (44). However, exceptions confirmed the original concern that comigration is insufficient evidence of interaction (44). The ability to separate and purify supercomplexes on sucrose gradients indicated that supercomplexes preexist in sample preparations before electrophoresis (44, 49). Moreover, the ability to purify supercomplexes allowed for their visualization and structural characterization by transmission electron microscopy and single-particle tomography at resolutions between 18 and 33 Å (43, 47, 49–52; reviewed in Reference 53). Three supercomplexes have been analyzed by this approach: supercomplex I_1III_2 from *Arabidopsis thaliana* (49) and *Bos taurus* (54), supercomplex III_2IV_2 from *Saccharomyces cerevisiae* (52), and supercomplex $I_1III_2IV_1$ from *B. taurus* (47, 50, 54). More interestingly, supercomplexes containing CI have been observed in tomograms of cristae isolated from *Podospora anserina* without the need for solubilization or further purification (55). The rapid development of cryoelectron tomography and superresolution optic microscopy techniques may soon enable supercomplexes to be observed in intact cells.

Respiratory Supercomplex Formation Requires Specific Factors and a Specific Microenvironment

Definitive confirmation that superassembly of respiratory complexes is a true phenomenon comes from the description of critical factors and conditions that impact superassembly and allow for its manipulation. Several of such factors and conditions are described here.

Assembly factors. The sequential assembly of subunits, complexes, and then SCs is finely regulated (44). A still-growing number of assembly factors and chaperones, some of them persisting as nonessential components of the complete complex, participate in complex assembly (19). The discovery of assembly factors has been driven by findings in yeast and by research into OXPHOS-related diseases (for a recent review, see Reference 56). Some of these factors directly assist in the assembly of individual complexes, as is the case of BCS1L, a factor required for the incorporation of Rieske protein (iron sulfur components of cytochrome bc_1 and $b6_f$ complexes) and Qcr10q into CIII. Mutations in *BCS1L* can cause a variety of syndromes, from the severe GRACILE (growth retardation, aminoaciduria, cholestasis, iron overload, lactic acidosis, and early death) syndrome to the less severe Björnstad syndrome (marked by hearing loss and hair abnormalities). Surf1 is an assembly factor for CIV, and mutations in the *Surf1* gene are linked to different manifestations of Leigh syndrome, a neurometabolic disorder. Other factors act in more indirect ways. One example is LRPPRC protein (leucine-rich pentatricopeptide repeat–containing protein), which is required for stability and translation of the COX1 subunit of CIV; LRPPRC deficiency causes the French-Canadian type of Leigh syndrome (57, 58). The actions of these factors may require intermediate partners; for example, proteins interacting with the structural proteins of the MINOS complex (Mitofilin/Fcj1) may be important for the assembly of respiratory chain complexes (59–62). The MINOS-interacting protein C1orf163, which interacts with Sam50 and Mitofilin, was renamed RESA1 (respiratory chain assembly 1) (63) because its depletion causes severe impairment of CIV assembly, with milder effects observed in other complexes. This short summary illustrates how the correct assembly and stabilization of complexes are highly regulated at multiple stages.

The organization of respiratory complexes into different and dynamic SCs led to proposals that additional assembly factors must be required to facilitate and regulate this process. Very recent

reports from three independent laboratories describe two related *S. cerevisiae* proteins (rcf1 and rcf2) required for the incorporation of the COX3 subunit into mature CIV, and these proteins may also be important for superassembly between CIII and CIV (64–66). Two mammalian orthologs have been identified for rcf1: HIG1A and HIG2A (66). Results from gene-silencing experiments show that HIG1A does not influence the formation of respiratory complexes or SCs but instead participates in the regulation of mitochondrial γ-secretase function (67). In contrast, HIG2A silencing impairs CIV assembly, as also occurs in yeast, and results in a moderate reduction in respirasome levels. These proteins should thus be regarded more as assembly factors for CIV than as assembly factors for SCs. A newly characterized protein, C11orf83, which binds cardiolipin and phosphatidic acid, interacts with the bc_1 core complex and is required for its assembly and stabilization (68). Depletion of C11orf83 in HeLa cells causes a decrease in the amount of CIII and impacts the CIII + CIV supercomplex, but not the CI + CIII + CIV supercomplex. This differential effect might be due to the preferential association of CI with CIII so that the reduction in the amount of CIII would primarily affect the fraction of CIII that is not bound to CI (68).

Cox7a2l (also known as Cox7RP) is the first bona fide factor identified for SC assembly in mammalian mitochondria. This protein is required for the stable interaction between CIII and CIV, but not for the individual assembly or function of either complex. Consequently, Cox7a2l has been renamed SCAF1 (supercomplex assembly factor 1) (98). Aside from SCAF1, no new specific assembly factors for SCs have been identified. The regulation of SC dynamics may require both positive and negative regulators. For example, MCJ/DnaJC15, a member of the DNAJC family of cochaperones, is a negative modulator of CI activity and impairs the formation of respirasomes (71). Mitochondria from MCJ/DnaJC15 knockout mice accumulate SCs, and their respiration is highly dependent on NADH. Under normal conditions, this increase is inconsequential; however, in fasting conditions, it facilitates lipid metabolism by increasing lipid degradation and gluconeogenesis. Moreover, MCJ/DnaJC15 knockout mice on a high-cholesterol diet have lower levels of cholesterol than do wild-type mice. More assembly factors for SCs will very likely be discovered. However, such factors will likely not be discovered through research into human diseases or research in yeast, because the lack of superassembly does not seem to severely impair respiration (*S. cerevisiae* lacks CI, and interaction between CIII and CIV in this organism seems to be tight and not dynamic).

Mitochondrial shape. Mitochondria have a highly structured architecture that adapts to the needs of cells to maintain mitochondrial function. For example, nutrient availability modulates both mitochondrial shape and the behavior of the mitochondrial network. Generally, nutrient depletion triggers mitochondrial elongation as a means of increasing the efficiency of energy production. Conversely, in conditions of nutrient excess, mitochondria fragment, increasing uncoupled respiration and reducing bioenergetic efficiency (for a comprehensive review, see Reference 119).

The relationship between mitochondrial dynamics and the assembly and function of the respiratory chain was recently confirmed by modulating mitochondria-shaping proteins and monitoring the structure of OXPHOS complexes (73). Several proteins are implicated in shaping the cristae architecture, and gene mutations or silencing affects both the mitochondrial shape and structures of respiratory chain complexes and SCs. Opa1, a dynamin-related GTPase, is an IMM protein that is involved in the maintenance of tight cristae junctions, forming high-molecular-weight oligomers that are disrupted during apoptosis (73). Apoptotic cristae remodeling and genetic ablation of Opa1 lead to a disorganization of the cristae architecture that impairs the assembly of SCs and affects mitochondrial metabolism and cell growth (73). Furthermore, the formation of high-molecular-weight oligomers enables Opa1 to respond to different energy demands. Under

starvation conditions, OPA1 oligomerization is increased, leading to cristae narrowing (139); this response is required to promote ATP synthase assembly and to sustain ATP production and cell survival (70). Numerous studies have demonstrated that partners and proteases of Opa1, which participate in cristae shaping, are needed for the correct formation and function of respiratory chain SCs. Furthermore, a large protein complex termed MICOS (mitochondrial contact site and cristae organizing system) (76) is a major player in the organization of the IMM architecture. The core proteins of the complex are Mic60 (Mitofilin/Fcj1), Mic10, Mic12, Mic26, and Mic27, but the complex also interacts with other components. MICOS interacts with respiratory complexes, and mutations in the MICOS complex core subunits lead to a severely altered IMM architecture and to impaired mitochondrial bioenergetics (77).

Lipids. SC formation, stabilization, and function are critically influenced by the lipid composition of the IMM, which is enriched in phospholipids: mainly cardiolipin, phosphatidylcholine, and phosphatidylethanolamine. Cardiolipin and phosphatidylethanolamine, both non-bilayer-forming phospholipids, are essential for the organization of protein-protein and protein-lipid microdomains, which are functional hot spots in mitochondria that influence superassembly between the respiratory complexes and create a lipophilic environment for interaction with ubiquinone (78). In yeast, cardiolipin depletion destabilizes SCs, but phosphatidylethanolamine depletion tends to favor the formation of larger SCs between CIII and CIV in mitochondria (79). The opposing effects of cardiolipin and phosphatidylethanolamine on SC stability have been ascribed to differences in charge; cardiolipin is anionic, and phosphatidylethanolamine is zwitterionic (79). In Barth syndrome (dilated cardiomyopathy, skeletal myopathy, neutropenia) patients, in whom cardiolipin remodeling is altered due to mutation of the *Tafazzin* gene, SCs are unstable, leading to the functional impairment of mitochondria (42). These changes have been demonstrated not only in cells from human patients, but also in yeast (79) and *Drosophila* (80). Moreover, studies in *A. thaliana* suggest that cardiolipin is able to strategically control the localization of functional protein complexes important for the coordination between assembly and degradation of respiratory chain SCs (81, 82). These complexes are formed by prohibitins (PHB1 and -2), a family of proteins assembled in the IMM with m-AAA proteases in long-ring complexes, together with SLP2, a mitochondrial protein belonging to the stomatin-like family. SLP2 helps form functional protein complexes important for the coordination between assembly and degradation of respiratory chain SCs. The importance of lipid composition in SC stabilization is supported by studies involving reconstituted proteoliposomes (83–85).

Respiratory Supercomplex Formation Precedes Superassembly

The dynamic assembly of complexes and SCs in intact cells can also be monitored by metabolic labeling of mtDNA-encoded proteins. Through the use of this approach, a gap of several hours was observed between the labeling of free complexes and the incorporation of labeled complexes into SCs (44). Another laboratory reported partially assembled CI interacting with CIII and CIV before its own assembly was finished. Moreno-Lastres et al. (86) concluded that completion of CI assembly requires interaction with CIII and CIV in what they termed the prerespirasome. Moreover, they proposed that the respiratory complexes and the respirasome are simultaneously assembled (86). The discrepancy between the two groups' observations is not relevant, because in the first case the metabolic labeling was performed in functionally respiring cells and in the second case cells were depleted of preexisting respiratory complexes and were therefore respiration deficient. When there is a deficit of respiratory complexes, intercomplex interaction may be accelerated, permitting both interaction between incomplete respiratory complexes

and detection of the prerespirasome reported by Moreno-Lastres et al. However, in the more physiological situation in which SCs assemble in fully functional mitochondria, the normal assembly path may predominate, and superassembly may proceed after the complex assembly is complete.

The observations are thus not incompatible, but interpretation of the observations is not trivial. Asynchronous assembly of complexes and SCs is compatible with the random collision and solid models because such a view accommodates the existence of free complexes and SCs without making any assumptions about which are the functional entities. In contrast, Moreno-Lastres et al.'s (86) interpretation is compatible only with the solid model because it views the respirasome as the functional respiratory unit. These differences suggest testable predictions. First, Moreno-Lastres et al.'s interpretation predicts that CI cannot be assembled in the absence of CIV or CIII, but this is not what is observed in human cells unable to assemble CIV, where free CI showing NADH dehydrogenase activity can be detected by BNGE (17). The same is true for mouse cells depleted of CIII and CIV (see below). These results provide evidence against the view that CI is an obligate scaffold for CIII and CIV assembly and that respiratory activity (including NADH dehydrogenase activity) is acquired only when CI, CIII, and CIV combine into SCs.

The Respirasome Respires, but Nonsuperassembled Complexes Are Also Fully Functional

The existence of respiratory SCs in vivo does not necessarily imply that these associations are needed for or are even involved in respiration. These SCs may have a completely different role, as is the case with the dimerization of the F_0F_1-ATP synthase. This synthase is not required for ATP synthesis activity but plays a critical structural role in the biogenesis and stability of the cristae membrane (87, 88) and is also postulated to be required for the formation of the mitochondrial permeabilization transition pore (89). The description of respiratory complex superassembly immediately led to assumptions of a function in respiration, despite the lack of direct evidence for these assumptions. The implication of the superassembly in respiration was automatic due to historical reasons and due to the fact that the characterization of SCs by BNGE in yeast (*S. cerevisiae*) revealed that virtually all CIV is associated with CIII, implying that the yeast III_2CIV_2 SC must respire (35). However, *S. cerevisiae* is unusual because it lacks CI, because NADH dehydrogenase activity is not linked to proton pumping, and because the enzyme does not superassemble with III_2CIV_2 (52). Interestingly, numerous reports show that, if mitochondria contain CI, most CIV is found in its free form. Moreover, this finding is independent of the species of origin and has been reported in humans (10); cows (35); mice (44); *Arabidopsis* (40); potato (40); spinach (39); beans (40); pea (90); sunflower (91); corn (92); and the fungi *Yarrowia lipolytica* (36, 93), *P. anserina* (94), and *Neurospora crassa* (95). Therefore, the proposal that SCs are the only true respiratory chain requires that (*a*) all free complexes and SCs lacking CIV be regarded as fragments generated by disintegration of the respirasome during purification and that (*b*) the III_2IV_1 SC observed by BNGE be incomplete because the expected SC was III_2IV_2 (35). Moreover, in a model that envisages the respiratory chain as a solid block, Schägger and coworkers hypothesized that all superassemblies obtained by BNGE, including the respirasome, are fragments of a larger structure in which respirasomes are linked by tetrameric CIV domains to form extended respiratory strings (36, 96, 97). However, a large body of results is difficult to accommodate within this supersolid model. A prerequisite for respiration is that the respirasome should contain CoQ and cyt *c* to respire. In fact, both electron carriers were reported to be part of the respirasome purified either from BNGE (44) or from sucrose gradients (47). Moreover, TEM revealed that cyt *c* is part of the respirasome attached to CIII (47).

It took 8 years after the description of supercomplexes to experimentally show that the respirasome delivers electrons from NADH to oxygen (44). However, this demonstration did not exclude the possibility that free respiratory complexes respire within the membrane. Evaluation of this possibility was enabled with the discovery of SCAF1, which is required for the superassembly of CIII and CIV but does not affect the function of either. A screening study of mouse strains showed that C57Bl/6J and Balb/cJ mice harbor a mutation in *SCAF1* that renders it inactive. As a result, mitochondria from these strains cannot build respiratory SCs that require direct interaction between CIII and CIV. SCAF1 thus defines three CIV populations (**Figure 3**): the fraction assembled with complexes CI and CIII in the respirasome, the fraction assembled with CIII alone, and a noninteracting fraction. Interestingly, the absence of functional SCAF1 does not cause major bioenergetic problems, and animals without functional SCAF1 are healthy and fertile, although they lack regulatory options for the fine-tuning of their bioenergetic performance (98, 99).

The above experiments demonstrate that CIV is equally functional in free form and when incorporated into superassemblies. This is also the case for CIII because, in SCAF1-deficient mice also lacking CI, CIII is detected mostly in the free dimer form, not in superassemblies (100), and yet these mitochondria are able to respire through succinate, to perform β-oxidation, and to synthesize pyrimidines. Assessment of the functionality of superassembled versus free CI is more difficult because most CI is found in SCs, either with CIII alone or with CIII and CIV. To complicate matters further, ablation of CIII, CIV, or cyt *c* makes CI very unstable (**Figure 2*b***) and results in its degradation (9–12). Interestingly, degradation of CI in the absence of CIII or CIV can be prevented by expressing alternative oxidase (AOX), a CoQ oxidase present in the mitochondria of plants, fungi, and some metazoans but absent in most animals (101). In cells expressing AOX, CI function in SC I_1III_2 can be investigated in cells lacking CIV, and free CI function can be investigated in cells lacking CIII. Both cells are able to respire upon permeabilization when supplied with glutamate or pyruvate, and this respiration is fully abolished by rotenone (a specific CI inhibitor) or salicylhydroxamic acid (an AOX inhibitor), but not by the specific CIII and CIV inhibitors antimicin A and KCN. Interestingly, the respiration rate is higher when CI is free than when it is superassembled with CIII (E. Perales-Clemente & J.A. Enríquez, unpublished results). Because respiratory complexes are equally able to function in free form or as part of superassemblies, there is no reason to suppose that both forms cannot contribute to the mtETC in a given cell, either sequentially or simultaneously.

A candid evaluation of all the experimental observations therefore directs us away from a trenchant position on the solid and fluid models as incompatible opposites and instead leads us to a more nuanced view that accepts the validity of the evidence supporting each model. But before analyzing the implications of this statement, we need to examine the concept of OXPHOS heterogeneity.

THE MULTIPLE STAGES OF OXPHOS SYSTEM HETEROGENEITY IN ITS DEMANDING PHYSIOLOGICAL CONTEXT

This section examines the central importance of asymmetry and heterogeneity for the function of the OXPHOS system. Despite the overwhelming experimental evidence for this view, such evidence is systematically ignored in current models and attempts to understand metabolism, OXPHOS-related disease in its broad definition, and the development of efficient treatments. When the concept of heterogeneity is taken into account, the apparently irreconcilable discrepancies between different structural models of the mtETC vanish because, once we no longer require all conditions to provide the same outcome, the results no longer appear contradictory.

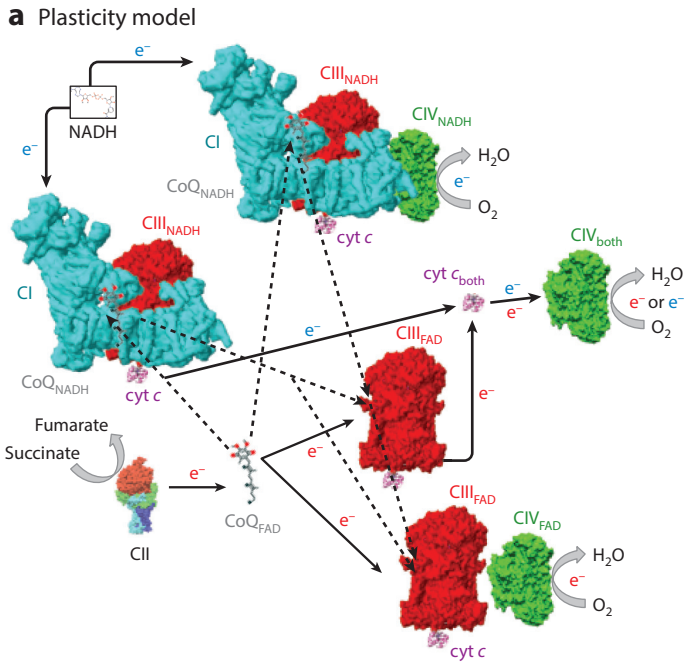


Figure 3

The plasticity model of the organization of the mitochondrial electron transport chain. (*a*) The respiratory chain components are modeled as opaque molecular surfaces from the structural models. The respirasome was modeled by modification of the model generated by Althoff et al. (47), but with bovine CI replacing the complex from *Thermus thermophilus* (18). The respiratory complexes can be functional either individually or as components of superassemblies. Most CI is superassembled with CIII dimers (CI_1CIII_2) or with CIII and CIV (the respirasome). CIII can also be found alone as a dimer or superassembled with CIV. CIII is thus segmented by its interaction with CI into a population dedicated to receiving electrons from NADH ($CIII_{NADH}$) and a CI-independent population that receives electrons from FAD ($CIII_{FAD}$). CIV is also segmented into a CIV_{NADH} population when in the respirasome, a CIV_{FAD} population when superassembled with dimeric CIII, and a CIV_{both} population when not superassembled. The interaction between CI and CIII sequesters a subpopulation of CoQ molecules preferentially dedicated to transferring electrons from NADH (CoQ_{NADH}). The superassembly of CIII and CIV defines two pools of cyt *c*: one in the respirasome that is preferentially dedicated to NADH-derived electrons (cyt c_{NADH}) and the other in CIII + CIV complexes (lacking CI) that is preferentially dedicated to transferring electrons from $FADH_2$ enzymes (cyt c_{FAD}). However, most CoQ (CoQ_{FAD}) and cyt *c* (cyt c_{both}) remain unbound to supercomplexes. Solid lines indicate the preferential routes for electrons. Intrinsic to its dynamic nature, the plasticity model proposes that the CoQ and cyt *c* segmentation is incomplete and that the functional pools are promiscuous (indicated by the *dotted lines*). This promiscuity can be generated by the dissociation of superassembled complexes or by the escape of CoQ or cyt *c* from the supercomplexes. Escape is enhanced by hyperreduction of the electron transport chain components. (*b*) Summary of the segmentation of electron transport chain components as a consequence of dynamic superassembly.

Functional Heterogeneity

The OXPHOS system is a complex and functionally unique structure that carries out three processes: It transfers electrons from reducing equivalents to oxygen; it uses the energy released by this electron transfer to pump protons at three specific sites, CI, CIII, and CIV, thereby generating a proton motive force (PMF) across the IMM; and finally it uses the PMF generated to synthesize ATP through the F_0F_1-ATP synthase (CV). These three processes are essential for energy production, but each also has additional functions. The PMF, for example, can be used to drive the import of critical metabolites into mitochondria or to generate heat instead of ATP (as occurs in brown adipose tissue). In fact, mitochondria contain specialized channels [uncoupling proteins (UCPs)] to uncouple the PMF from ATP synthesis by dissipating the proton gradient. Other futile cycles might also be used to generate heat, such as unproductive ATP consumption, but in this case the PMF and ATP synthesis would remain linked.

Electron transport is required for the function of enzymes that link the mtETC to diverse metabolic processes. All these enzymes, like CII, deliver electrons to CoQ through FAD but are unable to pump protons; they can be collectively considered as auxiliary enzymes of the respiratory chain or CII-type enzymes (102, 103). These enzymes include succinate dehydrogenase (SDH), required for the function of the TCA cycle; electron transfer flavoprotein–ubiquinone oxidoreductase, for β-oxidation; glycerol-3-phosphate dehydrogenase (G3PDH), for the shuttling of reducing equivalents from the cytoplasm; dihydroorotate dehydrogenase, for pyrimidine synthesis; choline dehydrogenase, for glycine metabolism; sulfide CoQ reductase, for sulfur and seleno-amino acid metabolism; and proline dehydrogenase, for arginine and proline metabolism. Due to the metabolic relevance of the oxidation and reduction of NADH and CoQ, many organisms have alternative NADH dehydrogenases and CoQ oxidases (AOXs) to detach these events from proton pumping when needed. Mammalian mitochondria, however, do not have these AOXs. As a consequence, a major difficulty that mammalian cells face in optimizing OXPHOS performance is that being efficient in one function may require suboptimal performance in another, and this discordance changes with cell type and in response to, for example, the organism's age and food regimen.

Genetic Heterogeneity

The OXPHOS system is also unique in being the only universal process in eukaryotic cells that requires components encoded in two genomes: mtDNA and nuclear DNA (nDNA). This arrangement implies a tight coevolution of the two genomes. However, the mutation rate is an order of magnitude higher for mtDNA than for nDNA (104), and recombination is marginal in mtDNA (105, 106). There is therefore an intrinsic tendency toward mtDNA-nDNA mismatch, highlighted by the rapidly acquired incompatibility between mtDNA and nDNA among closely related species (107–111). The mtDNA sequence also varies within a species, and mutations and polymorphisms accumulate over time without significant mixing by recombination (112), suggesting that a reasonable level of OXPHOS function can be maintained in the context of varying degrees of mismatch between mtDNA and nDNA OXPHOS genes. Thus, the function of a particular mtDNA haplotype should fluctuate in different nDNA contexts. Consequently, combination of a particular mtDNA haplotype with genetically distinct nDNA components would generate mETCs that are functional but with distinct performance. Diversity in the nDNA context between individuals arises by sexual reproduction (meiosis and recombination), with different combinations of available alleles for nDNA-encoded OXPHOS subunits produced by mixing and selection.

Within the same individual, nDNA diversity can be generated by differentiation with the expression of cell type–specific genes, including tissue-specific variants of structural OXPHOS

components (113). The particular genetic environment thus created may modify the OXPHOS requirements and may be either more or less permissive of minor discordance between genomes. An additional layer of complexity is that some OXPHOS structural subunits are expressed only in response to a defined stimulus, as in the case of hypoxia-inducible subunits (114). To make the situation still more complex, more than 2,000 single-nucleotide polymorphisms (SNPs) are believed to affect human nDNA-encoded OXPHOS genes (115), and some of them are proposed to have functional impact (116). SNPs raise the possibility that having two functionally distinct alleles of the same gene could generate two OXPHOS variants, and it is not known whether this diversity would confer any advantage to the cell, whether there would be any conflict between the variants, or whether one should predominate over the other.

This issue is addressed in Lane's postulate of mitonuclear match (117), an updated version of which proposes that the OXPHOS system is intrinsically incapable of evolving to a fixed and general optimum state because the variability of the two genomes originates through independent mechanisms and because the use of the system by different cell types and at different life stages can emphasize one or other of the multiple functions it performs. Thus, the cell can sense the mitonuclear match through changes in the activity of the OXPHOS system and can then optimize its performance by adjusting to match the cell-specific requirements. Requirements will thus depend on cell type, environmental conditions, and life stage.

Intracellular, Intramitochondrial, and Intracristae Heterogeneity

OXPHOS functional heterogeneity is also intracellularly required (118). The final decade of the last century saw impressive progress in our understanding of mitochondrial cellular dynamics, and this progress has forced a full-scale reevaluation of our understanding of mitochondria. Rather than being viewed as bacteria-like entities living, moving, and reproducing within the cytoplasm, mitochondria are better understood as extremely versatile and dynamic organelles able to divide but also to fuse, generate networks, and transit from elongated to highly fragmented forms in response to a diversity of intracellular and extracellular stimuli (119). This view of mitochondrial dynamism initially appeared to suggest that stable functional heterogeneity within the cytoplasm was not possible (120), but this idea was soon discarded with the delicate and precise determination of the timing of these processes, defining the mitochondrial life cycle (121). There are multiple examples of intracellular mitochondrial heterogeneity affecting, for example, membrane potential, composition, Ca^{2+} handling, and respiration capacity (for an extensive review, see Reference 118). The development of superresolution light microscopy and the refinement of immunogold labeling electron microscopy methodologies reveal that the distribution of protein components within the IMM is regionally determined and highly organized (122–125), as is illustrated by the analysis of the H^+-ATP synthase (126). Most notably, mitochondrial respiratory complexes and the ATP synthase are concentrated in the cristae and other IMM proteins, whereas UCP4 is excluded from them (123–125). Today it is clear that a complex molecular machinery is dedicated to building the asymmetric organization and composition of the IMM (127, 128), and the details of the profound impact of this asymmetry on OXPHOS function are beginning to emerge (73, 129). This evolving understanding requires an extension of the four classic compartments of mitochondria: the outer mitochondrial membrane (OMM), the IMM, the intermembrane space (IMS), and the mitochondrial matrix. The IMM is subdivided into the cristae-forming inner membrane and inner boundary membrane, whereas in addition to the IMS, one has to consider the cisternae lumen (**Figure 2*d***). Significantly, cristae can have different shapes (130, 131). They can adopt a tubular, prismatic, or even cuboid shape, but with a more or less fixed diameter (132), or they can adapt a flattened shape with a sac-like appearance (133). Moreover, more than one shape can often be

observed within the same cell (133, 134). Shape and curvature can have a substantial impact on the diffusion and distribution of lipid and protein components in the IMM (135, 136). Indeed, the structural organization of the IMM alters dynamically with functional changes (137).

The rich, dynamic structure of the IMM is fully interrelated with OXPHOS system structure and function (73, 137). Cristae shape and ATPase dimer formation are linked: In yeast mutants, in which the ATPase cannot dimerize, cristae are disorganized (87, 126, 138), whereas in mammalian cells, increased cristae density favors ATPase dimerization during autophagy (139). It has long been known that changes in the activity of the OXPHOS system trigger immediate alterations to mitochondrial shape; activation of mitochondrial respiration shifts mitochondria from orthodox to condensed forms and results in an expanded cristae space (140). Modern methodologies provide spectacular images of the changes in IMM topology during this transition, showing that changes in OXPHOS activity correlate closely with physical reorganization of the cristae (141). Whereas our knowledge of the molecular determinants of cristae shape and their role in apoptosis is increasing (reviewed in Reference 142), the relationship between cristae morphology and mitochondrial respiratory capacity still requires further investigation (73, 143).

The dynamic nature of cristae and their interrelationship with the OXPHOS system generate asymmetries and heterogeneity. For example, fluorescence microscopy analysis suggests that the mobility of mitochondrial respiratory complexes in the IMM is much slower than that of proteins of the OMM (144) and that CI and CII significantly differ in their redistribution within the IMM (145). More powerful approaches, such as quantitative immunoelectron microscopy, superresolution fluorescence microscopy, and single-molecule tracking, confirm that different mitochondrial proteins are asymmetrically distributed within the IMM in yeast mitochondria and that physiological changes affect this distribution (122). In animal mitochondria, cristae composition is markedly asymmetric, and this asymmetry is stable even after mitochondrial fusion with homogeneous interchange of OMM and matrix content (123, 124). Asymmetric distribution observed even within the cristae membrane, with higher concentrations of respiratory complexes in the sheet region (122, 124) and foci of F_0F_1-ATP synthase dimers at cristae tips (87, 126). This asymmetric distribution of IMM components determines the heterogeneity in mitochondrial composition and the concomitant compartmentalization in function. This compartmentalization is beautifully illustrated by the in situ determination of local pH gradients between CIV and CV, which distribute asymmetrically within cristae (146).

SUPRAMOLECULAR ORGANIZATION OF THE RESPIRATORY CHAIN AS A SOURCE OF HETEROGENEITY

The Plasticity Model: Supercomplexes as Dynamic Structures

The demonstration that respiratory complexes are able to participate in cell respiration both when free and when superassembled prompted proposals that free complexes and the full variety of associations observed by BNGE, some of them larger than the respirasome, probably coexist and that none should be discarded as a technical artifact without strong evidence. This inclusive view was expressed as the plasticity model of mtETC organization (**Figure 3*a***), in which the solid and fluid models are simply opposite extremes of a dynamic situation in which respiratory complexes can exist and function as free complexes and as part of a varied set of molecular associations (44). The stoichiometries of complexes within these associations and the differences in the stability of free and supercomplexed forms under different physiological conditions would create a landscape of structural options. The relative number of free and associated complexes varies between cell types and changes in response to physiological stimuli (see below). Mitochondria

from mouse fibroblasts grown in high-glucose medium and 21% oxygen contain CIV in the free, unassociated form (≈80%); associate with CI and CIII in the respirasome (CI + CIII + CIV; ≈15%); and associate exclusively with CIII (the III_2IV_1 SC; ≈5%). CIII is found as a free dimer and in the III_2IV_1 and CI + CIII + CIV SCs. CI exists mainly in association with CIII (CI + CIII or CI + CIII + CIV); free CI is rare, reflecting its instability and rapid degradation in the absence of CIII (9) or CIV (11, 12). A fundamental prediction of the plasticity model is that the mitochondrial respiratory chain will function in vivo with superassembled or free complexes. The model thus accommodates the lines of experimental evidence cited in support of the solid model and of the random collision model.

Indirect evidence supports the view that SC organization in living cells under physiological conditions is not fixed but rather exists in equilibrium with randomly dispersed complexes. In mice, overnight starvation reduced the amount of the $I_1III_2IV_1$ SC in liver mitochondria (98). In potato tuber mitochondria, hypoxia induced the dissociation of the $I_1III_2IV_1$ SC into free CI and the III_2IV SC (note that potato contains AOX, which permits the presence of stable free CI). This change was induced by the hypoxia-induced acidification of the medium; the effect was reproduced by acidification, but not by membrane potential alterations (147). A similar dissociation was observed in mouse fibroblasts (A. Guaras & J.A. Enríquez, manuscript submitted). A substantial increase in SC formation was observed upon in vitro differentiation of human mesenchymal stem cells into adipocytes (148) and during the maturation of mouse heart cardiomyocytes between E9.5 and E11.5 (149). SC distribution also changes with aging in rat brain and heart. In rat cortex mitochondria, there is an age-dependent (1.6-fold) decline in the overall proportion of CI exclusively detected in SCs; in contrast, insignificant changes are detected in the amounts of free III_2 (1.16-fold change) and IV_1 (1.26-fold change), and the III_2IV_1 SC is unaffected. Interestingly, the I_1III_2 SC shows a significantly stronger age-related decline (2.4-fold decline) than do SCs containing CIV ($I_1III_2IV_n$) (1.4-fold decline) (150). A significant decline in CI-containing SCs was also reported in aged hearts (151). Alterations to SC formation may also be a hallmark of pathological situations, as reported for Barth Syndrome (42) and heart failure (152).

Supercomplexes Organize the Flux of Electrons

The ability of free and superassembled respiratory complexes to respire raises the question of whether these structural alternatives have specific physiological roles and confer specific functional advantages. Four major and nonexclusive roles have been attributed to the organization of the mtETC into SCs: SCs may increase the efficiency of electron flux through substrate channeling or enhanced catalysis, they may prevent the generation of ROS by allowing for the sequestration of reactive intermediates, they may be required for structural stabilization of individual respiratory complexes, and they may provide a structural environment that allows for the assembly and activation of CI. Because any interpretation of SC function needs to take into account their dynamic nature, it is important to consider not only the potential function of superassembly but also the function of the free complexes and the changes associated with the transition between these forms.

Interaction between CI and CIII allows for functional segmentation of the CoQ pool. CoQ is required to transfer electrons from NADH- or FAD-dependent enzymes to respiratory CIII within the IMM. The cycle of CoQ reduction and oxidation is critical for energy production, redox balance, pyrimidine synthesis, and amino acid and lipid metabolism and is indirectly involved in apoptosis control and Ca^{2+} handling. In a recent landmark review, Lenaz & Genova (153) examined chemical, biochemical, metabolic, structural, and biophysical data going back to 1955 on the function of CoQ in mitochondria. These authors discussed the solid experimental observations

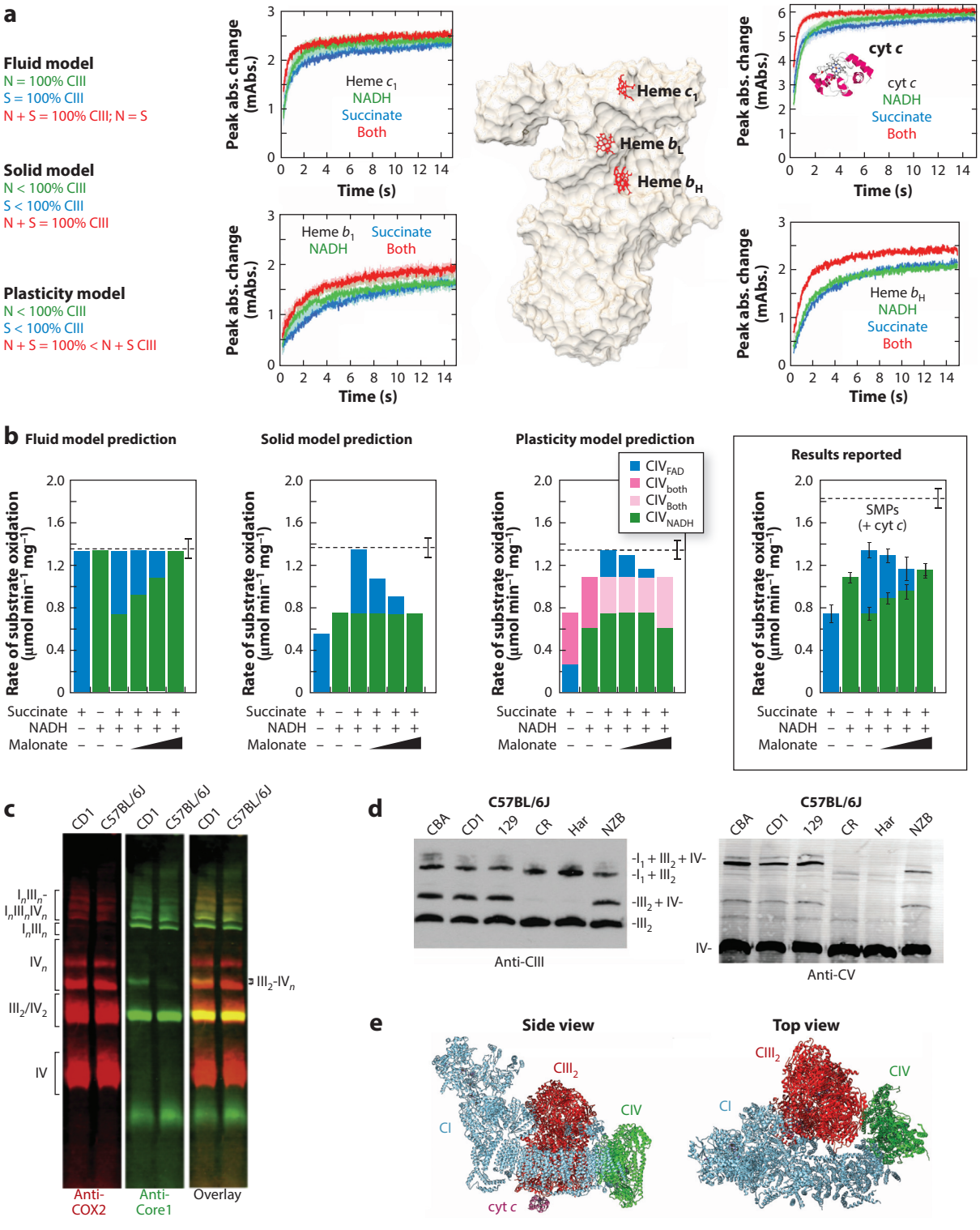
that do not agree with the single-pool concept for CoQ (154). The recent finding that purified SCs containing CI and CIII also carry CoQ in quantities sufficient to efficiently transfer electrons between CI and CIII linked the issue of the CoQ pool to respiratory SCs (44, 47). The fluid model of mtETC organization proposes a single CoQ pool, whereas the solid model proposes separate CoQ pools (103). However, the segmentation of pools or the existence of a single pool could theoretically be compatible with both structural solutions. Some studies of the reduction of CoQ in isolated mitochondria showed that both succinate and NADH can reduce a limited and specific fraction of the total CoQ pool (155, 156). In addition, it was estimated that a portion of CoQ (15% to 30% of the total pool) appeared to be bound to proteins (although the nature of the proteins was not determined), whereas the remaining fraction seemed to be free in the membrane (157). Rossignol and coworkers (158) demonstrated that not all CoQ in the IMM is equally available for succinate-dependent respiration. Measurement of reduced CoQ and cyt *c* in isolated state III mitochondria (ADP-stimulated respiration) showed that one fraction of CoQ was utilized during steady-state respiration; that another fraction was mobilizable, acting as a reserve used to maintain normal energy fluxes in response to a perturbation (e.g., inhibition of the respiratory complexes or mitochondrial disease); and that a third, nonutilizable fraction is unable to participate in succinate-dependent respiration. The succinate nonutilizable pool was estimated at 79% of total CoQ in muscle mitochondria and at 21% of total CoQ in liver mitochondria. Unfortunately, Rossignol and colleagues did not perform a similar analysis of NADH-dependent respiration. These results nevertheless provide compelling evidence against the existence of a single CoQ pool (158, 159).

More recently, Hirst's group investigated the partitioning of the CoQ pool by spectroscopic and kinetic methods (160). Their results clearly show that the reduction of the CIII-containing cytochromes is more pronounced when succinate and NADH are provided simultaneously than when only one substrate is used. Interestingly, the number of reduced CIII-containing cytochromes with both substrates is lower than the sum obtained by each substrate used separately. These results are incompatible both with the existence of a single CoQ pool and with two fully independent pools, one for NADH and the other for succinate. If the pure fluid model is identified with a single CoQ pool and the pure solid model with two fully independent CoQ pools, neither model can explain the observations of Hirst and colleagues. The plasticity model, however, can easily accommodate those results (**Figure 4*a***).

Research into the potential role of superassembly between CI and CIII in mouse fibroblasts has demonstrated that, when the amount of CIII is less than that of CI, electron transfer from succinate and glycerol-3-phosphate is impaired, whereas electron transfer from NADH is unaltered (98). This observation, incompatible with the existence of a single CoQ pool, could be explained by the fact that CI assembles with most of the available CIII, and this structural arrangement significantly favors the oxidation of NADH over that of the FAD-dependent enzymes SDH and G3PDH. These

Figure 4

The plasticity model reconciles apparently contradictory results. (*a*,*b*) Predicted outcomes of the experiments described in Reference 160 according to proposed models of mitochondrial electron transport chain organization, compared with the results observed. (*a*) Cytochrome reduction in anoxic conditions by submitochondrial particles (SMPs) fed with either succinate (S) or NADH (N). The three cytochromes of the CIII monomer are shown in red. The results obtained in Reference 160 are reproduced with permission. (*b*) Predicted and recorded rates of NADH and succinate oxidation in SMPs calculated from the simultaneous recording of oxygen consumption and NADH oxidation (160). The observed results are incompatible with the fluid and solid models but can be explained by the plasticity model. (*c*,*d*) Blue native electrophoresis of mouse heart mitochondria (165) or liver mitochondria (98) from the indicated mice strains. The analyses show the absence of supercomplex $III_2 + IV$ from C57BL/6J heart and liver and the absence of supercomplex $I + III_2$ IV from C57BL/6J liver but not heart. (*e*) Proposed structure of supercomplex $I + III_2 + IV$ obtained by docking the X-ray models of bovine complexes I, III, and IV onto the 3D map of supercomplexes obtained by single-particle electron cryomicroscopy (47).


a
Fluid model
N = 100% CIII
S = 100% CIII
N + S = 100% CIII; N = S
Solid model
N < 100% CIII
S < 100% CIII
N + S = 100% CIII
Plasticity model
N < 100% CIII
S < 100% CIII
N + S = 100% < N + S CIII
Peak abs. change (mAbs.)
Time (s)
Heme c_1
NADH
Succinate
Both
Heme b_1
Heme c_1
Heme b_L
Heme b_H
cyt c
Heme b_H
b
Fluid model prediction
Solid model prediction
Plasticity model prediction
Results reported
Rate of substrate oxidation (μmol min^{-1} mg^{-1})
Succinate
NADH
Malonate
CIV_{FAD}
CIV_{both}
CIV_{Both}
CIV_{NADH}
SMPs (+ cyt c)
c
CD1
C57BL/6J
I_nIII_n-
$I_nIII_nIV_n$
I_nIII_n
IV_n
III_2/IV_2
IV
III_2-IV_n
Anti-COX2
Anti-Core1
Overlay
d
C57BL/6J
CBA
CD1
129
CR
Har
NZB
$-I_1 + III_2 + IV-$
$-I_1 + III_2$
$-III_2 + IV-$
$-III_2$
IV-
Anti-CIII
Anti-CV
e
Side view
Top view
$CIII_2$
CIV
CI
cyt c

results led to the proposal of two functionally separate CoQ pools: the CoQ_{NADH} pool and the CoQ_{FAD} pool. The CoQ_{NADH} pool would be defined by the physical assembly between CI and CIII, which would favor transfer between NADH and cyt *c*; the remaining, freely diffusible CoQ (the majority) would be the CoQ_{FAD} pool. This proposal is supported by an independent report showing that human hybrid cells harboring an in-frame 18-bp microdeletion in mtDNA-encoded cytochrome *b* have severely reduced CI and CIII activity and increased CII activity but that there is a significantly stronger effect on CII + CIII activity than on CI + CIII activity (161). Another recent study showed that shortage of CoQ in brown adipose tissue reduces respiration driven by different substrates. G3PDH-dependent respiration is the most affected, followed by succinate-dependent and then CI-dependent respiration (162); palmitate oxidation is also severely reduced (162).

According to the plasticity model, there would be no significant cross talk between NADH oxidation and FAD oxidation, at least not until the electrons reach cyt *c*. Genova & Lenaz (163) made this prediction: "The nonhomogeneity of the ubiquinone pool with respect to succinate and NADH oxidation may be interpreted today in terms of compartmentalization of CoQ in the I–III supercomplex in contrast with the free pool used for connecting complexes II and III." The existence of two CoQ pools has profound implications for the understanding of central physiological responses such as the adaptation to fasting by liver mitochondria. Different mitochondrial respiratory substrates generate different proportions of NADH and FAD, and adaptation to a particular substrate or substrate combination requires an optimal equilibrium between the use of the NADH route and the use of the FAD route. Thus, fasting conditions, which force mitochondria to rely on fatty acids rather than on glucose, require adjustment of the proportion of respiratory SCs. How this adjustment is achieved and what the consequences would be if this adaptation were impeded remain to be investigated, and no obvious known mechanism can be invoked. Understanding this behavior is of paramount importance because similar adaptations are expected to be necessary when there is an increase in fatty acid supply, either in response to a high-fat diet or during fasting. Impairment of this adaptation may thus be relevant to pathological processes associated with obesity.

CI and CIII interaction minimizes ROS production. As many as ten possible sites of ROS production have been identified in mitochondria (164); sites in CI and CIII are especially relevant. Lenaz & Genova's group modulated the superassembly status between CI and CIII in purified mitochondria and reconstituted liposomal preparations (85). In both models, superoxide and H_2O_2 production by CI was significantly lower when it was superassembled with CIII, and combined CI+CIII activity was significantly higher in conditions that preserved SC formation (85).

CIII and CIV interaction allows for private electron pathways. Dynamic superassembly thus defines three populations of CIV: the fraction assembled with CI and CIII in the respirasome, the fraction assembled with CIII alone, and a noninteracting fraction. A screen of mouse strains by Lapuente-Brun and coworkers showed that C57BL/6J and BALB/cJ mice harbor a microdeletion in *Scaf1* that renders it inactive, and as a result mitochondria from these strains are unable to build respiratory SCs that require direct interaction between CIII and CIV (98). The Larsson group (165) questioned the lack of superassembly between CIII and CIV in C57BL/6J mice.

The first report on the SCAF1 microdeletion examined cultured fibroblasts and liver mitochondria that lack all CIV-containing SCs (III_2IV_1 and $I_1III_2IV_1$) (**Figure 4*c***and Reference 98). In contrast, Larsson's laboratory (165) worked with mitochondria from adult hearts and showed that SCAF1 mutants lack SC III_2IV_1 but retain putative SC $I_1III_2IV_1$ (**Figure 4*c***). The apparent discrepancy can be explained in a straightforward manner by Althoff et al.'s (47) structural studies of SC $I_1III_2IV_1$, which show that CI physically interacts with CIV and CIII (**Figure 4*c***). Therefore, in adult animals cardiomyocytes harbor much more abundant respiratory complexes

than in hepatocytes. The absence of functional SCAF1 prevents the formation of SC III_2IV_1, but some SC $I_1III_2IV_1$ may still be observed because CIII and CIV can be linked through their individual interaction with CI, despite being unable to form direct associations with each other. Analysis by quantitative proteomics and immunodetection confirms that both SCs $I_1III_2IV_1$ and III_2IV_1 contain SCAF1 but that the microdeletion induces SCAF1 degradation in the liver and heart, with the remnant SC $I_1III_2IV_1$ in the C57BL/6J heart being devoid of SCAF1. This remnant SC is therefore structurally different from the wild-type respirasome and is unstable (S. Cogliati, E. Calvo & J.A. Enríquez, unpublished). Therefore, apparently irreconcilable observations become perfectly compatible once we take into account the heterogeneity of the OXPHOS system.

Interestingly, the absence of functional SCAF1 does not cause major bioenergetic problems, and animals lacking functional SCAF1 are fertile and healthy, although they have lost regulatory options for the fine-tuning of their bioenergetic performance (98, 99). Thus, total cell respiration (driven by glucose, pyruvate, and glutamine) was significantly higher in cells lacking functional SCAF1, whereas CIV respiration driven by TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride) in intact cells was similar, regardless of SCAF1 expression. Respiration and the rate of ATP production were maximal with either substrate (pyruvate or succinate) in cells with nonfunctional SCAF1; however, to achieve maximal respiration in cells expressing functional SCAF1, both substrates had to be added simultaneously (98). Comparisons between liver mitochondria from C57BL/6J (SCAF1-deficient) mice and liver mitochondria purified from CD1 (SCAF1-functional) mice yielded similar results.

SCAF1 thus induces a structural segmentation of CIV that organizes it into functionally separate subpopulations (**Figure 3**): subsets of cyt *c* and CIV molecules dedicated to receiving electrons coming from either NADH (SC $I_1III_2IV_1$) or FAD (SC III_2IV_1) and a third CIV subpopulation able to receive electrons from both donors. This third subpopulation of free CIV seems to be the most abundant form, and because it can receive electrons from both FAD and NADH and requires free cyt *c*, its presence would explain the predominant interpretation of a pool behavior for cyt *c* (163).

The functional relevance of the superassembly of CIII and CIV was questioned in a study in *S. cerevisiae* (166). In this model, Trouillard and coworkers (166) designed a series of experiments aimed at determining whether cyt *c* is trapped in SCs, reasoning that superassembly would influence respiration only if this were the case. Interestingly, the authors demonstrated that a sizeable fraction of cyt *b* is prebound to the SC: ". . .the fraction of prebound cytochrome *c*. . .accounts for 16% of the entire cytochrome *c*, so that, with a stoichiometry of 4–5 cytochrome *c* per CcOx [cytochrome *c* oxidase], a large majority of CcOx would have a prebound cytochrome *c*."

They also beautifully show double kinetics for cyt *c* oxidation, namely that there are "two different types of cytochrome *c*, the fastest fraction being oxidized with a rate constant of $\sim 3.6 \times 10^3\ s^{-1}$ (time constant $\sim$280 μs). Although [this fast oxidation is] slower than the rate of O_2 binding to the CcOx at this [O_2] ($104\ s^{-1}$), we assign this fast oxidation to electron transfer from prebound cytochrome *c* to oxidized CcOx."

The relevant question here is not whether cyt *c* is physically trapped, but whether the superassembly functionally segments the cyt *c* pool. Unfortunately, the study could not be completed by analysis of a model with fully dissociated but functional CIII and CIV; such analysis would have enabled the authors to evaluate whether the double kinetics depend on superassembly (166). In summary, the data provided in this report support the existence of functionally distinct subpopulations of cyt *c* in some yeast strains, but the data do not show whether CIII and CIV superassembly is required to generate the functional segmentation of cyt *c*. These observations are in agreement with previous studies in *S. cerevisiae* that indicated that the mETC behaves as a single functional unit (167).

CODA

This review begins with an expression of concern about the scientifically unproductive dispute about which model, the solid model or the fluid model, better explains the corpus of data on the physical organization of the mitochondrial respiratory chain in the IMM. In this regard, quotations from original studies illustrate how the same results have been used to support opposing conclusions. However, the results must be allowed to speak for themselves; they might fit better or worse with a particular model, and sometimes they cannot be used to discriminate between two alternatives. This limitation merely reflects an intrinsic property of models; they are intellectual frameworks built to understand reality by accommodating experimental observations. If we forget this, the oversimplifications of our models force us to reject the implications of observations. Models are dispensable; observations and data should guide research progress.

DISCLOSURE STATEMENT

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