Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions vs. solid state electron channeling

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Lenaz G, Genova ML. Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions versus solid state electron channeling. Am J Physiol Cell Physiol 292: C1221–C1239, 2007. First published October 11, 2006; doi:10.1152/ajpcell.00263.2006.—Recent evidence, mainly based on native electrophoresis, has suggested that the mitochondrial respiratory chain is organized in the form of supercomplexes, due to the aggregation of the main respiratory chain enzymatic complexes. This evidence strongly contrasts the previously accepted model, the Random Diffusion Model, largely based on kinetic studies, stating that the complexes are randomly distributed in the lipid bilayer of the inner membrane and functionally connected by lateral diffusion of small redox molecules, i.e., coenzyme Q and cytochrome c. This review critically examines the experimental evidence, both structural and functional, pertaining to the two models and attempts to provide an updated view of the organization of the respiratory chain and of its kinetic consequences. The conclusion that structural respiratory assemblies exist is overwhelming, whereas the expected functional consequence of substrate channeling between the assembled enzymes is controversial. Examination of the available evidence suggests that, although the supercomplexes are structurally stable, their kinetic competence in substrate channeling is more labile and may depend on the system under investigation and the assay conditions.

THE MITOCHONDRIAL RESPIRATORY CHAIN

General Background

Shortly after the discovery of coenzyme Q (CoQ, ubiquinone) in beef heart mitochondria in David Green’s laboratory (23) and of its participation in electron transfer (24, 78), in the early 1960s, Hatefi et al. (75) found that externally added CoQ was reduced by NAD-linked substrates and that reduced CoQ was oxidized by mitochondrial preparations in a cyanide-sensitive manner (76). This finding led to resolution of the previously isolated NADH cytochrome c reductase (77) into two units (NADH CoQ reductase, called at the time DPNH CoQ reductase, and reduced CoQ cytochrome c reductase). Although at that time a number of investigators had succeeded in purifying enzyme systems catalyzing the oxidation of succinate by ferricytochrome c (60, 176, 177) and of ferrocytochrome c by oxygen (cytochrome-c oxidase) (35, 50, 63, 84, 162, 183, 186), the systematic resolution and reconstitution of four respiratory complexes from mitochondria were accomplished by Hatefi et al. (74), leading Green and Tzagoloff (61) to postulate that the overall respiratory activity is the result of both intracomplex electron transfer in solid state between redox components having fixed steric relation and, in addition, of intercomplex electron transfer ensured by rapid diffusion of mobile components acting as cosubstrates, i.e., CoQ and cytochrome c. This proposal contrasted the previous view derived from the spectrophotometric pioneering studies of Chance and Williams (20) and depicting the respiratory chain as a solid-state assembly of flavins and cytochromes in a protein matrix, but was substantially confirmed over the following years (19, 67, 110), leading to the postulation by Hackenbrock et al. (71) of the Random Diffusion Model of Electron Transfer. The organization of respiratory chains from different sources was then enriched in the following years of an increasing number of respiratory complexes either feeding electrons to CoQ or receiving electrons from CoQ (109). The composition of the principal oxidative phosphorylation (OXPHOS) components of the inner mitochondrial membrane is given in Table 1. It must be emphasized that the absolute values are dependent on the results of protein determination; therefore, comparison of molar ratios of individual components obtained from different laboratories seems to be more practical.

The electron transfer chain consists of four major multisubunit complexes designated as NADH CoQ reductase (complex I), succinate CoQ reductase (complex II), ubiquinol cytochrome c reductase (complex III), and cytochrome-c oxidase (complex IV). The best fit unit stoichiometry between complexes in beef heart mitochondria is 1 complex I:1.3 complex II:3 complex III:6.7 complex IV (157). In addition, there are 0.5 unit ATP synthase (also called complex V) and 3–5 units of the ADP/ATP translocase (catalyzing the equimolar exchange of ADP and ATP across the inner membrane) for each cytochrome oxidase, and there is one NADH/NADP+ transhydrogenase per complex I (19). Indeed, wide differences in cytochromes, CoQ, and pyridine nucleotide contents of mitochondria from differ-

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the content of acid-nonextractable flavin adenine dinucleotide (FAD) content. Based on half amount of cytochrome binding studies. Estimated from electron paramagnetic resonance (EPR) and from antibody titration. Based on flavin mononucleotide (FMN) content. Flavoproteins capable of feeding electrons to the respiratory chain can be envisaged on theoretical grounds and has indeed been proposed for the organization of the respiratory chain (105). In the prevalent view, the chain is organized in a liquid state. The large enzymatic complexes are randomly distributed in the plane of the membrane, where they move freely by lateral diffusion. Ubiquinone and cytochrome c are also mobile electron carriers, whose diffusion rates are faster than those of the bulkier protein complexes; their diffusion-coupled collision frequencies may be either higher or lower than any given reaction step within the complexes, and consequently electron transfer would be either reaction limited or diffusion limited. Alternatively, the components of the chain are envisaged to be in the form of aggregates, ranging from small clusters of few complexes to the extreme of a solid-state assembly. The aggregates may be either permanent or transient, but their duration in time must be larger than any electron transfer turnover to show kinetic differences from the previous model.

The higher organization of the respiratory chain components proposed earlier by Chance and Williams (20) got forgotten until recently, when Schägger and Pfeiffer (157) found structural evidence by blue-native (BN) electrophoresis of specific aggregations and introduced the model of the "respirasomes." Concomitantly, biochemical evidence for homo-oligomeric ATP synthase competent for ATP hydrolytic activity has been provided (97), and recently specific associations of ATP synthase with other OXPHOS components building up "ATP synthasomes" have been proposed (21).

The subject of the present review is a critical evaluation of the available evidence existing on the organization of the mitochondrial respiratory chain and of the two major models that have been proposed, also pinpointing the functional consequences of either mechanism of electron transfer.

STRUCTURAL AND BIOPHYSICAL EVIDENCE CONCERNING THE ORGANIZATION OF THE RESPIRATORY CHAIN

Evidence in Favor of Random Liquid-State Organization

The organization of the respiratory chain has represented a major research subject in the 1980s, culminating with acceptation of the Random Collision Model of Hackenbrock et al. (71) by the majority of investigators in the field. Ultrastructural studies (49) on the fine structure of lipid-depleted mitochondria showed that the microscopic appearance of the inner membrane does not change after lipid removal, indicating that protein-protein contacts are sufficient to keep the membrane in situ as a result of its very high protein-to-lipid ratio. The high protein to lipid weight ratio, however, does not mean that there is a high protein to lipid area ratio, since most protein complexes completely span the lipid bilayer and extend beyond it on both sides, so that the actual membrane area occupied by phospholipids is rather high (50–60% of total area) (71).

Differential scanning calorimetry, detecting the thermotropic behavior of lipids removed from direct contact with proteins, showed that the majority of phospholipids in the inner mitochondrial membrane behaves as a free bilayer (82). Freeze-fracture electron microscopy showed that the intramembrane particles are randomly distributed in the inner membrane (164).

Additional evidence for a random distribution of electron transfer complexes stems from the fact that antibodies against complex III and complex IV aggregate these complexes separately (72).

Direct investigation of the mobility of mitochondrial components by either electrophoretic relaxation or fluorescence recovery after photobleaching (FRAP) yielded lateral diffusion

<table>
<thead>
<tr>
<th>Complex I</th>
<th>Complex II</th>
<th>Coenzyme Q</th>
<th>Complex III</th>
<th>Cytochrome c</th>
<th>Complex IV</th>
<th>ATP Synthase</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Bovine heart</td>
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<tr>
<td>0.06–0.13 ( ^{ab} ) (1)</td>
<td>0.19 ( ^{b} ) (2)</td>
<td>6–8 (54)</td>
<td>0.25–0.53 ( ^{b} ) (3)</td>
<td>0.80–1.02 ( ^{b} ) (7)</td>
<td>0.60–1.00 ( ^{b} ) (6)</td>
<td>0.52–0.54 ( ^{b} ) (4)</td>
<td>19</td>
</tr>
<tr>
<td>0.08* (0.8)</td>
<td>0.11* (1.1)</td>
<td>0.30* (3)</td>
<td>0.35 (3.5)</td>
<td>0.80–1.13* (9.7)</td>
<td>12,62</td>
<td></td>
<td></td>
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<tr>
<td>0.20* (2)</td>
<td>3.4–4.0 (33)</td>
<td>0.34* (3)</td>
<td>0.45 (4)</td>
<td>131* (12)</td>
<td>157</td>
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<td>( ^{d} ) (1.1)</td>
<td>( ^{1} ) (1.3)</td>
<td>( ^{d} ) (3)</td>
<td>( ^{d} ) (6.7)</td>
<td>( ^{d} ) (3.5)</td>
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<tr>
<td>Rat liver</td>
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<tr>
<td>0.014* (1)</td>
<td>0.027* (2)/0.860* (63)</td>
<td>0.041* (3)</td>
<td>0.122* (9)</td>
<td>0.095* (7)</td>
<td>71</td>
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</tr>
<tr>
<td>0.078* (3)</td>
<td>0.236 (9.1)</td>
<td>0.222* (8.5)</td>
<td>159</td>
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</table>

Concentration values are expressed as nmol/mg protein. Mean molar ratios of the individual components are shown in parentheses; numbers were calculated by comparison to the content of Complex III whose amount, as obtained from the different authors, was normalized to a value of 3. Obtained from inhibitor binding studies. Estimated from electron paramagnetic resonance (EPR) and from antibody titration. Based on flavin mononucleotide (FMN) content. Determined by electrophoretic-densitometric approach. Based on 0.19 nmol heme a/mg protein and stoichiometry as indicated in parentheses. Determined by spectral analysis. Based on the content of acid-nonextractable flavin adenine dinucleotide (FAD) content. Based on half amount of cytochrome b content, as spectrophotometrically determined.
coefficients for mitochondrial membrane complexes in the range between $10^{-9}$ and $10^{-10}$ cm$^2$/s. The long-range diffusion measured by FRAP ($\mu$m) could be slower than short-range diffusion (nm), as the result of the high density of proteins (36). Accordingly, the diffusion coefficient of complex III was strongly enhanced by phospholipid enrichment of the membrane (71, 165); significantly, the long-range diffusion coefficient of phospholipids, although also increased, was affected to a much lesser extent.

Different authors (47, 71, 111), in agreement with the basic concepts of the Random Collision Model, have produced results showing that CoQ is a mobile carrier. However, there is some disagreement over the rate of CoQ diffusion, depending on the technique (i.e., fluorescence quenching or FRAP) used for measuring the lateral diffusion of ubiquinone homologues in the phospholipid bilayer. In the studies by Lenaz and coworkers, the diffusion coefficient ($D$) was determined to be in the $10^{-6}$ cm$^2$/s range; on the basis of these data, they concluded that the $D$ for ubiquinone is too high for diffusion to be rate limiting in mitochondrial electron transport (110), whereas the experimental $D$ values reported by Hackenbrock et al. (71) are in the $10^{-8}$ - $10^{-9}$ cm$^2$/s range.

The evidence for a random distribution of mitochondrial complexes, freely mobile in the inner membrane, was further supported by studies of rotational mobility (29), showing that the rotational correlation time of cytochrome-c oxidase is the same whether complex III and cytochrome $c$ are present or not in the same reconstitution system (90).

**Evidence in Favor of Solid-State Organization**

Circumstantial evidence against a random distribution of respiratory complexes comes from isolation of complex I-complex III (74) and complex II-complex III supercomplexes (188, 187), as well as cytochrome $b_6/f$-photosystem I units (14), indicating that such units may be preferentially associated in the native membrane.

The analysis of the exothermic enthalpy change of thio- denaturation of a protein-phospholipid vesicle containing both complex II and complex III (70) suggested the presence of a specific interaction between complexes II and III, but no such interaction was found for mixtures of complexes I-III and I-II. The same approach showed preferential interaction between cytochrome-c oxidase and the ATPase complex (140).

Because early reports of combined complexes used bile salts, which can lead to protein aggregations, and associations of complexes were not detected by antibodies within the membranes (19), not many researchers paid attention to a potential supramolecular organization of the respiratory chain. Stable supercomplexes of complexes III and IV isolated from *Paracoccus denitrificans* (8), thermophilic *Bacillus* PS3 (163), and thermoacidophilic archaean *Sulfolobus* (85) seemed to be special of these bacteria. Circumstantial evidence produced by Ozawa et al. (131) of a stable solid-state assembly of the entire OXPHOS system and by Hochman et al. (83) of the existence of dynamic aggregates in equilibrium with freely diffusing complexes was usually overlooked.

Much more recently, Cruciat et al. (25) and Schägger et al. (154, 156, 157) produced new evidence of multicomplex units in yeast and mammalian mitochondria, introducing a quantitative approach, namely, BN-PAGE.

In digitonin-solubilized mitochondria of *Saccharomyces cerevisiae*, which possess no complex I, a mild one-step separation protocol for the isolation of membrane protein complexes revealed two bands with apparent masses of 750 and 1,000 kDa containing the subunits of complexes III and IV (156). The smaller supercomplex (III$_2$IV$_1$) consisted of a complex III dimer and a complex IV monomer, whereas the larger supercomplex (III$_3$IV$_2$) represented a complex III dimer associated with two complex IV monomers.

Similar interactions of supercomplexes were also apparent in bovine heart mitochondria (157): a I$_1$III$_2$ supercomplex was found further assembled into various supercomplexes (respirasomes) comprising different copy numbers of complex IV (Fig. 1). Associations of complex II with other complexes of the OXPHOS system could not be identified under the conditions of BN-PAGE so far.

BN-PAGE has become a popular experimental strategy for the structural analysis of the protein-complex composition of the respiratory chain in different systems. On the basis of this procedure, the existence of respirasome-like supercomplexes was also reported for bacteria (172), fungi (99), and plant mitochondria (33, 41–43, 98), as well as for human mitochondria (155).

The I-III supercomplex proved to be especially stable on solubilization of isolated mitochondria by nonionic detergents, whereas complex IV-containing supercomplexes are of low abundance (bound complex IV is usually <10%) and complex II clearly is not associated, even in plants.

The first chromatographic isolation of a complete respirasome (I$_1$III$_2$IV$_2$) from digitonin-solubilized membranes of *Paracoccus denitrificans* indicated that complex I is stabilized by assembly into the NADH oxidase supercomplex because attempts to isolate complex I from mutant strains lacking complexes III or IV led to the complete dissociation of complex I under the conditions of BN-PAGE. Reduced stability of complex I in those mutant strains was also apparent from an almost complete loss of NADH CoQ oxidoreductase activity when the same protocol as for parental strain was applied (172).

Analyses of the state of supercomplexes in human patients with a single deficiency of complex III (155) and in cultured cell models harboring cytochrome $b$ mutations (1) also provided evidence that the formation of respirasomes is essential for the assembly/stability of complex I. Genetic alterations leading to a loss of complex III prevented respirasome formation and led to secondary loss of complex I; therefore, primary complex III assembly deficiencies presented as complex III/I defects. Conversely, complex III stability was not influenced by the absence of complex I.

In recent years, single particle image analyses by electron microscopy have been instrumental in providing evidence for the architecture of some respiratory supercomplexes that are highly stable to retain their enzymatic activity, even after purification from mammalian (153) or plant mitochondria (33) (Fig. 2).

Despite mounting evidence based on electrophoretic results showing that respiratory protein complexes specifically interact, forming supramolecular structures, characterization of the
supercomplexes by biochemical functional analysis is still poor.

**KINETIC ANALYSIS OF THE RANDOM DIFFUSION MODEL OF ELECTRON TRANSFER**

**“Pool” Behavior of CoQ and Cytochrome c**

The first proposal that CoQ functions as a mobile electron carrier was given by Green and Tzagoloff (61) on the basis of the isolation of discrete lipoprotein complexes of the respiratory chain, of which the quinone was a substrate in excess concentration over the prosthetic groups in the complexes. This was subsequently supported by the kinetic analysis of Kröger and Klingenberg (100); they showed that steady-state respiration in submitochondrial particles from beef heart could be modeled as a simple two-enzyme system, the first causing reduction of ubiquinone and the second causing oxidation of ubiquinol. If diffusion of the quinone and quinol species is much faster than the chemical reactions of CoQ reduction and oxidation, the quinone behaves kinetically as a homogeneous pool. According to this assumption, during steady-state electron transfer, the overall flux observed ($V_{obs}$) will be determined by the redox state of the quinone and proportional to either reduced quinone ($Q_{red}$) or oxidized quinone ($Q_{ox}$) concentration:

$$V_{obs} = V_{ox}(Q_{red}/Q_{ox}) = V_{red}(Q_{ox}/Q_{red})$$  \(\text{(1)}\)

where $V_{ox}$ is the rate of ubiquinol oxidation, $V_{red}$ is the rate of CoQ reduction, and $Q_i$ is total CoQ concentration (reduced plus oxidized).

Manipulation of Eq. 1 leads to the pool equation:

$$V_{obs} = (V_{red} \cdot V_{ox})/(V_{red} + V_{ox})$$  \(\text{(2)}\)

This expression was verified under a wide variety of input and output rates and establishes that CoQ distributes electrons randomly among the dehydrogenases and bc1 complexes, behaving indeed as a freely diffusible intermediate. The hyperbolic relation of electron flux on $V_{red}$ or $V_{ox}$ was confirmed in a variety of systems (68, 141, 146).

The concept of a mobile, laterally diffusing pool of CoQ molecules linking dehydrogenases and bc1 complexes is the natural consequence of these kinetic observations. Further evidence was provided by the characteristic effect of changing $V_{red}$ or $V_{ox}$ on inhibitor titration curves (101). In the case of the CoQ pool and titration of complex III by antimycin, $V_{obs}$ is related to $V_{red}$ and to $V_{ox}$ modified by a factor $x$, the fraction of complex III inhibited by antimycin:

$$V_{obs} = [V_{red} \cdot V_{ox} \cdot (1-x)][V_{red} + V_{ox} \cdot (1-x)]$$  \(\text{(3)}\)

According to this concept, pool behavior is characterized by a convex hyperbolic relationship between the integrated oxidation rate and the inhibitor concentration, whereas a linear relationship is expected by a stoichiometric association between the two enzymes (Fig. 3). The convexity of the inhibition curve in a system obeying to pool behavior is also a function of the ratio between $V_{red}$ and $V_{ox}$ (101) so that pool behavior may not be easily recognized at high $V_{red}$ over $V_{ox}$ ratios.

A large body of experimental evidence has validated the pool equation in a variety of mitochondrial systems (47, 68, 100). Most available data concern succinate oxidation in submitochondrial particles, whereas fewer data are available for NADH oxidation. In bovine heart submitochondrial particles, the observed rate of electron transfer between complex I and complex III was found to be comparable to that calculated from the pool equation (47). In other mitochondrial systems, the rate of complex I activity is strongly underestimated, due to the properties of CoQ analogs used as acceptors (40, 112) so that the pool equation is not directly applicable (56). As a consequence of this observation, most calculations based on absolute values of NADH CoQ reductase activity are to be taken with
Fig. 2. Structure of respiratory chain supercomplexes derived from single-particle image analysis. A: in the I\textsubscript{1}III\textsubscript{2} supercomplex of Arabidopsis, the surface of interaction between the 2 structure components seems to be within the membrane because the matrix-exposed hydrophilic portions of complex I (yellow) and of complex III (green, dotted line in top), are not in close contact. The membrane-embedded portion of complex III (space-filling structure) appears laterally connected to the membrane arm of complex I. [From Dudkina et al. (33), with permission of the National Academy of Sciences USA, copyright 2005.] B: x-ray structures of complex III\textsubscript{2} (red) and complex IV (green) superimposed on the electron projection maps of the bovine heart supercomplex I\textsubscript{1}III\textsubscript{2}IV\textsubscript{1}. Views perpendicular to the membrane (top, as looking from the intermembrane space) showed predominantly asymmetric triangular shapes, where the matrix arm of complex I can be assigned unambiguously to the sharpest corner of the image (white stain-depleted area on right) and the dimer of complex III appears attached laterally to the membrane arm of complex I. The complex IV monomer interacts with the other components by sharing only a small contact surface with complex III, whereas its main contact site is to the membrane arm of complex I. [From Schafer et al. (153), with permission of The American Society for Biochemistry and Molecular Biology; copyright 2006, permission conveyed through Copyright Clearance Center.]

If the CoQ concentration is not saturating for the activity of the reducing and oxidizing enzymes, Eq. 1 is modified (141) by feeding it in the Michaelis-Menten equation for enzyme kinetics, taking into account total CoQ concentration ([Q\textsubscript{t}]), the individual V\textsubscript{max} of the dehydrogenase and complex III, and their dissociation constants for CoQ. V\textsubscript{obs} is hyperbolically related to [Q\textsubscript{t}], and maximal turnovers of electron transfer are attained only at [Q\textsubscript{t}] saturating both V\textsubscript{red} and V\textsubscript{ox} (110).

\[
V_{\text{obs}} = \frac{[(V_{\text{mr}} \cdot V_{\text{mo}})/(V_{\text{mr}} + V_{\text{mo}})] \cdot Q_{\text{t}}}{((V_{\text{mr}} \cdot K_{\text{so}} + V_{\text{mo}} \cdot K_{\text{al}})/(V_{\text{mr}} + V_{\text{mo}})) + Q_{\text{t}}} \quad (4)
\]

where V\textsubscript{mr} and V\textsubscript{mo} are the maximal velocities of CoQ reduction and ubiquinol oxidation, respectively, and K\textsubscript{so} and K\textsubscript{al} are the dissociation constants for the dehydrogenase and for complex III, respectively. An additional complication in the saturation kinetics of CoQ may be in the fact that multiple quinone-binding sites may exist in the competent enzymes (e.g., site O or P and site I or N in complex III) (Trumpower (178)) and that oxidized and reduced forms of CoQ may bind to the same binding sites with different affinities and mutually compete with one another.

The factor in square brackets in the denominator of Eq. 4 is the apparent K\textsubscript{m} for Q\textsubscript{t} of the integrated activity (NADH oxidation or succinate oxidation). Direct titrations of CoQ-depleted mitochondria reconstituted with different CoQ supplements yielded a “K\textsubscript{m}” of NADH oxidation for Q\textsubscript{t} in the

extreme caution. We will reexamine this issue in Presence of errors or artifacts, below.

It might be argued that CoQ-pool behavior was shown only in reconstituted systems (39, 107, 126, 190) or in mitochondria where the relations of the inner membrane complexes were altered by swelling (158), freeze-thawing cycles (135), or sonication (100), whereas in intact mitochondria inner-outer membrane contacts and the quasi-solid organization of the matrix (167) may keep the integral proteins in a clustered immobilized arrangement. A study dealing with the presence of diffusible intermediates in the respiratory chain in intact phosphorylating mitochondria was accomplished by the double-inhibitor titration technique of Baum (5) by Stoner (171), who showed that, in intact coupled mitochondria in state 3 (i.e., synthesizing ATP), inhibition of complex III with myxothiazol makes succinate oxidase less sensitive to the complex II inhibitor 3’-hexylcarboxin, in accordance with the existence of a freely diffusible intermediate between the two steps (5). Unfortunately, in his study, Stoner did not perform similar titrations for search of a diffusible intermediate between complexes I and III.

As a conclusion, we may state with some certainty that, in beef heart mitochondria, succinate oxidation exhibits pool behavior, indicating the presence of CoQ as a diffusible intermediate between complex II and complex III. On the other hand, the same statement for NADH oxidation is supported by less clear-cut evidence.
range of 2–5 nmol/mg mitochondrial protein (39), corresponding to a Qₐ concentration of 4–10 mM in the lipid bilayer. The $K_m$ in the composite system is a poised function of $V_{max}$ and dissociation constants for CoQ of the complexes involved. This $K_m$ can be therefore varying with rate changes of the complexes linked by the CoQ-pool but is anyway an important parameter, in that it is operationally described as the Qt concentration yielding half-maximal velocity of integrated electron transfer $V_{obs}$ (113). Analysis of the literature shows that the physiological CoQ content of several types of mitochondria (4) is in the range of the $K_m$ for NADH oxidation and therefore not saturating for this activity.

In contrast to NADH oxidation, the $K_m$ for succinate oxidation for Qt was found to be one order of magnitude lower (39), although Norling et al. (126) had found similar values for the two systems.

The relation between electron transfer rate and CoQ concentration was seen for NADH oxidation in reconstituted systems and in phospholipid-enriched mitochondria (135, 158). Although NADH oxidative activities higher than the physiological rates could be attained by enriching the membranes with extra ubiquinone, the theoretical maximal $V_{obs}$ cannot be reached experimentally. The reason could be in the limited miscibility of ubiquinone with phospholipid bilayers; two-phase systems are formed just above the physiological CoQ concentration (170, 27, 127); clustered ubiquinone would be kinetically inactive, and clustering would impose an upper limit to the electron transfer rate in the CoQ region.

Despite the calorimetric and spin label evidence of Yu and colleagues (70), it seems that succinate oxidation obeys pool behavior because it is dependent on concentration of the CoQ pool (39, 158).

The substrate-like nature of CoQ is also shown by the fact that it exhibits saturation kinetics, not only when a short homologue is used as a substrate for an individual enzyme but also when the natural 2,3-dimethoxy-5 methyl-6-decaprenyl-1,4-benzoquinone (CoQ₁₀) is titrated in integrated respiration (i.e., NADH- and succinate-cytochrome c reductase) (39, 110, 141). A puzzling observation is that the $K_m$ for CoQ₁₀ of NADH cytochrome c reductase is much higher than that of succinate cytochrome c reductase (39): the latter is of the same order of magnitude as the concentration of respiratory enzymes, a

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Fig. 3. Schematic models showing ubiquinone as a mediator of the electron transport in the respiratory chain. Top: pool behavior. Coenzyme Q is shown as a freely diffusible interconnecting mediator (Q pool) among the electron donors (complex I) and the acceptors (complex III). Interaction of antimycin (AA) with the respiratory chain causes the "nonlinear" inhibition curve of substrate oxidation (due to multiple choices for quinol oxidation by the residual active molecules of complex III, as indicated also by the dotted arrows). In agreement with Eq. 3, the relation between the respiratory activity ($V_{red}/V_{ox}$) and the amount of antimycin relative to the antimycin-titer ($i/i_0$) depends on the ratio $V_{red}/V_{ox}$; the most hyperbolic curve is observed with the smallest value (i.e., $V_{red}/V_{ox} = 0.05$). Bottom: supercomplex arrangement. The serial mediation by ubiquinone (Q) only within the I-III supramolecular assembly would result in a linear relation of the respiratory activity to antimycin, $V_0 = V_{obs}$ in the absence of antimycin. $V_{ox}$, rate of ubiquinol oxidation; $V_{red}$, rate of coenzyme Q reduction; $V_0$, initial rate; $V_{obs}$, overall flux observed.
possible suggestion in favor of a stoichiometric association of complex II with complex III, which is, however, not experimentally found in the BN-PAGE investigations (157) or in metabolic flux control analysis (11) but was suggested by biophysical investigations (70).

Although the pool equation has been developed for CoQ between dehydrogenases and bc1 complexes, the same reasoning is valid for cytochrome c between bc1 complexes and cytochrome oxidase units (52).

Cytochrome c shares with ubiquinone the role of mobile substrate-like components of the respiratory chain. The role of cytochrome c mobility between complex III (cytochrome c reductase) and complex IV (cytochrome c oxidase) is even more intriguing than that of ubiquinone (105). Hackenbrock et al. (71) observed that, under physiological conditions of 150 mM ionic strength, cytochrome c is readily dissociated from the membrane and appears to undergo three-dimensional diffusion in the intermembrane space (65, 66); accordingly, duroquinol oxidase activity is increased by increasing ionic strength in parallel with the diffusion coefficient of cytochrome c passing from two-dimensional to three-dimensional diffusion. Although cytochrome c tends to collapse with the membrane surface randomly, it may be guided electrostatically as it approaches the electric field of the membrane integral complexes (95). Analysis by atomic force microscopy (AFM), used for obtaining information on phospholipid domains under bio-mimetic conditions, demonstrates the occurrence of laterally segregated regions in phospholipid matrices composed of mixed cardiolipin and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and indicates that cytochrome c, when injected in situ, shows preferential adsorption onto the lipid regions that are highly enriched in cardiolipin (30).

In reconstituted systems of cytochrome c reductase plus oxidase in the presence of excess cytochrome c, “pool behavior” is followed (52); likewise, the rotation rates of cytochrome-c oxidase reconstituted in lipid vesicles are not influenced by simultaneous incorporation of the reductase (90), suggesting that the two enzymes and cytochrome c are diffusing as independent entities. In cytochrome c-depleted beef heart mitochondria and submitochondrial particles (117), addition of exogenous cytochrome c exhibits saturation kinetics of both NAD-linked and succinate-linked oxygen uptake; the levels of cytochrome c giving saturation are similar to those found in intact beef heart mitochondria (19), indicating that a cytochrome c pool is needed for maximal electron transfer.

Deviations From Pool Behavior

Despite the wide acceptance of the CoQ pool as the mechanism for integrated electron transfer from dehydrogenases to cytochromes, deviations from pool behavior have also been described, raising doubts on the universal validity of the hypothesis (141). Important deviations from pool behavior were reported by Gutman (68) in beef heart submitochondrial particles oxidizing both NADH and succinate at the same time: because the predicted rates were smaller than the calculated rates, it was suggested that the two systems do not interact as closely as expected for a single homogeneous CoQ pool.

Other deviations from pool behavior occur at low ubiquinone concentrations (190), when the diffusion of the few quinone molecules over large distances may be hampered and one dehydrogenase may reduce only one or few complex III molecules within the distance scanned by diffusion, so that the system may approach solid-state behavior.

Likewise, deviations from pool behavior have been described at high membrane viscosity (79) obtained through either lowering the lipid content or having the lipid in the gel phase. The role of lipids in the arrangement of the respiratory chain will be analyzed in FORCES INVOLVED IN THE ORGANIZATION OF MEMBRANE PROTEINS: ROLE OF LIPIDS IN SUPERCOMPLEX FORMATION AND DISSOCIATION, below.

Isolated mitochondria were used to test the conclusions from inhibitor titration experiments that, in the yeast Saccharomyces cerevisiae, the respiratory chain consists of supermolecular assemblies. Under physiological conditions, neither ubiquinone nor cytochrome c exhibits pool behavior, as determined by the shape of the antimycin inhibition curve according to Eq. 3, implying that the respiratory chain in yeast is one functional and physical unit, with all respiratory complexes having a control coefficient of one on respiration (15). On the other hand, addition of high phosphate or trichloroacetate, acting as chaotrophic agents, restores pool behavior for both electron carriers. The authors concluded that the respiratory chain of yeast is organized as a supramolecular unit, but ascribed their findings to a special feature of the respiratory chain in yeast at difference with higher eukaryotes where pool behavior is normally found.

Stoner (171) could not clearly establish the existence of a diffusible intermediate between cytochrome c reductase and oxidase in intact mitochondria, in contrast with the clear-cut demonstration for a diffusible intermediate between complex II and complex III.

Flux Control Analysis of the Respiratory Complexes

Kinetic testing using metabolic flux control analysis is a powerful source of information on the supramolecular organization of enzyme complexes (88, 123). Metabolic control analysis predicts that, if a metabolic pathway is composed of distinct enzymes freely diffusible in a dynamic organization, the extent to which each enzyme is rate controlling may be different and the sum of all the flux control coefficients for the different enzymes should be equal to unity.

The flux control coefficient (C_i) of a step in a metabolic pathway is defined as the fractional change in the global flux through the pathway induced by a fractional change in the enzyme under consideration, and it can be expressed in mathematical terms by Eq. 5:

$$C_i = \frac{dJ_i/dI}{dJ_i/dI_{0}}$$

where (dJ_i/dI)_{0} is the corresponding change over the rate of the metabolic flux (J) (88).

On the other hand, in a supercomplex, the metabolic pathway would behave as a single enzyme unit, and inhibition of any one of the enzyme components would elicit the same flux control. In particular, in a system in which the respiratory chain is totally dissociated from other components of the OXPHOS apparatus (i.e., ATP synthase, membrane potential, and carriers), such as open nonphosphorylating submitochondrial particles, the existence of a supercomplex would elicit a flux
control coefficient near unity at any of the respiratory complexes, and the sum of all coefficients would be >1 (92).

Because flux control coefficients are mostly obtained by inhibitor titration of both total flux and the individual enzyme, their use for the assessment of pool behavior, although based on different theoretical grounds, has a practical exploitation very similar to that of inhibitor titrations. In fact, the presence of a lag in the inhibition of the integrated activity, as an indication that the inhibited step is not rate-limiting, should be common to both types of analyses.

The problem was addressed in mammalian and in plant mitochondria. The flux control coefficients of the complexes involved in NADH oxidation (I, III, IV) and in succinate oxidation (II, III, IV) were investigated with the use of bovine heart mitochondria and submitochondrial particles devoid of substrate permeability barriers (11). Both complexes I and III were found to be highly rate controlling over NADH oxidation, a strong kinetic evidence suggesting the existence of functionally relevant association between the two complexes. On the contrary, complex IV appears to be randomly distributed, although it is possible that if any stable interaction with complex IV exists in mammalian mitochondria it escaped detection, most likely because of a pronounced abundance of molecules in nonassembled form. Moreover, complex II is fully rate limiting for succinate oxidation, clearly indicating the absence of substrate channeling toward complexes III and IV (11). The major results are shown in Fig. 4 as threshold plots, i.e., plots of residual integrated activity (NADH oxidase or succinate oxidase) vs. extent of inhibition of the single step (enzyme complex).

Respiration has special features in plant cells; the mitochondrial respiratory chain is branched at the level of ubiquinone owing to the presence of at least five additional so-called alternative oxidoreductases, which participate in electron transfer (122, 144). Regulation of electron partitioning between alternative oxidoreductases, which participate in electron trans-

Fig. 4. Threshold plots of NADH oxidase (left) and succinate oxidase activity (right) in bovine heart mitochondria. Freehand threshold plots were drawn through data points (not shown, for clarity), representing the percentage of residual activity as a function of percent inhibition of complex I, complex II, complex III, and complex IV for the same inhibitor concentration, according to our results previously reported in Ref. 11. Respiratory activities were titrated in the presence of rotenone, carboxin, mucidi-n, and cyanide, respectively.

forces involved in the organization of membrane proteins: role of lipids in supercomplex formation and dissociation

The forces involved in protein organization in biomembranes have been analyzed in detail by Gil et al. (57). One major determinant of protein organization is the heterogeneous nature of the lipids in the membrane bilayer; because the integral membrane proteins are not fully deformable entities, their insertion in a lipid bilayer distorts the shape of the membrane locally. The lowest energy would be achieved in a system able to induce the least possible mismatch of the protein with the lipids, e.g., with the length of their acyl chains. For lipids in a fluid bilayer endowed with lateral mobility, integral proteins become surrounded by a lipid domain of boundary lipids (87), also called lipid annulus (81), composed of the lipid species providing the best match. The lipid annulus is not a static entity as was first inferred from biophysical studies showing lipid immobilization by integral proteins (87); rather, it is a dynamic one, where the exchange of lipid molecules with the surrounding bulk phase is very fast (119). The annular lipids must not be confused with those lipids that are usually detected in high-resolution crystal structures of membrane proteins and define nonannular or cofactor lipids (103). In some cases, special types of lipids having peculiar thermal behaviors aggregate to form clusters, such as those detected in plasma membranes and called lipid rafts (17, 31, 161). Lipids belonging to such clusters are more ordered than bulk lipids;
proteins in such regions are easily induced to clustering by stimuli triggering cascades of signal transduction.

At low protein concentrations in lipid bilayers, proteins are usually randomly dispersed (103), whereas at high concentrations they tend to aggregate. The forces involved in protein aggregation are both of entropic and enthalpic origins.

Lipid-mediated protein-protein interactions originate by hydrophobic mismatch: the same reasons inducing the formation of a lipid annulus to compensate for the mismatch by attracting the lipids having the best fit would be operative by attracting together proteins having mismatch of the same sense (13). For the same reason, however, proteins having hydrophobic mismatch of opposite sense should repel each other (57).

The possibility that a protein exhibits preference for a given lipid species so that a lipid annulus "wets" the protein surface may be another source of protein aggregation (103). The possibility that the wetting layer be shared by two or more proteins has been called capillary condensation and gives rise to aggregation phenomena (58) that are at a longer range scale than those involved in direct protein-protein interactions.

Wetting and capillary condensation were found to give rise to protein aggregation and to phase separation, in which one of the phases is rich in proteins, when approaching the thermodynamic conditions for a phase transition (58). As a function of increasing temperature, one observes sequentially a capillary condensate of a protein-rich phase of the preferred lipid, a densely packed protein array (two-dimensional protein crystal), and finally dispersion of the proteins.

In the mitochondrial inner membrane, integral proteins of the OXPHOS system are densely packed so that the average distance between complexes may be calculated to be few nanometers (105). In addition, the presence of different types of lipids would enhance wetting and capillary condensation as well as the possibility of phase separations. Thus the presence of protein aggregates of the transmembrane respiratory complexes would not be at all unexpected on theoretical grounds. The immobilization of the proteins would also be favored by the presence of outer membrane-inner membrane contacts (16), by the narrow tubular connections of the cristae (118), and by the high viscosity of the matrix, which would exert a slowing effect on the diffusion of proteins spanning the inner membrane, according to the Saffman-Delbruck relation (152). Saffman and Delbruck produced a hydrodynamic model for membrane diffusion of a cylindrical object embedded in a viscous continuum fluid sheet bounded by an aqueous fluid. Such a particle, simulating a membrane protein, is restricted to move laterally in the z-plane and to rotate around the z-axis. For lateral diffusion, the following equation was derived, clearly showing the retarding effect of the viscosity of the outer medium

\[ D_z = (kT/4\pi\eta h) \cdot (\log\eta/\eta' - a - \gamma) \]  

where \( D_z \) is the lateral diffusion coefficient (in cm²/s), \( k \) is Boltzmann’s constant, \( T \) is absolute temperature, \( h \) is the height of the cylinder, \( a \) its radius, \( \eta \) and \( \eta' \) are the viscosity of the membrane and of the outer medium, respectively, and \( \gamma \) is Euler’s constant (0.5772).

In addition, direct binding of matrix enzymes with respiratory chain complexes may occur, as proposed for complex I with several NAD-linked matrix dehydrogenases, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, malate dehydrogenase, and β-hydroxyacyl-CoA dehydrogenase (128, 174).

Changing the nature of the lipids would dramatically change their matching conditions to the proteins. This could be due to the presence and number of electrostatic charges, different length of the fatty acyl chains, different physical state, and different phases existing at the body temperature. Lipid peroxidation removes cis double bonds from the fatty acyl chains, inducing profound biophysical changes in the lipid bilayer (169), such as increased disorder and possible breakdown of membrane structure and of lipid-protein interactions (120), and presumably affecting the membrane thickness. Therefore, a change in membrane protein aggregation induced by lipid peroxidation may well be expected on theoretical grounds.

Early experiments reported by Ragan and Heron (142) provided evidence that purified complex I and complex III, when mixed as concentrated solutions in detergent and then codialyzed, combine reversibly in a 1:1 molar ratio to form a complex I-III unit (NADH-cytochrome c oxidoreductase) that contains equimolar flavin mononucleotide (FMN) and cytochrome c (clearly at difference with the supercomplexes found by BN-PAGE, where complex III is present as a dimer) (see Evidence in Favor of Solid-State Organization, above) and 2–3 mol of ubiquinone-10/mol of protein unit. Transfer of reducing equivalents to or from this unit by extra complex I or complex III molecules is slow and does not contribute to the overall rate of electron transfer from NADH to cytochrome c. Moreover, activation-energy measurements for NADH-cytochrome c oxidoreductase activity showed that oxidoreduction of endogenous ubiquinone-10 proceeds somewhat differently from the oxidation and the reduction of exogenous quinones, supporting the idea that the mobility of ubiquinone-10 in the complex I-III unit is highly restricted and suggesting that CoQ₁₀ is effectively trapped between the component complexes in an environment that may be partly protein and partly derived from the lipid annuli of those complexes.

However, CoQ₉-pool behavior could be restored, and complex I and complex III could be made to operate independently of each other by raising the concentrations of phospholipid and ubiquinone (~2-fold and 6-fold increase, respectively) in the concentrated mixture (80). Inclusion of phospholipid into the reconstituted system may have a number of effects on the physical state of the system. Heron and coworkers (80) have proposed that the principal difference lies in the relative mobility of the reconstituted complexes; when phospholipid in excess of that needed to form an annulus is absent, relative mobility is lost and complexes are frozen in their complex I-III assembly, favoring a stable orientation of the site of reduction of ubiquinone with respect to the site of oxidation. Apparently, the idea that the respiratory complexes are associated in fixed assemblies may sound incompatible with the presence of phospholipids in the natural inner mitochondrial membrane; nevertheless, it is worth mentioning that inner-outer membrane contacts and the quasi-solid organization of the matrix (167, 168) may keep the integral proteins in a clustered immobilized arrangement, thus favoring segregation of most of the phospholipids into separated patches.

Heron et al. (80) also reported that endogenous ubiquinone-10 leaks out of the complex I-III unit when extra phospholipid is present, causing a decrease in activity that could be alleviated by adding more ubiquinone. It is likely that the
function of the large amount of ubiquinone in the natural membrane may be, therefore, to maintain the ubiquinone-10 content in the supercomplex unit when it is formed (see Functional Relevance of Respiratory Supercomplexes, below).

An analogous system, obtained by fusing a crude mitochondrial fraction (R4B) enriched in complex I and complex III with different amounts of phospholipids and CoQ10 (111, 148), was used to discriminate whether the reconstructed protein fraction behaves as individual enzymes (CoQ-pool behavior) or as assembled supercomplexes depending on the experimental distances between the intramembrane particles. The comparison of the experimentally determined NADH-cytochrome c reductase activity with the values expected by theoretical calculation applying the pool equation showed overlapping results at phospholipid dilutions (wt/wt) from 1:10 on, i.e., for distances >50 nm, whereas at shorter distances between complex I and complex III, resembling the mean nearest neighbor distance between respiratory complexes in mitochondria (159, 179), pool behavior was not effective any more (10, 111). In the two experimental models, kinetic testing according to the metabolic flux control analysis validated the hypothesis of a random organization and of a functional association between complex I and complex III, respectively (unpublished results from our laboratory).

The formation of the supercomplex I-III is conditioned by the lipid component, but the role played by the lipid environment, in terms of its chemical composition, is not completely known.

All purified preparations of mitochondrial electron transfer complexes are isolated as lipoprotein complexes, the extent of associated lipid depending on the particular method used for isolation. Complete extraction of phospholipid from the lipoprotein complexes revealed a gross phospholipid composition reflecting that for the mitochondrial inner membrane. Predominant phospholipids present include cardiolipin, phosphatidylcholine, phosphatidylethanolamine, and lesser amounts of neutral lipids and phosphatidylinositol (48).

Two roles of phospholipid have been distinguished: 1) a dispersive solubilization effect that can be duplicated by appropriate detergents and 2) a catalytic effect that can be specifically fulfilled only by cardiolipin (53, 54, 103, 150, 181). Indeed, there are yet two more possible roles that may need to be met, particularly in the case of complex I and complex III. These roles might be to provide a sufficiently lipophilic environment for the interaction of the lipophilic electron carrier, ubiquinone, and to participate in linking together components of the respiratory chain.

The phospholipids in closest vicinity to the protein surface, as well as those in the free bilayer, are actually highly mobile and free to exchange, but cardiolipin was indicated as tightly bound, being more likely buried within the protein complexes (89, 102, 160). The absolute requirement of cardiolipin for cytochrome oxidase, complex I and complex III activities suggests that this phospholipid plays a crucial role in the coupled electron transfer process (54), but recent results seem also to indicate that cardiolipin stabilizes respiratory chain supercomplexes and the individual complexes. The availability of a cardiolipin-lacking yeast mutant (Δcrl1 null) provided the opportunity to demonstrate that mitochondrial membranes still contained the III2-IV2 supercomplex but that it was significantly less stable than supercomplexes in the parental strain.

Other phospholipids that are increased in the mutant, including phosphatidylethanolamine and phosphatidylglycerol, could not substitute for cardiolipin and could not prevent dissociation of supercomplexes, showing 90% of the individual homodimers of complex III and IV not organized into supercomplex under BN-PAGE conditions (138, 189). The putative direct protein-protein interaction of cytochrome oxidase and complex III is proposed to involve the domain formed by transmembrane helices of cytochromes b and c1 and the core components of complex IV, namely COX 1, 2, and 3 (138). In the structure of yeast, the membrane-imbedded domain of complex III forms a depression, and two phospholipid molecules, i.e., cardiolipin and phosphatidylethanolamine, are tightly bound in this cavity (102), suggesting that they can provide a flexible linkage between the above-mentioned subunits of complexes III and IV.

It is well documented that exposure of mitochondria to reactive oxygen species (ROS) can affect the respiratory activity via oxidative damage of cardiolipin, which is required for the optimal functioning of the enzyme complexes (133, 134, 137). Unpublished results from our laboratory suggest that dissociation of respiratory supercomplexes occurs in proteoliposomes, due to lipid peroxidation induced by 2,2′-azobis-(2-amidinopropane)dihydrochloride before reconstitution of a protein fraction enriched with complexes I and III (R4B) into the phospholipid vesicles.

FUNCTIONAL RELEVANCE OF SUPRAMOLECULAR ORGANIZATION

Enzymatic Advantage of Substrate Channeling

The term metabolon was introduced to describe enzyme-enzyme highly organized complexes in a sequential series of enzymes operating within a metabolic pathway (168). Evidence exists of such complexes for many metabolic pathways, including macromolecular biosynthesis, nitrogen base metabolism, lipid biosynthesis, glycolysis, fatty acid oxidation, urea cycle, and tricarboxylic acid cycle (168). Actually, according to this view, there are few, if any, free enzymes within cells. The tricarboxylic acid cycle enzymes have been particularly investigated. If mitochondria are disrupted by mild sonication or osmotic shock, then the “soluble” matrix enzymes appear to be organized in a large complex of proteins and can be easily sedimented at low speed (149). These complexes are stabilized by treatment with cross-linking reagents (32).

Different strategies are used to obtain the active sites of consecutive enzymes in close proximity, including isolation of the complexes (26, 121, 124) and genetic fusion of consecutive enzymes (115). The availability of the tridimensional structure of the enzymes of interest allows computer graphic modeling and docking studies, as it has been shown for mitochondrial malate dehydrogenase, citrate synthase, and aconitase (180). Among thousands of docking orientations tried, one was found to respond to the structural and experimental constraints previously obtained with yeast fusion proteins (116).

Is there a rate advantage for the cell in this organization of consecutive enzymes compared with a system where the enzymes are free in the bulk solution? The most likely reason for the organization would be to prevent the intermediates from escaping into solution where they may be sequestered by other enzymes for use in different metabolic pathways (166): in such
a way, the metabolic flow in the pathway is increased by the close proximity of the enzymes catalyzing consecutive reactions by ensuring the channeling of the intermediate (129). The existence of substrate channeling has been demonstrated by a great deal of studies, which largely used the addition of competing enzymes (44). If the intermediates in the enzyme complex diffuse to the added enzymes, then the rate in the complex would be lowered by competition. This was shown not to be the case, for example, for the citrate synthase and malate dehydrogenase complex, where the rate of the coupled reaction was only slightly inhibited, contrary to the result with the free enzymes, suggesting channeling of the intermediate oxalacetate (124).

In his study, Srere and colleague (174) also demonstrated that several dehydrogenases and other matrix enzymes bind to the mitochondrial membrane and in particular to complex I. It was calculated that the binding capacity of complex I for these enzymes is sufficient so that all mitochondrial malate dehydrogenase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase could be bound to complex I. The binding would result in a functionally coherent enzyme organization, since the NADH production and NADH oxidation would take place in an efficient coupled manner. A supramolecular assembly between the acyl-CoA dehydrogenases, ETF, ETF-CoQ reductase, and complex III was also suggested (173, 136).

A striking special feature of complex I of plants and green algae is the presence of up to five subunits that resemble γ-type carbonic anhydrases (CA). Very recently, a structural analysis by electron microscopy and mass spectrometry showed that CA2 is an integral membrane protein attached to the center of the membrane arm of complex I, forming part of a matrix-exposed spherical domain. The physiological role of the complex I-integrated CA domain is thought to be linked to proton translocation activity of this respiratory complex; CO₂ hydration might be favored by close vicinity to the membrane arm of complex I, which is involved in proton translocation across the inner mitochondrial membrane (175).

Using alamethicin-permeabilized rat liver mitochondria and external pyruvate plus lactate dehydrogenase as a competitive system for NADH, Kotlyar et al. (96) showed that NADH produced by matrix malate dehydrogenase was efficiently used by the exogenous system, thus showing strong competition with complex I. In other words, NADH produced by matrix dehydrogenase was released from mitochondria rather than being used by complex I. The authors concluded that this demonstrates the absence of NADH channeling between malate dehydrogenase and complex I.

If the inner membrane permeabilization has not modified the matrix protein organization, then the above results should mean that the rate advantage is not due to real substrate channeling but rather by maintaining close proximity between enzymes catalyzing consecutive reactions, thus favoring diffusion and collisional interactions over only short distances (96). This alternative to substrate channeling should be kept in mind when analyzing the presence of physiological supercomplexes in the OXPHOS system.

**Functional Relevance of Respiratory Supercomplexes**

Although, from a structural point of view, little doubt exists that respiratory chain complexes are organized, at least in part, as supramolecular aggregates, their functional significance is still obscure. The obvious consequence of such organization in providing contact surfaces for substrate (CoQ and cytochrome c) channeling (11) must be reconciled with experimental evidence of a pool function of CoQ and cytochrome c and of diffusional interactions at the basis of intercomplex electron transfer (47, 68, 69, 100, 101, 190).

The problem should be restricted to NADH oxidation, since, in the system supporting succinate oxidation, complex II does not physically interact with complex III in a supercomplex, as amply discussed above. The physiological implications of the interaction between complex I and complex III are not yet fully understood. It was speculated that they include enhancement of electron flow by direct channeling of ubiquinone, but the matter should be examined in better detail.

One remaining question is: would stoichiometric channeling of CoQ between complex I and complex III (131, 156, 157) be really incompatible with the pool behavior, since it would exclude the bulk of the CoQ pool from kinetic determination? Actually, in presence of preferential associations, the pool equation would be still valid if the rate of association/dissociation of the complexes were faster than the rate of electron transfer between complexes and CoQ molecules in the pool. An alternative possibility would be that, within a supercomplex, CoQ reduced by one enzyme has a way to dissociate in the pool to meet any other supercomplex, including the same one in a different site, for being oxidized.

On the other hand, the bound intercomplex quinone that allows electron flow directly from complex I to complex III may well be in dissociation equilibrium with the CoQ pool, so that its amount, at steady state, would be dictated by the size of the pool. This equilibrium would explain the saturation kinetics for total ubiquinone exhibited by the integrated activity of complex I and complex III (39) and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool (158). To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition must, however, require that the dissociation rate constants (kₘₐₜ) of bound CoQ be considerably slower than the rates of intercomplex electron transfer (k’ and k”) via the same bound quinone molecules (Fig. 5). To explain the high apparent Kₘ found for CoQ in NADH oxidase activity (39), kₘₜ for CoQ binding to complex I must be slow (55).

On the contrary, complex II kinetically follows pool behavior in reconstitution experiments (39) and in the double-inhibitor titration experiments (171), in complete accordence with the lack of supercomplexes found by both BN-PAGE (157) and flux control analysis (11). The isolation of discrete units having succinate cytochrome c reductase activity (73, 188) and the biophysical studies described in Ref. 70 appear in strong contrast with this indication. The only possible explanation so far is in the existence of very loose but specific contacts between complex II and complex III, not giving rise to any kind of channeling even in phosphorylating mitochondria (171). These contacts are lost even in frozen mitochondria and in submembrane particles, as shown by BN-PAGE (157), but are strongly nonphysiologically enhanced by some treatments used for mitochondrial respiratory chain fractionation. It would be interesting to apply BN-PAGE and flux control analysis to the succinate cytochrome c reductase units isolated by King and coworkers (177, 188).
Of course, the functional relevance of supercomplex organization in electron transfer may vary with the physiological conditions in the intact cells. Clearly under physiological conditions, the respiration rate is under control of energy-consuming processes (respiratory control). Under prevalent state 4 conditions (high ATP-to-ADP ratio), the electrochemical proton gradient is largely the rate-limiting step, so that operation of the respiratory chain as tunneling or diffusion may be of minor importance for the flux control. On the other hand, the situation may be dramatically different under conditions of high energy demand (state 3) or under uncoupling conditions. The latter may be more important in vivo than originally thought; it has been suggested that muscle and liver mitochondria may dissipate 30–50% of the electrochemical potential as heat (151) through action of the uncoupling proteins (147).

Recently, Piccoli et al. (139) evaluated the impact of the mitochondrial transmembrane potential ($\Delta \mu_{\text{H}^+}$) on the flux control exerted by cytochrome $c$ oxidase on the respiratory activity in intact cells. The results indicate that, under conditions mimicking the mitochondrial state 4 respiration, the control strength of the oxidase is decreased with respect to endogenous state 3 respiration. The authors suggested that such a change in control strength might be featured in terms of equilibrium between different organizational structures of the enzymatic complexes constituting the mitochondrial OXPHOS (139). Although the driving forces leading to the assembly/disassembly of the supercomplexes have not yet been defined, it is not unconceivable that, given the membrane-integrated nature of the single complexes, electrostatic-hydrophobic interactions may enter into play in response to $\Delta \mu_{\text{H}^+}$.

Possible Reasons for the Contrasting Results Obtained With Different Methods

Presence of errors or artifacts. Is there any possibility that either pool behavior experiments or flux control analysis of the NADH oxidase system is subjected to errors or misinterpretations?

As for flux control analysis, determination of the rate-limiting steps in very small submitochondrial particles might be thought to involve only few complexes I and III in the same membrane, thus making the channeling of CoQ obligated only between the very few enzymes present in the same membrane. The fact that identical results were obtained in submitochondrial particles and in mitochondria (11) strongly militates against this interpretation.

Examination of the literature does not provide as many examples of pool behavior of CoQ concerning NADH oxidation as those concerning succinate oxidation, where the discrepancy does not hold because succinate does not form a supercomplex. Particularly, studies of the quantitative adherence to CoQ pool behavior for NADH oxidation are usually not validated by direct measurement of $V_{\text{red}}$ (NADH-CoQ reductase), which is calculated indirectly from $V_{\text{obs}}$ (NADH oxidase) (see Refs. 56, 68, 190).

Furthermore, we have to consider that the maximal rate of complex I activity in mitochondria is considerably lower than that of complex III, mainly due to lower content (19) rather than to different turnover (46, 47). The data in the literature concerning complex III turnover may need to be revisited because it has been recently shown that the enzyme is dimeric but functionally behaves as a monomer (22). This finding would require the values found for the catalytic constant $k_{\text{cat}}$ of complex III to be doubled, since only half of the dimer is active at any time. The use of duroquinol as substrate for ubiquinol cytochrome $c$ reductase ($V_{\text{ox}}$) (68) represents another uncertainty, since duroquinol does not support maximal turnover of complex III compared with better substrates ubiquinol-2 and decyl-ubiquinol (46).

In a supercomplex containing stoichiometric amounts of complex I and of dimeric complex III, the total $V_{\text{obs}}$ would be

![Diagram](http://ajpcell.physiology.org/ by 10.220.32.247 on September 30, 2017)
determined by that of complex I \( (V_{\text{red}}) \), whereas the existence of pool behavior between the same units involved would elicit a \( V_{\text{obs}} \) considerably lower, according to the pool equation (Eq. 2). In the real situation, there is, however, an excess of complex III dimers not involved in the supercomplex formation (157). These would not significantly contribute to determination of flux control coefficients, since in these only the shapes of the titration curves, shown as normalized percentages, are compared. However, it cannot be excluded that they would contribute to the determination of \( V_{\text{ox}} \) in Eq. 2, since the experimental rates of ubiniquinol cytochrome c reductase activity, which are obtained in the presence of saturating amounts of exogenously added quinol, might include both free complex III and complex III in the supercomplex. Application of Eq. 2 under this condition would raise the calculated \( V_{\text{obs}} \), approaching the true rate of channeling in the supercomplex, and would erroneously indicate the presence of a pool behavior as a consequence of the strict correspondence between the experimental and the calculated rate values.

The demonstration of pool behavior by the sigmoidal inhibition curve of NADH or succinate oxidase activity in the presence of antimycin (101) may be complicated by the cooperativity of antimycin inhibition in the dimer of complex III (7, 22), so that other inhibitors should be preferred (190). Indeed, in bovine heart submitochondrial particles, we found a sigmoidal inhibition curve of ubiquinol cytochrome c reductase by antimycin that was overlapped by a sigmoidal inhibition curve of either NADH- or succinate-cytochrome c reductase. On the other hand, the ubiquinol cytochrome c reductase inhibition curve by either myxothiazol or mucidin was hyperbolic, as was the inhibition curve of NADH cytochrome c reductase. The inhibition curve of succinate cytochrome c reductase, although not sigmoidal, was sensibly less steep (E. Dalmonte, M. L. Genova, and G. Lenaz, unpublished observations). The reason for lack of sigmoidicity in the inhibition curve of succinate cytochrome c reductase may be searched in the relative ratio of \( V_{\text{red}} \) and \( V_{\text{ox}} \) (see Fig. 7 in Ref. 101).

Nevertheless, the studies of Kröger and Klingenberg (100) have clearly demonstrated that most CoQ in submitochondrial particles at steady state are reducible by either NADH or succinate and that only 10–20% is redox inactive. Because CoQ is in molar excess over the respiratory enzymes, this means that each dehydrogenase is able to reduce a large number of CoQ molecules, supporting the view of a homogeneous CoQ pool. Moreover, in the original experiment of Kröger and Klingenberg (100), \( V_{\text{red}} \) and \( V_{\text{ox}} \) were determined from total respiratory activity and the first-order rate constants of CoQ reduction and oxidation, respectively, and \( V_{\text{ox}} \) was also directly determined in a quenching-flow apparatus after an oxygen pulse. Thus the notion of CoQ as a diffusible intermediate also in NADH oxidation is difficult to discard and must be reconciled with the notion of supercomplexes on a functional basis.

Coexistence of pool behavior and channeling. In this discussion we assume that all of complex I is part of a supercomplex, whereas 30–50% of complex III is free, as indicated by the BN-electrophoretic data. Even if some CoQ molecules are bound within the supercomplexes, most quinone is certainly free in the lipids. Pool behavior would be observed only if complex I reduces both CoQ trapped in the supercomplex and CoQ in the pool at approximately the same rate. In this scenario, however, CoQ in the pool would compete with bound CoQ and decrease the actual extent of channeling. This view is therefore very difficult to support. In addition, Ragan and Heron (142) in their reconstitution studies have clearly demonstrated that, when a supercomplex I-III is formed, addition of an excess of complex III still allows electron transfer to occur only by channeling within the supercomplex and not by exchange with the pool. Thus coexistence of channeling and pool behavior could be found only in the presence of free units of both complex I and complex III.

Adherence to exclusively pool behavior without CoQ channeling. According to this interpretation, supercomplexes are structural units, but no substrate channeling occurs. The CoQ substrate is freely and rapidly exchanging with the pool. Indeed, quinone diffusion would be favored by close proximity of the complexes, increasing the probability of useful collisions with near neighbors rather than with distant complexes (see Ref. 96). This interpretation would explain CoQ pool behavior but also deviations from pool behavior reported when NADH and succinate are oxidized simultaneously (68) or at low CoQ concentration (190) or high lipid viscosity (79). The interpretation of Gutman (68) is of particular interest because he postulates a kind of compartmentation of CoQ pools that exchange between each other (spillover) at relatively low rates.

However, flux control analysis showing that both complex I and complex III are rate limiting in electron transfer cannot be explained by this interpretation. If the restriction of mobility of trapped CoQ in a supercomplex is very strong, then one could approach channeling conditions but would no longer observe pool behavior. The two modalities are obviously inconclicable.

Substrate channeling and pool behavior in different mitochondrial preparations. A final possibility is that supercomplexes are relatively labile entities. Thus different studies have analyzed respiratory activities at different states of organization of the chain. This may be the case for the study of Bianchi et al. (11) compared with the original studies of Kröger and Klingenberg (100, 101), since scrutiny of the mucidin inhibition curve for NADH oxidation does not show a convex shape, indicative of pool behavior as in the study of Kröger and Klingenberg (101). Further unpublished studies reported in Presence of errors or artifacts, above, clearly show lack of pool behavior for NADH cytochrome c reductase, whereas pool behavior may be envisaged for succinate cytochrome c reductase.

Thus the particles analyzed by Bianchi et al. (11) must be in a different organization state from those analyzed by Kröger and Klingenberg (101). The reason for this discrepancy is obscure and might be related to differences arising from diverse protocols in the separation procedures and in the assay conditions (i.e., the extent of coupling of the particles and the resulting energy state of the mitochondrial membrane would affect the supramolecular organization of the enzymatic complexes of the OXPHOS system).

The structural dissociation of functional supercomplexes may be gradual, being preceded by some extent of opening, allowing faster exchange of trapped CoQ with the pool (Fig. 6). The effect of chaotropic agents reported in yeast mitochondria (15) may be an example of such labile organization.

Obviously, although we are convinced that this interpretation is the only one tenable at the moment, further studies
are required for elucidation of these uncertainties, in particular analyzing the conditions for supercomplex association and dissociation and monitoring the functional changes induced.

**ROLE OF COQ POOL**

If we consider bound CoQ as stoichiometric with one site in the complexes that have been shown to contain bound CoQ (I, II, III) (104, 178, 182), in beef heart mitochondria we come up to no more than 0.35 nmol/mg protein, which would increase to ~0.5 nmol assuming more than one site to be fully occupied in complex I and complex III. Because the total CoQ content is higher than 3 nmol/mg (4, 19, 47, 56), we must assume that most CoQ (>84%) is free in the bilayer.

A great deal of biophysical data in the literature show that CoQ is dispersed in the phospholipids and is freely mobile by lateral diffusion; however, most of these data were obtained for CoQ inserted in liposomes (e.g., Refs. 45, 67). Nevertheless, some indirect evidence for the existence of a mobile CoQ pool in the mitochondrial inner membrane lipids stems from studies showing that CoQ homologues are endowed with lateral diffusion also when added to natural mitochondrial membranes. In particular, Rajarathnam et al. (143) showed by FRAP that a fluorescent derivative of CoQ$_{10}$ in fused mitochondria from rat
liver has a diffusion coefficient of $3.1 \times 10^{-9}$ cm$^2$/s, in line with the coefficient previously found for shorter derivatives by Hackenbrock et al. (71) but much slower than those found by Fato et al. (45) by fluorescence quenching of membrane probes by CoQ homologues.

Thus we may conclude that most certainly a mobile pool of CoQ exists in the inner mitochondrial membrane and that this pool is in equilibrium with protein-bound CoQ, as discussed above. A major function of the CoQ pool, therefore, must be to drive binding into sites formed at the border between adjacent complexes I and III to ensure correct channeling of electrons form one to the other complex. Because the dissociation constant of CoQ from the supercomplex must be high, as inferred from the apparent $K_m$ of the integrated activity and discussed above in Functional Relevance of Respiratory Supercomplexes, any decrease of CoQ concentration in the pool would decrease the amount of bound CoQ and therefore induce a fall of electron transfer. In this manner, free CoQ behaves as a reservoir for binding to the I-III supercomplex; in addition, free CoQ may be a reservoir also for other functions believed to require CoQ binding to specific proteins, such as uncoupling proteins (34) and the permeability transition pore (3, 185).

Furthermore, other electron transfer activities, such as succinate oxidation, are the result of interaction of complexes that do not show aggregation either by BN-PAGE or by kinetic flux control analysis: these activities necessarily need collisional interactions of CoQ molecules from the pool with both complexes II and III also randomly dispersed in the bilayer. Other activities such as GAPDH, ETF dehydrogenase, and dihydrolipoamide dehydrogenase, which are likely to be in minor amounts and strongly rate limiting in integrated electron transfer, are probably dictated by interaction through the CoQ pool. The only direct study that addressed this problem (145) demonstrated that in brown fat mitochondria the inhibition curve of glycerol phosphate cytochrome $c$ reductase is sigmoidal in the presence of myxothiazol and antimycin, suggesting the presence of a homogeneous CoQ pool between glycerol phosphate dehydrogenase and complex III. On the other hand, in *S. cerevisiae* mitochondria, Bunoust et al. (18) showed a very specific favorable competition of external NADH dehydrogenase (Nde2p) over mitochondrial glycerol phosphate dehydrogenase and complex III. On the other hand, in *S. cerevisiae* mitochondria, Bunoust et al. (18) showed a very specific favorable competition of external NADH dehydrogenase (Nde2p) over mitochondrial glycerol phosphate dehydrogenase for giving electrons to the respiratory chain, whereas the use of electrons from Nde1p and succinate dehydrogenase was shared to a comparable extent. The reason was ascribed to a supramolecular organization of GAPDH with NADH dehydrogenase(s) but not with succinate dehydrogenase.

Also reverse electron transfer from succinate to NAD$^+$, involving sequential interaction of complexes II and I by means of CoQ, must take place by collisional interactions in the CoQ pool, since no aggregation exists between complexes I and II. Gutman (68) provided evidence that energy-driven reverse electron transfer from succinate to NAD$^+$ indeed follows pool behavior.

This observation poses a particularly puzzling question: if complex I is completely or almost completely associated with complex III and the interaction of CoQ in the pool with the quinone-binding site in common between the two enzymes is necessarily slow (see Functional Relevance of Respiratory Supercomplexes), then how can ubiquinol reduced by complex II interact from the pool with the CoQ site in complex I at a rate compatible with the steady-state kinetics of reverse electron transfer? The intriguing idea that complex I may possess two different quinone-binding sites for direct and for reverse electron transfer, respectively, is compatible with the proposal by Grivennikova et al. (64) that two different routes exist for forward and reverse electron transfer within the complex. These two sites might become alternatively accessible depending on the magnitude of the membrane potential. Alternatively, one should postulate that the association rate constant of reduced CoQ from the pool to complex I in the supercomplex should be sufficiently fast to be compatible with the turnover of reverse electron transfer. It must be noted that the ATP-driven reverse electron transfer from succinate to NAD$^+$ occurs in the presence of a high mitochondrial transmembrane protonmotive force that, according to Ref. 139, might be the physiological signal and, at the same time, the trigger causing the structural reorganization of the enzymatic complexes of the mitochondrial OXPHOS system. The model hypothesis depicted by Piccoli et al. (139) from data on cytochrome oxidase might be extended to other enzymes of the respiratory chain, suggesting that also the I-III supercomplex would dissociate its constituting complexes under high $\Delta \mu_{H^+}$ condition, and this would no longer limit the access to the CoQ binding site in complex I.

The free diffusing CoQ is likely to also represent the main antioxidant species in the inner mitochondrial membrane, where it can break up the radical chain reaction of lipid peroxidation (37).

**PATHOPHYSIOLOGICAL IMPLICATIONS**

The involvement of mitochondria in a variety of pathological aspects and in aging (6, 28, 59, 106, 113, 184) has been largely ascribed to their central role in production of ROS and to the damaging effect of ROS on these organelles. In particular, damage to mitochondrial DNA would induce alterations of the polypeptides encoded by mitochondrial DNA in the respiratory complexes, with consequent decrease of electron transfer activity, leading to further production of ROS, thus establishing a vicious circle of oxidative stress and energetic decline (108, 130). This fall of mitochondrial energetic capacity is considered to be the cause of aging and age-related degenerative diseases (28, 106, 184). This vicious circle might be broken by agents capable of preventing a chain reaction of ROS formation and damage, such as CoQ in its reduced form (37).

The observation that lipid peroxidation disrupts the aggregation of complexes I and III (see above) has profound pathophysiological implications, since ROS produced by the mitochondrial respiratory chain induce a progressive peroxidation of mitochondrial phospholipids (37) and in particular of cardiolipin (133, 134) in aging and ischemic diseases, with demonstrated decreased activity of both complexes I and III (132, 137). It is tempting to speculate that, under the above conditions, a dissociation of complex I-III aggregates occurs, with loss of facilitated electron channeling and resumption of the less efficient pool behavior of the free ubiquinone molecules. Although no direct demonstration yet exists, dissociation of supercomplexes might have further deleterious consequences, such as disassembly of complex I and III subunits and loss of electron transfer and/or proton translocation. We could not even exclude that the consequent alteration of electron transfer may elicit further induction of ROS generation.
In accordance with this line of thought, the different susceptibilities of different types of cells and tissues to ROS damage may depend on, among other reasons, the extent and tightness of supercomplex organization of the respiratory chains, which depend on phospholipid content and composition of mitochondrial membranes.

Studies are needed to answer to these pressing questions.

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