The molecular machinery of Keilin's respiratory chain

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Keilin Memorial Lecture
Delivered at the University of Essex, on 3 July 2003
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Abstract

Keilin's classic paper of 1925 [Keilin (1925) Proc. R. Soc. London Ser. B 100, 129–151], achieved with simple, but elegant, techniques, describes the cytochrome components of the respiratory chain and their roles in intracellular respiration and oxygen consumption. Since that time, a tremendous amount of work has clarified the intricate details of the prosthetic groups, cofactors and proteins that comprise the respiratory chain and associated machinery for ATP synthesis. The work has culminated in advanced crystallographic and spectroscopic methods that provide structural and mechanistic details of this mitochondrial molecular machinery, in many instances to atomic level. I review here the current state of understanding of the mitochondrial respiratory chain in terms of structures and dynamics of the component proteins and their roles in the biological electron and proton transfer processes that result in ATP synthesis. These advances, together with emerging evidence of further diverse roles of mitochondria in health and disease, have prompted a new era of interest in mitochondrial function.

An historical perspective

In his historical account of the discovery of the respiratory chain and cytochromes, Keilin [1] modestly described his fortuitous observation of the characteristic four-banded visible spectrum of respiratory chain cytochrome in thoracic muscle of the adult fly stage of *Gasterophilus intestinalis*, a parasitic worm that he had been studying in relation to changes in haemoglobin content during its life cycle. In these early observations, he thought that the pigment was derived from haemoglobin in some way, and acknowledged its prior description by MacMunn in 1884. However, by examination of tissues of other organisms that did not contain haemoglobin, including baker's yeast, he soon realized that the pigment was widely distributed and could not be derived from haemoglobin. He went on to describe an even more fortuitous observation of the sudden appearance of the cytochrome spectrum in a suspension of aerated yeast cells that failed to show the spectrum on initial observation, and its reversible disappearance each time aeration was repeated. This key observation led him to the realization that the spectrum arose from multiple components, relevant to the biological oxidation processes of Thunberg, Wieland and Warburg, and central to the process of aerobic respiration. Keilin's classic paper of 1925 [2], achieved with simple but elegant techniques, described these findings and introduced the notion of distinct cytochromes *a*, *b* and *c* (although Keilin used the singular term cytochrome to define the entire system) that act together to form what was later called the respiratory or electron-transfer chain. In further work, he identified the cyanide- and carbon-monoxide-sensitive oxidase, resolved the two haem components of cytochrome oxidase which he termed cytochromes *a* and a_3 (by this time he had used the terms a_1 and a_2 for other bacterial pigments) and confirmed the observations of Okunuki that the band around 550 nm arose from two different *c*-type cytochromes, cytochrome *c* and c_1.

These seminal studies provided understanding of the electron-transfer-chain nature of the respiratory chain and its central roles in intracellular respiration and oxygen consumption that led to a tremendous amount of diverse work that has clarified further the intricate details of these processes. In a recent article [3], Slater has summarized the diverse studies of other groups that built on Keilin's work and which led to the identification of ubiquinone and the flavin, iron–sulphur and copper-containing respiratory prosthetic groups that cannot be observed easily by visible spectroscopy, the fractionation of the major protein complexes, the emergence of the link between respiratory electron flow,

Key words: electron transfer, energy coupling, Fourier-transform infrared spectroscopy (FTIR spectroscopy), mitochondria, respiratory chain.
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proton transfer and ATP synthesis, and the elucidation of intricate details of the underlying mechanisms. Most recently, major advances have been provided by advanced crystallographic and spectroscopic methods that provide structural and mechanistic details of this mitochondrial molecular machinery, in many instances to atomic level.

Electron transfer and ATP synthesis

The recognition of the link between respiratory and photosynthetic electron-transfer chains and the highly endergonic process of phosphorylation of ADP to produce ATP, and a view of the development of understanding from 1937 onwards of the nature of the link, was summarized by Mitchell in his 1978 Nobel lecture [4]. Although some ATP synthesis occurs by classical enzymological mechanisms in which an energy-releasing chemical transformation is strictly coupled to ADP phosphorylation within a single enzyme complex, the majority of ATP synthesis in non-photosynthetic aerobic organisms is catalysed by ATP synthases that are energetically linked to the respiratory electron-transfer chains. The energy of the exergonic electron-transfer reactions is not lost as heat, but is instead coupled to the generation of an electrochemical gradient of protons across the membrane in which they are located, which is used subsequently to drive the synthesis of ATP via the ATP synthases that are embedded in the same inner mitochondrial membrane.

The proposal that energy-generating electron-transfer processes can be coupled to ATP synthesis by means of an electrochemical proton gradient across the membrane was described by Mitchell in the 1960s [5,6], although development of the ideas has remained a controversial issue [7]. This chemiosmotic hypothesis had four basic proposals in its original form, namely (i) the electron-transfer components are arranged vectorially across a membrane in a manner such that electron transfer is linked to proton translocation across the membrane; (ii) ATP synthases are located in the same membranes in a manner that allows them to use the proton gradient to drive ATP synthesis; (iii) the membrane is impermeable to protons; and (iv) the membrane also contains proton-linked porter systems for osmotic stabilization and metabolite transport.

Importantly, Mitchell also developed the elegant and simple proposal of the vectorial redox loop in which it was postulated that the components of the redox chain alternated between electron carriers and hydrogen atom carriers. The hydrogen-atom carriers are effectively mobile in the membrane, and diffuse from a site of reduction (and proton uptake) on one side of the membrane to a site of oxidation (and proton release) close to the opposite membrane surface. Since a proton and an electron are transferred across the membrane together, the process is electroneutral. The loop is completed, however, by the transfer of the electron back across the membrane via electron carriers. In this case, an uncompensated charge is moved across the membrane and so generates an electric field across the membrane. The net result is the translocation of a proton across the membrane, and the conversion of redox energy into an electrochemical potential gradient of protons across the membrane, composed of a chemical potential difference of protons (the ΔpH) and an electric-field component ($\Delta\Psi$).

The classic example of such behaviour is seen in the role of ubiquinone in mitochondria, and the related menaquinone and plastoquinone in bacterial and photosynthetic systems respectively. A range of dehydrogenases and photosystems have quinone reduction sites that ensure that protons are taken up from one side of the membrane. The reduced quinone then dissociates from its reduction site and physically diffuses to proteins with oxidation sites linked to the other membrane surface where the protons are released. It was already envisaged when proposed that, in practice, the chemistry that occurred might be located in relatively small parts of the protein structures, with proton channels or wells connecting them to the appropriate aqueous surfaces. However, as the intricate details of the structures and mechanisms of the component reactions have emerged, it has become clear that additional complexities occur, some of which increase the overall energy-coupling efficiency and others perhaps necessary for precise control of the chemistry that is involved. Below, I provide an overview of present understanding and consider how this information prompts a re-evaluation of the textbook view of the balance of ATP synthesized by the glycolytic and respiratory pathways of aerobic glucose oxidation.

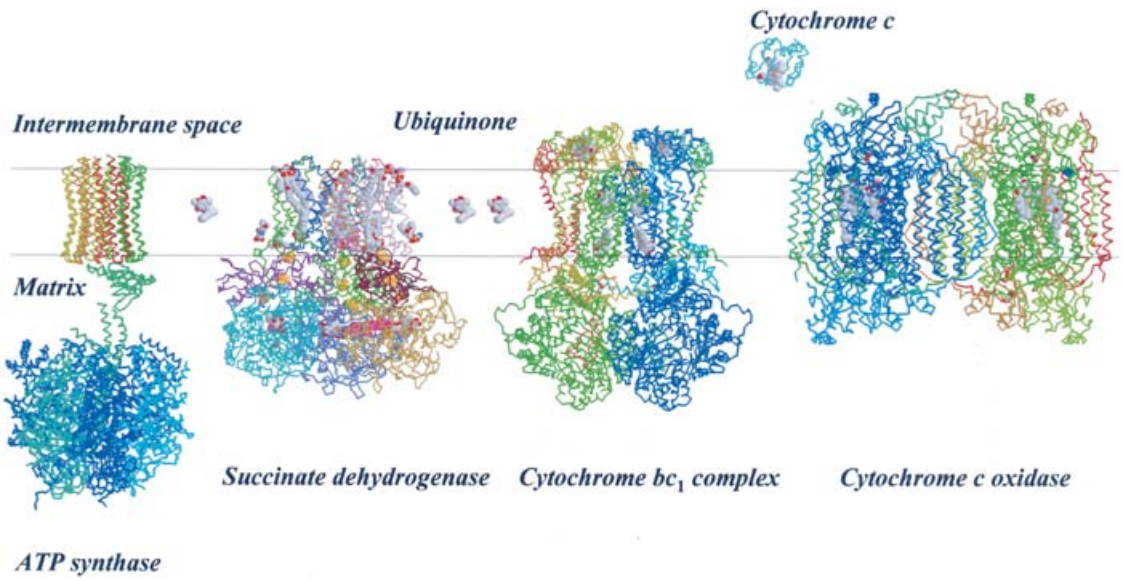
A current view of structures and mechanisms

In 1994, I summarized the state of knowledge regarding the possible structures of the major mitochondrial respiratory electron-transfer complexes [8]. Comparison of this information with the present level of atomic resolution information that is available (Figure 1) illustrates the phenomenal progress that has been achieved in the intervening period. Of the major complexes, only the structures of complex I and parts of the ATP synthase remain to be elucidated by high-resolution X-ray crystallography of appropriate three-dimensional crystals. The details of these atomic structures, together with the vast amount of new information gleaned by advanced spectroscopic methods, have had a tremendous impact on understanding of the structures of membrane proteins generally, and on the mechanisms operative in the respiratory and photosynthetic chains in particular.

Many of the key chemical and redox transformations take place in relatively small regions of the protein structure with channels in the protein structure allowing access of ions to this core. Candidates for such channels can now be visualized in the available structures, providing insights into the types of structures required for intra-protein ion movements, although it is still not possible to discern [9] whether such elements act as electrogenic ion wells [6], electroneutral ion channels or, possibly, structures that act as dielectric wells to lower the energy cost of transient unstable charged intermediates [10]. In any case, the precise

Figure 1 | Structural data on the respiratory chain and ATP synthase

Structures have been drawn using Protein Explorer. PDB files were: ATP synthase at 3.9 Å, 1QO1 [96]; succinate dehydrogenase trimer at 2.6 Å, 1NEK.mmol [97]; cytochrome bc_1 complex dimer at 3 Å, 1BGY [45]; cytochrome oxidase dimer at 2.3 Å, 2OCC [72]; cytochrome *c* at 1.9 Å, 1HRC [98].



properties of charged ions moving through such channels may be influenced by possible dynamic aspects. It is quite likely, for example, that the dielectric properties of the channel may alter between initiation of turnover and attainment of a steady state as fixed and movable charges redistribute. These considerations are discussed more specifically below.

An important consideration when exploring the possible mechanisms of coupling of ion and electron transfer in regions of low dielectric strength within proteins is the tendency towards local charge neutralization in order to minimize the energetic cost of formation of catalytic intermediates [11]. In the absence of appropriate solvent or protein-charge rearrangements that can achieve this, electron transfer into such systems can provide a strong driving force for uptake of a charge-compensating proton or, possibly, another cation. I have shown empirically that this occurs strictly in the catalytic intermediates of the binuclear centre of cytochrome oxidase [12] and have developed an abstract mechanism for coupling based on it [13] that may also be applicable in other coupled enzymes [11]. Further details are given in the discussions of specific coupling mechanisms below.

Complex I

For each glucose that is oxidized to CO_2 and water, two NADH are produced by glycolysis as pyruvate is formed, and a further eight are produced as pyruvate is oxidized by the citric acid. These NADH can be reoxidized by respiratory dehydrogenases. Although a variety of NADH dehydrogenases can be found in fungal, algal and plant mitochondria [14], the only type found as components of mammalian mitochondrial electron transfer chains is complex I. Electron transfer within complex I, in contrast with the simpler NADH dehydrogenases, is coupled to proton translocation across the membrane, greatly increasing the ultimate yield of ATP that can be produced.

Bovine complex I is remarkably complicated, with seven mitochondrially encoded subunits and at least 39 further nuclear-encoded subunits [15,16]. However, bacterial homologues of complex I [17–19] have only 14 subunits, seven of which are homologues of the mitochondrially encoded subunits and the other seven are homologues of nuclear-encoded mammalian subunits [20]. These 14 subunits presumably represent a common core catalytic structure. A number of reviews on polypeptides, topography and redox centres of the bovine and bacterial homologues have appeared recently [21–25]. Because the atomic structure of the complex is not yet solved, and because of the complexity and difficulty of monitoring of its redox centres, this enzyme remains the least well understood component of the respiratory chain. To date, only low-resolution structures of the complex have been obtained from electron microscopy images of single particles and two-dimensional arrays. The enzyme from various sources appears to be L-shaped with presumed membrane-embedded and matrix-located arms [24]. However, even this topology has been questioned recently, where an alternative 'horseshoe' conformation has been described [26].

Complex I contains non-covalently bound FMN together with several Fe_4S_4 and Fe_2S_2 iron–sulphur centres. In bovine enzyme, six distinct iron–sulphur centres can be distinguished by EPR spectroscopy [23]. However, analyses of the

polypeptide sequences of cofactor motifs, together with analyses of complex I from other sources, suggests that there may be as many as nine iron–sulphur centres [25]. Surprisingly, all of these prosthetic groups are located in subunits of the domain that extends into the mitochondrial matrix, rather than in the membrane-spanning arm. In fact, it would appear that the membrane domain has no redox centres, although some preliminary evidence has been presented for a possible redox centre of unknown chemistry [27].

Insights into structural organization have been obtained by subfractionation of purified enzymes from bovine and bacterial sources [28,29], together with sequence analyses for motifs and testing of specific locations for cofactor binding. This has been aided recently with the recognition that some parts of the structure are likely to have evolved from pre-existing modules that were incorporated into the complex [25,30]. It is clear that the 51 kDa subunit binds FMN and has the NADH-binding site and also houses one of the Fe_4S_4 centres, N3. It is associated with a 24 kDa subunit, likely to contain the Fe_2S_2 centre N1b, and a 75 kDa subunit, which probably contains three or four iron–sulphur centres. These three subunits have homology with the NADH oxidoreductase fragment of an NAD^+-reducing hydrogenase [30]. Of particular note is the homology with soluble NiFe hydrogenases, where it is apparent that their large and small subunits have homology with the 49 kDa and PSST (Pro-Ser-Ser-Thr) subunits of complex I. In addition, membrane-bound type-3 hydrogenases contain subunits that are homologous with the 49 kDa, PSST, 30 kDa, TYKY (Thr-Tyr-Lys-Tyr) and ND1 subunits, together with another that could be homologous with the ND2, ND4 or ND5 subunits (these latter three subunits are themselves weakly related to each other and to a class of bacterial Na^+/H^+ antiporters [31]). It may be concluded that the internal redox reactions involve electron transfer from NADH, via FMN, to centre N3, all within the 51 kDa subunit. Electron transfer is then connected through the other iron–sulphur centres to centre N2, the iron–sulphur centre of highest redox potential and the one that is likely to be the immediate donor to substrate ubiquinone. The hydrogenase homology has led to a prediction of the location of a catalytic core involving centre N2 between PSST and the 49 kDa subunit [25]. However, the number and location of the ubiquinone-binding site(s) remains elusive. The ND1 subunit in the hydrophobic arm has been suggested to provide a site for binding of ubiquinone, based on labelling with radioactive rotenone and DCCD (N,N′-dicyclohekylcarbodi-imide) [32] and on a very weak homology with a glucose–ubiquinone oxidoreductase [33]. However, mutants in the 49 kDa and PSST subunits implicate these subunits in ubiquinone and inhibitor binding, consistent with the predicted location of centre N2 between these same subunits [25], whereas sequence analyses have suggested that ubiquinone site(s) might be located on subunits ND4 and ND5 [34].

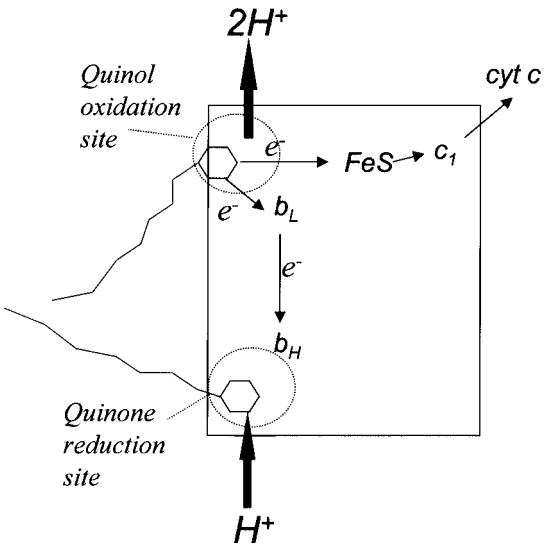
It is known that electron transfer through complex I is coupled to proton translocation and the consensus opinion is that the $H^+/2e^-$ ratio is 4 [35,36]. However, the lack of structural and functional data has hampered understanding of the coupling mechanism. Some have focused on mechanisms involving bifurcated electron flow around FMN [37], a 'mobile hydrogen atom carrier' function of FMN [38] and an $FMN^{\bullet-}/FMNH_2$ cycle [39]. Other have focused upon iron–sulphur centre N2 and multiple binding sites for ubiquinone [37,38]. However, none of these schemes has received significant experimental support to date. In fact, those schemes involving multiple sites for ubiquinone have been greatly weakened by the likelihood that complex I contains only one ubiquinone-binding site, albeit an extensive one [40], although the detection of multiple semiquinone signals by EPR spectroscopy [23] remains to be explained if only a single occupancy site is present. Most recently, prompted by the mechanical coupling of ATP synthase and other enzymes, by the reports that some forms of complex I are linked to sodium rather than H^+ translocation, and by the surprising finding that the 49 kDa subunit (and, therefore, the supposed ubiquinone-binding site) is actually located a considerable distance from the membrane components, Brandt et al. [25] have abandoned such chemical models in favour of one driven by large-scale long-range conformational changes.

Cytochrome bc_1 complex

The bovine mitochondrial cytochrome bc_1 complex is a member of a superfamily of homologous enzymes that occur widely in eukaryotic and prokaryotic respiratory and photosynthetic electron-transfer chains. Although the bovine enzyme is composed of eleven subunits [41], only three (iron–sulphur protein, cytochrome c_1 and cytochrome b) are common to the superfamily. These three subunits contain the four redox-active prosthetic groups (an Fe_2S_2 centre, haem C and two haems B), and again form a common catalytic core for its ubiquinol–cytochrome c oxidoreductase and associated proton-transfer activities. It is widely agreed that the electron- and proton-transfer pathway occurs essentially by the 'Q-cycle' mechanism (Figure 2) that was first envisaged by Mitchell [42]. Ubiquinol oxidation at the ubiquinol-binding 'Q_o' site results in a concerted electron transfer in which the first electron is transferred to the iron–sulphur protein and the second to the lower-potential haem B, termed b_L. The electron on the iron–sulphur protein is passed via cytochrome c_1 to the substrate cytochrome c. The electron on haem b_L moves across the membrane to reduce the higher-potential haem b_H and this in turn reduces ubiquinone to ubiquinol in two steps. The mechanism provides a very efficient means of utilizing the energy of electron transfer to form a protonmotive force across the membrane, resulting overall in two electrogenic proton translocations across the inner mitochondrial membrane for each pair of electrons eventually passed on to cytochrome c.

The Q cycle mechanism was formulated and largely proven even before the atomic structural data were available. Indeed, a relatively accurate structural model around the haem B prosthetic groups at 5–10 Å (1 Å = 0.1 nm) resolution had also been deduced from sequence and mutation studies. In

Figure 2 | Q-cycle mechanism of the cytochrome bc_1 complex
Details are given in the text. Two turnovers of the reaction shown are required for a complete cycle. This results in two ubiquinols oxidized at the quinol oxidation site, with liberation of four protons towards the cytoplasm, two electrons transferred to cytochrome *c* and one quinone re-reduced at the quinone reduction with uptake of two matrix protons.



contrast with complex I, the structures of several bovine, chicken and yeast *bc* complexes have since been solved at atomic resolution [43–46]. These atomic structures confirmed this predicted structure and mechanism and provide specific structural detail that was previously lacking. However, key details of the basic electron-/proton-transfer mechanism remain unclear. Particularly enigmatic are the chemical and physical factors that cause the strict bifurcation of electron transfer at the Q_o site [47,48]. For example, questions remain as to whether or not a remarkable long-range rotation of the globular protein domain of the iron–sulphur protein, identified from different headgroup positions in different crystal forms (Figure 3), is an essential feature for bifurcated electron transfer [49–51]. Certainly, it is necessary that the low-potential haem b_L is a sufficiently long distance from haem c_1 so that a 'short circuit' cannot occur, and this means that some physical movement of the iron–sulphur centre is required if it is to interact both with the ubiquinol–haem b_L complex and with the cytochrome c_1. We have argued that the strict bifurcation when the iron–sulphur–ubiquinol–haem b_L ternary complex is formed occurs simply as a result of the thermodynamics and relative rate constants of the complex [48], but others have suggested that domain movement may be essential to ensure bifurcation. Furthermore, on the basis of positions of various bound inhibitors, the Q_o site appears to be rather extensive. EPR lineshapes of the iron–sulphur protein have indicated that two ubiquinones might be bound within the Q_o site [52], a suggestion supported by some inhibitor-binding titrations [53] and by NMR monitoring of ubiquinone displacement [54]. These observations have led to proposals that two ubiquinols are bound within the Q_o site [55] or that two sequential binding positions for ubiquinone species might be operative [47]. Unfortunately, since the site is unoccupied in available crystal forms, this question remains unresolved. For both the Q_o and Q_i sites, and for the redox prosthetic groups, very little is presently known about which groups within the protein are specifically responsible for the extensive redox-linked protonation changes that are integral to the catalytic cycle. Furthermore, questions have been raised as to whether or not the bc_1 complex has a more complex reaction cycle involving concerted action of the two halves of the dimer, integrated by long-range conformational changes [56].

Cytochrome *c* oxidase

Cytochrome *c* oxidase, the modern name for Keilin's indophenol oxidase, is the final step of many membrane-bound electron-transfer chains [57,58], including that of the mitochondria. It catalyses the transfer of electrons from cytochrome *c* to molecular oxygen. The enzyme is arranged so that cytochrome *c* binds and donates electrons from the intermembrane space, but the protons are taken up from the opposite matrix-facing side. It was later discovered that each full catalytic cycle also results in four additional protons being taken up from the matrix and released to the intermembrane space, greatly increasing the efficiency of storage of released energy and giving an overall equation of:

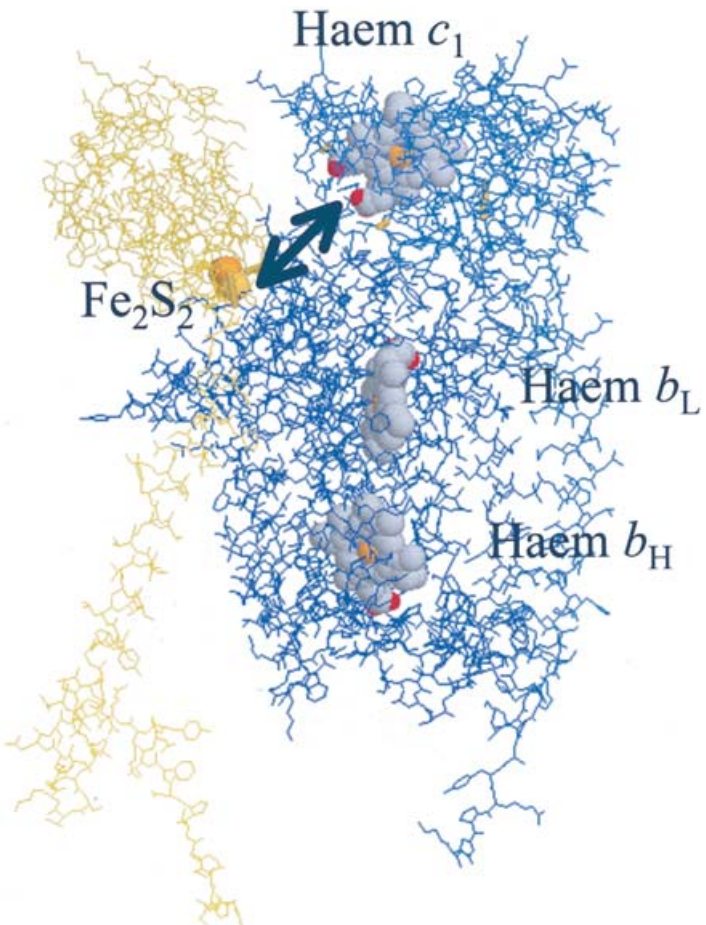
$$4\,\mathrm{cyt}\,c^{2+} + \mathrm{O_2} + 8\mathrm{H^+}_{\mathrm{matrix}} \rightarrow 4\,\mathrm{cyt}\,c^{3+} + 2\mathrm{H_2O} + 4\mathrm{H^+}_{\mathrm{cytoplasm}}$$

The mammalian mitochondrial cytochrome *c* oxidase is a very large structure that spans the inner mitochondrial membrane and is a dimer, with each monomeric unit being composed of 13 different polypeptides. However, again, sequence comparisons show that there is a wide range of simpler bacterial oxidases that are structurally related to cytochrome oxidase and which form a homologous 'superfamily'. All members contain the same three highly conserved subunits I, II and III, and it appears that these alone form a common 'catalytic core' that can catalyse all of the electron- and proton-transfer reactions, utilizing a series of metal cofactors. The first of these is made up of two copper atoms that form a bimetallic Cu^{2+}–Cu^{+} centre termed Cu_A, which transfer electrons from substrate cytochrome *c* and on to Keilin's haem *a*. However, Cu_A is positioned over the metal centres in subunit I so that the distances of the nearest copper atom to the haem *a* iron (19.5 Å) and to the haem a_3 iron (22.1 Å) are similar. Hence, although some other factors [59] may favour the generally supposed pathway of the electron firstly to haem *a*, this point may require further confirmation. The close contact of the haem edges (4.7 Å) promotes very fast electron transfer from haem *a* to Keilin's haem a_3, which forms, together with a further copper centre termed Cu_B, a 'binuclear centre' for oxygen binding and reduction.

Bovine mitochondrial and various bacterial oxidase structures (Figure 4) have been solved at atomic resolution [59–61]. Subunit II has two membrane-spanning α-helices and a

Figure 3 | Structure of the catalytic core of the bovine cytochrome bc_1 complex

The three core subunits of the iron–sulphur centre, cytochrome c_1 and cytochrome *b* are shown, together with their prosthetic groups. The arrow indicates the proposed movement of the iron–sulphur protein globular domain. Structure drawn using co-ordinates from 1BGY [45].



large globular hydrophilic domain that contains the Cu_A centre and provides much of the docking site for cytochrome *c*. The globular head sits over the cytoplasmic face of subunit I and Cu_A is close to the subunit II/I interface. Subunit I contains haem *a* and the binuclear centre. Several arrays of relatively hydrophilic amino acids and water molecules can be identified that could provide pathways for proton transfer through the protein. One of these (named the 'D' channel from an aspartic residue at its origin) leads from the matrix to a highly conserved glutamic residue (Glu^{242}) that is located close to the haem edges of both *a* and a_3. A second (named the 'K' channel from its conserved lysine residue) leads from the matrix phase to the binuclear centre itself. A third possible route, the 'H' channel, has been described in the bovine structure. A striking feature revealed by the crystal structures is a covalent link between a ring carbon of tyrosine and a ring nitrogen of a histidine ligand to Cu_B [60]. This creates a pentameric ring of the highly conserved amino acid sequence HPEVY, with the glutamic residue being Glu^{242} (Figure 5).

Despite the availability of high-resolution structural data, major questions remain as to the chemical nature of intermediates and the sites that are involved in associated protonation reactions. This arise in part from the complicated chemistry of oxygen reduction to water, which requires four electrons and four protons and must occur in a stepwise manner (Scheme 1). In each of these steps, the thermodynamics and chemistry of the intermediates are quite different. Two key stable catalytic intermediates are the 'peroxy' (P) and 'ferryl' (F) forms, both of which are considered to be $a_3{}^{4+}$ = O Cu_B (II) structures in which the O–O bond is already broken [62–71]. Formation of the F state and breakage of the O–O bond requires four electrons. In the P state, three are provided by the ferrous/ferryl and cuprous/cupric changes. It is likely that the additional electron is provided by the covalent tyrosine–histidine ligand to Cu_B, which forms a radical in P [72–78] that is re-reduced in F. However, this issue is not fully settled; for example, it appears that some radical redistribution to tryptophan residues close to haem a_3 can occur in certain conditions that cause P to convert into an alternative isoelectronic structure termed $F^{\bullet}$ [79].

A key aspect in consideration of the coupling mechanism of cytochrome oxidase is the empirically demonstrated fact

Figure 4 | Structure of the catalytic core of bovine cytochrome oxidase

The three core subunits I, II and III are drawn, together with the redox-active metal centres and bound magnesium, from co-ordinates in 2OCC [72]. The probable electron-transfer steps are shown with red arrows and possible proton pathways with yellow arrows.

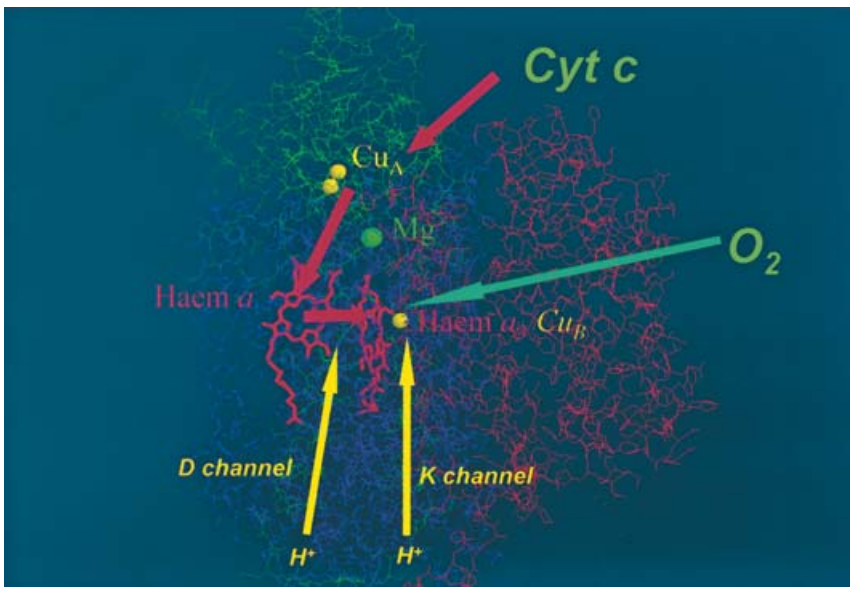
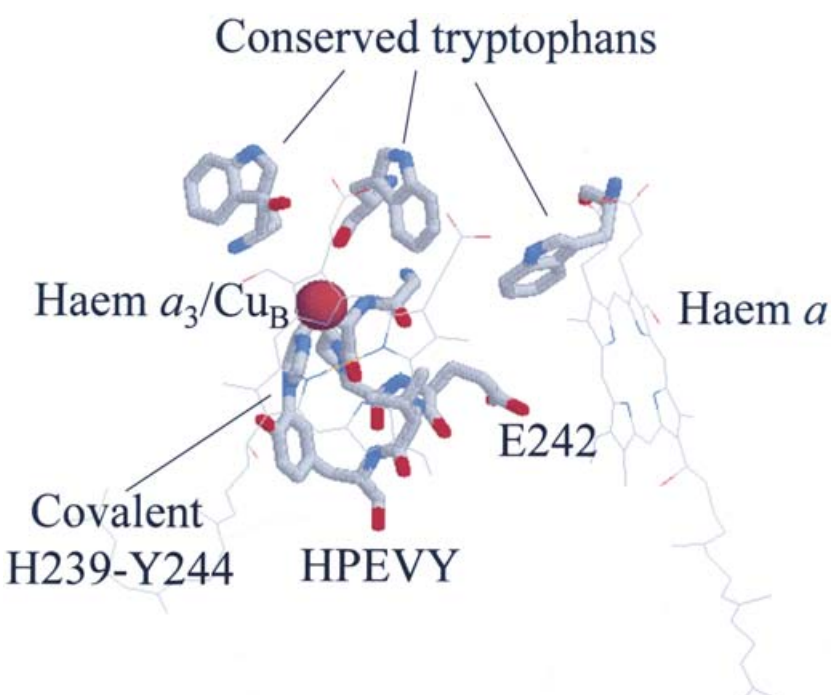


Figure 5 | Some key amino acids associated with the active site of bovine cytochrome *c* oxidase

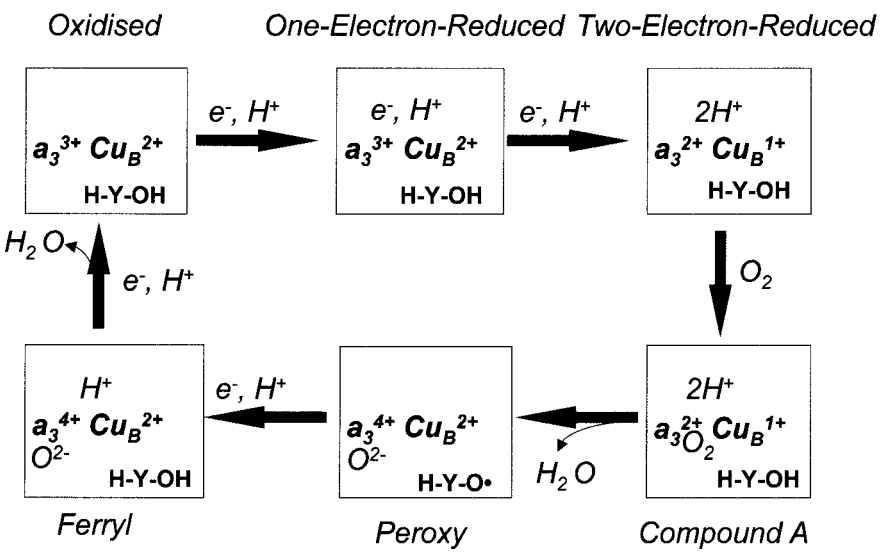
Haems *a* and a_3 and Cu_B (brown) are shown, together with the pentameric amino-acid-ring structure caused by the covalent tyrosine–histidine linkage, which incorporates a conserved glutamic acid, Glu^{242}.



that all stable electronation or ligand-binding changes in the binuclear centre are electroneutralized by protonation changes [80,81]. The notion of spatial separation of such countercharges from the oxygen chemistry was introduced to provide a basis for the coupling mechanism. In this 'glutamate trap' model, each electron transfer from haem *a* to the binuclear centre is accompanied by proton transfer via the 'D' channel and its glutamic acid to a charge compensating site,

Scheme 1 | Simplified reaction cycle of cytochrome *c* oxidase

On donation of an electron from cytochrome *c*, the oxidized enzyme becomes the one-electron-reduced (E) state, in which the electron is shared between the haem *a* and the binuclear centre. A subsequent electron transfer produces the two-electron-reduced (R) state, which binds oxygen to form an unstable oxygen–ferrous species similar to oxyhaemoglobin, and termed compound A [99]. This converts in the microsecond time range into the P state, a ferryl form that probably has a neutral radical on the covalent tyrosine–histidine. A third electron transfer from cytochrome *c* reduces the radical to form the F intermediate. The reaction is completed by a fourth electron transfer to F, which results in the reversion of the metal centres to their fully oxidized O state. Each electron donation is accompanied by proton binding, since the overall transitions between stable binuclear centre intermediates are electroneutral. In addition, four net proton translocations are linked to the cycle involving both binding of protons from the matrix and release of protons to the cytoplasm. Two of these are now thought to be associated with the O → R transition, and one each with the P → F and F → O steps. Details remain controversial of the locations of the bound protons in the stable intermediates, the ligand state changes of the binuclear centre metals (especially in relation to oxygen, hydroxide and water ligands), the pathways and transient binding sites of protons associated with the proton translocations, and the gating mechanism that controls the separation of substrate and translocated protons.



which is physically separated from the oxygen intermediates. Subsequent steps in which oxygen intermediates are themselves protonated via the 'K' channel were proposed to electrostatically repulse the charge-counterbalancing protons into the cytoplasmic phase, a necessity to maintain electrical neutrality of the stable binuclear site intermediates.

Despite the wealth of structural and spectroscopic data, and although most coupling models acknowledge the electroneutrality issue, the atomic details of the coupling mechanism have remained controversial. This has been complicated by the fact that the original assumption [82] that only the last two steps of the catalytic cycle are coupled, each to two proton translocations, has been superceded by a more recent view that proton translocations are linked to at least three, and possibly all four, of the reaction steps [76,83]. The fact that proton translocations are not restricted to the final two steps of the reaction cycle actually has little impact on the electrostatic model, since it requires adjustment only of the steps in the cycle at which protons destined for water formation are incorporated into binuclear centre – and these four protonations could conceivably be distributed between the catalytic steps in any manner. Indeed, I have suggested that the distribution of proton translocations associated with specific steps may be quite variable with different experimental conditions, even though the overall coupling efficiency and stoichiometry for one complete catalytic cycle would remain constant [79].

One important deduction from FTIR (Fourier-transform infrared) spectroscopy [84,85] is that Glu^{242}, originally assumed to become partly protonated on haem *a* reduction [13], is already protonated even in oxidized oxidase, so that the proton transfer to the trap site that is linked to haem–haem electron transfer should be a proton donation from Glu^{242}, which immediately becomes reprotonated via the 'D' channel. Hence other protonations that are weakly redox-linked to haem *a* [86] are unlikely to be crucial to the process.

A more difficult complication is the emergence of a body of data that suggests that the majority of translocated and substrate protons are moved through the 'D' channel and the Glu^{242} residue. Although not changing the abstract principles of the 'electroneutrality' model of coupling, this does raise considerable questions as to the nature of the gating process that controls the paths of the protons and prevents 'decoupling', which would occur if all protons could freely

contact the oxygen intermediates. The experiments designed to link specific protonation processes with specific channels are necessarily indirect and often involve some degree of interpretation, so that it is still not certain that the simple model of two separate channels for substrate and translocated protons is incorrect. Nevertheless, the possibility that the key control of proton pathways is located in the vicinity of Glu^{242} has led to some interesting proposals as to how this switch might operate, the most recent involving a redox-controlled change in the conformation of several water molecules that should be located close to Glu^{242} [87]. FTIR spectroscopy has also shown that the redox or conformation state of Glu^{242} is dependent specifically on the redox state of haem a/Cu_B [85,88], and this might reflect a movement intergral to its gating function. It might be pointed out that the pentameric HPEVY amino acid ring might also play some part, since chemical and conformational changes of the tyrosine–histidine pair might well be expected to induce conformational changes in Glu^{242}. Indeed, recent ATR (attenuated total reflectance)-FTIR studies of the catalytic intermediates indicate strong conformational perturbation of Glu^{242} in P that could be linked to tyrosine–histidine radical formation [89]. Hence, the IR approach may well provide the beginnings of an experimental method to understand further the changes in protonation and conformation that are occurring in this region of the protein that are likely to provide the atomic details of coupling mechanism in this class of enzymes.

A re-evaluation of yields of ATP synthesis from glucose oxidation

Aerobic glucose oxidation is normally broken down into stages of glycolysis (yielding two NADH and two ATP), the pyruvate → acetyl-CoA reaction (yielding two NADH) and the citric acid cycle [yielding six NADH, two $FADH_2$ (from succinate oxidation) and two GTP]. Hence, when combined with the early view of three 'coupling sites' for NADH oxidation and two for succinate oxidation, this suggested a net ATP production from one glucose molecule of eight ATP by glycolysis to pyruvate and a further 30 ATP by aerobic oxidation of pyruvate to CO_2 and water, and the total ATP/glucose value of 38 (assuming conversion of two GTP into two ATP) was often quoted in classical biochemical textbooks.

However, as the complexities of the respiratory chain and ATP synthase mechanisms have emerged, it became clear that these stoichiometries are not as straightforward as the early views suggested, and more recent textbooks have had to become rather less specific on the ATP/glucose issue – for example, a value of 30–32 is given in Lehninger Principles of Biochemistry [90] and "as many as 36" by Lodish et al. [91]. The more recent information on the mechanistic and structural aspects of the respiratory chain and ATP synthase now provide a rather clearer picture and allow these values to be re-evaluated more specifically.

Electron transfer from succinate to oxygen will result in six H^+ deposited outside (four due to bc_1 complex and two due to cytochrome oxidase), six H^+ taken up from the matrix (four due to bc_1 complex and two due to cytochrome oxidase) and six charges translocated (two due to bc_1 complex and four due to cytochrome oxidase). The majority view, although still perhaps open to possible question, is that electron transfer from NADH to ubiquinone through complex I is coupled to the net translocation of four H^+ and four charges across the membrane. The reduced ubiquinone is then reoxidized with the same outcome as succinate. Hence succinate oxidation is linked to six electrogenic proton translocations and NADH is linked to ten.

From the structure and stoichiometry of the *c* subunit ring of ATP synthase of yeast, it is apparent that a complete rotation of the motor element of the complex requires ten H^+ and that this should result in the synthesis of three ATP. Hence, if we assume that the mammalian ATP synthase rotor has the same *c* ring stoichiometry (although there is evidence that the ratio may vary in different organisms), we may assume that the H^+/ATP ratio of the ATP synthase is also 10:3. However, the exchange of ADP plus phosphate from the cytoplasm for an ATP from the mitochondrial matrix via their translocators effectively requires a further electrogenic proton. Hence the net synthesis of cytoplasmic ATP from ADP should operate at a ratio of 13:3 H^+/ATP. As a result, oxidation of NADH is expected to produce $3 \times 10/13 \approx 2.3$ ATP and oxidation of succinate $3 \times 6/13 \approx 1.4$ ATP. This would predict that the maximum yield of ATP from full oxidation of one glucose molecule should be: glucose → 2 pyruvate, 6.6 ATP; 2 pyruvate → 2 acetyl-CoA, 4.6 ATP; citric acid cycle, 18.6 ATP (with two ATP from two GTP). Oxidation of the whole ten NADH and two succinate would result in translocation of 112 H^+, giving an overall yield, with two ATP from two GTP, of 29.85 ATP/glucose (or 29.38 ATP/glucose if the GTP-derived ATP also has to be exported).

Prospects and conclusions

The advances in our understanding of the major components of Keilin's respiratory chain and associated ATP synthase have been tremendous, in terms of the range of advanced spectroscopic methods that have provided spatial and mechanistic details of the redox centres, the bioinformatic methods that have allowed structure prediction and recognition of components elements and modules in common with quite unrelated systems, and the crystallographic data that have transformed the understanding of membrane-protein structure at the atomic level. Even with this information, the above discussion highlights the fact that numerous questions remain unresolved, particularly in relation to catalytic-cycle intermediates, protonation processes and questions of dynamic aspects of individual amino acids and, in some cases, whole protein domains that might be key to the mechanism. FTIR spectroscopy has been used extensively to probe structural changes in individual cofactors and amino acids in proteins and is a technique that has now developed technically to a level that can be used to address these 'post-structural'

questions. Some recent uses have been described above and this spectroscopy seems set to provide further major contributions to the types of questions that remain. These developments place research on electron-transfer-chain proteins at the forefront of studies in membrane-protein chemistry. However, mitochondrial research is currently receiving renewed experimental interest from various other directions and understanding their basis in many cases requires understanding of the molecular machinery of respiration and ATP synthesis. One expanding area comes from the recognition of a possible link between a range of human disease states and mutations in mitochondrial DNA [92]. As might be expected, those tissues with a high energy requirement that rely extensively on aerobic metabolism are most severely affected. Lactic acidosis, muscular weakness, deafness, blindness, ataxia and dementia are common findings. A further area concerns free-radical generation. The enzymes of respiration have evolved to carry out their electron-transfer reactions in a way that minimizes electron-transfer side reactions that would result in formation of radical species. Such free-radical species are very reactive generally, and can cause considerable random chemical damage to DNA, lipids and proteins. Despite their safeguards, some radicals are inevitably produced by leakage of electrons to molecular oxygen in a way that results in reactive-oxygen species [93]. Although mitochondria contain enzymes to remove these, the constant, albeit very low, radical production may be responsible for damage to mitochondrial DNA and other cellular components, which is associated with disease and aging. Remarkably, mitochondria have also now been implicated in one pathway of apoptosis in some types of cells [94]. The details are still controversial, but it appears that the process may involve an increase in permeability of the outer mitochondrial membrane such that cytochrome *c*, a flavin-containing apoptosis-inducing factor and other proteins are released into the cytoplasm, where they act in concert with cytoplasmic factors to inhibit apoptosis inhibitors and to activate the caspase cascade. In a further development, attention has been drawn to the possible physiologically important action of the signalling molecule nitric oxide on the respiratory chain and other mitochondrial processes [95]. When Keilin identified the cytochrome components and their role in aerobic respiration, he provided a foundation that underpins these subsequent discoveries. I wonder if even he could have foreseen just how far these fields have since progressed, and have yet to progress, in providing a full understanding of the diverse cellular functions of the respiratory chain and the mitochondria in which they reside.

I am grateful to The Wellcome Trust in particular for continued support of the spectroscopic studies in my laboratory.

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Received 16 June 2003