Complex I (NADH:quinone oxidoreductase) is crucial to respiration in many aerobic organisms. In mitochondria, it oxidizes NADH (to regenerate NAD\(^+\)) for the tricarboxylic acid cycle and fatty-acid oxidation), reduces ubiquinone (the electrons are ultimately used to reduce oxygen to water) and transports protons across the mitochondrial inner membrane (to produce and sustain the protonmotive force that supports ATP synthesis and transport processes). Complex I is also a major contributor to reactive oxygen species production in the cell. Understanding the mechanisms of energy transduction and reactive oxygen species production by complex I is not only a significant intellectual challenge, but also a prerequisite for understanding the roles of complex I in disease, and for the development of effective therapies. One approach to defining a complicated reaction mechanism is to break it down into manageable parts that can be tackled individually, before being recombined and integrated to produce the complete picture. Thus energy transduction by complex I comprises NADH oxidation by a flavin mononucleotide, intramolecular electron transfer from the flavin to bound quinone along a chain of iron–sulfur clusters, quinone reduction and proton translocation. More simply, molecular oxygen is reduced by the flavin, to form the reactive oxygen species superoxide and hydrogen peroxide. The present review summarizes and evaluates experimental data that pertain to the reaction mechanisms of complex I, and describes and discusses contemporary mechanistic hypotheses, proposals and models.

Key words: electron transport chain, enzyme mechanism, mitochondrial, NADH:quinone oxidoreductase, proton-coupled electron transfer, proton translocation.
Table 1  The nomenclature for the 14 core subunits of complex I, the conserved cofactors bound by the hydrophilic subunits and the predicted TMHs which comprise the ND subunits in B. taurus (predicted using ConPredII [7], and not verified experimentally)

<table>
<thead>
<tr>
<th>Enzyme domain</th>
<th>B. taurus</th>
<th>Homo sapiens</th>
<th>Y. lipolytica</th>
<th>E. coli</th>
<th>T. thermophilus</th>
<th>Cofactors and TMHs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophilic arm (nuclear encoded in eukaryotes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75 kDa</td>
<td>NDUFS1</td>
<td>NUAM</td>
<td>NuoG</td>
<td>Nqo3</td>
<td></td>
<td>[2Fe–2S], 2 × [4Fe–4S]</td>
</tr>
<tr>
<td>51 kDa</td>
<td>NDUFV1</td>
<td>NUBM</td>
<td>NuoF</td>
<td>Nqo1</td>
<td></td>
<td>FMN, [4Fe–4S]</td>
</tr>
<tr>
<td>49 kDa</td>
<td>NDUFS2</td>
<td>NUCM</td>
<td>NuoCD</td>
<td>Nqo4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 kDa</td>
<td>NDUFS3</td>
<td>NUGM</td>
<td>NuoG</td>
<td>Nqo6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 kDa</td>
<td>NDUFV2</td>
<td>NUHN</td>
<td>NuoE</td>
<td>Nqo2</td>
<td></td>
<td>[2Fe–2S]</td>
</tr>
<tr>
<td>PSST</td>
<td>NDUFS7</td>
<td>NUIM</td>
<td>NuoB</td>
<td>Nqo6</td>
<td></td>
<td>[4Fe–4S]</td>
</tr>
<tr>
<td>TTYK</td>
<td>NDUFS8</td>
<td>NUKM</td>
<td>NuoQ</td>
<td>Nqo9</td>
<td></td>
<td>2 × [4Fe–4S]</td>
</tr>
<tr>
<td>Hydrophobic arm (mitochondrial encoded in eukaryotes)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND1</td>
<td>ND1</td>
<td>NU1M</td>
<td>NuoH</td>
<td>Nqo8</td>
<td></td>
<td>8 TMHs</td>
</tr>
<tr>
<td>ND2</td>
<td>ND2</td>
<td>NUGC</td>
<td>NuoN</td>
<td>Nqo14</td>
<td></td>
<td>8 TMHs</td>
</tr>
<tr>
<td>ND3</td>
<td>ND3</td>
<td>NUSM</td>
<td>NuoA</td>
<td>Nqo7</td>
<td></td>
<td>3 TMHs</td>
</tr>
<tr>
<td>ND4</td>
<td>ND4</td>
<td>NU4M</td>
<td>NuoM</td>
<td>Nqo13</td>
<td></td>
<td>13 TMHs</td>
</tr>
<tr>
<td>ND5</td>
<td>ND5</td>
<td>NU5M</td>
<td>NuoL</td>
<td>Nqo12</td>
<td></td>
<td>16 TMHs</td>
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<tr>
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<td>ND6</td>
<td>NUKM</td>
<td>NuoK</td>
<td>Nqo10</td>
<td></td>
<td>5 TMHs</td>
</tr>
<tr>
<td>ND4L</td>
<td>ND4L</td>
<td>NULM</td>
<td>NuoJ</td>
<td>Nqo11</td>
<td></td>
<td>3 TMHs</td>
</tr>
</tbody>
</table>

Figure 1  The reactions catalysed by complex I in the mitochondrion

NADH produced by the breakdown of carbohydrates, fats and proteins, is oxidized to NAD\(^{+}\), and two electrons are transferred, via an FMN and a chain of iron–sulfur clusters, to reduce Q to QH\(_2\). The redox reaction regenerates NAD\(^{+}\), provides electrons for the reduction of O\(_2\) to water, and provides the free energy for proton translocation across the inner membrane. Proton translocation contributes to the Δ\(\mu\) which is used, for example, to synthesize ATP. The fully reduced flavin in complex I also reduces O\(_2\) to superoxide, a cause of cellular oxidative stress. ET chain, electron transport chain; TCA cycle, tricarboxylic acid cycle.

(see Figure 2). The structure as a whole encases the cofactor cohort of the hydrophilic arm; a cluster ‘chain’ runs between the sites of NADH oxidation and quinone reduction, in a common configuration found in, for example, succinate dehydrogenase and fumarate reductase, nitrate reductase and some formate dehydrogenases. The large domain of the 75 kDa subunit in T. thermophilus and a small number of other organisms, including Escherichia coli, co-ordinates an additional [4Fe–4S] cluster, N7, which is not considered further in the present review; it is not conserved, and too distant (> 20 Å) from the other clusters to participate in catalysis [9].

The conserved cofactors of complex I are a non-covalently bound FMN (flavin mononucleotide) and eight iron–sulfur clusters, all bound by the hydrophilic domain (Table 1 and Figure 2). Prior to the structure, the clusters had been predicted correctly by sequence analyses, the overexpression of putative cluster-containing subunits, and EPR analyses (reviewed in [6,10]). No further permanently bound redox cofactors are known; previous suggestions of a novel cofactor, perhaps a quinoid group, in the membrane domain, have not been substantiated by MS [11]. The confinement of all the cofactors to the hydrophilic domain (Figures 1 and 2) is important in the formulation of mechanistic hypotheses for complex I, and distinguishes it from other proton-translocating redox enzymes, such as cytochrome bc\(_1\) and cytochrome c oxidase. The NADH-binding site is located in the 51 kDa subunit of complex I, adjacent to the flavin (see below), but the location (and number) of the quinone-binding sites remain unconfirmed; a putative binding site for the quinone headgroup is marked on Figure 2 (see below). Currently, there is significant controversy about how to map the structure of the T. thermophilus hydrophilic domain on to low-resolution electron microscopy structures of the intact complex. Brandt and co-workers contend that the whole domain is ‘lifted away’ from the membrane, placing cluster 4Fe[PS]/N2 35–60 Å away from the membrane interface, and requiring quinone to move substantially out of the membrane for catalysis [12,13].
Towards the molecular mechanism of respiratory complex I

Figure 2  The structure of the hydrophilic core subunits of complex I from T. thermophilus, and the chain of conserved cofactors [8]

The NADH and putative Q-binding sites (see text) are indicated. The subunits are named according to the nomenclature of B. taurus complex I. The conserved clusters (left, duplicate view but without the protein) are named according to their type (2Fe or 4Fe) and subunit, and then differentiated as all cysteine-ligated (C) or with one histidine ligand (H), or as clusters 1 or 2 (in TYKY). The non-conserved 4Fe cluster in the 75 kDa subunit has been omitted from the chain.

contrast, I (and others) contend that the hydrophilic domain is positioned with 4Fe[PS]/N2 close to the membrane (see Figure 1, same orientation in Figure 2), so that the quinone headgroup can approach 4Fe[PS]/N2, but remain in the membrane interface (reflecting its positions in succinate:ubiquinone oxidoreductase, cytochrome bc, and the bacterial reaction centre). In support of the latter model, a quinazoline inhibitor has been found to bind both the N-terminal section of the 49 kDa subunit and ND1 [14], and cross-links have been formed by the PSST and 49 kDa subunits with ND3 [15]. However, all of the evidence is circumstantial and the controversy will probably only be resolved when a high-resolution structural model of intact complex I becomes available.

The seven hydrophobic subunits of complex I comprise a large number of TMHs – the seven B. taurus subunits are predicted to contain 56 of them (see Table 1), although the predictions have not been confirmed experimentally, and the subunit lengths are not well conserved between species. For five of the ND subunits, fusion proteins (in Rhodobacter capsulatus [16] and Paracoccus denitrificans [17]) have been used to infer the number of TMHs (and the protein orientations), and the results match the predictions in Table 1. The arrangement of the ND subunits in the membrane domain has not been determined unambiguously, although ND4 and ND5 have been located at the distal end by single-particle analyses [18]. ND4, ND5 and ND2 are related to the Mrp family of Na+/H+ antiporters [16], and so, despite the distance of ND4 and ND5 from the hydrophilic arm, they are often suggested to participate in proton translocation (see below).

OVERVIEW OF COMPLEX I CATALYSIS

Under normal physiological conditions, mitochondrial complex I catalyses the two-electron oxidation of NADH and reduction of Q. The redox reaction (eqn 1) is thermodynamically very favourable ($\Delta E_{\text{pH7}} = +0.4$ V, although the physiologically relevant value of $\Delta E$ is determined also by the solution and the membrane conditions, the position of the Q-headgroup in the membrane [19], and by the NAD+/NADH and Q/QH2 ratios).

$$\text{NAD}^+ + 2\text{H}^+ + 2e^- \leftrightarrow \text{NADH} + \text{H}^+ \quad E_{\text{pH7}} = -0.33 \text{ V} \quad (20)$$

$$\text{Q} + 2\text{H}^+ + 2e^- \leftrightarrow \text{QH}_2 \quad E_{\text{pH7}} = +0.07 \text{ V} \quad (19)$$

For complex I in an energy-transducing membrane, the ‘favourable’ redox reaction is opposed by ‘unfavourable’ proton translocation, and the redox energy is transformed and conserved as $\Delta p$. It is generally accepted that, in mitochondria at least, complex I translocates four protons for each two-electron oxidation of NADH. Therefore the $\Delta p$ supported by complex I is thermodynamically limited to $\Delta p_{\text{lim}} = \Delta E/2$, as the system is never perfectly balanced some energy is always lost as heat (energy conservation is not completely efficient). As an estimation, in the mitochondrion $\Delta E \sim 0.35$ V so $\Delta p_{\text{lim}} \sim 0.175$ V at pH 7.8, [NAD$^+$] = 10[NADH] ([21] and references therein), and $[Q] = [\text{QH}_2]$ (value unknown)). In vitro, if $\Delta p > \Delta E/2$, complex I catalyses the reverse reaction also: NAD$^+$
reduction and QH₂ oxidation, driven by proton discharge across the membrane [22]. Although the general events which comprise the forward and reverse cycles are surely the reverse of one another (for example, NADH reduces the oxidized flavin, NAD⁺ oxidizes the reduced flavin), the two reactions are not necessarily microscopically the reverse of one another (the reaction is not at equilibrium so the forward and reverse cycles are not constrained to identical pathways) [23]. A useful analogy is presented by a fixed-gear bicycle: rotate the pedals forward and the bike moves forward, rotate the pedals backward and the bike moves backward, so in a general sense the bicycle is reversible. However, on more detailed viewing, for example of the chain on the teeth of the cogs, or the feet and ankles of the cyclist, it is obvious that the forward and backward motions are not the exact reverse of each other (a snapshot could easily reveal the direction of motion). Similarly, in complex I, the structures (and lifetimes) of transition states and reaction intermediates may vary according to the direction of catalysis. The present review discusses complex I catalysing the physiological ‘forward’ reaction; reverse catalysis by the mitochondrial enzyme has not been studied extensively and is probably non-physiological but, nevertheless, it may yield important and complementary mechanistic information.

If we follow a pair of electrons from their entry to complex I on a molecule of NADH, then catalysis comprises their transfer (as a pair) to the flavin, their transfer (one at a time) across the chain of iron–sulfur clusters, and their reunion as quinone is reduced to quinol. At an unknown point, but close to their arrival on the bound quinone, the electrons, which so far have retained most of their original potential energy, are harnessed to translocate protons across the membrane. Alternatively, an electron may ‘escape’ by hijacking a molecule of O₂. In the sections below, the mechanism of complex I is broken down into separate steps (NADH oxidation and O₂ reduction at the flavin site, intramolecular electron transfer, quinone reduction and proton translocation), although it is important to recognize that the separate steps do not, and cannot, occur independently.

NADH oxidation and the reactions of the FMN

In complex I, the primary function of the flavin is to oxidize NADH. The interconversion of NADH and NAD⁺ is catalysed by many flavoproteins, and generally considered (but not proven) to proceed by hydride transfer, rather than by transfer of two electrons and one proton (thus avoiding the highly unstable NADH⁻). To transfer the hydride, the nicotinamide ring of NADH stacks above the flavin isoalloxazine ring system, juxtaposing the hydride donor (C-4 of the nicotinamide ring) and acceptor (N-5 of the flavin) in a defined orientation, separated by ~3.5 Å [24]. Recently, structures of the hydrophilic domain of complex I from T. thermophilus with nucleotides bound [25] have revealed the expected juxtaposition (see Figure 3), along with specific interactions between the protein and the nucleotide (stacking interactions between three phenylalanine residues and the adenine ring, a number of hydrophobic interactions, and hydrogen bonds to the nicotinamide carboxamide, two ribose moieties and phosphates). However, the oxidation states of the bound nucleotides, flavin and clusters in these structures have only been inferred from the crystallographic conditions, and from spectroscopic data from different species of complex I. In the future, it will be crucial to elucidate structures in a variety of precisely defined states, to fully understand the determinants of any structural changes, and to address the structures of the reactive complexes.

The flavin in complex I (at least in B. taurus) has an unusually low potential (~0.38 V at pH 7.8; [26]), close to the potential of NAD⁺ (~0.35 V at pH 7.8; [20]). Therefore it catalyses both NADH oxidation and NAD⁺ reduction, and the thermodynamic reversibility of NADH:flavin oxido-reduction has been demonstrated electrochemically: the hydrophilic domain of B. taurus complex I, adsorbed to an electrode surface, establishes a Nernstian equilibrium between NAD⁺ and NADH [27]. Note that the ability of complex I to reduce NAD⁺ during reverse electron transfer [22] does not necessarily require the interconversion of NADH and NAD⁺ to be thermodynamically reversible (i.e. an infinitesimally small driving force, on either side of the equilibrium position, leads to net catalytic turnover; [28]). The implications of the electrochemical reversibility are that NADH:flavin oxido-reduction is kinetically fast and thermodynamically efficient.

Catalytic NADH oxidation by complex I (followed readily by NADH absorption at 340 nm) can be coupled to the reduction of a variety of electron acceptors to evaluate different aspects of the mechanism. For example, NADH:FeCN [ferricyanide, hexacyanoferrate(III)] [29] and NADH:APAD⁺ [21] oxido-reduction involve only the flavin: rotenone- or piericidin A-sensitive NADH:DQ (decylubiquinone) oxido-reduction comprises NADH oxidation, intramolecular electron transfer and quinone reduction, and, when complex I is incorporated in a vesicle, proton translocation across the vesicular membrane. NADH:FeCN oxido-reduction is the fastest known reaction: for complex I from B. taurus it achieves several thousand NADH per second [29]. In comparison, the highest rate of NADH:DQ oxido-reduction from purified B. taurus complex I so far is ~100 s⁻¹ [30]. Therefore NADH oxidation (comprising here NADH binding, hydride transfer and NAD⁺ release) is fast (kₐq(NADH)/kₚ(NAD⁺) > 1 × 10⁷ M⁻¹·s⁻¹ [21]) and not rate-limiting in NADH:DQ oxido-reduction. FeCN [29], APAD⁺ [21] and hydrophilic quinones such as Q-I [31] and O₂ [32], all react directly with the reduced flavin in complex I: they are all inhibited by high NADH concentrations, and can be described by Ping Pong or Ping Pong Pong mechanisms (see Figure 4). So far, kinetic analyses based on these reactions
have provided estimated dissociation constants for NADH from the reduced flavin (20 μM [33] and 160–260 μM [21]) and NAD⁺ from the oxidized flavin (800 μM; [33]), but these values refer to inhibited or product-bound states, and, due to their kinetic complexity, even these simple one-site reactions have so far eluded complete description. In addition, mutation of the conserved glutamate residue in the NADH-binding site of E. coli complex I has been used to suggest that its negative charge is important for determining nucleotide-binding affinities [34], and three inhibitors are known to inhibit NADH oxidation by complex I directly. Dipyridylmethylene forms a covalent adduct with the reduced flavin [35], and ADP-ribose (K_1 ADPR ∼ 0.3 mM and K_2 ADPR ∼ 500 μM [33]) and NADH (K_ox NADH-OH ∼ 0.3 mM and K_red NADH-OH ∼ 7 nM [33]) are NADH analogues. Finally, HAR (hexa-ammineruthenium III) is often used to evaluate the kinetic capability of the flavin site in complex I, because NADH:HAR oxidoreduction is relatively fast [36]. However, HAR reduction is not inhibited by high NADH concentrations, and its mechanism is not well understood (it has been proposed to occur by an ‘ordered’ mechanism): at present, HAR should not be used to quantify changes in the rates of specific reactions at the flavin, or to determine kinetic or thermodynamic constants.

THE REDUCTION OF MOLECULAR O₂ BY THE REDUCED FLAVIN

There has been much debate about the site(s) and mechanism(s) of O₂ reduction by complex I, leading to the production of superoxide and/or H₂O₂. Essentially every possibility has been discussed [the reduced flavin and flavosemiquinone, NAD⁺ radical, iron–sulfur clusters and SQ (semiquinone) intermediates]. Oxygen reduction by the reduced flavin [32] is now recognized as an important contributor that links superoxide production by complex I to the status of the mitochondrial NAD⁺/NADH pool, but a second site (either 4Fe[PS]/N2 or a SQ) may also contribute under some conditions [1]. A further possibility, the ‘conveyor molecule’ mechanism, is that superoxide production is increased by physiological or pharmacological agents that are reduced by the flavin, then reoxidized by O₂ in redox-cycling reactions; hydrophilic quinones have recently provided the first well-characterized (although non-physiological) example [31]. The present review focuses on the direct reaction of O₂ with the reduced flavin in complex I.

In complex I from B. taurus, O₂ reduction by the reduced flavin is much slower than NADH oxidation or NAD⁺ reduction (~0.5 NADH s⁻¹ in atmospheric O₂). Consequently, when complex I is incubated in a mixture of NADH and NAD⁺, an equilibrium is established between the oxidized and reduced flavins (the flavosemiquinone is unstable and present only in low amounts). Because O₂ reacts comparatively slowly with the reduced flavin, it does not perturb the equilibrium significantly, and the rate of reaction is the product of the reduced flavin and O₂ concentrations, and the corresponding bimolecular rate constant [32] (see Figure 5). In reality, the equilibrium position is a function of nucleotide binding also, because O₂ reduction is blocked by bound nucleotides (it is inhibited by high NADH concentrations [32,37], and solvent access to the flavin is blocked in the nucleotide-bound T. thermophilus structures; [25]). However, knowledge of the nucleotide-binding constants is too limited for a quantitative evaluation at present, so in Figure 5 the rate of O₂ reduction (H₂O₂ production) is plotted against the potential of the NAD⁺/NADH pool (expressed as E_set, from the Nernst equation for NAD⁺ reduction). The midpoint potential (E_1/2) of the curve is consistent with the reduction potential of the flavin determined by EPR [26] (rather than with any of the SQ or cluster potentials, see below), and the variation of E_1/2 with pH matches that expected for the flavin also (rather than matching a pH-independent cluster potential). Finally, the sigmoidal shape shows clearly that, in the rate-determining step, O₂ reacts with the fully reduced flavin, not with the flavosemiquinone.

In complex I from B. taurus, O₂ reduction by the (two-electron) reduced flavin generates superoxide (a one-electron reduced species) rather than H₂O₂, indicating that the ‘second’ electron is dissipated to the clusters, or that the nascent...
motifs. Reduction potentials and EPR signal assignments between species or reduction potential, or they may not. In any case, transferring E. coli spectra vary between species (for example, NADH-reduced 2Fe[24] is an evolutionary remnant, with no special role in the contemporary enzyme.

### INTRAMOLECULAR ELECTRON TRANSFER

In complex I, seven iron–sulfur clusters, one [2Fe–2S] cluster and six [4Fe–4S] clusters, form a chain which connects the flavin with the quinone-binding site, and the distal [2Fe–2S] cluster has no known role in energy transduction (see Figure 2 and Table 2). Essentially all extant information about the properties of the clusters in complex I has been derived using EPR spectroscopy [10]; reduced 2Fe and 4Fe clusters are paramagnetic, so the dependence of signal intensity on potential defines the reduction potential (see Table 2). When complex I from B. taurus is reduced by NADH, five EPR signals, from one reduced 2Fe cluster and four reduced 4Fe clusters, are apparent (see Figure 6). There has been extensive discussion about how to assign the five EPR signals to the eight clusters, based mostly on comparisons of spectra from overexpressed subunits and enzyme fragments with those from the isolated enzyme [10,39]. Note that in Figure 2 and Table 2, the names of the clusters are based on the structure; the clusters are often named by their proposed EPR signals (N1a, N1b, N2, etc.), but the original assignment is not correct [39] and this practice continues to confuse. In addition, the EPR spectra vary between species (for example, NADH-reduced E. coli complex I exhibits N1a, but not N5 [10,40]); the variations may result from ‘insignificant’ differences in cluster environment or reduction potential, or they may not. In any case, transferring reduction potentials and EPR signal assignments between species of complex I is not necessarily reliable.

In complex I, assignment of the 2Fe signals is straightforward. The overexpressed 75 kDa subunit exhibits a 2Fe signal very similar to that from the intact enzyme (signal N1b) [39,44], whereas the overexpressed 24 kDa subunit exhibits a different signal (N1a) that is observed in the reduced flavoprotein subcomplex (51 + 24 kDa), but not in intact B. taurus complex I (the cluster is not reduced by NADH) [42]. Therefore N1b is from 2Fe[75] and N1a is from 2Fe[24]. Signal N1a is observed in NADH-reduced E. coli complex I [40], as 2Fe[24] has a higher reduction potential in E. coli than in B. taurus [45]. The 4Fe signals N2 and N3 can be assigned with confidence, to 4Fe[PS] and 4Fe[51] respectively, since N2 interacts paramagnetically with a SQ intermediate [43], and N3 with the flavosemiquinone [26]. The assignment of N4 and N5 is still disputed [39,46]. Here, we consider that N4 is from one (or both) of 4Fe[TY]1 and 4Fe[TY]2, and N5 is from 4Fe[75]C [39]. Our assignment (see Table 2) is consistent with all extant data, but has not been demonstrated directly yet; here, for simplicity, we take the assignments of N4 to 4Fe[TY]1 and N5 to 4Fe[75]C as our model.

Combining the EPR signal assignment with data from redox titrations (see Table 2) [10,41] produces a reduction potential ‘profile’ for electron transfer along the cofactor chain (see Figure 7A). Cluster 4Fe[PS]/N2 has the highest potential. Clusters 4Fe[51]/N3, 4Fe[TY]/N4 and 4Fe[75]/N5 are all considered ‘isopotential’: in redox titrations they are all reduced at around −0.25 V (although the reduced-cluster stoichiometry has not been established unambiguously). Cluster 2Fe[75]/N1b is enigmatic because it is reduced over an unusually wide potential range [42], but its potential is certainly below −0.25 V. Further reduction of B. taurus complex I requires very low potential donors: new signals are observed at approx. −1 V [42], but they are not simple rhombic signals and are probably due to interactions between adjacent reduced clusters. Interestingly, signal N1a was not observed even at −1 V: the reduction of 2Fe[24]/N1a may be limited kinetically, because protein-film voltammetry studies found that its reduction potential is above −0.5 V [45]. In Figure 7A, the estimated potentials for 2Fe[24]/N1a and 2Fe[75]/N1b, and also for 4Fe[51]/H and 4Fe[TY]/2 (which are very probably oxidized in NADH), include a consideration of their positions between clusters that are reduced at higher potentials, so that electrostatic interactions decrease their apparent reduction potentials in redox titrations [47]. Finally, redox titrations rely on equilibrium potentials: the enzyme is in a ‘resting’ state that may not be catalytically relevant, and equilibrium potentials may not reflect the free-energy changes during turnover. With

### Table 2 The eight conserved iron–sulfur clusters in complex I

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Cluster</th>
<th>Ligation</th>
<th>EPR [39]</th>
<th>$E_{\text{red}}$ (V)</th>
<th>Comments on EPR signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 kDa</td>
<td>2Fe[24]</td>
<td>C · X4 · C · X35 · C · X26 · C N1a</td>
<td>est. -0.5</td>
<td>Not observed in intact B. taurus complex I</td>
<td></td>
</tr>
<tr>
<td>51 kDa</td>
<td>4Fe[51]</td>
<td>C · X4 · C · X3 · C · X35 · C N3</td>
<td>-0.25</td>
<td>Spin interactions with flavosemiquinone [26], $E_{\text{red}}$ from redox titration [10,41]</td>
<td></td>
</tr>
<tr>
<td>75 kDa</td>
<td>2Fe[75]</td>
<td>C · X3 · C · X3 · C · X13 · C N1b</td>
<td>est. -0.4</td>
<td>Reduced partially by NADH [42]</td>
<td></td>
</tr>
<tr>
<td>4Fe[75]</td>
<td>C · X3 · C · X3 · C · X43 · C N5</td>
<td>-0.26</td>
<td>N5 signal fast relaxing, $E_{\text{red}}$ from redox titration [10,41]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4Fe[75]</td>
<td>H · X4 · C · X2 · C · X26 · C</td>
<td>n.d.</td>
<td>Not observed by EPR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TYKY</td>
<td>4Fe[TY]1</td>
<td>C · X2 · C · X2 · C · X42 · C N4</td>
<td>-0.25</td>
<td>N4 may arise from 4Fe[TY]1 and/or 4Fe[TY]2; for simplicity it is attributed here to 4Fe[TY]1. $E_{\text{red}}$ (N4) from redox titration [10,41]</td>
<td></td>
</tr>
<tr>
<td>4Fe[TY]2</td>
<td>C · X2 · C · X3 · C · X26 · C</td>
<td>n.d.</td>
<td>Spin interactions with ubiquinolquinones [43]. $E_{\text{red}}$ from redox titration [10,41]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSST</td>
<td>4Fe[PS]</td>
<td>C · C · X35 · C · X35 · C N2</td>
<td>-0.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The ligation motifs are for the clusters in B. taurus complex I; there are small differences between species, for example, in T. thermophilus extra residues in the 2Fe[75], 4Fe[TY]1 and 4Fe[TY]2 motifs.

2 Cluster potentials are reported for B. taurus complex I.

3 Literature reports of N1a in B. taurus complex I result from confusion between N1a and N1b; this confusion has also caused problems with defining the stoichiometry and potential of N1b.
these points in mind, the alternating potential or ‘rollercoaster’ profile [48] in Figure 7(A) is consistent with rapid electron transfer along the chain (single low-potential clusters impede electron transfer much less than adjacent low-potential clusters) [49]. In Figure 7(A), the longest distance for transfer (4Fe[75]H to 4Fe[TY]1/N4), is compensated for by an increase in reduction potential ($k_{\text{rev}} = 1.8 \times 10^3$ s$^{-1}$ estimated by Dutton’s equation; [49]). The predicted rate-limiting step in forward electron transfer is 4Fe[51]/N3 to 2Fe[75]/N1b ($k_{\text{rev}} = 7.1 \times 10^6$ s$^{-1}$). During reverse-electron flow (from 4Fe[PS]/N2 to 4Fe[51]/N3) the rate-limiting step is predicted to be the longest distance transfer (4Fe[TY]1/N4 to 4Fe[75]H) ($k_{\text{rev}} = 4.3 \times 10^4$ s$^{-1}$; [49]). Recently, Verkhovsky et al. [50] used freeze-quenching to show that the two highest potential clusters in E. coli complex I (4Fe[PS]/N2 and 2Fe[24]/N1a) were reduced by NADH with an apparent time constant of $\sim 90 \mu$s (assuming no electron transfer occurs in the frozen sample), and that the second pair of reduced clusters (observed as signals N1b and N4, referred to as N6b) were formed more slowly ($\sim 1$ ms). The results are consistent with fast intramolecular electron transfer from the flavin to 4Fe[PS]/N2 ($< 90 \mu$s, it is not clear whether the rate-limiting step is hydride transfer, flavin reoxidation or intramolecular electron transfer) and with rate-limiting NAD$^+$ dissociation (within the NADH to 4Fe[PS]/N2 component) during turnover.

Figure 6  EPR spectra of complex I from B. taurus reduced by NADH

The spectra recorded at 12, 40 and 4 K (black) are compared with their simulated spectra (red). The inset for the 12 K spectrum (not on the x-axis scale) shows how the modelled 12 K spectrum comprises N1b (grey), N2 (green), N3 (blue), N4 (magenta) and N5 (orange). The g-values used were: N1b ($g_{x,y,z} = 2.024, 1.941, 1.926$), N2 ($g_{x,y,z} = 2.055, 1.926, 1.925$), N3 ($g_{x,y,z} = 2.041, 1.927, 1.865$), N4 ($g_{x,y,z} = 2.114, 1.930, 1.882$) and N5 ($g_{x,y,z} = 2.064, 1.928, 1.898$). Figure adapted with permission from [42]. ©2008 American Chemical Society.

It is likely that the cluster chain in complex I is already partially reduced during steady-state catalysis, so that a single electron pair does not traverse the complete chain during a single turnover. Figure 7(B) shows a model for rapid and efficient intramolecular electron transfer in complex I. At $-0.3$ V (the potential of the mitochondrial NAD$^+$/NADH pool), complex I contains four electrons, spaced alternately along the chain. NADH oxidation is reversible and fast (in comparison with Q reduction), and a mixture of states is established, including ‘poised’ states such as \{NADH:FMN\} (NADH bound to oxidized flavin) or alternative poised states such as \{NADH:FMN$^+$\} \{NADH:FMNH$^-$\} \{NADH:FMNH$^+$\} \{FMNH$^+$\}, as well as non-reactive states \{NAD$^+$:FMN$^-$\} \{NAD$^+$:FMN\} \{FMN$^-$\} \{FMN\}. Interestingly, if quinone binds to a non-reactive state, then it is unlikely to be reduced, provided that sufficient $\Delta p$ is present (see Figure 7A). Consequently, turnover only occurs when a poised enzyme binds Q, and NADH oxidation (or its equivalent) and Q reduction are enabled together. A SQ intermediate is observed in the presence of $\Delta p$ (see below); even though NADH is a two-electron donor and SQ formation requires only one electron, the flavin remains predominantly oxidized because the cluster chain retains the extra electron, and that the second pair of reduced clusters (observed as signals N1b and N4, referred to as N6b) were formed more slowly ($\sim 1$ ms). The results are consistent with fast intramolecular electron transfer from the flavin to 4Fe[PS]/N2 ($< 90 \mu$s, it is not clear whether the rate-limiting step is hydride transfer, flavin reoxidation or intramolecular electron transfer) and with rate-limiting NAD$^+$ dissociation (within the NADH to 4Fe[PS]/N2 component) during turnover.

Figure 7  Electron transfer reactions in complex I

(A) Potential energy profile for the substrates and cofactors in complex I (based on data from B. taurus). The substrate potentials are two electron potentials, and two values for Q are included, for $\Delta p = 0$ V and $\Delta p = 0.15$ V. The substrate potentials and the two flavin potentials, FMN1 (ox/semi) and FMN2 (semi/red) [26] are at pH $\sim 7.8$, and they include the coupled protonations. Cluster 2Fe[24]/N1a is not part of the chain. Values for 4Fe[51]/N3, 4Fe[75]/C/N5, 4Fe[TY]/1/N4 and 4Fe[PS]/N2 are from [41]; values for the other clusters are estimates. Edge-to-edge distances are indicated (in A˚). (B) Possible scheme for the transfer of two electrons to bound quinone, upon the oxidation of NADH (see text for details). Two electrons are highlighted in red for their identification.
analogy to the Grothuss mechanism of proton transfer) above the already high rates predicted by Dutton’s model and implied by the data of Verkhovskaya et al. (i.e. electron delivery to 4Fe[PS]/N2 is very unlikely to exert any rate-limiting effect on catalysis). In addition, provided that the individual electron transfers are fast and reversible, the redox potential energy of the NADH is transferred efficiently to the bound quinone (in analogy to efficient energy transfer by Newton’s cradle) – this may be key for thermodynamic reversibility in catalysis by complex I.

**QUINONE REDUCTION**

Quinone reduction and proton translocation by complex I are less well understood than NADH oxidation and intramolecular electron transfer; they both involve the enzyme’s membrane domain, for which there is currently no high-resolution structural model and only limited mechanistic information. The challenges for mechanistic studies are the lack of cofactors (few spectroscopic opportunities), the lack of independent catalytic activity and the experimental difficulties associated with the highly hydrophobic quinone substrate (typically Q-10), and with controlling and quantifying an electrochemical potential across the enzyme in a membrane. At present, extant data pertains mostly to identification of the quinone-binding site (or sites), and to observation and characterization of SQ intermediates.

A putative binding site for the quinone headgroup was identified in the structure of the hydrophilic domain of complex I from *T. thermophilus*, at the interface of the 49 kDa and PSST subunits; close to 4Fe[PS]/N2 and the membrane interface (see Figures 2 and 8) [8]. The site matches that proposed previously on the basis of mutations to the 49 kDa subunit in *R. capsulatus* [51], and site-directed mutants in the 49 kDa and PSST subunits of complex I from *Yarrowia lipolytica* have now been used to characterize it further [52]. A number of mutations affected NADH:DQ oxidoreduction and inhibitor binding [52–55], consistent with the proposed location of the binding site (see Figure 8).

However, there are additional considerations. First, the structurally characterized hydrophobic domain is cleaved from the hydrophobic domain, with the bottom of the putative Q-binding site and helices H1 and H2 in the cleavage plane, and there are significant stretches of adjacent sequence which are not defined structurally (in particular, the N-terminus of the 49 kDa subunit, and in PSST). Secondly, the decreased activities of the mutants do not necessarily mean that quinone binding or reduction is affected (alternatively, proton transfer, redox-coupled protonation or the secondary structure may be disrupted). Indeed, mutations of two residues in PSST which are distant from the putative binding site also decreased the activity significantly (see Figure 8) [54]. Nevertheless, residues in and around the putative quinone-binding site certainly do influence catalysis, showing that this region of the enzyme is important functionally [52].

Many diverse hydrophobic compounds inhibit complex I [57] and, because they inhibit NADH:quinone (but not NADH:ferricyanide) oxidoreduction they are termed ‘Q-site inhibitors’. Some of them, notably piericidin A, clearly resemble the quinone substrate, others, notably the acetogenins, display no apparent similarity. Q-site inhibitors may compete directly with the quinone substrate, others, notably the acetogenins, display no apparent similarity. Q-site inhibitors may compete directly with the quinone substrate, others, notably the acetogenins, display no apparent similarity. Q-site inhibitors may compete directly with the quinone substrate, others, notably the acetogenins, display no apparent similarity. Q-site inhibitors may compete directly with the quinone substrate, others, notably the acetogenins, display no apparent similarity. Q-site inhibitors may compete directly with the quinone substrate, others, notably the acetogenins, display no apparent similarity. 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and to the N-terminal section of the 49 kDa subunit underline the functional significance of this region of the enzyme [14].

SQ species have been detected during steady-state catalysis by complex I in tightly coupled submitochondrial particles, and differentiated by their relaxation rates and responses to membrane potential, pH and inhibitors [43,58,59]. SQNf is the fastest relaxing, and it is sensitive to uncouplers (which dissipate Δp), and to rotenone and piericidin A. SQNf is proposed to interact, by both spin–spin and dipolar interactions, with 4Fe[PS]/N2; it is considered to be ∼12 Å from the cluster, with the vector between
the two species close to the membrane plane, so that SQNf is ~5 Å closer to the membrane [43,58]. The proposed position of SQNf is consistent with the putative quinone-binding site described above. SQNf is insensitive to uncouplers and slowly relaxing (thus it is considered distant from the clusters), and it has been characterized thermodynamically [59]. At pH 7.8, the reduction potentials of SQNf are $E_{\text{Q/SQ}} = -0.045 \text{ V}$ and $E_{\text{QH2/Q}} = -0.063 \text{ V}$, so SQNf is thermodynamically stable; because $E_{\text{QH2/Q}} = -0.054 \text{ V}$ (lower than for free quinone), QNf is bound more tightly to complex I than QH2. There is no proposed location for SQN, although ND4 in E. coli complex I is a possibility because it was labelled by an azido-ubiquinone derivative [60] and is probably located at the distal end of the membrane arm. Finally, a third species, SQNx, which is also slowly relaxing, was described previously, but is now not considered to originate in complex I [59].

PROTON TRANSFER HYPOTHESES

First, the question of whether some complexes I transport Na+, instead of protons, across energy-transducing membranes has obvious implications for the mechanism of charge transfer. Steuber’s group co-reconstituted their preparation of complex I from Klebsiella pneumoniae with Na+-translocating ATP synthase from Ilyobacter tartaricus and demonstrated both NADH-supported ATP synthesis and ATP-supported NAD+ reduction [61]. It has since been claimed that Steuber's results stem from the presence of an unrelated Na+-translocating enzyme (Na-NQR) [62], but this claim has not yet been either proven or disproven. Steuber’s group have proposed that the complexes I from E. coli and Y. lipolytica translocate Na+ also [63,64], but in both cases the proposal has been attacked vigorously, using well-characterized and highly pure enzymes [65,66]. A Na+-translocating prokaryotic complex I would be reasonable, as some bacteria rely on Na+-motive forces to support, for example, ATP synthesis and the flagellar motor, but a Na+-translocating mitochondrial complex I seems extremely unlikely and this proposal is not generally considered viable. Therefore only proton translocation is considered for the rest of the present review.

There are several classes of energy-transduction mechanisms which should be considered for complex I. First, in a direct-coupling mechanism (exemplified, in the respiratory chain, by cytochrome c oxidase), proton translocation is initiated at the same active site as the electron transfer event that drives it. Secondly, in an indirect-coupling mechanism (exemplified, in the respiratory chain, by ATP synthase), a significant conformational change is used to transfer the potential energy of the driving reaction to a remote site, where proton translocation is initiated. Thirdly, in Q-cycle mechanisms (exemplified, in the respiratory chain, by cytochrome bc1), quinol is used as a mobile electron–proton carrier, to transport protons across the membrane dielectric. Although the three classes appear clearly distinguishable, in reality proposed mechanisms for complex I often fall between classes, or include components from more than one class. First, we consider possible relationships between complex I catalysis and quinone reduction.

Figure 9(A) depicts how Mitchell’s original Q-cycle mechanism applies to complex I: Q is reduced at the negative (matrix) side of the membrane by two electrons (from NADH) and two protons to form QH2. A second enzyme reoxidizes the QH2 at the positive side of the membrane, releasing two protons (to the intermembrane space). The system transports two protons and two electrons across the membrane (complex I does not ‘pump’ any protons) and it is conserved as a component of the following three mechanisms. Figure 9(B) depicts complex I catalysing by the ‘reductant-induced oxidation’ mechanism, that mirrors the mechanism of cytochrome bc1 [67]. Q is reduced, at the negative side, by one electron from NADH and one electron from a QH2 (first half-cycle) or a SQ (second half-cycle) bound at the positive side. Mechanism B predicts that each NADH oxidation results in the oxidation of one QH2 to Q (positive side), and the reduction of two protons to two protons (negative side), but when complex I was provided with NADH and two distinguishable quinone/quinol species, QH2 and QNf, no evidence for catalytic QH2 formation was observed [68]. The lack of cofactors in the membrane domain, and the proton-to-electron stoichiometry, present further obstacles to mechanism B, although it cannot be discounted unambiguously. Mechanism B transports four protons and two electrons across the
membrane; complex I pumps two protons per NADH. Figure 9(C) shows a modified version of mechanism B: at the negative side a SQ is reduced to QH₂ by one electron and two protons, and both the SQ and QH₂ are retained; the QH₂ is shuttled across the membrane and reoxidized at the positive side, by transfer of a single electron to a ‘substrate quinone’ and release of two protons. The SQ then returns to the negative side to repeat the half-cycle and finally reduce the substrate quinone. Mechanism C transports six protons and two electrons across the membrane; complex I pumps four protons per NADH and so mechanism C accounts for the experimentally observed stoichiometry. However, it requires an intramolecular shuttle for SQ and QH₂, and it still requires electron transfer across the membrane. Figure 9(D) presents a similar mechanism, but now the SQ and QH₂ intermediates are retained in the same site. Instead, proton access is switched between the negative and positive sides: SQ is always protonated from the negative side, QH₂ is always deprotonated to the positive side. Mechanism D also accounts for the experimentally observed stoichiometry of complex I, it does not require electron transfer across the membrane, and it comprises two SQ species, consistent with extant data. Mechanism D was described recently by Ohnishi and Salerno [69], and is related to a previous mechanism suggested by Dutton et al. [67]. However, it requires an unknown gating mechanism to impose directionality on the proton transfer events.

A second possible direct-coupling site in complex I is 4Fe[PS]/N2, because its reduction is coupled to proton uptake. The coupled-protonation has been proposed to occur on a nearby histidine residue: in *Y. lipolytica*, the 49 kDa subunit H226M mutant retained its ability to transport protons, but the reduction potential of 4Fe[PS]/N2 became independent of pH (over the measured range, pH 6–8) [56]; these results suggest that the redox-coupled protonation of 4Fe[PS]/N2 is not important in energy transduction. Alternatively, Friedrich and co-workers [70] proposed that two tyrosine residues are protonated upon 4Fe[PS]/N2 reduction, and that a glutamate residue is protonated upon 4Fe[PS]/N2 oxidation, but these observations could not be reproduced by Rich and co-workers [71]. Taken together, these observations do not form a strong case for a redox-linked protonation of 4Fe[PS]/N2 as the basis for proton translocation by complex I.

Recently, Berrisford and Sazanov [25] described a highly speculative redox-linked proton transfer mechanism for complex I, based on apparent changes in the electron density, and thus the ligation, of 4Fe[PS]/N2 in three states of the enzyme. They suggest that, although the oxidized cluster (State I) is ligated by four cysteine residues (Cys⁴⁵, Cys⁶⁸, Cys¹¹¹ and Cys¹⁶⁰ in the *T. thermophilus* PSST subunit), the reduced cluster is only ligated by three. Furthermore, when ‘cluster N6b’ (4Fe[TY]²) is oxidized, Cys⁴⁵ is dissociated and protonated (State II), but when 4Fe[TY]² is reduced, Cys⁴⁵ is dissociated and protonated (State III). The changes in electron density are certainly intriguing, but it is difficult to confidently assign the oxidation states of 4Fe[PS]/N2 and 4Fe[TY]² in the crystals: typically, 4Fe[PS]/N2 is the highest potential cluster in complex, but its potential in *T. thermophilus* has not been defined unambiguously and may be lower than usual [41,72], and the oxidation state of 4Fe[TY]² has been inferred using EPR signal N4 (although N4 may originate from 4Fe[TY]¹), and is either not observed or atypical in *T. thermophilus* [41,72]. However, the mechanism begins with the clusters oxidized (compare with Figure 7B), in State I. NADH is oxidized and 4Fe[PS]/N2 is reduced (State I→II), and then 4Fe[TY]² is reduced also (State II→III). One electron is transferred to bound quinone, and 4Fe[TY]² is reoxidized (State III→II), and then the second electron is transferred and 4Fe[PS]/N2 is reoxidized (State II→I). Each state change results in the dissociation and protonation, or deprotonation and re-association, of one of the cysteine ligands of 4Fe[PS]/N2, but, incredibly, none of the ligation changes are suggested to be involved directly in proton pumping! Instead, Berrisford and Sazanov [25] propose that all four protons are ‘pumped’ during quinol formation, one proton being transferred (from a protonated residue ‘loaded’ by Cys⁴⁵ deprotonation) to the periplasm by an unknown conformational change, and three protons being pumped in subunits ND2, ND4 and ND5 by further unknown conformational changes (see below). The conformational changes are coupled to 4Fe[PS]/N2 and 4Fe[TY]² reduction, and they originate in helices H1 and H2, and in a four-helix bundle in the 49 kDa subunit respectively, but the observed changes are small (see Figure 8). In addition, there are no well-defined examples of [4Fe–4S] clusters with cysteine ligands that dissociate and protonate upon reduction, and the suggested mechanism relies heavily on the pH-dependent reduction potential of 4Fe[PS]/N2 (it is difficult to reconcile with the proton-pumping activity of *Y. lipolytica* H226M; [56]). However, further work is required to better define the pKₐ and pHₑǎₜ values of 4Fe[PS]/N2. Currently, the best-defined values are from *Y. lipolytica* (pKₐ = 5.7, pHₑǎₜ = 7.3) [56], but they provide a ΔpK' value of less than 2; although the values are from equilibrium redox titrations, the separation is not sufficient for a strongly coupled proton pump.

So, what is the evidence for a significant conformational change during complex I catalysis? First, two different large changes in structure have been observed by electron microscopy: the ‘horseshoe’ conformation [73] and an ‘expansion’ over the whole structure upon enzyme reduction [74]. Both observations are now considered artefacts [74,75]. Furthermore, electron microscopy of the oxidized and reduced *E. coli* complexes I in ice (rather than negative stain) did not reveal any significant conformational change [75]. Secondly, several reports have described changes in the cross-linking pattern, particularly between the subunits of the hydrophilic arm of complex I, upon nucleotide binding and/or reduction, and they have been taken to represent conformational changes, or changes in the relative positions of the subunits (see, for example [74,76]). However, there are only minimal differences between the structures of the oxidized and reduced states of the hydrophilic arm of *T. thermophilus* complex I [25] (see Figure 8). Fourier-transform IR spectroscopy studies also suggested that oxidized and NADH-reduced complex I do not significantly differ structurally (the observed differences were fully consistent with the oxidation and reduction of a set of simple FeS proteins) [71]. Thirdly, and finally, ND4 and ND5 are located at the distal end of the membrane arm [18], and they are proposed to be important in proton translocation, because they are homologous with Na⁺/H⁺ antiporters [16], complex I is inhibited by several amiloride derivatives (known antiporter inhibitors [77]), and a photoaffinity analogue of the inhibitor fenpyroximate has been located on ND5 [78] (although this result has been challenged recently [14]). Coupling a redox reaction in the hydrophilic arm to proton translocation at the distal end of the hydrophobic arm would indeed require an unusual mechanism of long-range energy transfer. Such a mechanism could be based on a ‘conformational change’. Alternatively, Verkhovskaya and co-workers [79] suggested an ‘electrostatic transmission’ mechanism, mediated by conserved charges in the membrane domain. Whether such a mechanism could drive three protons across the membrane from ~100 Å away, remains to be established. Finally, a number of studies have mutated conserved residues in the membrane subunits of bacterial complexes I, and observed changes in Vₘₚₐₜ and Kₐₚ(Q) (see [79,80] and references therein), but the data are difficult to interpret in the absence of
CONCLUSIONS AND PERSPECTIVES

For many years, complex I was the poor relation of the respiratory chain enzymes, the refractory ‘black box’ which resisted structural or mechanistic characterization, the enzyme with little new to stir the imagination. Complex I, indeed, is an enzyme more challenging than most: difficult to purify and crystallize, lacking good spectroscopic handles, and comprising an unwieldy gamut of subunits and cofactors: but, finally, complex I is coming to heel. The first structural model for the hydrophilic domain of the enzyme has provided a solid starting point for mechanistic proposals which integrate structure with functional and spectroscopic data, and a further leap forward is to be anticipated when the structure of the hydrophobic domain, or of an intact complex I, is finally determined. However, structure is only part of the story (‘a picture of a racehorse does not tell you how it runs’) and solving the mechanism of a complicated redox enzyme such as complex I will certainly require innovative and advanced biophysical and biochemical approaches. Indeed, this is an exciting time for complex I – not only a challenging enzyme full of opportunities, but also an enzyme which may play an important role in many human diseases.

The present review has aimed to provide a balanced overview of experimental evidence pertaining to the mechanism of complex I, and a discussion of contemporary mechanistic hypotheses, proposals and models. Finally, current knowledge of the mechanism of complex I is summarized as follows. (i) NADH is oxidized by the FMN in a hydride transfer reaction, with the nicotinamide ring juxtaposed to the isoalloxazine ring system in a configuration common to many flavoenzymes. NADH:flavin oxidoreduction is fast (several thousand per second) and thermodynamically reversible; NAD+ dissociation may be rate-limiting in NADH oxidation. (ii) The reduced flavin reacts with O2 in a slow, bimolecular reaction, to generate ROS. The population of reduced flavin is determined by the concentrations of NADH and NAD+ (they set the potential of the flavin and block O2 access by binding to the active site). ROS production may be accelerated by conveyor molecules that induce redox-cycling reactions. (iii) Complex I contains eight iron–sulfur clusters. Seven of them form a chain between the flavin and the quinone-binding site. One of them, the distal cluster, is on the opposite side of the flavin. The ‘distal’ cluster has been suggested to act as a temporary electron storage device, to minimize the lifetime of the flavosemiquinone; the distal cluster has no apparent role in energy transduction. (iv) The seven clusters of the chain are spaced less than 14 Å apart, consistent with fast electron transfer along the chain. Five reduced clusters have been observed by EPR in mitochondrial complex I, and their reduction potentials measured. Controversy remains about how to assign the spectroscopic data to the structurally defined clusters, but, in the model most consistent with extant data, the cluster arrangement produces an alternating or ‘roller-coaster’ free-energy profile. The chain is pre-loaded with four electrons during turnover, increasing the apparent rate of electron transfer and aiding efficient energy conservation. (v) NADH oxidation and intramolecular electron transfer are both fast and reversible, and much faster than quinone reduction. Proton translocation is most probably coupled to quinone binding, quinol release or quinone reduction by the terminal iron–sulfur cluster. A possible quinone-binding site has been identified near to the terminal cluster, and SQ intermediates have been observed by EPR. (vi) The mechanisms of quinone reduction and proton translocation are not known; they are the subject of much speculation, but little data. Proton-coupled reactions at the terminal iron–sulfur cluster, quinone redox-cycling mechanisms and conformational changes are all possibilities that are currently under discussion.

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