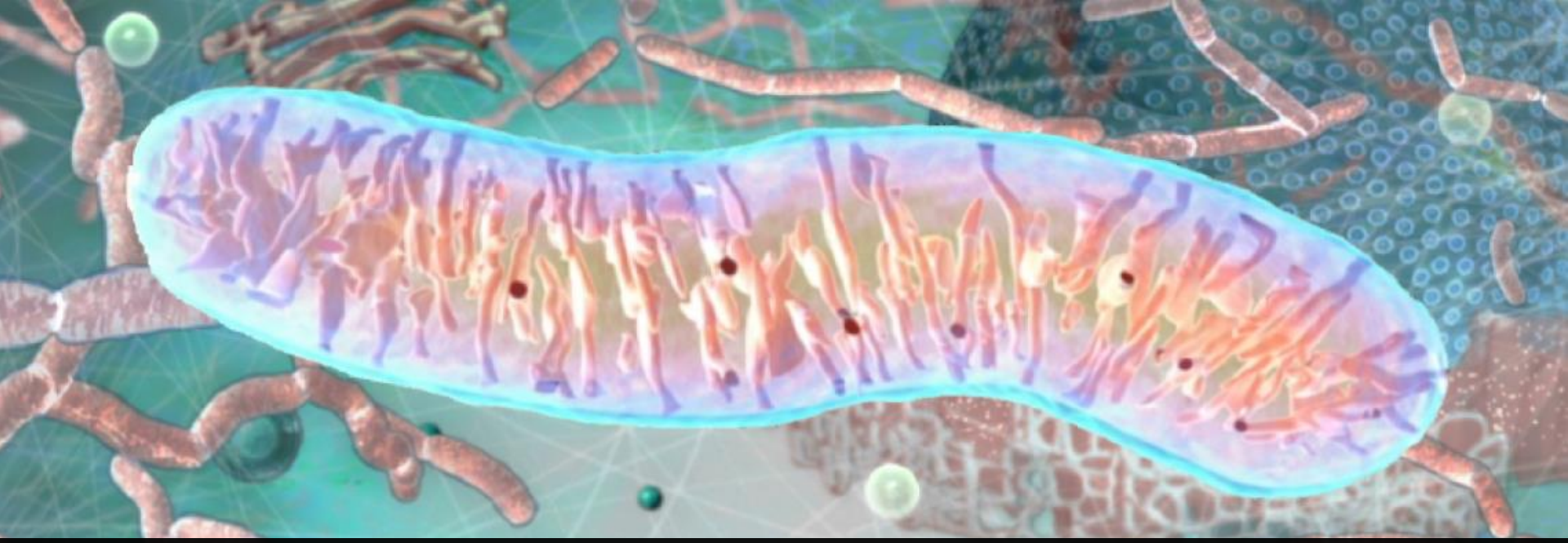


The enzymatic basis of energy-generation

Lecture 2: Respiration of organic compounds

Dr Chris Greening

Lecturer / Group Leader

Monash University

May 5 2016

Lecture 2: Respiration of organic compounds

I. Complex I: a respiratory supercomplex

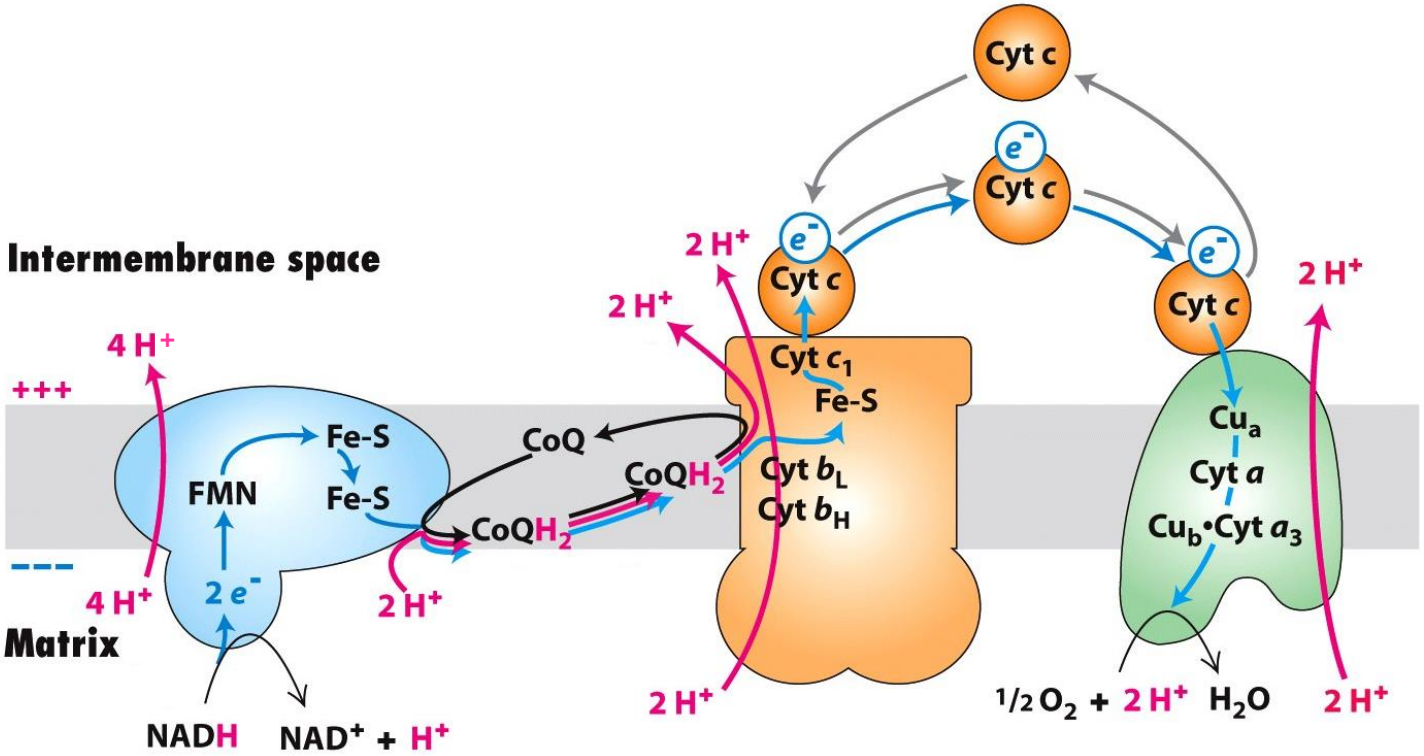
II. Complex III

III. Complex IV

IV. ETC plasticity

Organisation of the mitochondrial ETC

- Mitochondrial electron transport chains use the energy released from transmembrane electron transfers to pump protons and generate Δp. There are three linear proton-translocating complexes, complex I, complex III, and complex IV, and three side complexes.



Complexes of the mitochondrial ETC

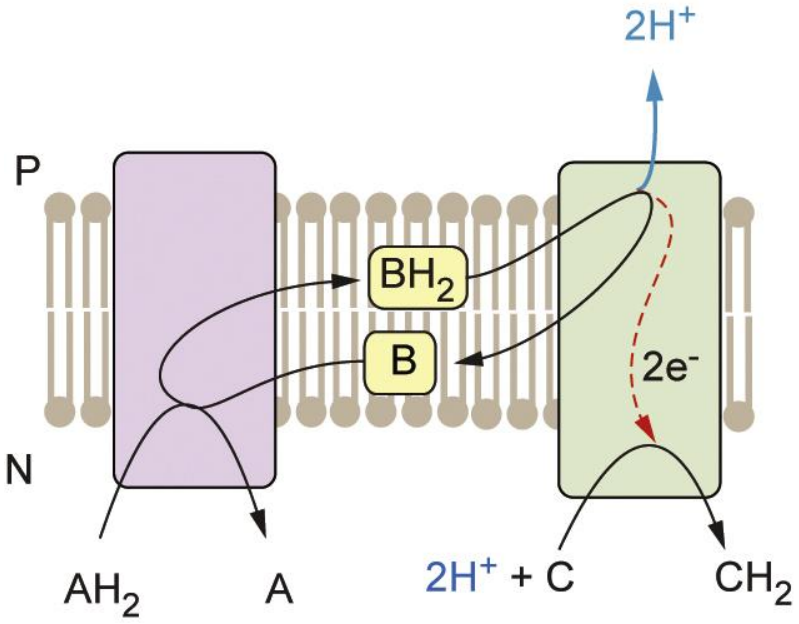
Main complexes	Full name	e^- donor	e- acceptor	Protons translocated
Complex I	NADH-ubiquinone oxidoreductase (NADH dehydrogenase)	NADH	Ubiquinone	4 H^+
Complex III	Ubiquinone-cyt *c* oxidoreductase (cytochrome bc_1 complex)	Ubiquinol	Cytochrome c_{ox}	2 H^+
Complex IV	Cytochrome *c*-O_2 oxidoreductase (cytochrome *c* oxidase)	Cytochrome c_{red}	O_2	4 H^+

Side complexes	Full name	e^- donor	e- acceptor	Protons translocated
Complex II	Succinate-ubiquinone oxidoreductase (succinate dehydrogenase)	Succinate	Ubiquinone	0 H^+
ETF dehydrogenase	Electron-transferring flavoprotein-ubiquinone oxidoreductase	ETF (reduced during fatty acid oxidation)	Ubiquinone	0 H^+
G3P dehydrogenase	Glycerol 3-phosphate dehydrogenase-ubiquinone oxidoreductase	Glycerol 3-phosphate	Ubiquinone	0 H^+

Two ways to generate Δ*p*

Scalar translocation:

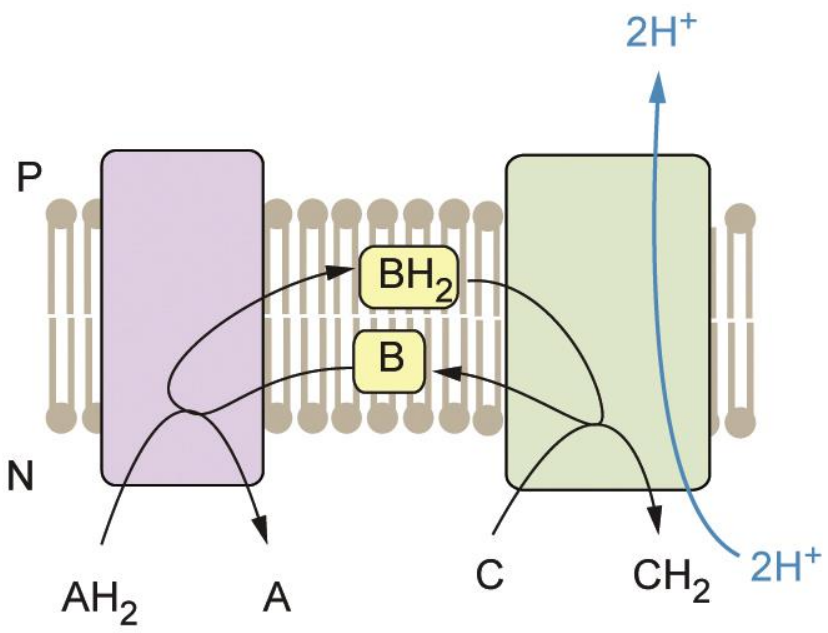
Charge displaced across the membrane by redox-loop mechanisms. Oxidation of BH_2 at P-side causes outward proton flow. Reduction of C at N-side causes inward electron flow.



e.g. Complex III, Complex IV

Vectorial translocation:

Protons are directly transferred from the N-side to the P-side *via* proton-translocating respiratory complexes.

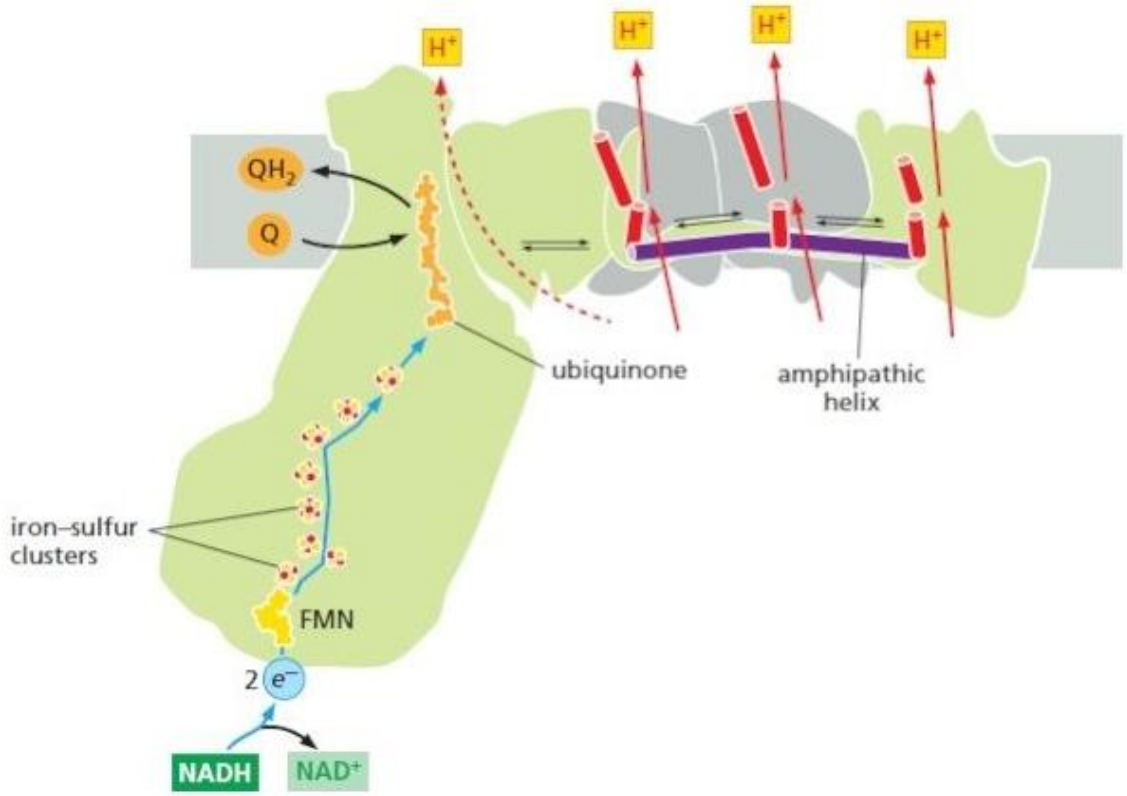


e.g. Complex I, Complex IV

Complex I: NADH dehydrogenase

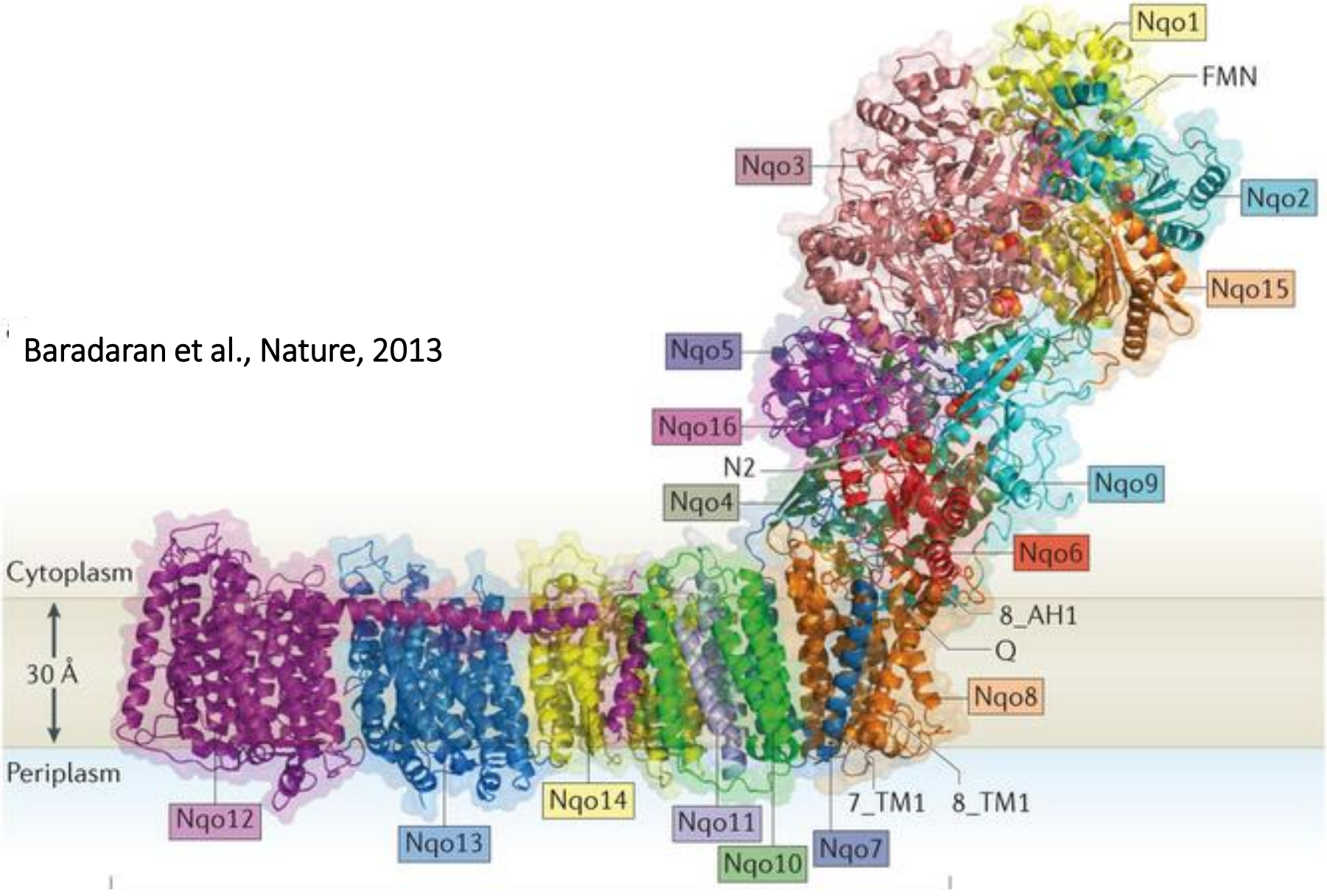
- Complex I is the first enzyme in mitochondrial and many bacterial electron transport chains. Contains hydrophilic arm (9 subunits) on N side, hydrophobic arm (7 subunits) in membrane.

- NADH (reduced in glycolysis and TCA cycle) oxidised by hydrophilic arm. Ubiquinone (lipid-soluble electron carrier) reduced at arm interface. Electrons funnelled by FMN and seven FeS clusters. Membrane arm directly pumps four protons for every $2e^-$.



3.3 Å crystal structure of bacterial complex I

Baradaran et al., Nature, 2013



Structure of Complex I took 15 years to solve

- Took so long due to two major difficulties associated with working with complex I:
  1. It is an integral membrane protein. Makes it v. challenging to purify and crystallise active enzyme.
  2. It really is complex. Mitochondrial enzyme contains 44 subunits, weighs 980 kDa.

- Leo Sazanov focused on the bacterial enzyme, which performs same function as the mitochondrial enzyme with "just" 16 subunits. He worked on two different enzymes (*E. coli, Thermus thermophilus*) and worked step-by-step from subcomplexes to the full complex.

- Systematically optimised expression, purification, and crystallisation procedures, paying much attention to detergent selection. At points, his team broke conventions. Often forced back to the drawing board.

Leader: Leonid Sazanov

The workflow that solved membrane domain

Expression

- Cultures of *E. coli* BL21 grown semi-aerobically in 30 L fermenter
- Samples harvested, physically lysed, and ultracentrifuged to form membranes

Purification

- Complex I purified in membranes by anion-exchange and size-exclusive chromatography
- Assayed by monitoring NADH-dependent oxidation of artificial e^- acceptor ferricyanide
- Activity and stability enhanced by solubilizing with *E. coli* total lipids and divalent cations

Crystallisation

- Crystallised in a complex detergent mixture and phased with selenomet derivative
- In total, 80,000 crystallization conditions tested, 1,000 crystals tested on synchrotron

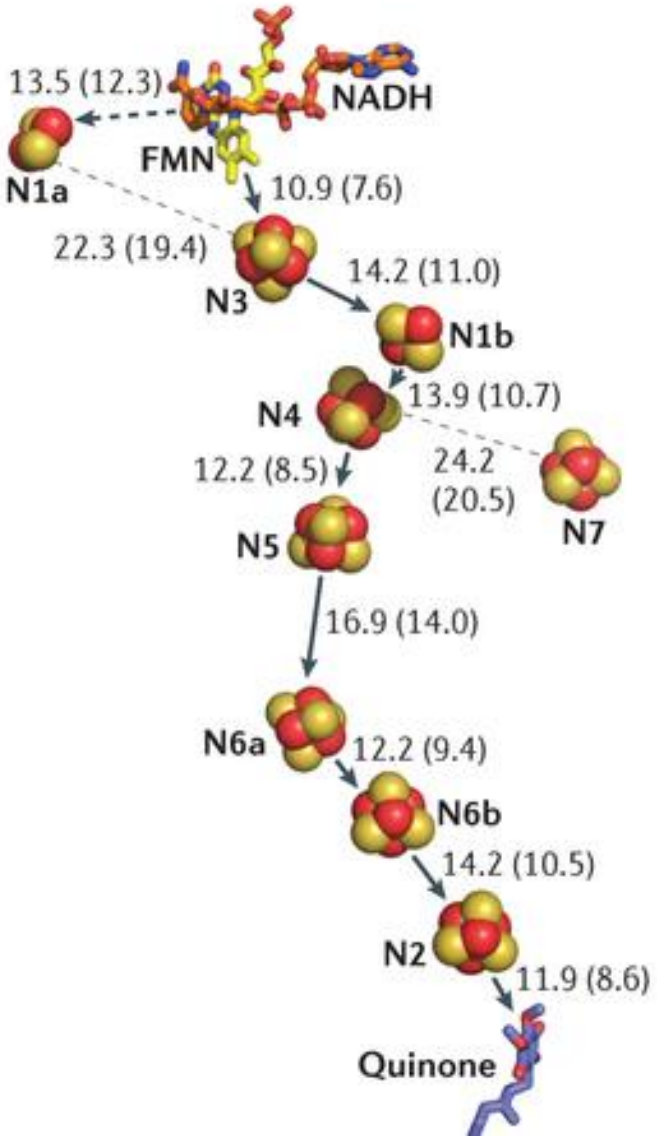
Efremov et al., Nature, 2011

Electron flow through the hydrophilic arm

1. NADH transfers $2e^-$ to FMN. FMN passes electrons on to FeS cluster N3 one-at-a-time. FMN is a $1e^-/2e^-$ gate that transiently forms $FMNH^\bullet$ radical. NADH, FMN, N3 all bind Nqo1 subunit.

2. Electrons flow through seven FeS clusters. These centres are 14 Å of each other and increase in E°' from -250 mV (N3) to -100 mV (N2). Two other centres (N1a, N7) too distant for e^- flow.

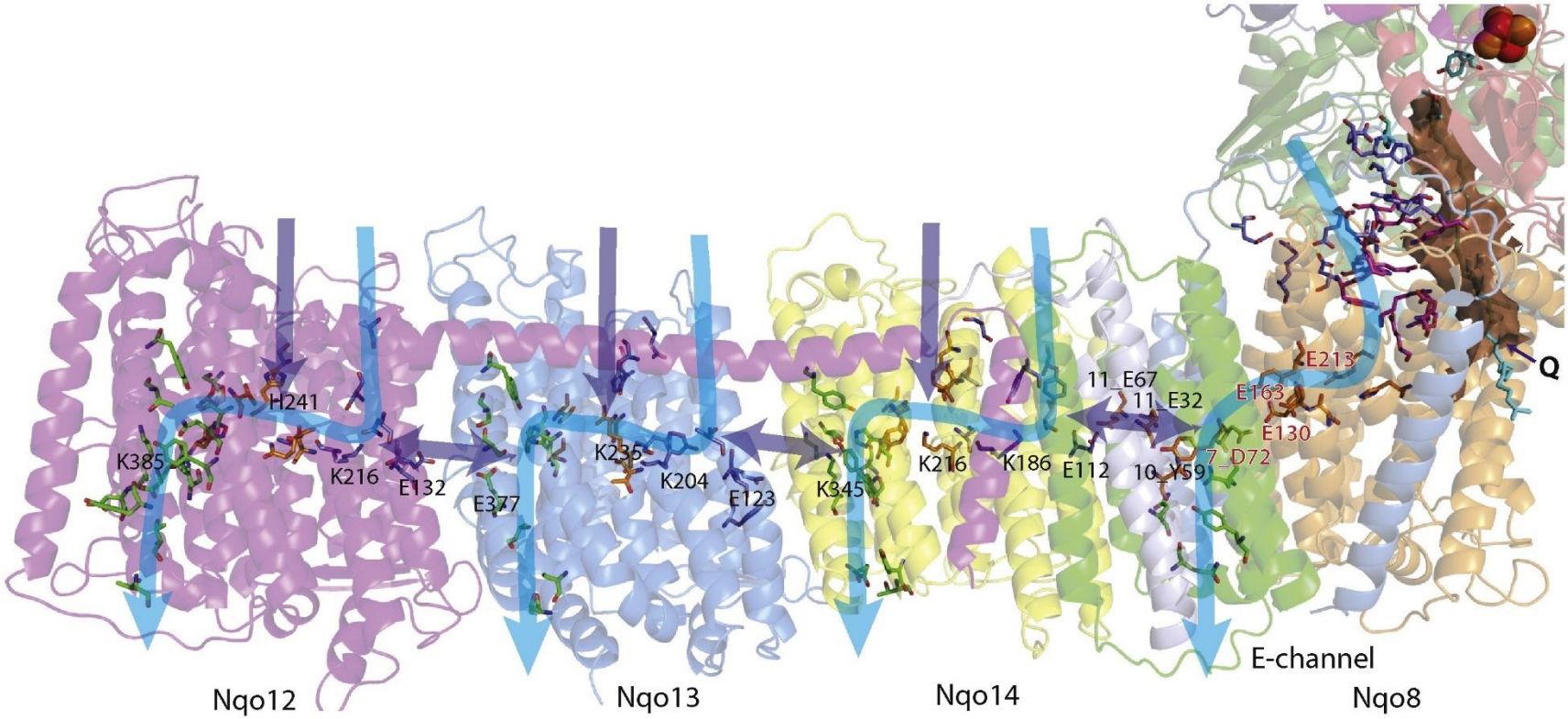
3. N2 donates e^- to ubiquinone one-by-one (UQ → $UQH^\bullet$ → UQH_2). UQ buried in a narrow channel formed by Nqo4 and Nqo6.



Proton pumping in the hydrophobic arm

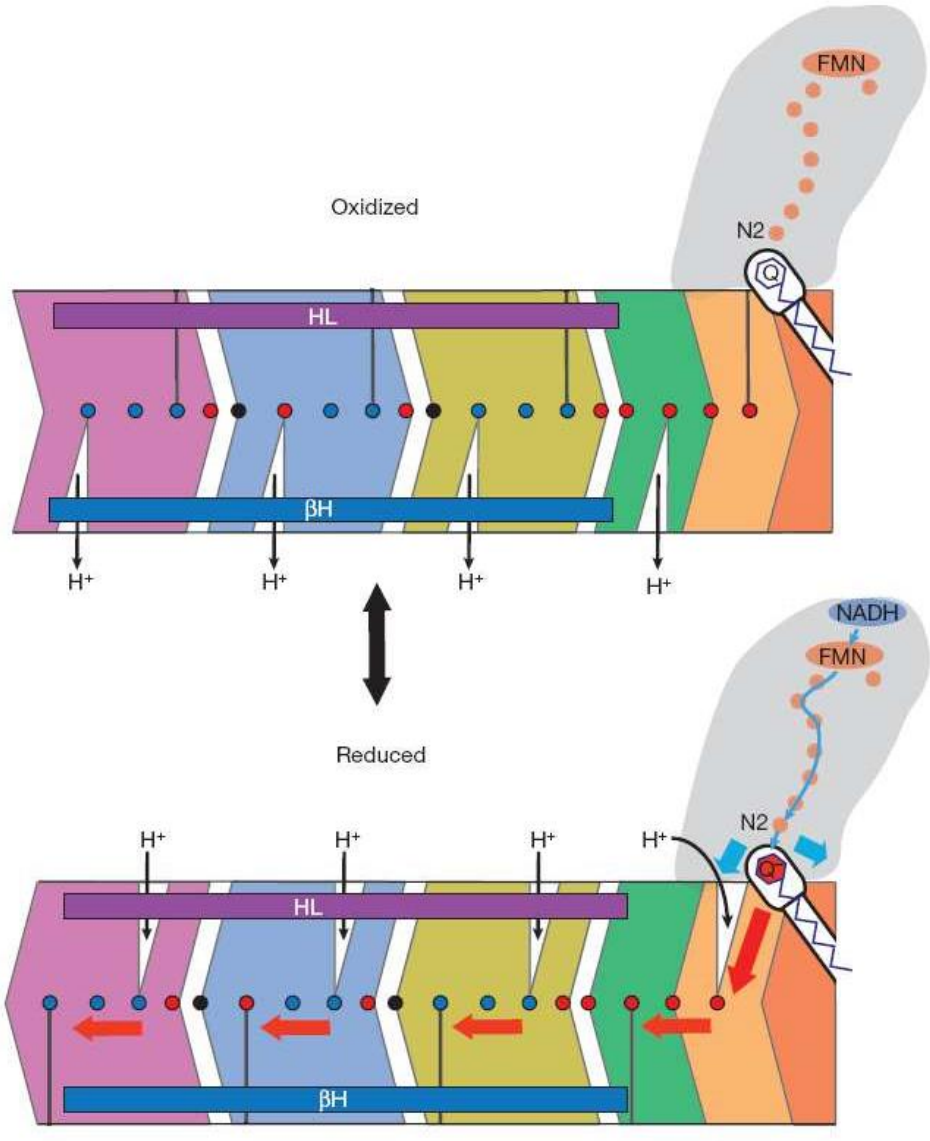
- Three antiporter-like proton pumps in hydrophobic arm: Nqo12, Nqo13, Nqo14. The fourth proton pumped at channel formed around Nqo8 at the hydrophobic/hydrophilic interface.

- Antiporter subunits contain pairs of symmetry-related five-helix bundles that serve as half-channels. Each contain Glu, His, or Lys residues that serve as protonation sites. Together create a central membrane-embedded axis of polar residues in a river of water.



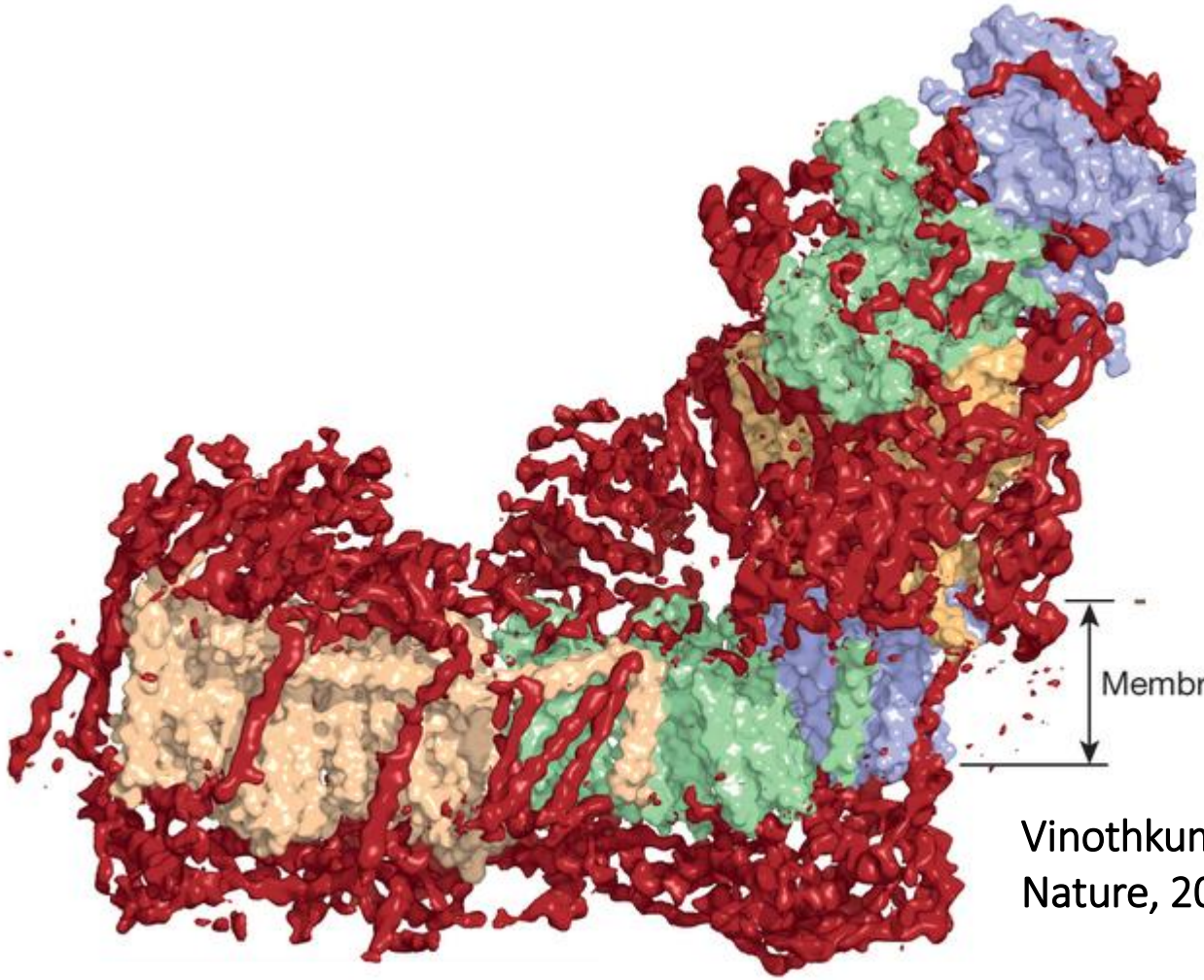
Proposed mechanism of coupling

- Lack of redox groups in hydrophobic domain shows coupling of e^- transfer to proton translocation must depend on long-range conformational changes.
- Ubiquinone dehydrogenation at tight binding site at arm interface results in changes in electrostatic interactions at the fourth (Nqo8) proton channel.
- Conformational changes propagate to antiporter-like subunits *via* central hydrophilic axis. Causes changes in solvent exposure and pK_a of key residues in half-channels resulting in proton translocation.



Cyro-EM of mitochondrial complex I

- Near-complete structure of mitochondrial complex I was solved to 5 Å by cryo-EM. Core subunits similar to bacterial complex I indicating conserved mechanism. However, supernumerary subunits also present (in red). Thought to help stabilise and protect enzyme.

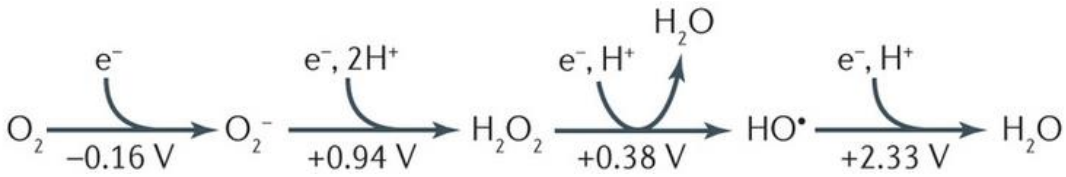


Vinothkumar et al., Nature, 2014

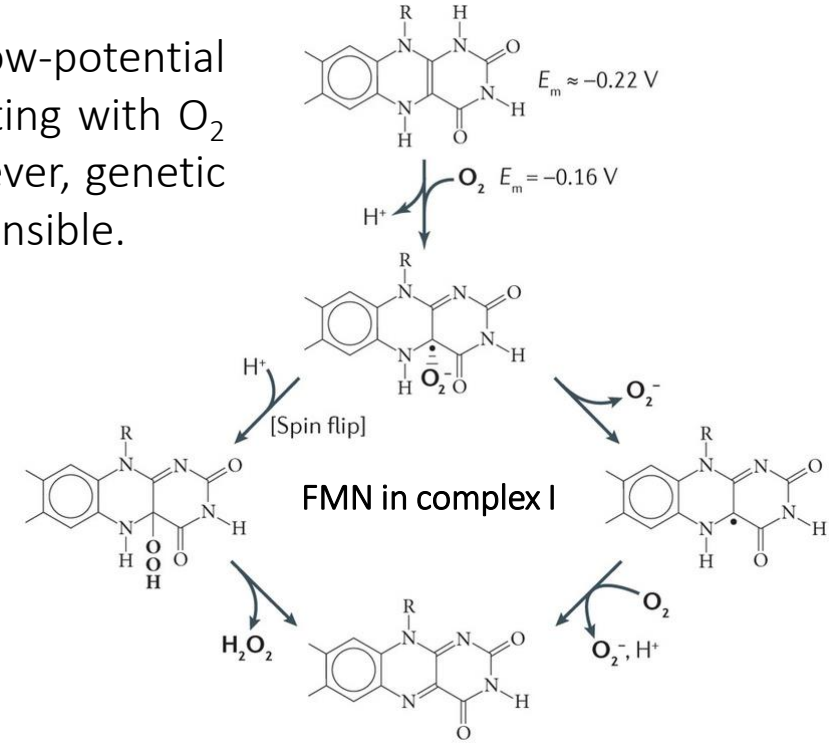
Leader: Judy Hirst

Complex I is the main source of oxidative stress

- Mitochondria are the main source of reactive oxygen species, i.e. superoxide ($O^{\bullet -}$), hydroxyl radicals ($OH^{\bullet -}$), and hydrogen peroxide (H_2O_2). These species cause oxidative DNA damage and are heavily implicated in ageing, cancers, and neurodegenerative diseases.



- Complex I is the main site of ROS generation. All low-potential sites capable of $1e^-$ reactivity are capable of reacting with O_2 to form $O_2^{\bullet -}$, i.e. FMN, FeS clusters, and UQ. However, genetic and biochemical studies suggest FMN mainly responsible.

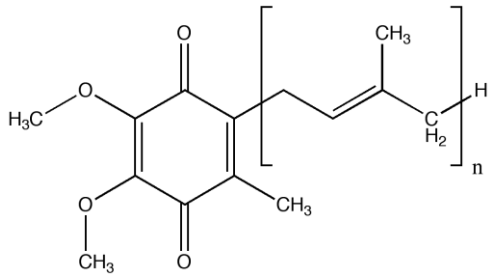


Environmental causes of Parkinson's disease

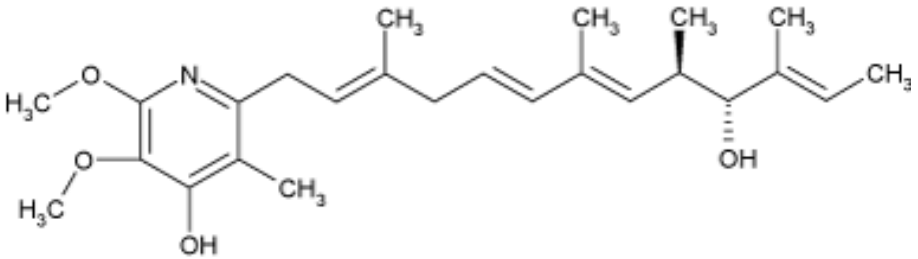
- Mitochondrial complex I dysfunction is the central cause of sporadic Parkinson's disease (PD) (Dawson et al., Science, 2003). Leads to ROS production that makes neurons vulnerable to glutamate excitotoxicity. Two main causes: environmental toxins and mitochondrial genetics.

- Multiple pesticides and toxins implicated in PD are specific inhibitors of Complex I, e.g. MPTP, paraquat, piericidin A, rotenone. Piericidin A and rotenone bind hydrophobic cavity of the ubiquinone-binding site to induce ROS probably by preventing e^- flow through complex.

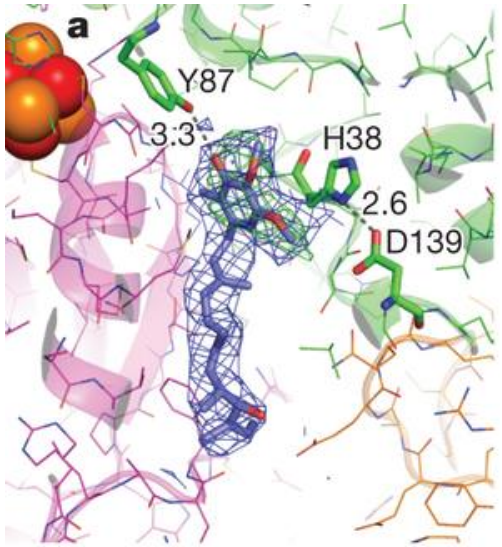
Ubiquinone:



Piericidin A:



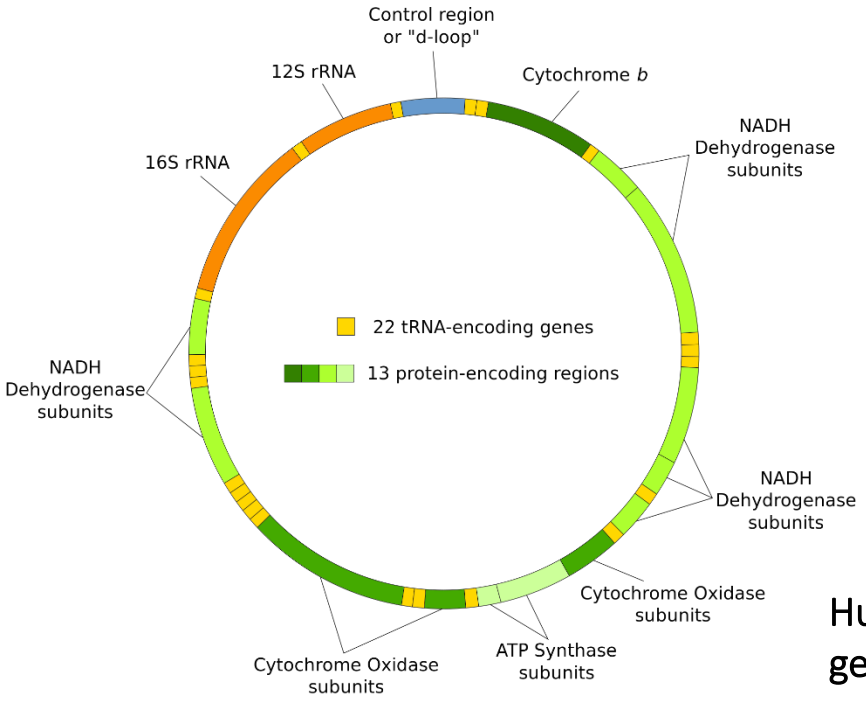
Irreversible competitive inhibition of ubiquinone-binding site by piercidin A:



Genetic causes of Parkinson's disease

- The 54 genes encoding mitochondrial Complex I are encoded between the two human genomes: the nuclear genome and the mitochondrial genome. mtDNA mutations more common due to lack of proof-reading and propagate due to maternal inheritance.

- Among 37 genes in human mtDNA, 7 encode proton-pumping subunits of Complex I. PD strongly linked to mutations in these genes, particularly *nqo8* gene, likely to decrease pumping efficiency. mtDNA mutations in complex I regulator α-synuclein also linked to PD.



Human mitochondrial genome map

Lecture 2: Respiration of organic compounds

I. Complex I

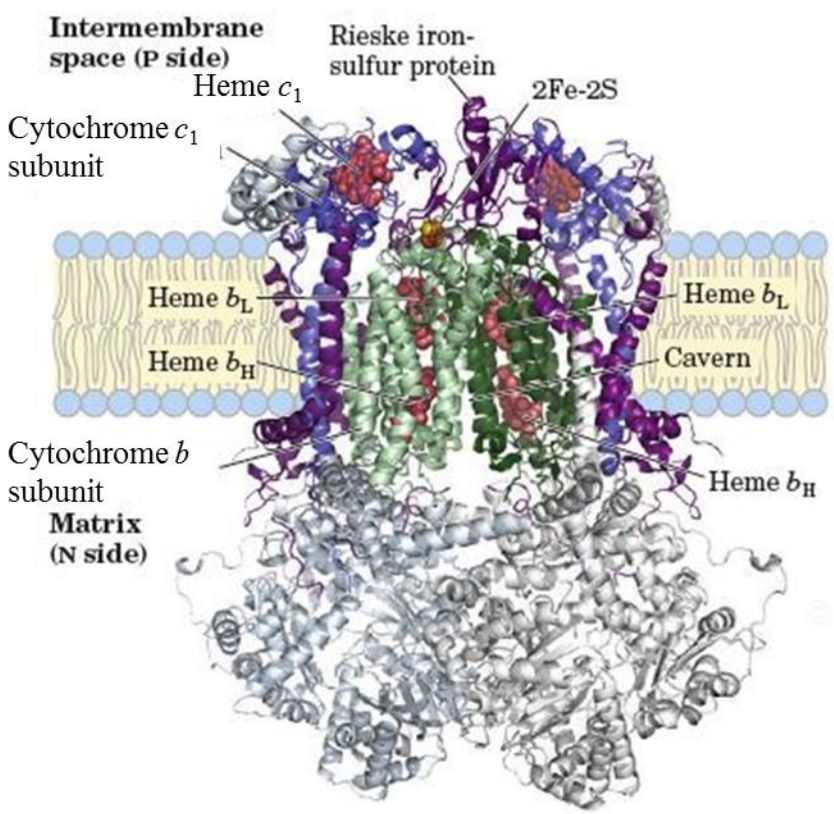
II. Complex III: bifurcating electrons

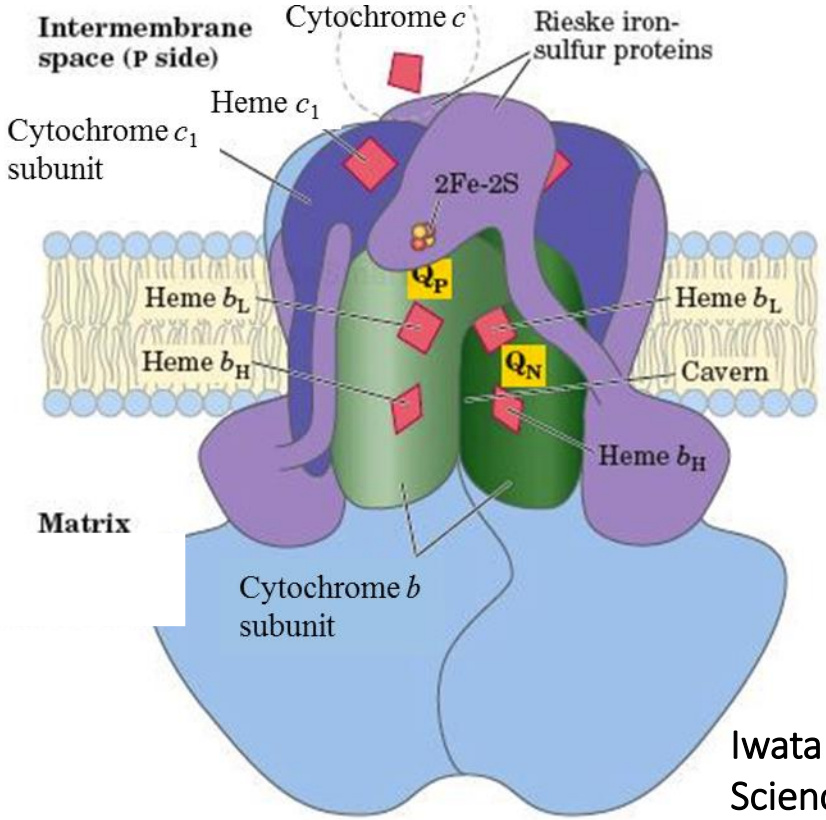
III. Complex IV

IV. ETC plasticity

Complex III: Cytochrome bc_1 complex

- Complex III forms of a dimer. The number of subunits in each monomer range from 3 in some bacteria to 11 in mitochondria. In all cases, three subunits participate in catalysis:
  - Cytochrome *b* subunit (green): contains two hemes (b_L, b_H) two ubiquinone binding sites (Q_P, Q_N)
  - Cytochrome c_1 subunit (dark blue): contains heme c_1, cytochrome *c* binding site
  - Iron-sulfur protein subunit (ISP; purple): contains [2Fe2S] cluster





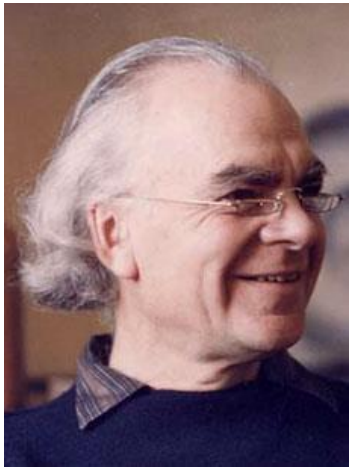
Iwata et al., Science, 1998

Complex III is an electron-bifurcating enzyme

- Peter Mitchell made a bizarre discovery on Complex III. Addition of the specific Q_P site inhibitor antimycin caused the complex to become disproprotionated. The heme *c* groups became oxidised as expected, whereas the heme b_L and b_H groups became reduced.

- On this basis, Mitchell proposed that electrons flowed nonlinearly through the complex in what he called the Q-cycle. The essence of this cycle is that electrons originating from the same donor are bifurcated, i.e. they flow in different directions.

- Theory was again controversial at its time, but late proven with elegant kinetic and structural studies. Once again, Mitchell's crackpoint ideas turned out to be right.

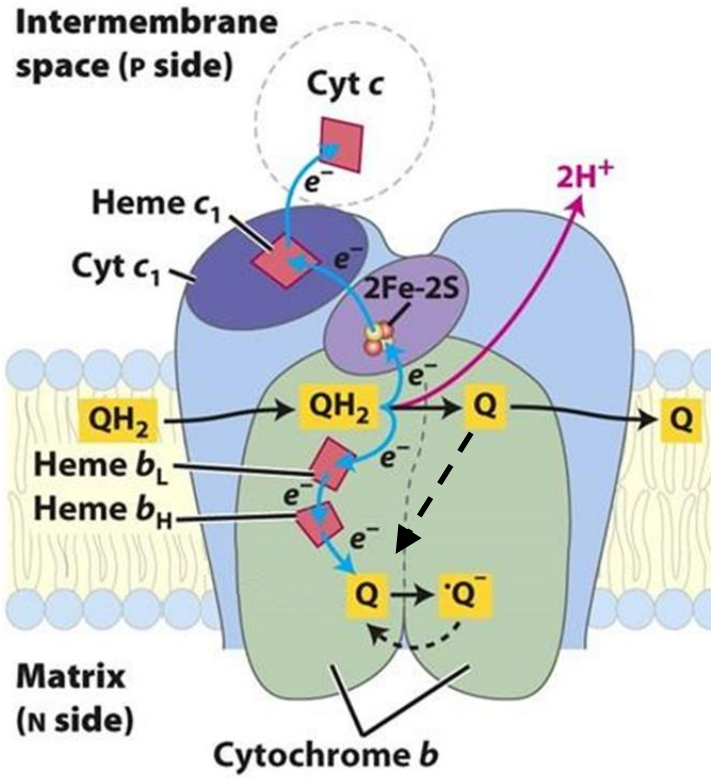


The electron-bifurcating step

- Following binding of UQH_2 at Q_P side, each of the two electrons is simultaneously transferred in different directions:

Electron transfer a: ½ UQH_2 (Q_P) → [2Fe2S] → Heme c_1 → Cytochrome *c*

Electron transfer b: ½ UQH_2 (Q_P) → Heme b_L → Heme b_H → UQ (Q_N) (product: $UQ^{\bullet -}$)



Energetic principles of bifurcation

- Whereas electron transfer a is energetically-favourable, electron transfer b is not.

$UQ + 2e^- + 2H^+ \rightarrow UQH_2$ $\quad E°' = +0.06$ V

Heme b_L-$Fe^{3+} + 1e^- \rightarrow$ Heme b_L-Fe^{2+} $\quad E°' = -0.10$ V

$(Fe^{III})_2 + 1e^- \rightarrow (Fe^{III})_1(Fe^{II})_1$ $\quad E°' = +0.30$ V

$\frac{1}{2}\ UQH_2 + (Fe^{III})_2 \rightarrow \frac{1}{2}\ UQ + (Fe^{III})_1(Fe^{II})_1$ $\quad \Delta E°' = +0.30 - 0.060 = +0.24$ V

$\frac{1}{2}\ UQH_2$ + heme-b_L-$Fe^{3+} \rightarrow \frac{1}{2}\ UQ$ + heme-b_L-Fe^{2+} $\quad \Delta E°' = -0.10 - 0.060 = -0.16$ V

- However, when the electron transfers simultaneously occur, the free energy change of the exergonic reaction can drive the endergonic reaction.

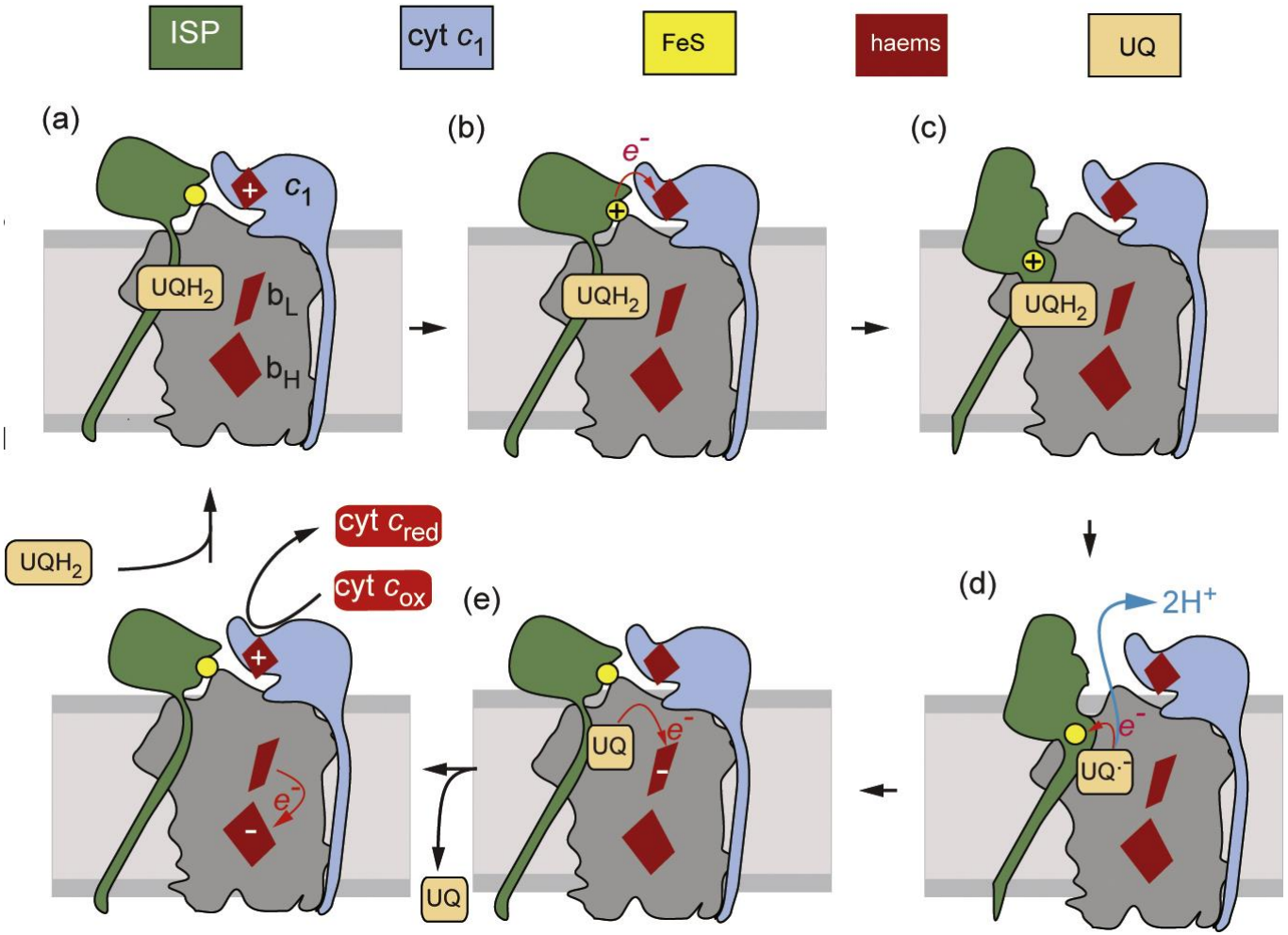
UQH_2 + heme-b_L-$Fe^{3+} + (Fe^{III})_2 \rightarrow UQ$ + heme-b_L-$Fe^{2+} + (Fe^{III})_1(Fe^{II})_1$

$\Delta E°' = -0.10 + 0.30 - 0.06 = +0.14$ V

$\Delta G°' = -2 \times 96.5 \times 0.14 = -27$ kJ mol^{-1}

Structural basis of bifurcation

- Comparison of crystal structures reveals that the iron-sulfur protein is highly flexible. It docks towards the Q_p when [2Fe2S] is oxidised and moves towards cyt c_1 when [2Fe2S] reduced.



Zhang et al., Nature, 1998

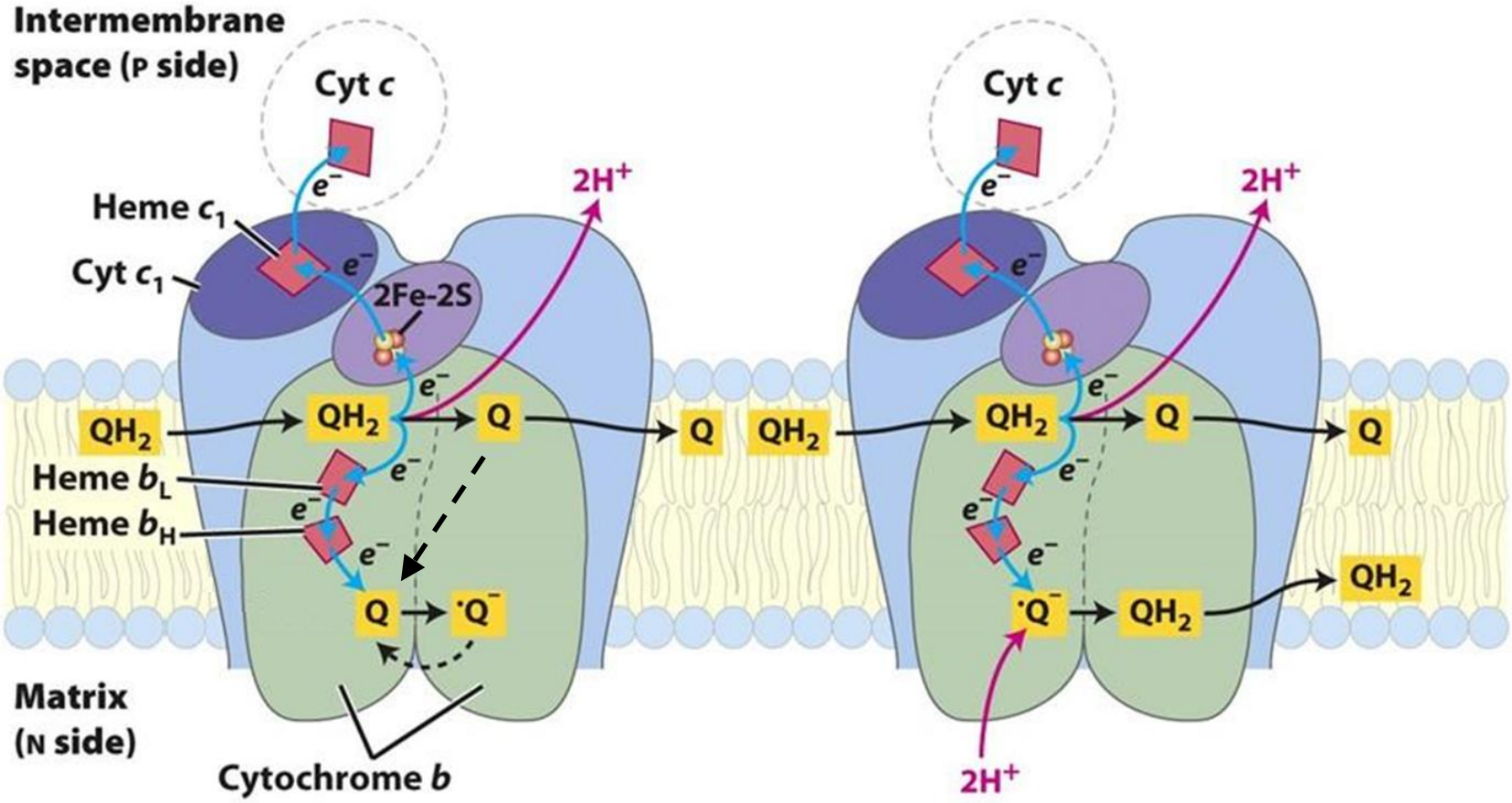
Structural basis of bifurcation

- It is proposed that ubiquinol initially donates its first e^- to oxidised [2Fe2S]. This initiates the iron-sulfur protein to move 20 Å towards cyt c_1 and hence become too distant to accept the second electron. Ubiquinol therefore forced to donate second e^- to heme b_L.

- Note these events happen at a sub-millisecond timescale leading to near-simultaneous e^- transfer. EPR shows semiquinone formation very transient and destabilised to prevent ROS.

- Equivalent electron-bifurcation processes are now known to drive many otherwise unfavourable redox processes. Only possible if the e^- donor can donate two electrons in different directions and is stable in semiquinone state, e.g. FAD, FMN, and quinones.

Completing the cycle



$$QH_2 + Q + \text{cyt } c \text{ (oxidized)} \longrightarrow \cdot Q^- + Q + 2H_P^+ + \text{cyt } c \text{ (reduced)}$$

$$QH_2 + \cdot Q^- + 2H_N^+ + \text{cyt } c \text{ (oxidized)} \longrightarrow QH_2 + 2H_P^+ + Q + \text{cyt } c \text{ (reduced)}$$

$$\text{Net equation: } QH_2 + 2 \text{ cyt } c \text{ (oxidized)} + 2H_N^+ \longrightarrow Q + 2 \text{ cyt } c \text{ (reduced)} + 4H_P^+$$

Q-cycle facilitates charge displacement

- In contrast to Complex I, protons are not directly pumped by Complex III. Instead, charge is displaced through a scalar mechanism. Benefit of this convoluted mechanism is that it provides a scalar mechanism to transfer +ve charge from N side to P side against a strong Δp.

- For every $2e^-$ transferred, four protons are released into the P-side. There are additionally two e^- transferred from the P-side to the N-side leading to two protons being taken up from N side. Results in an effective $2H^+/2e^-$ stoichiometry ($4H^+/2e^-$ in redox loop with Complex IV).

- There is evidence that the homodimer formation serves a mechanistic purpose as well as structural one. Strong evidence of quinones funnelled between the closely-packed monomers. Electron transfer between monomers also possible but unproven.

Lecture 2: Respiration of organic compounds

I. Complex I

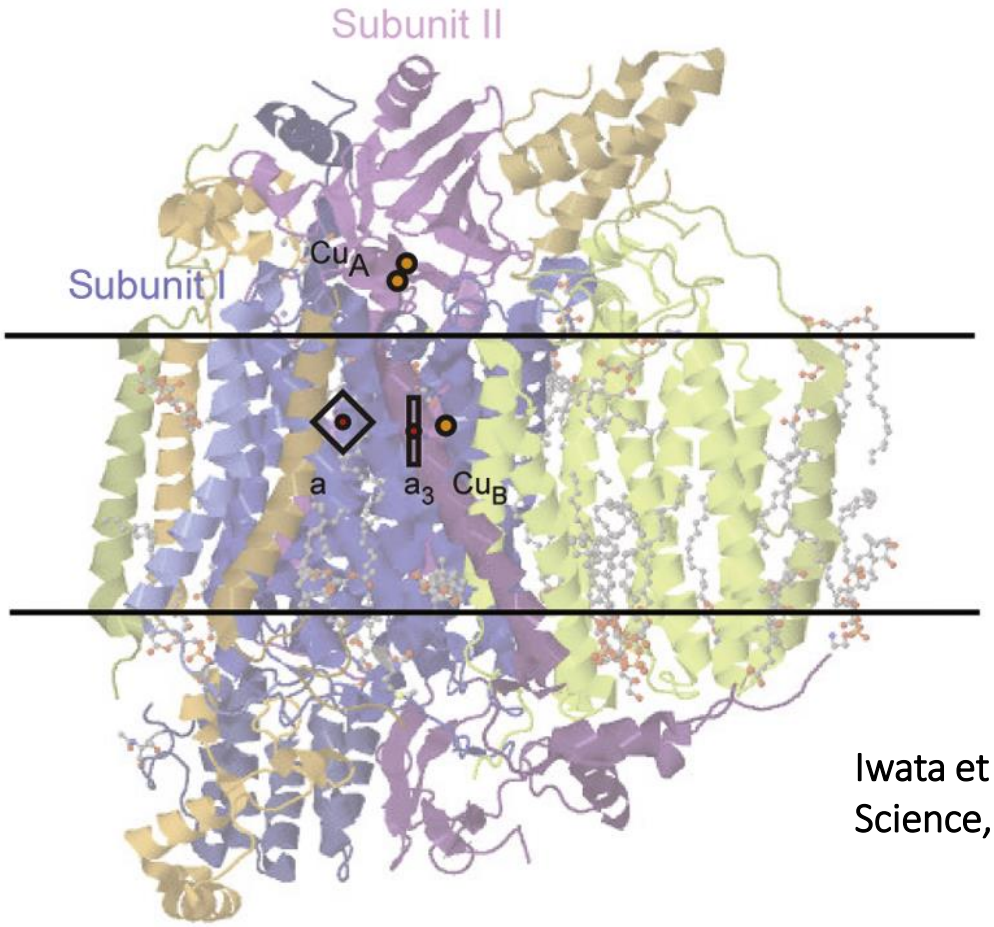
II. Complex III

III. Complex IV: reducing O_2

IV. ETC plasticity

Complex IV: Cytochrome *c* oxidase

- Complex IV couples the $4e^-$ reduction of the terminal e^- acceptor O_2 to the translocation of 4 protons ($4H^+/2e^-$). There are just two catalytic subunits. As with Complex I and III, the mitochondrial enzyme has supernumerary subunits, 11 in total.

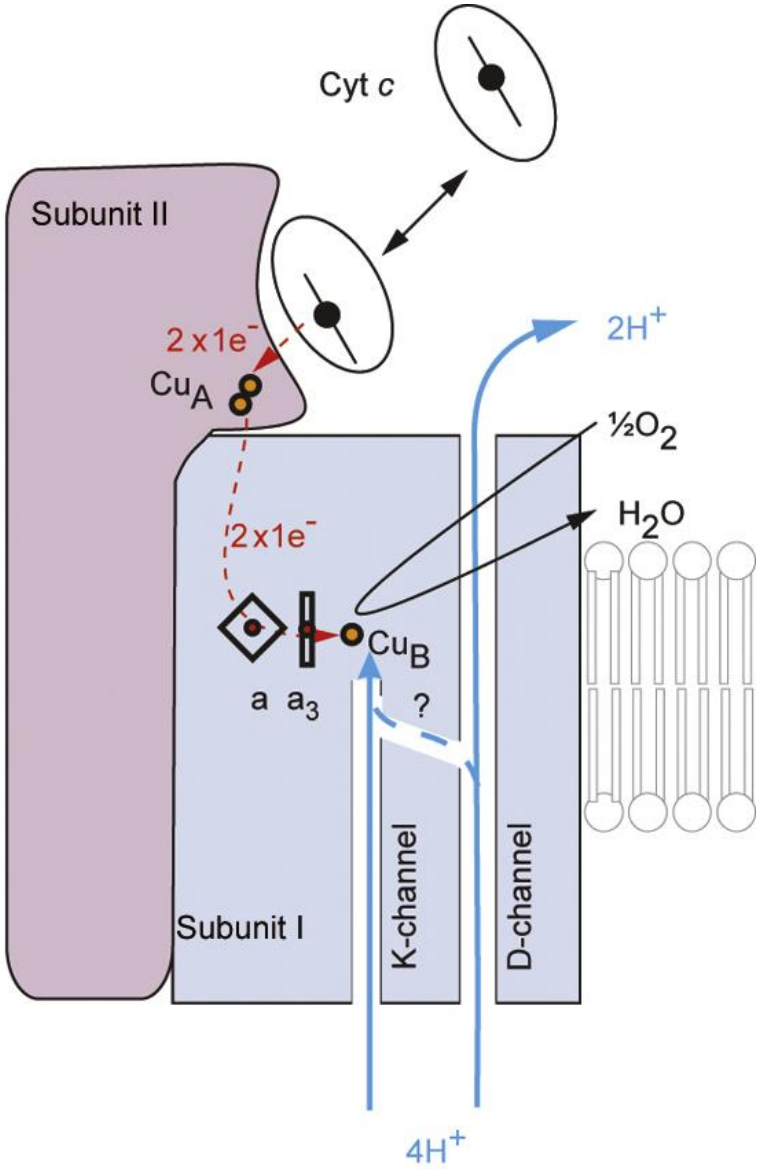


Iwata et al.,
Science, 1995

Electron transport through Complex IV

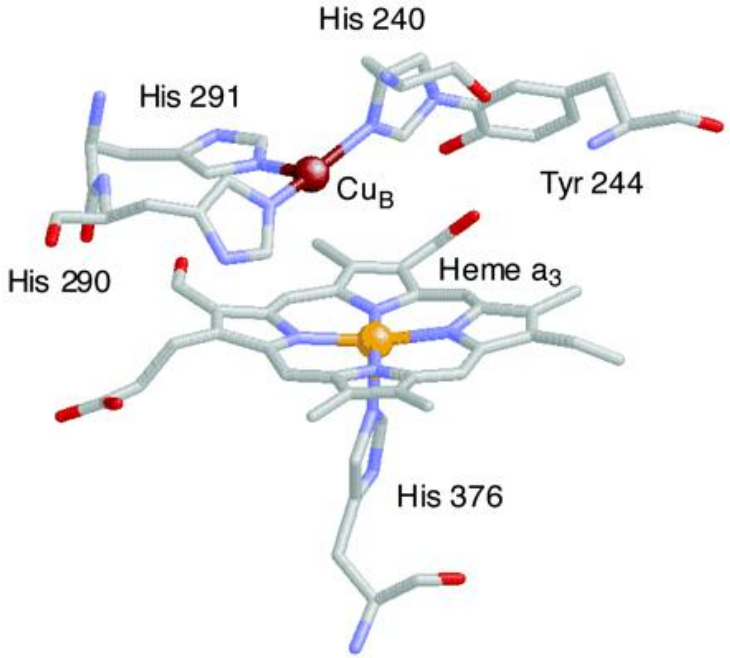
- There are four redox centres in Complex IV:
  - Subunit II: Contains two transmembrane helices and a globular domain that projects into P-phase. Globular domain contains binuclear copper site Cu_A and can transiently bind cytochrome *c*.
  - Subunit I: Contains eight transmembrane helices, three possible channels (K, D, and H), and three prosthetic groups (heme *a*, heme a_3, and mononuclear copper site Cu_B).

- Electrons all flow one-by-one through Complex IV in sequence: cyt *c* → Cu_A → heme *a* → heme a_3 / Cu_B. All sites in favourable distances (< 14 Å).



A bimetallic active site for O_2 activation

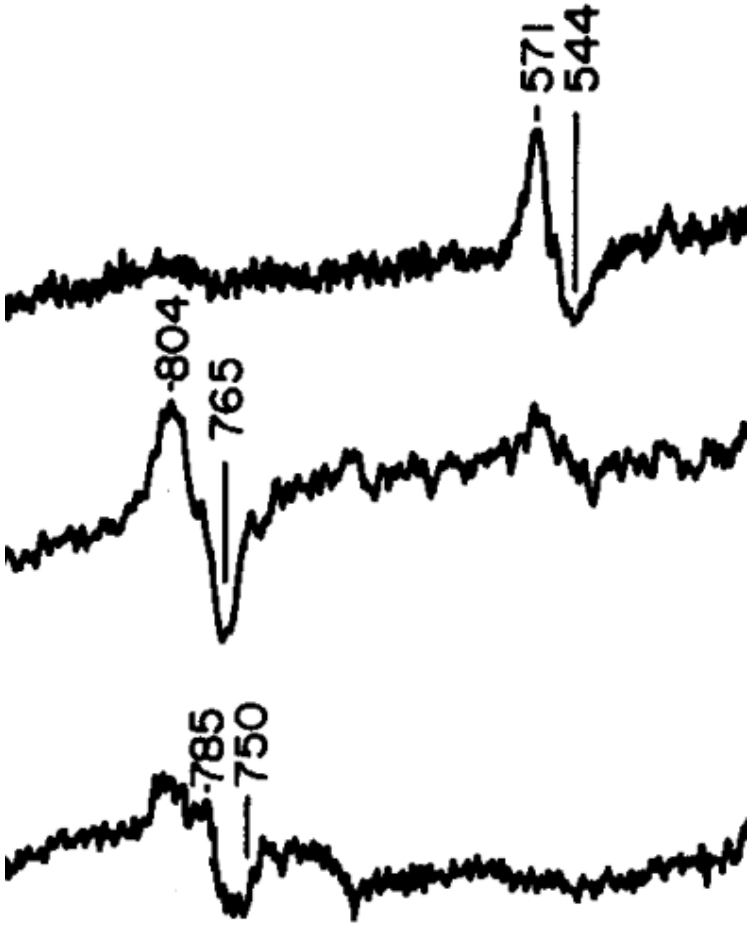
- Cytochrome *c* oxidase contains a unique bimetallic site for O_2 binding and reduction:
  - Heme a_3: contains a free axial ligand which serves as the coordination site for O_2
  - Cu_B: ligated by three histidine residues, contains a free ligand which binds reaction intermediate
  - Tyrosine: one of the Cu_B histidine ligands is covalently crosslinked to a distant tyrosine



- Due to differences in π-backbonding, CO, NO, and CN^- have a much stronger interaction with heme a_3 than O_2. Cyanide is a potent respiratory poison and works by Complex IV inhibition.

Resolving the catalytic cycle

- Time-resolved resonance Raman spectroscopy experiments using $^{16}O=^{18}O$ and $^{18}O=^{18}O$ detected distinctive bands in difference spectra corresponding to known Fe-O stretch bands. On this basis, three probable reaction intermediates were determined:



P species has 571/544 peaks corresponding to Fe^{II}-O_2 stretch.

Oxy

$a_3^{2+}-O_2$

Cu_B^{1+}

his - tyrH

P species has 804/765 peaks corresponding to Fe^{IV}=O stretch in more oxidised environment.

'P' species

$a_3^{4+}=O$

$Cu_B^{2+}-OH^-$

his - tyr•

F species has 785/750 peaks corresponding to Fe^{IV}=O stretch in more reduced environment.

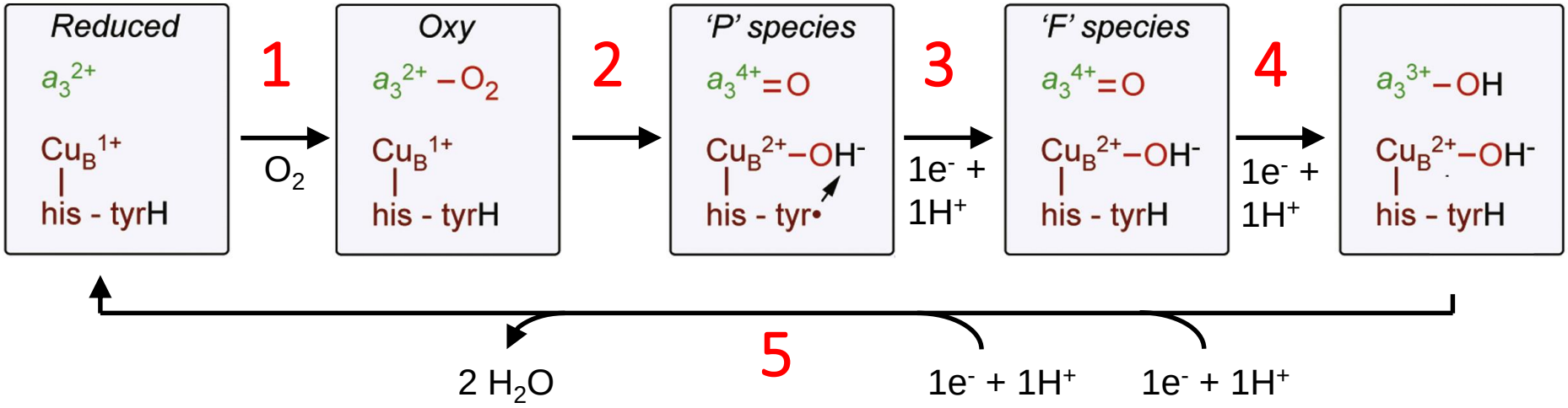
'F' species

$a_3^{4+}=O$

$Cu_B^{2+}-OH^-$

his - tyrH

The deduced reaction mechanism



1. All redox centres of the complex are in fully reduced state. O_2 binds at heme a_3.
2. O_2 reductively cleaved. Four electrons are donated: two from heme a_3 (Fe^{II} → Fe^{IV}), one from Cu_B (Cu^{I} → Cu^{II}), one from tyrosine (TyrH → $Tyr^{\bullet -}$). Heme a_3^{4+}=O and Cu_B^{2+}-OH^-.
3. The tyrosine is restored through delivery of $1e^-$ from cytochrome *c* and $1H^+$ from N-phase.
4. The heme a_3 is reduced (Fe^{IV} → Fe^{III}) through delivery of $1e^-$ from cytochrome *c* and $1H^+$ from N-phase.
5. The successive delivery of two further electrons and protons results in the ejection of H_2O. Leads to reduction of Cu_B (Cu^{II} → Cu^{I}) and heme a_3 (Fe^{III} → Fe^{II}) restoring complex back to reduced state.

Proton translocation pathways

- The last major question concerning Complex IV is how it couples electron transport to proton translocation. This occurs through two simultaneous mechanisms for a $4H^+/2e^-$ ratio:

  - Scalar translocation ($2H^+/2e^-$): The oxidised intermediates are reduced in $1e^- + 1H^+$ reactions. Electrons transferred from cytochrome *c* at the P-side. Protons are taken up from the N-side. This results in the effective translocation of $2H^+$ per $2e^-$ concomitant with H_2O production. Two water-filled, glutamate-lined cavities, the D and K channels, directly link the N side to the heme a_3 site.

  - Vectorial translocation ($2H^+/2e^-$): Like Complex I, Complex IV pumps protons through membrane. Exactly how is unclear and numerous mechanisms described (Yoshikawa et al., Chem. Rev., 2015).

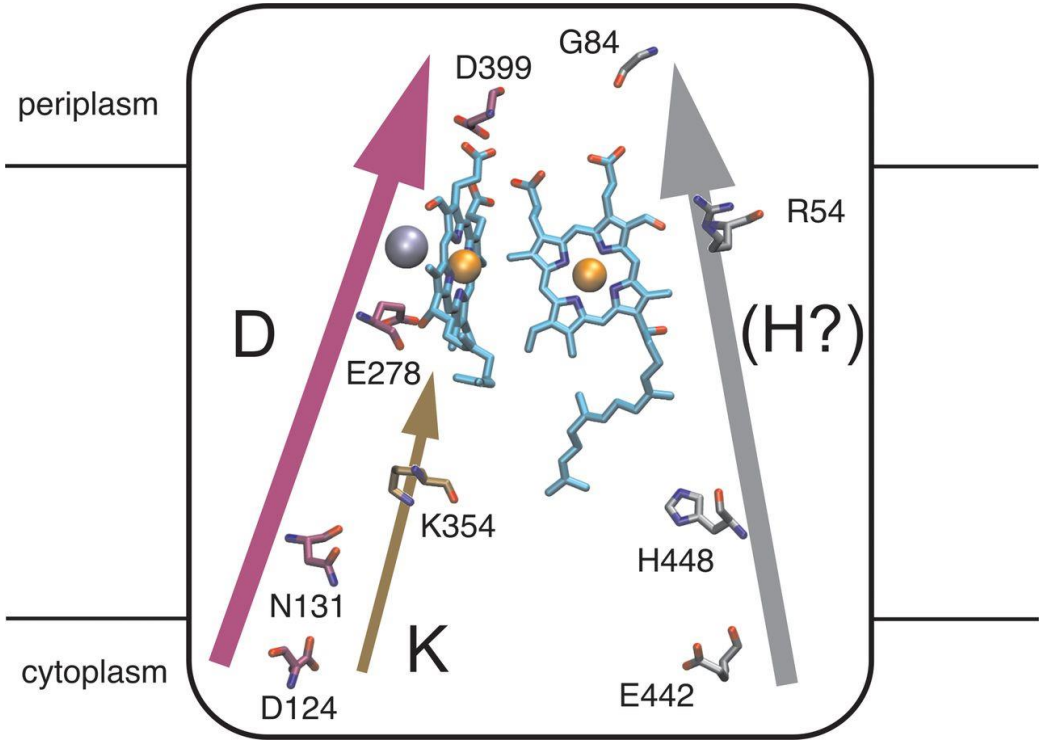
Proton pumping pathway is unresolved

D pathway hypothesis:

Protons pumped through D channel that passes through catalytic site. Redox chemistry at catalytic site changes pK_a of key Glu and Asp residues predicted to carry protons. Movements of bound water molecules may be important.

H pathway hypothesis:

Protons pumped through a possible side channel distinct from catalytic site. Redox chemistry at catalytic site causes conformational changes that drive proton pumping. Propionate side chain of heme *a* may serve as proton carrier.



Lecture 2: Respiration of organic compounds

I. Complex I

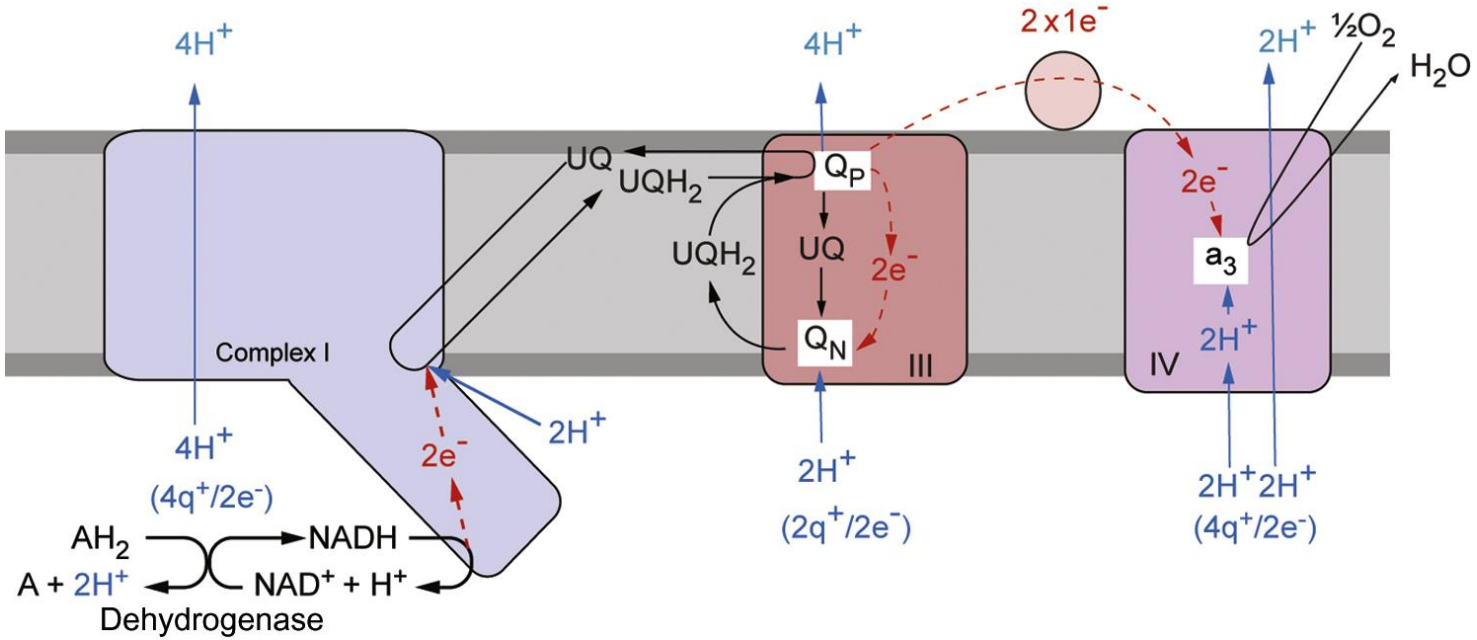
II. Complex III

III. Complex IV

IV. ETC plasticity

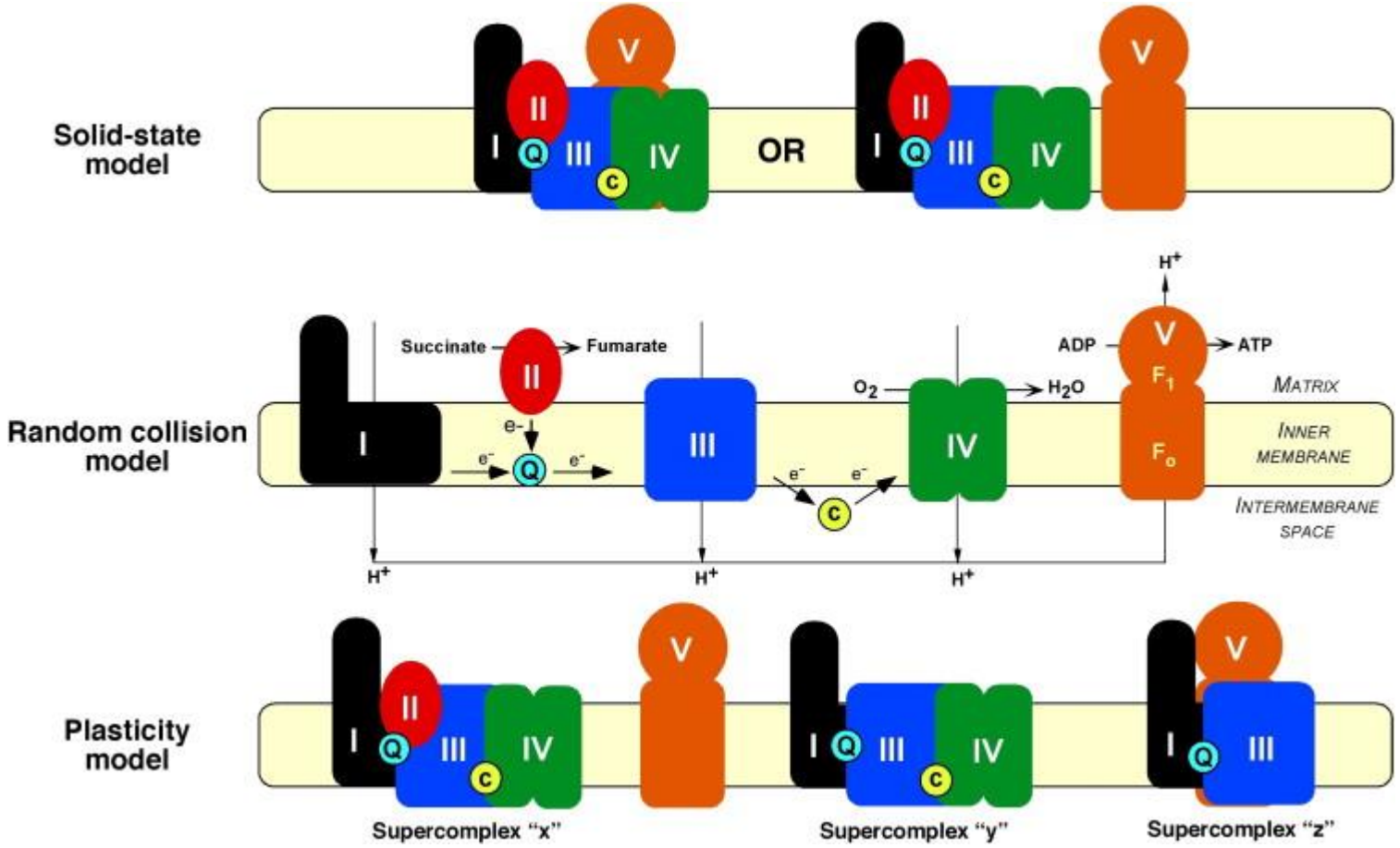
Counting up the protons

- In total, the equivalent of ten protons are translocated for every two electrons passed through the main mitochondrial electron transport chain ($10H^+/2e^-$). Six protons are pumped directly. Charge displacement at complex III in concert with IV leads to four protons moved.



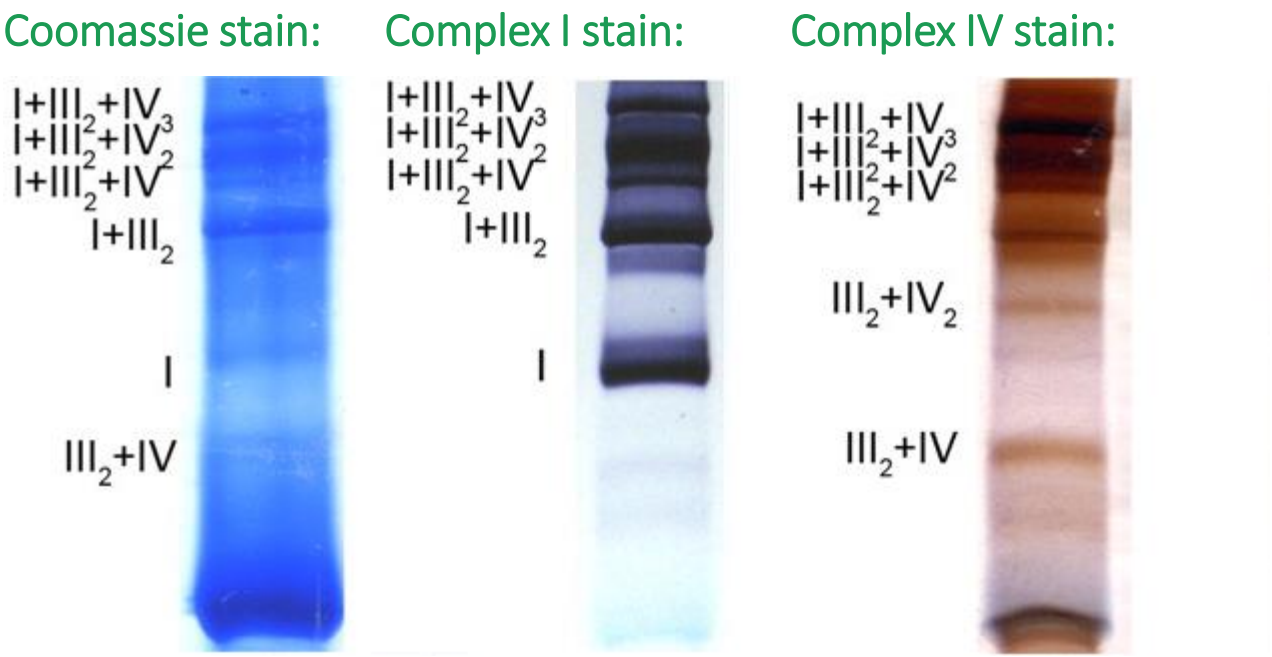
ETC supercomplex formation

- Three competing models of how the ETC is organised. Until recently, it was thought that ubiquinone and cytochrome *c* funnelled electrons between separated complexes by random diffusion. However, some recent studies that supercomplex formation can occur.



Respiratory supercomplex formation

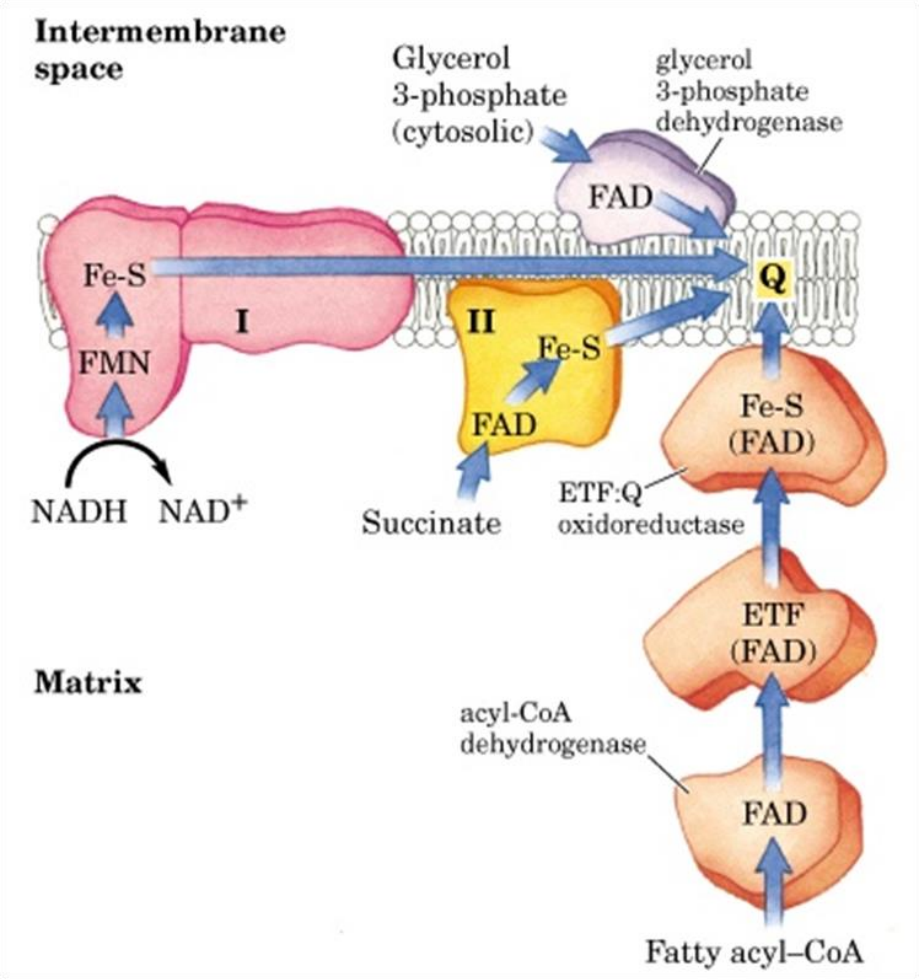
- Blue native polyacrylamide gel electrophoresis separates detergent-solubilised membrane proteins under nondenaturing conditions. This shows that a large proportion of the mitochondrial ETC complexes can associate into supercomplexes in optimally folded cristae.



- Conflicted evidence on functional significance of I + III_2 + IV_{1-4} supercomplex. It has been hypothesised that supercomplex formation may enhance rates and reduce ROS formation. Genetic and biochemical evidence, but contradicted by two recent kinetic studies.

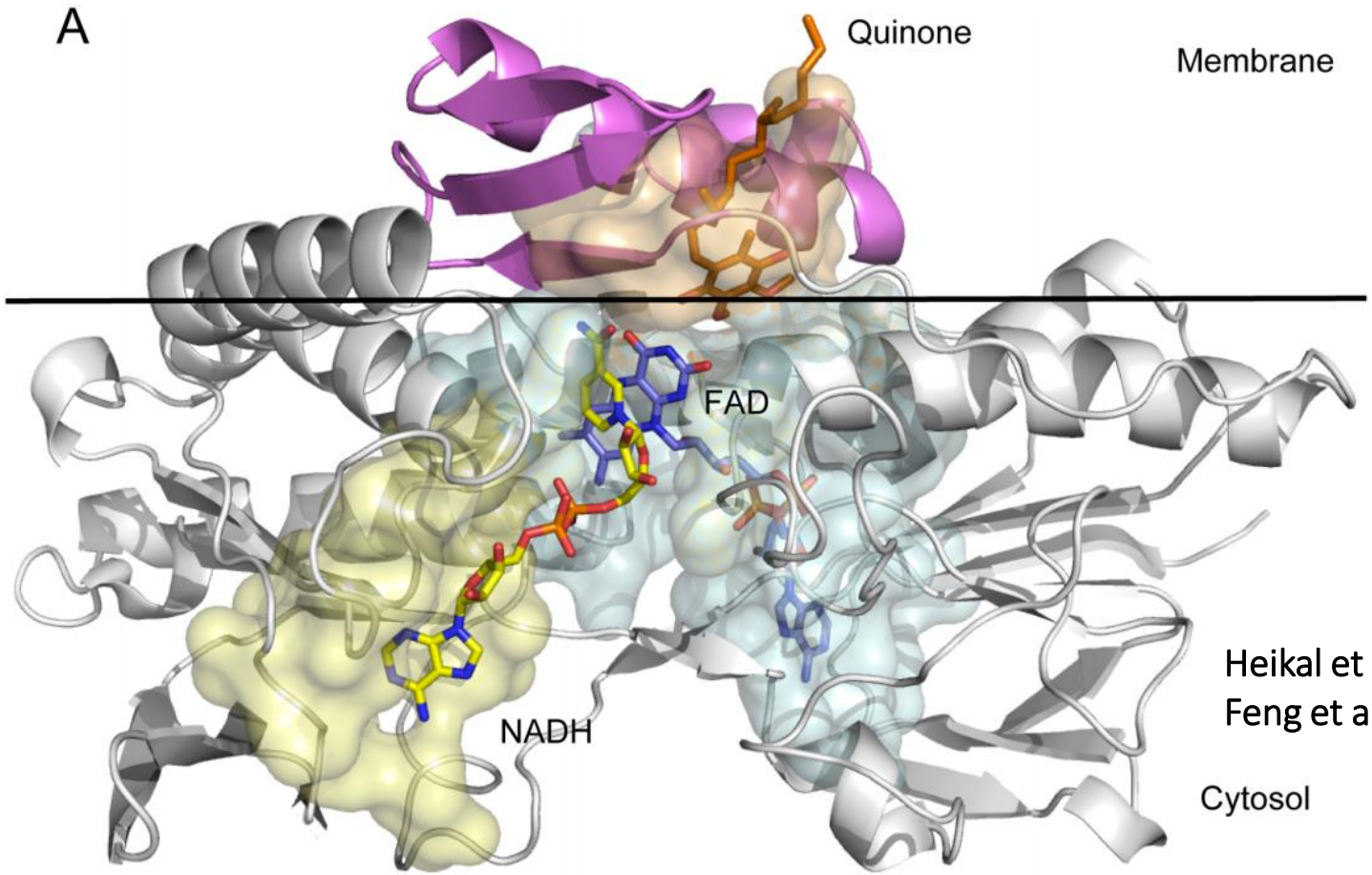
Not all primary dehydrogenases pump protons

- Other than Complex I, there are four primary dehydrogenases that input e^- derived from organic carbon oxidation into UQ pool:
  - Succinate dehydrogenase
  - ETF dehydrogenase
  - Glycerol 3-phosphate dehydrogenase
  - NADH dehydrogenase type II
- These do not pump protons, hence their ETC has overall $6H^+/2e^-$ ratios for their ETC. For first three complexes, the free energy change of e^- transfer is too low for proton pumping.



What about NADH dehydrogenase type II?

- NADH dehydrogenase type II (NDH2) is a monotopic membrane protein physically incapable of proton pumping. Absent from mammalian mitochondria, but present in fungi, plants, unicellular eukaryotes, and bacteria. Despite lower efficiency than Complex I, it has potential advantages such as lower ROS production and insensitivity to backpressure.

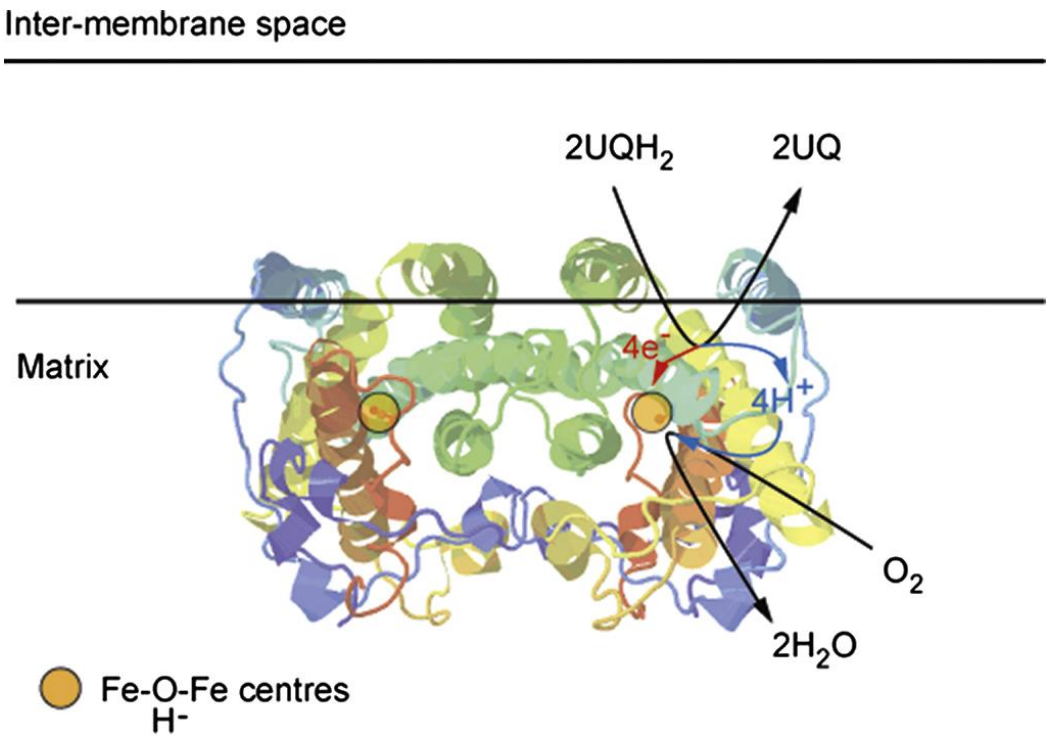


Heikal et al., Mol Micro 2014
Feng et al., Nature 2012

Not all terminal oxidases pump protons

- Most plants, fungi, and lower eukaryotes also contain an alternative oxidase (AOX) that couples UQH_2 oxidation to O_2 reduction. It is a monotopic protein with a diiron catalytic site.

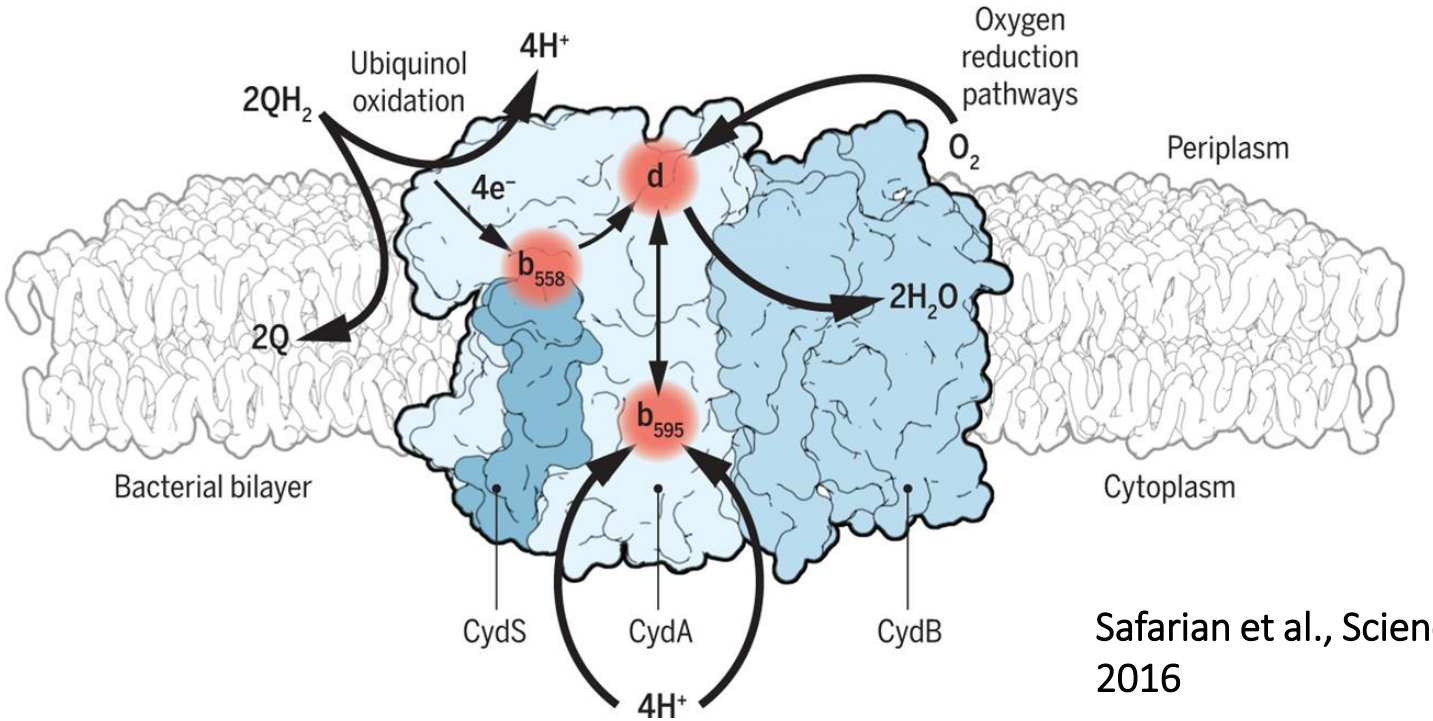
- Like NDH2, as a monotopic protein it does not directly generate Δp. Translocation efficiencies of $6H^+/2e^-$ when coupled to Complex I and $2H^+/2e^-$ when coupled to NDH2. Main functions are oxidative stress defences and thermogenesis (heat generation).



Shiba et al., PNAS, 2013

Tradeoffs of efficiency and affinity

- Many bacteria contain multiple terminal oxidases, which enable them to switch between aerobic and hypoxic growth. *E. coli* contains two distinct oxidases:
  - Cytochrome bo_3: A heme-copper oxidase functionally analogous to Complex IV but with modified hemes. More efficient oxidase ($4H^+/2e^-$) but micromolar affinity. Preferred aerobically.
  - Cytochrome *bd*: A unique bacterial complex. Cannot directly pump protons ($2H^+/2e^-$) but has a nanomolar affinity. Preferred and upregulated microaerobically.



Safarian et al., Science, 2016

Lecture summary

- The mitochondrial electron transport chain generates Δp by efficiently coupling electron transfer, from NADH to O_2, to vectorial and scalar proton translocation ($10H^+/2e^-$).
- Complex I is a multimeric complex that uses the energy yielded during an electron transfer cascade to transmit large-scale changes to four transmembrane proton pumps.
- Complex III operates through a Q-cycle by bifurcating the electrons from UQH_2. This facilitates charge displacement through a scalar mechanism.
- Complex IV uses an elaborate bimetallic centre to activate and reduce O_2. How this complex pumps protons, in contrast to other terminal oxidases, remains to be understood.
- Plasticity in electron transport chains enables plants and microbes to balance e^- flux and proton translocation in response to environmental change (e.g. hypoxia, oxidative stress).

Essay Question

Describe how transmembrane complexes use redox reactions to generate electrochemical gradients

Recommended reading

Recommended reading:

Nicholls DG & Ferguson SJ (2015). Bioenergetics 4. Elsevier Press.

Comprehensive, up-to-date textbook on bioenergetics.

Sazanov A (2015). A giant molecular proton pump: structure and mechanism of respiratory complex I. Nat Rev Mol Cell Biol 16, 375-388.

A solid up-to-date review linking structure and function from the leader in Complex I field.

All available for download at greeninglab.com