Proton-motive force, ATP synthetase, Coupling

I. Protonmotive force
A. Proof of proton gradient formation coupled to electron flow

Although scientists knew that electron transport led to the synthesis of ATP, for a long time they did not know how. Most people working on the question assumed that the mechanism would be similar to ATP production in glycolysis, and would therefore involve an intermediate with a high energy phosphate bond (substrate level phosphorylation). However, no one could find any evidence of such an intermediate.

The problem was solved by Peter Mitchell, who entered the scene with the radical proposal that the reason no one could find a high energy intermediate was because there wasn’t one; instead of using an intermediate compound, inorganic phosphate might be added directly to ADP by using the energy generated by setting up a gradient across the mitochondrial membrane.

In order to prove his hypothesis, Mitchell showed first that electron transport was coupled to the flow of protons out of the mitochondria, thereby producing a proton gradient. His experimental system consisted of a test tube filled with intact mitochondria in an unbuffered solution of KCl in an anaerobic environment. To these mitochondria, he added a substrate for the electron transport process, and when he added an aliquot of O\textsubscript{2}, he observed a change in the pH of the solution.

The first substrate he added was β-OH-butyrate, a compound that can be oxidized in the mitochondria to produce NADH and thus will initiate electron transport at Complex I. Upon addition of O\textsubscript{2}, he observed a steep decrease in pH (seen as an increase in the graph below, because the y-axis represents negative ΔpH), which indicated that the extramitochondrial environment was becoming acidified.

However, when he added rotenone, a rat poison that inhibits complex I (where NADH is oxidized and the first protons are pumped out), this acidification was blocked. From this result he concluded that electron flow is coupled to the flow of protons out of the mitochondria, and he noted that the ratio of H\textsuperscript{+} pumped out per O added (H\textsuperscript{+}/O - not O\textsubscript{2}) seemed to be about 6. With refinement, the ratio has been increased to 8 – 10.
When Mitchell added succinate, which initiates electron transport at Complex II, he observed a similar but smaller decrease in pH upon addition of \( \text{O}_2 \), as could be expected from the fact that succinate bypasses Complex I and thus leads to the transport of fewer protons (he observed an \( \text{H}^+/\text{O} \) ratio of about 4; now seen to be about 6 - 8). Repeating the experiment with rotenone had no inhibitory effect, as can be explained by the same reasoning; rotenone blocks at Complex I, which succinate bypasses. However, adding antimycin A, a drug that blocks Complex III (downstream of succinate’s entry), did inhibit the succinate response. From these experiments, he concluded that, in conjunction with the extraction of electrons from each substrate, protons are sequentially pumped out at each of the three positions (complexes I, III, and IV) as shown on the diagram in the last set of lecture notes. Further experiments where individual complexes were incorporated into liposomes further confirmed these results.

**B. Membrane potential**

Normally, mitochondria are present as a dilute solution, so pumping protons out of the mitochondria does not change the external pH (pH = 7), but does lead to an increase in the intramitochondrial pH from pH 7 in the absence of \( \text{O}_2 \) to pH 9 in its presence. This concentration gradient of protons between the inside and outside of the mitochondria also sets up a membrane potential across the mitochondrial inner membrane; since positively charged protons flow out, the outside becomes positively charged with respect to the inside of the mitochondrial inner membrane.

The following experiment demonstrates this membrane potential. First, respiring mitochondria were allowed to equilibrate in 5 mM KCl until the KCl concentration was 5 mM both inside and outside the mitochondria. A \( \text{K}^+ \)-specific ionophore (valinomycin) was then added to make the membrane specifically permeable to \( \text{K}^+ \) ions, effectively poking \( \text{K}^+ \)-specific holes in the membrane. \( \text{K}^+ \) could then flow into the mitochondria to dissipate the membrane potential generated by the proton gradient. The \( \text{K}^+ \) level reached an internal concentration of 200 - 300 mM.

Since \( \text{K}^+ \) entry is driven by the charge gradient, the relative concentrations of \( \text{K}^+ \) in and out of the mitochondria in this experiment can be used to measure the magnitude of the membrane potential that drove them in. Since the system reaches equilibrium,

\[
\Delta G = 0 = \Sigma v_i \mu_i \]

where \( \Sigma v_i \mu_i \) is the sum of the product of the number of molecules in the reaction (1 in this case) and the chemical potential of the molecule (\( \text{K}^+ \) in and outside the membrane). So:

\[
0 = \mu_{\text{Kin}} - \mu_{\text{Kout}}
\]

The chemical potential of \( \text{K}^+ \) in general, in or outside, is given by the equation:

\[
\mu_k = \mu^\ast_k + RT \ln[K] + zF \psi ,
\]

where \( \mu^\ast \) is the standard chemical potential, \( z \) is the charge of the ion, \( F \) is the Faraday constant, and \( \psi \) is the electrical potential.
So: \( 0 = \mu_{K_{\text{in}}} - \mu_{K_{\text{out}}} \)
\[ = (\mu^* K_i + RT\ln[K_i] + zF\psi_i) - (\mu^* K_o + RT\ln[K_o] + zF\psi_o) \]

Since the standard chemical potential is the same for K\(^+\) inside and outside, these terms cancel out in the above equation. Since in general \( \ln[A] - \ln[B] \) equals \( \ln([A]/[B]) \), the difference between the \( RT\ln[K] \) terms is equal to \( RT\ln([K_i]/[K_o]) \). Finally, since \( z \) and \( F \) are the same for both terms, the difference between the final terms is \( zF(\psi_i - \psi_o) \). \( \psi_i - \psi_o \) is the overall membrane potential. Thus, the equation with these substitutions is:
\[ 0 = RT\ln([K_i]/[K_o]) + zF(\psi_i - \psi_o) \]
which rearranges to the **Nernst equation**,

\[- (\psi_i - \psi_o) = (RT/zF)\ln([K_i]/[K_o]) \]

Thus we have found a way to relate the membrane potential generated by the proton gradient to the concentration gradient of the permeable ion, K\(^+\). In this case, the K\(^+\) gradient has dissipated the membrane potential generated by the H\(^+\) gradient.

\[ (\psi_i - \psi_o) = - (600/23)(2.3)\log(200/5) = (600/23)(2.3)\log(5/200) = - 96 \text{ mV} \]

Thus, the membrane potential produced by the proton gradient is about - 100 mV (negative inside). This can now be verified by directly measuring the membrane potential of giant mitochondria with electrodes where membrane potentials of - 100 to - 200 mV (inside) are measured.

**C. Source of proton-motive force**

The proton-motive force consists of a concentration gradient and an electrical gradient and is the source of energy for ATP synthesis. Since the relevant reaction is
\[ \text{H}^+_{\text{out}} \rightarrow \text{H}^+_{\text{in}} \],
the proton-motive force can be written as the difference between the inside and outside chemical potentials for H\(^+\):

\[ \Delta G = \mu_{H_i} - \mu_{H_o} = RT\ln([H_i]/[H_o]) + zF(\psi_i - \psi_o) \]

Since pH inside is 9 and pH outside is 7, and \( (\psi_i - \psi_o) \) is -100mV, or - 0.1V,
\[ \Delta G = -2.8 \text{ kcal/mol} - 2.3 \text{ kcal/mol} = \text{ about } -5 \text{ kcal/mol for a proton flowing down its gradient into the mitochondria.} \]

**II. ATP synthetase**

As shown on next page and in [V&V p. 588 Fig. 20-29](#), the ATP synthetase, the complex responsible for ATP production, has two components, \( F_0 \) in the membrane and \( F_1 \) in the mitochondrial matrix.
The enzyme’s function is coupled to proton flow; if protons flow in, it synthesizes ATP, while ATP hydrolysis drives protons out. The $F_1$ is formed of three $\alpha\beta$ dimers and one each of $\gamma$, $\delta$ and $\epsilon$. (See V&V p. 586 Table 20-2 for subunit composition.) All $\alpha$ and $\beta$ subunits have nucleotide binding sites, but the $\alpha$ sites are not functional in ATP synthesis. The three $\alpha\beta$ dimers are arranged in a circle around a central $\gamma$ subunit (Mr 34,000), which is composed of two long alpha helices that form a vertical axis perpendicular to the plane of the $\alpha\beta$ dimer circle (see Fig. 20-30). The $F_0$ consists of $a$, $b_2$, and $c_{9-12}$ subunits. (see also Stator-Rotor Figure on web page).

Experiments have been done to visualize the rotation of $F_1$ upon ATP hydrolysis. In the experimental setup, the $\alpha\beta$ dimers were immobilized on a glass surface through histidine tags engineered at the N termini of the $\beta$ subunits. A fluorescently labeled actin filament was attached to the $\gamma$ subunit of $F_1$ (lacking $\delta$ and $\epsilon$ subunits) through a streptavidin-biotin link (see F1ATPase Figure on web page). When ATP was added, the filament rotated. The measured rate of rotation was about 4 rev/s. At low ATP concentrations, stepwise rotations of 120° were observed. A total of 12 H+ are moved through per 4 ATP molecules hydrolysed.

Just like for the Na+/K+ pump (which can be run in reverse), a conformational change must occur to transform the gradient (the vector quantity) into ATP (a scalar quantity). The $\gamma$ subunit can rotate in the middle of the $\alpha\beta$ dimers and is required to gear the $H^+$ flux to ATP synthesis. When ADP and Pi bind to the $\beta$ subunit, it is easy for them to combine to form ATP, but it is difficult for the ATP to dissociate from $\beta$. The pivoting of $\gamma$ in the middle of the $\alpha\beta$ dimers allows the $H^+$ to flow in the right direction to force a conformational change in the $\beta$ subunits which leads to the release of ATP.
The coordination of ATP synthesis in each αβ dimer by rotation of the γ subunit is illustrated in V&V p. 590, Fig. 20-31. As the γ subunit rotates, the dimers progress from an empty state, O (open, no ATP), to a loose state, L (ADP and Pi loosely bound), to a tight state, T (ADP and Pi are compressed, resulting in the formation of ATP), and back to the empty state when ATP is released. Since protons flowing down their gradient produce about 5 kcal/mol of free energy (as calculated above), approximately 2 to 3 protons are required to synthesize a molecule of ATP (which requires 10 -12 kcal/mol); the exact number is variable due to leakage of protons across the membrane independent of ATP synthetase activity.

III. Coupling

In native mitochondria, the electron transport and ATP synthetase systems are tightly coupled, that is, in order to have a large electron flow, ATP synthesis must be occurring. The following experiment illustrates this. If mitochondria are suspended in the presence of O₂ and β-OH-butyrate, then the O₂ concentration only decreases very slowly (this is due to the fact that the inner mitochondrial membrane is slightly leaky to protons). Only when ADP and Pi are added does the rate of O₂ consumption increase (its concentration drops). When the ADP and Pi are used up (fully converted to ATP), the rate of O₂ consumption again levels off. (See also Fig. 20-12 in V&V p. 572) Normally, the ratio of Pi to O (not O₂) consumed (called the P:O ratio) is about 2.5 to 3.

One exception to the coupling of these systems occurs in the brown fat of young animals. Due to the presence of another protein in the inner mitochondrial membrane through which protons can flow, protons are able to reenter the mitochondria without flowing through the ATP synthetase. Rather than contributing to ATP production, the energy released by the collapse of the proton gradient generates heat. Newborn and hibernating animals keep warm the same way.