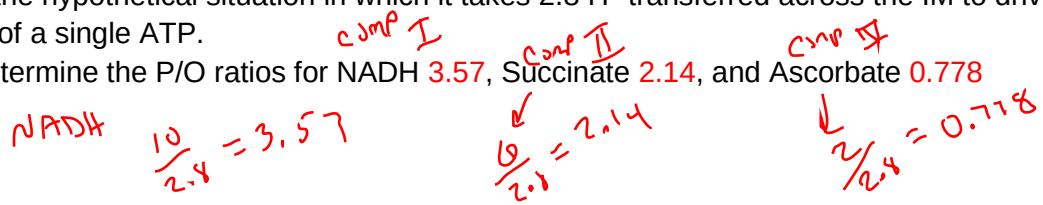
Problem Set 4 **(Due February 11th)**

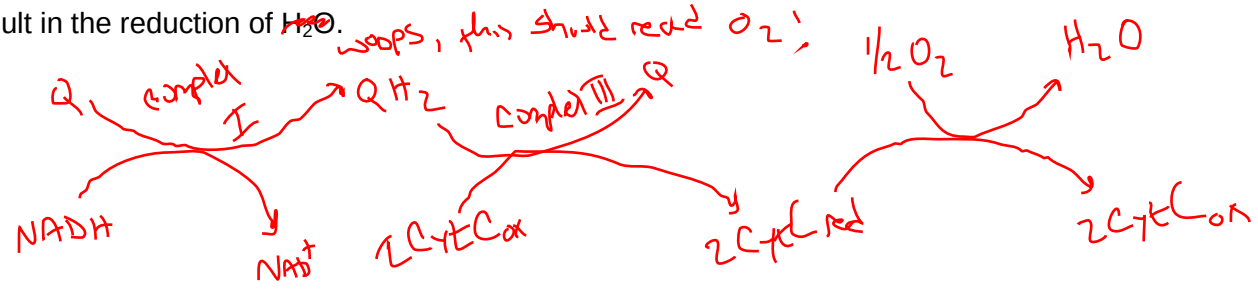
1. We discussed how a single equivalent of ATP is produced by pumping 4 H^+ from the matrix to the IMS.
   a. Based on this observation, justify the P/O ratios for NADH, Succinate, and Ascorbate. P/O ratio is the number of ATP made per oxygen atom reduced. When NADH is the electron source, a total of 10 H+ are pumped to the IMS (4 from Complex I, 4 from Complex 3, 2 from Complex 4), so 2.5 ATP for every Oxygen atom reduced (1 oxygen atom is reduced for every 2 electrons) can be made. When Succinate is the electron source, we lose out on the 4 protons from Complex 1 and have a total of 6 H+. This results in 1.5 ATP made per succinate (succinate also provides 2 electrons). When Ascorbate is the electron source, only complex IV is involved (Ascorbate transfers electrons directly to Cytochrome C), so only 2 protons are pumped giving us 0.5 ATP per Oxygen.
   b. Consider the hypothetical situation in which it takes 2.8 H^+ transferred across the IM to drive the synthesis of a single ATP.
      i. Determine the P/O ratios for NADH 3.57, Succinate 2.14, and Ascorbate 0.778



      ii. How many ATP would completely oxidation of pyruvate to CO_2 generate? 17.42
      Pyruvate oxidation to 3 CO_2 generates 4 NADH, 1 $FADH_2$ and 1 ATP (substrate level phosphorylation).

      NADH 4x3.57 = 14.28 $FADH_2$ = 2.14 and 1 ATP

2. Starting with NADH, show all relevant enzyme catalyzed reduction/oxidation events that occur and result in the reduction of ~~H_2O~~ O_2.



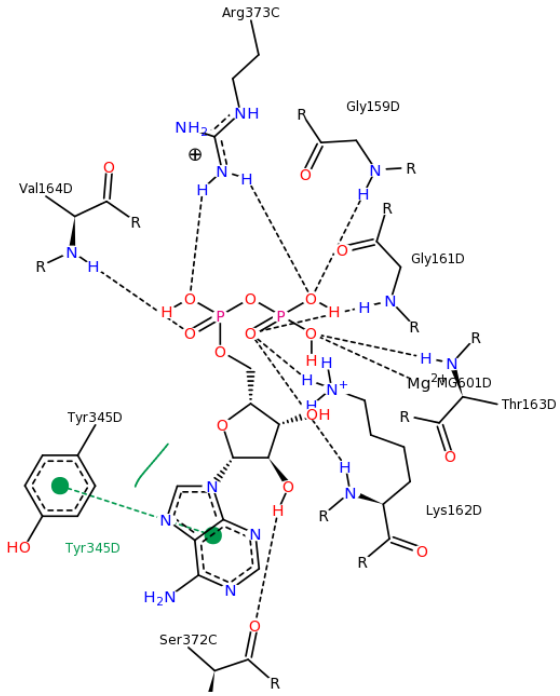
3. Clearly describe the role of tyrosine in the catalytic cycle of Cytochrome C Oxidase (Complex IV). In the transition from the oxy to the peroxy step, a hydrogen atom is abstracted from the tyrosine residue, resulting in a tyrosyl radical. The hydrogen atom is transferred to one of the oxygen atoms. In the next step, the radical is quenched by an incoming proton and electron.

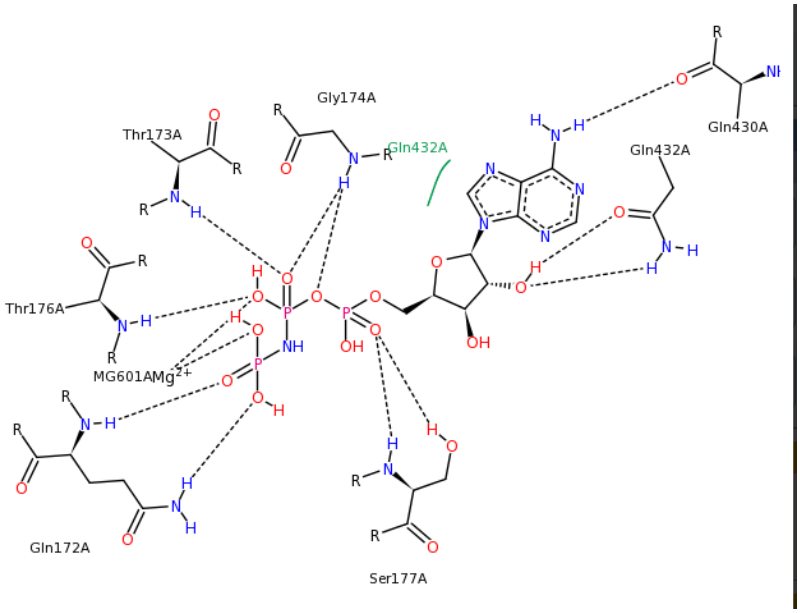
4. We will see next week that oxidation of a single glucose molecule to CO_2 should generate 32 ATP. However, in some tissue only 30 ATP equivalents are produced from the same oxidation process. How is this possible? Recall that there are two different shuttle mechanisms. The aspartate-malate shuttle effectively brings NADH (2 per glucose from glycolysis) into the matrix where it can enter the mETC at Complex I and generate 5 ATP (2.5 per NADH). The other shuttling mechanism (Glycerol-3-Phosphate Shuttle), however, does not transfer NADH into the matrix. Instead, the electrons for cytosolic NADH enter the mETC as $FADH_2$ (which reduces ubiquinone) and miss out on the 4 protons pumped by Complex I. This drops the P/O ratio to 1.5 so we only get 3 ATP made for these NADH.

5. Investigate the structure of the P_1-ATPase in pdbID1BMF. Examine the ADP site and ANP sites.

a. Is ANP a structural analogue of ADP or ATP? What is the difference and why is it a good analogue? ANP is an ATP analogue. In place of the oxygen between the β and γ phosphates, there is a nitrogen. This prevents hydrolysis of bond.

b. Compare and contrast these binding pockets. Please provide an image of the two sites and point out the most relevant similarities and differences.

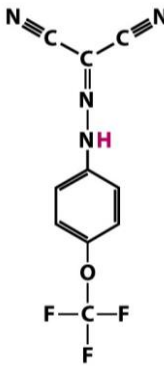




ADP ATP

These sites apparently use completely different residues to stabilize the substrates. This makes sense since ATP and ADP must have significantly different affinities.

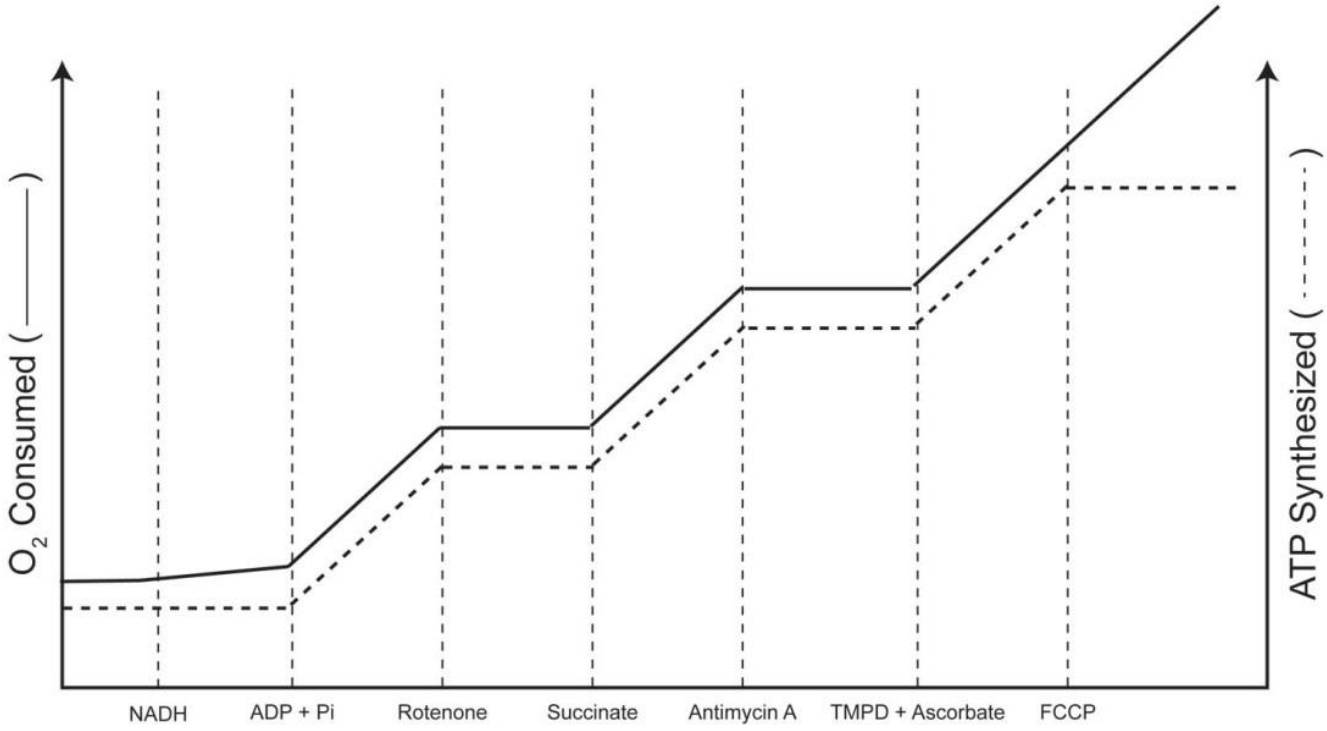
6. Addition of FCCP to respiring mitochondria will decouple electron transport and phosphorylation.



FCCP

a. Given the structure of FCCP (red proton is acidic), describe how this molecule can function as a decoupling agent. Uncoupling agents function to dissipate the proton gradient while still allowing electrons to flow through the electron transport chain. Under acidic conditions (as seen in the IMS), FCCP is protonated at the indicated nitrogen. When this molecule is protonate, it is neutral and not particularly polar. As such, it can easily diffuse through the Inner Membrane. When it arrives in the matrix, the basic conditions cause FCCP to be deprotonated. This results in a negative charge on the molecule and inhibits diffusion back to the IMS. So for each FCCP molecule, there is a net transfer of 1 H+ from the IMS to the matrix, which dampens the proton gradient and, as a result, prevents ATP Synthase from using the proton gradient for ATP synthesis.

b. Using the graph on the adjacent page, plot the O_2 consumption and ATP synthesized as a function of time when each of the indicated molecules are added. Please justify your graph based on the function of each compound.



c. How would the graph differ if CN^- was added to the experiment prior to NADH? Cyanide inhibits electron transport to O_2 in Complex IV. Adding CN at the beginning of the experiment would prevent O_2 consumption and H+ transport; therefore there would be no ATP Produced or O_2 Consumed.

7. Using the affinities for ATP and ADP to the (F_1F_0) ATPase given in Lecture 8, determine if the stabilization of ATP relative to ADP is sufficient to drive the otherwise endergonic condensation of ADP and Pi.

Dissociation constants

$K_{ATP} = 10^{-12}\ M^{-1}$

$K_{ADP} = 10^{-5}\ M^{-1}$

$\frac{K}{10^{12}}$

10^{5}

$\Delta G_{ATP} = -RT \ln K = -68.5\ kJ/mol$

$\Delta G_{ADP} = -RT \ln K = -28.5\ kJ/mol$

$\Delta\Delta G \cong -40\ kJ/mol$

Stabilization of ATP can overcome 40 kJ/mol

It takes 30.5 kJ/mol to $ADP + P_i \rightleftharpoons ATP + H_2O$

So yes, enough energy is

8. Please describe how the 3 conformations of the F_1F_0 ATPase are important for the synthesis of ATP. Make sure to include a discussion of relative energies for ATP and ADP in each conformation. The F_1F_0 ATPase has three binding sites for ATP/ADP. At any given time, one binding site is occupied with ATP, another is occupied with ADP and Pi (in rapid equilibrium with ATP as we discussed in class), and the third site is unoccupied. As protons are pumped into the matrix, the F_1 subunit rotates causing a conformational change of all three subunits. The subunit that had ATP now has negligible affinity for it and the ATP is released into the matrix. The site that was empty is now competent to bind ADP and Pi. The subunit that had ADP and Pi now tightly binds ATP (this stabilization of ATP is what drives the phosphorylation as seen above).

9. IF_1 is a small protein that plays an important role in the F_1F_0 ATPase catalyzed hydrolysis of ATP under hypoxic (O_2 deprived) conditions. Please explore this regulatory mechanism and describe how it works. Make sure to consider the role of pH and discuss why there is a concern the ATP will be hydrolyzed. This is discussed in your book on page 760. Under hypoxic conditions, O_2 is not present in sufficient amounts to serve as an efficient electron acceptor for electron transport. As such, electron transport stops and the proton gradient is not generated. It is very possible for ATPase to work in reverse such that protons are pumped out of the mitochondria, where they can be diluted into the cytosol, and ATP is hydrolyzed in the process. To prevent this, a small protein dimerizes under the acidic conditions – in the dimeric form, IF_1 is a potent inhibitor of the rotation of the γ subunit, therefore preventing ATP hydrolysis.

10. We have seen several examples of TCA Cycle enzymes (and will see many more in glycolysis) that respond to the concentration ratio of [NADH]/[NAD^+] and [ATP]/[ADP]. Based on what you know about mitochondrial ATP synthesis, please justify this observation. When chemical energy is abundant (e.g. lots of ATP and NADH), flux through glycolysis and TCA cycle is not a useful use of resources. Instead, the precursors are sent through anabolic pathways to produce energy stores.

11. Please familiarize yourself with the attached article and address the following questions:
    a. Atovaquone is used to treat malaria. Predict a way that this molecule can accomplish this. Atovquone inhibits complex III. A non-functional mETC puts a lot of pressure on organisms to survive without relying on aerobic respiration; as such, malaria is at a competitive disadvantage and dies.
    b. Examine the structures of atovaquone and stigmatellin. Predict how these inhibitors can competitively inhibit electron transport through Complex III. Both of these molecules have a 6 member ring that resembles Coenzyme Q. They can both likely bind directly to the ubiquinone binding site in a competitive manner.
    c. Figure 2 shows enzyme activity in the presence of increasing atovaquone. Describe how the enzyme activity was monitored. You do not need to report the experimental details, just discuss what technique was used and the basis for the signal. The reduction of Ctyochrome C was monitored by measuring the absorbance at 550 nm vs. 539 nm. One of these wavelengths corresponds to the reduced CytC and the other corresponds to the oxidized form.
    d. This manuscript presents evidence that atovaquone inhibits enzyme activity. Please discuss what evidence supports each of the following statements:
        i. Atovaquone binding at the Qp site strongly affects the Rieske iron sulfur cluster. Two pieces of evidence point to this conclusion. First, the EPR spectrum shown in Figure 3, which monitors the FeS cluster, indicates that the electronic environment has changed in the presence of atovaquone. The 2^{nd} piece of evidence is the modified reduction potential (Figure 4) of the FeS cluster in the presence of the inhibitor.
        ii. Atovaquone interacts with a Histidine that anchors the Fe_2S_2 cluster. This conclusion is drawn from an energy minimized structure (so a prediction) shown in figure 5. This structure shows the OH from Atovaquone interaction with the Rieske Histidine residue. The prediction is that this interaction locks Complex III in an inhibited conformation due to modified reduction potentials and proximity to other cofactors. Another piece of evidence comes in the text when the authors note a control calculation in which the hydroxyl group was removed; they found that the interaction was prevented. This is the same mechanism that Stigmatellin interacts with Complex III

iii. Complex III in bovine cells is resistant to atovaquone inhibition due to a single amino acid difference in the Qp site. Figure 7 shows that the difference between yeast and bovine enzymes in this area is the difference of a single amino acid; L→F, respectively. The authors show in Figure 6 that atovaquone is not a potent inhibitor when this amino acid is mutated from a leucine to a phenylalanine.