

Chemistry 5.07SC Biological Chemistry I
Fall Semester, 2013

Lectures 11 and 12. Biochemical Transformations III. Redox Cofactors: Oxidation and Reduction and a quantitative description of spontaneity.

Energy released from the oxidation of standard building blocks (sugars, fatty acids, amino acids) is harnessed in chemical bonds via NADH and used to make ATP. Oxidation and reduction in biological systems is carried out using two organic cofactors nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotides (FAD, FMN or on occasion riboflavin) and inorganic cofactors for electron transfer (ET). These cofactors play a central role in primary metabolism specifically the respiratory chain and the mechanism by which a proton gradient is generated and coupled for ATP production.

Outline:

- I. Examine the chemistry of the two organic cofactors NAD and FAD involved in redox chem.
- II. To introduce the inorganic cofactors involved in redox chemistry with focus on respiration.
- III. To provide a quantitative description for spontaneity with redox reactions.

I. There are two major organic redox cofactors:

A. NAD⁺

B. FAD (FMN)

A. NAD⁺/NADH (NADP⁺/NADPH)

1. Structure

The vitamin is nicotinic acid or nicotinamide (Niacin) and is a precursor to the actual cofactor. Deficiency in the vitamin is associated with the disease pellagra.

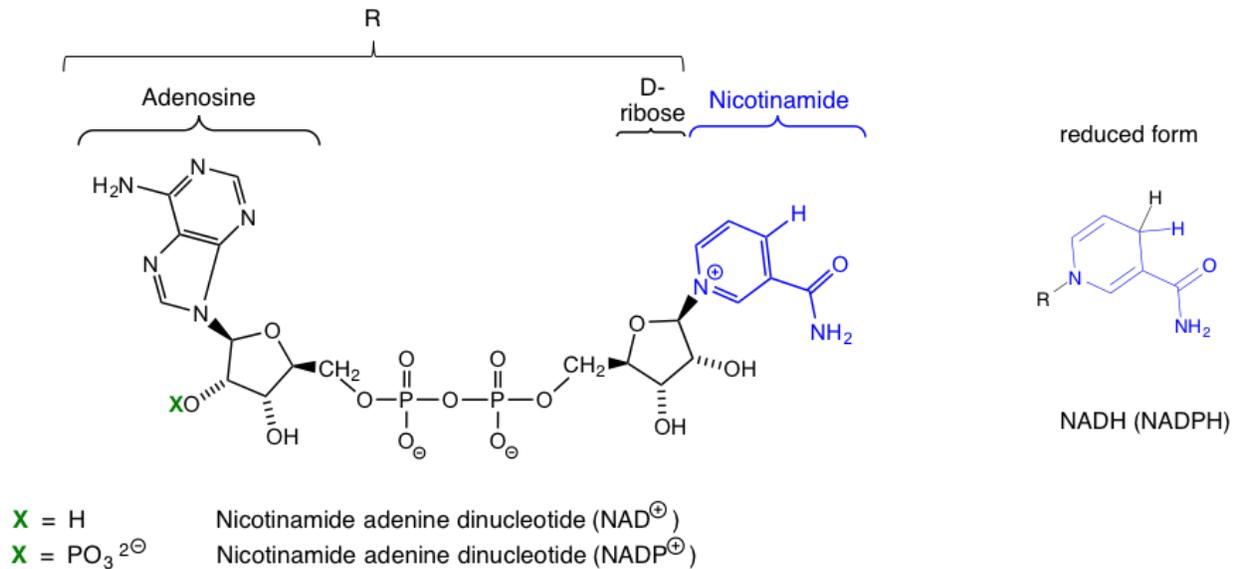


Figure 1. Structure of $NAD(P)^+$. $NAD(P)^+$ is composed of an AMP phosphodiester and a nicotinamide ribose 5'-phosphate. Note the only difference between NAD^+ and $NADP^+$ is a phosphate attached to the hydroxyl at C2' (green X). The business end of the molecule is the nicotinamide moiety. The oxidized form has a pyridinium ring and the reduced form has a 1,4-dihydropyridine ring.

Both of these forms have identical redox properties and chemical mechanisms. We will focus **ONLY** on the redox reactivity of this cofactor. The **$NADH/NAD^+$ and $NADP^+/NADPH$ ratios are of primary importance: the former in energy metabolism and the latter in reductive biosynthesis. Metabolic enzymes can in general readily distinguish between these two forms.**

2. Scope of reactions

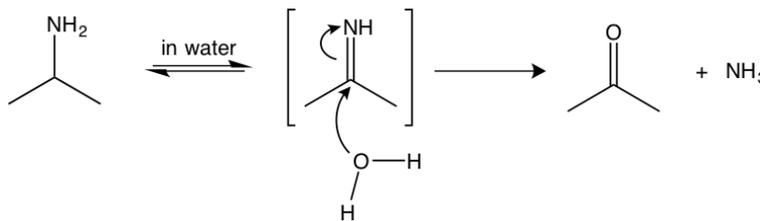
a. $NADH$ is of primary importance in energy metabolism (catabolic processes). It supplies reducing equivalents to reduce O_2 to H_2O in the respiratory chain and the energy released generates a proton motif force used to make ATP. $NADPH$ on the other hand is used predominantly in biosynthesis (anabolic processes) and maintaining the redox status inside the cell.

b. reactions in primary metabolism:

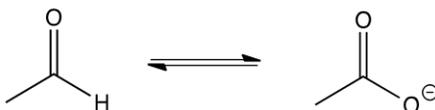
i. Reduction of a ketone to an alcohol with examples in the glycolysis pathway and Krebs cycle – lactate dehydrogenase (LDH), malate dehydrogenase (MDH), alcohol dehydrogenase (ADH). The reactions are reversible and pH dependent.



ii. Oxidation of an amine to an imine that hydrolyzes to a ketone and ammonia. An example is glutamate dehydrogenase (GDH) that is a major enzyme involved in nitrogen metabolism.



iii. Oxidation of an aldehyde to an acid. An example is glyceraldehyde 3-phosphate (GAP) dehydrogenase (GAPDH) that you will see in the glycolysis pathway.

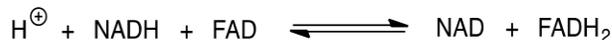


iv. Oxidation of saturated fatty acid thioesters to unsaturated fatty acid thioesters. Note the oxidation requires an activated hydrogen on the carbon ($C\alpha$) to the thioester.

This reaction plays a major role in fatty acid biosynthesis and degradation.



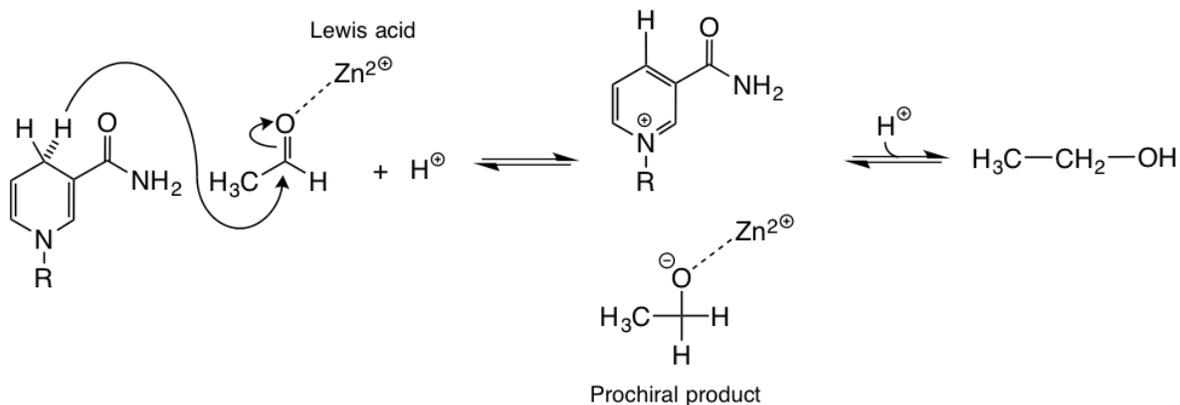
v. Redox chemistry with the second organic redox cofactor FAD. Example: respiratory chain.



3. Mechanism of the reactions shown above. The mechanism of this cofactor for ALL reactions involves a **hydride (H^-)** and **proton (H^+)** transfer. An example of a typical reaction is the reduction of an aldehyde to alcohol. This is one of the endings in the glycolysis pathway in yeast, the mainstay of the beer and wine industry (fermentation, metabolism without O_2). The alcohol dehydrogenase (ADH) reaction is reversible and the proton makes the overall reaction

pH dependent. Thus by changing the concentration of one of the substrates $[H^+]$, the equilibrium of the reaction can be shifted. $NAD^+/NADH$ are often used in coupled assays to measure enzyme activity as NADH has absorption at 340 nm with a large extinction coefficient ($\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$), while NAD^+ has no absorption at this wavelength. Note that in the ADH reaction that in addition to the redox cofactor there is a Zn^{2+} , which we previously encountered in the aldolase reaction. It acts as a Lewis acid to polarize the carbonyl for nucleophilic attack. In this case the Nu is the hydride (H^-).

NOTE: H^- does not attack the O of the carbonyl, it attacks the C of the carbonyl. Think about Lecture 9 on carbonyl chemistry. In general, H^- and H^+ transfer occur in different steps of the reaction as shown above.

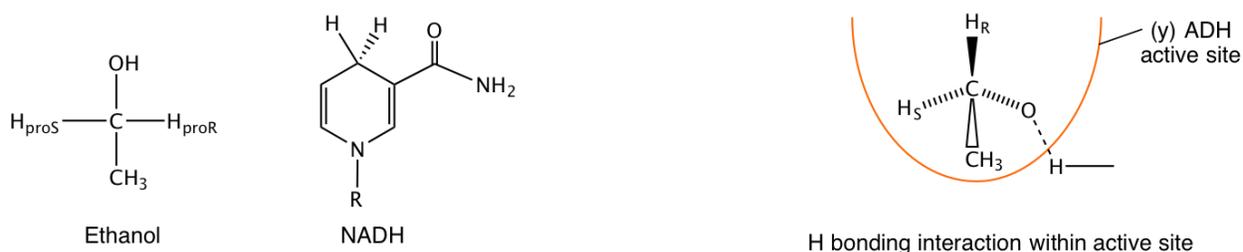


4. Stereochemistry: Dehydrogenases are absolutely stereospecific. These enzymes can catalyze the conversion of substrate to product a million times without making a mistake stereochemically. Below is described the classic Westheimer and Vennesland (W/V) experiment. Recall our discussions in Lecture 2 about the chirality of amino acids. All proteins are made up of chiral amino acids (except glycine) and thus the proteins themselves provide chiral binding sites for their chemistry. The W/V experiments demonstrated that an enzyme **can distinguish between prochiral hydrogens** of its substrate as a result of its chiral binding site. For example, yeast (y)ADH can distinguish between the two protons of the CH_2 in CH_3CH_2OH and the 2Hs at C4 of NADH.

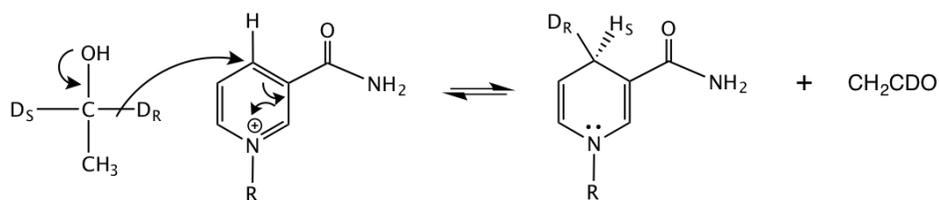
Digression on prochirality: We will NOT discuss the W/V experiment in Lecture. But as chemists I hope you will all think about the beauty of this experiment and the wonder of protein catalysts. The concept of prochirality is critical to understanding the TCA cycle since citrate has prochiral arms (this is covered in later lectures in more depth). Understanding prochirality was vital to determining the steps of the Krebs's cycle.

Westheimer and Vennesland (W/V) Experiment

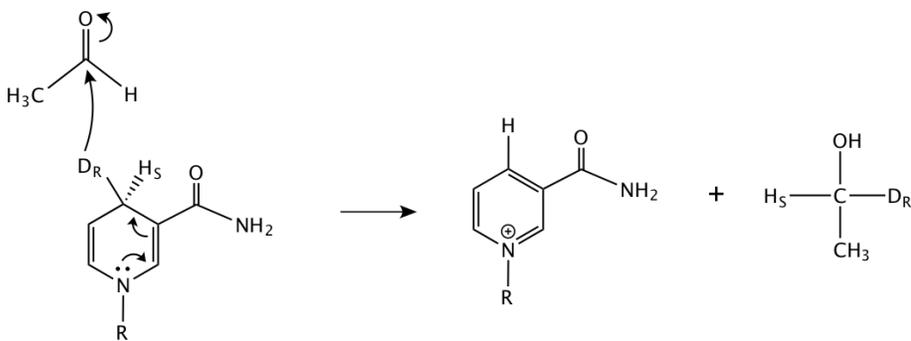
Both of the yeast (*y*) ADH enzyme substrates, ethanol and NADH at C-4 position, have prochiral H's.



W/V hypothesized that (*y*)ADH can distinguish between the two prochiral hydrogens. Their method to demonstrate this involved using a stable isotope of H with an additional neutron known as Deuterium (D). In their model, only one of the Ds would be transferred (D_R is shown below), leaving a chirally labeled NADH. D_R has been stereospecifically transferred to C₄.



To test their model, the [D_R]-labeled NADH was incubated with acetylaldehyde and the fate of D_R was examined.



NOTE: H (hydride) is transferred to the C of the carbonyl NOT the oxygen.

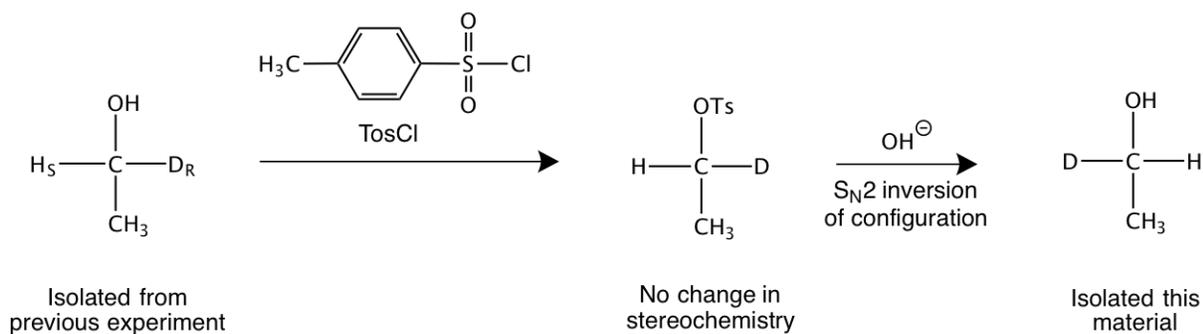
Results:

100% of D label was transferred to acetaldehyde.

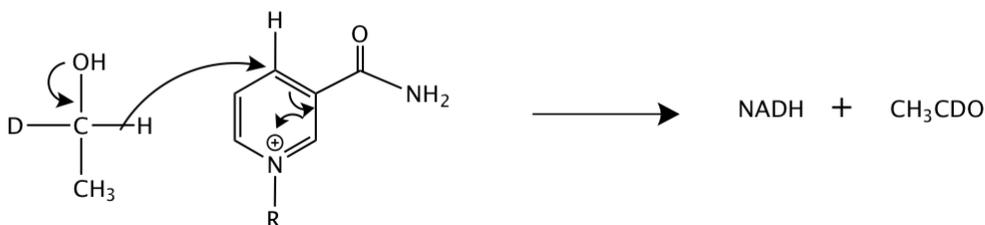
0% of D label was present in NAD.

W/V concluded that (y)ADH can distinguish between the two faces of NAD.

But what about ethanol? Can (y)ADH distinguish the prochiral H's of ethanol?



Third Experiment:



Now only H is transferred to NAD⁺.

Conclusion: The enzyme can distinguish between the two prochiral H's of NADH and EtOH!

End digression.

5. Redox properties of NAD⁺



NAD⁺ **only** undergoes **two electron reductions inside the cell**, as the one electron reduction process is too far up hill (thermodynamically unfavorable).

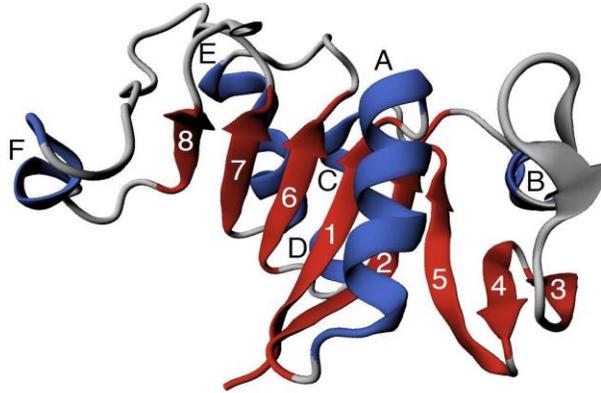
a. The reduction potential of NAD⁺ is NOT modulated by the protein environment in contrast with most redox active cofactors and is -0.32 v.

b. NAD⁺/NADH act as substrates with enzymes, that is, they bind and dissociate after each turnover. This behavior contrasts with the FAD cofactors that are always tightly or covalently bound to the enzyme.

c. NAD⁺ is O₂ stable in contrast with most redox cofactors (FAD and the metal-based cofactors that use iron and copper). Note O₂ has two unpaired electrons that reside in its antibonding orbitals. This property makes O₂ unreactive with sugars, amino acids etc as their electrons are all paired. However, both flavins and metals can carry out chemistry one electron at a time. Thus they can react with O₂; the chemistry is energetically favorable.

d. NAD⁺/NADH have a conserved binding domain called a Rossmann fold (see below and Lecture 3) and is easily recognized by conservation of specific residues many of them glycines. The cofactor is almost always bound in an extended conformation. Note the Rossmann fold contains two “nucleotide” binding motifs (β₁αβ₁β); one for nicotinamide ribose phosphate and one for the AMP form of the NAD⁺ (see Figure 2).

A.



B.

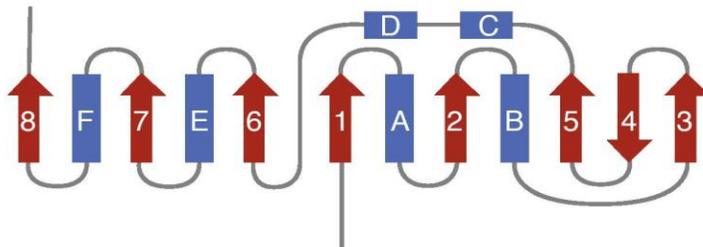


Figure by O'Reilly Science Art for MIT OpenCourseWare.

B. PDB: [4P8R](#).

Seattle Structural Genomics Center for Infectious Disease, Abendroth, J., Lorimer, D., Edwards, T.E. Structure of a glycosomal glyceraldehyde 3-phosphate dehydrogenase from *Trypanosoma brucei*.

Figure 2. Rossmann fold has two nucleotide binding sites – $\beta\alpha\beta\alpha\beta$ repeats. A. 3D reconstruction of the Rossmann fold in glyceraldehyde-3-phosphate dehydrogenase. B. Plumbing diagram of the Rossmann fold.

B. The second organic redox cofactor is FAD (FMN). See the Lexicon also. The chemistry of this cofactor is complex and much less intuitive than NAD^+ . FAD and FMN are the common forms used in cells. Riboflavin (RF) is used much less frequently.

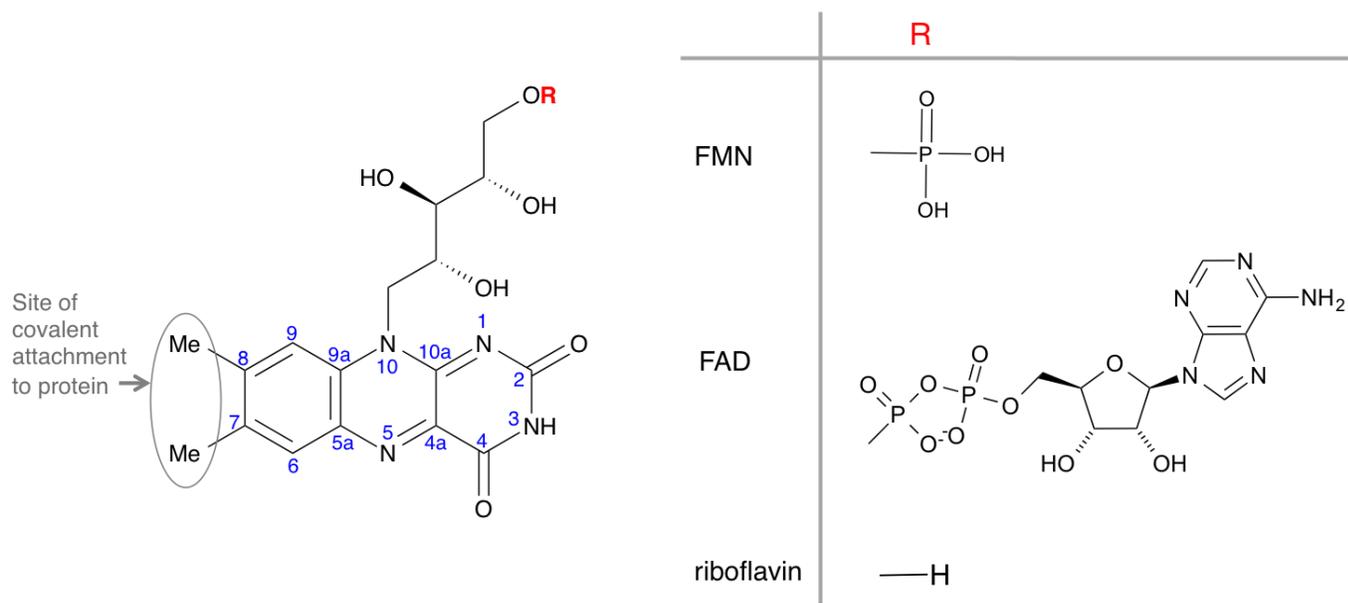


Figure 3. Three forms of flavin (FMN, FAD, riboflavin).

1. This cofactor is the major mediator between 2 e⁻ donors and 1 e⁻ acceptors. In the following figure you can see the available redox states. They all have different visible spectra and can thus be identified by their unique features. The oxidized flavin (FAD or FMN) is planar and bright yellow, while the reduced flavin (FADH₂ or FMNH₂) is butterfly shaped (non-planar and is colorless). The two one e⁻ reduced states are also colored with the color depending on their protonation state.

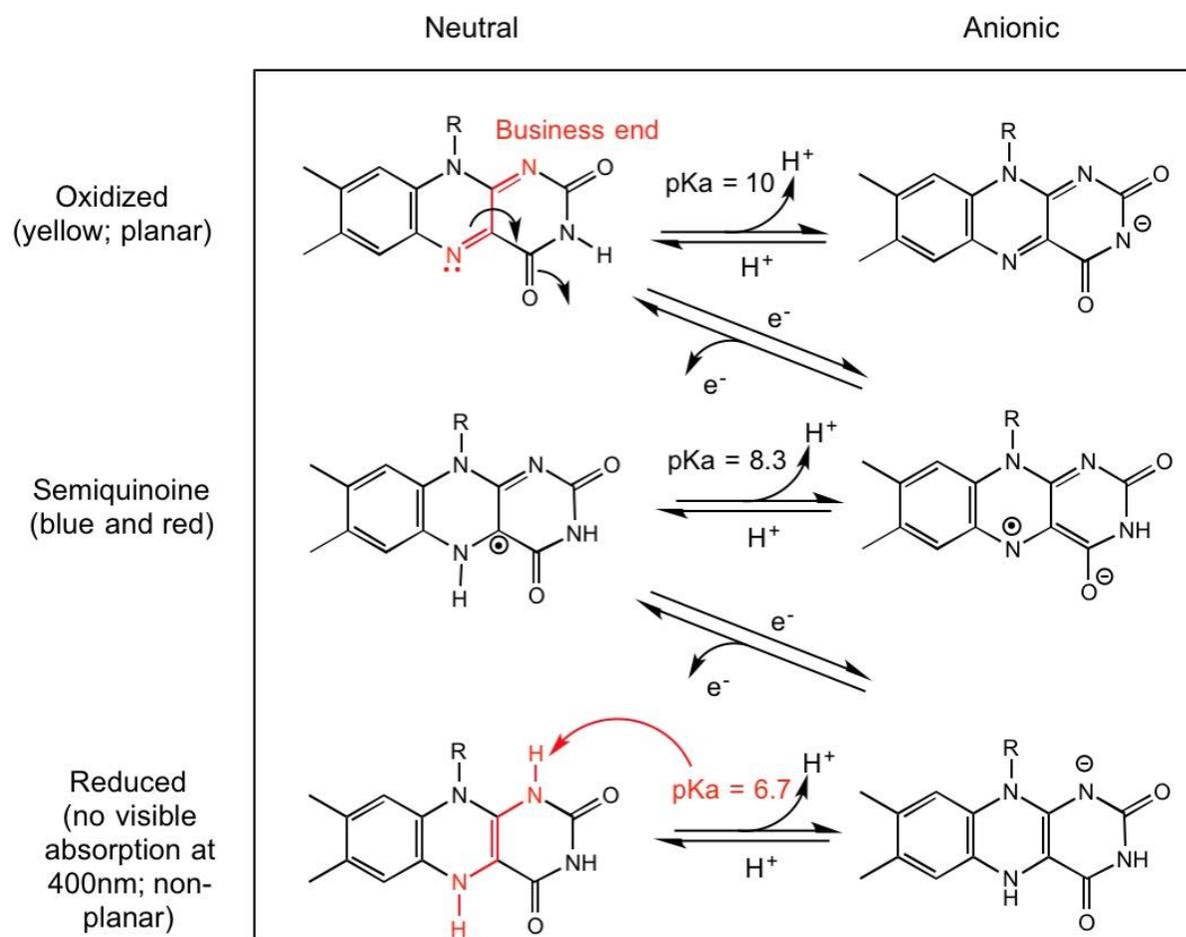


Figure 4. Flavin in its different redox states.

2. Structure: the flavin ring structure is called an isoalloxazine. Many structures of flavin requiring proteins have been solved at atomic resolution. In contrast to NAD⁺, there is NO ubiquitous FAD binding site such as the Rossmann fold.

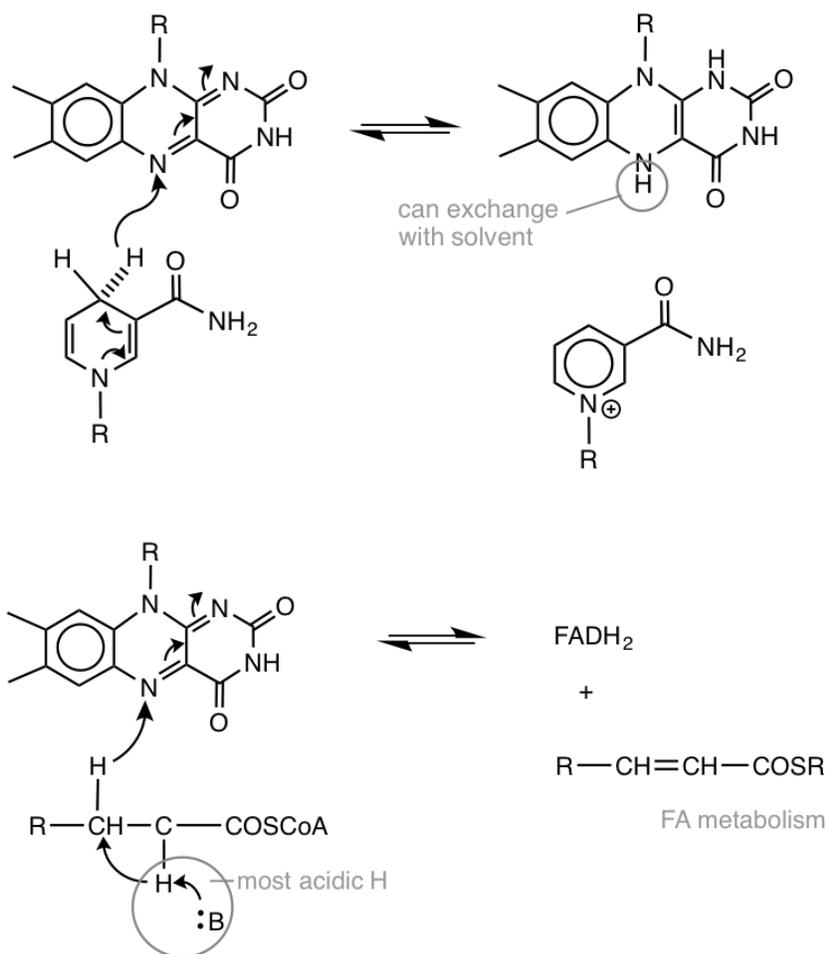
3. Scope of reactions: These are very similar to those described above for NAD⁺/NADH.

4. Mechanisms: Multiple mechanisms are involved in flavin-dependent reactions. However, based on hundreds of reactions studied in detail, generalizations can be made.

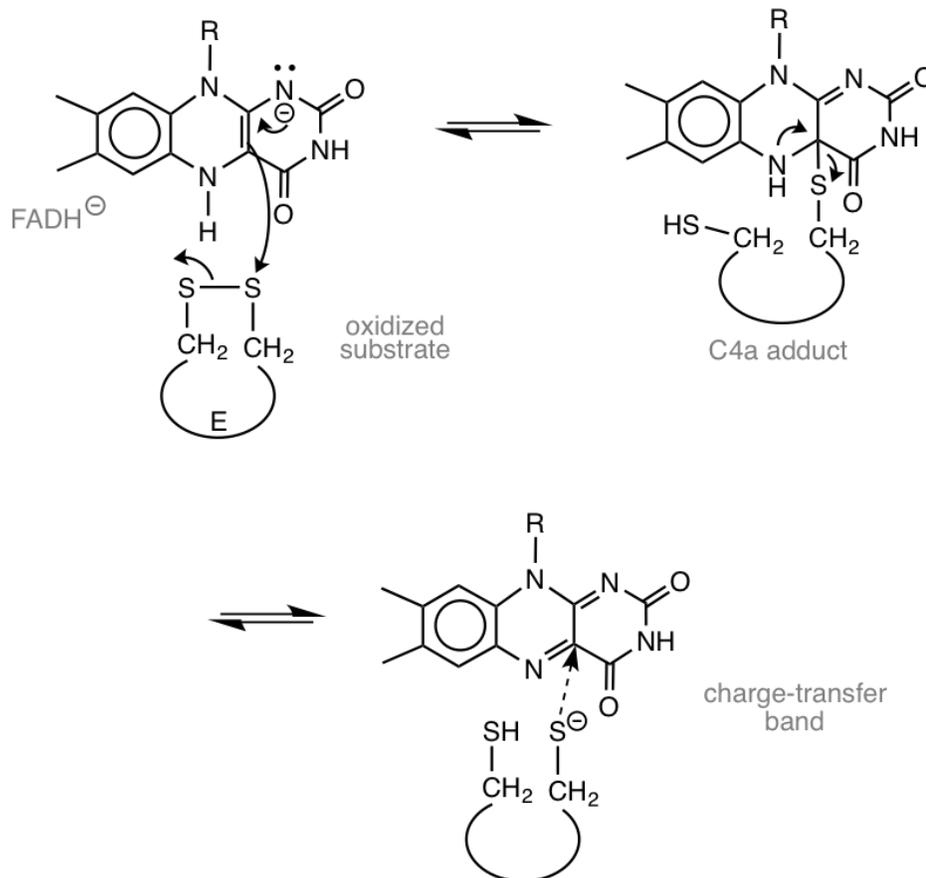
a. 2 e⁻ chemistry. H⁻ (hydride transfer) occurs to the N5 position of the flavin. Two e⁻ chemistry also occurs at the C4a position of the flavin. We will see that both reactions are used in primary metabolism (an example is the pyruvate dehydrogenase (PDH) reaction that bridges the glycolysis pathway and TCA cycle).

b. 1 e⁻ chemistry. Chemistry also occurs 1 e⁻ at a time. This chemistry is possible with flavins because the semiquinone radical forms (the anionic red form and the neutral blue form) are stable due to extensive electron delocalization over the isoalloxazine ring. One can draw many resonance structures of the planar semiquinone radicals shown above.

Examples of N5 chemistry: As shown below, the top reaction will be observed in the respiratory chain and the bottom reaction in fatty acid metabolism. Note that in the latter case, the most acidic proton is adjacent to the thioester and is always the one that is removed by a general base catalyst (GBC).



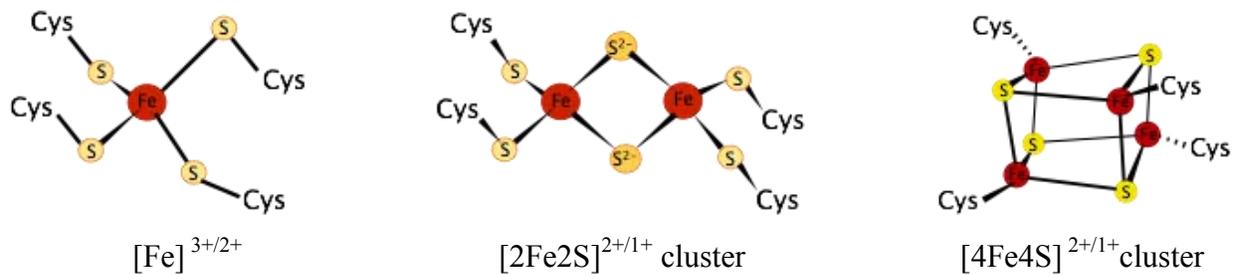
Example of C4a chemistry from the pyruvate dehydrogenase (PDH) macromolecular machine.



II. Inorganic redox-based cofactors. Metal clusters play an important role in catalysis and a central role in respiration, photosynthesis, nitrogen fixation, and many enzymatic reactions. Thirty to 35 % of all proteins use redox-inactive (structural role) or redox-active metallo-cofactors (catalytic role). Recall the Zn²⁺ that we examined with the class II aldolase and ADH dehydrogenase reaction. Zinc is d¹⁰ and is redox inert; the Zn²⁺ functions as a Lewis acid. However, harnessing the energy of NADH oxidation via the respiratory chain, requires redox-active cofactors that are predominantly iron and copper.

1. Structure: The following cofactors are widely used in biology: iron sulfur cluster (FeS) clusters and hemes.

Iron Sulfur Clusters (the indicated charge $[]^{n+}$ is based on the oxidation state of the Fe either +2 or +3 and the sulfides (S^{-2})). These clusters undergo one electron reactions.



Hemes Recall that we have previously seen hemes when discussing Hb and Mb. Hemes in Hb and Mb function as reversible binders of O_2 . Hemes in the respiratory chain are involved in electron transfer with other metallo-proteins and their iron changes redox state between Fe^{2+} and Fe^{3+} .

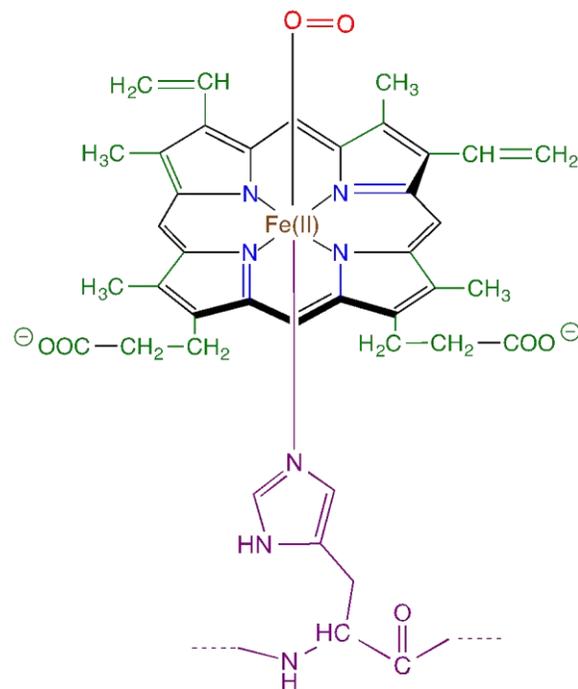
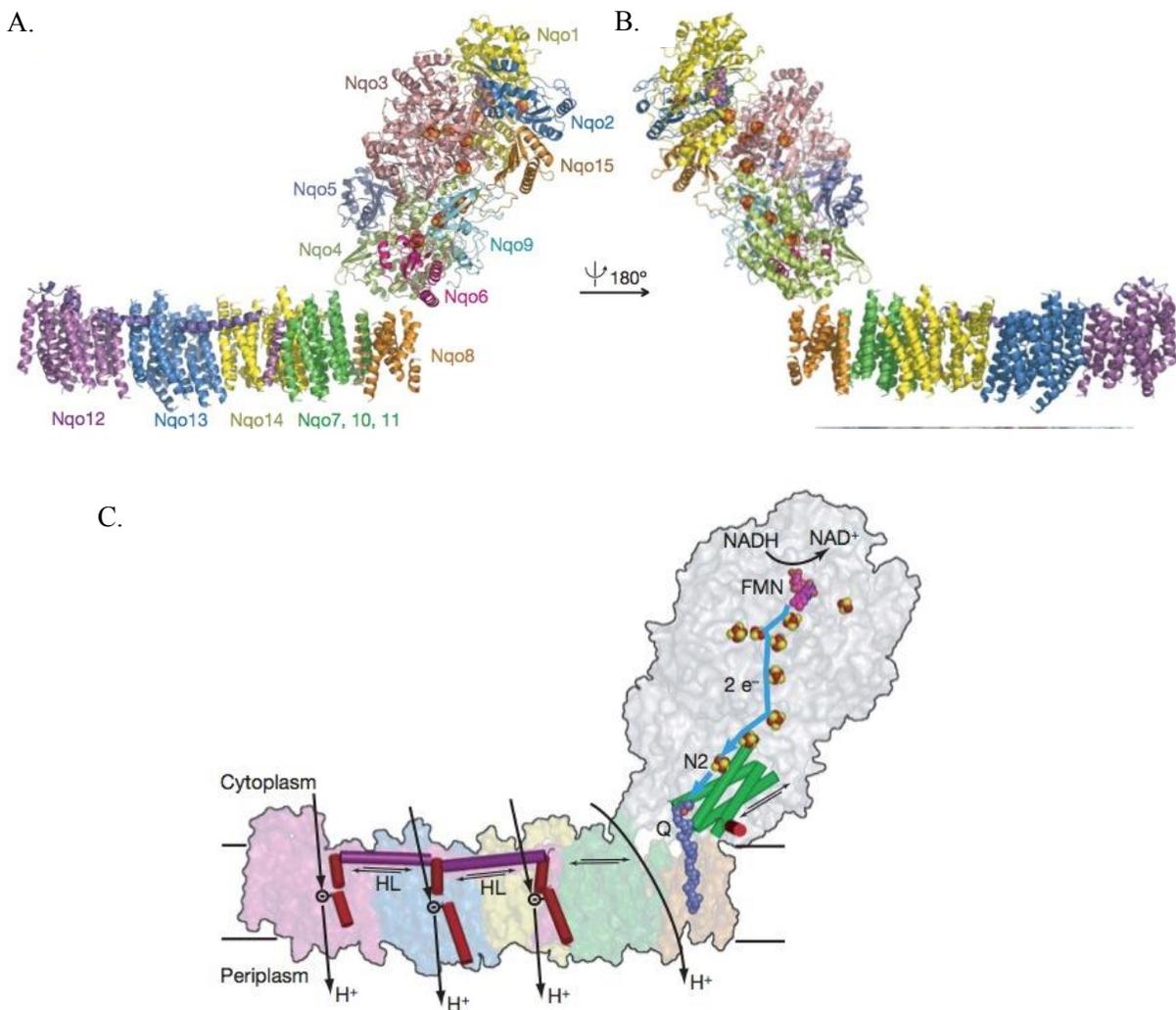


Figure 5. Heme or protoporphyrin IX in Hb and Mb. The equilateral ligands are “N” from the pyrroles; the axial ligands vary depending on the protein.

Example: An example of long range electron transfer is shown below based on a recent structure and much biochemical analysis, Nature 2010 465, 441-5. Complex I (NADH:ubiquinone oxidoreductase) is one the largest protein assemblies known and plays a

central role in energy production by the mitochondrial respiratory chain. Many mutations in complex I subunits are associated with human neurodegenerative diseases.



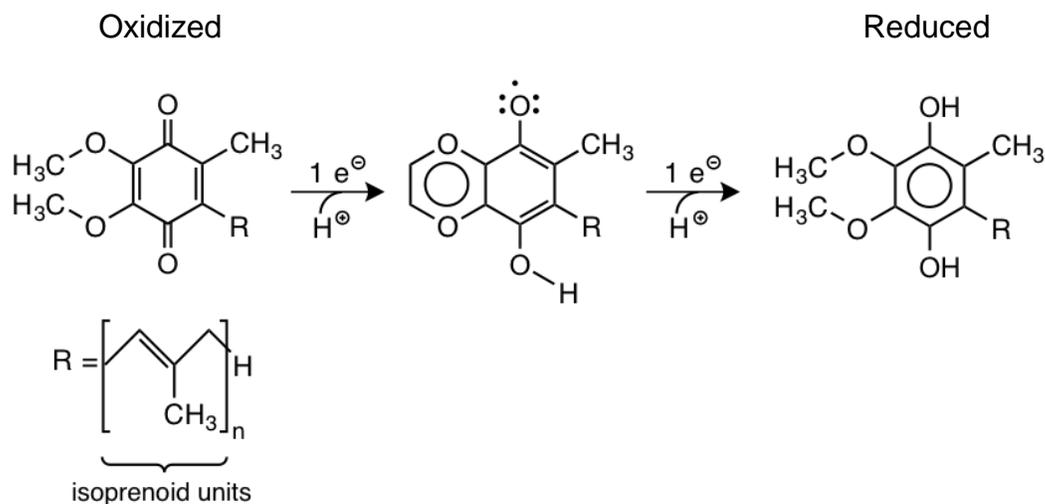
Courtesy of Macmillan Publishers, Ltd. © Nature.

Source: Figure 3ab, 4 from Efremov, Rouslan G., Rozbeh Baradaran, and Leonid A. Sazanov. "The architecture of respiratory complex I." *Nature* 465, no. 7297 (2010): 441-445.

Figure 6. A. and B. Side views, in the membrane plane of the entire complex I from *Thermus thermophilus*. FeS clusters are shown as red and yellow spheres. C. NADH and FMN (magenta) donates two electrons by electron transfer (blue line for pathway), one at a time, to the chain of Fe-S clusters (red and yellow spheres) and finally passed from the N2 cluster to the quinone (dark blue). Electron transfer is coupled to conformational changes in the four helix bundle domain (green helices) and helix (red). These changes are transmitted to HL (magenta) that allows transport of three protons. The fourth proton is transported at the interface of the two domains. The hydrophilic domain surface is shown in grey, whereas the membrane domain surface is colored as in A. and B.

This complex is a large L-shaped membrane bound enzyme. The reaction involves transfer of electrons over 90 Å from NADH that binds to the hydrophilic domain, to ubiquinone in or near the hydrophobic membrane bound domain (purple, Q).

The cofactors are FMN, FeS clusters and a quinone.



A structure of a quinone in the oxidized state (left, above). Quinones can undergo reduction by 1 e^- or 2 e^- steps to a semiquinone (middle, above) or hydroquinone (right, above) as we discussed for flavins.

2. Oxidation/reduction by electron transfer (ET). The types of ET reactions shown in the structure (Figure 6) are prevalent in the respiratory chain in the other complexes in the mitochondria, in the photosynthetic reaction centers I and II, in nitrogenase, the enzyme that reduces N_2 to NH_3 , in hydrogenase that catalyzes the reversible interconversion of H^+ and H_2 etc. ET is distinct from the organic transformations we have discussed thus far and is a major reaction type in inorganic biotransformations. The chemistry of ET is also distinct from H transfer reactions (where H transfer encompasses transfer of a **proton (H^+)**, **hydride (H^-)** or **hydrogen atom (H^\bullet)**). The distinction arises as the mass of an electron is 1/2000 the mass of a H. An electron can be described as both a particle and a wave. The wave properties of electrons allow them to behave quantum mechanically, that is, they are able to “tunnel”, transfer an electron over distances of 10 to 15 Å without any intermediate. Due to the mass differences, protons (hydride etc) can tunnel only over ~ 0.7 Å. Think about all the bond lengths in the reactions we have thus far studied and you will realize that electrons involved in chemical

transformations over 10 to 15 Å are unique. Another distinction between ET and H transfer are the rate constants associated with these processes. Rate constants for ET (k_{ET}) are very fast, 10^8 s^{-1} , while typical enzymatic reactions involving C-H bond cleavage occur with rate constants of 1 to 10^2 s^{-1} . Based on Marcus/Levitch theory (equation below), the rate constant for ET under optimized conditions falls off exponentially with distance. Typically it decreases by a factor of 10 for every 1.7 Å. The rate of ET is also dependent on the driving force for the reaction (ΔG°) and the reorganization energy (λ) of the electron donor and acceptor and their environment. ΔG° and λ are a direct consequence of protein structure. The first term H_{AB}^2 is

$$k_{\text{ET}} = \sqrt{\frac{4\pi^3}{h^2 \lambda RT}} H_{\text{AB}}^2 \exp\left\{-\frac{(\Delta G^\circ + \lambda)^2}{4\lambda RT}\right\}$$

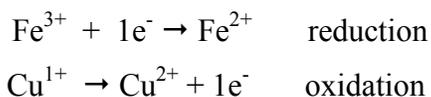
the electronic overlap between the donor and the acceptor. Looking at hundreds of proteins with metal cofactors one can make the generalization that Nature has figured out how to space redox cofactors 10-15 Å apart so that ET occurs by **TUNNELING---NO INTERMEDIATES**. For those interested in more details beyond the scope of this discussion see Nature Chem. Biol. 5, 543-550 (2009).

III. Quantitative Description of ET and Spontaneity and the relationship to free energy.

(revisit your notes on oxidation and reduction from Freshman Chemistry)

Definitions: **reductant** (reducing agent) gives up electrons; **oxidant** (oxidizing agent) accepts electrons.

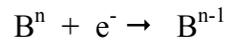
All redox reactions are divided into two half reactions: one describing the reduction step and the other the oxidation step.



One can then ask the question can Cu^{1+} reduce Fe^{3+} ? Is the reaction spontaneous? Can we make the reaction spontaneous? These questions are very similar to the ones we asked when discussing ATP and the use of free energy changes to describe the spontaneity of a reaction. As with free energy, we will note the mechanisms by which Nature can alter spontaneity.

Biochemists describe all transformations in terms of reduction potentials and use different standard states from chemists.

In general if $A^n + e^- \rightarrow A^{n-1}$ n is the oxidation state



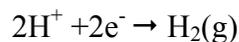
then one can ask, is $A^n + B^{n-1} \rightarrow A^{n-1} + B^n$ spontaneous?

Recall that $\Delta G = \Delta G^\circ + RT \ln [A^{n-1}][B^n]/[A^n][B^{n-1}]$ In this case ΔG is $-w_{\text{electrical}}$ where $w_{\text{electrical}} = nF\Delta E$. n is the number of moles of electrons; F is a faraday or the electrical charge of one mole of e^- (96,500J V^{-1} or cal mol^{-1}) and ΔE is the electron potential difference where the units of E are volts (V), the number of joules of work (J) required to transfer 1 coulomb of charge, C. This information gives rise to the **Nernst Equation** (instead of having group transfer of phosphate as seen above with ATP, we now see group transfer with electrons).

$$\Delta E = \Delta E^\circ - RT \ln [A^{n-1}][B^n]/[A^n][B^{n-1}]$$

since $\Delta G = -nF\Delta E$, a positive ΔE defines a spontaneous reaction

As with ΔG , one needs a reference point or standard state. The biochemists' standard state differs from a chemist's standard state by assigning the pH to 7. Reduction potentials, like free energies, are defined with respect to an arbitrary standard state (hydrogen half reaction shown below). The chemists standard state is the hydrogen half reaction:



in which the H^+ is in equilibrium with H_2 at a Pt electrode. This half reaction is arbitrarily assigned to a standard reduction potential E° of 0 V at pH 0, 25°C and 1 atm. For biochemists $E^{\circ'}$ is also 0 v and therefore at the biochemists standard state of pH 7,

$$E^{\circ'} = -0.42 \text{ v}$$

See if you can determine the source of -0.42 v. One needs to think about how pH can effect reduction potential. To do this one needs a H^+ component of the half reaction.

For the general reaction: $M^n + 1e^- + H^+ \rightarrow M^{n-1}$

$$\begin{aligned} E^\circ &= (-RT/nF) [M^{n-1}]/[M^n][H^+] = (-RT/nF) [M^{n-1}]/[M^n] + (RT/nF) \ln[H^+] \\ &= (-RT/nF) [M^{n-1}]/[M^n] - (RT/nF) \text{ pH} \end{aligned}$$

where **RT/nF is 0.059v** and includes the ln to log conversion.

For every pH unit (factor of 10) one observes a change in potential of - 59 mv.

Therefore from pH 0 to 7, the standard reduction potential $E^{\circ'}$ becomes - 0.42 v (0.059×7), the standard state for the biochemist.

The more positive $E^{\circ'}$, the greater the tendency of the species to be reduced.

Thus in the accompanying Table, O_2 wants to be reduced (0.8v), while H_2 wants to be oxidized.

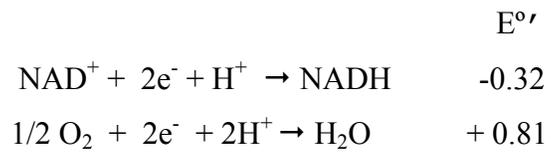
This propensity for reduction is the basis for O_2 as an end point of our respiratory chain.

Redox reactions in biology vary from -600 mv to + 815 mv (Table 1).

Standard E° values (at 25° C and pH 7)		
Half-Reaction		E° (V)
$1/2 O_2 + 2 H^+ + 2 e^-$	\Rightarrow H_2O	0.816
$Fe^{3+} + e^-$	\Rightarrow Fe^{2+}	0.771
$NO_3^- + 6 H^+ + 6 e^-$	\Rightarrow $1/2 N_2 + 3 H_2O$	0.75
$NO_3^- + 2 H^+ + 2 e^-$	\Rightarrow $NO_2^- + H_2O$	0.421
$NO_3^- + 10 H^+ + 8 e^-$	\Rightarrow $NH_4^+ + 3 H_2O$	0.36
$NO_2^- + 8 H^+ + 6 e^-$	\Rightarrow $NH_4^+ + 2 H_2O$	0.34
$CH_3OH + 2 H^+ + 2 e^-$	\Rightarrow $CH_4 + H_2O$	0.17
fumarate + $2 H^+ + 2 e^-$	\Rightarrow succinate	0.031
$2 H^+ + 2 e^-$	\Rightarrow H_2 (pH 0)	0.00
oxaloacetate + $2 H^+ + 2 e^-$	\Rightarrow malate	-0.166
$CH_2O + 2 H^+ + 2 e^-$	\Rightarrow CH_3OH	-0.18
pyruvate + $2 H^+ + 2 e^-$	\Rightarrow lactate	-0.185
acetaldehyde + $2 H^+ + 2 e^-$	\Rightarrow ethanol	-0.197
$SO_4^{2-} + 8 H^+ + 6 e^-$	\Rightarrow $S + 4 H_2O$	-0.20
$SO_4^{2-} + 10 H^+ + 8 e^-$	\Rightarrow $H_2S + 4 H_2O$	-0.21
$FAD + 2 H^+ + 2 e^-$	\Rightarrow $FADH_2$	-0.219
$CO_2 + 8 H^+ + 8 e^-$	\Rightarrow $CH_4 + 2 H_2O$	-0.24
$S + 2 H^+ + 2 e^-$	\Rightarrow H_2S	-0.243
$N_2 + 8 H^+ + 6 e^-$	\Rightarrow $2NH_4^+$	-0.28
$NAD^+ + H^+ + 2 e^-$	\Rightarrow $NADH$	-0.320
$NADP^+ + H^+ + 2 e^-$	\Rightarrow $NADPH$	-0.324
$2 H^+ + 2 e^-$	\Rightarrow H_2 (pH 7)	-0.414
$CO_2 + 4 H^+ + 4 e^-$	\Rightarrow $1/6$ glucose + H_2O	-0.43
$Fe^{2+} + 2 e^-$	\Rightarrow Fe	-0.85

Table 1. Biologically-relevant redox reactions.

An example you will use over and over again from the respiratory chain:



net reaction:



since $\Delta G^{\circ'} = -nF\Delta E^{\circ'} = -(2)(96,500)(1.13)$

$$= -218 \text{ kJ/mol}$$

How much ATP can you make from this overall process?

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