**Redox Cofactors**

\[ E^{0^+} \]


- 0.82 \( \text{O}_2 + 4\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O} \)
- 0.40 \( \text{cytochrome a}_2 (\text{Fe}^{3+}) + e^- \rightarrow \text{cytochrome a}_2 (\text{Fe}^{2+}) \)
- 0.30 \( \text{O}_2 + 2\text{H}_2\text{O} + 4e^- \rightarrow 2\text{H}_2\text{O}_2 \)
- 0.24 \( \text{cytochrome c} (\text{Fe}^{3+}) + e^- \rightarrow \text{cytochrome c} (\text{Fe}^{2+}) \)
- -0.20 \( \text{FAD} + 2\text{H}^+ + 2e^- \rightarrow \text{FADH}_2 \)
- -0.32 \( \text{NAD(P)}^+ + \text{H}^+ + 2e^- \rightarrow \text{NAD(P)}H \)

**Nicotinamide (pyridine dinucleotide)**

Bugg, Chapter 6 pp. 123 - 129

\[
\begin{align*}
\text{R} &= \text{H} \\
\text{NADH, NAD}^+ \\
\text{R}^+ \text{PO}_3^{2-} &= \text{NADPH, NADP}^+ \\
\end{align*}
\]

Vitamin B\textsubscript{3}, nicotinic acid, niacin

\[ E^{0^+} \] \( \text{NAD}^+/\text{NADH} \) couple = -0.32 V vs NHE

very strong reducing agent

\( \text{NAD(P)}^+ \leftrightarrow \text{NAD(P)}H \) mostly involved in 2e- chemistry

\( \text{H}^- \) (hydride) source
Reaction types catalyzed by NAD(P)⁺ / NAD(P)H

NAD(P)⁺ / NAD(P)H is a carbon hydride source!?

Electronegativities

<table>
<thead>
<tr>
<th>Element</th>
<th>Electronegativity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>2.1</td>
</tr>
<tr>
<td>Li</td>
<td>1.0</td>
</tr>
<tr>
<td>Be</td>
<td>1.6</td>
</tr>
<tr>
<td>Na</td>
<td>0.9</td>
</tr>
<tr>
<td>K</td>
<td>0.8</td>
</tr>
<tr>
<td>Mg</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>1.5</td>
</tr>
<tr>
<td>Si</td>
<td>1.8</td>
</tr>
<tr>
<td>P</td>
<td>2.1</td>
</tr>
<tr>
<td>S</td>
<td>2.5</td>
</tr>
<tr>
<td>Cl</td>
<td>3.0</td>
</tr>
<tr>
<td>Br</td>
<td>2.8</td>
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<tr>
<td>I</td>
<td>2.5</td>
</tr>
<tr>
<td>F</td>
<td>4.0</td>
</tr>
<tr>
<td>O</td>
<td>3.5</td>
</tr>
<tr>
<td>N</td>
<td>3.0</td>
</tr>
<tr>
<td>C</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Examples of carbon hydride sources:
Eschweiler-Clark Reaction

\[
R\text{-NH}_2 + H_2C\equiv O \rightarrow R\text{-N} = CH_2 \rightarrow R\text{-N} + CH_3 \quad \text{CO}_2
\]

Cannizzaro Reaction

\[
2 \text{Ph-C\text{-H}} + \Theta \text{OH} \rightarrow \text{Ph-C\text{-OH}} + \text{PhCH}_2\text{OH}
\]

Ethanol metabolism:

Alcohol Dehydrogenase:

- if run in T\textsubscript{2}O or D\textsubscript{2}O, no T or D incorporation in NADH
- if run with H\textsubscript{3}CCD\textsubscript{2}OH, complete D incorporation in NADH
- Results consistent with a hydride-transfer (H\textsuperscript{-}) mechanism and not a proton-transfer (H\textsuperscript{+})
Active site of horse liver alcohol dehydrogenase

Evidence for a hydride-transfer vs an electron-transfer mechanism
Cyclopropyl carbinyl radical ring opening as a probe for radical intermediates

\[
\text{cyclopropyl carbinyl radical (radical clock)} \quad \overset{k \approx 10^8 \text{ s}^{-1}}{\longrightarrow} \quad \text{4-butenyl radical}
\]

Product consistent with a hydride-transfer mechanism
If an electron-transfer mechanism:

\[
\begin{align*}
\text{CO}_2\text{H} & \quad + \quad e^- \\
\text{CO}_2\text{H} & \quad \rightarrow \\
\text{CO}_2\text{H} & \quad + \quad e^-
\end{align*}
\]

\[
\begin{align*}
\text{CO}_2\text{H} & \quad 2 \quad H^+ \\
\text{CO}_2\text{H} & \quad \rightarrow \\
\text{CO}_2\text{H}
\end{align*}
\]

Chemical model for the electron-transfer mechanism

Stereochemistry of alcohol dehydrogenase:

**pro-chirality**

H’s are enantiotopic, chemically equivalent
Enzymes are chiral and create a chiral environment around the substrate even though they are not covalently bound to each other.

Stereochemistry of alcohol dehydrogenase: hydride transfer is stereospecific

Stereochemistry inverted
Conformation of the nicotinamide cofactor determines if the pro-R or the pro-S hydrogen is transferred from the cofactor.

Axial H of NAD(P)H is preferentially transferred.

Conformation of NAD\(^+\) cofactors

NAD\(^+\) from Lactate Dehydrogenase (Pro-R specific)

NAD\(^+\) from Glyceraldehyde-3-phosphate Dehydrogenase (Pro-S specific)
Mechanism of Aldehyde dehydrogenase

Covalent catalysis:

Non-covalent catalysis:

These are mechanistically equivalent to a Cannizarro Reaction

Regeneration of NADH from NAD⁺ (glycolysis):

Glyceraldehyde-3-phosphate Dehydrogenase (G3PDH)
**Flavin Coenzyme:** Vitamin B₃, one- and two-electron transfer
Bugg, Chapter 6, pp. 129-142

![Flavin structures](image)

tricyclic flavin ring system: isoalloxazine

---

**Flavin Redox Chemistry**

![Flavin redox reactions](image)

\[ E^{\circ \ast} \approx -0.2 \text{ V vs NHE for free flavins (two-electron couple)} \]
The protein environment can have a large influence on the redox potential of the cofactor.

\[
\begin{align*}
& \text{Fl}_{\text{ox}} \quad \text{k}_m \quad [\text{Fl}_{\text{ox}} \cdot \text{Enzyme}] \\
& 2e^-, H^+ \quad \uparrow \quad \Delta G_1 \quad \downarrow \quad \text{E}^{\circ}\', \quad \Delta G_2 \quad 2e^-, H^+ \\
& \text{FlH}_{\text{red}}^\cdot \quad \text{k}_m' \quad \Delta G_3 \quad [\text{FlH}_{\text{red}}^\cdot \cdot \text{Enzyme}] \\
& \Delta G_2 \quad \Delta G_3 \quad \Delta G_4
\end{align*}
\]

thermodynamic cycle: \( \Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4 \)

\( \Delta G \) and \( \text{E}^{\circ}\' \) are related by the Nernst equation

\[
\Delta G^\circ = -nF\text{E}^{\circ}\' = -\frac{RT}{nF} \ln K_{eq}
\]

\( n \) = number of electrons

\( F \) = Faraday constant = 23.063 Kcal\( \text{V}^{-1} \text{eq}^{-1} \)

= 96.542 KJ\( \text{V}^{-1} \text{eq}^{-1} \)

---

Flavoenzymes are classified according to their re-oxidation mechanism by \( \text{O}_2 \)

Direct re-oxidation

- \( \text{substrate} \)
- \( \text{ox} \text{idized} \text{substrate} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{Fl}_{\text{sq}} \)
- \( \text{Fl}_{\text{red}} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{H}_2\text{O}_2 \)
- \( \text{O}_2 \)

or

- \( \text{substrate} \)
- \( \text{ox} \text{idized} \text{substrate} \)
- \( \text{Fl}_{\text{red}} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{Fl}_{\text{red}} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{H}_2\text{O}_2 \)
- \( \text{O}_2 \)

Indirect re-oxidation

- \( \text{substrate} \)
- \( \text{ox} \text{idized} \text{substrate} \)
- \( \text{Fl}_{\text{red}} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{Fl}_{\text{red}} \)
- \( \text{Fl}_{\text{ox}} \)
- \( \text{H}_2\text{O}_2 \)
- \( \text{O}_2 \)

---

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Flavoenzymes:

1. **Oxidases**: reduced flavin cofactor re-oxidized directly by O₂

2. **Dehydrogenase**: reduced flavin re-oxidized by another group, i.e.,

   \[
   \begin{align*}
   &\text{FlH}_{\text{red}} \quad \text{Fl}_{\text{ox}} \quad O_2 \quad H_2O_2 \\
   &\text{R-S-S-R} \quad 2 \text{R-SH} \quad \text{R-S-S-R}
   \end{align*}
   \]

3. **Mixed Function Oxidase**: reduced flavin reacts with O₂ to give a flavin-4a-hydroperoxide (Fl-OOH) which oxidizes the substrates by transferring an oxygen atom to the substrate. Overall, O₂ is “split” with oxygen atom being incorporated in the oxidized substrate, the other oxygen atom ends up as water.

4. **Electron-Transfer Flavoproteins** (ETF):

Oxygen:

\[
\begin{align*}
&\text{triplet} \quad 3 \left[ \begin{array}{c}
\cdot \\
\cdot \\
\cdot
\end{array} \right] \\
&\text{singlet} \quad 1 \left[ \begin{array}{c}
\cdot \\
\cdot \\
\cdot
\end{array} \right]
\end{align*}
\]

spin: \( s = 2n + 1 \)

spin of an electron = ± 1/2

For oxidases and mixed function oxidases:

\[
\begin{align*}
&\text{Flavin-4a-hydroperoxide} \\
&\text{pK}_a \ H_2O_2 \sim 11.6
\end{align*}
\]

For mixed function oxidases, the flavin 4a-hydroperoxide is the oxygen-transfer (oxidizing) agent
For mixed function oxidases (monooxygenases), the oxidized flavin must first be reduced to 1,5-dihydroflavin (FlH$_{\text{red}}$).
Reducing agent is often NAD(P)H and is often supplied as a separate enzyme.

\[
\text{NAD(P)H} \rightarrow \text{NAD(P)}
\]

Nicotinamide $E^{\circ} \sim -0.32 \text{ V}$
Flavin $E^{\circ} \sim -0.20 \text{ V}$

\[
\text{Nicotinamide} \quad \text{NAD} \quad \text{Flavin} \quad \text{FAD}
\]

\[
\text{p-hydroxybenzoate hydroxylase}
\]

\[
\text{electrophilic oxygen}
\]

\[
\text{H}_2\text{O}^{+} + \text{H}_2\text{O}
\]
Pterins: truncated flavin coenzymes

\[
\begin{align*}
\text{Biopterin} & \quad \text{pteridine reductase} \quad \text{NADPH, H}^+ \\
\rightarrow & \\
\text{Dihydrobiopterin} & \quad \text{dihydropteridine reductase}
\end{align*}
\]

Bugg, p. 141-142

Phenylalanine hydroxylase: tetrahydrobiopterin dependent mixed function oxidase

Electrophilic substitution analogous to p-hydroxybenzoate hydroxylase

Mechanism of the NIH (Jerina-Daly) Shift:
Cyclohexanone Monooxygenase: Flavin-4a-hydroperoxy anion as a nucleophilic oxidant

\[
\begin{align*}
\text{Cyclohexanone} & \xrightarrow{H^+} \text{Flavin-4a-hydroperoxy anion} \\
 & \xrightarrow{pK_a H_2O_2 \approx 11.6} \text{Baeyer-Villager Reaction}
\end{align*}
\]

Glutathione Reductase: NADPH-dependent flavin dehydrogenase

\[
\begin{align*}
\text{Glutathione} & \xrightarrow{2e^-, 2H^+} \text{G-S-G} \\
\text{Glu} \quad \text{Gly} \quad \text{Cys} & \quad = \text{G-SH} \\
\text{NADPH} & \quad \text{NADPH} \\
\text{Fl} & \quad \text{FlH}_{\text{red}} \\
\text{G-S-H} & \quad \text{G-S-G} \\
\text{HS-G} & \quad \text{GS-SG} \\
\text{O}_2 & \quad \text{H}_2\text{O}_2
\end{align*}
\]
**Glutathione Reductase:** mechanism involves a covalent adduct at the 4a-position of the FAD cofactor

\[
\text{Cys}_{41}-\text{Cys}_{46}\]

\[
\text{G-S-S-G}
\]

pdb code: 1GRA
**Mechanism of Flavoenzyme Catalysis:** D-amino acid oxidase

C-nucleophile mechanism. Amino acid enolate adds to the flavin 4a-position. The flavin then acts as a leaving group.

\[ \text{pK}_a \approx 25 \]

X-ray structure shows NO active site base

**Mechanism of Flavoenzyme Catalysis:** D-amino acid oxidase (con’t)

N-nucleophile mechanism

Hydride transfer mechanism: concerted, singled step mechanism, no covalent intermediate. Hydride adds directly to the flavin N5-position.
Kinetic Isotope Effect (KIE): influence of a substitution of an isotope on the rate of a reaction. Gives information on the rate limiting step of the reaction.

\[
C-H (D) \rightarrow [H^+ \cdots H^+] \quad \ddagger \quad \nu = \frac{1}{2} \pi c \left[ \frac{f}{m_x m_y} \left( \frac{m_x}{m_x + m_y} \right) \right] \frac{1}{2}
\]

The magnitude of the KIE is dependent on the mass difference of the isotopes.

Energy required to break the C-H or C-D bond

\[\Delta G^\ddagger\] for the heavier isotope (C-D) is greater, which translates into a slower reaction rate.

KIEs are expressed as: \[\frac{k_H}{k_D}\]

Transition state of the hydride transfer mechanism:

A C-H and N-H bonds are being broken in the transition state.

Kinetic isotope effect data is consistent with two bonds being broken in the transition state for D-amino acid oxidase.
Crystal structure of D-amino acid oxidase supports the hydride transfer mechanism

pdb code: 1C0L

Succinate and Acetyl CoA Dehydrogenase

Anion mechanism

\[ R - C = X + \text{substrate} \rightarrow \text{product} + R - C = X \]

where \( X = \text{O}^- \) or SCoA
Succinate and Acetyl CoA Dehydrogenase (con’t)

Hydride-transfer mechanism

\[
\begin{align*}
\text{B:} & \quad R \quad \text{N} \quad \text{N} \quad \text{H} \\
\text{O} & \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{H} \\
\text{R} & \quad \text{H} \quad \text{X} \\
\text{X} & \quad \text{H} \\
\end{align*}
\]

\[
\begin{align*}
\text{B:} & \quad R \quad \text{N} \quad \text{N} \quad \text{H} \\
\text{O} & \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{H} \\
\text{R} & \quad \text{H} \\
\end{align*}
\]

Succinate and Acetyl CoA Dehydrogenase (con’t):

Electron-transfer mechanism

- Flavins do not abstract hydrogen atoms (H•)
- Covalently inactivated enzyme

\[
\begin{align*}
\text{B:} & \quad R \quad \text{N} \quad \text{N} \quad \text{H} \\
\text{O} & \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{H} \\
\text{R} & \quad \text{H} \\
\end{align*}
\]

\[
\begin{align*}
\text{B:} & \quad R \quad \text{N} \quad \text{N} \quad \text{H} \\
\text{O} & \quad \text{N} \quad \text{O} \quad \text{N} \quad \text{H} \\
\text{R} & \quad \text{H} \\
\end{align*}
\]

223
There is good evidence for the N-nucleophile mechanism for MAO (slide 215).

Porphyrin vs. Corrin

Heme: Ligand for Fe(III) is a porphyrin, which is a fully conjugate ligand

Vitamin B_{12}: Ligand for Co(III) is a corrin, which is NOT a fully conjugate ligand
Heme (Cytochrome P450)-dependent Monooxygenases:
over 500 different isozyme- Superfamily
Bugg, Chapter 6, pp. 143-147

Overall mechanism of P450 hydroxylation

R-H + O2 → R-OH

R-H + O2 → R-O⁻ + H⁺
**Active site of cytochrome P450 enzymes**

**P450 cam**  
*pdbe code: 1AKD*

**P450 2B4**  
*pdbe code: 1PO4*

T252V or T252A mutants lose hydroxylase activity  
T252X retains ~ 33% of the WT activity  
suggest an intervening water molecule(s)  
as the H⁺ source  
X= O-methylthreonine

**Mechanism of the hydroxylation step:**  
oxygen rebound mechanism (J. Groves)
Mechanism of the hydroxylation step (con’t):
Concerted oxygen insertion (M. Newcomb)

\[
\begin{align*}
H-O-O&-H \quad \xrightarrow{H^+} \quad H-O-\overset{=}O-H \quad \xrightarrow{H_2C-CH_3} \quad H_3C-H_2C-OH
\end{align*}
\]

protonated hydrogen peroxide:
a very strong oxidant

\[
\begin{align*}
\text{proton may be from a conserved threonine. Not acidic enough?}
\end{align*}
\]

resembles protonated hydrogen peroxides

Ultra-fast Radical Clocks as mechanistic probes for P450 hydroxylation reactions: consistent, in part, with the oxygen insertion mechanism

\[
\begin{align*}
\text{major product} & \quad \sim 48:1 \\
\text{~ 2-3 : 1}
\end{align*}
\]
**Cytochrome P450 mediated epoxidation reactions:**
activation of pro-carcinogens

- Benzo[a]pyrene
- Aflatoxin B₁

**Mechanism cytochrome P450 epoxidation of alkenes:**
Oxygen rebound mechanism

Concerted mechanism:
Mechanistic probe for P450 alkene epoxidation:

Not Observed: suggests concerted mechanism

Vitamin B\textsubscript{12} Dependent Enzymes: Bugg, Chapter 11, pp 240-244

Mutase- catalyzes a rearrangement of the substrate
Conversion of vitamin B$_{12}$ to co-enzyme B$_{12}$

Methylmalonyl CoA Mutase:

Methylmalonyl CoA

succinyl CoA

Co-enzyme B$_{12}$
Methylmalonyl CoA Mutase (con’t):

\[
\begin{align*}
\text{Methylmalonyl CoA} & \quad \xrightarrow{\text{Methylmalonyl CoA Mutase}} \\ \text{succinyl CoA} & \quad + \text{Co-enzyme B}_{12}
\end{align*}
\]

![Methylmalonyl CoA Mutase diagram](image)

Glutamate mutase

\[
\begin{align*}
\text{L-glutamate} & \quad \xrightarrow{\text{Glutamate mutase}} \\ \text{(2S,3S)-3-methyl-L-aspartate} & \quad + \text{Coenzyme B}_{12}
\end{align*}
\]

![Glutamate mutase diagram](image)

pDB code: 19C