Enzymatic Oxidation–Reduction Reactions 33

Life is interposed between two energy levels of the electron.

Albert Szent Gyorgyi

The driving force, for all life as we know it, is derived from reduction–oxidation (redox) reactions. Most biological oxidations are often coupled to cellular energy production. Typically carbon compounds (such as carbohydrates) are oxidized to carbon dioxide, while oxygen is reduced to water. Enzymes play a significant role in connecting the series of redox reactions ultimately involving oxygen. In mitochondrial electron transport chain, electrons are passed from NADH along a series of electron acceptors/donors (oxidants and reductants) to O_2. Molecular oxygen is the final oxidant (terminal electron acceptor) of aerobic metabolism. Biological reductions, on the other hand, are employed to store energy in chemical forms for later use. In photosynthetic organisms, reduction of carbon dioxide (to carbohydrates) is powered by sunlight, while water is oxidized (to oxygen). This broad canvas of redox reactions serves to drive pumps, maintain concentration gradients across membranes, and generate metabolites that have high group transfer potential and/or are energy rich. Not surprisingly, oxidoreductases form a significant group (EC 1.x.x.x) of well-represented enzymes (Chap. 4).

33.1 What Are Oxidation–Reduction Reactions?

Oxidation–reduction reactions involve transfer of electrons between two chemical species. A compound which loses electron(s) is oxidized while the compound that gains electron(s) is reduced. A compound that donates its electron(s) is called a *reductant* or a *reducing agent*. Conversely the electron-accepting molecule is the *oxidizing agent* or *oxidant*. The oxidation and reduction events must occur together –

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that is, an oxidant has to be present to accept the electron(s) from a reductant. In other words, for a molecule to be reduced, some other molecule has to get oxidized. In this sense redox reactions may be compared to proton transfers in acid–base chemistry. An acid can lose/donate a proton only when a base accepts it. No oxidant can gain electrons without another substance (reductant) losing electrons. A complete oxidation–reduction reaction is thus a combination of two half reactions (*redox couples*). When the two half reactions are combined, the component with greater tendency to gain electrons (the oxidant) gets reduced at the expense of the other (the reductant – which loses electrons).

Reduction Potential – Measure of Tendency to Lose Electrons We rank acid strengths based on their pKas; the lower the pKa, the stronger is that acid (Chap. 30). Similarly, the tendency to gain/lose electrons may be used to rank order compounds. The strength of an oxidizing agent can be measured electrochemically by dissolving it in water and measuring the voltage required to reduce it. This in fact defines a scale of *standard reduction potentials* (or *redox potentials*) for each oxidant/reductant. Table 33.1 contains a selective list of redox couples and their standard reduction potentials. A large positive standard reduction potential indicates high electron affinity of that compound and that it is a strong oxidant. Its conjugate reductant is a poor electron donor and a weak reducing agent. For example, the conjugate redox pair of $½O_2/H_2O$ has the highest positive standard reduction potential (+0.816 V; where V denotes volts). This makes O_2 the strongest available oxidizing agent and H_2O (its conjugate reductant) the weakest reducing agent. At the other end of the spectrum, $2H^+/H_2$ redox pair (−0.421 V) has a large negative standard reduction potential.

Table 33.1 Standard reduction potentials for few biologically relevant redox couples

Redox couples (shown in the direction of reduction)	E'° (V)
$½O_2 + 2H^+ + 2e^- \rightarrow H_2O$	+0.815
$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$	+0.300
Cytochrome c (Fe^{3+}) + $1e^- \rightarrow$ Cytochrome c (Fe^{2+})	+0.254
Dehydroascorbate $+2H^+ + 2e^- \rightarrow$ Ascorbate	+0.060
$2H^+ + 2e^- \rightarrow H_2$	**0.000**[a]
Oxaloacetate $+2H^+ + 2e^- \rightarrow$ Malate	−0.170
FAD $+2H^+ + 2e^- \rightarrow FADH_2$	−0.180[b]
Pyruvate $+2H^+ + 2e^- \rightarrow$ Lactate	−0.185
GSSG $+2H^+ + 2e^- \rightarrow$ 2 GSH	−0.230
Lipoate $+2H^+ + 2e^- \rightarrow$ Dihydrolipoate	−0.290
$NAD^+ + 2H^+ + 2e^- \rightarrow NADH + H^+$	−0.320
$NADP^+ + 2H^+ + 2e^- \rightarrow NADPH + H^+$	−0.320
$2H^+ + 2e^- \rightarrow H_2$	−0.421[a]

All the E'° values are for standard conditions of unit activity (1 M concentration) at 25°C and pH 7.0 ($[H^+] = 10^{-7}$ M); Gases are at 1 atmospheric pressure. [a]By convention, the standard hydrogen electrode at pH 0 ($[H^+] = 1$ M) has an E° of zero. However, at pH 7.0 its measured E'° value is − 0.421 V. [b] This E'° value is for free $FAD/FADH_2$ redox couple. Depending upon the active site microenvironment in a given flavoprotein, this value varies from − 0.450 V to + 0.150 V

Reduction Potentials and Reaction Thermodynamics Reduction potentials measure the affinity for electrons. The standard reduction potential is measured in volts (V) and denoted as $\mathbf{E}^\circ$. The hydrogen electrode (representing $2H^+/H_2$ redox pair) is assigned an arbitrary $\mathbf{E}^\circ$ value of 0.00 V (Table 33.1), and all others are ranked relative to it. Many redox couples of biological interest involve protons. Standard reduction potentials for biological redox couples are thus more meaningful when expressed at pH 7.0 (near physiological conditions and not at pH = 0). And therefore such reduction potentials (defined at pH 7.0 and 25 °C) are shown as $\mathbf{E}'^\circ$ (and not as $\mathbf{E}^\circ$). Accordingly, when $[H^+]$ is 10^{-7} M (i.e., pH is 7.0), the $\mathbf{E}'^\circ$ of $2H^+/H_2$ redox pair (the hydrogen electrode) becomes 0.421 V.

The reduction potential (**E**) not only depends on the chemical nature of a given redox couple ($\mathbf{E}'^\circ$ as listed in Table 33.1) but also on their relative concentrations (activities). This relationship is described by the *Nernst equation*:

$$\mathbf{E} = \mathbf{E}^\circ + \frac{\mathbf{RT}}{\mathbf{n}\boldsymbol{F}} \ln \frac{[\textbf{electron acceptor}]}{[\textbf{electron donor}]}$$

where **n** is the number of electrons transferred per molecule and ***F*** is the Faraday constant (23.063 kcal$\times V^{-1}\times mol^{-1}$). The actual **E** value thus depends on the concentrations of oxidized and reduced species of the redox couple. We note that Nernst equation and the free energy relationship between ΔG, ΔG° and the composition of the reaction mixture ($\Delta G = \Delta G^\circ + RT \ln \Gamma$; see Chap. 10) are analogous.

Electrons spontaneously flow from a compound with lower reduction potential to that with a higher reduction potential. Therefore, under standard conditions, they are transferred from reduced component of any conjugate redox pair in Table 33.1 to the oxidized component of any conjugate redox pair above it. By convention, the tendency of electron flow (indicated by $\Delta\mathbf{E}'^\circ$) for any oxidation–reduction reaction between two redox couples may be calculated as follows:

$$\Delta\mathbf{E}'^\circ = \mathbf{E}'^\circ_{\textbf{electron acceptor}} - \mathbf{E}'^\circ_{\textbf{electron donor}}$$

For example, electrons can spontaneously flow from H_2 (−0.421 V) to O_2 (+0.816 V) – i.e., the process is thermodynamically feasible ($\Delta\mathbf{E}'^\circ$ = +1.237 V). The more positive the $\Delta E'^\circ$, the greater is the tendency for electron flow between the two redox couples. In this sense, ΔE for a redox reaction and its corresponding ΔG are related by

$$\Delta\mathbf{G}^\circ = -\mathbf{n} \times F \times \Delta\mathbf{E}'^\circ \qquad (\textbf{and also}, \Delta\mathbf{G} = -\mathbf{n} \times F \times \Delta\mathbf{E})$$

where, again, **n** is the number of electrons transferred per mole (equivalents per mole) in the redox reaction and ***F*** is the Faraday constant (23.063 kcal × ×V^{-1} × mol^{-1}). A positive ΔE therefore means that ΔG is negative and the reaction is spontaneous (i.e., thermodynamically favorable; Chap. 10). By analogy, *the*

criterion of spontaneity for a given oxidation–reduction reaction is ΔE (and not $\Delta E'^{\circ}$). All these concepts are illustrated with the help of lactate dehydrogenase reaction in the box below.

Redox Chemistry of Lactate Dehydrogenase Reaction

The following reversible reaction is catalyzed by lactate dehydrogenase:

$$\textbf{Pyruvate} + \textbf{NADH} + \textbf{H}^{+} \rightleftarrows \textbf{Lactate} + \textbf{NAD}^{+}$$

Viewed from left to right, pyruvate is reduced to lactate, while NADH is oxidized to NAD^+ in this reaction. In the reverse direction (from right to left), NAD^+ is reduced to NADH at the expense of lactate oxidation. *Oxidation–reduction reactions are thus always coupled*. For convenience, however, we can describe them as two half reactions (redox couples), both written in the direction of reduction as shown below:

$$\begin{aligned} &\text{Reduction of pyruvate}: && \text{Pyruvate} + 2\text{H}^{+} + 2\text{e}^{-} \rightarrow \text{Lactate} \\ &\text{Reduction of NAD}^{+}: && \text{NAD}^{+} + 2\text{H}^{+} + 2\text{e}^{-} \rightarrow \text{NADH} + \text{H}^{+} \end{aligned}$$

The E'° values for these two redox couples, under standard conditions, may be obtained from Table 33.1. The electrons flow from NAD^+/NADH couple (-0.320 V) to pyruvate/lactate couple (-0.190 V) because the latter is at higher positive standard reduction potential. This means pyruvate/lactate half reaction goes as shown while the NAD^+/NADH couple undergoes oxidation. The NADH is the reducing agent and pyruvate is the oxidizing agent. The $\Delta E'^{\circ}$ for this reaction may be accordingly calculated:

$$\begin{aligned} \Delta \text{E}^{'\circ} &= \text{E}'^{\circ}_{\text{pyruvate/lactate}} - \text{E}'^{\circ}_{\text{NAD/NADH}} \\ &= -0.190\ \text{V} - (-0.320)\text{V} \\ &= +0.130 \end{aligned}$$

The ΔG° for the reaction may now be obtained as the two are related. It is a two-electron reduction ($n = 2$) and therefore

$$\Delta G^{\circ} = -n \times F \times \Delta E^{\circ} = -2 \times 23.063 \times 0.130 = -5.996\,\text{kcal} \times \text{mol}^{-1}$$

Since the ΔG° is negative (-5.996 kcal$\times$mol^{-1}), *reduction of pyruvate by NADH is a spontaneous process under standard conditions* (at 25°C and pH 7.0 and with pyruvate, lactate, NAD^+, and NADH all at 1.0 M).

Lactate dehydrogenase (rat skeletal muscle) is exclusively cytosolic. The approximate concentrations of lactate (5.0 mM), pyruvate (0.5 mM), NAD^+ (0.5 mM), and NADH (0.001 mM) in this compartment are reported.

(continued)

Reduction potentials for the two redox couples under these conditions (at 25°C) may now be calculated as shown.

$$E_{\text{Pyr/Lac}} = E'^{\circ} + \frac{RT}{nF} \ln \frac{[\text{Pyruvate}]}{[\text{Lactate}]} = -0.190\,\text{V} + \left(\frac{2.303 \times 1.987 \times 298}{2 \times 23063} \log \frac{[0.5]}{[5.0]} \right) \text{V}$$

$$= -0.190\,\text{V} + 0.0296 \times \log(0.1)\,\text{V} = -0.190\,\text{V} - 0.0296\,\text{V} = -0.2196\,\text{V}$$

Similarly,

$$E_{\text{NAD/NADH}} = E'^{\circ} + \frac{RT}{nF} \ln \frac{[\text{NAD}^+]}{[\text{NADH}]}$$

$$= -0.320\,\text{V} + \left(\frac{2.303 \times 1.987 \times 298}{2 \times 23063} \log \frac{[0.5]}{[0.001]} \right) \text{V}$$

$$= -0.320\,\text{V} + 0.0296 \times \log(500)\,\text{V} = -0.320\,\text{V} + 0.0799\,\text{V} = -0.240\,\text{V}$$

The NAD^+/NADH couple (−0.240 V) has a more negative reduction potential than that of pyruvate/lactate couple (−0.2169 V). Accordingly, NADH reduces pyruvate but the ΔG for this reduction ($n = 2$) is different. This may now be calculated as before:

$$\Delta G = -n \times F \times \Delta E = -2 \times 23.063 \times (-0.2196\,\text{V} + 0.240\,\text{V})$$
$$= -0.941\,\text{kcal} \times \text{mol}^{-1}$$

This ΔG value is different from ΔG° ($-5.996\ \text{kcal} \times \text{mol}^{-1}$; the standard free energy change) for this redox reaction – it indicates the actual free energy change at the physiological concentrations of the redox couples prevailing in the muscle.

If the pyruvate/lactate ratio in the liver is 1:100, then $E_{\text{Pyr/Lac}}$ will be −0.249 V. This is more negative than that of NAD^+/NADH couple (−0.240 V), and hence lactate now reduces NAD^+ (ΔG for the reaction as written above will be $+0.415\ \text{kcal} \times \text{mol}^{-1}$). The redox reaction actually goes from right to left in the liver! It may be reiterated that *the criterion of spontaneity for a reaction is ΔE (or ΔG) and not ΔE′° (or ΔG°)*. Reversible feature of lactate dehydrogenase reaction forms the basis of functional Cori's cycle.

Effect of pH on Standard Reduction Potentials We noted that the standard hydrogen electrode at pH 0 ($[H^+] = 1$ M) has an E° of zero by convention (Table 33.1). And at pH 7.0, its measured E'° value is −0.421 V. In general, pH does influence the E° value of all redox couples that involve H^+ ions in their half reactions. The NAD^+/NADH couple is a common example of this kind. As long as the pH of the reaction is maintained (recall that at standard condition, pH is 7.0), its

E'° value is −0.320 V. At any other pH, the reduction potential of NAD^+/NADH couple will change. We can calculate the effect of pH on E by suitably including [H^+] term in the Nernst equation. For unit increase in pH, the E becomes more negative by 0.059 V (for one electron reduction). For NAD^+/NADH couple (where $n = 2$), however, the ΔE will be 0.0295 V for each pH unit change.

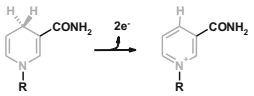
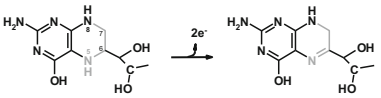
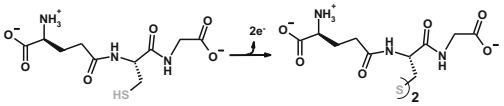
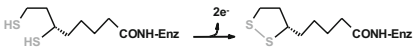
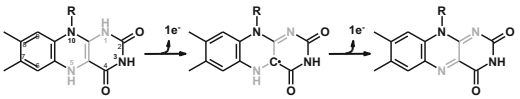
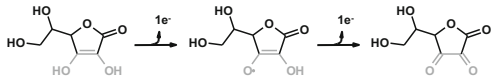
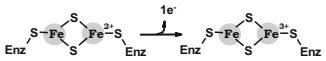
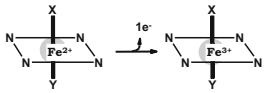
33.2 How Enzymes Influence Redox Reaction Rates

We noted above that reduction of pyruvate by NADH is a spontaneous process under standard conditions. However, when pyruvate and NADH are mixed together, no reaction occurs. While this reaction is thermodynamically allowed (i.e., spontaneous with ΔG negative), there exists a kinetic barrier. This is where enzymes come into picture. As with any other reaction, an enzyme can facilitate and catalyze redox reactions. *Overcoming kinetic barriers of redox reactions* is their foremost feature.

At times the reduction potential difference between two reactants (redox couples) may be large – making the reaction difficult. Redox couples with intermediate reduction potentials – *mediators* – often facilitate such reactions. They act as "go between" the two reactants. Obviously such mediators themselves are redox-active groups/compounds. Except for the thiols (reversible oxidation–reduction of cysteine⇄cystine; Table 33.1), there is not much on the polypeptide chain for an enzyme to offer. Further, when a substrate is oxidized, electrons have to be moved out. There are no obvious electrophilic groups in the amino acid side chains of proteins. Thus redox enzymes without exception show obligate requirements for either (a) an organic coenzyme to act as an electron acceptor or (b) a redox-active transition metal to pass the parcel of electrons to another acceptor molecule. A number of nonprotein coenzymes, cofactors, and prosthetic groups are recruited for this purpose. This is the second feature through which enzymes contribute to redox catalysis. More common examples of these are listed in Table 33.2.

Enzymes are able to modulate redox potentials of bound substrates and/or cofactors. This is done by virtue of their ability to create unique microenvironments – the third important feature of redox enzymes. Two examples of *redox modulation* illustrate this point well. The E'° value of bound FAD/$FADH_2$ couple varies from −0.450 V to +0.150 V, depending upon the flavoprotein active site (Table 33.1). When the active site preferentially binds FAD over $FADH_2$, then the E'° of this redox couple becomes more negative. Conversely, if $FADH_2$ is favored over FAD, the E'° tends to become more positive. These conclusions simply follow from the Nernst equation (see box above – Redox chemistry of lactate dehydrogenase reaction – for a similar calculation). Cytochromes provide yet another example where enzyme protein brings about a shift in the reduction potential by differential binding. They all contain heme – an iron atom stuck between four N atoms of a porphyrin. But the fifth and the sixth ligands donated by the polypeptide greatly influence the reduction potential of the bound heme. This forms the basis for the "bucket brigade"

Table 33.2 Cofactors participating in enzymatic oxidation–reduction reactions

Cofactor/prosthetic group (enzyme example)	Reduced form → oxidized form[a]
Nicotinamide adenine dinucleotides (lactate dehydrogenase)	 NAD(P)H NAD(P)$^+$
Biopterin (phenylalanine hydroxylase)	 5,6,7,8-Tetrahydrobiopterin 7,8-Dihydrobiopterin
Glutathione (glutathione peroxidase)	 2 GSH GSSG
Lipoamide (dihydrolipoyl dehydrogenase)	 Dihydrolipoate Lipoate
Flavins (FAD and FMN) (succinate dehydrogenase, glutathione reductase)	 1,5-Dihydroflavin Flavin semiquinone Oxidized flavin
Ascorbic acid (vitamin C) (prolyl hydroxylase; 2-ketoglutarate decarboxylating)	 Reduced Ascorbate radical Oxidized
Iron–sulfur clusters (Fe-S)$_n$ (where $n = 2$ or 4) (dihydroxylating dioxygenase; ferredoxins)	 (Fe-S)$_2$ cluster
Heme (cytochrome oxidase)	
Transition metal ions (like Cu, Fe, and Mo) (laccase; catechol dioxygenase)	

[a]The number of electrons transferred in oxidation step(s) is shown. While proton transfers may accompany oxidation, this inventory is not explicitly shown. Groups/atoms relevant to oxidation are marked in gray. See Table 31.1 (Chap. 31) for details of R groups in NAD(P), FAD, and FMN. Amino acid side chains, water, O_2, etc. provide the fifth and sixth ligands (X and Y) to heme iron

of cytochromes lining up the electron transport chains. Cytochrome a_3 (+0.385 V) of cytochrome oxidase can easily steal an electron from cytochrome c (+0.235 V) in the mitochondria.

33.3 Mechanisms and Modes of Electron Transfer

The carbon encountered in an enzyme substrate exists in a range of oxidation states. For example, various oxidation states of a one-carbon compound are shown below (Fig. 33.1). Considering their electronegativities (H < C < O), the notional number of electrons present on carbon decreases by two in each step. More reduced compounds are richer in H than O, and conversely more oxidized compounds are richer in O than H.

Biological oxidation reaction may be broadly viewed as (a) removal of electrons from a substrate molecule or (b) its direct combination with oxygen, where oxygen atom accepts the electron(s). We will look at reactions involving molecular oxygen a little later. A substrate molecule is oxidized when electrons are transferred from it to an acceptor. Such transfers occur in multiples of *single-electron currency – the reducing equivalent*. In practice this may be through $1e^-$ transfers or $2e^-$ transfers. A vast majority of organic substrate oxidations are $2e^-$ transfer events. The two electrons may be transferred in a single step or they may be moved one at a time (two $1e^-$ transfer steps). Single-electron transfers generally have high-energy barriers and require stabilization of a radical. *If two $1e^-$ steps are involved, then a free radical intermediate must form.* Free radicals may be detected by their typical EPR signals. Few structures like flavins and ascorbic acid (Table 33.2) help stabilize such reactive species. Other chemical apparatus available for enzymes to do this are quinoid structures of some coenzymes (vitamins E and K and coenzyme Q) and redox-active transition metals (like Fe, Cu, and Mo). *Quinoproteins* are a recently characterized group of enzymes (quinoproteins, copper-quinoproteins, and quinohemoproteins) whose catalytic mechanisms involve free radical intermediates on quinone-containing prosthetic groups. Pyrroloquinoline quinone (PQQ) is one such prosthetic group found in a number of bacterial dehydrogenases and oxidases (Duine 1999; Klinman 1996).

The more common $2e^-$ transfers can occur by two distinct mechanisms: a) Enzymatic dehydrogenations offer the first example where hydride (H^-, a proton

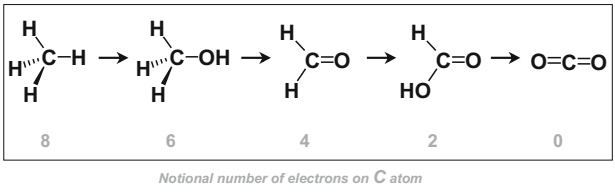


Fig. 33.1 Various oxidation states of one carbon compounds. Oxidation state of carbon increases from left (methane) to right (carbon dioxide)

with two electrons) transfer may be the oxidation step. Reactions of nicotinamide cofactors (NAD and NADP) apparently go through hydride transfer step(s). b) In the second option, a substrate proton is first abstracted. A substrate carbanion is therefore expected to form, at least transiently. In a subsequent step, $2e^-$ transfer from the carbanion to an acceptor takes place. Some of the flavoenzymes operate through this mechanism.

33.4 Pterine and Folate Cofactors

Pterines and folic acid derivatives (Table 33.2) in their many forms perform diverse roles in nature ranging from pigments (butterfly colors) to cofactors for numerous redox and one-carbon transfer reactions (Benkovic 1980). The unique chemistry of the pteridine heterocycle is responsible for this extraordinary diversity of function. The pteridine ring system resembles the isoalloxazine ring of the flavine coenzymes (see Table 33.2). Pterines are important redox cofactors (and include folic acid, tetrahydrobiopterin, and molybdopterin). The active form of biopterin is 5,6,7,8-tetrahydrobiopterin. This is oxidized to 7,8-dihydropterin during hydroxylation of the aromatic ring of phenylalanine by phenylalanine hydroxylase (see Table 33.4 below). A similar role is played by biopterin during the formation of 3,4-dihydroxypheylalanine from tyrosine. Folate differs structurally from biopterin in that the substituent on C6 of the pteridine ring (see Table 33.2) consists of *p*-aminobenzoic acid further linked to polyglutamic acid. Tetrahydrofolate (THF) is a versatile cofactor that participates in redox chemistry and also functions to transfer "one-carbon" units in several oxidation states (Table 33.3). The "one-carbon" units are covalently attached to THF at its N^5, N^{10}, or both N^5 and N^{10} positions (Matthews and Drummond 1990). The one-carbon units enter the THF pool from serine, by the action of serine hydroxymethyltransferase (see Table 35.4 in Chap. 35), at N^5,N^{10}-methylene-tetrahydrofolate. The only other one carbon (methyl group) transfer chemistry involves coenzyme B_{12}.

Table 33.3 Enzyme reactions of different "one-carbon" tetrahydrofolate derivatives

Tetrahydrofolate (THF) derivative[a]	Enzyme reaction
N^5-Methyl-THF	Homocysteine methyltransferase
N^5,N^{10}-Methylene-THF	Serine hydroxymethyltransferase, thymidylate synthase
N^5-Formyl-THF, N^{10}-Formyl-THF	Formylmethionyl-tRNA synthase, GAR transformylase, AICAR transformylase
N^5-Formimino-THF	Glutamate formiminotransferase
N^5,N^{10}-Methenyl-THF	N^5,N^{10}-Methenyl-THF cyclohydrolase

[a]The oxidation states of "one-carbon" units correspond to methanol (methyl-THF), formaldehyde (methylene-THF), and formate (formyl-THF, formimino-THF, and methenyl-THF) as shown in Fig. 33.1

Table 33.4 Enzyme active sites for molecular oxygen to react

Prosthetic group	Examples
Heme iron	Cytochrome oxidase, cytochrome P_{450}
Non-heme iron	Monooxygenases and dioxygenases, prolyl hydroxylase (2-oxoglutarate dependent), superoxide dismutase
Copper (monometallic)	Amine oxidase
Copper (bimetallic)	Laccase, oxidases, superoxide dismutase
Manganese	Photosystem II (oxygen-evolving complex), superoxide dismutase
Flavin (FAD or FMN)	D-Amino acid oxidase, oxygenases, oxidases
Biopterin	Phenylalanine hydroxylase

The importance of folate as a cofactor is obvious as it participates in the biosynthesis of several amino acids and nucleotides. Both 7,8-dihydropterin and 7,8-dihydrofolate are reduced back to their active tetrahydro-states by dihydrofolate reductase. This enzyme is a key target for cancer chemotherapy (inhibited by methotrexate; see Chap. 21 Irreversible inhibitions). Similarly, sulfanilamide (a sulfa drug and a structural analog of *p*-aminobenzoic acid) is a medically valuable antibacterial agent (Table 28.4 in Chap. 28).

Nicotinamide and riboflavin are by far the most common redox cofactors used by enzymes in nature. We will describe the salient features and associated chemical mechanisms for them in some detail below. The support for their proposed mechanisms has come from actual enzyme reactions as well as from corresponding model reactions.

33.5 Nicotinamide Cofactors

Nicotinamide cofactors frequently participate in enzymatic redox reactions. They take part in oxidations and reductions via their pyridine ring (of the nicotinamide) and hence are sometimes also termed pyridine nucleotides. Change in absorbance (at 340 nm) associated with their oxidation–reduction is a convenient way of monitoring these enzyme reactions (Chap. 12). Nicotinamide adenine dinucleotide (NAD^+) was the first enzyme cofactor to be discovered in the conversion of glucose to ethanol by yeast (by Harden and Young in 1904). Subsequently, nicotinamide adenine dinucleotide phosphate ($NADP^+$) was identified (by Warburg and Christian in 1934) as the redox cofactor responsible for glucose-6-phosphate oxidation in erythrocytes. Total chiral synthesis of NAD^+ was accomplished in 1957 by Todd's group. $NADP^+$ differs from NAD^+ in having an extra phosphate group (see Table 31.1 for structures). $NADP^+$ sports the additional phosphate group on its 2′OH of adenosine moiety. Despite this structural difference, both NAD^+/NADH

and $NADP^+$/NADPH redox pairs have identical reduction potentials (−0.320 V, Table 33.1). Physiologically, however, NAD^+/NADH redox couple functions largely as an electron acceptor (oxidizing agent) and is preferred in catabolic reactions. $NADP^+$/NADPH couple on the other hand is the choice reductant (electron donor) in biosynthetic steps of metabolism.

Enzymes display a conserved structural motif to bind NAD^+ – called the Rossmann fold. Active sites of many dehydrogenases bind NAD^+ in an extended conformation. In the bound state, the orientation of nicotinamide group can be either *anti* (away from; as in malate dehydrogenase) or *syn* (toward; as in glyceraldehyde-3-phosphate dehydrogenase) position with respect to its N-glycosidic bond. In a majority of enzymes, the pyridine nucleotide cofactor is bound reversibly (more like a substrate). However UDP-galactose epimerase is known to contain stoichiometric, tightly bound NAD^+ at its active site.

NAD(P)H is a powerful biological reducing agent but is stable in air. Reduced pyridine nucleotides (both NADH and NADPH) do not react with oxygen – in this sense they are distinct from reduced flavins which do (see below). The NAD^+/NADH couple is a common participant in all dehydrogenases wherein >CH-XH is oxidized to >C = X (where X is N or O). As with some reductase reactions, this redox couple also is used to reduce C = C double bonds. The redox chemistry with NAD^+/NADH couple is believed to proceed with a *hydride transfer* step. Oxidation of the C-H bond is often shown as its heterolytic cleavage – accompanied by a hydride transfer to C-4 position of NAD^+. Failure to equilibrate this transferable hydrogen with solvent (water) protons is suggestive of this mechanism. However this does not constitute a final proof of hydride transfer – because some enzymes can exclude water from their active site during catalysis. When a hydride from NAD(P)H is transferred to carbon atom, acid–base dissociation of the resultant C-H bond is exceedingly slow. But whenever the hydride is transferred to a hetero-atom (which is electronegative), then it exchanges rapidly with solvent protons. Isotopic labeling studies coupled with observed deuterium kinetic isotope effects have led to the general acceptance of a hydride transfer mechanism. Such a mechanism for lactate dehydrogenase reaction is depicted in Fig. 33.2

The pyridine ring is puckered and non-planar in the reduced state (NADH or NADPH), whereas it is planar when oxidized. The C-4 of NADH has two nonequivalent hydrogens (labeled A and B); either one can move out as a hydride during its oxidation. As shown in Fig. 33.2, lactate dehydrogenase is A-side specific. Technically this means the H from *proR* position of C-4 of NADH is selectively transferred to pyruvate by this enzyme. In the reverse direction, the hydride is transferred to the *re* face of NAD^+ (which is planar). With respect to their choice of C-4 hydrogen on pyridine nucleotides (either NAD or NADP), dehydrogenases may be either A-side specific or B-side specific. For instance, glyceraldehyde-3-phosphate dehydrogenase is B-side specific – it picks up the H from *proS* position of C-4 of NADH (this corresponds to *si* face of NAD^+, in the reverse reaction). The trans-hydrogenase of animal mitochondria – transfers hydride between NADH and $NADP^+$ – is *proR* specific (A-side) for one and *proS* specific (B-side) for the other substrate.

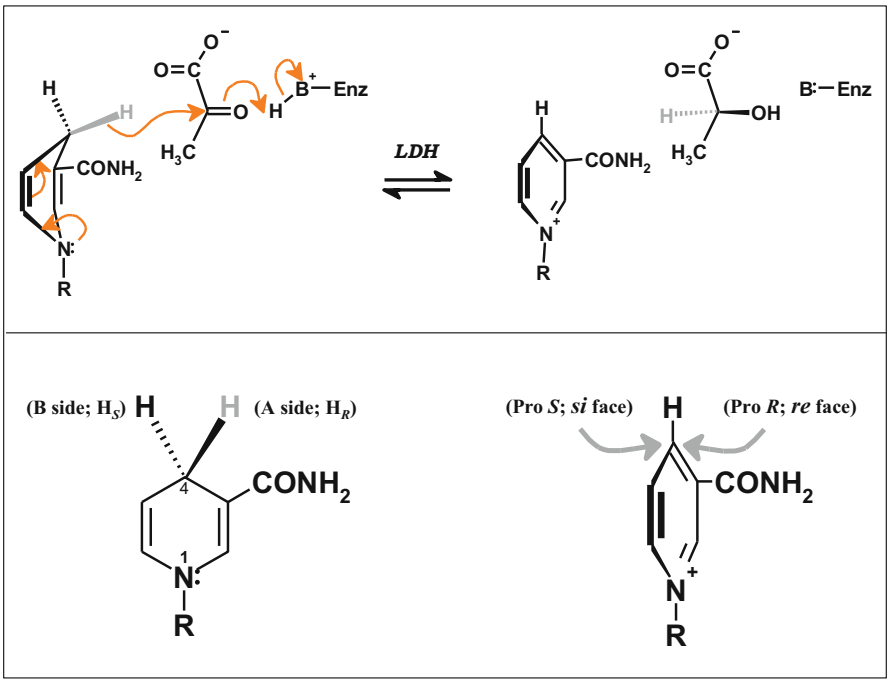


Fig. 33.2 Hydride transfer in the lactate dehydrogenase (LDH) reaction mechanism. This enzyme reaction is specific for the C-4 *R* hydrogen (bottom panel: H in gray, A side specificity) of NADH. In the reverse direction, it transfers the hydride to C-4 *proR* position (*re* face) of NAD^+

Lastly, it is worth noting that pyridine nucleotides (and NAD^+ in particular) have important non-redox roles in metabolism as well. It is the substrate for poly (ADP-ribose) polymerase (PARP), *E. coli* DNA ligase, and toxins of diphtheria and cholera. In all these cases, ADP-ribose of NAD^+ is transferred to different acceptors.

33.6 Flavins and Flavoenzymes

The two common flavin coenzymes (FMN and FAD) are chemically modified versions of riboflavin (vitamin B2). The ribitol side chain when phosphorylated is FMN or when attached through a diphosphate to adenosine gives FAD (see Table 31.1 for structures). Riboflavin itself was first isolated from eggs and its structure elucidated in 1935. Extensively conjugated, tricyclic isoalloxazine ring system of riboflavin imparts it (and its cofactors) the bright yellow color. More importantly, this isoalloxazine ring system forms the redox-active structure of FMN and FAD. The oxidized cofactor absorbs in the visible region with one peak around 450 nm (Fig. 33.3). This 450 nm peak is absent in the corresponding $2e^-$ reduced form (1,5-dihydroflavin). The oxidized form of flavin is planar, while the

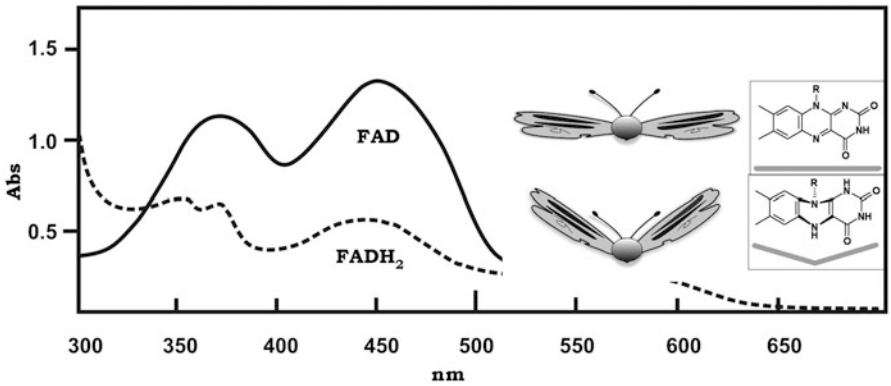


Fig. 33.3 Absorption spectra of reduced and oxidized FAD. Spectral properties, particularly those of the reduced flavin, are greatly influenced by their microenvironment at the active site. Representative spectrum of reduced FAD in an enzyme-bound form is shown; $FADH_2$ (1,5-dihydroflavin), in free solution and in air, is unstable. Corresponding spectral features of FMN and $FMNH_2$ are almost identical

1,5-dihydroflavin is bent with the two outer ring planes forming an angle (of ~30°) along the N5–N10 axis. One can visualize enzyme active sites selectively stabilizing one of these two forms – a possible basis for shifting the reduction potential of the flavin cofactor. For instance, D-amino acid oxidase binds FAD about 10^7 fold tighter than $FADH_2$ (1,5-dihydroflavin). Unlike with nicotinamide coenzymes, flavin cofactors are much more tightly bound to flavoenzymes (K_D ranging from 10^{-7} M to 10^{-14} M). In some cases the flavin (through a methyl C on its C-8) is covalently bound to the apoenzyme (e.g., through His residue of succinate dehydrogenase).

FAD (as also FMN) can exist in three distinct oxidation states – FAD, $FADH^{\cdot}$ (semiquinone radical), and $FADH_2$ (1,5-dihydro FAD). Since flavin semiquinone is reasonably stable (at least in enzyme-bound form), it can be a significant intermediate in flavoenzyme catalysis. Flavins are able to participate both in $1e^-$ and $2e^-$ transfer reactions. They are crucial as adapters/mediators between $2e^-$ oxidations (of organic compounds) and $1e^-$ oxidations (like in electron transport chains). This feature also allows them to react with molecular oxygen (see below). On the contrary, pyridine nucleotides are always restricted to $2e^-$ (hydride) transfer reactions. Interestingly, 5-deazaflavin (where the flavin N-5 is replaced by a C atom) behaves more like a nicotinamide cofactor – capable of only $2e^-$ transfers. Factor F_{420} ($E'^{\circ} = -0.360$ V) found in methanogenic bacteria is an example of naturally occurring 5-deazaflavin.

Flavoenzymes may be classified according to the nature of (a) electron acceptors that accept electrons from the reduced flavin and (b) electron donors that transfer electrons to the oxidized flavin nucleus (Fraaije and Mattevi 2000; Joosten and van Berkel 2007; Mattevi 2006). Different substrates like alcohol (D-lactate dehydrogenase), aldehyde (glucose oxidase), amine (D-amino acid oxidase), C-C bond (acyl-CoA dehydrogenase), and thiols (glutathione reductase) may donate electrons to

reduce the enzyme-bound flavin. When we analyze the molecules that receive electrons from a dihydroflavin (enzyme-bound $FADH_2$, for instance), two broad categories may be observed. One group using molecular oxygen (O_2) as the electron acceptor includes oxidases, monooxygenases, dioxygenases, and metallo-flavoenzymes. We shall address this group in the next section. The second category includes all flavoproteins – like succinate dehydrogenase and glutathione reductase – that do not use O_2 as electron acceptor. These dehydrogenase mechanisms represent a major class of reactions wherein flavin coenzymes take part. Varied experimental evidence (from model reactions and enzyme examples) supports different roles for its participation in these reactions. Possible mechanisms for such substrate dehydrogenations are:

(a) Direct hydride transfer from the substrate to oxidized flavin (to its N-5)
(b) Nucleophilic attack by substrate hetero-atom (other than C) at C4a (the bridge C between C-4 and N-5) of oxidized flavin
(c) Nucleophilic attack by substrate carbanion at N-5 of oxidized flavin
(d) A radical mechanism involving $1e^-$ transfers

Glutathione reductase is a well-characterized enzyme among the flavoprotein dehydrogenases. It regenerates glutathione (GSH) from its oxidized disulfide (GS-SG) by the following reaction:

$$\mathbf{GS-SG + NADPH + H^+ \rightleftarrows 2\ GSH + NADP^+}$$

Based on their reduction potentials (Table 33.1), equilibrium position favors the reduction of GS-SG (oxidized glutathione; disulfide) to GSH (reduced; thiol) at the expense of NADPH. The hydride from NADPH is first transferred to the bound flavin (to its N-5) of glutathione reductase. Bound $FADH_2$ further transfers the two electrons to reduce a disulfide – of two cysteine residues at the enzyme active site. These cysteine thiols reduce GS-SG to two molecules of GSH. In turn, the active site disulfide is regenerated for the next catalytic cycle (Fig. 33.4). Other flavoenzymes with a similar chemical mechanism (but with different disulfide substrates) include dihydrolipoamide dehydrogenase, thioredoxin reductase, and trypanothione reductase.

Like glutathione reductase, many other flavoenzymes utilize NAD(P)H to reduce their bound flavin coenzyme. However, some of them eventually transfer these electrons to molecular oxygen. These will be the subject of our next topic.

33.7 Reactions Involving Molecular Oxygen

Molecular oxygen (O_2, also known as dioxygen) is the ultimate electron acceptor in aerobic metabolism. Oxygen atom has eight electrons with the configuration $1s^2,2s^2\ 2p^4$ (Chap. 29, Table 29.1). However, in nature it exists as a diatomic molecule with a double bond (bond order is 2) between the two O atoms. Interestingly enough it

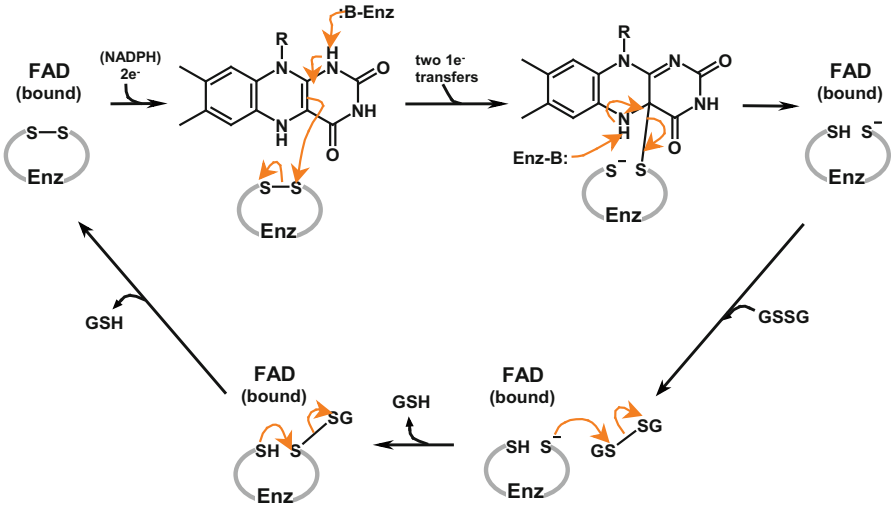


Fig. 33.4 Glutathione reductase reaction mechanism. Reduction of the enzyme disulfide requires the transfer of $2e^-$ from NADPH via the bound FAD (top reactions). The two active site cysteine thiols in turn reduce GSSG (bottom reactions). All proton transfer steps are not explicitly shown for the sake of clarity

exists as a diradical in the ground state (O_2 has a triplet ground state). Its two unpaired electrons in the valence orbitals have parallel spins making it diamagnetic. The low reactivity of O_2 is closely related to its triplet ground state with the following molecular orbital description:

$$(2s\sigma)^2(2s\sigma*)^2(2p\sigma)^2(2p\pi)^4(2p\pi*)^{1,1}$$

The peculiar electronic configuration of O_2, according to Pauli's exclusion principle, dictates that it can accept only unpaired electrons. Therefore electrons must be transferred to O_2 one at a time. Such single-electron transfers are in obvious contrast to the transfer of electrons in pairs seen in most redox reactions, discussed above. Special cofactors are required to transfer electrons from a two-electron donor to one-electron acceptor (and *vice versa*!). FAD is one such cofactor that is capable of participating in both $1e^-$ and $2e^-$ redox reactions.

Reactions of molecular oxygen usually occur at a prosthetic group on the enzyme (Malmstrom 1982). Dioxygen reacts with a fully reduced flavin (such as bound $FADH_2$) to yield a flavin-peroxide. The C4a of the isoalloxazine is where this peroxy adduct forms (Fig. 33.5). The final fate of such peroxides is decided by the nature of the active site of that particular enzyme. The redox active metal center of an enzyme may also react with molecular oxygen. The metal center may (a) transfer electrons to bound O_2 or (b) activate the organic substrate so that it can react with O_2, also bound at the active site.

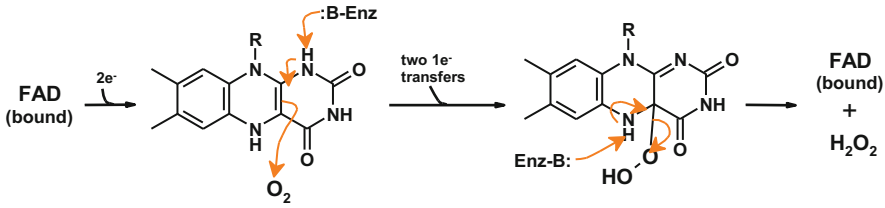


Fig. 33.5 **The FAD peroxy adduct of D-amino acid oxidase and formation of hydrogen peroxide**

Enzymatic insertion of atoms from dioxygen into organic substrates was first demonstrated, independently by Hayashi and Mason, in the 1950s. In their pioneering work, oxygen isotopes (^{18}O in particular) were exploited. Since then more than 200 enzymes are known to use molecular oxygen as one of their substrates. These O_2-utilizing enzymes are classified into *oxidases* and *oxygenases*. In an oxidase-catalyzed reaction, O_2 functions only as an electron acceptor. In D-amino acid oxidase, for instance, bound $FADH_2$ reduces molecular oxygen to hydrogen peroxide. In general, the successively reduced products of molecular oxygen include the following:

$$\underset{\text{(Dioxygen)}}{O_2} \xrightarrow{e^-} \underset{\text{(Superoxide)}}{O_2^{-\cdot}} \xrightarrow{e^-, 2H^+} \underset{\text{(Hydrogen peroxide)}}{H_2O_2} \xrightarrow{2e^-, 2H^+} \underset{\text{(Water)}}{2H_2O}$$

In oxygenase-catalyzed reactions, however, one or both the atoms of O_2 are incorporated into organic substrates. Oxygenases in turn may be (a) *dioxygenases*, which catalyze the insertion of both atoms of O_2 into the organic substrate, or (b) *monooxygenases* (mixed function oxidases), which catalyze the insertion of one atom of O_2 into the organic substrate while the other one is reduced to water.

Participation of protein R-groups (amino acid side chains) alone is not enough to catalyze the reactions involving O_2. All enzymes that activate/reduce O_2 are conjugated proteins. Associated cofactors/prosthetic groups at the reaction centers include flavins, heme, iron (Fe^{3+}/Fe^{2+}), or copper (Cu^{2+}/Cu^{+}). Non-heme iron prosthetic groups are common in oxygenases and some oxidases. Phenylalanine hydroxylase uses tetrahydro-biopterin along with Fe^{2+} for its activity. Table 33.4 lists the prosthetic groups exploited by enzymes acting on molecular oxygen. The list will however be different, and gets expanded, when the reactants are either superoxide or hydrogen peroxide, instead of O_2.

Metal ions in redox catalysis: Metal ions contribute to enzyme catalysis by shielding/stabilizing charges and by enhancing the nucleophilicity of water (Chap. 6). Multivalent metal ions can act as super (Lewis) acids. In addition, redox-active metal ions directly participate in electron transfer chemistry. Reduction potential of a redox-active metal center can be modulated, and it depends on the nature of ligands coordinating the metal ion. Metal ions can be classified as hard acids, soft acids, and borderline cases. Hard acids prefer a hard ligand, while soft acids prefer softer ligands (Irving–Williams stability series and Pearson's Hard-Soft-

Acid-Base classification). For instance, Fe^{3+} (hard acid) prefers hard ligands (with O) than Fe^{2+} (borderline case, preferring N,S-containing ligands). Clearly ligand preference versus the actual ligands present influences the reduction potential (and metal reactivity) of a redox-active metal. This forms the basis of redox manipulation at active sites – enzymes offer appropriate side chain residues to modulate the metal center chemistry. A more detailed treatment of this subject may be found in specialized books on bioinorganic chemistry.

Binding of metal cofactor (regardless of whether it is redox-active or not) to the apoenzyme can be followed by one or more techniques. Metal ion binding may be tested by (a) kinetic competition with other metal ions and use of chelators; (b) gel filtration, equilibrium dialysis, and/or ultracentrifugation; (c) difference spectral titrations (with UV-visible, fluorescence, optical rotatory dispersion, or circular dichroism); and (d) resonance techniques (like nuclear magnetic resonance, proton relaxation rates, electron spin resonance, or electron paramagnetic resonance). The actual ligands coordinating the metal center may be directly visualized by X-ray structure analysis. Depending on their strength of metal binding, enzymes may be grouped into *metallo-enzymes* and *metal-activated enzymes*. This distinction, however, is purely arbitrary and is based on the magnitude of the binding constant – when the binding is strong ($K_D < 10^{-8}$ M), it is a metallo-enzyme, but when metal is weakly bound ($K_D > 10^{-8}$ M), it is a metal-activated enzyme. In practice, however, we see a continuum of metal ion-binding constants, and it is difficult to classify borderline cases.

Metal ion-requiring enzymes may be distinguished into three groups depending on who donates the coordinating ligands to the metal. The metal ion is bound to the enzyme via the substrate (E-S-M) in a substrate bridge complex. Instead, the enzyme may independently bind the substrate and the metal – the enzyme bridge complex (M-E-S). Thirdly, we can visualize a metal bridge complex, where enzyme makes contact with both the metal and the substrate, individually and in combination. These different binding types can be experimentally verified by the set of analytical techniques listed above.

33.8 Summing Up

Oxidation–reduction reactions provide the driving force to all biological reactions. A reaction with positive reduction potential ΔE (and hence negative ΔG) is thermodynamically feasible. Like with all other reactions, enzymes hasten redox reaction rates by lowering kinetic barrier. When required, they recruit cofactors and help in tuning their redox potential, facilitate electron transfers, modulate oxygen reactivity, and exert control over the nature of redox substrate used.

Pyridine nucleotides and flavin coenzymes are two frequently encountered molecules in redox enzyme chemistry. They serve two distinct purposes. NAD (P) is adept at $2e^-$ transfers (hydride transfers) and is stable in O_2 environment. FAD (and FMN) is able to participate in both $1e^-$ and $2e^-$ transfers – hence act as

step-down or step-up adapters in redox reactions. Reduced flavin can therefore react with molecular oxygen.

Not all oxidation–reduction reactions occur at the carbon atom. Examples include nitrate reductase and sulfate reductase. Nitrogenase – containing flavin, molybdenum, and iron–sulfur center – performs a multi-electron reduction of nitrogen ($N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$). Multicomponent enzyme systems like dihydroxylating dioxygenases even contain a mini-electron transport chain (NADH → FAD → FeS cluster → non-heme Fe) where electrons ultimately flow to substrates.

It is not surprising that nature has evolved a range of enzymes, coenzymes, and cofactors to perform redox reactions – as they fuel all the carbon-based life processes. Many radical and redox chemistries pose frontiers in enzymatic reaction mechanism where reaction intermediates and pathways need investigation.

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