CHAPTER 2

Antioxidant Molecules and Redox Cofactors

2.1 GLUTATHIONE

JOSEPH J. BARYCKI

Redox Biology Center and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska

The tightly controlled maintenance of the intracellular redox environment is essential for normal cellular function. Oxidative stress (i.e., in the form of increased ROS), which is generated by essential metabolic reactions such as mitochondrial

Redox Biochemistry, edited by Ruma Banerjee

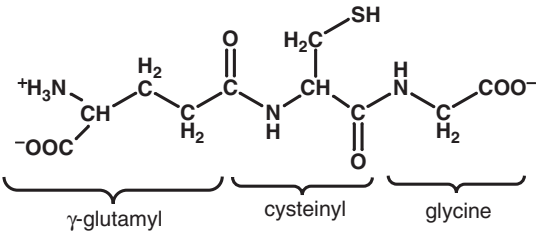


Figure 2.1. Structure of GSH.

energy production and oxidation of toxins by cytochrome P450, is dissipated by the concerted efforts of antioxidants that help buffer the redox environment. This section focuses on the major nonprotein thiol-based redox buffer of the cell. Glutathione (GSH) is a tripeptide comprised of glutamate, cysteine, and glycine (γ-glutamylcysteinylglycine) and is vital for normal cellular function (Fig. 2.1). Its homeostasis is modulated to a large extent by two enzymes: glutamate cysteine ligase, which catalyzes the committed step in GSH biosynthesis, and γ-glutamyltranspeptidase, which initiates GSH reclamation (Fig. 2.2). Since GSH can potently modulate cell signaling in an oxidation-dependent fashion, it is critical for the cell to have efficient ways of replenishing reduced levels. Reduced GSH pools are maintained by glutathione reductase, a pyridine nucleotide disulfide reductase that transfers electrons from NADPH to oxidized GSH. This enzyme provides the crucial direct link between the pool of electron-rich metabolic intermediates, like NADPH, and the array of cysteine-containing proteins that depend on thiol regeneration for sustained activity.

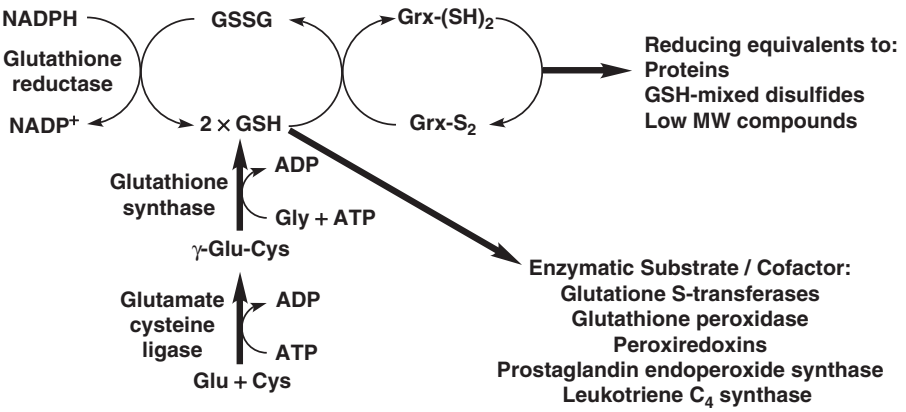


Figure 2.2. The dithiol/disulfide redox couple of reduced and oxidized glutathione is the major thiol-based redox buffer in many organisms. Grx-$(SH)_2$ and Grx-S_2 denote reduced and oxidized glutaredoxin, respectively.

2.1.A Biological Functions

GSH is found primarily in eukaryotes and gram-negative bacteria, although a select number of gram-positive bacteria have also been shown to utilize it. In eukaryotic systems, approximately 90% of the intracellular GSH pool resides in the cytoplasm, with the remainder in organelles such as the mitochondria, the endoplasmic reticulum, and the nucleus. However, the biosynthesis of GSH appears to occur exclusively in the cytosol. Cellular GSH concentrations are estimated to range from 0.5 to 10 mM in most cell types with the vast majority of GSH being in the reduced formed. The predominant oxidized form of GSH, GSSG, results from the cross-linking of two molecules of GSH via a disulfide bond. The ratio of GSH to GSSG is often used to express the redox status of a cell. Typically, the GSH:GSSG ratio is >10:1 in the cytosol and mitochondria and as low as 1:1 in the endoplasmic reticulum. The lower GSH:GSSG ratio in the endoplasmic reticulum facilitates formation of disulfide bonds during folding of secretory and membrane proteins.

GSH is involved in numerous biological processes. These functions include the storage and transport of cysteine, leukotriene and prostaglandins biosynthesis, maintenance of protein structure and function, and the regulation of enzyme activity through the reduction of disulfide bonds or by glutathionylation. However, its primary function appears to be in the maintenance of intracellular redox homeostasis by affording protection against reactive oxygen and nitrogen species as well as electrophilic xenobiotics. As an introduction to these diverse functions, several GSH-dependent processes have been highlighted.

Protection against ROS generated as a result of aerobic metabolism is largely afforded by the combined efforts of several enzyme systems that will be discussed in detail in Chapter 3. Superoxide dismutases catalyze the conversion of two superoxide ($O_2^{\cdot -}$) molecules into hydrogen peroxide (H_2O_2) and oxygen. H_2O_2 can then be converted to water by catalase, glutathione peroxidases, or peroxiredoxins. Glutathione peroxidases are a family of selenocysteine-containing enzymes that can convert H_2O_2 to water or lipid peroxides to their corresponding alcohols concomitant with the generation of GSSG (Eqs. (2.1) and (2.2)).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \tag{2.1}$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \tag{2.2}$$

Peroxiredoxins catalyze the same molecular transformations albeit by a different catalytic mechanism. The GSSG generated by glutathione peroxidases and peroxiredoxins is rapidly reduced by an NADPH-dependent glutathione reductase. Under normal cellular conditions, glutathione reductase can maintain a high GSH:GSSG ratio. However, in cases of extreme oxidative stress, GSSG can rapidly be exported to help preserve the GSH:GSSG ratio. In addition, increased GSSG levels can lead to formation of mixed disulfides between GSH and protein thiols, thus lowering GSSG levels while protecting free sulfhydryls within proteins.

Members of the glutathione transferase enzyme family can also convert organic peroxides to their corresponding alcohols and water and generate GSSG. However,

the primary function of this enzyme family is to catalyze the conjugation of GSH to electrophilic substrates. Glutathione transferases have broadly been classified as cytosolic, mitochondrial, and microsomal. The latter class is now referred to as membrane-associated proteins in eicosanoid and GSH metabolism. Cytosolic and mitochondrial glutathione transferases are distantly related and share common structural features, whereas the microsomal enzymes appear to be evolutionarily distinct. Glutathione transferases activate the free sulfhydryl of GSH by lowering its apparent pK_a, thus making it a better nucleophile. The activated GSH can then attack nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. In addition to reducing the reactivity of the electrophilic compound, conjugation to GSH can increase solubility. GSH conjugates can then be exported out of the cell by transmembrane multidrug resistance-associated proteins. In addition to detoxification, glutathione transferases are important in the biosynthesis of leukotrienes and prostaglandins and their diverse functions will be discussed in Chapter 3, Section 3.5B.

GSH can nonenzymatically form complexes with a variety of heavy metals, including mercury, lead, arsenic, gold, silver, zinc, and copper. Its free sulfhydryl is the main coordination site for metal binding but additional favorable interactions can be made with other functional groups within the tripeptide. GSH has been implicated in the storage and transport of metals, and as a cofactor in redox reactions involving metals. Glutathione can also reduce dehydroascorbate to ascorbate, another cellular antioxidant discussed in Chapter 2, Section 2.2. Glutathione-dependent dehydroascorbate reductase activity has been assigned to members of the glutaredoxin and glutathione transferase enzyme families. In addition, glutaredoxin can accept electrons from GSH and transfer reducing equivalents to a variety of proteins, low molecular weight compounds, and glutathione-mixed disulfides, thus expanding the impact of the GSH:GSSG redox system.

2.1.B Biosynthesis

GSH is synthesized from its constituent amino acids by the successive action of two ATP-dependent enzymes (Fig. 2.2): glutamate cysteine ligase and glutathione synthetase. Glutamate cysteine ligase catalyzes the rate-limiting step in GSH biosynthesis, conjugating glutamate via its γ-carboxylate to cysteine. The proposed catalytic mechanism proceeds via the phosphorylation of the γ-carboxylate of glutamate by ATP. The amino group of cysteine, serving as a nucleophile, attacks the γ-glutamyl phosphate intermediate to yield γ-glutamylcysteine. Based on sequence analysis, three distinct groups of glutamate cysteine ligases have been identified (γ-proteobacteria; nonplant eukaryotes; α-proteobacteria and plants) that may have evolved from a distant common ancestor. Although strong sequence conservation is observed within a group, insignificant sequence homology exists between these three groups. Despite these differences, all three groups of glutamate cysteine ligases are predicted to have similar catalytic mechanisms and likely have common structural motifs.

As the committed step to GSH biosynthesis, the reaction catalyzed by glutamate cysteine ligase is tightly regulated. GSH biosynthesis is often limited by free cysteine

availability and glutamate cysteine ligase is feedback regulated by the end product, GSH. Transcriptional regulation, as well as post-translational modifications of the enzyme, ensures exquisite control of intracellular GSH levels. In several eukaryotic systems, additional regulatory control is afforded by the presence of a modifier subunit. In these systems, glutamate cysteine ligase is a heterodimer comprised of a catalytic subunit and a modifier or regulatory subunit with corresponding molecular weights of ~70 kDa and ~30 kDa, respectively. Although the catalytic subunit contains each of the substrate binding sites and is capable of catalysis alone, it exhibits a high K_m for glutamate. The addition of the modifier subunit decreases the K_m, making it a more efficient enzyme at physiological concentrations of glutamate. In addition, the formation of the complex lessens product inhibition by GSH. The expression of the catalytic and modifier subunits is not tightly coordinated and differential tissue distribution of the two subunits has been observed, which may be involved in tissue-specific regulation of GSH levels. In addition, the stimulatory effects of the modifier subunit on catalysis may depend on the presence of an intersubunit disulfide bond with the catalytic subunit, thus linking the redox state of the cell with GSH biosynthesis. However, it remains unclear if this disulfide is a requirement for activation.

Escherichia coli glutamate cysteine ligase is a monomeric enzyme and structures of the apo form of the enzyme and in complex with a transition-state analogue have been determined (Fig. 2.3). A six-stranded antiparallel β-sheet is central to the protein structure and this partial barrel comprises much of the active site. In the complex

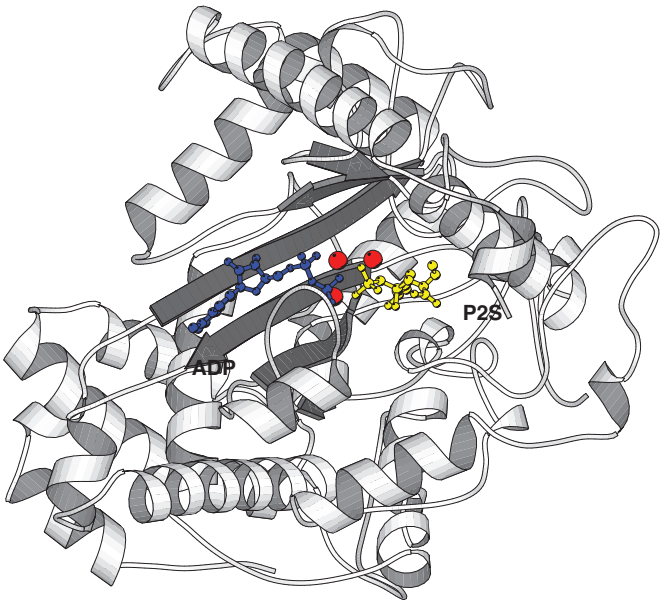


Figure 2.3. Crystal structure of *E. coli* glutamate cysteine ligase. The ribbon representation illustrates the overall fold of the monomeric enzyme. Bound in the enzyme active site are ADP, 3 Mg^{2+} ions (red spheres), and the mechanism-based inhibitor, (2*S*)-2-amino-4-[[(2*R*)-2-carboxybutyl](phosphono)sulfon-imidoyl] butanoic acid (P2S). (PDB ID 1VA6)

structure, ADP is located adjacent to the inhibitor. Three magnesium ions are clustered near the phosphates of ADP and the phosphorylated transition-state analogue. It has been proposed that this magnesium cluster increases the reactivity of the γ-phosphate group of ATP and stabilizes the resulting γ-glutamylphosphate intermediate. The cysteine binding site is adjacent to the glutamate binding site and undergoes a significant conformational change upon ligand binding. Interestingly, there does not appear to be a general base located near the α-amino group of L-cysteine. Overall, the observed structure of the *E. coli* glutamate cysteine ligase is consistent with the proposed enzyme mechanism.

The resultant γ-glutamylcysteine is coupled to glycine by glutathione synthetase. Similar to the situation observed for glutamate cysteine ligase, glutathione synthases from bacteria share limited sequence homology to eukaryotic homologs and appear to have different quaternary structures. The *E. coli* glutathione synthetase is a functional homotetramer, whereas both the human and yeast glutathione synthetases are homodimers. Despite these differences, all three homologs are members of the ATP-grasp superfamily and are thought to proceed via similar catalytic mechanisms. An acylphosphate intermediate is generated by the transfer of the γ-phosphate of ATP to the carboxylate of γ-glutamylcysteine. The α-amino group of glycine can then attack this phosphorylated intermediate, displacing the inorganic phosphate to form GSH.

Support for this mechanism has been provided by crystal structures of several glutathione synthetase complexes. Human glutathione synthetase bound to its product GSH, ADP, a sulfate ion, and two magnesium ions has been reported (Fig. 2.4). In this complex, the sulfate ion mimics the γ-phosphate of ATP and is located between the β-phosphate of ADP and the α-amino group of the glycine portion of GSH. Similar to glutamate cysteine ligase, two magnesium ions are located in the enzyme active site and likely facilitate binding of ATP, activate the γ-phosphate, and stabilize the acylphosphate intermediate. Comparisons between unliganded and ligand-bound structures of glutathione synthetase also suggest that large conformational changes accompany formation of the Michaelis complex.

Glutathione synthetase rapidly converts γ-glutamylcysteine to GSH *in vivo* and the catalytic activity of glutamate cysteine ligase is generally thought to be rate limiting in glutathione biosynthesis. The liver is the primary site of GSH biosynthesis and, in this tissue, glutathione synthetase activity is severalfold higher than glutamate cysteine ligase activity. However, in other tissues, such as skeletal muscle, the relative activities of the two enzymes are comparable, suggesting glutathione synthetase activity may affect intracellular GSH levels. As such, comparatively limited research has focused on the regulation of glutathione synthetase activity. Current studies are focused on the transcriptional regulation of glutathione synthetase, as little evidence is available to suggest glutathione synthetase activity is regulated post-translationally.

2.1.C Degradation

A significant, but overlooked, component of GSH homeostasis is precursor availability. Cysteine is often limiting in GSH biosynthesis and is obtained from the diet, the transsulfuration pathway, which converts homocysteine to cysteine, or the salvage of

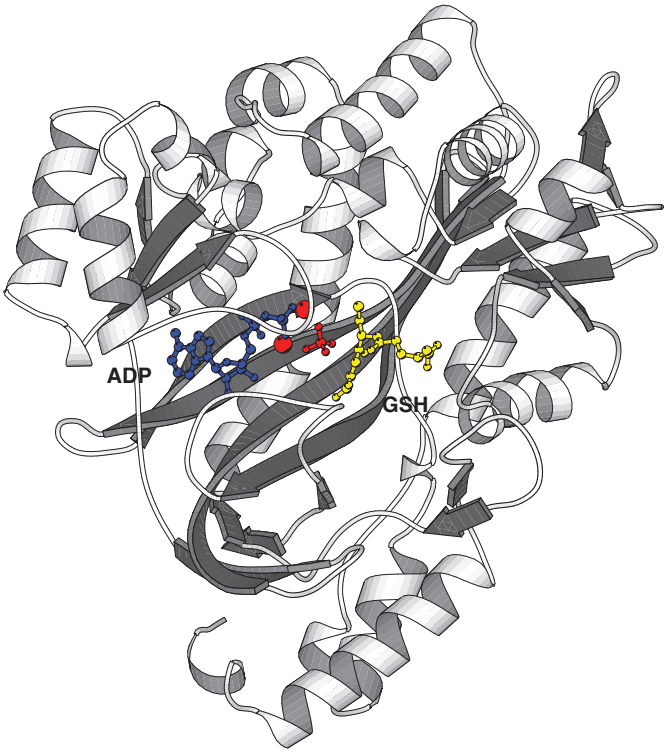


Figure 2.4. Crystal structure of human glutathione synthetase. The ribbon representation illustrates the overall fold of a single subunit of the dimeric enzyme. Bound in the enzyme active site are ADP, 2 Mg^{2+} ions (red spheres), and glutathione. (PDB ID 2HGS)

excreted GSH and its conjugates. The γ-glutamyl cycle proposed by Alton Meister and co-workers describes the reclamation of GSH (Fig. 2.5). γ-Glutamyltranspeptidase catalyzes the first step in the glutathione salvage pathway and cleaves γ-glutamyl amide bonds. The cleaved glutamate can either be transported directly into the cell or be transferred to an amino acid or peptide acceptor before import. Once inside the cell, glutamate is released from the γ-glutamyl peptide by the concerted activities of γ-glutamylcyclotransferase and 5-oxoprolinase. The cysteinylglycine is cleaved by a membrane dipeptidase to generate cysteine and glycine that can be imported into the cell. Cysteine conjugates arising from the degradation of GSH conjugates are funneled into the mercapturic acid pathway.

Mammalian γ-glutamyltranspeptidases are embedded in the plasma membrane by a single N-terminal transmembrane anchor and are heterologously glycosylated. However, the enzyme does not need to be tethered to the membrane or glycosylated to have complete activity *in vitro*. The catalytic mechanism proceeds via a γ-glutamyl-enzyme intermediate, with a conserved threonine residue serving as the nucleophile. The acyl-enzyme intermediate can be hydrolyzed to generate

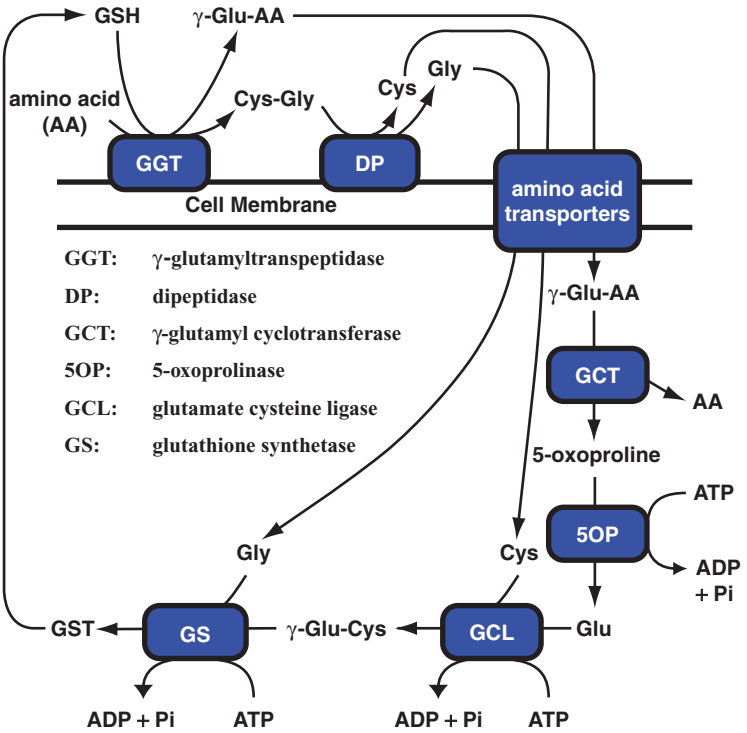


Figure 2.5. The γ-glutamyl cycle. Described by Alton Meister and co-workers, the γ-glutamyl cycle describes the salvage of GSH and GSH conjugates.

glutamate. Alternatively, the α-amino group of an amino acid or the N-terminal amino group of a small peptide can attack the acyl-enzyme intermediate, resulting in transpeptidation of the glutamate from GSH to the acceptor molecule. Bacterial γ-glutamyltranspeptidases are located in the periplasmic space and appear to have limited transpeptidase activity compared to their eukaryotic homologs. In contrast to the GSH biosynthetic enzymes, γ-glutamyltranspeptidases from these diverse species share considerable sequence homology.

γ-Glutamyltranspeptidase is synthesized as an inactive 60 kDa polypeptide and cleavage of the proenzyme yields a fully active heterodimer comprised of a 40 kDa and a 20 kDa subunit (Fig. 2.6). Processing of γ-Glutamyltranspeptidase is thought to be an intramolecular autocatalytic event. A mechanism has been proposed in which processing proceeds via an N-O acyl shift, with a conserved threonine serving as the nucleophile. The peptide bond immediately preceding the threonine residue is broken, leading to activation of the enzyme. This threonine residue at the N terminus of the newly formed 20 kDa subunit can then serve as a nucleophile in the catalytic mechanism of the enzyme as discussed previously. Based on its unique autoprocessing activity, enzymatic activity, and its structure (Fig. 2.7), γ-glutamyltranspeptidase has been identified as a member of the emerging N terminal nucleophile hydrolase

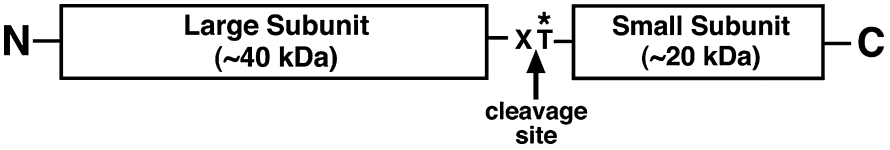


Figure 2.6. Schematic of γ-glutamyltranspeptidase. γ-Glutamyltranspeptidase is synthesized as an inactive ~60 kDa polypeptide. Autocleavage of the peptide bond immediately prior to a conserved threonine residue results in an active heterodimer.

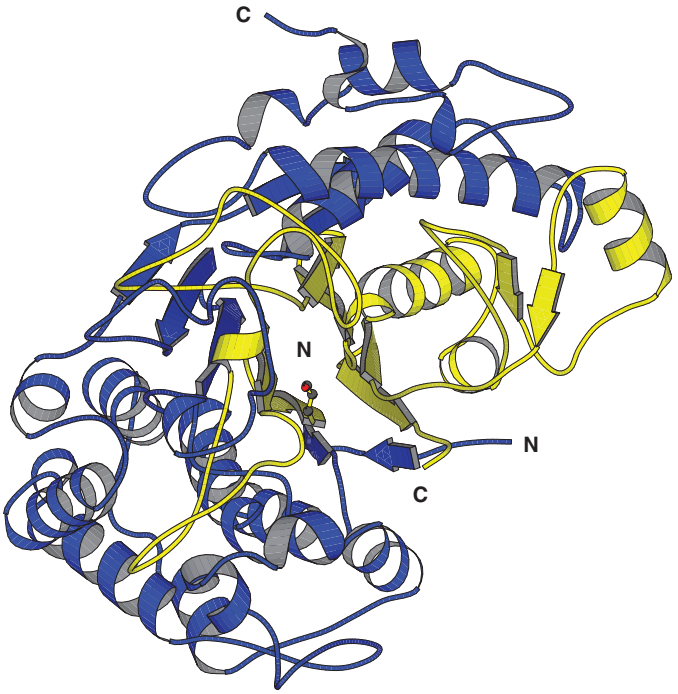


Figure 2.7. Crystal structure of *Helicobacter pylori* γ-glutamyltranspeptidase. The ribbon representation illustrates the overall fold of the active heterodimer. The 40 kDa and 20 kDa subunits are colored in blue and yellow, respectively. The conserved threonine residue at the new N terminus of the processed enzyme is shown in ball and stick representation.

superfamily. Members of this enzyme family are autocatalytically processed to yield an active enzyme, with the resulting N terminus acting as the nucleophile in amide bond hydrolysis reactions. Autoprocessing of γ-glutamyltranspeptidase leads to a large conformational change, with the loop preceding the catalytic threonine moving >35 Å, thus relieving steric constraints that likely limit substrate binding. In addition, cleavage of the proenzyme results in the formation of a threonine–threonine dyad required for efficient catalysis.

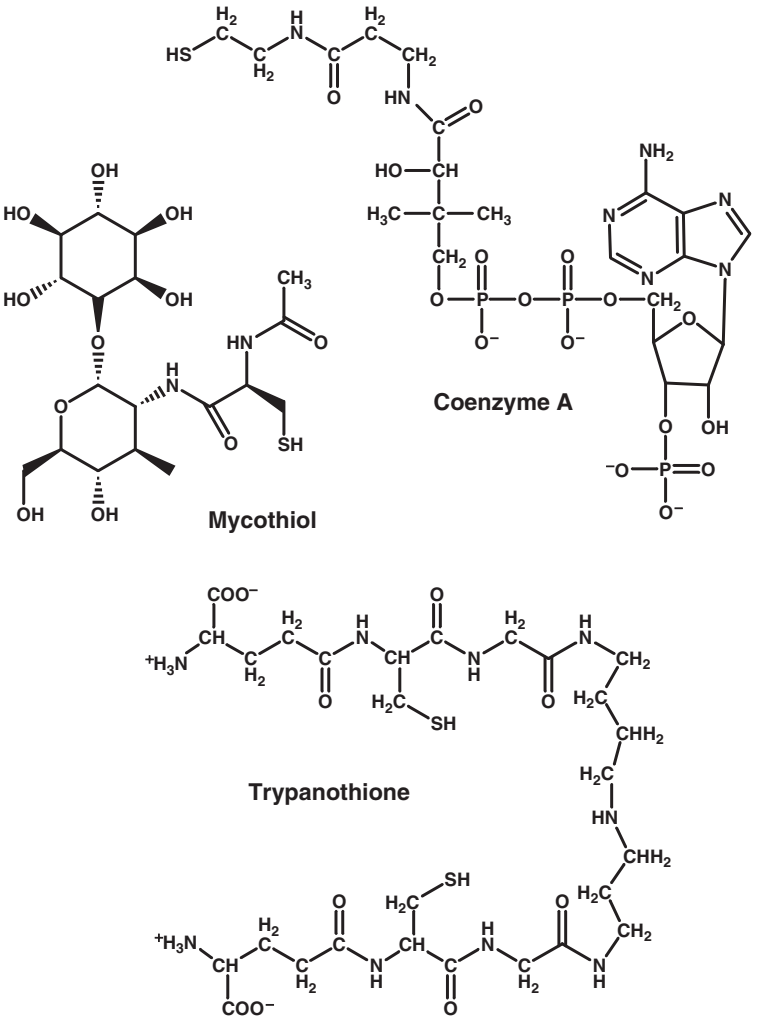


Figure 2.8. Structures of coenzyme A, mycothiol, and trypanothione.

Studies with γ-glutamyltranspeptidase-deficient mice indicate that one of the primary functions of GSH is the storage and transport of cysteine. These mice appear normal at birth, exhibit slower growth, do not mature sexually, and have shorter life spans. They are also considerably more susceptible to cataract formation and damage by ROS. Supplementation with N-acetylcysteine, a cysteine precursor, largely restores the wild-type phenotype, suggesting that the primary function of γ-glutamyltranspeptidase in normal cells is the recovery of cysteine for use in protein and GSH synthesis, which is important for growth and oxidative protection. In agreement with these findings, γ-glutamyltranspeptidase is found to be upregulated in several cancer types and has been shown to accelerate tumor growth and increase

tumor resistance to damage induced by chemotherapy and radiation treatment. This is likely due to the enhanced ability to salvage cysteine from circulating GSH.

γ-Glutamyltranspeptidase has been well characterized; however, the details of the remainder of the GSH salvage pathway are less defined. Cysteinylglycine and its conjugates are cleaved by membrane-associated dipeptidases to their constituent amino acids, which can then be transported across the membrane. In mammalian systems, aminopeptidase M and cysteinylglycinase have been reported to catalyze this reaction. Leucyl aminopeptidase, a cytosolic enzyme, also exhibits a strong preference for cysteinylglycine and its conjugates, suggesting that an unidentified cysteinylglycine transporter may contribute to GSH salvage. In addition, significant extracellular γ-glutamylcysteine arises from the transpeptidase activity of γ-glutamyltranspeptidase and a γ-glutamylcysteine transporter has been identified. Studies designed to elucidate this vital metabolic pathway remain an active area of research.

2.1.D Other Thiol-Based Redox Buffers

Although GSH is the most prevalent thiol-based redox buffer, alternative low molecular weight thiols that serve equivalent functions have been identified. Trypanothione, N^1,N^8-bis(glutathionyl)spermidine, was initially isolated in the African trypanosome, *Trypanosoma brucei*, and is synthesized by cross-linking two molecules of GSH via spermidine (Fig. 2.8). Another variation on this strategy involves mycothiol (1D-*myo*-inosityl 2-(*N*-acetylcysteinyl)amido-2-deoxy-*a*-D-glucopyranoside), which has *N*-acetylcysteine as its redox-active thiol group. Coenzyme A, which is used as a cofactor in numerous biological processes, has also been shown to serve as a redox buffer in *Staphylococcus aureus*. Trypanothione, mycothiol, and coenzyme A can cycle between a reduced and an oxidized form, and each system has a functional reductase that is structurally related to glutathione reductase. In addition to these well characterized low molecular weight thiols, additional variations of thiol/disulfide redox systems have been identified from numerous sources.

SELECTED REFERENCES

1. Copley, S.D., and Dhillon, J.K. (2002). Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. *Genome Biol* 3:research0025.1-0025.16.
2. Dickinson, D.A., and Forman, H.J. (2002). Glutathione in defense and signaling: lessons from a small thiol. *Ann. NY Acad. Sci.* 973:488–504.
3. Lu, S.C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J.* 13:1169–1183.
4. Sies, H., and Packer, L. (2005). Glutathione transferases and gamma-glutamyl transpeptidases. *Methods Enzymol.* 401:408–501.
5. Wang, W., and Ballatori, N. (1998). Endogenous glutathione conjugates: occurrence and biological functions. *Pharmacol. Rev.* 50:335–356.

2.2 ASCORBATE

HAN ASARD

Department of Biology, University of Antwerp, Antwerp, Belgium

Ascorbate (vitamin C) is well known for its radical-scavenging capacity and is the most effective water-soluble antioxidant in human plasma. It is the terminal small molecule antioxidant in many biological systems and probably the most abundant free radical scavenger in many cell types.

In addition to its role as an antioxidant, the moderately positive standard redox potential of the ascorbate/monodehydroascorbate couple makes ascorbate an excellent one-electron donor to a large variety of enzymes, in particular, oxygenases and hydroxylases. Its role as a cofactor for the enzyme prolyl, hydroxylase, lies at the basis of the discovery and isolation of ascorbate in 1932 by Albert Szent-Györgyi and Charles King as the "anti-scorbutic (scurvy preventing) factor." Soon thereafter, its chemical nature as a glucose derivative was elucidated by Walter Haworth. Major advances in understanding the importance of ascorbate in human health were made by Linus Pauling in the 1960s and 1970s. His work continues to fuel debates on the optimal daily intake of vitamin C for humans.

2.2.A Ascorbate Chemistry

Ascorbic acid (L-*threo*-hex-2-enono-1,4-lactone) is a diacid ($pK_1 = 4.2$ and $pK_2 = 11.8$), which exists almost exclusively as the monoanion at physiological pH values. The conjugated structure of the five-membered lactone ring (Fig. 2.9), containing an ene-diol group, allows stabilization of the free radical one-electron oxidation product, monodehydroascorbate, by delocalization of the unpaired electron.

Monodehydroascorbate is sufficiently stable and can be detected by electron paramagnetic resonance (EPR) in many biological samples. Increased monodehydroascorbate concentrations are considered to be a reliable marker for oxidative stress conditions. Oxidation of monodehydroascorbate yields dehydroascorbate, which is unstable at physiological pH. Hydrolysis of the O—C_1 ester bond in the lactone ring results in the generation of 2,3-diketo-L-gulonic acid and the irreversible loss of cellular ascorbate. However, the dominant mode of decay of ascorbate is through disproportionation of monodehydroascorbate (Fig. 2.9).

Ascorbate is a chain-breaking antioxidant that reacts readily with reactive species such as hydroxyl ($OH^{\cdot}$), alkoxyl ($RO^{\cdot}$), and peroxyl ($ROO^{\cdot}$) radicals, but also with tocopheroxyl ($TO^{\cdot}$) and urate ($UH^{\cdot -}$) radicals. An important reactivity of ascorbate is its ability to reduce the tocopheroxyl radical to tocopherol. Tocopherols (vitamin E, see Section 2.3) are lipid-soluble antioxidants essential for preventing the propagative peroxidation of membrane lipids under oxidative stress conditions. A key to the regeneration of lipophilic tocopherols by hydrophilic ascorbate is the exposure of the phenolic group of tocopherol to the lipid–water interface. Consistent with the antioxidant properties of ascorbate, the standard redox potential of 0.28 V (pH 7.0)

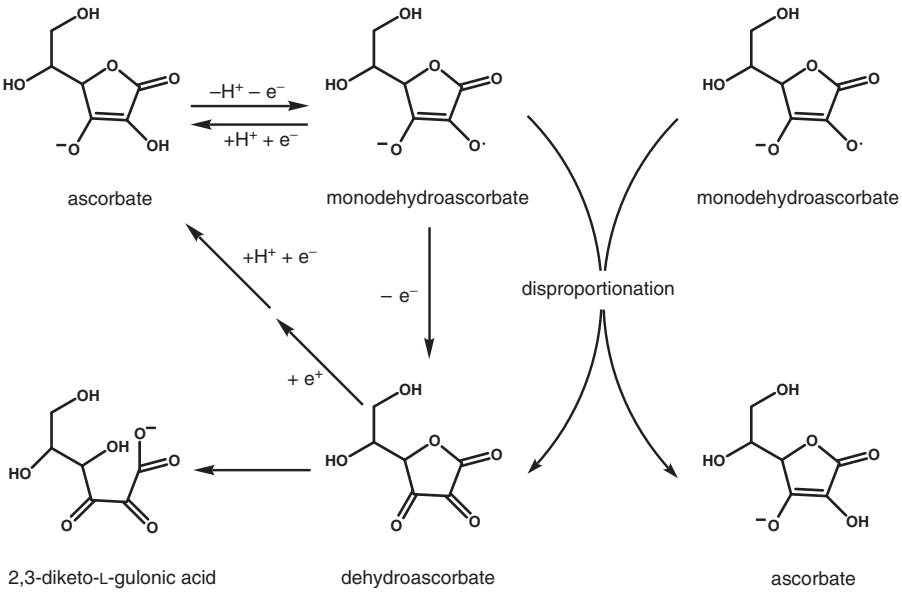


Figure 2.9. Ascorbate chemistry. Oxidation/reduction, protonation/deprotonation, and disproportionation reactions of ascorbate.

for the ascorbate/monodehydroascorbate couple is more negative than that of most common free radical redox couples.

Paradoxically, ascorbate can also function as a pro-oxidant by reducing transition metals such as Cu^{2+} and Fe^{3+}. The ascorbate mono- and dianions are readily oxidized by trace amounts of metals commonly present in buffers used in biochemical research. Ferrous iron formed by the oxidation of ascorbate can then catalyze the generation of the hydroxyl radical ($OH^{\cdot}$) from H_2O_2 in Fenton-like reactions. Because the diacid form of ascorbate is much less reactive, ascorbate is often extracted from biological samples and foodstuffs in acidic media and in the presence of metal chelators, and it is recommended to make stock solutions of ascorbate in the acid form.

2.2.B Ascorbate Biosynthesis

Although the immediate precursor for ascorbate biosynthesis is invariably an aldone-1,4-lactone, the biosynthetic pathway varies (Fig. 2.10). In mammals, the aldonolactone is L-gulono-1,4-lactone, which is derived from UDP-glucose. L-Gulono-1,4-lactone is converted to 2-keto-L-gulono-1,4-lactone by the endoplasmic reticulum-associated L-gulono-1,4-lactone oxidase (Fig. 2.10). The keto form spontaneously converts to ascorbate, generating H_2O_2 as a by-product. Ascorbate biosynthesis occurs in the endoplasmic reticulum of the liver of most mammals and in the kidney of egg-laying mammals, reptiles, and amphibians. Loss of the lactone oxidase activity in humans and other primates, teleost fish, guinea pigs, fruit bats,

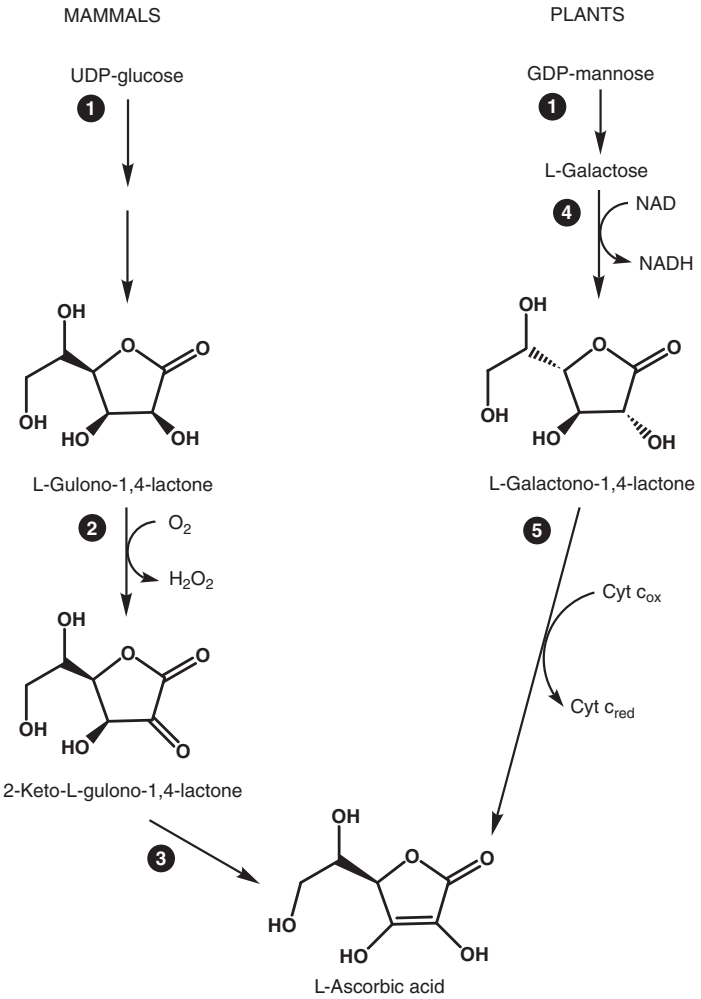


Figure 2.10. Principal ascorbate biosynthetic pathways in mammals and plants. ① Multiple conversions; ② L-gulono-1,4-lactone oxidase; ③ spontaneous reaction; ④ L-galactose dehydrogenase; and ⑤ L-galactono-1,4-lactone dehydrogenase.

and a few other groups has made them dependent on dietary vitamin C intake. The inability to synthesize ascorbate in these species may have been advantageous, in that it reduces the levels of H_2O_2 in the liver.

In plants, ascorbate is synthesized from the relatively rare sugar L-galactose via L-galactono-1,4-lactone in the so-called Smirnoff–Wheeler–Running pathway (Fig. 2.10). This two-step oxidation is catalyzed by the NAD^+-dependent L-galactose dehydrogenase and L-galactono-1,4-lactone dehydrogenase. The latter enzyme uses

cytochrome *c* as an electron acceptor and is localized in the mitochondrial inner membrane. L-Galactose is derived from GDP-L-galactose, which itself probably originates through epimerization from GDP-mannose. A point mutation in one of the enzymes synthesizing GDP-mannose results in plants with very low ascorbate levels (*vtc1* mutants). L-Galactono-1,4-lactone can also be derived from UDP-D-galactose, and it is therefore possible that other minor biosynthetic pathways occur in plants. Obviously, engineering plants to produce higher levels of ascorbate is an attractive biotechnological target and various approaches are actively being pursued to achieve this goal. Plants respond to various oxidative stress conditions by increased levels of ascorbate.

Apart from animals and plants, the presence of ascorbate and ascorbate analogues has been documented in several other eukaryotic organisms. Most fungi synthesize the ascorbate analogue, D-erythroascorbate, and in some fungi, 6-deoxyascorbate or D-araboascorbate is found. Ascorbate, however, appears to be absent from prokaryotes.

2.2.C Ascorbate Recycling

Because ascorbate plays a pivotal role in defense against reactive free radicals, there is a redundancy in mechanisms for regenerating it from monodehydroascorbate and dehydroascorbate. Single-electron reduction of monodehydroascorbate is primarily mediated by cytochrome b_5-dependent monodehydroascorbate reductase, and by the thioredoxin reductase system using NADPH as the ultimate electron donor. Mammalian NADH-dependent monodehydroascorbate reductase has a high affinity for monodehydroascorbate ($K_m \sim 4\ \mu M$) and is localized in the mitochondrial outer membrane and in microsomal membranes. A mutation in this enzyme is linked to type II methemoglobinemia. An alternative mechanism for reduction of monodehydroascorbate to ascorbate exists in chromaffin cells from the adrenal medulla. In these cells, a transmembrane electron shuttle provides electrons from cytosolic ascorbate to monodehydroascorbate inside chromaffin granules supporting ascorbate-dependent dopamine and norepinephrine hydroxylation. The protein responsible for this reaction is a di-heme *b*-type cytochrome (cytochrome b_{561}). Monodehydroascorbate reduction has also been detected at the surface of erythrocyte membranes, where it probably helps to maintain blood plasma ascorbate levels. New evidence suggests that a similar cytochrome b_{561} may be involved in this reaction.

Dehydroascorbate is rapidly reduced to ascorbate in most mammalian cells. Direct reduction of dehydroascorbate by two molecules of GSH is thermodynamically feasible and has been demonstrated in cell-free systems. In addition, several GSH- and NADPH-dependent dehydroascorbate reductases have been identified in mammalian cells, including glutaredoxin, thioredoxin reductsae, and protein disulfide isomerase.

Plants also contain GSH-dependent dehydroascorbate reductases and proteins with dehydroascorbate reductase activity, such as the Kunitz-trypsin inhibitor. However, the major sources of electrons for the reduction of monodehydroascorbate in plants are GSH in the so-called ascorbate–GSH pathway (or Halliwell–Foyer–Asada

pathway) and water. In the photosynthetic electron transport chain, electrons are derived from the photooxidation of water by photosystem II and are transferred to monodehydroascorbate by ferredoxin. The ascorbate–GSH cycle involves an NAD(P)H-dependent monodehydroascorbate reductase and a GSH-dependent dehydroascorbate reductase. GSH is recycled by an NAD(P)H-dependent glutathione reductase. This system is particularly well studied in the chloroplast but is also operational in other plant cell compartments such as mitochondria and peroxisomes. Increased activities of the ascorbate recycling enzymes under oxidative stress conditions suggest an important role for these reactions in regulating cellular ascorbate homeostasis.

2.2.D Ascorbate Transport

Because of its size and charge, ascorbate does not readily permeate lipid bilayers and needs to be transported from its location of synthesis at the mitochondria and endoplasmic reticulum to other cell compartments. Vitamin C redistribution in animal and plant organs is accomplished by facilitated diffusion and active transport mechanisms for ascorbate and dehydroascorbate.

The structural similarity between glucose and dehydroascorbate is sufficient for the mammalian GLUT-type facilitative glucose transporters to translocate dehydroascorbate but not ascorbate. The affinities and transport capacities of GLUT1 and GLUT3 for glucose and dehydroascorbate are similar. Hence, dehydroascorbate uptake competes effectively with glucose transport and is sensitive to regulation by insulin. In addition, glucose-insensitive dehydroascorbate uptake pathways have been demonstrated in various cell types but the transporters have not yet been identified. Less is known about dehydroascorbate transport in plant cells. Dehydroascorbate uptake has been demonstrated in tobacco cell cultures, and facilitated dehydroascorbate and glucose transport occurs in plant-derived mitochondria. Transport of ascorbate across plant plasma membranes and chloroplasts probably occurs through a glucose-insensitive system.

Ascorbate uptake in animal cells utilizes the large electrochemical sodium gradient across the cell membrane. This transport is mediated by the recently cloned sodium-dependent vitamin C transporters, SVCT1 and SVCT2. SVCT transporters are highly specific for L-ascorbate and are widespread in animal organs. Transport of ascorbate by SVCT is electrogenic and therefore very sensitive to changes in the membrane potential. For obvious reasons, there is significant interest in understanding the regulation of SVCT expression, distribution, and post-translational modifications. For example, decreased levels of SVCT expression are implied in the reduced capacity of the elderly to absorb ascorbate from the diet.

2.2.E Importance of Ascorbate in Stress and Disease

Its antioxidant capacities have raised a great deal of interest in the role of ascorbate in human health and disease. For example, oxidative damage to DNA is believed to be an

important cause underlying cancers, and increased levels of ascorbate in cell cultures have proved effective in reducing free radical-induced mutation rates. However, a large number of the physiological effects of ascorbate are actually mediated by its role as a cofactor for a variety of redox enzymes. The role of ascorbate in preventing scurvy is mainly based on its function as a cosubstrate for 2-oxoglutarate-dependent dioxygenases, which incorporate oxygen into organic substrates. Dietary deficiency of ascorbate limits hydroxylation of prolyl residues in collagen by the dioxygenase, prolyl hydroxylase, causing collagen instability and the typical scurvy symptoms. Proline hydroxylation is also essential in the post-translational modification of several other proteins such as the transcription factor hypoxia-inducible factor 1α, responsible for oxygen sensing in mammals. Hypoxia-inducible factor 1α controls the expression of a large number of proteins, some of which are of great importance in angiogenesis, ischemia, and cancer. This illustrates that the role of ascorbate in preventing disease clearly exceeds its function as a free radical scavenger.

SELECTED REFERENCES

1. Bánhegyi, G., Braun, L., Csala, M., Puskás, F., and Mandl, J. (1997). Ascorbate metabolism and its regulation in animals. *Free Rad. Biol. Med.* 23:793–803.
2. Smirnoff, N., and Wheeler, G.L. (2000). Ascorbic acid in plants: biosynthesis and function. *Crit. Rev. Plant Sci.* 19:267–290.
3. Wilson, J.X. (2005). Regulation of vitamin C transport. *Ann. Rev. Nutrition* 25:105–125.

2.3 OTHER ANTIOXIDANTS

JULIE M. STONE and MARK A. WILSON

Redox Biology Center and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska

Antioxidant molecules can be divided into different nonmutually exclusive categories based on their functions (enzymatic or nonenzymatic), their physical properties (water-soluble or lipid-soluble), and, for humans, their sources (endogenous or exogenous). Some antioxidant molecules are enzymes (Chapter 3) or serve as cofactors for enzymes (Section 2.4). The major nonenzymatic cellular redox buffer systems rely on the water-soluble antioxidants GSH (Section 2.1) and ascorbic acid (Section 2.2). However, numerous less abundant antioxidants also serve important functions in redox homeostasis and are important for cellular function and disease prevention.

In this section, we focus on relatively common small molecule antioxidants, including some produced endogenously in animals and others that are acquired exogenously through diet. Vitamin E (tocopherols and tocotrienols) and vitamin A (or provitamin A

β-carotene and related carotenoids) are major lipid-soluble plant-derived antioxidants essential for normal growth, development, and reproduction and are described in some detail. Other antioxidant molecules are discussed only briefly. These antioxidant molecules function not only in quenching chain reactions, scavenging ROS, and preventing lipid peroxidation, but also influence cellular functions by binding to specific receptors to initiate signal transduction cascades, inhibiting or activating enzymes, and regulating gene expression.

2.3.A Lipid-Soluble Antioxidants

Some of the most abundant small molecule antioxidants, particularly GSH and ascorbic acid, are water soluble and perform their ROS scavenging activity primarily in the aqueous environment of the cytosol and within the various compartments and organelles of the cell. However, ROS can also damage lipids, particularly via free radical-mediated reactions that result in lipid peroxidation. Uncontrolled lipid peroxidation causes widespread disruptions in the cell by compromising membrane integrity and function and can lead to cell death. The threat of ROS damage to lipids requires that the cell has defense mechanisms specific to the hydrophobic environment of the cell and organelle membranes. A major part of this response is mediated by lipid-soluble small molecule antioxidants such as vitamin E and β-carotene, which quench free radicals and prevent lipid peroxidation chain reactions. We discuss the properties of some of the most important antioxidants in this section.

2.3.A1 α-Tocopherol. α-Tocopherol is the most biologically active of the eight forms of vitamin E. The name derives from the Greek *tokos*, meaning "offspring," and *pherein*, meaning "to carry," as vitamin E was first identified as being essential for reproductive function in rats. The other forms of vitamin E (β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienols) are closely related in chemical structure to α-tocopherol, and all share the common two-ring (chromanol) core and the 16 carbon aliphatic side chain (Fig. 2.11). These various compounds differ from each other in the identity of the substituents on the chromanol core, and the tocotrienols are further distinguished from the tocopherols by the presence of three double bonds in their isoprenoid side chain. Adding to this complexity is the fact that α-tocopherol also contains three chiral centers, making a total of eight stereoisomers, although the most abundant naturally occurring form of the compound is the *R,R,R* form. All of the tocols (as this class of compounds is called) are closely related and share vitamin E activity; however, it is important to emphasize that α-tocopherol is not strictly synonymous with vitamin E.

α-Tocopherol is a potent lipid-soluble antioxidant that is synthesized exclusively by plants. The lipid solubility of α-tocopherol is a consequence of the hydrophobic phytyl side chain, which anchors the antioxidant chromanol ring to the membrane. The antioxidant function of α-tocopherol is a consequence of its ability to quench free radicals by becoming a stable radical itself, which is a common mechanism among many radical-scavenging antioxidants. α-Tocopherol forms a stable radical species

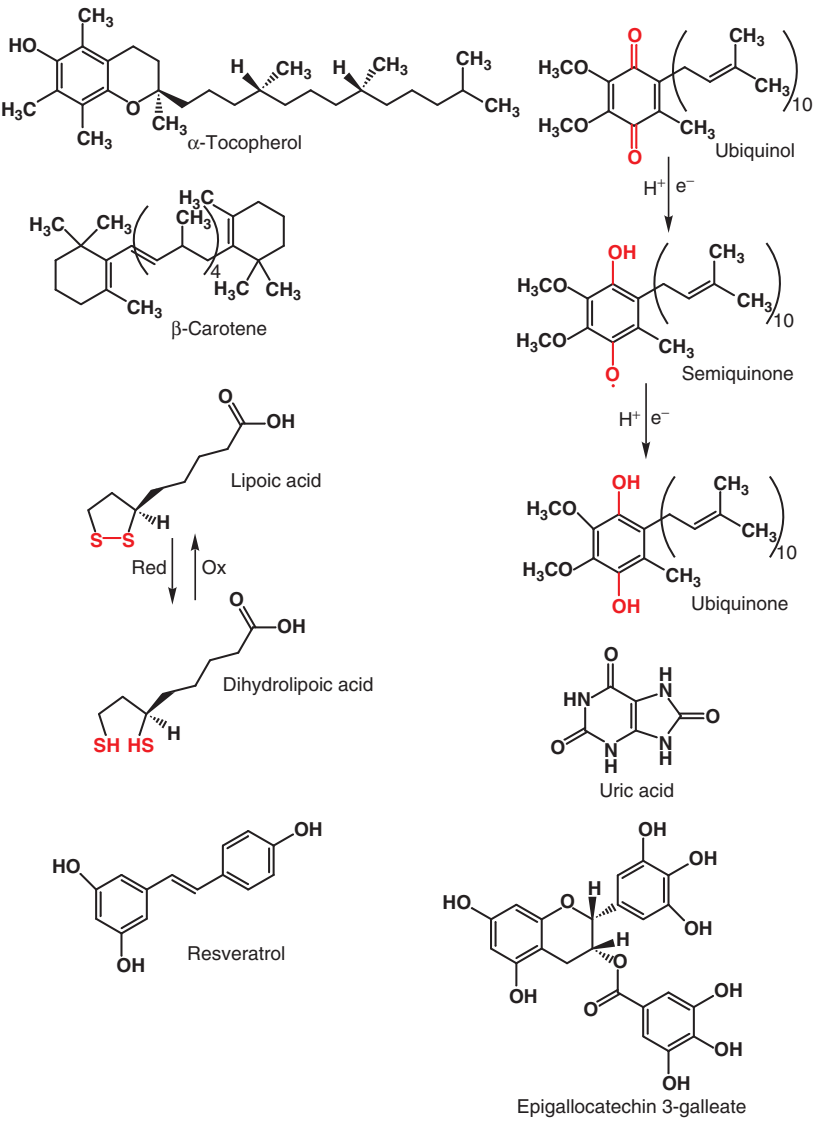


Figure 2.11. Structures for common small molecule antioxidants.

(the tocopheroxyl radical) by delocalizing the unpaired electron that is generated by hydrogen atom abstraction from the exocyclic phenolic oxygen through resonance with the aromatic moiety of the chromanol ring. By sacrificially donating the equivalent of a hydrogen atom to a reactive radical, the tocopheroxyl radical effectively sequesters the free electron in a less reactive (and therefore less damaging) form on its own chromanol moiety. The tocopheroxyl radical can be reduced to regenerate

α-tocopherol by other antioxidants, including ubiquinol, ascorbic acid, and, indirectly, dihydrolipoic acid.

2.3.A2 Carotenoids and Vitamin A. Carotenoids are the most abundant pigmented plant-derived compounds produced in photosynthetic organisms with more than 600 carotenoid compounds identified. These compounds, which impart the red and yellow colors in fruits and vegetables, are also the source of certain colors in birds, insects, and marine invertebrates. Carotenoids are isoprenoids consisting primarily of eight joined isoprene units with a rigid backbone due to the presence of 3 to 15 conjugated double bonds (Fig. 2.11). Modifications include cyclization of the carbon skeleton at one or both ends. The absorption spectrum is determined by the chromophore length, affecting the color of the molecules. A systematic nomenclature exists, but the trivial names based on their original sources are still most commonly used. For example, β-carotene was isolated from carrots and is probably the most studied carotenoid (Fig. 2.11), because of its ability to be cleaved to form retinal (vitamin A) in the animal intestine. In the human intestine, cleavage is catalyzed by the recently cloned β-carotene 15,15′-cleavage enzyme using a monooxygenase-type reaction to produce retinal. In plants, carotenoids are essential; they directly participate in photosynthesis and reactions to limit the vast amounts of ROS produced in chloroplasts. Oxygen-containing carotenoids (the xanthophylls) are integral components of photosystem II and the light-harvesting complex. Zeaxanthin is responsible for absorption of excess excitation energy and conversion to heat in a process termed thermal energy dissipation preventing formation of potentially harmful ROS. Several carotenoids also participate in protection against photoinduced damage. The relative efficiency of individual species at preventing lipid peroxidation chain reactions has been tested in liposomes and has been found to be in the order: lycopene $>$ α-tocopherol $>$ β-carotene $>$ lutein. Carotenoid radicals can be reduced by both α-tocopherol and ascorbic acid, which might account for the observed synergistic interactions between these three important vitamins (A, C, and E).

2.3.A3 α-Lipoic Acid/Dihydrolipoic Acid. α-Lipoic acid is a sulfur-containing antioxidant and an essential cofactor in both the pyruvate dehydrogenase and the α-ketoglutarate dehydrogenase multienzyme complexes. α-Lipoic acid is not a vitamin, as it is synthesized in the mitochondrion, although the biochemistry of its synthesis is still incompletely understood. The general chemical architecture of α-lipoic acid is similar to many lipid-soluble antioxidants in that it contains a redox-active moiety linked to a hydrocarbon side chain that imparts lipid solubility to the molecule. Unlike most other lipid-soluble antioxidants, however, α-lipoic acid is amphipathic (soluble in both aqueous and nonaqueous environments) due to the hydrophilic carboxylic acid group at the end of its hydrocarbon side chain. α-Lipoic acid has a single chiral center, and the naturally occurring form is the *R*-enantiomer.

α-Lipoic acid contains a disulfide linkage that forms a five-membered ring, and this disulfide can be reduced to a dithiol with concomitant ring opening to form dihydrolipoic acid (Fig. 2.11). Unlike many other antioxidants, both the oxidized

(α-lipoic acid) and the reduced (dihydrolipoic acid) forms of the molecule can serve as antioxidants, albeit with different specificities for particular ROS. In general, dihydrolipoic acid is considered the superior antioxidant, because it can participate in thiol-disulfide oxidation–reduction reactions (two electron oxidation/reduction) to form α-lipoic acid. In addition, dihydrolipoic acid plays a key role in maintaining cellular redox homeostasis by reducing dehydroascorbic acid to generate lipoic acid and ascorbate.

2.3.A4 Ubiquinol/Ubiquinone. Ubiquinol and its oxidized form, ubiquinone, are so-named because they are ubiquitous in the cells of aerobic organisms. One of the major roles of the ubiquinol/ubiquinone redox pair is to shuttle electrons in the electron transport chain between mitochondrial complexes II and III during oxidative phosphorylation. In this capacity, ubiquinone accepts two reducing equivalents (hydrogen atoms) from complex II and is reduced to ubiquinol. Ubiquinol then diffuses through the lipid membrane to complex III, where it is oxidized back to ubiquinone, thus passing reducing equivalents down the electron transport chain. Consistent with the critical role of ubiquinol/ubiquinone in oxidative phosphorylation, approximately 80% of ubiquinol/ubiquinone in the cell is found in the mitochondria. Importantly, ubiquinone can also accept one electron to form a stable radical anion semiquinone. As with α-tocopherol, the formation of a stable radical is a common mechanism by which antioxidants quench reactive free radicals; thus, ubiquinone can act as a lipid-soluble antioxidant.

Like the other lipid-soluble antioxidants that we have discussed, ubiquinol contains a redox-active group (benzoquinol) attached to a hydrophobic side chain (Fig. 2.11). This hydrocarbon side chain is composed of multiple five-carbon isoprene units, and the most common form of ubiquinol in humans contains a side chain with ten isoprene units. Hence, the dominant human form of ubiquinol is sometimes called coenzyme Q_{10}, where the subscript indicates the number of isoprene units in the side chain. This long isoprenyl side chain is highly hydrophobic and anchors ubiquinol to the membrane. Unlike other more amphipathic lipid-soluble antioxidants like α-lipoic acid or α-tocopherol, the strong hydrophobicity of ubiquinol keeps the molecule completely confined in the lipid bilayer.

2.3.B Water-Soluble Antioxidants

The prevalence of so many lipid-soluble antioxidant molecules (described in Section 2.3.A) point to the importance of protecting cellular and organellar membranes from lipid peroxidation. The water-soluble antioxidant enzymes and the major redox buffers, ascorbic acid and GSH, are considered to be the primary small molecule antioxidants used in the aqueous environments of the cell. However, there are lesser known water-soluble small molecules that appear to be important as well in protecting against oxidative stress and disease, which are described in this section.

2.3.B1 Flavonoids. Plants produce a wide spectrum of secondary metabolites referred to as polyphenols. Polyphenolics are subdivided into the nonflavonoids (e.g.,

hydroxybenzoic acids, hydroxycinnamic acids, and stilbenes) and the flavonoids (flavonols, flavanals, isoflavones, and anthocyanins). The plant-derived flavonoids are composed of more than 4000 different species that have two aromatic benzene rings linked through three carbons that can form an oxygenated heterocycle. Quercetin (Fig. 2.11) is representative of the most abundant flavonoid class, the flavonols. Isoflavones (phytoestrogens), found in high abundance in legumes, are also under intense scrutiny for their potential health benefits. The antioxidant activities of flavonoids are attributed to chelation of redox-active metals to prevent peroxyl radical and lipid peroxidation, scavenging of hydroxyl and peroxyl radicals, and quenching of superoxide radicals and singlet oxygen.

BOX 2.1 GREEN TEA AND ANTIOXIDANT POLYPHENOLS

Green tea has been a staple beverage in Asian diets for millennia. Chinese legend claims that green tea was discovered in 2737 BC by the Emperor Shen Nung, who had the sensible habit of boiling water before he drank it. One day some leaves from a nearby tea plant fell into the emperor's boiling water, thus spontaneously brewing the first cup of green tea. The emperor was so favorably impressed with the resulting drink that he proclaimed it to have been sent from Heaven.

Though the veracity of this legend is questionable, Emperor Shen Nung would be very pleased with the subsequent success of his beverage. Tea is the second most consumed beverage in the world, the first being water. Tea was used as a currency in China, its consumption has been elevated to the level of an elaborate ritualistic ceremony in Japan, and its trade helped both finance the expansion of the British Empire and to ignite British colonial unrest that culminated in the American Revolution. Many varieties of tea exist, but they are all derived from leaves of the same plant, *Camellia sinesis*. The various types of tea differ in how the tea leaves are treated after being harvested. "Black" teas are made from processed and oxidized leaves, while "green" tea is made from freshly dried, unoxidized leaves. While both have health benefits, green tea is generally thought to have the superior effect on health, particularly in the prevention of cancer.

Researchers have identified a class of antioxidants called polyphenols that appear to be responsible for much of the health benefits of green tea. The most active of these polyphenols in green tea is (−)-epigallocatechin 3-galleate (EGCG, Fig. 2.11), although there are several other related compounds in green tea that have similar structures and some biological activity. The specific mode(s) of action of EGCG are still being investigated, but a variety of proteins that bind EGCG have been identified and include the 90 kDa heat shock protein (Hsp90), dihydrofolate reductase, DNA methyltransferases, the laminin receptor,epidermal growth factor receptor, and telomerase. This varied

and growing list of protein targets of EGCG testifies to the complex biological activity of this natural product in multifactorial diseases such as cancer, neurodegeneration, and heart disease.

BOX 2.2 THE FRENCH PARADOX AND RESVERATROL/SIRTUINS

Which diet should we follow to live long and healthy lives — Mediterranean, French, Japanese, low-fat, low-carb, or calorie-restricted? How do cultural dietary traditions impact an individual's longevity and incidence of chronic and degenerative disease? The "Free Radical Theory of Aging" purports that a healthy lifestyle and antioxidant consumption can reduce the consequences of aging. Plants produce a wide spectrum of secondary metabolites important for their own health that also function as antioxidants in humans who ingest them.

Are the foods we eat (and the antioxidants they contain) key to maintaining health and warding off disease? Perhaps. A possible molecular basis for the "French Paradox"—the low incidence of heart disease in the high-fat-diet-eating French—has surfaced only in the past few years: resveratrol (*trans*-3,5,4′-trihydroxystilbene, Fig. 2.11)—a nonflavonoid polyphenolic antioxidant produced in grape skins, soy, and peanuts to fight against plant disease and a component of an ancient oriental medicine (Ko-jo-kon) used to treat blood vessel, heart, and liver diseases—may be the key molecule. Resveratrol has received a great deal of attention in both the scientific and popular literature and has been touted to protect against a wide range of age-related diseases including Alzheimer's and heart disease. But how? The answer might lie in its ability to affect gene expression. Caloric restriction (and increased longevity) was linked to extra copies of sirtuin genes in both yeast and the nematode worm *Caenorhabditis elegans*. Sirtuins are NAD^+-dependent protein deacetylases that can control gene expression and stabilize repetitive DNA sequences and are now implicated in aging, cell survival, and apoptosis. A screen for small molecule compounds that increase the catalytic rate of sirtuins revealed several effective plant polyphenols, including quercitin and resveratrol.

How does resveratrol solve the French Paradox? Resveratrol is abundant in grape skins and effectively extracted in red wine processing. So, washing down rich French dishes swimming in butter-laden sauces with a glass of red wine might just be the secret to longevity. Ironically, a diet high in fat may actually enhance the uptake of some important lipid-soluble antioxidants too. Discovery of "new" plant-derived antioxidants is transpiring at a staggering pace, and more "magic bullets" may be on the horizon!

2.3.B2 Uric Acid. Uric acid is a modestly water-soluble antioxidant with the ability to neutralize a broad spectrum of ROS, particularly singlet oxygen and free radicals. Uric acid is an end product of purine catabolism and is one of the most abundant circulating antioxidants, with a blood plasma concentration in humans of about 300 μM. Uric acid can accept a single electron to form a stable radical, and thereby neutralize a variety of ROS species.

Humans, apes, and certain New World monkeys have significantly higher serum uric acid levels than other primates due to an evolutionarily recent mutation that functionally inactivates urate oxidase. Urate oxidase converts uric acid to allantoin, which is more water soluble and a much poorer antioxidant than uric acid. It has been speculated that the resulting increase is serum uric acid concentrations in the hominids may contribute to their longer life span and lower age-specific cancer rates than other mammals.

2.3.C Antioxidants and Human Health

Antioxidants have diverse biochemical roles and therefore are important in human health. Vitamin E is an essential nutrient with deficiencies in humans leading to a variety of diseases including mild hemolytic anemia and spinocerebellar diseases, which usually manifest as ataxia (poorly coordinated movement) and neuropathy (nerve damage or conduction problems). Vitamin E deficiency is rare, however, as most adults receive their recommended daily allowance of vitamin E in their diet, and the majority of cases of vitamin E deficiency are diagnosed in children with congenital defects in fat absorption.

In both plants and animals, carotenoid deficiency is more detrimental than tocopherol deficiency. Vitamin A deficiency leads to various eye diseases, including xerophthalmia (childhood blindness), age-related cataract formation, and macular degeneration. Two carotenoids, lutein and zeaxanthin, accumulate in the macula of the eye, where they are thought to scavenge free radicals and protect against light-induced damage. Vitamin A deficiency remains a problem in developing countries, where humanitarian efforts to genetically engineer enhanced carotenoid content in several crop plants have met with mixed success. The major challenges that need to be addressed with crops engineered for enhanced carotenoid content are consumer distaste for "yellow" rice, the fact that retinal-forming enzymes are repressed in the guts of malnourished individuals, and the paucity of fat (to aid absorption) in their diets.

α-Lipoic acid/dihydrolipoic acid has a complex role in human health, as they are both essential enzyme cofactors and potent antioxidants. The essential role of α-lipoic acid in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase multienzyme complexes makes it vital for energy metabolism, and it is no coincidence that α-lipoic acid is synthesized in mitochondria, where these multienzyme complexes are located. The best-characterized benefit from α-lipoic acid supplements is ameliorating some of the neurological complications of diabetes, for which it is approved in Germany.

Ubiquinol/ubiquinone (coenzyme Q_{10}) can be synthesized by humans and is therefore not a vitamin by definition. Nevertheless, coenzyme Q_{10} has enjoyed a long period of popularity as a dietary supplement. Many exaggerated claims of the sweeping

preventative and regenerative powers of coenzyme Q_{10} should be regarded with suspicion, and there is comparatively little research that has been done to determine if coenzyme Q_{10} supplementation has clinical value. One exception is that coenzyme Q_{10} supplementation is generally regarded as beneficial for the treatment of congestive heart failure.

Flavonoids are reported to have antioxidant, anti-inflammatory, and estrogen hormone-like properties. Certain flavonoids, like quercitin, may protect against atherosclerosis or cardiovascular disease. Isoflavones, such as obtained from soy, are also known as phytoestrogens, because they can bind to the estrogen receptor and mimic the effects of endogenous estrogen hormones.

Uric acid is produced by the body in significant quantities, and its modest water solubility can cause serious health problems. Excess uric acid can crystallize in the body, especially in the synovial fluid in the joints of the lower extremities, causing a painful inflammatory arthritic condition known as gout. In addition, the poor water solubility of uric acid can cause problems in the kidney and urinary tract by forming kidney stones. Uric acid kidney stones represent only about 10% of the total cases of kidney stones, however, which are more commonly composed of insoluble calcium oxalate or phosphate crystals.

SELECTED REFERENCES

1. Carotenoids and Dietary Lipids (2005). Special issue *Biochimica et Biophysica—Molecular Basis of Disease* 1740(2).
2. Dellapenna, D., and Pogson, B.J. (2006). Vitamin synthesis in plants: tocopherols and carotenoids. *Annu. Rev. Plant Biol.* 57:711–738.

2.4 REDOX COENZYMES

RUMA BANERJEE

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan

DONALD F. BECKER

Redox Biology Center and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska

2.4.A Flavin

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) participate in a variety of enzyme-catalyzed reactions as noncovalently or covalently bound redox cofactors. A distinguishing property of flavins is their ability to couple one-electron and two-electron transfer reactions between substrates and different electron carriers. Thus, flavins equilibrate between quinone (oxidized), semiquinone (one-electron reduced), and hydroquinone (two-electron reduced) species with reversible electron transfer occurring across the N(5) and N(1) atoms of the isoalloxazine ring of the flavin (Fig. 2.12). The redox potential of free FAD is −0.219 V

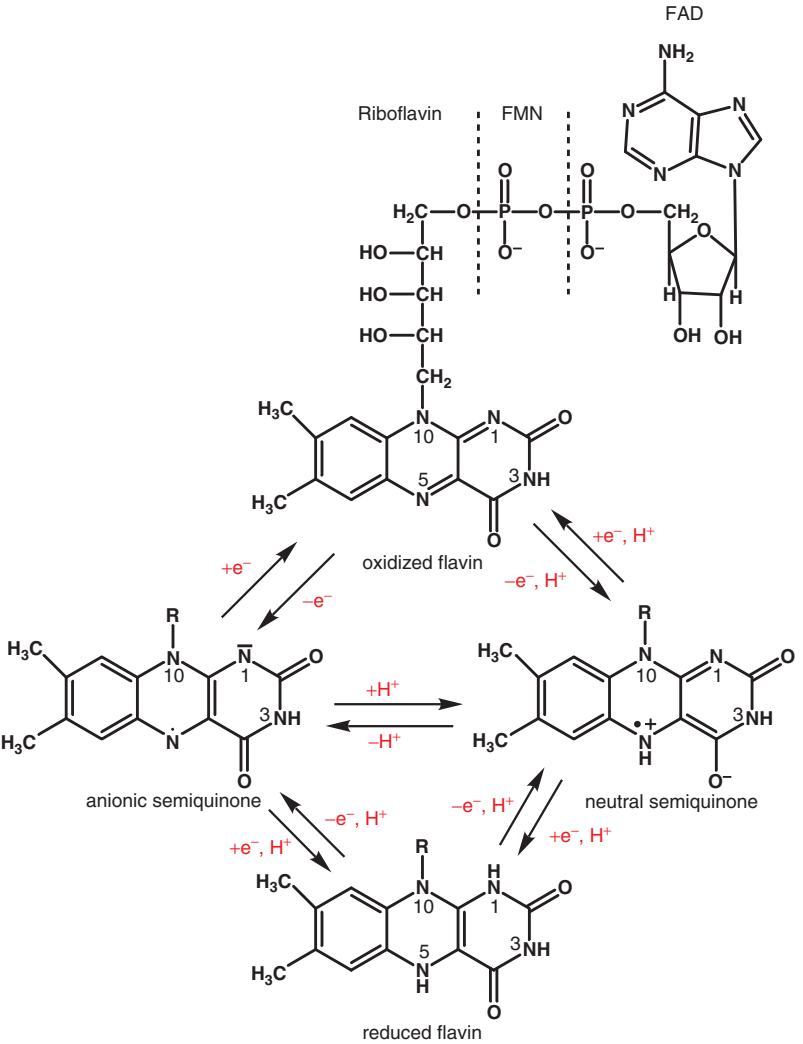


Figure 2.12. Flavin redox states.

(pH 7.0) in solution while enzyme-bound flavin has a redox potential range generally of +0.1 to −0.4 V (versus the normal hydrogen electrode), which contributes to the involvement of flavoenzymes in a remarkable variety of reactions such as dehydrogenation, electron transfer, dehalogenation, hydroxylation, luminescence, DNA repair, and disulfide reduction. Reactions catalyzed by flavoenzymes can be divided into oxidative and reductive half-reactions as shown in Fig. 2.13 for medium chain acyl-CoA dehydrogenase, which catalyzes the first step in fatty acid oxidation.

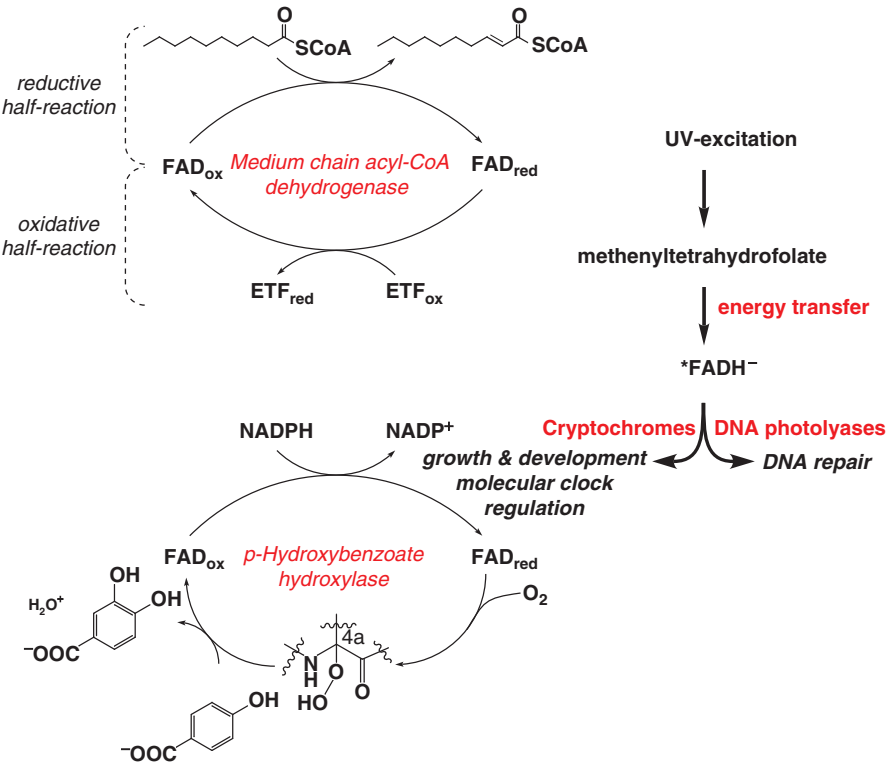


Figure 2.13. Flavin coenzyme-catalyzed reactions. Flavoenzymes shown are medium chain acyl-CoA dehydrogenase, *p*-hydroxybenzoate hydroxylase, and the cryptochrome/photolyase family.

The reductive half-reaction describes the reduction of the flavin by an electron donor such as the substrate octanoyl-CoA for medium chain acyl-CoA dehydrogenase. The oxidative half-reaction involves the oxidation of the reduced flavin by an electron acceptor such as molecular oxygen or, in the case of medium chain acyl-CoA dehydrogenase, the electron transfer flavoprotein.

The reactivity of enzyme-bound reduced flavin coenzyme with molecular oxygen is significantly influenced by the active site environment and substrate/product complexation. Reaction of reduced flavin with molecular oxygen proceeds initially through a one-electron reduction of oxygen to form a flavin semiquinone and $O_2^{\cdot -}$ pair. Subsequent events then lead to the formation of a flavin C(4a)-peroxide or H_2O_2/ $O_2^{\cdot -}$ products. Monooxygenases are a group of flavoenzymes that form a C(4a)-peroxide intermediate from which one oxygen atom is inserted into the substrate while the other oxygen atom is reduced to water. A well characterized example of this class

of flavoenzymes is *p*-hydroxybenzoate hydroxylase, which utilizes NADPH as an electron donor to generate reduced flavin for subsequent hydroxylation of aromatic substrates (Fig. 2.13). Flavoenzymes that react with molecular oxygen during catalytic turnover and generate H_2O_2 are called oxidases such as D-amino acid oxidase, which directs the oxidative deamination of D-amino acids to α-keto acids and ammonia. Electron transferases such as flavodoxin react with molecular oxygen in the absence of a physiological electron acceptor to form $O_2^{\cdot -}$. Dehydrogenases also generate $O_2^{\cdot -}$ but the reactivity is considerably lower than other classes of flavoenzymes, most likely to help ensure the efficient transfer of electrons to the correct physiological acceptor such as the electron transfer flavoprotein for medium chain acyl-CoA dehydrogenase. The ability of reduced flavins to perform the one-electron reduction of molecular oxygen to $O_2^{\cdot -}$ implies that flavins generally contribute to intracellular oxidative stress.

In addition to the diverse catalytic power of flavoenzymes, several flavoenzymes are involved in regulatory and signaling pathways. An example is the programmed cell death pathway or apoptosis in which the apoptotic inducing factor is involved. The apoptotic inducing factor is a mitochondrial flavoenzyme that exhibits NADH oxidase and DNA binding activities. When localized in the inner mitochondrial membrane, the NADH oxidase function of the apoptotic inducing factor appears to enhance oxidative phosphorylation, but at the appropriate signal, the apoptotic inducing factor is trafficked to the cell nucleus, where it binds chromatin, leading to its condensation, a morphological hallmark of apoptosis. In diazotrophic bacteria, the flavoprotein NifL regulates nitrogen fixation according to the intracellular redox environment. When the flavin is oxidized, NifL inhibits NifA, the transcriptional activator of the nitrogen fixation genes, by forming a NifL–NifA complex. Under reducing conditions, NifL does not bind to NifA, allowing NifA to activate expression of the nitrogen fixation genes. Another interesting class of flavoenzymes is the structurally related cryptochromes and DNA photolyases that are activated by blue light. These flavoenzymes harvest light energy by absorbing a photon at a second cofactor, which, depending on the organism, is either 5,10-methylenetetrahydrofolate (see Fig. 2.19) or 8-hydroxy-7,8-didemethyl-5-deazariboflavin, and by transferring the excitation energy to the reduced FAD ($FADH^-$) (Fig. 2.13). Cryptochromes are involved in photosensitive signaling pathways that set the circadian clock and regulate plant growth and development. In bacterial DNA photolyases, light excitation of the reduced flavin triggers electron transfer to the cyclobutane pyrimidine dimer to repair the damaged thymine bases. Another group of light-sensing flavoproteins is the phototropins that help mediate adaptive responses to blue light in plants.

Riboflavin (vitamin B_2) synthesis occurs in several lower organisms and in plants but not in mammals. The biosynthesis of riboflavin originates with GTP and ribulose-5-phosphate and involves the capsid-forming enzyme lumazine synthase. Riboswitches involving flavin-binding RNA aptamers have been found in bacteria that regulate riboflavin synthesis genes in response to cellular flavin levels.

In humans, riboflavin is the precursor for the biosynthesis of FMN and FAD coenzymes. Flavokinase phosphorylates riboflavin to generate FMN, which is then adenylated by FAD synthetase to form FAD. It is generally accepted that

riboflavin deficiency results in increased oxidative stress due to lower activity of FAD-dependent glutathione reductase and xanthine oxidase resulting in lower concentrations of reduced GSH and uric acid, respectively. In fact, glutathione reductase activity is sometimes used to evaluate riboflavin nutritional status.

2.4.B NAD

The pyridine nucleotide, nicotinamide adenine dinucleotide (NAD), was originally described as a low molecular weight compound that is required for fermentation of yeast. NAD and its phosphorylated derivative, NADP, are now recognized as universal energy carriers performing reversible two-electron transfers in a variety of essential metabolic reactions. NAD^+ (oxidized form) participates primarily in oxidative reactions by accepting electrons from energy-rich substrates at the 4 position of the nicotinamide ring (ultimately leading to ATP formation), while NADPH (reduced form) serves mainly as an electron donor in reductive biosynthetic reactions (Fig. 2.14). The standard redox potentials for the two-electron reduction of the NAD^+/NADH and $NADP^+$/NADPH couples are -0.320 V and -0.324 V (pH 7.0). However, the intracellular ratio of $NADP^+$/NADPH is quite low, indicating that the actual cellular redox potential of the $NADP^+$/NADPH couple is considerably more negative (≤ -0.37), favoring the principal function of NADPH as an electron donor. In addition to bioenergetics, NAD participates in signaling pathways by serving as a precursor to calcium releasing agents and as a substrate for protein modifications such as poly-ADP-ribosylation of transcription factors by poly-ADP-ribose polymerases and deacetylation of histones by sirtuins in the nucleus. NADPH has a critical role in oxidant production and antioxidant defense in mammals via the enzyme actions of NADPH oxidase and glutathione reductase, respectively (see Chapter 3, Section 3.3). In neutrophils (white blood cells), NADPH oxidase catalyzes the oxidation of NADPH

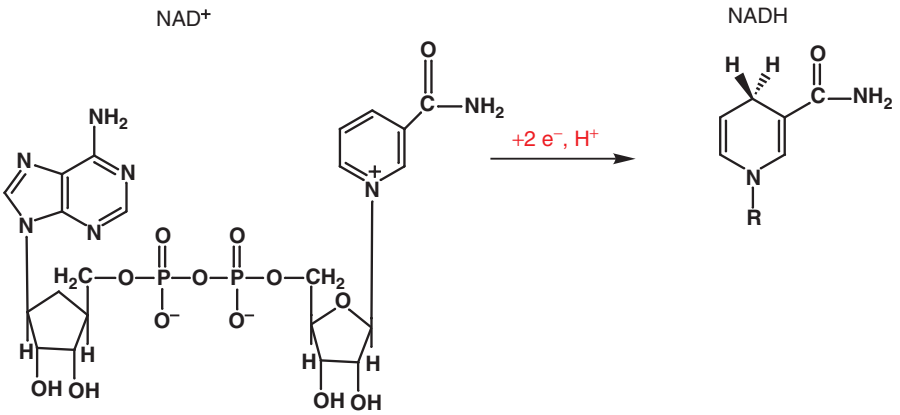


Figure 2.14. Structure of NAD^+/NADH.

by molecular oxygen to generate $O_2^{\cdot -}$, which helps kill invading microorganisms in phagocytic cells.

NAD biosynthesis in mammals can occur *de novo* from tryptophan but the salvage pathways involving either nicotinic acid (pyridine 3-carboxylic acid) or nicotinamide are the primary contributors of NAD. NAD deficiency is manifested as a disease known as pellagra, which is characterized by diarrhea, dermatitis, and dementia. Pellagra was first described in the middle of the18th century and presently occurs mainly in developing countries and poor population groups. Niacin (vitamin B_3) is the dietary form for NAD supplements and appears useful for preventing and treating atherosclerosis. Niacin supplementation decreases lipoprotein plasma levels of LDL and VDL while increasing HDL/HDL_2 levels.

2.4.C Quinones

Quinones were first discovered as enzymatic cofactors in the bacterial pyrroloquinoline quinone (PQQ)-dependent enzymes, glucose dehydrogenase and methanol dehydrogenase. PQQ, an *ortho*-quinone, is noncovalently bound to the enzyme through ionic interactions with its carboxylic groups. Biosynthesis of PQQ is absent in plants and animals, while in bacteria it is assembled from glutamate and tyrosine. Following the characterization of PQQ-dependent enzymes, other quinine-dependent enzymes were discovered in bacteria, yeast, plants, and animals but the structural identities of the quinone cofactors remained uncertain. Eventually, four additional quinonoid cofactors were identified but, unlike PQQ, these quinones are generated post-translationally from oxidation of side chain aromatic rings of specific tyrosine or tryptophan residues within the polypeptide chain and remain covalently bound to the enzyme. These quinone cofactors are classified according to the type of covalent linkage as topaquinone (TPQ), lysine tyrosylquinone (LTQ), tryptophan tryptophylquinone (TTQ), and cysteine tryptophylquinone (CTQ). Similar to flavins, quinone cofactors support two- or one-electron transfers and involve two half-reactions (Fig. 2.15). The reductive half-reaction involves the formation of the reduced quinone cofactor while the oxidative half-reaction is characterized by transfer of electrons from the reduced quinone cofactor to an acceptor such as ubiquinone or cytochrome *c* as in the case of alcohol dehydrogenases or molecular oxygen such as in copper amine oxidase. The redox potentials of the various quinone cofactors are generally in the range of 0.06–0.13 V (versus normal hydrogen electrode, pH 6.8–7.5).

Quinoproteins that derive their own quinone cofactor are found widely in nature. The physiological roles of these enzymes are not fully defined in mammals but copper amine oxidase (TPQ-dependent) appears to have roles in regulating glucose uptake and cell adhesion. Also in humans, LTQ-dependent lysyl oxidase helps form the extracellular matrix catalyzing the cross-linking of collagen and elastin. Diseases involving connective tissue disorders, liver fibrosis, and breast cancer have been linked to malfunctions in lysyl oxidase. In contrast to the covalently linked

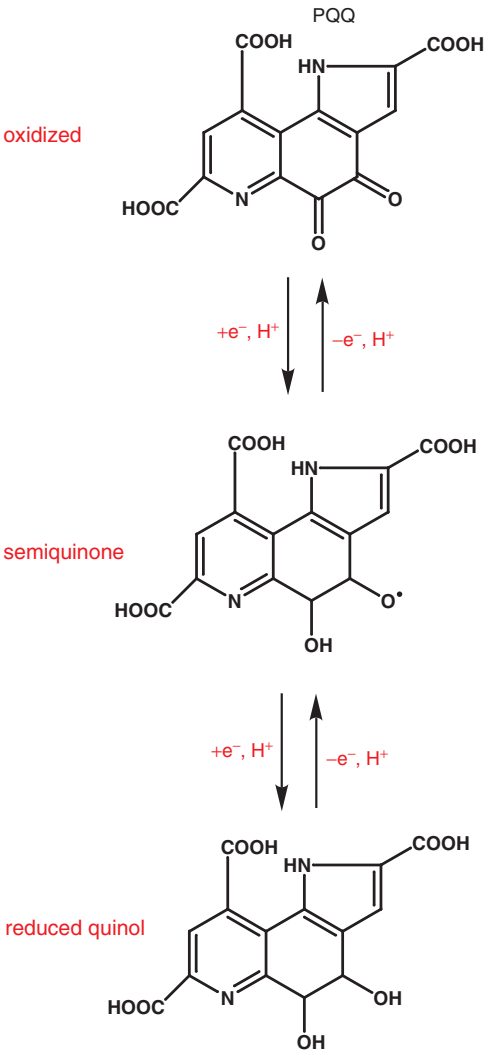


Figure 2.15. Redox states of pyrroloquinoline quinone (PQQ).

quinone cofactors, PQQ has traditionally been thought to be an essential cofactor only in bacteria. More recently, however, a PQQ-dependent dehydrogenase involved in mammalian lysine metabolism has been reported. These findings led to the proposal that PQQ should be classified as a new B vitamin but further evidence is needed to confirm whether PQQ functions as a cofactor in humans. Even so, PQQ is found in human milk and it is generally accepted that PQQ has positive nutritional effects on growth, development, and reproductive health as well as functioning as a

radical scavenger, which contribute to the antioxidant benefits of green tea that contains PQQ.

2.4.D Pterins and Molybdopterins

Pterins are redox cofactors that contain two fused heterocyclic rings, the pteridine ring, which is a structure that is also seen in folic acid and in the isoalloxazine ring in flavins. Tetrahydrobiopterin and molybdopterin serve as cofactors in hydroxylations of aromatic rings, in NO biosynthesis, and in monooxygenation reactions. Pterins can exist in multiple redox states (Fig. 2.16). The one-electron oxidation of tetrahydrobiopterin leads to formation of a radical, an intermediate that has been observed in nitric oxide synthase as discussed later. The two-electron oxidation product, dihydrobiopterin, exists as one of two tautomers and each is recognized by a different reductase. Thus, the quinonoid form generated during aromatic amino acid hydroxylation is a substrate for dihydropterin reductase, whereas 7,8-dihydrobiopterin is reduced by dihydrofolate reductase. Biopterin is the four-electron oxidized product

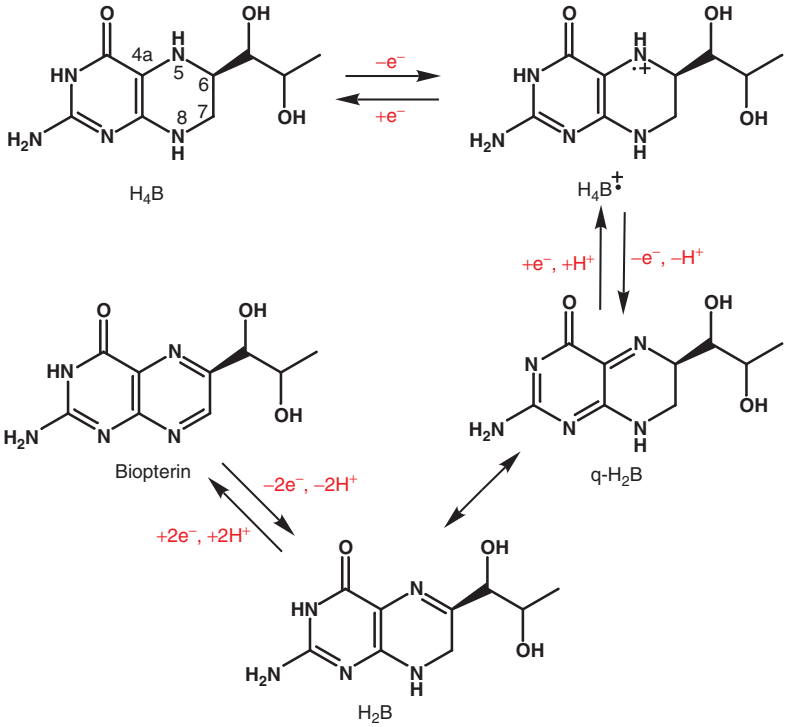


Figure 2.16. Redox states of biopterin. H_4B, H_2B, and q-H_2B denote tetrahydrobiopterin, dihydrobiopterin, and quinonoid dihydrobiopterin, respectively.

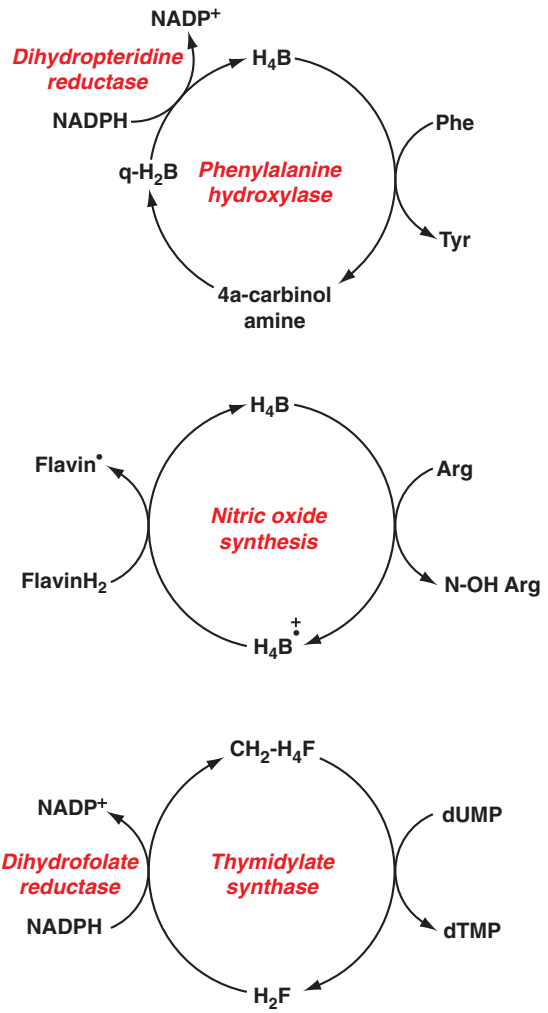


Figure 2.17. Redox reactions catalyzed by tetrahydrobiopterin and methylenetetrahydrofolate. This figure is modified with permission from Dennis Stuehr. The figure is adapted from a similar one published by C.C. Lei et al. (2003).

of tetrahydrobiopterin. The lability of tetrahydrobiopterin in solution to oxidation by oxygen and reactive oxygen species (viz., $O_2^{\cdot -}$, peroxynitrite, and H_2O_2) has led to the suggestion that it may function as an antioxidant.

The pterin-dependent aromatic amino acid hydroxylases are monooxygenases that catalyze the addition of one oxygen atom from molecular oxygen to the substrate and reduce the other atom to water (Fig. 2.17). In addition to tetrahydrobiopterin, these

proteins require mononuclear iron, which is essential for catalysis. In analogy to the chemistry of flavin-dependent hydroxylases, in which the hydroxylating species is believed to be the 4a-peroxyflavin (see Fig. 2.13), a 4a-peroxy intermediate is postulated to form in the pterin-dependent hydroxylases. The essential role of ferrous iron in these enzymes suggests that, as in cytochrome P450-dependent hydroxylases, a high valence iron-oxo intermediate may be the actual hydroxylating agent, although this has not been established unequivocally. The product of the hydroxylase reaction is the two-electron oxidized quinonoid dihydrobiopterin, which is released and needs to be reduced by dihydropteridine reductase to tetrahydrobiopterin prior to participation in another turnover cycle. Mutations in phenylalanine hydroxylase lead to phenylketonuria, an inborn error of metabolism that is routinely screened for in all newborns in the United States and treated by a low phenylalanine-containing diet. Hyperphenylalaninemia can also result from a deficiency in dihydropteridine reductase, which is treated additionally by provision of 5-hydroxytryptophan and L-3,4-dihydroxyphenylalanine, products of the tryptophan hydroxylase and tyrosine hydroxylase reactions, respectively. These reactions also require active dihydropteridine reductase and their products are the precursors of the neurotransmitters, serotonin and norepinephrine, respectively.

A modified pterin cofactor that coordinates molybdenum or tungsten is found associated with enzymes that catalyze the transfer of an oxygen atom to or from substrates with a concomitant two-electron oxidation or reduction of the cofactor. The molybdopterin cofactor consists of a tricyclic pyranopterin with a metal-coordinating *cis*-dithiolene in the pyran ring (Fig. 2.18). In some molybdopterin enzymes, the coordination sphere of the molybdenum/tungsten can involve two molybdopterin moieties, which can be further derivatized with nucleotides (e.g., GMP).

Molybdopterin-containing enzymes catalyze important reactions in global carbon, nitrogen, and sulfur cycles, are mostly found in bacteria, and fall into two families named after representative members: (1) aldehyde oxidase/xanthine dehydrogenase and (2) nitrate reductase/sulfite oxidase. The two classes differ in the coordination environment of the molybdenum. Thus, a terminal sulfur or a strictly conserved cysteine serves as molybdenum ligands in the first and second families, respectively (Fig. 2.18). The reactions catalyzed by representative molybdopterin enzymes are shown in Eqs. (2.3)–(2.5).

$$\text{Aldehyde oxidoreductase:} \quad \mathrm{RCHO + H_2O \rightarrow RCOOH + 2H^+ + 2e^-} \tag{2.3}$$

$$\text{Nitrate reductase:} \quad \mathrm{NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O} \tag{2.4}$$

$$\text{Sulfite oxidase:} \quad \mathrm{SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-} \tag{2.5}$$

The common mechanistic theme in these reactions is that the oxygen atom for "oxo transfer" is derived from water and that the molybdenum cycles between the oxidized Mo(VI) and the reduced Mo(IV) states (or the corresponding oxidation states for tungsten). Completion of the catalytic cycle requires restoration of the molybdenum to the oxidation state found in the resting enzyme. For this, additional prosthetic groups such as iron–sulfur clusters, FAD/NADH, or heme are associated

with molybdopterin-containing enzymes that restore the molybdenum to the correct oxidation state at the end of the catalytic cycle. Since iron–sulfur clusters and hemes are obligate one-electron donors/acceptors, it follows that interconversion between Mo(VI) and Mo(IV) occurs in two one-electron steps. Given the proximity of the pterin to the single-electron donors, it is possible that a pterin radical intermediate may be involved in regenerating the active form of the enzyme. In fact, a pterin radical is observed by EPR spectroscopy in purified aldehyde dehydrogenase.

Tetrahydrobiopterin is also used as a cofactor by nitric oxide synthase, which generates nitric oxide from arginine and oxygen and contains a complex array of additional cofactors: NADPH, FAD, FMN, and heme. The crystal structure of nitric oxide synthase revealed that the tetrahydrobiopterin is too remote from the substrate-binding site to be directly involved in oxygen activation for the two successive monooxygenase reactions catalyzed by this enzyme. However, kinetic and spectroscopic studies have established a role for electron transfer from tetrahydrobiopterin to the heme during oxygen activation (Fig. 2.17). The product of one-electron transfer, the tetrahydrobiopterin radical, has been observed by EPR spectroscopy

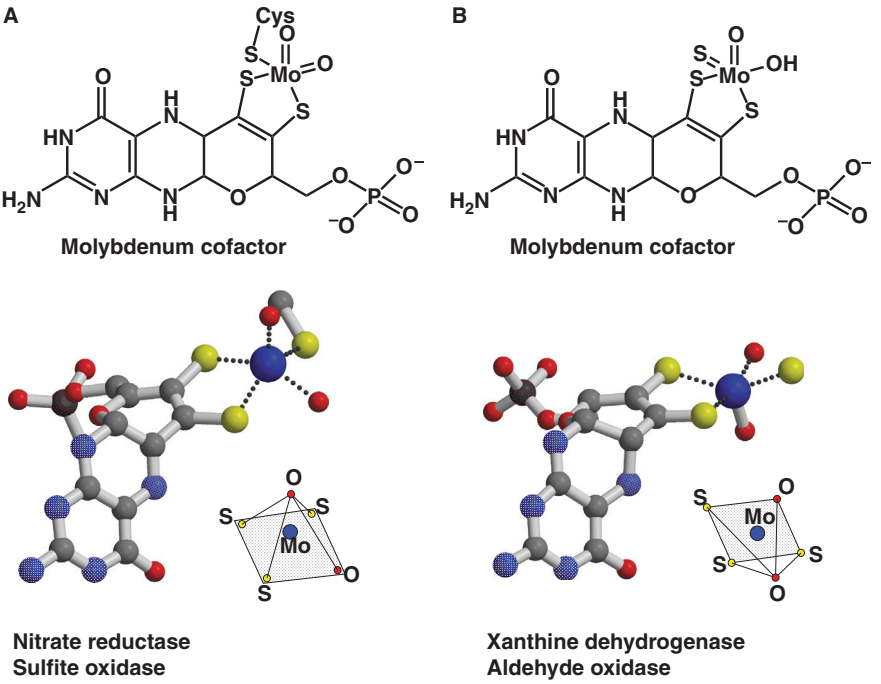


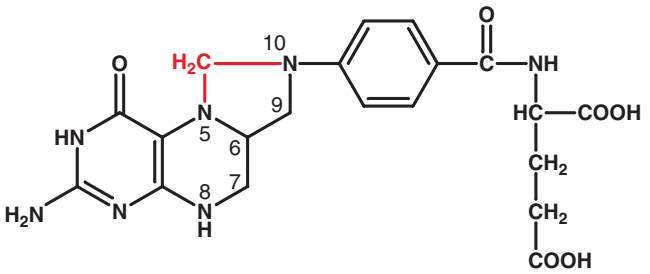
Figure 2.18. Structures of two classes of molybdopterin cofactors. In the first subclass shown in (A) the molybdenum atom is coordinated by a conserved cysteine residue, whereas in (B) it is ligated to a terminal sulfur. This figure is reproduced with permission from *Annu. Rev. Plant Biol.* (2006) 57:623. The authors thank Drs. G. Schwarts and R. Mendel for making this figure available for use.

and its formation is correlated with the disappearance of the Fe(II)–O_2 intermediate on heme and subsequent formation of the argininine hydroxylation product, N^{ω}-hydroxy-L-arginine. Thus, the role of tetrahydrobiopterin in nitric oxide synthase is in the electron relay from NADPH to heme that is required for oxygen activation.

2.4.E Folic Acid

Folic acid is a member of the B-vitamin family and represents the oxidized form of the biologically active cofactor, tetrahydrofolic acid (Fig. 2.19). The synthesis of tetrahydrofolic acid occurs in microbes and plants and involves a series of steps that bring the pterin, *p*-aminobenzoate, and glutamate moieties together in a pathway that has been heavily targeted by antibiotics including the sulfa drugs. In methanogenic bacteria, a structural variant of this cofactor, tetrahydromethanopterin, is found. The primary function of tetrahydrofolate in nature is to serve as a carrier of one-carbon units at various oxidation states, which are donated in biosynthetic reactions for generation of amino acids, purines, and pyrimidines. The one-carbon unit is carried either at the N^5 (methyl, formimino, formyl), N^{10} (formyl), or between the N^5 and N^{10} (methylene, methenyl) positions. In the majority of these one-carbon transfer reactions, the tetrahydrofolate carrier does not undergo a redox change. The exception is the thymidylate synthase-catalyzed reaction, which converts dUMP to dTMP. In this reaction, a one-carbon unit at the oxidation state of a methylene group is transferred from 5,10-methylene tetrahydrofolate to C5 of dUMP followed by a hydride transfer from the C6 position of the cofactor to the exocyclic methylene of the intermediate (Fig. 2.17). This leaves the coenzyme in the oxidized dihydrofolic acid state, which is not useful as a one-carbon carrier and needs to be reduced and returned to the tetrahydrofolate cycle. This reduction is catalyzed by dihydrofolate reductase.

Given the importance of the products of the thymidylate synthase and dihydrofolate reductase-catalyzed reactions to rapidly growing cells, it is not surprising that



5,10 Methylenetetrahydrofolate

Figure 2.19. Structure of methylenetetrahydrofolate. This is the tetrahydrofolate derivative that participates in the redox reactions catalyzed by photolyase and by thymidylate synthase.

these two enzymes have been heavily targeted by pharmacological agents. Hence, 5-fluorodeoxyuridylate, which is converted intracellularly to 5-fluorodeoxyuridine monophosphate, is a mechanism-based inhibitor of thymidylate synthase and is used clinically as an antitumor agent. Methotrexate, trimethoprim, and pyrimethamine are inhibitors of dihydrofolate reductase and are effective as anticancer, antibacterial, and antimalarial agents.

Tetrahydrofolate serves a novel photoantenna function in conjunction with a subclass of photolyases that repair pyrimidine dimers generated by UV light-induced damage to DNA. As shown in Fig. 2.13, photolyases contain an essential flavin, FAD, which catalyzes the photoreactivation reaction. Methylenetetrahydrofolate (Fig. 2.19) or, in some organisms, 8-hydroxy-7,8-didemethyl-5-deazariboflavin functions to increase the efficiency of the reaction, particularly under conditions of limiting light. Both photoantenna cofactors have higher extinction coefficients and absorption maxima at longer wavelengths relative to that of the two-electron reduced FAD, which is the active form of the flavin photocatalyst, making them more efficient for capturing photons. Thus, the role of methylenetetrahydrofolate in DNA photolyase is to absorb blue light photons and to transfer the excitation energy to the flavin acceptor for use in pyrimidine dimer repair.

SELECTED REFERENCES

1. Massey, V. (2000). The chemical and biological versatility of riboflavin. *Biochem. Soc. Trans.* 28:283–296.
2. Fischer, M., and Bacher, A. (2005). Biosynthesis of flavocoenzymes. *Nat. Prod. Rep.* 22:324–350.
3. Mattevi, A. (2006). To be or not to be an oxidase: challenging the oxygen reactivity of flavoenzymes. *Trends Biochem. Sci.* 31:276–283.
4. Modjtahedi, N., Giordanetto, F., Madeo, F., and Kroemer, G. (2006). Apoptosis-inducing factor: vital and lethal. *Trends Cell Biol.* 16:264–272.
5. Berger, F., Ramirez-Hernandez, M.H., and Ziegler, M. (2004). The new life of a centenarian: signalling functions of NAD(P). *Trends Biochem. Sci.* 29:111–118.
6. Dubois, J.L., and Klinman, J.P. (2005). Mechanism of post-translational quinone formation in copper amine oxidases and its relationship to the catalytic turnover. *Arch Biochem. Biophys.* 433:255–265.
7. Sancar, A. (2003). Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem. Rev.* 103:2203–2237.
8. Wei, C.-C., Crane, B.R., and Stuehr, D.J. (2003). Tetrahydrobiopterin radical enzymology. *Chem. Rev.* 103:2365–2383.
9. Schwartz, G., and Mendel, R.R. (2006). Molybdenum cofactor biosynthesis and molybdenum enzymes. *Annu. Rev. Plant Biol*. 57:623–647.
10. Mure, M. (2004). Tyrosine-derived quinone cofactors. *Acc. Chem. Res.* 37:131–139.