

Chapter 3

Coping with Oxygen

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3.1 The Impact of Oxygenation on an Anaerobic World

Sometime before 2.7 BYA, a new and biologically toxic substance began to appear in the environment. Biologically produced dioxygen, O₂, probably first began to accumulate in small pools or layers above cyanobacterial mats. These photosynthesizers must have already developed ways to at least partially deal with dioxygen and, with greater difficulty, the reactive oxygen species (ROS) derived from it (see Chap. 1 and below). But for primitive anaerobes in the vicinity, these new substances must have been especially toxic. Nevertheless, it is clear that they evolved ways to cope with the new threats. One way was to simply avoid dioxygen altogether. There is evidence for a long period in which the deep ocean remained anoxic even after dioxygen began to accumulate in surface layers and in the atmosphere (Kasting 1991). Thus, anaerobes were able to hide and “buy time” to evolve defenses. Some still use such refuges today, in deep oceans and lakes. The existence in these early ages of various chemical sinks (such as iron Fe²⁺) for

oxygen meant that the overall concentration in the oceans did not rise to significant levels for hundreds of millions of years (Fig. 2.6). In that period, organisms developed many and varied ways to cope with ROS. These are the major topics of this chapter.

3.2 Production of Reactive Oxygen Species

In order to understand how organisms learned to fight the toxicity of dioxygen one has to know how ROS are created and what makes them so dangerous. Their basic chemistry has been discussed in Chap. 1, so we shall emphasize here the biological aspects. How does dioxygen give rise to ROS *in vivo*?

To reiterate a point from Chap. 1: dioxygen is paramagnetic with two unpaired electrons with parallel spin. This constitutes a barrier to many chemical reactions. However, O_2 can be reduced in the presence of paramagnetic transition metals such as iron and copper in a series of steps. The stepwise reduction of O_2 to H_2O produces several reactive species including hydrogen peroxide (H_2O_2), the superoxide anion radical $O_2^{\cdot-}$, and the hydroxyl radical $OH^{\cdot}$ (Fig. 3.1). Hydroxyl radicals are the most dangerous, for they react with almost every biomolecule. Results from reactions of $OH^{\cdot}$ include: amino acid modifications such as

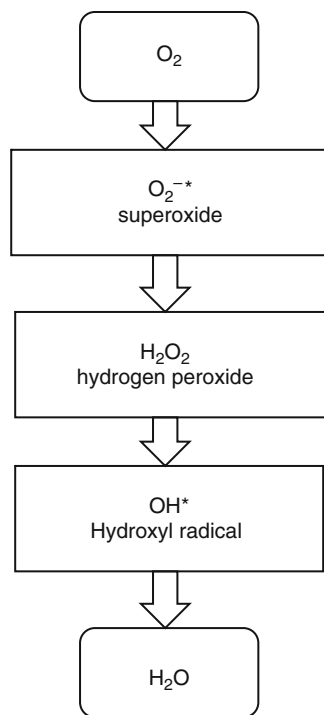


Fig. 3.1 ROS production accompanying the stepwise reduction of dioxygen to water. Note that an electron is added at each step

meta-tyrosine and ortho-tyrosine from phenylalanine, initiation of lipid peroxidation, and oxidation of nucleic acids. In the earliest ages of O_2 production, ROS were probably produced abiotically in the sea by a variety of adventitious metal-catalysed reactions. We have noted in Chap. 2 that the anoxic primeval ocean must have been rich in ferrous iron, an excellent catalyst. There are a number of metals in addition to iron, including copper, chromium, vanadium and cobalt which are capable of redox cycling in which a single electron may be accepted or donated by the metal. This action catalyzes reactions that produce reactive radicals. Two important reactions should be remembered from Chap. 1: the Fenton's reaction and the Haber–Weiss reaction. In these reactions hydroxyl radicals are produced from hydrogen peroxide in the presence of ferrous iron. All enzymes that either produce ROS or deal with them contain one of these metals. On the other hand proteins have been evolved which complex these metals, so as to control the charge transfer. Without these protecting enzymes, organisms will encounter the problem known as “oxidative stress” (see Chap. 8), which is an imbalance between oxidants and antioxidants in favor of the oxidants. This may potentially lead to the damage of biomolecules and therefore be dangerous for life.

In higher organisms, however, most of the free radicals are not obtained from the environment, but are created in *mitochondria*; the power plants of cells where dioxygen is reduced to produce energy (see Chap. 4). Within mitochondria, there seems to be a competition between the useful metabolic process of transporting the electrons along the respiratory chain or losing them to the immediate environment, generating reactive compounds (Hamanaka and Chandel 2010; Scherz-Shouval and Elazar 2010). Up to 2% of the electrons going through the respiratory chain end up forming superoxides or other radicals. A critical point here is the lifetime of radicals that are a part of the electron transport chain (see Table 1.1). Those that are slow in transferring an electron to the next step in the chain stand a significant chance of transferring it to form an ROS instead.

Another site of radical production is in *peroxisomes* which are organelles specialized in the oxidation of fatty acids: During β -lipid degradation, electrons are transferred from a cofactor $FADH_2$ to molecular oxygen. The superoxide anions created react to form hydrogen peroxide via enzymes like superoxide dismutase (SOD) (Fig. 3.1). Paradoxically, higher organisms seem to be most susceptible to ROS. This is certainly true for humans (see Chap. 8). For example, hydroxyl radicals have a major role in arteriosclerosis by destroying lipoproteins and the endothelial tissue (van Kuijk and Dratz 1987). But hydroxyl radicals can also cause damage due to lipid peroxidation (Fig. 3.2) of membranes (Stadtman 1992). Interestingly, only unsaturated fatty acids undergo lipid peroxidation, while saturated fats do not. On the other hand, it should also be noted that dioxygen is needed to synthesize unsaturated fatty acids – commonly known as the “good” fatty acids. Why is it so important to have unsaturated fatty acids? Most likely they establish the particular properties of membranes – the fluidity and permeability for several substrates in contrast to the stiff membranes made of saturated fatty acids.

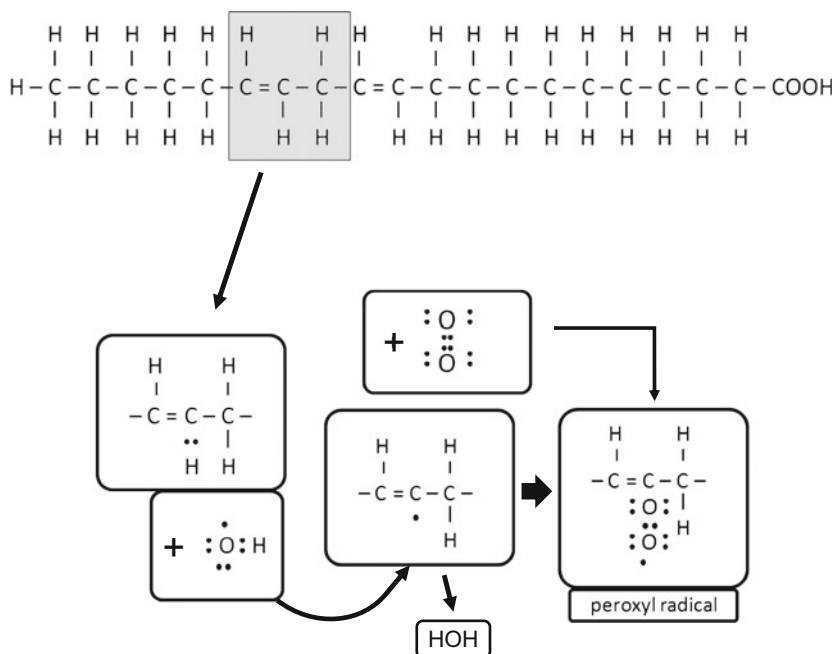


Fig. 3.2 An example for lipid peroxidation: oxygen radicals damage cellular membranes. Unsaturated fatty acids are common targets

Not only macromolecules but also cofactors such as the important metabolic reductant NADPH are vulnerable to ROS. For example, singlet oxygen reacts very rapidly with NADPH resulting in hydrogen peroxide and NADP^+ (Bodaness and Chan 1977).

We have introduced a few very important reactions whereby ROS are created and in which way they attack biological targets. There are several other pathways for producing ROS in smaller amounts: via xanthine oxidase, NADPH oxidases, cytochromes P450, and monooxygenase oxidases.

The creation of ROS is not limited to animals but also occurs in plants. During the transfer of high energy electrons within the photosynthetic process, some electrons can escape the chain to produce ROS (Krieger-Liszkay 2005). Here, a class of antioxidants found only in plants, a special class of carotenoids, was evolved to provide protection.

Despite their deleterious nature, ROS in physiological concentrations have become important for life. Through evolution, organisms have learned how to use them for intra- and intercellular signal transmission and regulation of cellular redox states. The remarkable fact is that during evolution higher organisms became dependent on ROS. ROS started to play important roles in cell signaling. Thus, to maintain proper cellular homeostasis, a balance must be held between ROS production and consumption (see Chap. 8). We shall also discuss these aspects later in this chapter.

The problems which anaerobes and aerobes have with ROS are fundamentally different. It seems that early anaerobic life on Earth had to cope with ROS created in the environment; either by inorganic photolysis or emerging photosynthesizers, whereas aerobes have had to deal primarily with the self-generated ROS. Utilizing dioxygen may provide a lot of energy, as we shall see in Chap. 4, but it also carries a considerable danger. Different strategies were evolved in the form of enzymes and chemical compounds to reduce this danger.

3.3 Coping with Reactive Oxygen Species

Most of the life on Earth with which we are familiar evolved in the presence of dioxygen, and had to adapt to this potentially dangerous substance. This was accomplished by evolving a large battery of antioxidant systems. Some of these systems are present in all life forms, from bacteria to mammals, indicating the appearance of at least traces of dioxygen early in the history of life. Indeed, photosynthetically generated oxygen may have appeared in local sites long before the first noticeable rise in atmospheric dioxygen about 2.5 BYA (Chap. 2). Even earlier, traces of dioxygen generated by inorganic photolysis of water must have been present. Here we will concentrate on a few antioxidation systems and their functions. There are two kinds of antioxidants – small molecules that scavenge oxygen by reacting with ROS and enzyme systems that detoxify them. There are also systems that combine small molecules and specific enzymes.

3.3.1 *Scavenger Molecules*

There exists a class of readily oxidized molecules that can scavenge ROS before they cause damage to the various biological molecules, or prevent oxidative damage from spreading, e.g. by interrupting the radical chain reaction of lipid peroxidation (Fig. 3.2). There are two major groups of such antioxidants. One type is soluble in water, and therefore found in cytoplasm and blood. The other group occurs in lipids and helps protect cellular membranes. An excellent review with over 150 references is provided by Wikipedia (<http://en.wikipedia.org/wiki/Antioxidant>).

Ascorbic acid, better known as vitamin C (Fig. 3.3), is a monosaccharide occurring in almost all organisms. A deficiency causes a serious disease called scurvy. Unfortunately we humans have to obtain vitamin C from food since we cannot synthesize it. Vitamin C is the major water-soluble antioxidant. It is very effective in scavenging a wide array of ROS and free radicals. It seems to protect low density lipoproteins (LDL) against oxidation even more effectively than vitamin E (see Fig. 3.3 and below). In fact, it recycles vitamin E radicals and vice versa (Fig. 3.3). In the cell there is also an interplay with glutathione (GSH) (see

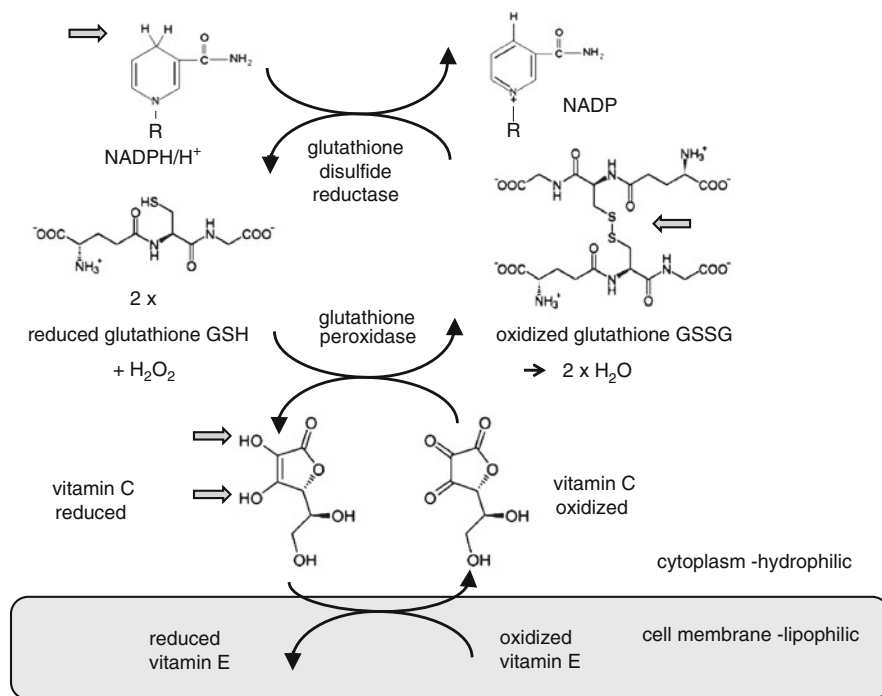


Fig. 3.3 Interplay of vitamin E, vitamin C, glutathione and NADP. The *arrows* point to the important sites of reactions. Note that H₂O₂ (or organic peroxides) can oxidize glutathione resulting in the glutathione redox cycle

below), which reduces ascorbic acid (Ramsey and Sharpless 2006; Wells et al. 1990). Ascorbic acid is also important in plants and occurs in high concentrations. It is a substrate for the antioxidant enzyme ascorbate peroxidase, thereby being important for oxidative stress resistance (Shigeoka et al. 2002). Vitamin C became very famous through the work of Linus Pauling, who suggested consuming several grams per day to delay aging. In which way and to what degree vitamin C is really involved in such protection remains under discussion.

Alpha-Tocopherol or *vitamin E* is the most prominent among several isoforms of tocopherol. Although it occurs in most organisms, in humans it is by far the most abundant lipid-soluble antioxidant, being present in most membranes. It protects cell membranes against oxidation by reacting with lipid radicals in the lipid peroxidation reaction (Herrera and Barbas 2001). Vitamin E taken each day is thought to substantially lower the risk of heart disease. It seems that vitamin E protects LDL against oxidation, which is a critical step in the development of atherosclerosis. The oxidized form of vitamin E can be recycled to the reduced form by oxidation of ascorbate in the cytoplasm (Fig. 3.3) or retinol or ubiquinol in the membrane (Wang and Quinn 1999). It is still uncertain as to which other protective functions vitamin E may be involved in, such as neuroprotection (Sen et al. 2006).

Beta-carotenoids are typically found in the plant membranes, where they serve for the protection of the photosynthetic apparatus against ROS created by the high energy electrons activated by the captured light. They include lipid-soluble beta-carotene (a vitamin A precursor) and related substances called carotenoids, and alpha-carotene or lycopene (the red color in tomatoes), lutein, and zeaxanthine. It seems that carotenoids are much weaker antioxidants than vitamin E. Thus, whether they are also effective in humans is still a matter of controversy.

Resveratrol. Natural antioxidants are very popular in contemporary society. Among these is a polyphenol named resveratrol. Its presence, especially in red wine, has led to a recommendation to justify drinking wine. Whether, in fact, resveratrol is actually acting as an antioxidant in the human body is a matter of discussion (Sardi 2004). Plants evolved a very effective system using resveratrol to counter oxidative damage especially in parts which are exposed to air and sun light. It appears also to serve as a protection against infections by fungi or parasites. Resveratrol is especially found in high concentrations in the skin of berries. As a consequence, it occurs in white grape drinks up to 200 µg/l and in red grape drinks up to about 1,000 µg/l: However, much higher concentrations are found in wine, being concentrated to up to 50 mg/l during the wine making process. How much resveratrol has to be taken to produce observed effects? It was shown that it causes a longer life in animal experiments, but extrapolating the data to human suggest that one would have to drink more than 30 glasses of red wine per day to have the same concentration as in the study. Obviously this would not be good for the liver. In addition, the action of resveratrol in humans is not really understood despite current intense investigation.

Many other antioxidants could be mentioned, but the principal is clear; each reacts with either dioxygen or ROS. Another way to deal with such threats is via dedicated enzyme systems. Enzymes have the advantage over scavenger molecules that they are not consumed as they deactivate ROS. One enzyme molecule can be used as a catalyst over and over, but a scavenger such as ascorbic acid is either lost or must be regenerated after reaction.

3.3.2 *Enzymes for Detoxification of ROS*

There are a number of enzymes distributed widely over the whole range of organisms that appear to exist mainly to detoxify ROS. Here, we shall use as an example superoxide dismutase (SOD). A wider view can be found in the book by Lane (2002). SODs are found in all aerobic cells and some extracellular fluids (Fridovich 1995, 1997). They catalyze the conversion of two molecules of superoxide into hydrogen peroxide and dioxygen (see Chap. 1). The benefits here are that H_2O_2 can in turn be broken down into water and dioxygen by another efficient enzyme (catalase) and even if not, hydrogen peroxide will not cause immediately damage since it is substantially less toxic than superoxide. SOD accelerates the detoxifying reaction roughly 10,000-fold over the non-catalyzed reaction. It was recently

Table 3.1 Superoxide dismutases

Type	Organisms	Localization
FeSOD	Prokaryote	Cytosol
	Few plants	Mitochondria
MnSOD	Prokaryote	Cytosol
	Eukaryote	Mitochondria
NiSOD	Prokaryote	Streptomyces
Cu,ZnSOD	Eukaryotes (some bacteria)	Cytosol
Cu,ZnSOD	Eukaryotes	Extracellular

There are three groups of enzymes that exhibit significant sequence/structure similarities, and are presumed to have a common origin

discovered that the gene for SOD is defective in patients with amyotrophic lateral sclerosis (ALS), better known as Lou Gehrig's disease. The patients suffer muscle atrophy and paralysis.

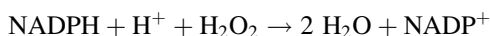
As shown in Table 3.1, there are several kinds of SODs. All are metal-containing enzymes with bound iron, manganese, copper or zinc donating the essential anti-oxidant activity. SODs must have been evolved early since they are present in all aerobic organisms (Fridovich, 1981). SODs are inducible enzymes - exposure of bacteria or vertebrate cells to higher concentrations of dioxygen results in rapid increases in the concentration of SOD (see Chap. 8). The different forms of SOD fall into several seemingly independent classes. The Fe-SOD and Mn-SOD are found mainly in prokaryotes or in eukaryotic mitochondria. They are structurally quite similar. The Cu/Zn-SODs are primarily eukaryotic proteins, one class of which is cytosolic, the other is extracellular. They appear to be unrelated to the Fe- or Mn-SOD's classes. Mn-SOD occurring in mitochondria of eukaryotic cells presumably has been transferred via endosymbiosis from an aerobic bacterium. The Cu/Zn-SOD has been detected in the cytoplasm of eukaryotic cells and some gram-negative bacteria (Fridovich 1997). It has to be distinguished from the Cu/Zn-SOD occurring in the extracellular fluids. It seems that most aerobic bacteria do not have this SOD, perhaps because Fe-SOD and Mn-SOD were adequate for the same purpose. To further complicate the matter, nickel-containing SOD has been found in prokaryotes such as the bacterium *Streptomyces*. Both, Fe-SOD and Mn-SOD (as well as Ni-SOD), are thought to be evolved before aerobic photosynthesis was present. Although this collection sounds confusing, its occurrence can be understood by evolution. First of all, it implies that some very low levels of oxidants were present even in the primitive environment, perhaps from UV photolysis of water. Recall from Chap. 2 that iron and manganese were prevalent in the ancient anaerobic ocean, while copper and zinc became abundant only after the great oxidation event. This may explain why FeSOD and MnSOD are found in the more ancient prokaryotes and mitochondria that derived from them, while Cu/Zn-SOD occurs in higher bacteria and in the younger cytosols of eukaryotes.

Another prominent enzyme, *catalase*, is needed to finish the work of SOD. It is found in the peroxisomes of eukaryotic cells when it degrades hydrogen peroxide to water and dioxygen. Its importance can be deduced from a special property

evolved: catalase belongs to that class of enzymes with the most efficient catalytic activity. It should be noted that strictly anaerobic methane bacteria – still present on Earth – evolved catalase to survive oxidative conditions.

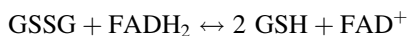
3.3.3 Antioxidant Enzyme Systems

As we already have seen, some antioxidants do not work alone. Often cascades or cycles are involved consisting of several partners such as an enzyme, cofactor and vitamin. The most prominent of such is the GSH system (Fig. 3.3), which consists of several steps but in summary catalyses the following reaction which essentially detoxifies H_2O_2 :

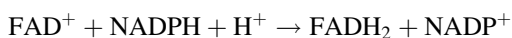


This system consist of GSH, glutathione-disulfide-reductase and glutathione peroxidase (Hashida et al. 2000).

Glutathione (GSH) (Fig. 3.3) is present in almost all cells and may well be the most important intracellular defense molecule against damage by ROS. It occurs in all known aerobic organisms. It is a peptide of three amino acids: glutamyl-cysteinyl-glycine. The cysteine provides an exposed free thiol group (SH) which can transfer two electrons to ROS neutralizing them. Thus, this very reactive thiol group provides an excellent target for radical attack. Reaction with radicals oxidizes two GSH molecules to form a disulfide-linked dimer, denoted as GSSG (see Fig. 3.3). But the reduced forms GSH are then regenerated from GSSG in a redox cycle involving glutathione-disulfide-reductase (named GSSG-reductase; Forni and Willson 1986) consuming NADPH as electron donors. For the regeneration another cofactor is needed: FAD/FADH₂, a part of the glutathione-disulfide-reductase.



FADH₂ will be regenerated from FAD⁺ by NADPH/H⁺



It seems likely that GSH plays a major role in maintaining the cell's redox state, since high concentrations (about 5 μM) is found in cells due to the presence of active glutathione-disulfide-reductase (Fig. 3.3). The ratio of GSH/GSSG in cells is higher than 10:1 which seems to be enough to neutralize the dangers from ROS.

A confirmation for the importance of the glutathion system was recently shown. Programmed cell death (apoptosis) was induced when GSH was withdrawn from cells (Seiler et al. 2008).

Apoptosis can be induced by lipid peroxides which activate the apoptosis activating factor (AIF). It seems that with increasing oxidative stress more and

more GSH and glutathione peroxidases are used to fight various oxidation processes and therefore are now not available for reducing lipid peroxidases. Thus, suicide of a cell seems to be more advantageous than allowing a conversion to a tumor cell. Interestingly, additional vitamin E seems to inhibit the apoptosis.

Glutathione peroxidases as a part of the GSH system possess a selenocysteine at the active site in which selenium replaces the usual sulphur. Like catalase these enzymes catalyse the degradation of hydrogen peroxide; they also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants (Fig. 3.3).

Thioredoxin, a small oxidoreductase ($M_r = 12$ kDa) exists in many isoforms, and is found in all known organisms. The key to its function lies in a conserved motif of a short peptide with four amino acids, cysteine–glycine–proline–cysteine. It can cycle between a reduced state with two –SH groups provided by the cysteines which can again neutralize ROS by transferring electrons to yield an oxidized form carrying a disulfide bridge. The reduced form is then recovered after reduction by an enzyme *thioredoxin reductase* using NADPH as an electron donor.

Aging is thought to be connected with the accumulation of oxidized proteins (Stadtman 1992). The major candidates for oxidation within proteins are the sulphur containing amino acids, cysteine and methionine (see Chap. 8). In eukaryotic cells proteasomes are mostly responsible for degradation of the oxidized proteins in the cytosol and nucleus. In addition, specific enzyme systems (the glutaredoxin/glutathione/reductase system and the thioredoxin/reductase system) evolved to reverse the oxidation that has formed disulphide bridges. Also important are methionine sulfoxide reductases which are able to reduce methionine sulfoxide back to methionine within the proteins. This may fail when the concentration of hydrogen peroxide becomes so high that even methionine sulfoxide reductase as well as catalase will also be oxidized. A consequence of this, graying hairs of elderly people, will be discussed in Chap. 8.

When were these defense mechanisms evolved? Because some of these mechanisms are found in all organisms, these must have arisen before the potential for aerobic respiration (see Chap. 4) had appeared. But the early existence of aerobic photosynthesis together with the evolution of the key enzymes like cytochrome oxidase when there was not significant O_2 in the environment suggests an alternative evolution (Castresana and Saraste 1995). It is not generally accepted, however, that aerobic metabolism itself evolved at such an early date. These early defenses against adventitious dioxygen could then have been adapted by the first creatures living in truly aerobic environments.

3.4 How to Avoid Reactive Oxygen Species?

The best strategy to avoid ROS is to avoid their production. This has meant different things at different periods in evolution. At the earliest times, before dioxygen was a significant component in the environment, the major source would have been photolysis of water by high-energy UV radiation. An easy way for organisms to

avoid high energy radiation is not to live at the surface of the ocean but in a depth of at least several meters. On land the best strategy is to find shadowed places or/and to use the day and night cycle. This problem disappeared by the time (see Fig. 2.6) the ozone layers formed. But by then, there was of course, significant photosynthetically produced dioxygen, in the oceans and atmosphere.

Alternatively membrane-bound enzymes like cytochrome oxidase, coupled with an NADH, might have reduced O_2 right at the cell surface. Cytochrome oxidase and cytochrom c appear to be ancient, but how old we do not know. During the earliest periods of rising dioxygen (say 2.9–2.5 BYA) avoidance of dioxygen by anaerobes would probably have been easy. Dioxygen was being generated by photosynthesizers only in restricted environments – mainly shallow ocean fringes and perhaps terrestrial ponds. Vast regions on Earth, especially in deep oceans must have remained anoxic, if for no other reason than that oxygen diffusing into them from the above-mentioned sources would have been instantly reduced by the abundant reducing agents present (Fe^{2+} , H_2S , etc).

Even today, there exist extensive environments such as some deep ocean region that are essentially anoxic and harbor mainly anaerobic microbes. Such must have persisted throughout geological history. It seems likely that ancient anaerobes developed oxygen-sensing chemotaxis to avoid oxygen-rich regions (see below). Later, facultative aerobes may have (and still do) used such sensors to seek oxygen (see Sect. 3.6).

3.5 Evolving Defense Strategies

As worldwide O_2 levels inexorably rose, stronger defences were required. One, of course, was the evolution of antioxidants and enzyme systems to destroy ROS, as described above. It is very difficult to date antioxidants, but enzymes such as Fe-SOD and Mn-SOD appear to be very ancient. However, it is becoming clear that a wide variety of alternative defence strategies have evolved over the eons.

3.5.1 Aggregation for Defense

Experiments have suggested another important way of avoiding ROS. Ma and Eaton (1992) were puzzled by the fact that the high diffusivity of H_2O_2 should mean that bacteria like *Escherichia coli* should not be able to protect themselves against H_2O_2 , even though they contained high concentrations of catalase. They discovered that dilute suspensions of *E. coli* were, in fact vulnerable. However, in concentrated cultures where bacteria clumped together, there was surprising resistance. Apparently, the “surface” members in the aggregate of cells can protect the “interior” members from ROS. This raises the remarkable suggestion that protection against ROS might have been one of the evolutionary driving forces towards

the development of multi-cellular eukaryotes! If so, oxygen has played an even larger part in our evolution than hitherto suspected.

3.5.2 Melanin

Another way to avoid the problem of ROS is to metabolize dioxygen before it can form radicals. On Earth, phenols were already available in great amounts. Using dioxygen, monophenols could easily be hydroxylated to diphenols such as DOPA or dopamine, by enzymes such as phenoloxidase (tyrosinase, catecholoxidases, laccase, and other oxidases) (Fig. 3.4). Dioxygen is split, with one oxygen atom being attached to the benzoic ring at various positions. Tyrosinase for example attaches an oxygen atom in the intimate neighborhood (*o*-position) of the first bound oxygen (Matoba et al. 2006; Decker et al. 2006). The other oxygen atom is incorporated into water. After oxidation the result will be a reactive quinone, a benzol ring with two aggressive keto groups. Because of their severe reactivity with, for example pathogenic invaders, they are considered as a part of the innate immune system (Bogdan 2007; Jiang et al. 2007). On the other hand, quinones can spontaneously polymerize to form polymeric nets called melanin. The color of melanin depends on the incorporation of a cysteine derivate which results in reddish pheomelanin, alternatively dark eumelanin is formed, when it is not incorporated (Fig. 3.4).

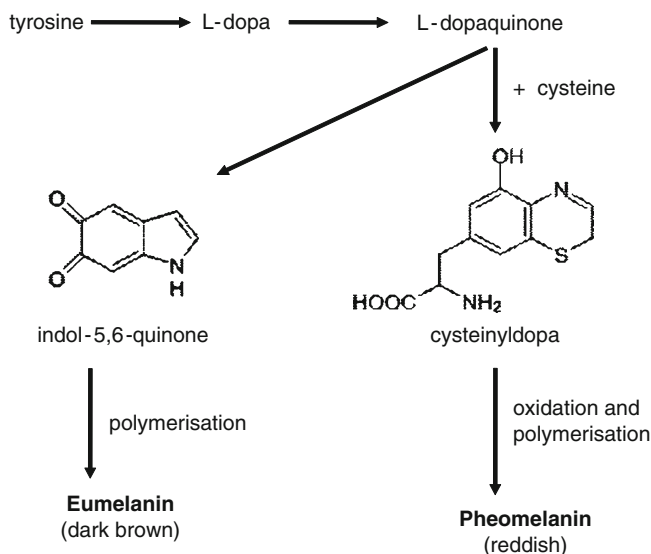


Fig. 3.4 Scheme of melanin synthesis. The conversion of tyrosine to L-dopaquinone is catalysed by the enzyme tyrosinase. Eumelanin colors the hair brown to black, while incorporation of cysteine results in a more reddish pheomelanin

Melanin is also needed for the sclerotization of arthropods, forming a resistible cuticle after molting. In wound healing in arthropods melanin would close open breaks in the exoskeleton. Should bacteria or other pathogens invade such organisms melanin will encapsulate them as a part of the primary immune response (Claus and Decker 2006). These phenomena are also found in fungi, plants and fruits. When an apple is attacked by a worm, melanin nets are formed to guide the worm out of the apple, bypassing the core. Thus, the apple seeds are protected.

Melanin has been conserved during evolution. In humans, melanin occurs in the retina of the eye, colors the hair and protects the skin from energy rich UV light (Oetting et al. 2003; Plonka et al. 2009; Wood et al. 2009). In the retina, melanin protects this area containing very high oxygen concentration from the potential formation of oxygen radicals. Mutation of the conserved environment of the active site would eliminate the function of tyrosinase to form melanin resulting in albinism (Schweikardt et al. 2007). Once formed, melanin can still neutralize ROS as a strong antioxidant due to its large amounts of double bonds. Melanin is an example of a self-stabilizing co-evolution between different strategies. Here metabolizing oxygen may either result in the synthesis of antioxidants or in shields against radiation.

Another enzyme belonging to this protein family is laccase, which in plants facilitates the binding of oxygen to the other free position of monophenols (Claus 2008, 2009). The result will also be a polymer net such as lignin, a major constituent of woody tissues. It appears that due to resulting advantages, phenol oxidation appeared early in evolution and spread to every kingdom. In all cases metal ions had to have been involved, in most cases these were iron or copper.

3.5.3 Oxygen Transport Proteins Prevent Creation of Oxygen Radicals

A more physiological strategy in animals is to keep the dioxygen concentration low in and around cells. Normally very small organisms take up dioxygen by diffusion. In animals with dimensions above about 1 cm, a circulation system with oxygen transport proteins (OTP) is needed (see Chap. 5). The functions of OTP have become optimized to deliver dioxygen, but not provide too much. In other words, only as much dioxygen will be released from OTP to the blood to diffuse into the cell as the cell needs.

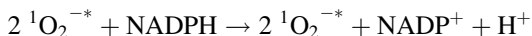
It is clear that the OTPs themselves are fashioned so as to allow the transport of considerable amounts of dioxygen without the creation of ROS. The most common OTP's are hemoglobins and hemocyanins (see Chap. 5). How do they prevent dioxygen from forming radicals? Hemoglobins carry heme groups within a protein matrix which is almost open to the surrounding. In its center there is an iron atom which binds onto one side the dioxygen molecule in a well protected cavity. A very short channel protects the iron and its bound dioxygen against the influence of the environment. Thus, even though erythrocytes carry much dioxygen, it is not free to react and form ROS. In the case of hemocyanin, the active site which involves a pair

of copper atoms is protected by the protein matrix as well as via a special folding motif of the protein (see Chap. 5).

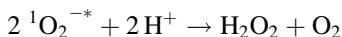
Despite the protective function of the OTP, oxidation at the binding site sometimes occurs, forming the so-called “met” form of the protein. In the red blood cells of animals, which maintain a high concentration of dioxygen, a very active reduction system (met-hemoglobin reductase) is present to keep the concentration of met-form low.

3.6 Reactive Oxygen Species as Cellular Signals

So far, we have presented ROS as substances which, over life’s history, had to be protected against. But evolution has ways of even turning unfavorable aspects to advantage. A number of ROS have come to play a signaling role in various biological systems in both plants and animals (Hancock et al. 2001; Finkel and Holbrook 2000). For example, ROS are involved in inducing programmed cell death (apoptosis) or necrosis. They also play roles in regulating the expression of genes and activation of cell signaling cascades. We shall center on H_2O_2 as an example of a widely used signal. In cells where it is needed, it is synthesized via enzymatic oxidation of NADPH in two steps



The crucial enzyme here is NADPH oxidase. As we have learned previously superoxide can be reduced to hydrogen peroxide by SOD in the second step:



H_2O_2 has two advantages as a signal molecule. First, it is relatively stable (Table 1.1). Second, it is readily diffusible in cells and tissues and through membranes as well. Thus, it can act as a messenger to modulate both intracellular and intercellular processes. In many cases, this is through the regulation of gene transcription. Gene regulations proceed through the action of transcription factors, proteins which interact with the nuclear DNA. In some still unknown way, H_2O_2 appear to activate certain transcription factors, or favor their transport into the nucleus. More detail on the physiological significance of ROS signaling will be presented in Chap. 8.

3.7 Dioxygen as a Signal: Oxygen Sensor

The varying responses and needs of organisms for dioxygen have led to the evolution of molecules that act as dioxygen sensors. For strict anaerobes, including Archean organisms, a sensor for avoidance of dioxygen (a chemiotactical sensor) or for generation of antioxidants is most appropriate. For facultative aerobes or

aerobes, sensors can act positively by helping the organism to seek dioxygen. This may have been an important function in the early years of aerobic metabolism, when dioxygen was scarce and unevenly distributed. Those aerobes that could sense regions of high O₂ concentrations would have a metabolic advantage. There are even some sensors that act to concentrate microbes in regions of optimum dioxygen concentrations - neither too low nor too high (Szurmant and Ordal 2004).

Most dioxygen sensors are membrane proteins comprised of two portions – a dioxygen-binding domain which is often (but not always) of the hemoglobin type, coupled to an effector domain, often an enzyme such as a kinase which can start a phosphorylation cascade. It has even been suggested that all present-day hemoglobins evolved from such molecules (Vinogradov et al. 2007; see Chap. 5). Binding of dioxygen to the heme in the first domain causes a conformational switch; this is transmitted to the enzymatic domain, activating or inactivating it, frequently initiating a cascade of signals that modify cell motility.

Another way was evolved by the bacterium *E. coli*. Under O₂-limiting conditions, a fumarate and nitrate reduction transcriptional regulator, FNR, contains a [4Fe-4S]²⁺ cluster, generating a dimeric protein controlling the activation of the appropriate genes. Exposure to O₂ results in conversion of the cluster into a [2Fe-2S]²⁺ form leading to dissociation of the active dimer into inactive monomers. Thus, bacteria evolved the ability to sense environmental O₂ for protection and choosing the appropriate respiration, anaerobe or aerobe (Unden et al. 1995; Crack et al. 2008; Green et al. 2009).

Oxygen sensing in higher organism often serves very different functions. Frequently, the necessity is to maintain optimal intracellular oxygen levels. For example, low levels of blood oxygen (hypoxia) in humans trigger a complex response in which specialized kidney cells are stimulated to produce erythropoietin. This travels to bone marrow cells and stimulates the release of more cells carrying hemoglobin, countering the hypoxia (see, for example Semenza 2008; see Chap. 8).

3.8 Summary: Reactive Oxygen Species and Life

Summarizing the major ideas in this chapter, we find that important for proper understanding of the role of oxygen in life is the fact that ROS can be both dangerous and useful substances. Nature seems not to have completely rejected these potentially toxic molecules since dioxygen has become abundant on Earth. Rather, ways were evolved to control them and to even make them essential for life. The consequence today is that ROS can play various, even opposing roles during different cellular processes. However, the benefits and the harms potential in ROS molecules dictate a careful balance between their production and controlling them by antioxidants and specific enzymes to maintain almost homeostatic conditions. Otherwise oxidative stress would result in a severe threat to the cells of even multicellular organisms (see Chap. 8).

The problem of dealing with ROS is fundamentally different in anaerobes and aerobes. In the former, avoidance or detoxification is the major strategy. Aerobes, however, have a built-in source of ROS – the oxidative metabolism to be discussed in the following chapter. To cope, they must regulate ROS. For a long time ROS were considered only to be highly toxic by-products of that metabolism that are not useful to the organism (see above). Scandalios (2002) has summarized the discussion during recent years between those who consider ROS to be negative “by-product” molecules and those who favor the idea that ROS became useful molecules for higher organisms. Furthermore, as we shall see in the next chapter the advantage of aerobic metabolism is so great that it overrides the disadvantage of ROS production. This means that during evolution the overall benefits of ROS creation outweighed the negative aspects.

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