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REVIEW

Oxidative Stress and Mechanisms of Protection Against It in Bacteria

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Abstract—In the review contemporary data on the effects of oxidative stresses of various kinds in bacteria are summarized. A general theory of oxidative stress, peculiarities of oxidative stress in eukaryotes and prokaryotes, and natural and induced oxidative stresses are described. Data on the mechanisms of protection against oxidative stress are given, including prevention of the generation of oxidative stress, prevention of propagation of free radical chain reactions, and the mechanisms of repair of damaged DNA. The regulation of effector genes via redox-sensitive iron-containing proteins is analyzed. Special attention is given to the expression of so-called antioxidant and associated enzymes as protection mechanisms and to the space–time organization of the response of bacteria to oxidative stress.

Key words: oxidative stress, antioxidants, bacteria, regulon, reactive oxygen species, *SoxRS*, SoxR, OxyR, σ^S

GENERAL CONCEPTS OF OXIDATIVE STRESS THEORY

Oxygen is essential for most living organisms and, with the exception of a rather small group of anaerobic bacteria, they depend on its presence in the environment. First, they need it for the generation of energy in the form of ATP during the process of oxidative phosphorylation. This process is associated with the reduction of an O_2 molecule to water, and it is the main process providing energy to aerobic organisms. Whereas in eukaryotic organisms oxidative phosphorylation occurs in mitochondria, in bacteria it occurs in the plasma membrane because they do not have intracellular membrane structures. The importance of the role of oxidative phosphorylation is supported by data showing that over 90% of oxygen used by the human body is consumed by mitochondrial cytochrome oxidase [1]. This enzyme catalyzes four-electron reduction of oxygen to water according with the scheme:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O.$$

Oxygen is used by many enzymes as substrate as well. For example, in kidney there are about thirty enzymes that use oxygen for metabolizing different compounds like amines, prostaglandins, purines, amino acids, carnitine, etc. [1]. Reactive oxygen species (ROS) like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) accompany these processes. ROS are generated during many other cellular activities including oxidation of small molecules (such as flavins, catecholamines, hydroquinones) by microsomal cytochromes P-450 and b_5, microsomal flavin dependent reductases, xanthine dehydrogenases, and monoamine oxidases and by the leakage of electrons from the electron transport chain [2, 3]. In vertebrates, the rate of generation of ROS is tightly associated with the rate of oxygen consumption and is proportional to the number of mitochondria [4]. In rat liver and pigeon heart, at physiological oxygen concentrations about 1-4% of the O_2 consumed is transformed to ROS due to leakage from the electron transport chain [5-8]. ROS-induced damage occurs mainly where transition metal ions (especially iron and copper) are found [3]. However, in fact, all cellular components are attacked by ROS. Their interaction with proteins results in the modification of amino acids—oxidation of thiol groups of cysteine and methionine, the imidazole ring of histidine, and the rings of tyrosine, phenylalanine, and tryptophan, etc. [9, 10].

Reactive oxygen species also attack DNA, producing chain breaks, modification of the carbohydrate parts and

Abbreviations: ROS) reactive oxygen species; HPI, HPII) hydroxyperoxidase I and II; CoQ) coenzyme Q; Mn-SOD, Fe-SOD, Cu,Zn-SOD) superoxide dismutases containing manganese, iron, and copper plus zinc ions; GSH, GSSG) reduced and oxidized forms of glutathione.

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nitro bases, and this may lead to point mutation [3]. Polyunsaturated fatty acids are especially sensitive to ROS attack, and this initiates a chain reaction that forms lipid peroxides (lipid-OOH) and so-called lysolipids. Since ROS are very harmful for cell function, there is a rather complicated many level system for protection against their toxic effects. All systems of protection against ROS are divided into three groups: 1) prevention of ROS generation; 2) free radical chain termination and detoxication of radicals by antioxidant enzymes and quenchers; 3) repair of damaged elements.

Binding of transition metal ions by specific and unspecific proteins such as ferritin, transferrin, albumin, and others is an important protection mechanism against ROS [11]. Low molecular weight antioxidants include α-tocopherol (vitamin E), ascorbic acid (vitamin C), and uric acid. They operate mainly chain breaking, stopping their propagation [3]. Glutathione quenches hydroxyl radicals and singlet oxygen and is a substrate for some enzymes; it regenerates vitamins E and C [3, 11].

In addition, superoxide dismutase (SOD) (which metabolizes superoxide anion) and catalase and glutathione peroxidases (which catabolize hydrogen and lipid peroxides, respectively) are enzymes that participate in the protection against ROS. Antioxidant properties have been found in proteins whose main role is different than protection against ROS, for example, myoglobin and hemoglobin [12]. Secondary enzymes of antioxidant protection comprise another group that is connected with glutathione. Glutathione-S-transferases catalyze the conjugation of glutathione with nucleophilic xenobiotics or cellular components modified by ROS. This results in detoxication of ROS-modified compounds. NADPH-dependent glutathione reductase reduces oxidized glutathione, oxidizing NADPH. Finally, the latter compound is reduced by glucose-6-phosphate dehydrogenase.

It is interesting to note that some bacteria possess a rather unusual mechanism for protection against superoxide anion. Real SOD activity was not found on the cultivation of *Lactobacillus plantarum* in the presence of high concentrations of manganese (better in the presence of Mn(II)) (cited after [13]). The protective compound(s) is(are) sensitive to EDTA treatment and can be removed by dialysis. These bacteria do not grow at low concentrations of manganese in the medium, which indicates that some low molecular weight compound(s) containing manganese function(s) as superoxide dismutase.

The situation when for some reason the generation of free radicals is higher than the rate of their detoxication is called "oxidative stress" [11, 14]. Under these conditions, the free radicals that are formed can modify all kinds of cellular components. If not metabolized, modified compounds are accumulated by the cell. Under normal conditions the products of free radical attack on different cellular components, like lipids, proteins, nucleic acids, etc. are accumulated as well [3, 9, 11, 15, 16].

FEATURES OF OXIDATIVE STRESS IN PROCARYOTES AND EUKARYOTES

The structural organization of a cell is an important regulator of oxidative stress. For instance, in eukaryotic cells the sources of ROS and the systems for their detoxication are localized in specific compartments. Of course, cellular components that are located close to places of ROS generation will be most subject to damage; this is especially seen in locations where free transition metal ions are present [3]. This situation is well demonstrated in DNA damage. The process is very important because DNA modification can result in point mutations and malignant cell transformation [17]. In fibroblast cell cultures, treatment with hydrogen peroxide inducing oxidative stress first modifies mitochondrial but not nuclear DNA [18]. Moreover, although nuclear DNA is repaired during 1.5 h, mitochondrial DNA is not. Calculations suggest that mitochondrial DNA is damaged 10^4 times more frequently than nuclear DNA [14]. It should be noted that mitochondrial components are subjected to oxidative stress more intensively than nuclear elements because ROS are generated mainly by mitochondria. However, the consequences of these injuries are very different, since DNA damage to generative tissues are passed to offspring. Mitochondrial DNA is not so important in heredity.

In prokaryotes, such strict spatial organization of oxidative processes cannot be expected because of the absence of structural organization of their cells. In fact, damage to DNA occurs via the same mechanisms as in eukaryotes. Thus, superoxide anion can both directly or directly modify cellular elements or extract iron from the protein-bound state, and therefore intensify the oxidative stress effect [19].

NATURAL OXIDATIVE STRESS

Aerobic conditions lead to the generation of ROS. This raises the question of the mechanisms of the generation of ROS. As discussed above, mitochondria are the main source of ROS in eukaryotes [6, 7, 20]. Analogous research has been carried out with the bacterium *E. coli*. It turns out that the rate of generation of superoxide anion and hydrogen peroxide depends on the stage of culture development. The transition of a bacterial culture from lag-phase to exponential phase resulted in 5-10-fold increase in hydrogen peroxide generation [21]. The stationary concentration of hydrogen peroxide was about 0.1-0.2 μM and did not depend on the stage of development of the culture. The stabilization is a result of cooperation between hydrogen peroxide producing and removing systems, particularly catalase activity. However, it is possible to change the stationary concentration of hydrogen peroxide by modifying catalase activity using special techniques.

There is still no generally accepted view on the location of generation of ROS in the electron transport chain [21-23].

The calculated rate of ROS generation in exponentially growing *E. coli* cultures is close to that found in mammalian cells [21]. It followed the theoretical 2 : 1 stoichiometry for superoxide/hydrogen peroxide ($2O_2^- + 2e^- + 4H^+ \rightarrow H_2O_2 + O_2$). This means that most of the hydrogen peroxide generated in bacterial cells arises as from superoxide ion metabolism [21].

The stationary concentration of hydrogen peroxide in bacterial cells call be changed experimentally. It is increased in *E. coli* mutants with reduced catalase activity or ones that are not able to enhance catalase activity by its synthesis in response to oxidative stress [24]. In a strain deficient in HPI catalase in log-phase cell culture, the level of hydrogen peroxide was increased. It should be noted that the levels of products of free radical modification of proteins or lipids were not modified by HPI catalase activity deficiency in any stage of bacterial culture development. However, in a strain deficient in a regulatory protein of expression of HPI catalase (OxyR), the stationary hydrogen peroxide concentration was increased in all three stages of culture development. Although in this strain the level of carbonyl proteins was not different from control, in lag-phase and exponential phase the accumulation of TBA-reactive substances was noted. A strain deficient in alkylhydroxyperoxidase was not different in the level of hydrogen peroxide comparative to the wild type, but the level of TBA-reactive substances was increased in lag-phase. The frequency of mutation in *E. coli* strains deficient in HPI catalase is close to that of the wild strain. However, strains deficient in OxyR or alkylhydroxyperoxidase at all stages of cell culture development showed increased mutation frequency [24].

EXOGENOUS OXIDATIVE STRESS

The mechanisms of this kind of stress are not different from natural stresses. Externally added reagents are involved in redox processes, generating superoxide anion and products of its transformation. Being an uncharged molecule, exogenous hydrogen peroxide freely enters the cell. Much data have been accumulated on the consequences of its effects and on prevention and repair of damage caused by it [24-26]. Figure 1 shows a general scheme of stress reactions. Redox reactions generate superoxide anion, which can be reduced to hydrogen peroxide. It oxidizes OxyR protein, which activates the expression of one of the catalases (HPI catalase, hydroxyperoxidase I in *E. coli*), alkylhydroxyperoxide reductase (Ahp), glutathione reductase (GR), and about five additional proteins. An analogical chain reaction produces superoxide anion, but it regulates the expression of other group of proteins including Mn-dependent superoxide dismutase and glucose-6-phosphate dehydrogenase. It should be noted that the set of effector genes depends on the stage of culture development, and some effector proteins depend on several or least two. For example, the expression of HPI catalase in exponential phase is regulated by OxyR and in stationary phase by rpoS. Analogically, effector proteins of the *soxRS* regulon are controlled by soxQ and/or marA (Fig. 1).

It should be noted that some effector proteins are enzymes. For example, superoxide anion finally results in an increase in Mn-SOD expression. The growth of SOD activity raises the concentration of hydrogen peroxide, which is also harmful for the cell. Therefore, in *E. coli* the activity of HPI catalase is increased via activation of OxyR. As a result, the formed superoxide is not metabolized to hydroxyl radical but to water and molecular oxygen. The *soxRS* regulon includes one more enzyme, nitroreductase A [27]. This enzyme catalyzes two-electron reduction of organic nitrogenous compounds, which makes them unavailable for redox cycling and superoxide anion production. In addition to nitro compounds, this enzyme with low substrate specificity reduces quinones, ferricyanide, and 2,6-dichloroindophenol. This makes clear the protective role of nitroreductase A; it prevents the generation of superoxide anion by nitro compounds [27].

Other groups of effector proteins include enzymes associated with the maintenance of reduced glutathione level—glutathione reductase and glucose-6-phosphate dehydrogenase. The latter provides NADPH for the reduction of glutathione by glutathione reductase. Another group includes the enzymes reducing or degrading products of free radical modification of cellular components. Among these are endonuclease IV and alkylhydroxide reductase. The former repairs damage in DNA, and the second reduces organic peroxides. The role of other inducible proteins in cell protection is not so obvious.

Depending on the kind of oxidative stress, not only different amounts of proteins can be modified, but also different species may appear. For example, it was shown that the set of oxidized proteins depended on the method of induction of oxidative stress [28]. When anaerobically grown *E. coli* cells were exposed to hydrogen peroxide stress, alcohol dehydrogenase E, elongation factor G, the heat shock protein Dna K, and oligopeptide-binding protein A were identified as the major protein targets. A similar pattern was found when cells were grown under aerobic conditions in the presence of different iron concentrations. The stress induced by hydrogen peroxide under aerobic conditions resulted in the oxidation of the β-subunit of F_0F_1-ATPase, and the other changes were similar to those found under anaerobic conditions except that alcohol dehydrogenase E was not synthesized in the presence of oxygen. The generation of superoxide anion results in a more specific pattern in which elongation factor G and the β-subunit of F_0F_1-ATPase were significantly affected [28].

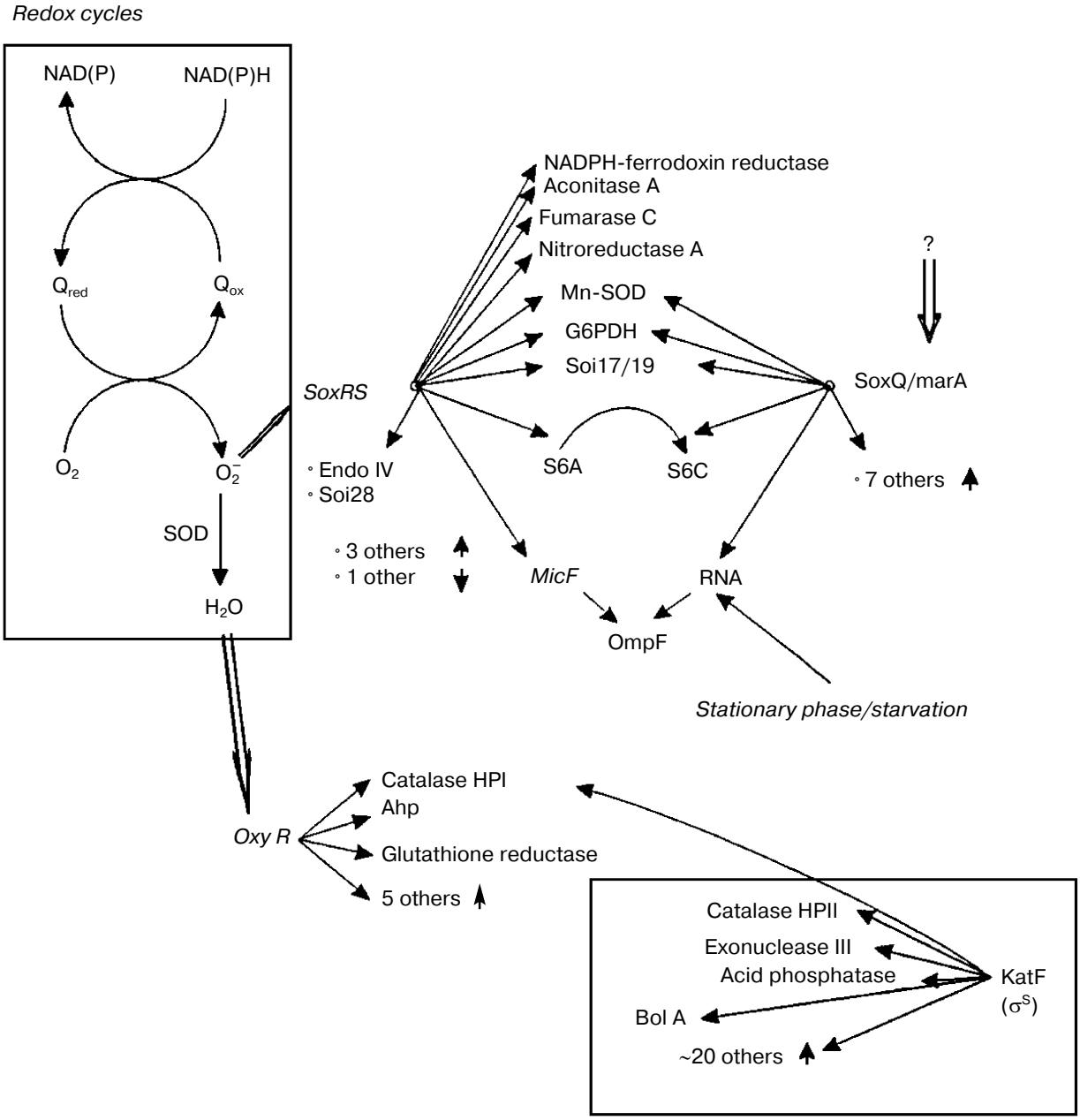

Fig. 1. Regulons responsible for initiation of the cell response to external active oxygen species. The operation of two ROS-regulated regulons, *soxRS* and *oxyR*, is shown. Abbreviations: Endo IV) endonuclease IV; G6PDH) glucose-6-phosphate dehydrogenase; S6A, S6C) unmodified and modified forms of ribosomal protein S6; OmpF) outer membrane porin (modified from [25]).

MECHANISMS OF PROTECTION AGAINST OXIDATIVE STRESS

This problem was briefly discussed already. The protection mechanisms can be divided into three groups: 1) prevention of ROS generation; 2) quenching of chain propagation; 3) repair of damage. More detailed analysis of the problem can be found in works of V. P. Skulachev (Belozersky Institute of Physico-Chemical Biology, Moscow) [29, 30]. Here we will just give a short description of the mentioned hypothesis developed mainly for multicellular organisms. Skulachev suggests that soft uncoupling of the mitochondrial transport chain may prevent one electron reduction of the oxygen molecule. The situation occurs in mitochondria when ADP reserves are exhausted (so-called transition from state 3 to state 4). The transition is accompanied by three events: 1) sharp decrease in the rate of oxygen consumption by mitochon-

drial cytochrome oxidase due to the increase in proton potential; therefore the oxygen level increases; 2) the level of reduction of flavins, CoQ, and nonheme-containing proteins is increased; since the concentration of both substrates of "parasitic" reactions is increased, the generation of superoxide anion and the products of its transformation is increased as well; 3) the transition from state 3 to state 4 results in increase in reduction of electron carriers and a change in their quality, especially CoQ. In state 4, the coenzyme is in its reduced form, $CoQH_2$. Ubisemiquinone ($CoQ^{\cdot-}$) is a short living intermediate form arising when complex III non-heme iron is reduced. The $CoQ^{\cdot-}$ formed is rapidly oxidized to CoQ by cytochrome b_1. In state 4, cytochrome b_1 is highly reduced since its oxidation is prevented by high transmembrane electrochemical proton potential. Therefore, in state 4 $CoQ^{\cdot-}$ becomes a long living component. This long living element has more negative redox potential than $CoQH_2$. It seems to play a major role in one electron reduction of oxygen [17, 29].

So, the increase in ROS generation in multicellular organisms activates the protection system. Soft uncoupling was described above as a preventative mechanism. Thyroxine and triiodothyronine as well as their metabolites act as uncouplers. This idea is supported by several experimental works on mitochondria isolated from control and hypo- and hyperthyroid animals (cited after [30]). It must be noted that there is a mechanism counteracting the uncoupling affect of thyroid hormones. This role is ascribed to male steroid hormones.

When soft uncoupling is not enough, other mechanisms for prevention of ROS generation are activated. For example, under some specific conditions pores permeable for compounds with molecular weight up to 1.5 kD open in the inner membrane of mitochondria [29]. They are specifically sensitive to very low concentration of cyclosporin A. The large size of these pores results in fast equilibration of gradients of all low molecular weight compounds including protons and substrates of oxidative phosphorylation. As a result, the rate of respiration is sharply increased to a level limited only by the activity of enzymes and quantity of participants, but not by transmembrane gradients of hydrogen and substrates. Interestingly, pore formation can be induced by some products of one electron reduction of oxygen and is in some way connected with calcium transport, which is accumulated in mitochondria before pore formation. The formation of long-living pores is the next step of protection.

In the case when the cell is not able to prevent further ROS accumulation, the mechanism of programmed cell death, or apoptosis, is started. This means that multicellular organisms have a specific mechanism of elimination of harmful cells that helps the remaining cells to avoid transformation. For instance, in injured cells malignant transformation can be started and further spread to other cells [29, 30].

The prevention of ROS generation in bacteria is much less studied. The greatest attention has been given to reduction of oxygen level in nitrogen fixing bacteria ([31] cited after [29]). Nitrogenase is sensitive to the presence of oxygen in the medium, and therefore a mechanism to maintain low oxygen concentration is in need. The nitrogen-reducing bacterium *Azotobacter vinelandii* has a specific terminal oxidase *d* which seems to provide low oxygen level in the nitrogen fixing area.

Azotobacter vinelandii possesses at least two types of terminal oxidases (cited after [30]). Cytochrome oxidase *o* is an energy-producing enzyme that is analogous to mitochondrial cytochrome oxidase; it translocates protons through the membrane. Cytochrome oxidase *d* does not operate as a proton pump, but it generates a proton potential using a scalar process that takes place at both membrane sides. In *A. vinelandii*, the affinities of oxidase *d* and oxidase *o* for oxygen are 4.5 and 0.33 μM, respectively. Therefore, the highly active oxidase *d* does not affect the energy-forming oxidase *o* because it is not able to reduce the oxygen concentration to the level where it can affect the function of oxidase *o* [29].

It follows that unicellular as well multicellular organisms have well developed systems for prevention of increased levels of ROS. However, there are cases when preventative mechanisms cannot provide protection, and in these cases other mechanisms should be activated. Therefore, we will now analyze the mechanisms of detoxification of ROS and products resulting from their effects.

Among other mechanisms for protection against ROS formation, the most attention has been given to the second of the mentioned mechanisms, the inhibition of chain propagation. Soviet scientists have made major contributions to the theory of chain reactions and especially to the investigation of lipid peroxidation processes. These problems are very well described in the domestic and international literature and therefore will be not analyzed here, but interested readers can find relevant information in some review articles [3, 32-37].

The last category of protective mechanisms against ROS-produced injuries is the formation of enzymes that repair damage—repairases. Concerning lipids, these are peroxidases that decompose lipid peroxides, lipases that hydrolyze damaged lipids, etc. In fact, there is no information on the repair of proteins except for the reactivation of the enzyme aconitase—a component of the tricarboxylic acid cycle. It has been found that aconitase can be reversibly inactivated by superoxide and peroxynitrite [38-40]. This specific property of aconitase is a good marker of free radical injury to cells. There is much more information on the mechanisms of free radical damage to proteins and further accumulation and degradation of oxidized products. For example, it was shown that oxidized forms of proteins are hydrolyzed more rapidly by proteases than native ones [9, 16, 40, 41].

Now we will analyze one of the most interesting and rapidly developing areas of study of protection against ROS—to repair of damaged DNA molecules. This issue is especially important because it is critical for the survival of injured cells. *E. coli* have been the main subject for studies of DNA repair after is modification by ROS. Therefore, in this specific microorganism the fine mechanisms of repair processes are the best investigated.

The processes of DNA damage can be divided into two stages: the formation of primary products that are mainly unstable, and the formation of end products which are formed as a result of hydrolysis and reorganization of the primary products. Most of the information presented here is taken from reviews of American authors [24, 26, 42]. For convenience of analysis, the types of damage are separated into damage to nitrogen-containing bases and damage to deoxyribose. DNA-Glycosylases initiate the repair by hydrolyzing bonds between the base and carbohydrate of a modified or "incorrect" base and result in formation of areas without bases. *E. coli* possesses a thymine glycosylase (molecular mass 27 kD) named endonuclease III that specifically affects the duplexes of DNA damaged by X-ray and UV. *E. coli* formamidopyrimidine glycosylase (31 kD) releases fragmented purine lesions (formamidopyrimidines, or FAPy) from methylated, alkali-treated DNA. Protein MutY is also a part of the defense system against mutagenesis in these bacteria. It efficiently removes adenine from 8-oxoG-containing DNA base pairs.

E. coli exonuclease III (30 kD) has near-absolute preference for double-stranded substrates, is inhibited by metal chelators such as ethylenediamine tetraacetic acid (EDTA), and stimulated by magnesium ions. It is induced by rpoS. Special interest should be given to endonuclease IV of this bacterium. Its activity is induced 10-fold in response to certain superoxide-generating agents or by nitric oxide. Therefore, the enzyme is part of the *soxRS* regulon. Purified endonuclease IV (30 kD) is heat-stable and very active as well as 3′-PGA diesterase and DNA-specific 3′-phosphatase. Endonuclease IV in concert with exonuclease III account for virtually all of the DNA-specific 3′-phosphatase activity in *E. coli* [26]. Information on repair enzymes in *E. coli* is given in Table 1. It should be noted that the number of copies per cell varies widely.

The separate group of endonucleases of *E. coli* includes endonucleases UvrA, UvrB, and UvrC acting on a vast array of different types of DNA damage induced by UV light. The same enzymes cleave fragments without nitro bases, so all of the glycosylases also fix fragments without nitro bases and deoxyribose after ROS attack. These enzymes show phosphomono- and diesterase activities. Wide substrate specificity puts them at the intersection of many repair pathways. Common systems of repair recognizing many kinds of damage to DNA in *E. coli* include proteins UvrA, UvrB, and UvrC, which direct a complicated system of reactions for localizing and excising from both sides of a site of DNA damage. Appropriate helicases, polymerases, and ligases eliminate damaged oligonucleotides and terminate the repair process. Table 2 shows the set of damaging agents and DNA adducts which are substrates of UvrABC nucleases. It is seen that the complex recognizes large and small adducts. These nucleases are inhibited mainly by intercalating agents. A mechanism for the operation of the UvrABC system has been proposed [42]. This so-called recombinant repair mechanism can correct any DNA damage as long as at least one intact copy remains in the damaged area.

Little attention has been given to the formation of less oxidation-sensitive forms of enzymes. In Fridovich's laboratory it was found that in *E. coli* cells oxidative stress increased the activity of fumarase C [43-45]. Tetrameric fumarase C, like aspartase and argininosuccinase, does not contain iron–sulfur centers, unlike the dimeric fumarases A and B, and this provides its high stability [43-45].

Table 1. Repair enzymes for oxidative DNA damage induced by oxidative stress in *E. coli* (modified from [25])

Enzyme	Specificity	Copy number in *E. coli*
Exonuclease III	AP endo (II); 3′-repair Inducible (*katF/rpos*)	1000-10000
Endonuclease IV	AP endo (II); 3′-repair Inducible (*SoxRS*)	50-1000
Endonuclease III	TG-glycosylase; β-lyase	500
FAPy glycosylase	FAPy/8oxoG glycosylase	400
(MutM)	β-lyase; dRPase	
MutY protein	Adenine glycosylase (8oxoG:A preference)	30

Note: Abbreviations: AP) apurinic; AP endo (II)) class II (hydrolytic) AP endonuclease; 3′-repair) 3′-PGA diesterase/3′-phosphatase; TG-glycosylase) thymine glycol glycosylase; β-lyase) class 1 AP endonuclease; FAPy) formamidopyrimidine; 8oxoG) 8-oxo-7,8-dihydroguanine (8-hydroxyguanine); dRPase) 5′-deoxyribophosphodiesterase.

Table 2. UvrABC nuclease substrates (modified from [42])

Damaging agent	Adduct(s)
Bulky adducts	
N-Acetoxy-2-acetylaminofluorene	C8-Guanine
Benzo[A]-pyrene diol epoxide	N2-Guanine
N,N′-Bis(2-chloroethyl)-N-nitrosourea	Bifunctional alkylation
Cyclohexylcarbodiimide	Unpaired T and G′ residues
Cisplatin and *trans*-platinum	N7-Guanine
Mitocyn C	O-6-Methylguanine
4-Nitroquinone-1-oxide	C8, N2-Guanine
Psoralen	C-5, C-6-Thymine
Pyrimidine dimer	C-5, C-6-Pyrimidine
Thymine glycol	C-5, C-6-Thymine
Non-bulky adducts	
N-Acetoxy-2-acetylaminofluorene	C-8-Guanine
N′-Methyl-N-nitrosoguanidine	O-6-Methylguanine
Apurinic/apyrimidinic sites	Abasic sites
Not repaired	
Phosphotriesters	
Loops in DNA	
Dihydrothymine	
Urea residues	
Inhibits repair	
Actinomycin D	
Caffeine	
Ethidium bromide	
Chloroquinone	

EXPRESSION OF ANTIOXIDANT ENZYMES AND ANTIOXIDANT-ASSOCIATED ENZYMES AS A MECHANISM OF PROTECTION AGAINST OXIDATIVE STRESS

Superoxide dismutase and peroxidases form the first line of defense against ROS. First found in mammalian liver, superoxide dismutase [46] was found several years later in *E. coli* cells [47]. Since that time, this bacterium became the most popular subject for the study of antioxidant systems. To date, it is known that *E. coli* cells have three types of superoxide dismutase. A manganese-containing form (Mn-SOD) was the first identified in these bacteria. This enzyme is coded by the *sodA* gene, whose transcription is controlled by mechanisms that depend on metal ions. At least six global effectors of gene *sodA* transcription have been identified and some of these were described above. Thus, products of the loci of the *soxRS* and *soxQ* genes, genes *fur*, *arcA*, and *fnr* as well as IHF protein are the main regulators of *sodA* gene expression. Products of *sox* activate the expression and the others suppress it. The activity of Mn-SOD is sensitive to external oxidative stress. In contrast, the activity of SOD containing iron (Fe-SOD) does not depend on the growing conditions [48]. It is produced under both aerobic and anaerobic conditions and therefore is called a constitutive form, in contrast to the inducible Mn-SOD, which is absent under anaerobic conditions and is rapidly produced when oxygen is supplied. Under aerobic conditions, the formation of hybrid forms containing subunits of Mn-SOD and Fe-SOD is possible [49]. Also, it is possible to change the level of both superoxide dismutases by modifying the availability of the ions which are incorporated into their active sites. For example, manganese (II) ions increase the level of Mn-SOD and iron ions (II) the content of Fe-SOD [50]. Chelating agents induced Mn-SOD and significantly increased the level of enzyme induction by manganese. Paraquat increases the intracellular level of ROS [51] and has a synergistic effect with manganese ions. The highest levels of Mn-SOD were registered by combined treatment of cells with manganese ions, chelating agents, and paraquat. All these effects depended on the presence of oxygen. The authors suggest that oxygenation and intracellular superoxide anion formation stimulates the formation of Mn-SOD because superoxide oxidizes manganese (II) to (III), which efficiently competes with iron (II) for the apoSOD.

Recently, a new SOD form was found in *E. coli* and other bacteria; it is called Cu,Zn-SOD because of it contains both copper and zinc ions [43, 52-55]. This enzyme is thought to be localized in periplasm. Mutants of the bacterium *Brucella abortus* that do not contain Cu,Zn-SOD were less viable in mice, which suggests its participation in pathogenesis (cited after [52]). To evaluate the role of Cu,Zn-SOD in *E. coli*, the inhibitor of the enzyme diethyl dithiocarbamate (DDC) was used. This compound extracts copper (II) ions from the active center of Cu,Zn-SOD, and does this as at the level of a single cell or a whole organism. Experiments were carried out on strains that do not contain the other dismutases. DDC inhibited aerobic growth of these strains of *E. coli*, but did not affect the growth of mutants with other SODs. Bacteria containing Fe-SOD as well as cells with added SOD mimetic MnTMPyP (a manganese-containing por-

phyrin) were not sensitive to DDC. DDC increased the induction of fumarase C, which is a part of the *soxRS* regulon. Taken together, these data indicate that Cu,Zn-SOD is important for protection against superoxide in *E. coli* deficient in other SODs. From this it was concluded that probably Cu,Zn-SOD is not strictly a periplasmic enzyme, or there is a mechanism communicating oxidative stress signal from periplasm to cytosol [52].

Naturally, the operation of SOD increases the level of hydrogen peroxide. The latter is not very toxic, but in the presence of superoxide anion and transition metal ions, it can be converted into the very toxic hydroxyl radical. Catalases dismutate hydrogen peroxide to molecular oxygen and water. Therefore, we will now analyze the regulation of their activity.

In *E. coli* cells two catalase types have been identified, HPI and HPII (hydroxyperoxidase I and hydroxyperoxidase II), although some information on the existence of third HPIII form appeared [56]. However, most publications deal with two first catalase types. Catalase HPI is bifunctional catalase-hydroxyperoxidase containing two protoheme IX groups connected in a tetramer of identical subunits with molecular weight 80 kD [57]. Catalase HPII is a monofunctional catalase with six heme *d* isomers connected in a hexameric structure of subunits with molecular weight 84.2 kD [58, 59]. Although an important part of the protection system of *E. coli* and other bacterial cells, catalases are not necessary for cell growth under normal conditions. Many bacterial strains survive without measurable catalase activity. However, catalase is an important component for selective advantage. In the presence of hydrogen peroxide, cells with catalase activity survive better than those without it (cited after [60]). Thus, it was found that HPI improves the reducing transport processes, both those dependent on and those independent of proton motive force [61], this perhaps being connected with its periplasmic localization.

The two *E. coli* catalases are regulated in different ways. In exponentially growing cultures, introduction of hydrogen peroxide or compounds increasing its level resulted in a several fold increase in the activity of the *katG* gene coding HPI catalase. In contrast, HPII is not sensitive to this operation. Recent data has shown that addition of hydrogen peroxide, in addition to activating HPI catalase synthesis, increases the synthesis more than 33 other proteins increasing cell resistance to higher, usually lethal, hydrogen peroxide concentrations [26]. These 34 proteins comprise the *oxyR* regulon. The important role of HPI in cell protection has been shown with strains with increased levels. These strains resist higher hydrogen peroxide concentrations [61].

This raises the question, if there are many effector proteins whose activity is increased by oxidative stress, are there some general places of oxidative signal reception? Special proteins that can be reversibly oxidized when the cytoplasm becomes more oxidized have been identified.

REDOX SENSITIVE IRON-CONTAINING PROTEINS AS INTRACELLULAR SENSORS OF OXIDATIVE STRESS

SoxR—a sensor for superoxide anion. After the description of the *soxRS* and *oxyR* regulons, researchers searched for intracellular sensors activating them. In Demple's laboratory (Harvard School of Public Health, Boston, USA) in *E. coli* cells, the genetic locus called *soxR* (superoxide response) positively regulating 9 of about 40 superoxide-activated proteins was found [62]. The proteins were induced on the transcription level. The set included at least three proteins with antioxidant properties—manganese-containing superoxide dismutase (metabolizing superoxide anion), endonuclease IV (repairing radical-induced damage to DNA), and glucose-6-phosphate dehydrogenase (producing NADPH). Induction of the *soxR* regulon also reduced the level of OmpF protein of the outer membrane and modified the level of small ribosomal protein S6. The latter is a component of the small ribosome subunit. OmpF and small ribosome proteins are responsible for bacterial resistance to various antibiotics. Therefore, it was concluded that the described regulon operates as a part of the inducible protection system against xenobiotics [62]. In a cell, the *soxR* regulon can be induced by nitric oxide radical, which may play a role in resistance to macrophages [63, 64].

It was found that the product of the *soxR* gene, SoxR protein, binds to DNA at a specific site and activates the *soxS* gene, this resulting in increase of SoxS protein (Fig. 2) [65, 66]. This protein activates genes which are a part of the general response of *E. coli* cells to increased generation of superoxide anion. SoxS limits the transcription of *soxS in vivo* and binds to *soxS* promoter *in vitro* [67]. The protein binds promoters of the following genes: *micF*, *zwf*, *nfo*, and *sodA*. The binding of SoxS with *micF* and *zwf* promoters accelerates subsequent interaction RNA-polymerase with them [68, 69]. Therefore, it was concluded

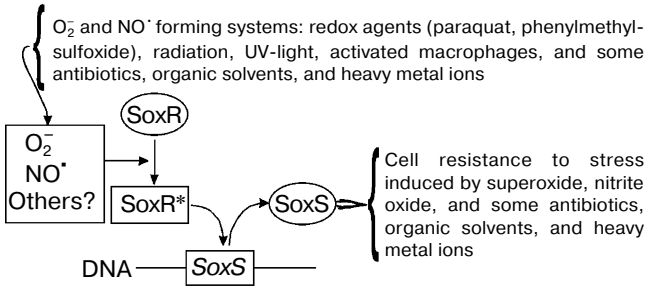

Fig. 2. Induction of the SoxRS regulon and resulting increase in cell resistance to external factors. Intracellular signal converts SoxR protein into an active form that on binding to DNA activates SoxS synthesis (modified from [59]).

that the small regulatory protein SoxS with molecular mass 12.9 kD is a direct inducer of *soxS* genes of the response to oxidative stress in bacteria, although it is impossible to exclude the participation of other proteins in the transcription activation [67]. Modern ideas on the topic are sketched in Fig. 3.

What is known about the activating mechanisms of SoxR? The purified protein contains 107 amino acid residues and has high analogy with another regulatory protein, MarA, which also plays some role in cell resistance to antibiotics [64]. Both proteins are homological to the C-terminus of the XylS-AraC protein family and are even closer to the N-terminus of Rob protein, which contains about 100 amino acids. Investigation of the interaction of proteins with DNA of *E. coli* has lead to the conclusion that these DNA areas can be occupied by different proteins, inducing the synthesis of the same proteins [64]. At the same time, the specificity may be a result of time–space organization of synthesis, binding, and degradation of a large family of related proteins [64].

Purified SoxR protein is a dimer and contains a non-heme iron. The apoprotein can be obtained by treatment by thiols (glutathione [70] or mercaptoethanol [71]) in the presence of oxygen. It was surprising to find that both the apo- and iron-containing forms of SoxR have approximately the same high affinity to DNA [70]. Therefore, it was concluded that iron is not required for binding, for formation of the appropriate conformation, or for bond formation between DNA and protein. Iron induces the specific structure of the promoter site of SoxR–*soxS* complex that stimulates the initiation of RNA polymerase containing factor σ^{70} [72]. SoxR binds *soxS* promoter in a way that is rather unusual for transcription activation. It covers the space about 18 base pairs between –35 and –10 elements of promoter, which are separated by the unusual length in 19 base pairs instead the common 17 base pairs. Therefore, the distance between these elements in *soxS* promoter is not optimal. It is suggested that activated SoxR compensates for this deficiency by inducing a structural reorganization as was proposed for MerR protein, which regulates the bacterial respond to the presence of mercury (cited after [72]). The structural difference between complexes of promoter *soxS* and activated and non-activated forms of SoxR was found by the "finger print" technique [71, 73]. Bacterial mutants with directed base deletions with the distance reducing to 16-18 pairs expressed and increased activation of *soxS* promoter that could be registered as an additional activation on induction of oxidative stress by paraquat [74]. Therefore, it was concluded that the formation of "non-optimal" distance in the promoter is a key in the mechanism of the SoxR activating effect.

SoxR is a protein containing non-heme iron and existing as a homodimer. Each monomer contains a [2Fe-

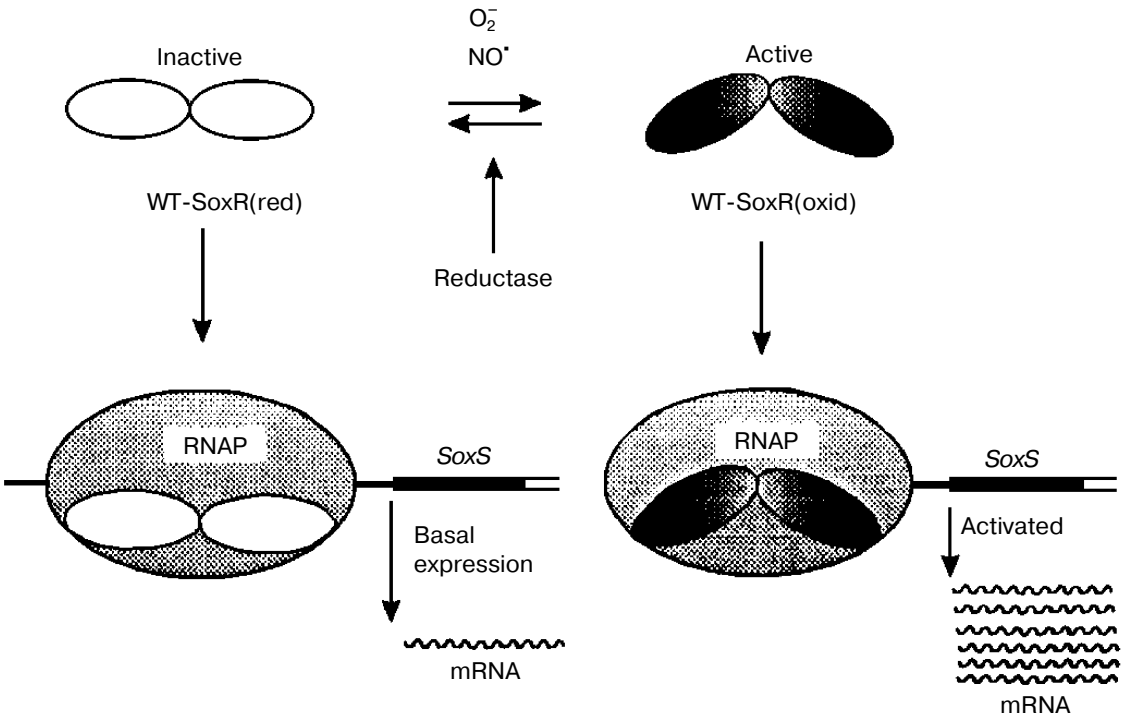

Fig. 3. Model of redox regulation of activity of SoxR protein. Intracellular signal converts SoxR protein into active form that on binding to DNA activates SoxS synthesis. The inactive form also binds to DNA but does not significantly activate the synthesis of particular RNA (modified from [59]).

2S] cluster responsible for the regulation of its activity as a transcriptional factor [72, 75, 76]. Titration of purified SoxR protein showed that its transition potential at pH 7.6 is about −285 ± 10 mV. Its activity disappeared completely at potential −380 mV and was restored in full at potential −100 mV. Therefore, it was concluded that one electron oxidation–reduction of the [2Fe-2S] cluster regulates the transcriptional activity of SoxR [76]. Electron spin resonance spectroscopy indicated that *in vivo* in the protein from "wild" strains, depending on conditions, has from 40 to more than 95% of the [2Fe-2S] clusters in their reduced form [72]. It is suggested that in cells the reduced state of [2Fe-2S] centers is maintained by a specific reductase, but this enzyme has not yet be identified.

The results of model experiments on the interaction of SoxR with the promoter were elegantly confirmed *in vivo* with specially constructed mutants. The mutant genes of the *soxRS* regulon were permanently activated without stress-inducing agents [62]. SoxR protein was purified from these mutants and sequenced [77]. This protein had the activated conformation in the absence of superoxide generating compounds. However, when all these three constitutive SoxR forms (with modification in three different regions of the polypeptide chain) were purified, it was discovered that *in vitro* they possess the transcriptional activity that disappears on reduction. This indicates that the mutant proteins are sensitive to reduction–oxidation and are not locked in the active conformation. Direct investigation *in vivo* indicated that constitutive activity of mutant proteins is a consequence of their high sensitivity to oxidizing agents. Electron spin resonance spectroscopy showed that in wild-type cells from 40 to 95% of the [2Fe-2S] centers are reduced, but in the mutants of this protein less that 4% the of centers are reduced [72]. The midpoint oxidative–reductive potential of the mutant proteins was shifted to −65 mV, which is in good agreement with its increased sensitivity to oxidation [72].

As discussed above, the level of SoxR protein reduction seems to be supported by an enzymatic method. A series of constitutively active SoxR protein mutants was investigated, and these mutants differed in the composition of the carboxylic terminal cluster containing four cysteine residues [78]. Since this specific site is responsible for binding of [2Fe-2S] clusters with the protein, it was postulated that this site participates in its association with the reductase(s). SoxR protein can be activated by nitrosylation as well as through modification of [2Fe-2S] clusters with the formation of dinitrosyl-iron–dithiol adducts [79].

Recently, *in vitro* experiments showed that the transcription of the *soxR* gene, which is positioned on the chromosome head-to-head with the *soxS* gene, activates the intergenic region, and the gene is repressed by SoxR protein [80]. Activated and non-activated SoxR binds to the site equally well, exerting nearly constant repression of *soxS*. Activated SoxR protein stimulated *soxS* promoter more than 30-fold. Functional *soxR* promoter depresses *soxS* transcription when SoxR is not activated and enhances its transcription when SoxR is activated [80]. Consequently, SoxR is a highly polar transcription switch regulated by redox signals, which maximizes the changes in *soxS* expression.

OxyR—a sensor for hydrogen peroxide. As discussed above, OxyR protein plays the role of a sensitive sensor responding to an increase in hydrogen peroxide level. It is a member of the LysR family of transcription activators; it is a rather well characterized tetramer [80]. The protein exists in two forms, oxidized and reduced, and only the oxidized form activates the transcription [81]. Direct oxidation of OxyR is therefore responsible for the activation of this regulon [81-83]. Both OxyR forms bind to DNA, but it seems they do have different binding specificities. The reduced can bind the *oxyR* and *mom* promoters, but not the *katG* and *ahpC* promoters, and contacts ATAG nucleotide repeats in two pairs of adjacent major grooves separated by one helical turn. Oxidized OxyR has been found to bind all OxyR-regulated promoters that have been tested, and it binds in four adjacent major grooves. Differences in binding may allow OxyR to carry out different functions under different conditions. Therefore, OxyR can repress the *oxyR* and *mom* promoters during normal growth and activate *katG* and *ahpC* in response to oxidative stress. OxyR activates transcription by increasing the binding of RNA polymerase to the promoters and has recently been shown to require specific surfaces on the carboxy-terminal domain of the α-subunit of RNA polymerase to activate transcription ([81, 84] cited after [80]).

An increase in hydrogen peroxide induces the oxidation of the Cys-199 residue and then the Cys-208 residue of OxyR [85]. This results in the formation of an intramolecular disulfide bond (Fig. 4). The two cysteine residues are highly conserved, and their directed substitution inactivates the protein. Therefore, it was concluded that the formation of an intramolecular disulfide bond between cysteine residues 199 and 208 induces conformational changes activating the transcription factor OxyR.

The activation of OxyR by hydrogen peroxide is a transient process. In wild-type strains, the amount of synthesized *oxyR* RNA reaches a maximum 10 min after introduction of hydrogen peroxide and returns to the initial level after 60 min. However, the amount of OxyR protein is not changed by hydrogen peroxide treatment. These observations indicate that the protein is deactivated by the reduction of the intramolecular Cys-199–Cys-208 disulfide bond [85]. A set of isogenic strains defective in components of the system reducing disulfide bonds, such as glutathione reductase (*gorA*, grxA (GR1)), glutathione synthetase (*gshA*), thioredoxin (*trxA*), and thioredoxin reductase (*trxB*) was created in the laboratory of G. Storz (National Institute of Child Health and

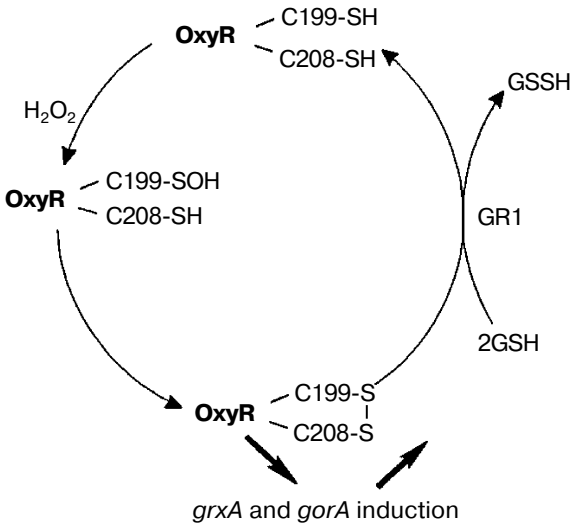

Fig. 4. Model of redox regulation of the activity of protein OxyR. It is suggested that hydrogen peroxide induces intramolecular oxidation of cysteine residues that activates OxyR. The protein can be inactivated by enzymatic reduction of the oxidized cysteine residues (modified from [85]).

Human Development, Bethesda, USA). After 30 min hydrogen peroxide treatment, the level of *oxyS* RNA in *gorA*$^-$ and particularly *grxA*$^-$ and *gshA*$^-$ mutants was increased compared to the initial strain. A *trxA*$^-$ mutant behaved similarly to the initial strain, and *trxB*$^-$ strain exhibited a more rapid decrease in OxyS expression. The latter finding is connected with an increased level of GR1 in this strain. Since it is well known that GR1 catalyzes the reduction of intramolecular disulfide bonds using glutathione, it is supposed that this specific enzyme is responsible for the deactivation of OxyR (Fig. 4). Model experiments *in vitro* with oxidized OxyR and GR1 in the presence of glutathione confirmed this suggestion. The inactive form could be deactivated by hydrogen peroxide in the presence of glutathione. Therefore, it was finally concluded that enzymatic reduction of the intramolecular disulfide bond is responsible for inactivation of activated OxyR [85].

The redox potential of the OxyR regulatory protein was measured using the reversible reaction between OxyR and GSH/GSSG [86]. For this, OxyR was incubated with different concentrations of GSH/GSSG and *in vitro* relative activity of OxyR was measured, evaluating transcription activation. At GSH/GSSG ratio higher than 5 : 1, a sharp and significant reduction in transcription activity was found. The titration data fit well with the idea of reduction and oxidation of four monomers interacting cooperatively, this suggesting that in solution a tetrameric OxyR form exists. Therefore, the process of reduction can be finally represented by the equation:

$$\text{OxyR}_{ox} + 8\text{GSH} \overset{\text{GR1}}{\leftrightarrow} \text{OxyR}_{red} + 4\text{GSSG}.$$

On calculation of the titration data, the OxyR redox potential was found, -185 ± 5 mV. Its value is 90 mV higher than the one found in *E. coli* cytosol. Consequently, in cytosol under normal conditions OxyR is mainly in the reduced (inactive) form. The redox potential of OxyR is also higher than that for all known disulfide reductases of *E. coli*. For this reason, purified thioredoxin from these bacteria inactivates OxyR under *in vitro* conditions. Therefore, it is supposed that another enzymatic system different from GR1 is responsible for OxyR inactivation *in vivo* [85]. It is interesting to note that the OxyR response is reversible, meaning that its activation by hydrogen peroxide induces activities that allow for OxyR inactivation.

Available information on the regulation of expression of effector OxyR genes is summarized in Fig. 4. Under normal conditions, the cysteine residues responsible for the activity of OxyR are reduced. Increase in hydrogen peroxide concentration results in the oxidation of cysteine 199 and then cysteine 208. The formation of an intramolecular disulfide bond between the two cysteine residues activates the protein and its binding to specific DNA sites that express the effector genes. Since OxyR redox potential is much higher than that of cytoplasm, the enzymatic reduction of disulfide bonds takes place in parallel with glutathione oxidation. Moreover, the activation of OxyR contributes to the reversibility of the process via an increase in the activity of specific reductases. As a result, the amount of oxidized (activated) form decreases and consequently the synthesis of RNA of the specific genes is reduced. Therefore, Storz et al. considered that like the activity of OxyR responds to the redox state of reduced and oxidized forms of thiol compounds, SoxR responds to the ratio of oxidized and reduced redox nucleotides ($NADP^+$/NADPH). It is suggested that the difference in potentials of two main intracellular buffers (NADPH/$NADP^+$ and GSH/GSSG) provides the possibility of protein regulation with different sensitivities to oxidation/reduction centers [85].

Other regulators of activity of genes of protective enzymes in bacteria and eukaryotes. Factor σ^S is another regulator of *E. coli* that helps the bacterium to survive oxidative stress [80, 86-90]. It is coded by *katF* (known in different alleles as *nur*, *appR*, *csi-2*, *abrD*, and *rpoS*). Biochemically, it was shown that it is an alternative transcriptional sigma factor expressed in stationary phase or during starvation, and it was therefore renamed σ^S (stationary (starvation) sigma factor). Its activity is controlled at transcriptional and posttranscriptional levels. The σ^S factor controls the regulon of at least 30 genes. Not all proteins of the regulon are characterized yet, but it is known that they increase survival time under deficient conditions by providing a set of protective mechanisms. For instance, under treatment by high hydrogen peroxide

concentrations and other extreme treatments, σ^S factor regulates the expression of katG (HPI, hydroxyperoxidase I), katE (hydroxyperoxidase II), dps (nonspecific DNA-binding protein), xthA (exonuclease III), and gorA (glutathione reductase) [87]. As noted above, *katG*, *dps*, and *gorA* genes are also activated by OxyR, which means that *E. coli* possesses at least two regulons responsible for protection from hydrogen peroxide: OxyR and σ^S functioning in exponential and in stationary phases, respectively. The transcription, translation, and stability of σ^S are modulated by different signals including starvation signal ppGpp, homocysteine lactone as a signal of density, cAMP, and UDP-glucose. The expression of *sodA* is regulated not only by SoxS and MarA, but also by arcA (aerobic respiration control), Fnr (fumarate nitrate reductase), Fur (ferric uptake regulation), and IHF (integration host factor). It is seen how complicated mechanisms are involved in expression regulation of genes responsible for the survival of cells under oxidative stress conditions.

The regulation of oxidative stress-induced proteins has been studied much less in eukaryotes than in prokaryotes. For instance, in yeast Yap1 and Yap2 proteins have been described; they are functional and structural analogs of c-fos and c-jun factors of mammals, and they respond to hydrogen peroxide [80]. These proteins induce the expression of genes involved in the synthesis of amino acids and are also activated by UV light. Proteins Gcn4, Ace1, Mac1, Hap1, Hsf1, Skn7, and some others are regulator proteins involved in the reaction of yeast cells on the introduction of oxidants. This problem is much less studied in higher eukaryotes. Thus, NF-κF factor is sensitive to many unrelated signals, including oxidative stress [80, 89]. Therefore, there is the opinion that the generation of some activated oxygen species may be the common link in coordinated response to different kinds of stress. The transcriptional factor AP-1 (activator-protein-1) consists of two products of protooncogenes, *c-fos* and *c-jun*. They form a complex activating many effector genes [80]. Different proteins like Ref-1, TRX, and some others are regulatory factors in higher eukaryotes, but their role as regulators of reaction to oxidative stress is still insufficiently studied.

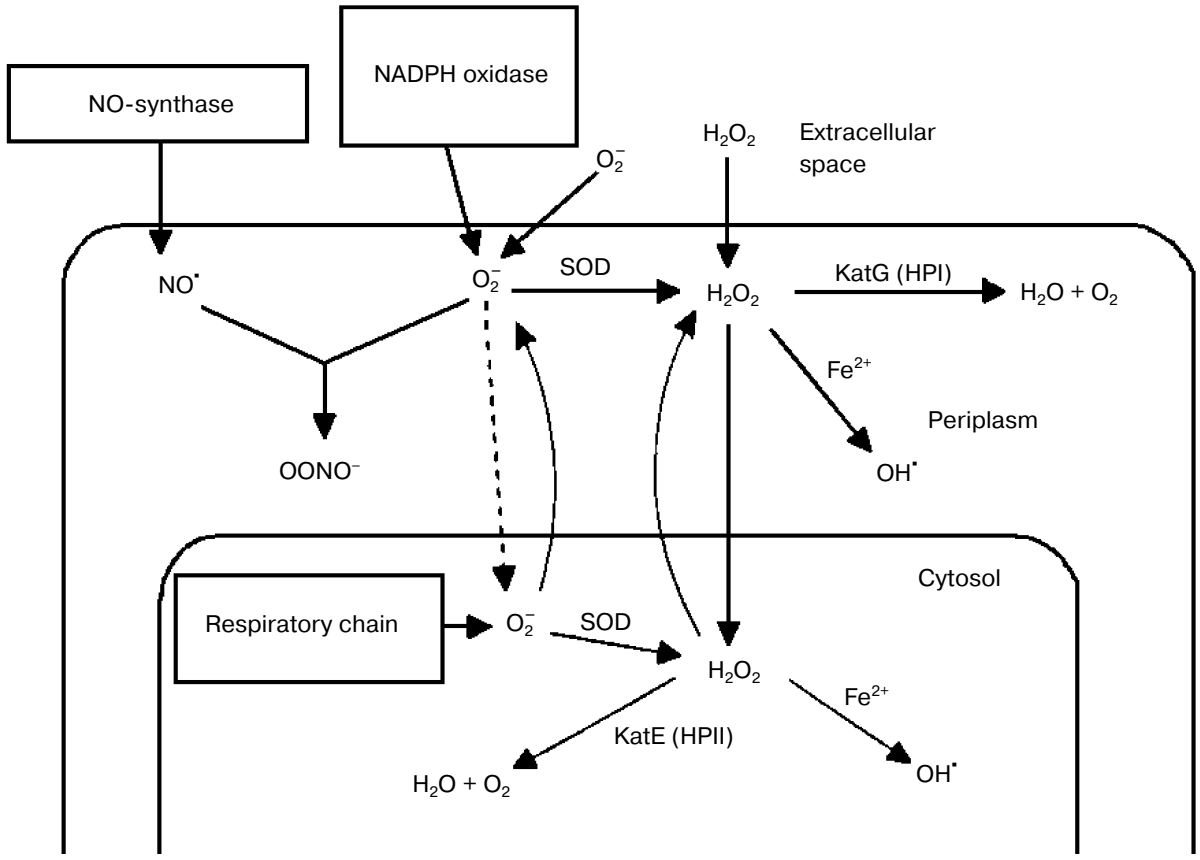

Fig. 5. Space–time coordination of the bacterial cell response to different oxidative stresses. External reactive oxygen species enter the periplasm and here can be either inactivated or enter the cell. In the cell, they again can be either inactivated or result in a cell response. Some of the ROS entering the cell or generated in the cell may leave the cell (modified from [64]).

TIME–SPACE ORGANIZATION OF RESPONSE TO OXIDATIVE STRESS

Figure 5 summarizes the available information on the localization and realization of some particular reactions with ROS in *E. coli*, *Salmonella typhimurium*, and possibly other bacteria. Exogenously introduced hydrogen peroxide, superoxide anion, or peroxynitrite radical rather freely penetrate the cell wall and reach the periplasm. The origination of ROS seems unimportant. They may be exogenous chemical compounds generated by enzymes of other cells. For example, the macrophage attack is an important element of protection of animals against bacteria. The products of reactions catalyzed by macrophage NADPH oxidase and nitroxide synthetase are the main defense weapons [64, 91]. So, ROS enters the periplasm. The polar compounds peroxynitrite or superoxide anion cannot easily cross the cell membrane. Only hydrogen peroxide, freely crossing the lipid phase of the membrane because of its electroneutrality, reaches the cytosol. But in the periplasmic space there are enzymes metabolizing superoxide anion and hydrogen peroxide, in *E. coli* Cu,Zn-SOD and KatG (HPI). Together they can detoxify superoxide anion and hydrogen peroxide. It cannot be excluded that here in the presence of some amount of iron the formation of hydroxyl radicals takes place. However, some part of hydrogen peroxide and small amounts of superoxide reaches the cytoplasm. Here they are catabolized by SOD and KatE (HPII) and activate specific proteins like OxyR and SoxR and directly or after transformation into hydroxyl radical destroy cell components. Some part of ROS may return to the periplasm or even to leave the bacteria. ROS-modified DNA can be repaired by specific enzymes, lipid peroxides can be metabolized by peroxidases, and oxidized proteins can either be accumulated or hydrolyzed by more or less specific proteases.

In conclusion, one more aspect of the problem connected with oxidative stress that has serious practical importance in addition to theoretical interest should be noted. Many antibiotics used in medicine kill bacteria by inducing oxidative stress. Low antibiotic concentrations induce antioxidant systems and this results in increased resistance of the bacteria, which leads to the use of increased doses of antibiotics. In addition, the increase in antioxidant potential can generate new bacterial strains resistant to antibiotics, particularly those that affect bacteria via the generation of ROS. Thus, practical uses may come from theoretical works on oxidative stress connected with the study of molecular aspects of the resistance of bacteria to antibiotics.

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