Redox Proteins as Targets for Drugs Development Against Pathogens

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Abstract: Antimicrobial drug resistance in pathogens is an increasing human health problem. The rapid loss of effectiveness in antibiotics treatments and the accumulation of multi-resistant microbial strains are increasing worldwide threats. Moreover, several infectious diseases have been neglected for years and new antimicrobial treatments are lacking. Redox metabolism has already been proved to be a useful target for drug development. During the last years a significant number of electron carriers, enzymes, proteins and protein complexes have been studied and some of them were found to be essential for survival of several microbial pathogens. This review will focus on three major redox metabolic pathways which may provide promising strategies to fight against pathogens: the non-mevalonate pathway for isoprenoids biosynthesis, the iron metabolism and the iron-sulfur proteins. The common attractive link of all these processes is the plant-type ferredoxin-NADP⁺ reductase, an enzyme that participates in numerous electron transfer reactions and has no homologous enzyme in humans. Research in these redox pathways will open new perspectives for the rational design of drugs against infectious diseases.

Keywords: Isoprenoids biosynthesis, heme oxygenase, ferredoxin-NADP(H) reductase, ferredoxin, iron-sulfur clusters, iron-sulfur proteins, drug targets, pathogenicity.

1. INTRODUCTION

The widespread and intensive use of antimicrobial agents for human treatment and veterinary purposes has increased the number of multi-resistant microbial strains [1,2]. Most of the first generation antibiotics will be unsuitable for the treatment of microbial infections in next years. Antibiotic resistance of any antimicrobial drug will inevitably appear between one to ten years from the time it begins its clinical use [3]. Moreover, the existence of multi and pan-drug resistant microbes [4] increases the human health risk particularly for those that are in hospitals or suffering immunodeficiencies. Antibiotic-resistant bacteria can be transferred from reservoirs to healthy people increasing the risk of infections, the use of antibiotics and adding extra costs of health and social care [2,5].

The current situation will put future patients in real danger and concerted international actions are urgently needed [6]. Moreover, several infectious diseases have been neglected for years [7] and no enough knowledge is available for development of specific antimicrobial drugs. In other cases, complexity of the organisms has exceeded the effort to find new drugs and appropriate tools to fight them are still lacking. These important health problems are widespread all over the world and in some cases worsened as a consequence of environmental conditions, poverty, unsafe water, poor sanitation, and proliferation of animal reservoirs and vectors. Consequently, new strategies and better antimicrobial drugs to overcome the above-mentioned threats need to be developed [6,8].

Redox metabolism has already been proved to be a useful target for drugs development. It involves multiple enzymes and processes. 14.9% of non-redundant entries of the Enzyme Data Bank March 2012 release, which contains 20,639,311 entries, are classified as oxidoreductases. To this group it should be added those enzymes in which there is not net electron exchange between substrates and products but a redox reaction occurs during the catalytic process, as in the case of some transferases, isomerases, synthases, hydratases, etc. Interestingly, some of these metabolic pathways and their enzymes are essential for several microbial pathogens which make them attractive targets for drugs development. The use of redox metabolism as target for antimicrobial drugs is also supported by the relevant differences that have been observed between redox pathways from humans and some infectious agents.

Moreover, many drugs are supplied as inactive forms that redox enzymes convert to compounds that act on the final targets [9,10]. On the contrary, some xenobiotics become inactive after oxidation-reduction by the cell [11]. Consequently, avoiding these redox reactions may help to strengthen the effect of some drugs. On the other hand, a number of antimicrobial agents display their effects by inducing the generation of oxygen reactive species in conjunction with redox enzymes of the host. This strategy was proved to be useful against several microbial infections and cancer [12].

Understanding of microbial and host redox metabolisms could help in tailoring new drugs obtained by a rational design. In this article we review a number of findings related to different redox metabolic pathways and the usefulness as potentially attractive drug targets.

2. THE NON-MEVALONATE PATHWAY FOR ISOPRENOIDS BIOSYNTHESIS AS DRUG TARGET

2.1. Isoprenoids Biosynthesis

Isoprenoids are a broad group of natural compounds with carbon skeleton assembled from five-carbon building blocks derived from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). Isoprenoids are broadly distributed among living organisms, both eukaryotes and prokaryotes. They are functionally important for many aspects of cell metabolism, for membrane structure and function. Many relevant biological isoprenoids roles have been described in photosynthesis, respiration, hormonal regulation of metabolism, regulation of growth and development, defense against pathogens, intracellular signal transduction, vesicular transport within the cell and as coenzymes [13]. Isoprenoids also serve as attractants for pollinators, seed dispersers [14], natural antibiotics and herbivore repellents as well as toxins in plants [15,16]. Several isoprenoids are known to influence membrane structure [16,17]. The specific precursor of all isoprenoids, mevalonate (MVA), was found to be synthesized by the condensa-
tion of three acetyl-CoA molecules via acetoacetyl-CoA and 3-hydroxy-3-methyl-glutaryl-CoA, yielding after phosphorylation and decarboxylation IPP [13]. However, it had been found an MVA-independent pathway for IPP formation in bacteria [18], green algae [19] and higher plants [20, 21]. What is more, genome analysis of Leptospira interrogans allows us to suggest that a similar metabolic pathway is present in this pathogen [22, 23]. However, some differences may exist. It has been shown that IPP in chloroplasts is synthesized from pyruvate and glyceraldehyde-3-phosphate (GA-3P) and not from MVA [18, 19].

2.2. Biosynthesis of the Isoprenoids Precursor IPP via the DOXP/MEP Pathway

After decarboxylation of pyruvate, the acetaldehyde is added to the carbonyl group of GA-3P yielding 1-deoxy-D-xylulose 5-phosphate (DOXP) as the first intermediate [21, 24]. The starting enzyme of this pathway is 1-deoxy-D-xylulose 5-phosphate synthase (Fig. 1). The second enzymatic step, the reduction of DOXP to 2-C-methyl-D-erythritol 4-phosphate (MEP) is catalyzed by dxr gene product, namely DOXP-reductoisomerase in the presence of NADPH and Mn²⁺. The further biosynthetic step consists in the conversion of MEP to 4-diphosphocytidyl-2-C-methyl-D-erythritol in a CTP-dependent reaction by the ispD gene product, a 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol synthase. The following two steps of the pathway were initially identified using bioinformatics methods. The ispE gene product, a 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol kinase, catalyzes the fourth step, the phosphorylation of CDP-methyl-D-erythritol (CDP-ME) at the 2-hydroxy group in an ATP-dependent reaction yielding 2-C-methyl-D-erythritol-2-phosphate. The ispF gene product catalyzes the next step in the pathway, the conversion of 2-C-methyl-D-erythritol-2-phosphate into 2-C-methyl-D-erythritol-2,4-cyclo-diphosphate. This product is accumulated under stress conditions in several bacteria [25].

The last two steps leading to IPP and DMAPP with (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) as intermediate are less well understood. The involved enzymes GcpE and LytB were characterized as oxygen sensitive iron-sulfur proteins most likely containing [4Fe-4S] clusters [26–29]. Both reactions depend on the transfer of two electrons. In Escherichia coli it was demonstrated that flavodoxin together with ferredoxin (flavodoxin)-NADP⁺ reductase and NADPH can serve as electron donor for both GcpE and LytB [28–30] while in the cyanobacterium Thermosynechococcus elongatus ferredoxin was identified as electron donor.
donor for GcEP [31]. Although not yet proven, it has been hypothesized that the ferredoxin and ferredoxin-NADP+ reductase (FNR) identified in Plasmodium falciparum, Toxoplasma gondii and other Apicomplexa may play a role in this process [32]. LytB leads to the simultaneous formation of IPP and DMAPP in a ratio approximately 5:1 from HMBPP. This ratio resembles the requirement of an excess of IPP for the downstream synthesis of isoprenoids [33].

The MEP pathway is found operative in most eubacteria [18], algae [20,21,24,34], cyanobacteria and diatoms [35], but is absent in animals, fungi, archaeabacteria and certain bacteria like streptococci, staphyloccoci and lactobacilli [36]. Although higher plants possess both the MEP pathway as well as the MVA pathway for isoprenoids synthesis [21,37] distinct compartmentalization occurs [38–43]: the MVA pathway takes place in the cytosol and mitochondria (e.g. for sterols and ubiquinone) while the MEP pathway, for synthesis of both IPP and DMAPP, occurs in plastids (e.g. for isoprene, plastotoquione-9, carotenoids and phyto) [34,44].

2.3. The DOXP/MEP Pathway as an Antimicrobial Drug Target

Interestingly, it has been found that isoprenoids are synthesized exclusively via the DOXP/MEP pathway in various pathogenic bacteria like E. coli, Mycobacterium tuberculosis, Mycobacterium leprae, Helicobacter pylori, Vibrio cholerae and Bacillus anthracis and in the apicoplast of the apicomplexan protozoan P. falciparum. Since the amino acid sequences of the constituent enzymes of the DOXP/MEP pathway are highly conserved (except HMBPP synthase that has a large plant-specific internal domain of unknown function) [45] and have no known human counterparts [43], these enzymes offer the potential for the development of novel antibacterial, antimalarial and herbalicidal compounds, and perhaps without toxicity to humans.

The DOXP pathway of P. falciparum has attracted considerable interest as a target for new antimalarial drugs [46–48]. Fosmidomycin, a natural antibiotic, was found to inhibit the DOXP reductoisomerase enzyme [49]. The antimalarial effectiveness of fosmidomycin was evaluated in patients suffering from acute uncomplicated P. falciparum infections. The treatment with fosmidomycin was very well tolerated and efficient in initial parasite infection and fever clearance [50]. However, parasites reappeared in several patients after termination of the treatment. Based on the proven clinical efficacy of fosmidomycin the characterization of additional P. falciparum derived enzymes of the DOXP pathway is highly desirable. These enzymes could finally serve as targets for other new antimalarial drugs, which may be used in combination with fosmidomycin. Such a combination able to shut down the DOXP pathway at two different steps is expected to result in a pronounced synergistic increase in efficacy [51].

Despite substantial progress, the quest for therapeutically significant inhibitors of DOXP-reductoisomerase has not yet led to the development of drugs that are clinically useful. Several inhibitors of DOXP-reductoisomerase have been identified, with fosmidomycin and its acetyl analogue being the most potent to date [52].

3. THE IRON METABOLISM AS DRUG TARGET

3.1. Iron as Nutrient

Iron is an essential nutrient for most organisms. The ability of this transition metal to easily accept and donate electrons allows it to participate in numerous redox reactions. Chemical versatility of iron makes it a valuable prosthetic group for incorporation into proteins as a biocatalytic center or as an electron carrier [53]. Therefore, iron is involved in a wide variety of important biological processes, such as oxygen storage and transport, respiration, photosynthesis, DNA synthesis, cell signaling and defense [54].

The majority of iron in mammals is as heme. This molecule is made up by a heterocyclic protoporphyrin IX ring and an iron atom in its ferrous reduction state. While heme is an important cofactor for many proteins, it also has the potential to cause toxicity at high concentrations. Accumulation of free heme in cells will result in cellular damage, oxidative stress and tissue injury because under aerobic conditions it is able to generate reactive oxygen species. In addition, heme is a small lipophilic molecule that can easily incorporate into membranes, disrupt lipid bilayers and destabilize the cytoskeleton [55,56]. Therefore, in mammals heme is mainly found bound to hemo proteins, in particular hemoglobin and myoglobin [57].

Pathogenic microorganisms, specially, need iron to colonize their hosts and cause disease. However, the level of free iron in mammals is very low as a strategy to avoid infections [58]. Although mammals have developed multiple methods of preventing microorganism from acquiring iron, pathogens have devised a variety of mechanisms to extract iron from the host iron-containing compounds [59].

3.2. Iron Acquisition in Pathogens

A common mechanism in bacteria is to produce and secrete compounds known as siderophores to chelate iron from the environment with high affinity. Another mechanism is to capture iron from iron-binding proteins such as transferrin, lactoferrin or ferritin, via outer membrane receptors. A third mechanism is to extract iron from host’s heme or heme-containing proteins [56,60]. The latter two mechanisms are found only in pathogens.

In gram-negative bacteria have been identified three classes of heme acquisition systems [56,60]. The direct heme uptake system involves binding of heme or heme-containing protein to an outer membrane receptor and its transport via a TonB-dependent mechanism across the periplasm. Once in the periplasmatic space, the heme forms a complex with a transport protein which shuttles it to an ATP-binding cassette transporter anchored to the inner membrane. Heme is then passed into the cytoplasm in an ATP-dependent process [56,60]. Pathogens that have this system include Pseudomonas aeruginosa [61], V. cholerae [62], Yersinia enterocolitica [63] and Shigella dysenteriae [64]. The second uptake system involves a secreted heme-binding protein called hemophore, an outer membrane receptor and an ATP-binding cassette transporter. Hemophores are able to take up free heme or extract heme from hemoproteins present in the external medium and bring it to the cell surface for uptake through a TonB-dependent specific outer membrane receptor [60]. The hemophore mediated heme uptake system has been identified in P. aeruginosa [61], Serratia marcescens [65] and Haemophilus influenzae [66]. A third heme acquisition system is the bipartite hemoglobin receptor HpuAB, identified in Neisseria spp. [67,68]. This system is composed of a TonB-dependent outer membrane receptor (HpuB) and an accessory outer membrane lipoprotein (HpuA). The HpuAB complex let the utilization of hemo globin porphyrin as nutrient iron source [69].

Gram-positive heme uptake systems consist in a specific receptor that shuttles heme to an ATP-binding cassette transporter, which carries the heme across the cytoplasmatic membrane and delivers the porphyrin ring into the cytoplasm [56,60]. One of the most studied heme acquisition system in gram-positive bacteria is the iron-regulated surface determinant system (Isp) of Staphylococcus aureus [70], which is also found in other gram-positive pathogens. The Isp system includes four proteins anchored to the cell wall (IspABCH), a transpeptidase (SrtB), a membrane transport system (IspDE and two cytoplasmic hemoprotein binding proteins IspG, IspI) [60]. It has been shown that S. aureus is able to bind human hemo proteins, remove the heme molecule and degrade it leading to the formation of free iron that is used as a nutrient source [70]. Other gram-positive bacteria, such as Corynebacterium diphtheriae [71,72] and Streptococcus pyogenes [73,74], can also utilize heme and hemoglobin as iron source. Additionally, it has been reported one hemophore-mediated heme uptake system in B. anthracis. This gram-positive bacterium secretes two hemophores, IspX1 and
IsdX2, which are able to extract heme directly from hemoglobin [75]. Heme is then imported into the cytoplasm through the Isd system.

It has been suggested that heme (in the form of hemoglobin) is likely to be the sole iron source for *L. interrogans* [76], mainly because the spirochete is hemolytic, displays chemotaxis towards hemoglobin [77] and possess multiple genes for putative heme acquisition and utilization [78]. Although little is known about the iron acquisition systems in *Leptospira*, it is proposed that the bacterium may secrete hemolysins to lyse red blood cells and make iron available as heme for further uptake [78].

In order to scavenging iron from heme or heme binding compounds, microbes must be able to access the iron atom contained within the porphyrin ring. The final step of this process requires enzymatic degradation of heme which usually involves its oxidative cleavage by a family of monooxygenases known as heme oxygenases [79] and an abundant source of reducing equivalents.

### 3.3. Heme Oxygenase Structure, Function and Mechanism

Heme oxygenase (HO) is the only enzyme which employs heme as both a substrate and a prosthetic group, although it is not considered an hemoprotein *per se* [80]. This enzyme, first characterized in eukaryotes, catalyzes the conversion of heme to biliverdin IXα, carbon monoxide (CO) and free iron (Fe2+) utilizing reducing equivalents. In mammals, biliverdin is reduced to bilirubin by a biliverdin reductase, finally conjugated to glucuronic acid and excreted [81].

Genes encoding heme oxygenases have been identified in a wide range of organisms including mammals [82], higher plants [83], algae [84,85], cyanobacteria [86], fungi [87,88], and some pathogenic microorganisms [76,89–92]. The first crystal structure of a heme oxygenase was obtained in 1999 for the human HO-1 in complex with heme [93]. Currently, several heme oxygenases structures are available [94–103]. Studies on structural aspects propose that the fold of heme oxygenases is a single compact domain consisting of mostly α-helices. The heme is sandwiched between two helices called proximal and distal, and is coordinated with a neutral imidazole group of histidine and an oxygen atom of water as the axial ligands [104].

Mammalian heme oxygenase exists as two isoforms, an inducible HO-1 and a constitutive HO-2. Both enzymes have a C-terminal hydrophobic domain that is involved in the binding to the microsomal membrane [105,106]. These enzymes play important roles in different physiological processes. HO-1 is implicated in iron homeostasis and defense against oxidative and cellular stress, while HO-2 seems to be involved in neurotransmission by the generation of CO as a physiological messenger molecule [106]. In photosynthetic organisms heme oxygenases are soluble enzymes that participate in the biosynthesis of photoreceptive pigments. Cyanobacteria and algae utilize the biliverdin IXα produced by HO as a precursor of phycochromes used for light-harvesting during photosynthesis [86,107]. In higher plants, biliverdin is reduced to phycobchromes, the chromophore of the phytochrome family of photoreceptors [108,109]. In pathogenic microorganisms the major role of heme oxygenases has been attributed to iron acquisition from host heme during infection [110], although some of them have been identified to protect against heme toxicity [60].

Heme degradation proceeds through a complex reaction that requires the input of seven electrons delivered by NADPH and three molecules of oxygen (Fig. 2). The first step of the catalysis involves the heme binding to HO at a specific pocket to form the ferric heme-HO complex, followed by the reduction of the ferric-heme iron to the ferrous state using one electron. Immediately, molecular oxygen binds the complex to yield an oxy-ferrous form (Fe3+-O2) [111]. The subsequent one-electron reduction and protonation of the ferrous-O2 heme gives a ferric hydroperoxide (Fe3+-O2- OOH). Lastly, the distal oxygen of the Fe3+-OOH attacks the porphyrin ring on the α-meso-carbon to convert heme to ferric α-

Fig. (2). Heme degradation pathway catalyzed by heme oxygenase. α, β, γ, and δ represent α-, β-, γ-, and δ-meso-carbons of heme, respectively.
meso-hydroxyheme. The next step of heme breakdown involves the oxidation of the ferric \( \alpha \)-meso-hydroxyheme to ferrous-verdoheme and CO by consuming one molecule of oxygen and one reducing equivalent [113]. Degradation of verdoheme to ferric-biliverdin requires both oxygen and three reducing equivalents [114]. Ferric-biliverdin is finally reduced to the ferrous state consuming one electron followed by the rapid release of free iron (Fe\(^{2+}\)), and the slow dissociation of biliverdin from the protein, step that has been proposed to be the rate limiting [116]. In general, the IX\(\alpha\) isomer of biliverdin is produced, although it has been reported that \( P. \ aeruginosa \) heme oxygenase displays selectivity for the \( \delta \)-meso heme carbon and generates \( \delta \)-biliverdin (70%) and \( \beta \)-biliverdin (30%) [91,117].

The mechanism of heme cleavage is broadly conserved between heme oxygenases from most organisms. However, the source of reducing equivalents is highly variable. In mammals the electrons required to complete the heme oxygenase reaction are derived from NADPH cytochrome P450 reductase [111,118], a flavoprotein that incorporates one FAD and one FMN as prosthetic groups. In contrast, plants, cyanobacteria and bacteria heme oxygenases seem to be ferredoxin-dependent. It has been suggested that the redox system NADPH/FNR/ferredoxin could be the electron donating partner of these enzymes [108,119,120]. In addition, it is proposed the requirement for a second (auxiliary) reductant, such as ascorbate or trolox, to carry out the full activity of bacterial HO [108]. Nevertheless, there is a report showing that catalytic activity of \( P. \ aeruginosa \) heme oxygenase is efficiently supported by FNR without the need of a ferredoxin [121].

3.4. Heme Oxygenase as an Antimicrobial Drug Target

Since iron is required for pathogen survival and infectivity, iron uptake and utilization became an attractive target for antimicrobial drug development [122]. Considering that many pathogenic microorganisms utilize heme oxygenase to acquire iron from the host's heme, it seems to be logical that the inhibition of this enzyme may prevent pathogen colonization of mammals. Therefore, HO may provide a potential target for new antimicrobials.

An important consideration of any drug target is that its function should be essential for microorganism survival. Notably, heme oxygenase has been shown to be required for growth in some microbial pathogens. A knockout of the heme oxygenase gene in \( P. \ aeruginosa \) (\( \textit{pigA} \)) disables the bacteria to use heme iron and consequently shows severe growth defects [91]. Similar observations have been made in the pathogen \( L. \ interrogans \) [76]. Growth of a mutant in the \( L. \ interrogans \) heme oxygenase gene (\( \text{LB 186} \)) was impaired when there was only hemoglobin in the medium, suggesting that in the absence of this protein the spirochete cannot effectively utilize heme as an iron source. In addition, when the role of \( L. \ interrogans \) heme oxygenase in pathogenesis was investigated there has been shown that the enzyme contributes significantly to virulence in the hamster model of infection [123]. Collectively, these studies indicate that decreasing the amount of functional heme oxygenase is of crucial importance for the survival of these pathogens.

3.5. Heme Oxygenase Inhibitors

Many progress have been made in finding new strategies for inhibiting heme oxygenases. It has been proposed that metal substituted porphyrins, such as Zn\(^{2+}\) protoporphyrin IX and Sn\(^{4+}\) protoporphyrin IX, are effective competitive inhibitors of mammalian heme oxygenase [124]. Those compounds bind to the HO active site via metal coordination to the proximal histidine and replace the natural substrate heme. However, because of their identical heterocyclic nucleus they are unspecific and have been found to inhibit other heme dependent enzymes [125]. More recently, azole-based compounds have shown inhibition of the mammalian heme oxygenase in a non heme competitive manner [126]. These molecules derive from the chemical azalanstat and do not affect nitric oxide synthase or soluble guanylyl cyclase except at high millimolar concentrations, but are potent cytotoxins P-450 inhibitors [127]. There is also evidence for the use of isocyanides as uncompetitive inhibitors of heme oxygenase. They completely stopped heme degradation by coordination to the verdoheme intermediate [125].

A significant consideration in the development of novel antibiotics targeting microbial heme oxygenase is its specificity for the mammalian enzyme. Computer-aided drug design was used to identify small-molecule inhibitors of the apo-form of \( N. \ meningitidis \) and \( P. \ aeruginosa \) HO [124]. It has been hypothesized that finding inhibitors able to bind the heme free protein instead of compounds that coordinate heme iron would increase the selectivity toward bacterial heme oxygenase. By \textit{in silico} screening of a large number of compounds several molecules that bind to HO at the heme binding pocket were identified. These compounds may preclude HO to be active with its natural substrate. Eight compounds were shown to bind to \( N. \ meningitidis \) and \( P. \ aeruginosa \) heme oxygenases \textit{in vitro} with binding affinity in the micromolar range. Furthermore, many of them also inhibited HO activity \textit{in vivo}. They were not toxic to cells and could decrease the production of biliverdin in a recombinant \( E. \ coli \) system. The compounds tested were also able to cross the cell membrane and specifically inhibit the growth of \( P. \ aeruginosa \) under iron limitation conditions where heme is the sole iron source. Further testing should be undertaken to ensure selectivity of these inhibitors toward bacterial versus mammalian enzymes and they may be optimized to improve their biological activity.

4. IRON-SULFUR CLUSTERS AND PATHOGENICITY

4.1. Iron-Sulfur Clusters in Nature

Iron-sulfur clusters are prosthetic groups involved in a variety of regulatory processes and redox reactions [128,129]. They are widely distributed in nature performing a myriad of functions. Chemical activity of iron and sulfur with variations in composition in a concerted association with the protein scaffold offer different redox potentials, oxidation states and physical accessibilities of clusters to the environment, making them one of the most important molecular building blocks in the bacterial versus mammalian enzymes and they may be optimized to improve their biological activity.

4.2. Iron-Sulfur Clusters Biogenesis

The understanding of the iron-sulfur clusters biosynthesis is an important topic for science and medicine. Research in this area involves the biogenesis of metal cofactors, the intracellular traffic of metal, the participation of other cofactors and proteins and the characterization of genes, their operons and products [129].

The general mechanism of clusters assembly is based on the presence of a protein scaffold which initially binds sulfur atoms from a cysteine desulfurase and iron atoms from a donor to synthesize a [2Fe-2S] or [4Fe-4S] cluster. Subsequently, the cluster is transferred to an apo-protein target with the assistance of some intermediaries and molecular chaperons [131] (Fig. 3). In prokaryotes, three distinct iron-sulfur cluster assembly mechanisms have been described: the iron-sulfur cluster (ISC), the nitrogen fixation (NIF) and sulfur mobilization (SUF) systems. NifU and NifS proteins, which belong to the NIF system, were identified as required for maturation of nitrogenase enzyme in \( Azotobacter \ vinelandii \) and other organisms, as well as in maturation of other iron-sulfur proteins. NIF proteins involved in the formation of iron-sulfur clusters can also be found in organisms that do not fix
Sulfur donor repressor, which is encoded as part of the iron-sulfur cluster assembly needs to be tightly regulated. In E. coli, the operon iscRUA-iscBA-fdx, which encodes for a set of essential proteins for iron-sulfur cluster biosynthesis, was also identified [131]. This operon has been found in E. coli and in the genome of other organisms as well. Other genes playing important roles in iron-sulfur cluster formation were identified. The sufAB-CDSE operon encodes for a set of proteins whose expression is regulated by oxidative stress and/or iron starvation.

Phylogenetic distribution and metabolic insertion of these iron-sulfur clusters biogenesis systems are complex [132]. As mentioned above, ISC appears to be a housekeeping iron-sulfur cluster assembly system, while SUF is specifically adapted to synthesize iron-sulfur cluster in harsh environmental conditions such as oxidative stress and iron starvation. In cyanobacteria the SUF pathway is the major system for iron-sulfur cluster assembly compared to the ISC pathway. On the contrary, in E. coli the relative importance of SUF decreases in relation to ISC [133,134]. In eukaryotes, iron-sulfur cluster assembly exhibits a higher degree of complexity because of sub-cellular organization, in which different systems exist. It is remarkable that ISC is found in eubacteria and most eukarya whereas SUF is found in bacteria, archaea, plants and parasites [133, 134].

Despite of differences in function and localization of these iron-sulfur cluster assembly pathways, there are some similarities between them. IscS and SufS proteins are structurally similar to NifS. All three systems contain a cysteine desulfurase [135]. IscU is homologous to the N-terminal domain of NifU and contains three conserved cysteine residues that are essential for its function as a scaffold protein [128,133,135]. SufA and IscA bind iron and coordinate its delivery to iron-sulfur cluster in IscU [134]. The ISC machinery contains three additional components, two essential chaperons HscB and HscA, and a nonessential [2Fe-2S] ferredoxin [135]. Although the biochemical properties of the SUF-specific components are still under study, it is known that SufE interacts with SufS and stimulates its cysteine desulfurase activity [136]. The SufB, SufC, and SufD proteins are associated as a stable complex, and SufC has been shown to display ATPase activity [135,137].

Iron-sulfur cluster assembly needs to be tightly regulated. In E. coli, the isc operon is under the transcriptional control of the IscR repressor, which is encoded as part of the iscRSUA operon and auto-regulates its own expression as well as that of iscSUA [137–139]. IscR is a member of the Rrf2 transcriptional regulators family and possesses a winged helix-turn-helix DNA binding domain. IscR contains three cysteine residues and was shown to be an iron-sulfur protein with a [2Fe-2S] + cluster [138,139]. Likewise, the [2Fe-2S] cluster in IscR can interconvert from [2Fe-2S] + to [2Fe-2S] 2+ in response to oxidizing or reducing conditions. The regulatory activity of IscR iron-sulfur cluster may depend on the oxidation/reduction state of cluster as in the case of the SoxR superoxide-sensing transcription factor. However, the effect of a similar redox behavior in IscR DNA binding activity is unknown [133,139].

Iron binding to the iron-sulfur cluster is a process that has not yet been completely elucidated. Frataxin is a protein that in humans is localized in mitochondria and it seems to be involved in iron-sulfur cluster assembly. Frataxin homologues in prokaryotes and eukaryotes are postulated to deliver iron to the scaffold protein IscU [133,139–142]. However, the general mechanism by which frataxin allows incorporation of the metal into iron-sulfur proteins is not yet completely understood [142].

The other important element for clusters assembly is the sulfur. This is an essential element for life since it is involved in numerous metabolic processes [143]. In its reduced form plays a central role in biosynthesis of the amino acids cysteine and methionine [143].

Sulfur incorporation into iron-sulfur clusters is performed by cysteine desulfurases such as IscY and SufS, which acquire sulfur from free L-cysteine by a pyridoxal-5'-phosphate-dependent desulfuration [141–143]. Sulfur is transiently bound in the persulfide form to an active-site cysteine of desulfurase. Subsequently, it is transferred to the scaffold protein. In the SUF system there is an intermediary protein, SufE, which forms a complex with the SufS cysteine desulfurase for sulfur mobilization to the scaffold protein [136,142].

4.3. Iron-Sulfur Clusters and Pathogenesis

Currently, iron-sulfur clusters have been widely studied. Defects in their formation and/or activity are now recognized as cause of several human diseases [130]. Disruption of iron-sulfur cluster biogenesis is deleterious to cell leading to diseases such as Friederich’s ataxia or sideroblastic anemia. Accumulation and dysfunction in iron-sulfur clusters biogenesis are postulated to deliver iron to the scaffold protein IscU [133,139–142]. However, the general mechanism by which frataxin allows incorporation of the metal into iron-sulfur proteins is not yet completely understood [142].

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assembly allows the control of both compounds availability in bacteria. Consequently, these proteins have been related to virulence mechanisms of several pathogens, the efficiency with which these organisms gain access to their host, infect and colonize a new host [145,146]. Strategies used to ensure bacterial success in pathogenicity is an important field of research. The identification of factors involved in such strategies is crucial in diagnosis and drug development against pathogenic microbes and infectious diseases.

The capability of some microbes to access their hosts is largely influenced by some iron-sulfur proteins which act as sensors [128,145,147]. In E. coli cells, the soxRS regulon protects against superoxide and nitric oxide due to oxidation of the SoxR sensor, a [2Fe-2S]-containing transcriptional regulator that triggers the protection response [148]. Besides, ferredoxins are not major sources of iron storage but are critical in regulating the amount of free iron and in the detection of its availability in the host [145]. Altering the above mentioned processes will affect bacterial survival. Likewise, ferredoxins are the source of reducing power for several metabolic processes in bacteria, donating electrons initially provided by the FNR [149]. As some of these processes are unique and have been pointed as essential for bacterial survival, altering or knocking them will consequently affect the virulence mechanisms in some pathogenic organisms [133]. In this context, it has been observed that bacteria need to finely regulate homeostasis of the NADP(H) pool to enable proper deployment of their defensive response being glucose-6-phosphate dehydrogenase and FNR, among other enzymes, the ones that perform this task [148,150].

We have previously reported that despite being a bacterium L. interrogans contains a plastidic class FNR with high catalytic efficiency, at difference from the bacterial class FNRs [23]. The inclusion of a plastidic FNR in Leptospira metabolism and in its parasitic life cycle is not currently understood. Our studies also showed that in L. interrogans the FNR exchanges electrons with a bacterial-type [4Fe-4S] ferredoxin, process which has not been previously observed in nature. Ferredoxins usually work as electron hubs providing reduction equivalents for multiple metabolic pathways. It is expected that this FNR/ferredoxin couple participates in a variety of metabolic reactions in Leptospira. A very low potential [4Fe-4S] ferredoxin from P. aeruginosa with characteristics of a housekeeping protein, abolished bacterial growth when its gene was deleted, unless a plasmid copy of the gene was provided to mutant strain [151].

In Mycobacterium, some genes involved in sulfur metabolism have consistently been identified as up-regulated in response to oxidative stress, nutrient starvation, dormancy adaptation, and during macrophage infection. Mutants in sulfur metabolism genes are severely impaired in their ability to persist and cause disease. Moreover, the SUF assembly system is presumed to play a key role in the pathogenicity of this bacterium. Sulfur metabolic pathways and iron sulfur cluster assembly can be used as potential antimicrobial targets for this pathogen [143,152]. In other organisms such as Bacillus subtilis, genomic analysis of iron-sulfur cluster biosynthesis genes suggests that only SUF system is present. This was pointed as an essential system for the bacterium growth [141] and consequently constitutes an interesting drug target. In this context, genomic analysis of Shigella flexneri, a facultative intracellular pathogen of humans indicated that sufABCDSE operon is expressed during the infection stage, presumably to sense the environment and initiates an appropriate metabolic strategy [153]. Likewise, studies on formation of essential proteins for H. pylori viability indicate that proteins involved in iron-sulfur clusters assembly such as NifU and NiS are absolutely required for virulence and are responsible for the assembly of many (non-nitrogenase) iron-sulfur proteins [146].

5. FNR, A COMMON ATTRACTIVE LINK

Plant-type FNRs are ubiquitous, hydrophilic and monomeric enzymes that contain noncovalently bound FAD as a prosthetic group. They catalyze the reversible electron transfer between NADP(H) and different low potential one-electron donors (e.g., ferredoxin, flavodoxin, heme oxygenase and iron). These flavoenzymes are present in plastids, bacteria, cyanobacteria and apicoplasts of intracellular parasites, and participate in processes as dissimilar as photosynthesis, steroid hydroxylation, nitrate reduction, anaerobic pyruvate assimilation and fatty acid desaturation. They have been also involved in xenobiotic detoxification, amino acid and deoxyribonucleotide synthesis, iron-sulfur cluster biogenesis and in the regulation of several metabolic pathways. In animals, yeast mitochondria and in a few bacteria, another flavoenzymes displaying FNR activity, the adrenodoxin reductases, have been characterized. These enzymes are evolutively unconnected to plant-type FNRs, do not share sequence similarities and belong to the disulphide oxidoreductase family of flavoproteins, whose prototype is glutathione reductase [154,155]. Plant-type and mitochondrial-type FNRs share some properties as NADP(H) binding and catalysis, but the reaction geometry with the nucleotide is different [155,156], encouraging the search for compounds that inhibit one enzyme without affecting the other.

Despite ample variations in amino acid sequences between plant-type FNRs, the chain topologies of all of them are highly conserved. The protein molecule is made up of two structural domains, the carboxyl terminal region includes most of the residues involved in NADP(H) binding, whereas the large cleft between the two domains accommodates the FAD group (see reviews on theses enzymes [157–159]).

FNR is involved in a direct or indirect way to all the metabolic processes that we have discussed in this work.

Non-mevalonate pathway for isoprenoids biosynthesis has been proposed as an attractive target for development of antibacterial compounds, especially for the fact that is present in clinically important pathogens and is not present in humans [23,32,160]. FNR was implicated in electron transfers occurring at the last two steps of this pathway [28–32].

As mentioned before, iron is required for pathogen survival and infectivity and many pathogenic microorganisms utilize the heme oxygenase enzyme to acquire this essential nutrient from host’s heme. In bacteria, electrons required for the heme oxygenase reaction are derived from ferredoxin. It has been suggested that the redox system NADPH/FNR/ferredoxin could be the electron donating partner of these enzymes [108,119,120]. Moreover, in the case of P. aeruginosa heme oxygenase the catalytic activity is efficiently supported by FNR without the need of a ferredoxin [121].

Iron-sulfur proteins play an essential function in iron and sulfur regulation and consequently in the development and protection against infectious diseases [146]. In particular, ferredoxins are critical in regulating the amount of free iron in eukaryotes and have ability to detect the availability of this compound in host environment [145]. In addition, ferredoxins are the source of reducing power for several metabolic processes in bacteria, donating electrons initially provided by FNRs [149]. Several of these processes have been considered as essential for bacterial survival and implicated in virulence mechanisms in some pathogenic organisms [145]. Different authors have suggested that the synthesis of iron-sulfur clusters in the apicoplast of some organisms is an essential event that could not be replaced by mitochondrial machinery and implies at least five ferredoxins. Based on this, it is likely that alteration of the redox system FNR/protein substrate could produce an important effect on more than one essential metabolic pathway [161]. These
effects could be induced either by inhibiting the reductase activity or affecting the interaction between the reductase and some of its substrates. Recent studies have proposed inhibition models by altering ferredoxins active sites (iron-sulfur clusters) assembly [162, 163].

The FNR insertion in these relevant metabolic pathways makes this enzyme a potential antimicrobial target to investigate in different pathogens. Moreover, as the enzyme participates as a redox hub (Fig. 4), it may be expected that knocking down its activity will produce a profound effect on pathogen survival. Several inhibitors have been described for FNR enzymes and the potential to development of new ones has been analyzed [32].

In summary, it is evident that research in these redox pathways will open new perspectives contributing to the rational design of drugs for infectious disease control.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by grants from CONICET and Agen- cia de Promoción Científica y Tecnológica (ANPCyT, Argentina)

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl pyrophosphate</td>
</tr>
<tr>
<td>DOXP</td>
<td>1-deoxy-D-xylulose-5-phosphate</td>
</tr>
<tr>
<td>FNR</td>
<td>ferredoxin-NADP+ reductase</td>
</tr>
<tr>
<td>GA-3P</td>
<td>glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>HMBPP</td>
<td>(E)-4-hydroxy-3-methyl-but-2-enyl diphosphate</td>
</tr>
<tr>
<td>HO</td>
<td>heme oxygenase</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>ISC</td>
<td>iron-sulfur cluster system</td>
</tr>
<tr>
<td>Isd</td>
<td>iron-regulated surface determinant system</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical Formula</th>
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<tbody>
<tr>
<td>MEP</td>
<td>2-C-methyl-D-erythritol 4-phosphate</td>
</tr>
<tr>
<td>MVA</td>
<td>mevalonate</td>
</tr>
<tr>
<td>NIF</td>
<td>nitrogen fixation system</td>
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<tr>
<td>SUF</td>
<td>sulfur mobilization system</td>
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REFERENCES


Krapp AR, Humbert MV, Carrillo N. The soxRS response of Escherichia coli can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content. Microbiology 2003; 149: 311-4.


