POSTĘPY MIKROBIOLOGII – ADVANCEMENTS OF MICROBIOLOGY
2022, 61, 2, 81–93
DOI: 10.2478/am-2022-0008

ENZYMATIC AND NON-ENZYMATIC RESPONSE DURING NITROSATIVE STRESS IN *ESCHERICHIA COLI*

Rohan Nath[a], Swarnab Sengupta[a], Arindam Bhattacharjee[*]

Department of Microbiology, University of North Bengal
[a] Contributed equally

Received in November 2021, accepted in April 2022

Abstract: Nitrosative stress is an adverse physiological condition mediated by an excessive level of reactive nitrogen species (RNS). RNS react with the different macromolecules *in vivo* and result in the inactivation of these molecules. But the mechanism to counteract the effect of nitrosative stress is poorly understood. *Escherichia coli* is one of the best understood and well-studied microorganism. Although several studies have been reported on *Escherichia coli* to characterize the effect of various stress response but fewer works are there to see the effect of nitrosative stress. *Escherichia coli* encounter numerous stresses during its growth, survival, and infection. They respond to various stress conditions by activating common regulator proteins and thiols. These stress conditions result in the accumulation of these regulator proteins and thiols that allow cells to adjust to specific stress situations, conferring stress tolerance and survival. In this review, different enzymatic and non-enzymatic mechanisms to counteract the effect of nitrosative stress in *Escherichia coli* have been discussed and a hypothesis for the working mechanism of hybrid cluster protein that helps to combat nitrosative stress has been proposed. Here, we have tried to give a clear scenario about the mode of action of stress-responsive elements present in *Escherichia coli*.

1. Introduction. 2. *Escherichia coli* and nitrosative stress. 2.1. Protein damage caused by nitrosative stress. 2.2. DNA damage caused by nitrosative stress. 3. Nitrosative stress response components in *Escherichia coli*. 3.1. Flavohemoglobin. 3.2. Cytochrome *c* peroxidase (Ccp1). 3.3. Flavorubredoxin. 3.4. Catalase. 3.5. Superoxide dismutase (SOD). 3.6. Cytochrome *c* nitrite reductase (NrfA). 3.7. Hybrid cluster protein (HCP). 3.8. Tripeptide glutathione. 3.9. Cytochrome *bd* oxidase. 4. Summary

Keywords: *Escherichia coli*, nitrosative stress, protein tyrosine nitration, reactive nitrogen species, stress response

Abbreviations:
Ccp: Cytochrome *c* peroxidase, **flavoHbs/Hmp**: Flavohemoglobin, **flavoRbs**: Flavorubredoxin, **GSH**: Reduced glutathione, **GSNO**: S-Nitrosoglutathione, **GSSG**: Oxidized glutathione, $\mathbf{k_m}$: Michaelis-Menten constant, **ROO**: Rubredoxin O_2 oxidoreductase, **ROS**: reactive oxygen species, **SNP**: Sodium nitroprusside

1. Introduction

In biology, a deleterious factor or any condition adversely affecting living cells' growth or survival could be stress [117]. Stresses like osmotic, cold, acid, heat, metal, oxidative, nitrosative, etc., challenge these microorganisms in their natural habitat [5, 12, 22, 43, 76, 78, 87, 108, 113]. In case of oxidative stress, the cellular redox homeostasis changes due to the elevated production of different reactive oxygen species (ROS) like superoxide (O_2•), peroxyl (ROO•), singlet oxygen (O), semiquinone radical (HQ•), alkyl radical, alkoxyl (RO•), ozone (O_3) hydroxyl (OH•) [43]. While nitrosative stress mediates by reactive nitrogen species (RNS), which formed due to reactions between NO• and ROS like peroxynitrite ($ONOO^-$), alkyl peroxynitrites (ROONO), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), nitrous acid (HNO_2), nitronium ion (NO_2^+), nitroxyl anion (NO^-), nitrosonium ion (NO^+), nitryl chloride (NO_2Cl) which can quickly react with different macromolecules *in vivo* [54, 60, 79, 96]. "Nitrosative stress" was coined by Stamler (1996), where reactive nitrogen species (RNS) were involved in mediating the stress. NO• is a small lipophilic molecule that also acts as a cell-signaling molecule *in vivo* [5, 12, 54, 60, 78, 79, 96]. A low amount of NO• helps in cell signaling and defense [80]. However, the elevation in NO• can cause an environment hazardous to microorganisms. This elevated level of NO• creates a toxic condition, which directly alters the stability of various lipids, proteins, enzymes of critical metabolic pathways, replication, transcription, and translation machinery, which makes it difficult for the microorganisms to survive [70, 73]. NO• synthesis within the cell during the catalytic conversion of

* Corresponding author: Arindam Bhattacharjee, Department of Microbiology, University of North Bengal, Raja Rammohunpur, Siliguri, Darjeeling, West Bengal, India, 734013; aribiochem@gmail.com, abmicrobio@nbu.ac.in
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L-arginine to L-citrulline by nitric oxide synthases (NOSs) is carried out [79]. Research related to NOS in prokaryotes has shown a similar molecular activity compared to their mammalian counterparts. NO• is also generated during the nitrite respiration in several bacteria, including *E. coli*, by the activity of nitrite reductase [21].

To counteract the effect of reactive nitrogen species, *E. coli* activates a sensing mechanism that can lead to a programmed molecular response within the cell, otherwise known as "nitrosative stress response," which is crucial for their survival and adaptation [53, 62]. Reports on cellular components like flavohemoglobin (HmpA), flavorubredoxin, cytochrome *c* nitrite reductase (NrfA), cytochrome *c* peroxidase, catalase, low molecular weight thiols, cytochrome *bd* oxidase, like enzymes are known to act as the stress response enzyme under nitrosative stress in different microorganisms, but the mechanisms are not well established [7, 19, 25, 35, 36, 101]. It is vital to characterize the counteracting mechanisms of microorganisms like *E. coli*, to understand the physicochemical condition of the cells during nitrosative stress.

Although *E. coli* has one of the most well-understood biochemical systems, very minimal studies concerning nitrosative stress are present. Thus, in this review, the response of *E. coli* under nitrosative stress was extensively studied. The mechanism of action of these components under nitrosative stress needs more studies [19, 25]. In this paper, we have attempted to characterize various cellular components of *E. coli* and their mechanism of action, which are involved in counteracting the nitrosative insults.

2. *Escherichia coli* and nitrosative stress

As an opportunistic pathogen, *E. coli* must enter the host and adapt to its environment while the host tries to eliminate the invading pathogens. Host organism secretes many types of antibacterial and antiviral molecules via their defense systems like the activated macrophages and neutrophils. Reactive species like NO• and ROS, and RNS are released [17, 88]. So the *E. coli* have to survive under the presence of ROS and RNS that can specifically damage bacterial lipids, proteins, and DNA. Along with the host cell, even bacterial cells could produce NO•.

In some cases, the denitrifying bacteria produces NO• as an obligate metabolic intermediate where the carbon (C) to NO_3^- ratio is strictly maintained [94, 95]. However, denitrifying bacteria are not very common in the gastrointestinal tract, where C to NO_3^- ratio is very high. Meanwhile, *E. coli* in the gut can reduce NO_3^-/NO_2^- to ammonia [21, 102, 111], thus increasing the cells protection against nitrosative stress.

2.1. Protein damage caused by nitrosative stress

NO• is capable of inhibiting and modulating enzymes containing metal centers such as the [4Fe-4S] cluster of fumarate and nitrate reductase (FNR), heme iron center of guanylate cyclase and the $[2Fe\text{-}2S]^{2+}$ of SoxR, carbonic anhydrase, catalase [24, 92]. Another inhibitory effect, i.e., nitration of tyrosine, is caused by RNS [47, 81]. Nitrosative agents directly bind and inhibit metabolic enzymes such as aconitase [51], terminal respiratory oxidases, etc., in *E. coli* [11]. Proteomic studies on the *E. coli* YtfE protein revealed a vital role in repairing the iron-sulfur cluster proteins [56]. In a cellular environment, NO• readily reacts with superoxide anion ($O_2^{\cdot -}$) to produce peroxynitrite ($ONOO^-$) [83]. Peroxynitrite readily reacts with carbon dioxide (CO_2) to produce $ONOOCO_2^-$, which decomposes to give carbonate radical anion and NO_2^-. This form of peroxynitrite can nitrate aromatic amino acids of protein like the tyrosine residues. Peroxynitrite is very toxic and can react to modify cysteine, tryptophan, and methionine residues of proteins [3]. One of the significant concerns of protein modification by the nitrating agent is protein tyrosine nitration (PTN) which may lead to the inactivation of most proteins in microorganisms [11, 24, 47, 51, 81].

Protein tyrosine nitration is a well-known effect of nitrosative stress within the cell. Nitrosants target the side chains of tyrosine in proteins [81], resulting in the formation of 3-nitrotyrosine. This specific reaction may lead to indiscriminate damage and irreversible modifications of various proteins, including those involved in stress responses like SODs. Usually, manganese superoxide dismutase (MnSOD) uses $O_2\bullet^-$ as the critical substrate, but in the presence of the nitrating agent, pKa of -OH of tyrosine gets decreased in such a way that may lead to a change in the redox status resulting in inhibition of the enzyme [82]. In *E. coli*, AtpA, AceF, FabB, GapA, IlvC, and TufA proteins contain RNS sensitive cysteine molecules [14]. In *E. coli*, dihydrolipoyltransacetylase (AceF) can scavenge environmental toxins such as arsenic in the cell. RNS can bind and modify the two vicinal thiol groups of AceF. That may lead to the inactivation of the protein [14]. In type II fatty acid biosynthesis, a carrier protein, namely 3-oxoacyl (acyl carrier protein) synthase I (FabB), plays a vital role where the FabB protein contains a highly conserved cysteine molecule that is vital for its catalytic activity. Modification of this conserved site leads to an interruption in fatty acid synthesis and thermoregulation of membrane fluidity [14].

2.2. DNA damage caused by nitrosative stress

At the time of nitrate to nitrite metabolism, reactive NO• and HNO_2 are formed. NO• does not directly react with DNA, but when it oxidizes to N_2O_3, it becomes

a robust nitrating agent, then it can damage DNA. N_2O_3 reacts with nucleotides having exocyclic amino groups like guanine, cytosine, and adenine, converted to uracil, xanthine, and hypoxanthine, respectively [97]. The exocyclic nucleotide amines can generate unstable N-nitroso (–N–N=O) derivatives, later deaminated. If not repaired by a cellular repair mechanism, these damaged bases are deleterious for the cell [97]. Cellular secondary amines and amides can be nitrated and may produce alkylating agents that lead to mutation in different sites of DNA. Nitrosative mutagenesis is very prominent when cells from a nitrate-dependent atmosphere shift to oxygen-dependent respiration [111]. Scientific reports have linked the overproduction of NO•, which subsequently reacts with superoxide anion to form N_2O_3 during this period, causing nucleobase deamination like the formation of 2'-deoxyxanthosine (dX) and 2'-deoxyoxanosine (dO) from guanine, etc. [28, 111].

3. Nitrosative stress response components in *Escherichia coli*

Although not many components of nitrosative stress response in *E. coli* are fully understood, both enzymatic and non-enzymatic components are involved in combating the hostile situation. Some of those mechanisms involved in the nitrosative stress response will be discussed below in this paper.

3.1. Flavohemoglobin

Flavohemoglobin (HmpA) is a 44 kDa monomer in which two different modules get fused to form a multidomain protein [115]. It was the first flavoHb's family protein, discovered in *E. coli* in 1991. An 1191 bp DNA fragment encodes this protein, and this was the first globin sequence obtained in a microorganism [19, 25, 101]. Regulation of HmpA protein expression by the FeS protein Fnr, which acts as a positive transcription factor [10]. While Fnr protein expression by exogenous signals like redox, oxidative, nitrosative, and anoxic stress is a potent transcription regulator [61]. Nitrating agents like NO•, SNP, GSNO, etc., have been shown to induce *hmpA* transcription [2, 101]. While in another experiment carried out by Adolfsen et al. [2] showed that HmpA delayed the detoxification of NO• until oxidative stress agent H_2O_2 was present. Their experiment showed that H_2O_2 downregulated *hmpA* transcription. To understand the molecular mechanism behind this suppression, they used integrated computational modeling and wet-lab data, which showed that the presence of H_2O_2 repressed the transcription and translation of HmpA protein. The mechanism showed a distant similarity with carbon catabolite repression (CCR), where glucose is the preferred carbon source and is consumed first by the cell, followed by consuming other less preferred carbon sources [2]. The exact mechanism of action needs further research.

To understand the importance of the flavohemoglobin protein in uropathogenic *E. coli*, where these cells in the urinary tracts face high NO• concentrations, generated as part of the host immune response. These experiments showed that isolates from patients with UTI showed a high expression of flavohemoglobin, and it shows the ability of the bacterium to colonize the urinary tract. Results showed *hmpA* mutants were less competitive in colonizing the urinary tracts and were less viable under heavy NO• exposure [13, 101].

Other HmpA protein regulators are MetR protein and NsrR protein [9, 61, 101]. Both of them regulate the HmpA expression in the exact opposite way. MetR is a positive regulator where *metR* mutant results in downregulation of HmpA protein while at the same time *nsrR* mutant shows constitutive HmpA protein expression, thus indicating that NsrR protein negatively regulates *hmpA* transcription [9].

Since most aerobic organisms produce ROS and NO• themselves; hence, they also possess flavohemoglobins (flavoHb) that help them in the detoxification of NO• at O_2 concentrations as low as 35 μM [79]. Interestingly, even the anaerobically growing organisms like the *E. coli* in gut microflora or those present in the urinary tract show efficient NO• detoxification systems. Justino et al. [57], in their article, demonstrated that *E. coli* growing under anaerobic conditions showed NO• detoxification by the protein YtfE and NrdHIEF gene cluster, along with the upregulation of flavohemoglobin (HmpA) and flavorubredoxin (NorV); they also found maximal HmpA expression only after 30–45 minutes under anaerobic condition [57]. Since HmpA protein can detoxify NO• in both aerobic and anaerobic conditions, it has been under the radar of redox biologists. X-ray structure of flavoHb shows that flavoHb's has a C-terminal NAD-binding and FAD-binding domain and a N-terminal globin domain. Purified HmpA possesses heme band FAD, confirmed by the crystal structures of HmpA [115]. Under anaerobic conditions, the heme portion of HmpA protein, i.e., Fe(III), is quickly reduced to Fe(II) by the action of physiological substrates NADPH through electron transfer from FAD. This event enables HmpA to react with NO• and results in highly stable nitrosyl species formation. Again NO• can be reduced to NO^- by transferring one electron from Fe(II). The NO^- is then separated from the complex and converted to N_2O via dimeric species. However, under aerobic conditions, NO• mainly reacts with $Fe(II)O_2$, where preliminary a volatile nitrosyldioxyl complex (Fe[II]OONO) forms and nitrate as the final product [57, 101, 115] (Fig. 1). Kim et al. [114] proved

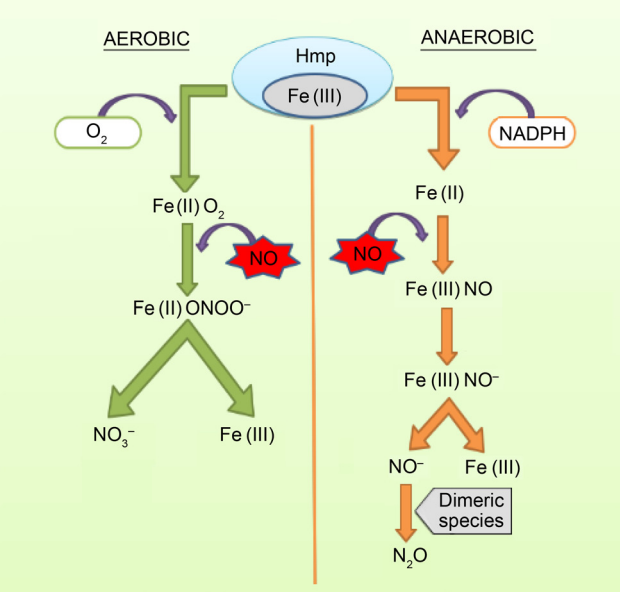


Fig. 1. Mechanism of action of flavohemoglobin under aerobic and anaerobic condition in presence of nitrating agent

Under aerobic condition Fe(II) present in Hmp remains associated with O_2 to form Fe(II) O_2 complex. Under nitrosative stress in presence of NO, it forms nitrosyldioxyl complex (Fe[II]OONO) and this complex ultimately breaks down to nitrate (NO_3^-) and heme group returns back to its original state [Fe(III)]. Under anaerobic condition, NO• reacts with Fe(II) and Fe(III)$NO^-$is formed. Fe(III)NO^- is again broken down to NO^- and then it is converted to N_2O via dimeric species.

spectrophotometrically that HmpA could bind NO• reversibly. The kinetics of the reaction of NO• and HmpA is estimated using stopped-flow analysis. After the flow of an anoxic mixture of HmpA and NO• stops, the maximum absorbance was at 422.5 nm, 534 nm, and 568 nm. It indicated the formation of nitrosyl complexes [114]. The mechanism of action of HmpA has some similarities with the action of cytochrome *c*, a vital stress response enzyme. Cytochrome *c* reacts with NO• of SNP in the Fe(II) state [23]. Similarly, HmpA reacts with NO• in the Fe(III) state, and nitrosyl complex formation occurs. Ferric-nitrosyl spectra predominate at high O_2 concentrations. This observation indicates that HmpA can nullify the activity of reactive NO• [45]. The NO• mediated inactivation of aconitase, reported earlier, is also diminished by the activity of flavoHb in the presence of O_2. Experimentally it has been proved that under oxic conditions, elevated flavoHb can protect aconitase activity in gas mixtures containing 240 ppm NO•. This NO• metabolism for the protection and survival of *E. coli* is crucial [39].

3.2. Cytochrome *c* peroxidase (Ccp1)

Cytochrome *c* peroxidase (Ccp1) is the first heme enzyme analyzed with the help of its crystal structure. This enzyme can rapidly bind with cytochrome *c*. It also shows the ability to react with hydroperoxides which oxidize the enzyme. It has a highly α-helical structure with a cylindrical shape. The heme group is present in a hydrophobic pocket in the protein, coordinated by the Nε2 atom of H175, the only axial ligand to the heme iron. The sixth coordinate position remains vacant or engaged by a water molecule is available for binding to the peroxide substrate. Ccp1 heme contains a high-spin (S = 5/2) paramagnetic Fe(III) atom in the ground state. During the catalytic cycle, Fe(III) atom then oxidizes to a Fe(IV) = O oxyferryl intermediate [25, 32, 59]. Some reports revealed that Ccp1 is efficiently active against H_2O_2 and efficiently active against $ONOO^-$ [32]. A recent report suggests that in *E. coli*, Ccp1 can use H_2O_2 as the terminal electron acceptor under anoxic conditions [59]. Peroxynitrous acid [ONOO(H)] and its conjugate base ($ONOO^-$) both undergo non-enzymatic and enzymatic reactions. Peroxynitrous acid is responsible for the production of sulfonic and sulfinic acids with the help of oxidized cysteine residue present in critical metabolic enzymes such as aconitase [44] and glyceraldehyde-3 phosphatedehydrogenase (GAPDH) [67]. Peroxynitrite also promotes the nitrotyrosine formation of manganese superoxide dismutase (MnSOD), a critical antioxidant enzyme [116].

Cells must be able to identify and detoxify ONOO(H) because of its potential toxicity. Like H_2O_2, ONOO(H) also binds to the heme moiety and forms an intermediate termed CompoundI (CmpdI) by oxidizing two-electron of peroxidases. The heterolytic cleavage of the ONOOH results in the formation of NO_2^- which cannot diffuse from the active site of the enzymes like horseradish peroxidase (HRP), myeloperoxidase (MPO), and lactoperoxidase (LPO) but gets readily oxidized by the action of CmpdI to give CmpdII and NO_2•. This CmpdII then transports NO_2• out from the enzyme active site (Fig. 2). So, not surprisingly,

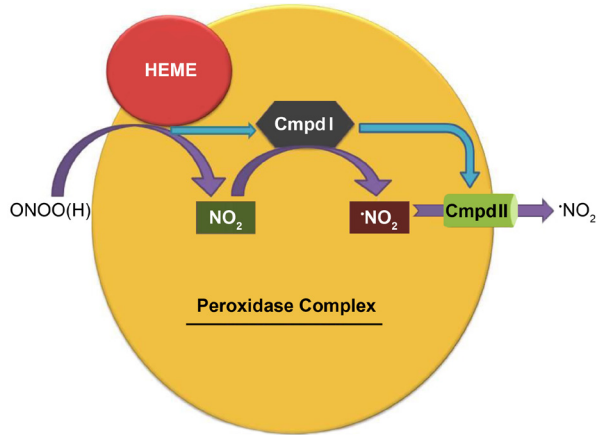


Fig. 2. Mechanism of action of cytochrome *c* peroxides

Sequential transformation of peroxynitrite ($ONOO^-$) to nitrite NO_2). The nitrite (NO_2) formed by the action of Compound I (Cmpd I) diffuses out of the cell by the action of CompoundII (Cmpd II).

ONOOH also quickly reacts with H_2O_2 metabolizing enzymes that oxidize NO_2^- to $\bullet NO_2$, including bacterial peroxiredoxins [16, 116].

Although H_2O_2 and ONOOH are efficient substrates of catalase and peroxidase, their stabilities differ significantly. H_2O_2 is reactive until redox-active metal centers or thiols will deactivate it. This peroxide is an appropriate secondary messenger in cells [34, 100, 105].

Cytochrome *c* peroxidase (Ccp1) reduces H_2O_2 efficiently. Ccp1 is a well-known H_2O_2 scavenger in *E. coli*. To form CmpdI, the ferric heme of Ccp1 rapidly reacts with H_2O_2, and CmpdI comes back again to its resting stage by the action of two molecules of CycII. Reports suggest H_2O_2 and ONOO(H) involve similar Ccp1-dependent peroxide stress responses in microorganisms [41]. Inorganic peroxides can rapidly react on peroxidases, and peroxides are oxidized [29, 41, 106], making them feasible to act as secondary messengers because of their high specificity, as mentioned in the review article Forman et al. [33]. Reports suggest that the high reactivity of ONOOH towards Ccp1 inhibits signaling. So it is assumed that H_2O_2 and ONOO(H) can elicit a similar antioxidant response when reacting with the sensor Ccp1 [18, 68].

3.3. Flavorubredoxin

Nitric oxide is present throughout the biosphere. In the human body, tightly regulated NO• production generates sufficient NO• to affect neoplastic tissue, pathogens, and gut microflora [31]. Even the nanomolar level of NO• could potently block the terminal oxidase and respiration [40] and changes the regulatory mechanism of the citric acid cycle by destroying its [4Fe-4S] centers. Also, significant secondary toxicity of NO• may occur via reactivity of $ONOO^-$, NO^-, NO_2^-, nitrosothiols, and dinitrosyl-iron [55, 58, 75]. *E. coli* can combat this situation by the activity of flavorubredoxin [85].

In *E. coli*, *yga*A encodes a transcription regulator named b2709, renamed NorR. It regulates the anaerobic NO• reduction and detoxification. Regulation of flavorubredoxin by *yga*K (redesignated as *nor*V), contains a NO•-binding non-heme di-iron center [45]. Facultative eubacteria and anaerobic archaea, including *Desulfovibrio* sp., *Methanococcus* sp., *Treponoma* sp., *Salmonella* sp., *Clostridium* sp., and the photosynthetic cyanobacterium *Synechocystis* sp. also possess the protein flavoRb [50, 71, 115]. *E. coli* flavoRb shares more than 34% amino acid similarity with rubredoxin-oxygen oxidoreductase (ROO) of *Desulfovibrio gigas* [93]. The main distinguishing feature of ROO from flavoRb in that the rubredoxin domain is present as a separate protein. The electrometric properties and Flavin mononucleotide (FMN) stoichiometry proved the presence of the di-iron center in flavoRb of *E. coli* [107]. Reports suggest that rubredoxin oxidoreductase (Rbo) can also reduce ROS, combating oxidative stress in anaerobes [64]. The protective effect is due to the Rbo reduction of the superoxide oxidized [4Fe-4S] clusters. Again another report suggested that ROO and flavoRb can detoxify NO• in anaerobes lacking nitric oxide reductase (Nor), where O_2 reducing respiratory chains are clearly expressed [45]. Reports have shown that these enzymes can function as NO• and/or O_2 reductases, but *E. coli* flavoRb can only use NO• as the substrate, whereas ROO can use both the NO• and O_2 [85]. The critical catalytic site for NO• reduction is di-ferrous (Fe^{2+}–O–Fe^{2+}) center. The hypothetical mechanism involves the reduction of NO• by flavoRb via supplying two electrons at the di-ferrous center, which may result in the formation of two nitroxyl anions. The two molecules of nitroxyl anion can bind to form N_2O and water [37]. Another hypothesis suggests that NO• can reduce to Fe^{3+}–O–Fe^{3+}–$NO_2^-(H^+)$, an intermediate. This intermediate can again react with the second NO• to form N_2O [85] (Fig. 3). This NO• metabolism is crucial for the protection and growth of *E. coli* [45]. The rapid and irreversible O_2-mediated inactivation of NorV in *E. coli* via the formation of intermediates has been reported [38, 50]. Baptista et al. [7] reported that flavorubredoxin is a potent NO• detoxifier under anaerobic conditions. Prolonged exposure to both oxidative and nitrosative stress simultaneously renders it inactivated. Electron paramagnetic resonance (EPR) spectroscopy data showed that the transcription activator NorR lost its activity; thereby, NorR ATPase could not

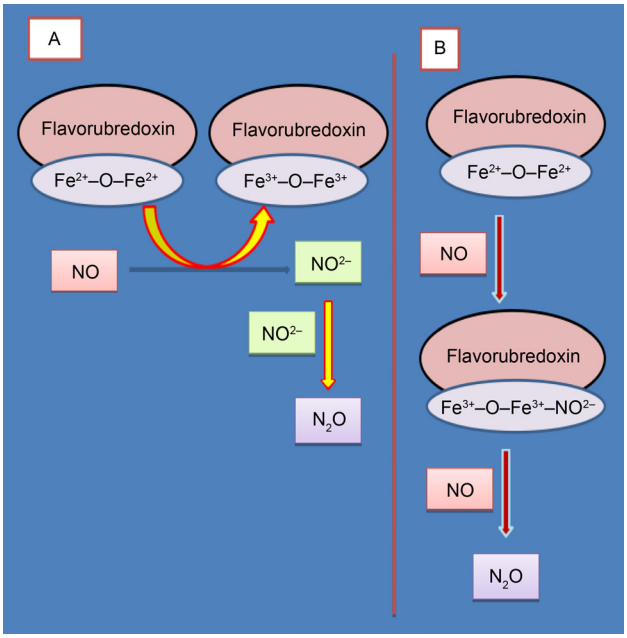


Fig. 3. Mechanism of action of flavorubredoxin

NO• can be reduced to N_2O by two mechanisms involving the [Fe^{2+}-O-Fe^{2+}]. (A) via formation of NO^{2-}, (B) Formation of N_2O from NO• via formation of [Fe^{3+}-O-Fe^{3+}-NO^{2-}] as intermediate.

activate flavorubredoxin under double stress conditions [7]. Another report on NO• detoxification showed that the HmpA mode was the dominant system under aerobic conditions while the NorV mode of detoxification was more dominant under anaerobic conditions. Under microaerophilic conditions, *E. coli* NO• detoxification systems HmpA and NorV systems could not optimally function as both systems are severely compromised. Both systems lose approximately 60% of their combined NO• detoxification capacity [84]. *E. coli* has also been isolated from the urinary tract of humans, where they act as opportunistic microbe. Isolates from patients were analyzed, where they showed to have gained a competitive advantage by developing a mechanism to grow under anaerobic conditions in the presence of low concentrations of endogenously generated NO• by reprogramming its transcriptome. Differential gene expression analysis proved that NO• plays a critical role in their pathogenesis. These adaptations might have helped these microorganisms to colonize the bladder during UTIs successfully. Hence, both flavohemoglobin and flavorubredoxin play a vital role in NO• detoxification to keep the level of NO• in check, and at the same time, these low levels of NO• induce the required pathogenic genes required for colonization [69].

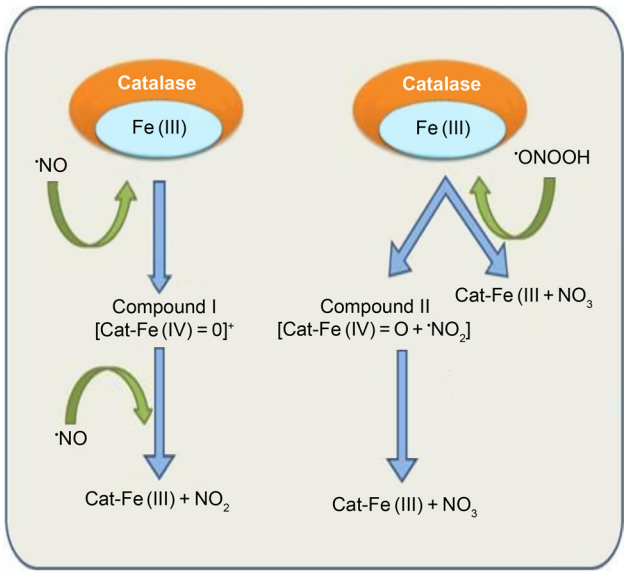


Fig. 4. Mechanism of action of catalase

Catalase has the Fe(III) center which helps in quenching of nitric oxide and peroxynitrite. When catalase encounters NO•, Compound I ([Cat-Fe(IV) = O]$^{+\cdot}$) is formed due to oxidation by NO•. When such Compound I again reacts with another NO•, nitrite (NO_2^-) is formed as end product. On the other hand, when catalase encounters peroxynitrite ((ONOOH), Compound II ([Cat-Fe(IV) = $O^{+\cdot}NO_2$]) is formed which breaks down to nitrite (NO_3^-).

3.4. Catalase

Catalase is a well-known stress response enzyme conserved from prokaryotes to eukaryotes. The primary role of catalase is to detoxify H_2O_2, which helps the cell combat oxidative stress [99]. When oxygen is present in the environment, aerobic bacteria utilize molecular oxygen as a terminal electron acceptor. In the electron reduction step, reactive oxygen species like superoxide (O_2•) and hydrogen peroxide (H_2O_2) form during the conversion of oxygen to water. Hydrogen peroxide gets further degraded into water and oxygen molecules in the presence of catalase. In *Saccharomyces cerevisiae*, catalase not only acts upon hydrogen peroxide but can also detoxify peroxynitrite ($ONOO^-$) [86]. After the generation of peroxynitrite, it can readily react with protein and nucleic acid, leading to cell damage and even death [82, 83]. Some reports speculated that if the concentration of peroxynitrite crosses a certain threshold level, catalase may emerge as protective machinery against peroxynitrite [86].

There are two different catalases in *E. coli*, namely HPI (hydroperoxidase I) and HPII (hydroperoxidase II). HPI, the product of the *kat*G gene, is transcriptionally induced during logarithmic growth in response to low concentrations of hydrogen peroxide [72]. HPI is a tetrameric enzyme that contains a protoheme IX prosthetic group larger than the typical 84 kDa subunits. HPII is a mono-functional catalase but is uncommon in most other respects, including its hexameric structure, the heme-D-like prosthetic group that gives the enzyme its characteristic green color, and the 93 kDa size of the subunit.

The catalase peroxy-nitrito complex formation occurs during the reaction between catalase and peroxynitrite is hypothesized. However, Gebicka and Didik [41] proposed that decay of peroxynitrite does not occur via the formation of compound I ([Cat-Fe(IV) = O]$^{+\cdot}$). Unlike the decay of H_2O_2, the decay of peroxynitrite involves the formation of Compound II ([Cat-Fe(IV) = $O^{+\cdot}NO_2$]) or peroxynitrite directly converts to nitrate by the action of catalase. Then Compound II is reduced by the action of •NO_2 (Fig. 4). Reports suggest that the increasing catalase concentration reduces peroxynitrite-mediated protein tyrosine nitration. It is one of the most substantial evidence of the peroxynitrite scavenging activity of catalase [98]. *In vitro* experiment showed that in the presence of 20 mM NO•, the Soret band of native catalase peak shifted from 406 to 426 nm with an isosbestic point at 415 nm under aerobic and anaerobic conditions. Two new peaks appeared at 540 nm and 575 nm indicating the formation of nitric oxide-catalase complex and ferrous-nitrosyl complex, respectively [15]. Thus, catalase can scavenge the NO• by forming compound II is clearly understood. While NO• can react with compound I of catalase and oxidize to •NO_2. These mechanisms are mainly involved in reducing nitrosative stress effect in *E. coli* [41].

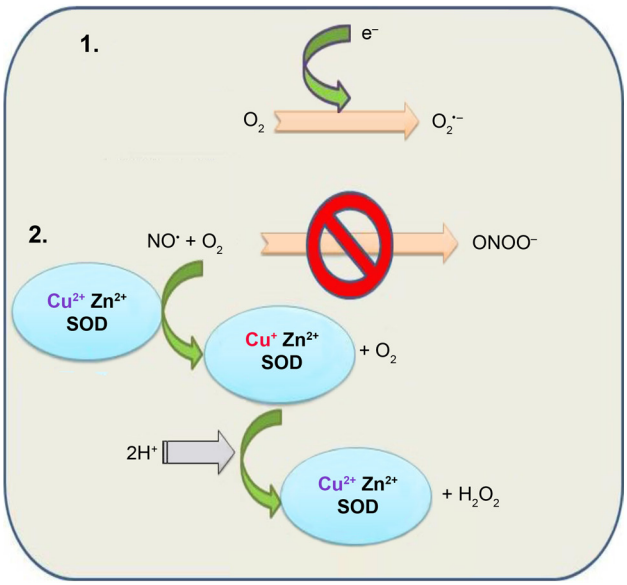


Fig. 5. Mechanism of action of CuZn-SOD

(1) Generation of superoxide (O_2^-) from oxygen by the addition of one electron. (2) The formed superoxide is used up by $Cu^{2+}Zn^{2+}$SOD where Cu^{2+} is reduced to Cu^+ with the formation of molecular oxygen (O_2). The reduced Cu^+ of CuZn-SOD is again oxidized to Cu^{2+} with the formation of H_2O_2. In this way CuZn-SOD inhibits the formation of $ONOO^-$ from NO and O_2^-.

3.5. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is an essential cellular metalloenzyme in prokaryotic and eukaryotic cells that helps to combat or minimize the potential cytotoxicity of the superoxide free radicals. SODs are well known for dismutating superoxide free radicals [52]. The designation of SODs is according to the presence of metal in their metallo-active core. In general, SODs are present in the cytoplasm and periplasmic space of *E. coli*. In *E. coli*, researchers have identified three types of SODs, namely manganese containing SOD (Mn-SOD), iron-containing SOD (Fe-SOD), and copper-zinc containing SOD (CuZn-SOD). Different studies reported that SODs could also act on RNS [65]. This characteristic stress response role is present in CuZn-SOD [1, 49].

CuZn-SOD was first characterized in 1969 by Fridovich and McCord [52]. They found that a blue copper protein could rapidly catalyze superoxide dismutation. Cu/Zn-SOD is a dimeric enzyme that contains zinc and copper, where the histidine imidazole bridges the enzyme's active sites zinc and copper [65]. The zinc present at the active site is bound by aspartate and two histidine molecules with a bridging imidazole, whereas the copper is bound to a water molecule and three additional histidines. In CuZn-SOD, Zn plays a role in stabilizing the protein, and Cu plays an essential role in catalysis via changing its oxidation state Cu^{2+}/Cu^+ [49]. This enzyme is present in the periplasmic space of *E. coli*, and *sodC* gene encodes this protein. A study reported that CuZn-SOD is synthesized exclusively in the aerobic stationary phase in *E. coli*. Though prokaryotic SOD is structurally different from eukaryotic SOD, the function is similar in both cases [1, 49].

CuZn-SOD cannot directly react with the nitric oxide. Instead, it reacts with the superoxide or ROS. CuZn-SOD inhibits the RNS formation by interacting with the different ROS. So it can lower the ROS level in the cell. Thus, NO• cannot react with the ROS; thus, the formation of different RNS within the cell decreases. Simultaneously dismutation reaction of superoxide by both the oxidation and reduction processes are carried out. The copper (Cu) core of the CuZn-SOD is mainly responsible for the oxidation-reduction reaction. In the first step, one superoxide molecule converts to one molecule of oxygen but simultaneously, Cu^{2+} of CuZn-SOD reduces to Cu^+. In the second step, Cu^+ of CuZn-SOD oxidizes back to its normal form while O_2 is reduced to H_2O_2 [1] (Fig. 5). This catalysis reaction directly interferes with the peroxynitrite formation by scavenging free *in vivo* superoxide, available for peroxynitrite formation [1].

3.6. Cytochrome *c* nitrite reductase (NrfA)

E. coli can convert nitrate to ammonium in its membrane with the help of two enzymes, namely periplasmic nitrate reductase and periplasmic nitrite reductase (NrfA). This NrfA can convert nitrate to ammonium by reducing nitrate. However, it can also catalyze the five electron reduction of NO• and two-electron reduction of hydroxylamine [6, 109]. *nrf* operon is mainly responsible for encoding NrfA and NrfB in the *E. coli* chromosome [20]. During the anaerobic growth of *E. coli*, it synthesizes at least five types of *c*-type cytochrome in response to stringent nutritional signals. During anaerobic growth and in the presence of trimethylamine-N-oxide (TMAO), TorC cytochrome formation occurs. Two other *c*-type cytochromes, namely periplasmic *c*-type nitrate reductase and pentaheme cytochrome *c*, are the products of *nrfA* and *nrfB* gene, respectively, located at the *E. coli* chromosome [77]. Nitrite strongly induces the expression of these cytochromes, while nitrate is a weak inducer.

The *nrf* operon encodes the NrfA protein [109]. Cytochrome *c* nitrite reductase embedded in periplasm uses nitrite as a terminal electron acceptor and formate as an electron donor. NrfA activity is strongly suppressed by high nitrate concentration because nitrate is a more potent oxidizing agent, and when available, it is the prime source of energy. *E. coli* holds three protein complexes that can reduce nitrate to nitrite. The *nar*GHJI operon and *nar*ZYWV operon encode cytoplasmic reductases, whereas nap operon encodes the periplasmic nitrate reductase of *E. coli* [74, 109].

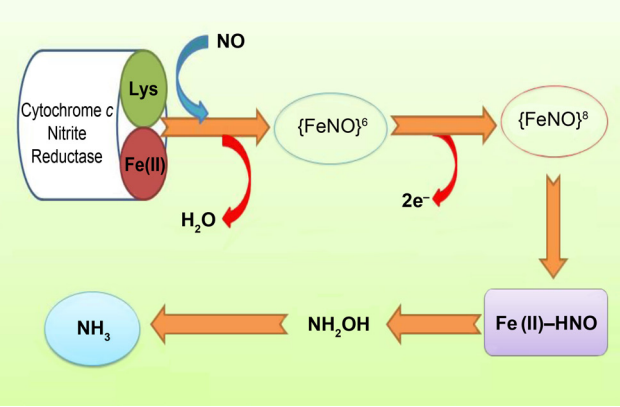


Fig. 6. Mechanism of action of cytochrome *c* nitrite reductase

Cytochrome *c* nitrite reductase encounter NO• and $\{FeNO\}^6$ is formed with the removal of water, followed by the formation of $\{FeNO\}^8$ by removing 2 electrons. After that Fe(II)-HNO complex is formed which reduced to hydroxylamine (NH_2OH). Hydroxylamine is again reduced to ammonia (NH_3).

Characterization of the function of cytochrome *c* nitrite reductase by the analysis of reaction intermediates crystallographic structure and density functional calculation. This enzyme converts nitrite to ammonia without forming any potential reaction intermediate. It carries a unique lysine-coordinate heme group (Fe-Lys) [30] which facilitates nitrite reduction by cleaving the N-O bond heterolytically. The cleavage of the N-O bond occurs when the nitrogen interacts with reduced Fe(II) but not with the Fe(III). This step generates a molecule of water and $\{FeNO\}^6$ species. Another H-bonding network weakens the N-O bond by introducing an electronic asymmetry in the nitrite molecule. Then, NO• is reduced rapidly by cytochrome *c* nitrite reductase via the addition of two electrons in *E. coli.* After that, a constructive two-electron two-proton step leads to the formation of hydroxylamine. Hydroxylamine is further dehydrated to give ammonia and water molecule with the help of the two-electron reduction step (Fig 6). The catalytic cycle is closed with a single electron reduction [30, 104].

3.7. Hybrid cluster protein (HCP)

Hybrid cluster protein is commonly known as "Prismane protein," abundant in *E. coli* and most anaerobic, aerobic, and facultative organisms as a stress response enzyme [103]. An extensive study on this enzyme because of its unusual property of the iron-sulphur cluster is done [104]. Generally, in these proteins, a unique [4Fe-4S] or [2Fe-2S] cluster is present, while at the same time, a novel type of hybrid cluster, [4Fe-2S-2O], has also been reported. This novel protein [4Fe-2S-2O] cluster contains 4Fe atoms, two bridging oxo groups, two bridging sulfides, and an unidentified Fe-like fifth bridging atom [103]. The [4Fe-4S] cluster is similar to those found in ferredoxins. HCPs are related to the carbon monoxide dehydrogenase protein family [27, 66]. *In vitro* studies confirm that HCP produces NH_3 and H_2O due to NH_2OH degradation. [112]. A recent report suggested that HCP inhibits the inactivation of cytoplasmic proteins like aconitase and fumarase from nitrosative stress [110]. Experiments on aconitase and fumarase showed that these enzymes completely lost their catalytic activities when 1.5 μM NO• was mixed every 30 min to cultures of the HCP mutant grown anaerobically. The activity of aconitase and fumarase in hcp^+ is higher than hcp^- mutant. This report also suggested that an NADH-dependent reductase, Hcr is needed for HCP activity. HCP interacts with Hcr and HCP-Hcr complex forms. This complex inhibits the accumulation of NO• via reduction of NO• to N_2O [110]. Balasiny et al. [5] checked the activity of aconitase and fumarase in hcp^+ytfE^+ and hcp^-ytfE^- strains with nitrosative stress under anaerobic conditions. They found that hcp^+ytfE^+ strains showed higher aconitase and fumarase activity. This result clearly explained the prediction that YtfE chelates the NO• from nitrosylated Fe-S cluster proteins, and HCP actively converts this cytoplasmic NO• to N_2O. They also demonstrated how YtfE is involved in HCP induction. YtfE releases NO•, which relieves the NsrR repressor from the HCP promoter [5]. Thus, at lower concentrations of NO• which is insufficient to induce the activation of *norV*, *hmpA*, *nrfA*, both YtfE, and HCP coordinately remove the toxic NO• from the cells under anaerobic conditions. However, even under low concentration NO• when aerobic and micro-aerobic conditions are present, the nitric oxide reductase activity of HCP in *E. coli* is inhibited by flavohemoglobin in an Hcr-independent pathway [91].

The mechanism of action of HCP needs further studies. Nevertheless, it can nullify the effect of RNS via its [4Fe-2S-2O] and [2Fe-2S] center. Recent reports predict that the iron center Fe(II) gets oxidized to Fe(III) in the presence of NO• or RNS, and NO• gets reduced to N_2O [112]. Breakdown of NH_2OH through [2Fe-2S] center is a possible explanation. The reduction of hydroxylamine into ammonia and water may occur via the formation of the $[2Fe^{3+}–2S]$ compound. However, further study is needed to know the mechanism fully. Here we propose a model for the mechanism of action of HCP (Fig. 7).

3.8. Tripeptide glutathione

γ-L-glutamyl-L-cysteinyl-glycine is commonly known as glutathione (GSH). Tripeptide glutathione is the main low-molecular weight thiol in *E. coli.* It helps in scavenging toxic compounds such as naturally occurring electrophile methylglyoxal. GSH is also necessary

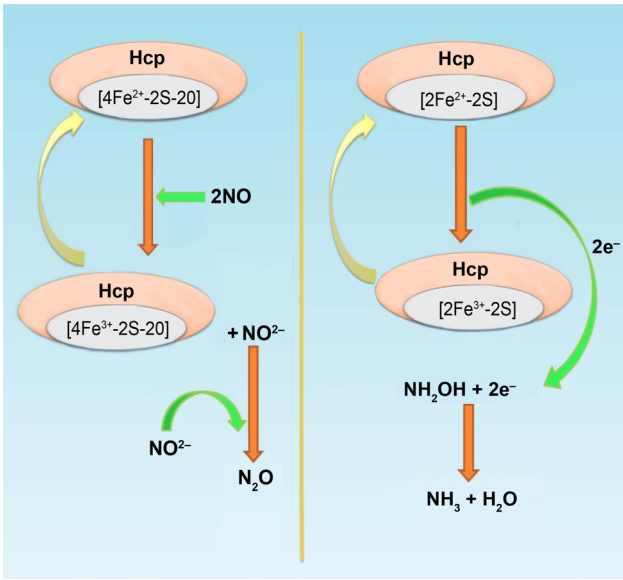


Fig. 7. Predicted mechanism of action of hybrid cluster protein
This predicted figure shows the mode of action of Hcp via two different mechanisms. In one pathway, the [4Fe^{2+}-2S-2O] containing Hcp can react with NO and Fe^{2+} is oxidized to $Fe^{3+}$with the formation of NO^{2-}. NO^{2-} can again react with another NO^{2-} and N_2O is formed as the end product. In another mechanism, [2Fe^{2+}–2S] containing Hcp can react with NO and NH_2OH (hydroxylamine) is formed which may get converted to ammonia (NH_3). In this reaction also Fe^{2+} is oxidized to Fe^{3+} but it can again come back to its normal state.

for several cell processes, like controlling the redox status within the cell [46]. Under normal physiological and stress conditions, the reduced form of GSH is higher than the oxidized form of glutathione (GSSG). GSSG may form during the catalysis of GSH peroxidase (Gpx) or direct oxidation of GSH. GSSG may further be reduced back to GSH with the help of both of glutaredoxin (Grx)/thioredoxin (Trx) system and NADPH-dependent GSH reductase (Gsr) system. The change in the ratio between the reduced and oxidized form of glutathione indicates the redox stress environment within the cell [26]. The review article of Aquilano et al. [4] discusses glutathione's protective role against stress conditions by acting as a co-factor for several detoxifying enzymes, scavenging hydroxyl radicals and singlet oxygen directly.

Nitrosative stress leads to the generation of RNS like NO• and $ONOO^-$, which can damage cellular proteins and nucleic acids. It also affects the redox status of the cell. Studies showed that intracellular GSH could react with NO•/$ONOO^-$. When NO• concentration is high in the cells, then the cysteine molecule of GSH reacts with NO•. GSH acts as a NO• buffering molecule leading to the formation of GSNO. GSNO also acts as an endogenous NO• reservoir [4]. After its formation, GSNO may get transported outside the cell either by the GSH transporter system [118] or denitrosylation enzymes such as GSNO reductase and thioredoxin (Trx) [89]. Another study demonstrated that the incomplete oxidation of GSH under $ONOO^-$ stress conditions might lead to the generation of thiyl radical. This radical initiates an oxygen-dependent chain reaction that results in the formation of peroxyl radicals (one of the potent RNS) which can react with GSH. Thus, the amount of RNS decreases in the cell. Studies also reported that GSNO reductase (GSNOR), first discovered in *E. coli*, can also break down GSNO and remove its hazardous effect. GSNOR converts GSNO to GSSG and NH_3 [63] (Fig. 8). A very recent report proved that in *cydD E. coli* mutant, the intercellular concentration of GSH increases which helps in combating nitrosative stress. The NO• mediated stress tolerance was similar in both *cydD* mutant and wild type. Thus it can be assumed that low molecular weight thiols can restore heme incorporation into a cytochrome complex [48].

3.9. Cytochrome *bd* oxidase

Peroxynitrite is a potent nitrating agent where it directly damages DNA, protein modification/oxidation, and lipid oxidation that imbalances the cell redox homeostasis. The first report on peroxynitrite decomposition by cytochrome *bd* oxidase was by Borisov et al. [12]. A comparison between cytochrome *bd* oxidase and cytochrome *c* oxidase showed that the former was more resistant to $ONOO^-$ where the possible explanation of this resistance is due to the absence of any redox-active copper centers in the enzyme [12]. While it was resistant to $ONOO^-$, results also indicated that it could successfully

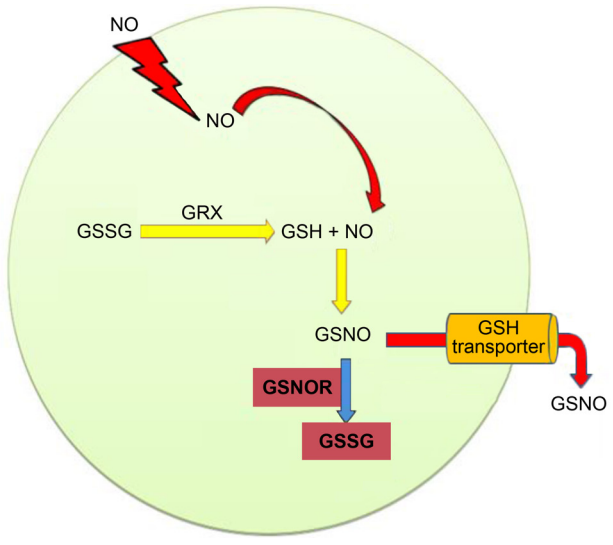


Fig. 8. Basic working principle of GSH under nitrosative stress
Under normal physiological condition, GGSG (oxidized glutathione) converts to GSH (reduced glutathione) by the action of GRX (glutaredoxin). Under nitrosative stress, GSH reacts with NO and stores the NO in the form of GSNO. It can again be transported outside the cell via GSH transporter or GSNO can be broken down to GSSG by the activity of GSNOR (blue arrow).

metabolize $ONOO^-$ to NO•. Time resolved absorption spectroscopy showed peroxynitrite decomposition was proportional to the enzyme concentration, and it increased with the electron flux at an apparent turnover rate of as high as ~ 10 mol $ONOO^-$ (mol enzyme)$^{-1}$ s^{-1}.

Recent reports on uropathogenic *E. coli* (UPEC) experiments on cytochrome *bd* oxidase have shown that this enzyme is also crucial for host colonization of the bladder under nitrosative stress in a microaerobic environment. At the same time, it also helps *E. coli* cells to survive inside macrophages. This enzyme also plays a vital role in biofilm formation, where transcriptomic data showed the upregulation of *cyd*ABX in mature UPEC biofilms [8, 42]. However, a high concentration of NO• renders it inactive [90].

4. Summary

Several reports on characterizing the effect of nitrosative stress in different organisms are present. Although *E. coli* is one of the best understood biochemical systems, very few reports are available to characterize nitrosative stress response. Here we have tried to compile and represent some of the major components involved in nitrosative stress response and their probable mode of action in *E. coli.* To overcome the stress condition, *E. coli* employs various defense systems like flavohemoglobin, which detoxifies NO• under aerobic and anaerobic conditions. Under the anoxic condition, NO• reacts with the reduced Fe(II) portion of HmpA to form highly stable nitrosyl species and gets converted to N_2O. While in the aerobic condition, the reactive NO• gets converted to nitrate as the final product. Another antioxidant system regulation by the enzyme Ccp1 shows the oxidation of H_2O_2 and peroxynitrous acid via the formation of intermediates CompoundI (CmpdI) and CompoundII (CmpdII) with subsequent release of NO_2•. Flavorubredoxin, coded by *norV* gene, is another protein that can detoxify NO• under anaerobic conditions. Hypothetically its mode of action involves NO• reduction via di-ferrous center to form two nitroxyl anions, which then bind with each other to form N_2O and water. This mechanism is strictly anaerobic because of the transcription factor NorR, which is sensitive to the presence of oxygen.

Catalase is an enzyme that protects *E. coli* against peroxynitrite, which gets decomposed to NO_2• via the formation of CompoundII ([Cat-Fe(IV) = $O^+ \cdot NO_2$]). *In vitro* experiments with NO• and catalase indicated the formation of nitric oxide-catalase complex. Catalase is also involved in the detoxification of ROS like H_2O_2. Similarly, another cellular enzyme, superoxide dismutase (SOD), helps *E. coli* overcome nitrosative stress by interacting with different ROS. The SOD directly interferes with peroxynitrite formation by scavenging free superoxide ions. Thus, when ROS levels get decreased inside a cell, RNS cannot be produced since the formation of RNS requires both NO• and ROS. Other enzymes like cytochrome *c* nitrite reductase (NrfA) enzyme detoxifies NO• by adding two electrons that lead to the formation of hydroxylamine. Hydroxylamine then further gets converted to ammonia and water.

On the other hand, the spectrophotometric analysis showed that cytochrome *bd* oxidase could decompose peroxynitrite. A unique mode of action in the hybrid cluster protein model shows that very low concentrations of NO• induce this cluster of proteins. YtfE & HCP complex chelates lower concentrations of NO• under anaerobic conditions. The iron centers of this complex reduce the NO• or RNS to N_2O is hypothesized, as its mode of action needs further research.

The non-enzymatic detoxification system, like the thiols of the cells, actively detoxifies NO•. Here, the GSH helps reduce oxidized proteins that lead to the formation of GSNO, where it either gets transported outside the cell or gets converted to GSSG and NH_3 by GSNOR.

We have tried to compile the different functional strategies utilized by *E. coli* to counteract the nitrosative stress. Thus, more original research is needed to understand the complete mechanism of action under nitrosative stress response in *E. coli.* This review may prove helpful for such studies in the future.

Acknowledgement

The authors acknowledge Subham Saha (PG student) for helping compile the research materials and the University of North Bengal for providing essential infrastructure to carry out this research.

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