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

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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.


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

Abbreviations:
Ccp: Cytochrome c peroxidase, flavoHbs/Hmp: Flavohemoglobin, flavoRbs: Flavorubredoxin, GSNO: S-Nitrosoglutathione, GSSG: Oxidized glutathione, 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•-), peroxy (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•), nitroxy anion (NO•), nitrosionium ion (NOO•), nitryl chloride (NO.Cl) 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...
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• ratio is strictly maintained [94, 95]. However, denitrifying bacteria are not very common in the gastrointestinal tract, where C to NO• ratio is very high. Meanwhile, *E. coli* in the gut can reduce NO•/NO• 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-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* Yfe protein revealed a vital role in repairing the iron-sulfur cluster proteins [56]. In a cellular environment, NO• readily reacts with superoxide anion (O2•−) to produce peroxynitrite (ONOO−) [83]. Peroxynitrite readily reacts with carbon dioxide (CO2) to produce ONOOCO2−, which decomposes to give carbonate radical anion and NO3−. 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 O2•− 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*, dihydrolipoxytransacylase (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• are formed. NO• does not directly react with DNA, but when it oxidizes to N2O3, it becomes
a robust nitrating agent, then it can damage DNA. N\(_2\O\) 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 (\(-N\equiv N\equiv O\)) 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\(_2\O\), 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 flavoHbs 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\(_2\O\)\(_2\) was present. Their experiment showed that H\(_2\O\)\(_2\) 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\(_2\O\)\(_2\) 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 flavoubredoxin (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 flavoHbs 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\(_2\O\) via dimeric species. However, under aerobic conditions, NO\(^-\) mainly reacts with Fe(II)O\(_2\), where preliminary a volatile nitrosylidoxyl complex (Fe(II)OONO) forms and nitrate as the final product [57, 101, 115] (Fig. 1). Kim et al. [114] proved
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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 O2 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 O2. 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 Ne2 atom of H175, the only axial ligand to the heme iron. The sixth coordinate position remains vacant or engaged by a water molecule 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 H2O2 and efficiently active against ONOO− [32]. A recent report suggests that in E. coli, Ccp1 can use H2O2 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-phosphate dehydrogenase (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 H2O2, ONOO(H) also binds to the heme moiety and forms an intermediate termed Compound I (CmpdI) by oxidizing two-electron of peroxidases. The heterolytic cleavage of the ONOOH results in the formation of NO2− 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 Compound I to give CmpdIII and NO•. This CmpdII then transports NO• out from the enzyme active site (Fig. 2). So, not surprisingly,
ONOOH also quickly reacts with \( \text{H}_2\text{O}_2 \) metabolizing enzymes that oxidize \( \text{NO}_2^- \) to \( \bullet\text{NO}_2^- \) including bacterial peroxiredoxins [16, 116].

Although \( \text{H}_2\text{O}_2 \) and ONOOH are efficient substrates of catalase and peroxidase, their stabilities differ significantly. \( \text{H}_2\text{O}_2 \) 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 (Ccpl) reduces \( \text{H}_2\text{O}_2 \) efficiently. Ccpl is a well-known \( \text{H}_2\text{O}_2 \) scavenger in \( E.\text{coli} \). To form Cmpdl, the ferric heme of Ccpl rapidly reacts with \( \text{H}_2\text{O}_2 \), and Cmpdl comes back again to its resting stage by the action of two molecules of CycII. Reports suggest \( \text{H}_2\text{O}_2 \) and ONOO(H) involve similar Ccpl-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 Ccpl inhibits signaling. So it is assumed that \( \text{H}_2\text{O}_2 \) and ONOO(H) can elicit a similar antioxidant response when reacting with the sensor Ccpl [18, 68].

3.3. Flavorubredoxin

Nitric oxide is present throughout the biosphere. In the human body, tightly regulated \( \text{NO}^+ \) production generates sufficient \( \text{NO}^+ \) to affect neoplastic tissue, pathogens, and gut microflora [31]. Even the nanomolar level of \( \text{NO}^+ \) could potently block the terminal oxidase and respiration [40] and changes the regulatory mechanism of the citric acid cycle by destroying its \([4\text{Fe}-4\text{S}]\) centers. Also, significant secondary toxicity of \( \text{NO}^+ \) may occur via reactivity of ONOO\( \text{O}^- \), \( \text{NO}^+ \), \( \text{NO}_2^- \), nitrosothiols, and dinitrosyl-iron [55, 58, 75]. \( E.\text{coli} \) can combat this situation by the activity of flavorubredoxin [85].

In \( E.\text{coli} \), \( ygaA \) encodes a transcription regulator named b2709, renamed NorR. It regulates the anaerobic \( \text{NO}^+ \) reduction and detoxification. Regulation of flavorubredoxin by \( ygaK \) (redesignated as \( \text{norV} \)), contains a \( \text{NO}^+ \)-binding non-heme di-iron center [45]. Facultative eubacteria and anaerobic archaea, including \( \text{Desulfovibrio sp.} \), \( \text{Methanococcus sp.} \), \( \text{Treponema sp.} \), \( \text{Salmonella sp.} \), \( \text{Clostridium sp.} \), and the photosynthetic cyanobacterium \( \text{Synechocystis sp.} \) also possess the protein flavoRb [50, 71, 115]. \( E.\text{coli} \) flavoRb shares more than 34% amino acid similarity with rubredoxin-oxxygen oxidoreductase (ROO) of \( \text{Desulfovibrio gigas} \) [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 \( E.\text{coli} \) [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 \([4\text{Fe}-4\text{S}]\) clusters. Again another report suggested that ROO and flavoRb can detoxify \( \text{NO}^+ \) in anaerobes lacking nitric oxide reductase (Nor), where \( \text{O}_2 \) reducing respiratory chains are clearly expressed [45]. Reports have shown that these enzymes can function as \( \text{NO}^+ \) and/or \( \text{O}_2 \) reductases, but \( E.\text{coli} \) flavoRb can only use \( \text{NO}^+ \) as the substrate, whereas ROO can use both the \( \text{NO}^+ \) and \( \text{O}_2 \) [85]. The critical catalytic site for \( \text{NO}^+ \) reduction is di-ferrous (Fe\( ^{2+}-\text{O}^{-}\text{Fe}^{2+} \)) center. The hypothetical mechanism involves the reduction of \( \text{NO}^+ \) 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 \( \text{N}_2\text{O} \) and water [37]. Another hypothesis suggests that \( \text{NO}^+ \) can reduce to Fe\( ^{2+}-\text{O}^{-}\text{Fe}^{2+}-\text{NO}_2^- \) (H\( ^+ \)), an intermediate. This intermediate can again react with the second \( \text{NO}^+ \) to form \( \text{N}_2\text{O} \) [85] (Fig. 3). This \( \text{NO}^+ \) metabolism is crucial for the protection and growth of \( E.\text{coli} \) [45]. The rapid and irreversible \( \text{O}_2 \)-mediated inactivation of NorV in \( E.\text{coli} \) via the formation of intermediates has been reported [38, 50]. Baptista et al. [7] reported that flavoRb oxidoreductin is a potent \( \text{NO}^+ \) 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](image-url)
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.

3.4. Catalase

Catalase is a well-known stress response enzyme conserved from prokaryotes to eukaryotes. The primary role of catalase is to detoxify H₂O₂, 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 (O₂•⁻) and hydrogen peroxide (H₂O₂) 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 peroxymonosulphite (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 katG 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.

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]⁺) is formed due to oxidation by NO•. When such Compound I again reacts with another NO•, nitrite (NO₂⁻) is formed as end product. On the other hand, when catalase encounters peroxynitrite ((ONOOH), Compound II ([Cat-Fe(IV) = O•-NO₂⁻])) is formed which breaks down to nitrite (NO₂⁻).

![Fig. 4. Mechanism of action of catalase](image-url)

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]⁺) is formed due to oxidation by NO•. When such Compound I again reacts with another NO•, nitrite (NO₂⁻) is formed as end product. On the other hand, when catalase encounters peroxynitrite ((ONOOH), Compound II ([Cat-Fe(IV) = O•-NO₂⁻])) is formed which breaks down to nitrite (NO₂⁻).
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. CuZn-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\(_2\)O\(_2\) [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 nitrite. However, it can also catalyze the five electron reduction of NO\(_{2}^-\) 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 *narGHJI* operon and *narZYWV* operon encode periplasmic reductases, whereas nap operon encodes the periplasmic nitrate reductase of *E. coli* [74, 109].
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]⁺ 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 NH₃ and H₂O due to NH₂OH 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 N₂O [110]. Balasiny et al. [5] checked the activity of aconitase and fumarase in hcp⁺ and hcp⁻ strains with nitrosative stress under anaerobic conditions. They found that hcp⁻ 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 N₂O. 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 N₂O [112]. Breakdown of NH₂OH through [2Fe-2S] center is a possible explanation. The reduction of hydroxylamine into ammonia and water may occur via the formation of the [2Fe⁺−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
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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 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 thyl 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 NH₃ [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...
metabolize ONOO\textsuperscript{-} to NO\textsuperscript{•}. 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 \( \sim 10 \text{ mol ONOO}^\cdot \text{(mol enzyme)}^{-1} \text{s}^{-1} \).

Recent reports on uropathogenic \textit{E. coli} (UPEC) experiments on cytochrome \textit{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 \textit{E. coli} cells to survive inside macrophages. This enzyme also plays a vital role in biofilm formation, where transcriptomic data showed the upregulation of \textit{cydABX} in mature UPEC biofilms [8, 42]. However, a high concentration of NO\textsuperscript{•} renders it inactive [90].

4. Summary

Several reports on characterizing the effect of nitrosative stress in different organisms are present. Although \textit{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 \textit{E. coli}. To overcome the stress condition, \textit{E. coli} employs various defense systems like flavohemoglobin, which detoxifies NO\textsuperscript{•} under aerobic and anaerobic conditions. Under the anoxic condition, NO\textsuperscript{•} reacts with the reduced Fe(II) portion of HmpA to form highly stable nitrosyl species and gets converted to N\textsubscript{2}O. While in the aerobic condition, the reactive NO\textsuperscript{•} gets converted to nitrate as the final product. Another antioxidant system regulation by the enzyme Ccp1 shows the oxidation of H\textsubscript{2}O\textsubscript{2} and peroxynitritic acid via the formation of intermediates CompoundI (CmpdI) and CompoundII (CmpdII) with subsequent release of NO\textsubscript{2}. Flavorubredoxin, coded by \textit{nor}V gene, is another protein that can detoxify NO\textsuperscript{•} under anaerobic conditions. Hypothetically its mode of action involves NO\textsuperscript{•} reduction via di-ferrous center to form two nitroxylnilions, which then bind with each other to form N\textsubscript{2}O 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 \textit{E. coli} against peroxynitrite, which gets decomposed to NO\textsuperscript{•} via the formation of CompoundII ([\textit{Cat}-Fe(IV) = O\textsuperscript{•}\cdot NO\textsubscript{2}]). \textit{In vitro} experiments with NO\textsuperscript{•} and catalase indicated the formation of nitric oxide-catalase complex. Catalase is also involved in the detoxification of ROS like H\textsubscript{2}O\textsubscript{2}. Similarly, another cellular enzyme, superoxide dismutase (SOD), helps \textit{E. coli} overcome nitrosative stress by interacting with different ROS. The SOD directly interferes with peroxynitritate 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\textsuperscript{•} and ROS. Other enzymes like cytochrome c nitrite reductase (NrfA) enzyme detoxifies NO\textsuperscript{•} 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 \textit{bd} oxidase could decompose peroxynitrite. A unique mode of action in the hybrid cluster protein model shows that very low concentrations of NO\textsuperscript{•} induce this cluster of proteins. YtfE & HCP complex chelates lower concentrations of NO\textsuperscript{•} under anaerobic conditions. The iron centers of this complex reduce the NO\textsuperscript{•} or RNS to N\textsubscript{2}O is hypothesized, as its mode of action needs further research.

The non-enzymatic detoxification system, like the thiols of the cells, actively detoxifies NO\textsuperscript{•}. 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\textsubscript{3} by GSNO.

We have tried to compile the different functional strategies utilized by \textit{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 \textit{E. coli}. This review may prove helpful for such studies in the future.

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References

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