Nitric oxide and nitrosative stress tolerance in bacteria

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Abstract
Nitric oxide is not only an obligatory intermediate in denitrification, but also a signalling and defence molecule of major importance. However, the basis of resistance to NO and RNS (reactive nitrogen species) is poorly understood in many microbes. The cellular targets of NO and RNS [e.g. metalloproteins, thiols in proteins, glutathione and Hcy (homocysteine)] may themselves serve as signal transducers, sensing NO and RNS, and resulting in altered gene expression and synthesis of protective enzymes. The properties of a number of such protective mechanisms are outlined here, including globins, flavohemoglobin, diverse enzymes with NO- or S-nitrosothiol-reducing properties and other redox proteins with poorly defined roles in protection from nitrosative stresses. However, the most fully understood mechanism for NO detoxification involves the enterobacterial flavohaemoglobin (Hmp). Aerobically, Hmp detoxifies NO by acting as an NO denitrosylase or ‘oxygenase’ and thus affords inducible protection of growth and respiration, and aids survival in macrophages. The flavohaemoglobin-encoding gene of *Escherichia coli*, *hmp*, responds to the presence of NO and RNS in an SoxRS-independent manner. Nitrosating agents, such as S-nitrosoglutathione, deplete cellular Hcy and consequently modulate activity of the MetR regulator that binds the *hmp* promoter. Regulation of Hmp synthesis under anoxic conditions involves nitrosylation of 4Fe-4S clusters in the global transcriptional regulator, FNR. The foodborne microaerophilic pathogen, *Campylobacter jejuni*, also expresses a haemoglobin, Cgb, but it does not possess the reductase domain of Hmp. A Cgb-deficient mutant of *C. jejuni* is hypersensitive to RNS, whereas *cgb* expression and holoprotein synthesis are specifically increased on exposure to RNS, resulting in NO-insensitive respiration. A ‘systems biology’ approach, integrating the methodologies of bacterial molecular genetics and physiology with post-genomic technologies, promises considerable advances in our understanding of bacterial NO tolerance mechanisms in pathogenesis.

Key words: bacterial stress response, flavohaemoglobin, flavohemoglobin, haemoglobin, nitric oxide, nitrosating agent.

Abbreviations used: GSNO, S-nitrosoglutathione; Hcy, homocysteine; SNP, sodium nitroprusside.

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Perspective
Biochemists and microbiologists have been fascinated by the ability of microbes to transform the oxyanions of nitrogen to gaseous products since the 1880s, even earlier than H.G. Wells’ prophetic novel ‘In the Days of the Comet’. However, it is only comparatively recently that NO has attracted so much attention. Reviewing this area 100 years later, Zumft et al. [1] pointed out uncertainties regarding the functional role and position of NO, particularly whether N2O is formed directly by nitrite reductase or by the intermediary action of an NO reductase. It would have been imprudent to contest the view that ‘progress clearly requires more biochemical and genetic studies in NO metabolism’.

Contributions to this meeting show that NO is an obligatory intermediate in the nitrogen cycle, that NO arises in bacteria through the action of various nitrite reductases, and that it is reduced to N2O by various NO reductases. However, other pathways for both NO appearance and NO disappearance exist. Thus NO in microbes can arise from the activity of respiratory nitrite reductases and perhaps from NO synthases with homology to the well-characterized mammalian enzymes that catalyse the oxidation of arginine to NO and citrulline in the presence of oxygen and certain cofactors. In this contribution, I review briefly mechanisms by which bacteria detoxify NO, outside the nitrogen cycle, necessarily restricting references to reviews, illustrative examples and recent papers. Because the chemistry of NO and its redox-related partners is complex, however, such a description must also address the sources, fates and biological effects of nitrosating agents and other sources of ‘nitrosative stress’.

NO, nitrosative stress and ‘reactive nitrogen intermediates’
NO is a signalling and defence molecule of major importance in biological systems [2]. At concentrations of approx. 10^{-7} M, NO controls blood pressure in mammals and is a messenger in the central and peripheral nervous systems. Sustained NO generation by the immune system at higher concentrations inhibits key enzymes including terminal oxidases and other haem enzymes that bind dioxygen, and Fe-S centres in enzymes such as aconitase. Toxic effects may also arise from reactions involving nitrosation (see below) or the potentially toxic peroxynitrite, formed from reaction of NO with...
superoxide anion [3]. NO, being a lipophilic radical, diffuses across cell membranes and through the cytoplasm. NO is soluble in water (1.6 mM at 37°C) but does not react with it. If generated in a cellular environment at approx. 10⁻⁷ M, NO should have a lifetime of approx. 30 min if it were oxidized to NO₂. In practice, its lifetime may be only a few seconds, as NO reacts rapidly with diverse targets, particularly iron centres, thiols and superoxide. Thus, the relationship between the chemistry and the biological activity of NO is complex, as NO generates new species with modified activity and functions. These products may store NO or exert toxic effects while their formation may initiate redox or conformational changes.

We have emphasized [4] that the redox-related reactive species NO⁺ (nitrosium), NO and NO⁻ (nitroxyl) have unique chemistries [3] and are distinct from each other, reflecting the presence of nitrogen in oxidation states N(III), N(II) and N(I) respectively. Care should be taken to maintain this distinction and to design and interpret experiments accordingly. Inspection of recent experimental work in this area shows that investigators are using a wide range of NO-related compounds and treatments, designed to induce nitrosative stress. These compounds can give quite different biological effects according to their concentration, the availability of oxygen and metal ions in particular, pH, the reactivity of nucleophiles and other variables. In use are S-nitrosothiols, SNPs (sodium nitroprusside) and commercially available NO releasers, such as the NONOates (diazenium-dimido) and NOCs that release NO with specified half-lives varying from a few minutes to many hours. The SNP anion [Fe(CN)₅(NO)]²⁻, with an NO⁺ nitrosoyl group, is an effective nitrosating agent at neutral pH values to S and N nucleophiles. It is often used as, or stated to be, an NO releaser, but this can occur only indirectly through the nitrosation of a thiolate group. The product may then decompose to release NO and oxidized thiol RS or the NO may remain co-ordinated to the Fe centre, which is probably reduced with the reformation of the NO⁺ group. The S-nitrosothiols RSNOS [usually GSNO (S-nitrosoglutathione), S-nitrosoacetylpenicillamine and S-nitrosocysteine] may undergo homolysis to give RS and NO, in some cases in a metal-catalysed reaction. They can also transfer the NO⁺ group to a nucleophile, such as a thiolate, by trans-nitrosation [5]. GSNO, for example, nitrosates thiolates readily. In vivo, the NO⁺ group undergoes several transnitrosations, and there may be an equilibrium distribution of NO⁺ between protein thiol groups and small thiolate molecules. Since nitrosation involves the transfer of the NO⁺ group to a nitrosating agent, to a nucleophilic receptor, such as an amine or, more significantly, a thiol, NO cannot itself act as a nitrosating agent.

The diversity of NO and nitrosative stress tolerance mechanisms in bacteria

NO and its congeners exert toxic effects, and microbes have evolved a number of mechanisms for coping with these species. Table 1 illustrates the scope and depth of research in this area. Here, we focus on the globins and particularly flavohaemoglobinins, since they illustrate all the essential and exciting features of this research area: (i) their synthesis is up-regulated by NO and related species, (ii) their activity consumes NO and thus protects NO-sensitive processes such as respiration and (iii) there is ample genetic evidence from knockout mutants for a definable contribution of certain globins to ‘NO’ resistance.

Haemoglobins – not just for oxygen

Haemoglobins are arguably the best studied of all proteins and recent insights in globin function seem destined to prolong that interest. Microbial globins have been known since Keilin’s time but their functions remained mysterious. We now recognize three main classes of microbial globins, all of which contain members involved in NO detoxification.

Single-domain bacterial haemoglobins

The first bacterial globin to be identified and sequenced was the haemoglobin of Vitreoscilla, an obscure bacterium whose soluble haemoprotein (Vgb) is significantly increased under microaerobic conditions. This protein comprises a single domain, unmistakably globin-like, and protohaem; it lacks the reductase domain seen in the flavohaemoglobins (see below). Despite the evidence that expression of this protein in heterologous hosts can confer some protection from nitrosative stress, the generally accepted view is that Vgb facilitates oxygen utilization, perhaps by directly interacting with a terminal oxidase (for reviews see [11,12]). More persuasive evidence for NO detoxification by a small, single-domain has recently been obtained for Cgb of Campylobacter jejuni. [10] A Cgb-deficient mutant of C. jejuni was hypersensitive to nitrosating agents (GSNO and SNP) and a NO-releasing compound (spermine NONOate). Expression of Cgb was strongly and specifically induced following exposure to nitrosative stress. In the absence of preinduction by exposure to nitrosative stress, no difference was seen in the degree of respiratory inhibition by NO or the half-life of NO when cells of the wild-type and the cgb mutant were compared. However, for cells in which the Cgb expression was induced by GSNO, the period of respiratory inhibition by NO was significantly longer in the cgb mutant compared with the wild-type and this correlated with the NO disappearance. We conclude that, unlike the archetypal single domain globin Vgb, Cgb forms a specific and inducible defence against NO and nitrosating agents.

Truncated globins

The truncated globins are a recently discovered, widespread group of globins, shorter by 20–40 residues than other single domain globins. The globin fold is formed from only four α-helices arranged in a two-over-two α-helical sandwich. Although certain trHbs supply pathogens with O₂, others appear to be involved in NO stress tolerance. In Mycobacterium bovis, the truncated globin, HbN, protects...
Table 1 | Proteins with roles in tolerance to NO and nitrosative stress

<table>
<thead>
<tr>
<th>Class</th>
<th>Protein</th>
<th>Organism(s)</th>
<th>Function/reactions catalysed</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globins</td>
<td>Myoglobin, haemoglobin</td>
<td>Higher animals</td>
<td>Transient formation of peroxynitrite-globin; quantitative formation of nitrate without nitration of globin</td>
<td>Physiological role in NO tolerance? NO scavenging?</td>
<td>[6,7]</td>
</tr>
<tr>
<td></td>
<td>Truncated globin (HbN)</td>
<td><em>Mycobacterium bovis</em></td>
<td>Conversion of NO into nitrate</td>
<td>NO-inducible NO uptake; mechanism unknown</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Vitreoscilla globin (Vgb)</td>
<td><em>Vitreoscilla</em> sp.</td>
<td>NO consumption, accumulating nitrate</td>
<td>Mechanism unknown; globin confers growth tolerance to SNP</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Single-domain globin (Cgb)</td>
<td><em>Campylobacter jejuni, C. coli</em></td>
<td>Confers enhanced resistance to NO and nitrosating agents</td>
<td>Mechanism unknown</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>Flavohaemoglobin (Hmp)</td>
<td><em>E. coli</em>, <em>Salmonella, B. subtilis, Erwinia, many others</em></td>
<td>Enzymic detoxification of NO by conversion into nitrate</td>
<td><em>hmp</em> expression up-regulated by NO and nitrosating agents</td>
<td>[4,11,12]</td>
</tr>
<tr>
<td>Reductases</td>
<td>Flavorubredoxin (NorVW)</td>
<td><em>E. coli</em></td>
<td>NO reduction and detoxification</td>
<td>Up-regulated in response to NO and nitroprusside</td>
<td>[13–15]</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c nitrite reductase (NrfA)</td>
<td><em>E. coli</em></td>
<td>NO reduction and detoxification</td>
<td>Nrf-strains show higher NO sensitivity</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>GSH-dependent formaldehyde dehydrogenase</td>
<td><em>E. coli</em>, yeast, mammalian cells</td>
<td>GSNO reductase</td>
<td>Controls cellular levels of S-nitrosothiols and S-nitrosylated proteins</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Cytochrome and quinol oxidases</td>
<td>Mitochondria of higher organisms, bacteria</td>
<td>NO reduction at Cu₆⁺</td>
<td>Activity may be restricted to oxidases in haem-Cu₆⁺ family; physiological significance not clear</td>
<td>[18,18a]</td>
</tr>
<tr>
<td>Others</td>
<td>Hybrid cluster protein</td>
<td><em>E. coli</em></td>
<td>Hydroxylamine reductase?</td>
<td>Up-regulated by nitrosating agents</td>
<td>[19,20]</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c’ (CycP)</td>
<td><em>Rhodobacter capsulatus</em></td>
<td>NO reductase, forming N₂O</td>
<td>CycP mutants are hypersensitive to nitrosothiols and NO</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>AhpC (peroxiredoxins)</td>
<td><em>Salmonella, M. tuberculosis</em></td>
<td>Confers enhanced resistance to peroxynitrite and nitrosating agents, but not to NO donors</td>
<td>Mechanism unknown</td>
<td>[22,23]</td>
</tr>
</tbody>
</table>
aerobic respiration from the effects of NO and, in vitro, HbN stoichiometrically oxidizes NO to nitrate [8]. Evidence for an NO-protective role also comes from expressing HbN in heterologous host cells, M. smegmatis and Escherichia coli, under the control of the vgb promoter [24]. It is assumed that function in vivo of such heterologously expressed single-domain globins depends on association with a host reductase to create, perhaps transiently, a protein with electron transfer capabilities for NO metabolism, as in flavohaemoglobins.

**Flavohaemoglobins**

The best-understood microbial globins are the flavohaemoglobins. The *E. coli* flavohaemoglobin (Hmp) was the first microbial globin for which a gene sequence was obtained. Hmp is a 44 kDa monomer having a haem domain 46% identical with Vgb. The C-terminal domain closely resembles ferredoxin-NADP⁺ reductase in having highly conserved binding sites for FAD and NAD(P)H. Purified Hmp possesses haem B and FAD, their presence confirmed by the crystal structures of flavohaemoglobins [11,12]. This reductase domain transfers electrons from NAD(P)H to haem-bound ligands and is essential for Hmp functions [25].

The first direct evidence that a flavohaemoglobin was involved in protection against NO and its congeners was the finding that *hmp* transcription is up-regulated by NO, SNP, GSNO and related species [26]. Subsequently, an NO-resistant mutant of *E. coli* was shown to exhibit an O₂-dependent NO-consuming activity referred to as ‘NO dioxygenase’ attributed to ‘hmp’ [26a]. It is now the accepted view that Hmp detoxifies NO, supported by the following key observations. Indeed, no other physiological role for flavohaemoglobins has been established. For pre-2004 references, see [11,12]. The main observations are (i) null *hmp* mutants of *Salmonella* and *E. coli* are hypersensitive to killing by GSNO; (ii) the haem of Hmp is readily reducible by physiological substrates (NAD(P)H) through electron transfer from FAD, and Hmp catalyses redox chemistry with NO and O₂ at the haem; (iii) under anoxic conditions, Hmp reduces NO to NO− with subsequent formation of N₂O, although the physiological significance of this is questioned; (iv) the level of NO resistance of respiration in *E. coli* correlates with the level of Hmp. Respiration in cells preinduced by treatment with SNP is totally resistant to NO concentrations up to 50 μM, whereas respiration of an *hmp* mutant is highly sensitive to submicromolar NO; (v) null *hmp* mutants of *Salmonella* and *E. coli* are hypersensitive to killing by human macrophages; and (vi) the *hmp* gene is up-regulated by NO or nitrosating agents. Indeed, *hmp* is consistently one of the most highly up-regulated genes observed in genome-wide transcription profiling of *E. coli*, *Salmonella* and *Bacillus subtilis* cultures exposed to nitrosating agents [27,28] (J. Flatley, J. Barrett, S. Pullan, M. Hughes, J. Green and R. Poole, unpublished work) or in *Salmonella* following infection and induction of NO synthesis in J774 cells.

Although our understanding of *hmp* up-regulation is incomplete, several mechanisms have been identified and studied so far [4]. One involves the interaction between S-nitrosothiols and homocysteine (Hcy) and illustrates clearly how the use of a nitrosating agent can effect cellular changes independent of NO. In this model, Hcy is nitrosated by GSNO (which has been experimentally demonstrated), thus depriving MetR (a LysR family DNA-binding protein) of its co-regulator. One gene activated by MetR with Hcy as cofactor is *glyA*, which, in *E. coli*, is adjacent to *hmp* and divergently transcribed from it. Increased Hcy levels, achieved either by exogenous Hcy or in certain *met* mutants, decrease *hmp* expression. However, in the absence of Hcy, MetR binds at a site proximal to *hmp*, and up-regulates *hmp* transcription. It is important to recognize that this mechanism may not explain *hmp* regulation by NO itself. First, NO induces *hmp* expression anoxically under which conditions NO will not nitrosate Hcy (although the presence of metal ions, for example, might allow NO⁺ formation from NO). Secondly, although the S-nitrosoHcy generated on reaction with GSNO breaks down to release NO, which could itself be the inducer, the reaction of Hcy with SNP (which also induces *hmp*) forms a more stable species from which NO is not released.

Anoxygenically, the global regulator Fnr is also involved in the regulation of *hmp* since an *fnr* mutation enhances *hmp-lacZ* expression [26]. The [4Fe-4S]⁺ cluster of Fnr is oxygennable and controls protein dimerization and site-specific DNA-binding. However, NO also acts anaerobically with the Fe-S cluster of purified Fnr forming a dinitrosyl-iron cysteine complex [29]. Fnr inactivated by O₂ or NO binds specifically to an Fnr box centred at +5.5 within the *hmp* promoter (P₉₉) but with lower affinity. Dose-dependent up-regulation of P₉₉ *in vivo* by micromolar NO concentrations of pathophysiological relevance is abolished by mutation of *fnr*, and NO also modulates expression from model Fnr-regulated promoters. Thus, Fnr can respond not only to O₂, but also to NO, with major implications for global gene regulation in bacteria.

In *Salmonella* too, Hmp provides NO-inducible protection from S-nitrosothiols and acidified nitrite, but here the NO-sensing mechanism involves the Fe-responsive regulator Fur. Interestingly, a recent genome-wide profile of the response of aerobically grown *E. coli* to the same agents demonstrated that, for some genes, the NO sensing was mediated by the modification of transcription factors containing iron or redox-active cysteine residues, including NorR and Fur [27]. A role for Fur may, however, require revisiting since in our experiments, the choice of a chemically defined medium appears to negate the effect of GSNO on Fur (J. Flatley, J. Barrett, S. Pullan, M. Hughes, J. Green and R. Poole, unpublished work).

Aerobically, Hmp catalyses a reaction in which NO is transformed to give the innocuous NO₃⁻ ion. Although it is clear that Hmp of *E. coli* catalyses the overall reaction:

\[2\text{NO} + 2\text{O}_2 + \text{NAD(P)H} \rightarrow 2\text{NO}_3^- + \text{NAD(P)} + \text{H}^+\]

the reaction mechanism is disputed. Gardner et al. [13] envisaged the reaction of oxygen with ferrous haem to give oxy-Hmp, which in turn reacted with NO to form nitrate.
However, at biologically relevant O₂ and NO concentrations, Hmp preferentially binds NO, not O₂, which then reacts with O₂ to form nitrate. In effect, the reaction involves the oxidation of haem-bound nitroxy (NO⁻) equivalent and has been called an O₂ nitroxylation or denitrosylase reaction [30]. The formation of a haem-bound nitroxy equivalent in this enzyme, as well as in the nematode Ascaris globin and haemoglobin, suggests that the peroxidase-like active site that we have observed in resonance Raman examination of Hmp has evolved for handling of NO.

Conclusions and outlook
What can we expect next? Although much has been learned of bacterial responses to the potentially toxic effects of NO and other forms of nitrosative stress, our knowledge is rudimentary. We know something about the two major NO-detoxifying enzymes, the flavohaemoglobin and the flavorubredoxin, of the most intensively studied microorganism. We might smugly assume that other proteins in these classes will be found to have similar functions in other organisms and recently a fungal pathogen has been shown to utilize both a flavohaemoglobin and GSNO reductase [31]. But there remain some pathogens, for example of plants, which must encounter NO in their lifestyles about which next to nothing is known. And what would be the consequences of damage from NO and other forms of nitrosative stress if the proteins that we have identified thus far were ineffective? How severe are the consequences of NO-related damage? Which proteins and cellular processes are the first casualties and is damage repaired? Post-genomic methods are throwing up genes and proteins that seem to be important in NO stress that we simply do not understand.

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