Nitrate reduction pathways in mycobacteria and their implications during latency

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Mycobacterial persistence has gained a lot of attention with respect to developing novel antitubercular drugs, which could drastically reduce the duration of tuberculosis (TB) therapy. A better understanding of the physiology of Mycobacterium tuberculosis, and of the metabolic state of the bacillus during the latent period, is a primary need in finding drug targets against persistent TB. Recent biochemical and genetic studies of nitrate reduction in mycobacteria have revealed the roles of distinct proteins and enzymes involved in the pathway. The differential degree of nitrate reduction among pathogenic and non-pathogenic mycobacterial species, and its regulation during oxygen and nutrient limitation, suggest a link between nitrate reduction pathways and latency. The respiratory and assimilatory reduction of nitrate in mycobacteria may be interconnected to facilitate rapid adaptation to changing oxygen and/or nitrogen conditions, increasing metabolic flexibility for survival in the hostile host environment. This review summarizes the nitrate metabolic pathways operative in mycobacteria to provide an insight into the mechanisms that M. tuberculosis has evolved to adapt successfully to the host environment.

Introduction

Tuberculosis (TB), caused by Mycobacterium tuberculosis, is the second leading infectious cause of mortality worldwide (Cegielski et al., 2002; Dye et al., 1999). It is estimated that two billion people, one-third of the world’s population, are already infected with M. tuberculosis (Glaziou et al., 2009). Every year, approximately eight million people develop active disease and two million die of TB (World Health Organization, 2008). More than 95% of these deaths occur in the developing world, where the disease is predominantly found (Borgdorff et al., 2002). In addition, the public health impact of M. tuberculosis has become increasingly severe because of the HIV epidemic (Gandhi et al., 2006). Being dually infected with HIV and TB worsens the outcome of both diseases, as is evident from the fact that TB is the leading cause of death among HIV-positive individuals (World Health Organization, 2007). Current chemotherapy (directly observed treatment, short course; DOTS) to treat TB uses a combination of rifampicin, isoniazid, ethambutol, streptomycin and pyrazinamide (Gbayisomore et al., 2000). The requirement of this antibiotic regimen to continue for a lengthy period of time increases the probability of non-compliance as well as the threat of the development of drug resistance by M. tuberculosis. A lengthy period of treatment of more than 6 months is required in order to achieve a sputum-negative clinical stage. The existence of a differential population of replicating and non-replicating bacilli forces the current regimen of available drugs to be continued until the relapse rate reduces to less than 5%. The latent form of TB is therefore the major hurdle in developing effective chemotherapy against TB (Dick, 2001; Gomez & McKinney, 2004; Stewart et al., 2003).

Efforts towards developing a drug against the latent or persistent stage of M. tuberculosis are hindered by its poorly understood metabolism. There has been considerable recent interest in studying persistence and dormancy in mycobacteria, and now we have begun to understand the mechanisms by which the bacilli themselves stop replicating upon encountering adverse conditions but still keep their ability to resume growth when conditions become favourable (Wayne & Sohaskey, 2001; Cosma et al., 2003). There is now good evidence that the initiation of latency can be triggered by depletion of nutrients, shifts in pH and depletion of oxygen in granuloma structures formed by the host as an attempt to fight the infection (Aly et al., 2006; Betts et al., 2002; Höner zu Bentrup & Russell, 2001; Parrish et al., 1998; Rustad et al., 2008, 2009; Schnappinger et al., 2003). To circumvent nutritional shortages, M. tuberculosis has developed alternative means for generating energy. The pathogen increases the breakdown and utilization of fatty acids, which are abundant in the caseous environment of the granuloma, as a source of carbon and energy during infection in the lungs (McKinney et al., 2000;
Wayne & Lin, 1982). Several genes involved in lipid metabolism have been studied for their role during persistent \textit{M. tuberculosis} (Ehrt & Schnappinger, 2007; Russell et al., 2010). Some of these genes were later found to be essential for growth and persistence \textit{in vivo} (Liu et al., 2003; Muñoz-Elias & McKinney, 2005; Muñoz-Elias et al., 2006; Pandey & Sassetti, 2008; Savvi et al., 2008; Shi & Ehrt, 2006). The carbon metabolism of \textit{M. tuberculosis} thereafter became a major area of research to address the persistence-related issues of the disease. More recent evidence from central carbon metabolism analysis has highlighted the importance during bacterial growth arrest of metabolic pathways involved in energy generation and nutritional adaptation (Rhee et al., 2011; Shi et al., 2010).

The importance of the nitrate metabolic pathways of \textit{M. tuberculosis} was established based on the evidence that a shift from the aerobic replicating to the hypoxic non-replicating stage of TB bacilli in the granuloma induced the expression of enzymes related to nitrate reduction (Höner zu Bentrup & Russell, 2001; Shi et al., 2005). There has recently been interest in the concept that nitrate reduction pathways play an important role during the adaptation of \textit{M. tuberculosis} to an anaerobic microenvironment and therefore in the establishment of latency. Nitrate respiration could provide energy for mycobacterial metabolism during the latent stage in the absence of oxygen in the caseous necrotic granuloma. Recent reports have accumulated evidence to elucidate the molecular mechanism of nitrate metabolism in \textit{M. tuberculosis}. Here, we review the pathways and enzymes involved in nitrate reduction to help understand the latent stage and identify potential targets against persistent \textit{M. tuberculosis}.

The nitrate respiration pathway in mycobacteria

Studies of mycobacterial nitrate reduction were earlier limited to a role in the classification and identification of the genus \textit{Mycobacterium}, after an extensive study 40 years ago showed that \textit{M. tuberculosis} reduces nitrate to nitrite, whereas \textit{Mycobacterium bovis} and \textit{M. bovis} BCG have no discernible nitrate reductase (NR) activity (Virtanen, 1960; Wayne & Doubek, 1965). An \textit{in vitro} model of dormancy later showed increased nitrate reduction by \textit{M. tuberculosis} when subjected to gradual depletion of oxygen (Wayne & Hayes, 1998). It was then hypothesized that this hypoxically induced nitrate reduction probably serves a respiratory function in supporting the hypoxic shiftdown of \textit{M. tuberculosis} from aerobic growth to non-replicative persistence. The respiratory function of the nitrate reduction induced during hypoxic shiftdown to dormancy was further supported when the complete genome sequence of \textit{M. tuberculosis} revealed the presence of genes homologous to the anaerobic NR of \textit{Bacillus subtilis} (Camus et al., 2002; Cole et al., 1998). Four genes, \textit{narG}, \textit{narH}, \textit{narJ} and \textit{narL}, clustered together as \textit{narGHJI} in an operon, were found to encode an anaerobic NR in \textit{M. tuberculosis} (Fig. 1). Later, \textit{M. bovis} BCG was also discovered to carry the same gene cluster, and a single nucleotide polymorphism (SNP) within the promoter of the \textit{narGHJI} operon was demonstrated to be the reason for its decreased NR activity compared with \textit{M. tuberculosis}.

\textbf{Fig. 1.} The nitrate respiration pathway in mycobacteria. The organization of the electron transport chain for nitrate respiration in the plasma membrane is shown. Respiratory NR (NarGHJI) is composed of three subunits: a catalytic \(x\) subunit (NarG), a soluble \(\beta\) subunit (NarH) and a quinol-oxidizing \(\gamma\) subunit (NarI) (Moreno-Vivian et al., 1999). \(x\) and \(\beta\) subunits are anchored to the cytoplasmic side of the membrane by the \(\gamma\) subunit. A \(\delta\) polypeptide, which is not part of the final enzyme, participates in the assembly and stability of the \(x\beta\) complex prior to its membrane attachment. The enzyme uses the quinol (Q) pool as the physiological electron donor and generates a proton motive force by a redox loop mechanism. NarI oxidizes quinols at the periplasmic side of the membrane, releasing two protons into the periplasm. Electrons are passed to NarG via the Fe–S centre of NarH to reduce nitrate, with consumption of two cytoplasmic protons. Nitrite produced by the reduction of nitrate is exported outside the cell via the transport protein NarK2 anchored in the plasma membrane. Cyt, cytochrome.
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*tuberculosis* (Stermann *et al.*, 2003, 2004). Indeed, the characterization of polymorphic nucleotides in the *narGHJI* promoter in different subspecies of the *M. tuberculosis* complex subsequently showed that all *M. tuberculosis* strains are not necessarily confined to the −215T *narGHJI* SNP, implicating the differential prevalence of ‘ancestral’ and ‘modern’ *M. tuberculosis* families (Goh *et al.*, 2005). Later it was found that an increase in NR activity during hypoxic shiftdown was not due to the induced expression of *narGHJI* but due to the induction of the nitrate transporter encoded by *narK2* (Hutter & Dick, 2000; Sohaskey & Wayne, 2003). The differential activity of *narK2* was also responsible for the decreased NR activity of *M. bovis* BCG compared with *M. tuberculosis* (Sohaskey & Modesti, 2009). This evidence clarified that *NarGHJI* is constitutively expressed in both *M. tuberculosis* and *M. bovis* BCG under both aerobic and hypoxic conditions, and that it is NarK2 which regulates the NR activity. The expression of *narK2* is induced during hypoxia, which allows the transport of nitrate into and nitrite out of the cell.

Further experiments showing (1) inhibition of NR activity by potassium ferricyanide even in the absence of oxygen, and (2) induction of NR activity by nitric oxide even in the presence of oxygen, suggested that NarK2 senses the reox state of the cell and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing, conditions (Sohaskey, 2005). The reducing environment created by the absence of oxygen or the presence of nitric oxide increases the levels of reduced components of the electron transport chain such as cytochrome oxidase (Fig. 1). As a result, a decreased electron flow could be a part of the signal required for NarK2 activity. NarK2, as a membrane protein, is ideally located to sense components of the electron transport chain. The quinones/quinols of *M. tuberculosis*, which are non-protein components of electron transport and are freely diffusible in the membrane, could serve not only as the electron source for NR but also as an indicator of oxygen levels. Unexpectedly, the transcription of both *narGHJI* and *narK2X* is independent of nitrate and nitrite levels. NR activity in *M. tuberculosis* appears to be independent of the substrate concentration, as determined by mRNA levels and assays of cell extracts. However, it was found that the presence of nitrate in the medium enhanced the survival of *M. tuberculosis* when it was subjected to sudden anaerobiosis (Sohaskey, 2008). This indicated that NR might play an important role during the sudden interruption of aerobic respiration caused by hypoxia or nitric oxide in the in vivo environment. In addition, nitrate respiration could also help *M. tuberculosis* to generate resistance against acid and nitrogen species stress, as demonstrated in a recent study (Tan *et al.*, 2010). In the absence of nitrate during hypoxia, mild acidic conditions promoted the dissipation of the proton motive force, rapid ATP depletion and cell death. Survival of *M. tuberculosis* under hypoxia was restored when nitrate, an effective terminal electron acceptor for anaerobic respiration, was added to the medium. Nitrate respiration was found to be equally important in protecting hypoxic non-replicating *M. tuberculosis* against the reactive nitrogen species which are likely to be encountered by the pathogen in the infected host.

Also identified in the *M. tuberculosis* genome during sequencing was a gene designated *narX*, which has been proposed to encode a ‘fused NR’ (Hutter & Dick, 1999). This proposal was made because the predicted product of *narX* would be a protein with homology to parts of the NarG, NarJ and NarL proteins, although its actual function is unknown. Although there are two sets of genes in *M. tuberculosis*, *narGHJI* and *narX*, that show homology with other prokaryotic NR genes, only *narGHJI* has been found to be responsible for nitrate reducing activity in culture. This was proved when insertional inactivation of the *narGHJI* locus eliminated the production of nitrite, and this activity could be restored by complementation with a plasmid-borne copy of the genes (Sohaskey & Wayne, 2003). Insertion in *narX* had no effect on the reduction of nitrate.

**Role of the respiratory NR in the survival and pathogenesis of mycobacteria**

Evidence for the role of NR in virulence was reported when immunodeficient SCID mice infected with the *M. bovis* BCG *narG* mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain (Weber *et al.*, 2000). Nevertheless, the mutant induced tissue damage in the lungs of immunocompetent mice, although it was cleared from many organs, unlike the wild-type strain. *M. bovis* BCG lacking anaerobic nitrate NR (*NarGHJI*) failed to persist in the lungs, liver and kidneys of immunocompetent (BALB/c) mice (Fritz *et al.*, 2002). In SCID mice, the bacilli caused chronic infection despite the disruption of *narG*, even though the growth of the mutant was severely impaired in the lungs, liver and kidneys. The persistence and growth of the *narG* mutant of BCG in the spleens of both mouse strains appeared largely unaffected, and indicated that the role of the enzyme in pathogenesis is tissue-specific. The dependence on NR for persistence in a tissue-specific manner was supported by evidence of different microenvironments in various organs in terms of the availability of oxygen (Tsai *et al.*, 2006; Via *et al.*, 2008). The limited presence of oxygen in certain organs provided evidence of a close resemblance between Wayne’s *in vitro* hypoxic dormancy model and the *in vivo* latent stage of TB in host tissues (Lim *et al.*, 1999; Wayne & Hayes, 1996; Wayne, 2001). Contrary to these findings, another study demonstrated that an *M. tuberculosis* mutant defective in NR was able to persist in the lungs of chronically infected mice to an extent similar to that of wild-type *M. tuberculosis* (Aly *et al.*, 2006). This study showed experimentally that *M. tuberculosis*-induced granulomas in mice were not severely hypoxic, even after 1 year of aerogenic infection. Mice chronically infected with *M. tuberculosis* displayed a wide range of oxygen partial
pressures in different parts of the lungs, which, however, rarely approached the anoxic conditions consistently found in necrotizing tumours. On the other hand, rabbit, guinea pig, non-human primate and human tuberculous granulomas were found to be severely hypoxic in similar experimental settings (Tsai et al., 2006; Via et al., 2008). Rabbit, guinea pig, non-human primate or human models of M. tuberculosis infection would therefore be more appropriate to study the latency phenotype seen during human infection. It would be interesting to see how an M. tuberculosis strain defective in nitrate reduction (narG knockout strain) would survive and persist in rabbit/guinea pig/non-human primate lung granulomas compared with wild-type M. tuberculosis.

Mycobacterium smegmatis, which follows a similar pattern of oxygen depletion-induced dormancy in Wayne’s model, was later also identified to carry a similar respiratory NR (Dick et al., 1998; Khan & Sarkar, 2006). Application of NR-specific inhibitors had a profound effect on the survival of M. smegmatis in Wayne’s in vitro dormancy model, which further suggested a significant role for nitrate metabolism during the latent period of TB. The effect of these inhibitors on the survival of M. smegmatis during dormancy was not seen when nitrate was absent from the medium, which indicated that the presence of nitrate was an obligatory requirement for the functioning of NR during anaerobic respiration. However, the inhibitors used in the latter study may not have been very specific and could have exerted their effect on the viability of M. smegmatis during dormancy due to their off-target action. The existence of an alternative respiratory mechanism and/or fermentation pathway could not be ruled out, since M. smegmatis was able to survive in the absence of nitrate during the dormant stage in the above studies. Observations from the Khan and Sarkar study also indicate that in the absence of oxygen, nitrate respiration is preferred above other alternative mechanisms if nitrate is present in the medium (Khan & Sarkar, 2006). It would be interesting to know how a narG mutant of M. smegmatis would survive in Wayne’s hypoxic dormancy model in the presence and absence of nitrate. This should also answer the question about the availability of alternative respiratory chains during hypoxic dormancy.

Sources of nitrate in the host cell as a substrate for NR

Apparently, nitrate as a substrate for NR can also be provided in sufficient amounts in the lungs, liver and kidneys of infected individuals. The reported estimates of net nitrate synthesis by mammalian tissue vary greatly, and range from 0.15 to 1 mM per day (Kelm, 1999). Within tissue, nitrate is mainly a product of spontaneous degradation of nitric oxide. Nitric oxide, in contrast, is produced enzymically by three different nitric oxide synthetases (Stuehr, 1999). An inducible nitric oxide synthase is expressed in response to inflammatory and proinflammatory mediators (Bogdan et al., 2000). A variety of cells, including hepatocytes, can be induced to synthesize nitric oxide (Brown, 1999). Significant amounts of nitrate are detected in the urine of mice infected with bacteria, suggesting that nitrate is available in the kidney, especially in animals undergoing an inflammatory process (Flesch & Kaufmann, 1991). It is intriguing to speculate that the inflammatory process due to mycobacterial infection in the lungs, liver and kidneys might increase the amount of nitrate within granulomas and thereby provide an additional supply of this nutrient for the anaerobic metabolism of the pathogen. Proof of the presence of nitrate in host cells was confirmed when THP-1 macrophages infected with M. tuberculosis were shown to be able to produce nitrite even without an exogenous supply of nitrate in the growth medium (our unpublished data). Nitrite as an end-product of nitrate reduction can only be detected during the late stage of infection of THP-1 macrophages with M. tuberculosis, indicating the limited availability of oxygen in the host cell when the bacterial burden rises.

The nitrate assimilation pathway in mycobacteria

It has been known for a long time that pathogenic and saprophytic mycobacteria can utilize nitrate as a source of nitrogen (Hedgecock & Costello, 1962). However, the molecular basis of nitrate assimilation in mycobacteria remained unknown. Assimilation of nitrate in other bacteria involves a multistep pathway, which requires a series of enzymes functioning in combination (Fig. 2) (Berks et al., 1995; Hochstein & Tomlinson, 1988; Moreno-Vivian et al., 1999). Nitrate is first converted into nitrite with the help of an NR, and subsequently is converted into ammonia by nitrite reductase (Nir). Ammonia is normally assimilated into glutamine via glutamine synthetase (GS). In the bacterial system, this nitrogen could then be distributed for various cellular nitrogen requirements of through glutamine 2-oxoglutarate aminotransferase (GOGAT). Although genes with homology to a distinct assimilatory NR have not been identified in the mycobacterial genome, a role for the respiratory NR NarGHJI is suspected in the assimilation of nitrate. This is based on the fact that NarGHJI is expressed constitutively and mediates the reduction of nitrate not only under anaerobic conditions but also under aerobic conditions. The extent of nitrate reduction under aerobic conditions is much lower than that of anaerobic nitrate reduction. This could be explained by the fact that under aerobic conditions, NarK2 will not be able to transport nitrate inside the cell to make it available for NarGHJI. Even so, a low level of nitrate could be transported inside the cell through diffusion under aerobic conditions, which may be responsible for the basal level of nitrate reduction seen under aerobic conditions. The proposal that NarGHJI also serves as an assimilatory function found further support when a similar respiratory enzyme was identified in M. smegmatis and inhibition of this enzyme blocked the assimilation of nitrate (Khan et al., 2008). The capability for nitrate assimilation of M. smegmatis was shown in this study by using nitrate, nitrite or ammonia as the sole nitrogen source,
along with the respective inhibitors of the enzymes involved in the pathway. Most significantly, this assimilation pathway remained active even when the organism was shifted to an oxygen depletion-induced dormant stage. An inhibitor of the enzymes involved in the pathway also had a significant effect on the viability of the dormant bacilli, and hence indicated the importance of this pathway during the persistent stage. Later studies showed that a narG mutant of *M. tuberculosis* failed to grow on nitrate, which further proved that the nitrate assimilation pathway is also functional in virulent mycobacterial strains and that a respiratory NR mediates this assimilation (Malm et al., 2009). This study also identified that a nirBD-encoded nitrite reductase is responsible for the reduction of nitrite to ammonia, as a nirB mutant failed to grow on both nitrate and nitrite. The expression of nirBD was found to be regulated by a response regulator, GlnR, as a glnR mutant also failed to grow on nitrate and nitrite. A specific binding site for GlnR within the nirB promoter was identified and confirmed by electrophoretic mobility shift assay. Therefore, it was recognized that NarGHJI and NirBD mediate the assimilatory reduction of nitrate and nitrite, respectively, and that GlnR acts as a transcriptional activator of nirBD.

The involvement of GS in the assimilation of nitrate was also proved when l-methionine sulfoximine (L-MSO), an inhibitor of GS, was observed to block the reduction of nitrite in *M. smegmatis*. However, other nitrogen sources could be assimilated even after the blockage of glutamine dehydrogenase by using the alternative enzyme glutamate dehydrogenase (Khan et al., 2008). This underlines the significance of GS in the assimilation of nitrate when the availability of other nitrogen sources is restricted under nutrient-starved conditions in chronically infected tissues. Studies regarding the availability and capability for utilization of other nitrogen sources during chronic infection are needed to provide a deeper insight into the possible link between latency and nitrogen assimilation, in order to identify future drug targets. In addition, the *in vitro* findings on the assimilation of nitrate during the active and dormant stages of tubercle bacilli need to be verified in appropriate animal models that have a closer resemblance to dormancy *in vivo*.

**Conclusions**

For understanding mycobacterial pathogenesis and persistence, the presence of essential nutrients in the host and their metabolism by the pathogen remain an area of considerable interest. Recent progress on nitrate metabolic pathways, which are operative in different mycobacterial species and under different physiological conditions, have helped in understanding the biology of mycobacteria during the latent stage. These reports support the emerging paradigm that the respiratory reduction of nitrate through NarGHJI of *M. tuberculosis* could provide energy for the latent stage survival of the pathogen. Given that the latent stage is assumed to be anaerobic, the reduction of nitrate to nitrite may generate the ATP necessary to keep the pathogen alive in the absence of oxygen as a terminal electron acceptor. Therefore, NarGHJI might play a major role in maintaining the viability of the pathogen and could be an attractive drug target to kill dormant tubercle bacilli. The regulation of respiratory nitrate reduction by the redox state of the cell opens up the prospect of targeting the mycobacterial component NarK2, which controls the transport of nitrate into and nitrite out of the cell. Evidence that the nitrate assimilatory pathway is operative during the active replicating and the non-replicating stages of mycobacteria has also clarified that nitrate can serve as a source of nitrogen when the availability of other nitrogen sources is limited under nutrient-deprived conditions. Ongoing studies of the availability of nitrogen sources to *M. tuberculosis* during the acute and persistent stages of the disease in different tissues will clarify the significance of the *in vitro* findings to *in vivo* infection. In future, further investigation of downstream pathways and the enzymes involved in the complete nitrogen metabolism of *M. tuberculosis* during its survival in the latent stage will strengthen our understanding of the nitrate reduction pathways. In conclusion, the present *in vivo* and *in vitro*
evidence suggests that nitrate reduction pathways could play a vital role during the survival of the latent stage of *M. tuberculosis*, and that targeting enzymes involved in these pathways may help in developing novel antitubercular drugs.

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