FEBS Letters 580 (2006) 4031–4041

Oxygen binding and NO scavenging properties of truncated hemoglobin, HbN, of *Mycobacterium smegmatis*

Amrita Lama, Sudesh Pawaria, Kanak L. Dikshit*

Institute of Microbial Technology, Sector 39 A, Chandigarh 160036, India

Received 28 April 2006; revised 2 June 2006; accepted 13 June 2006

Available online 22 June 2006

Edited by Stuart Ferguson

Abstract **Unraveling of microbial genome data has indicated that two distantly related truncated hemoglobins (trHbs), HbN and HbO, might occur in many species of slow-growing pathogenic mycobacteria. Involvement of HbN in bacterial defense against NO toxicity and nitrosative stress has been proposed. A gene, encoding a putative HbN homolog with conserved features of typical trHbs, has been identified within the genome sequence of fast-growing mycobacterium, *Mycobacterium smegmatis*. Sequence analysis of *M. smegmatis* HbN indicated that it is relatively smaller in size and lacks N-terminal pre-A region, carrying 12-residue polar sequence motif that is present in HbN of *M. tuberculosis*. HbN encoding gene of *M. smegmatis* was expressed in *E. coli* as a 12.8 kD homodimeric heme protein that binds oxygen reversibly with high affinity ($P_{50} \sim 0.081$ mm Hg) and autooxidizes faster than *M. tuberculosis* HbN. The circular dichroism spectra indicate that HbN of *M. smegmatis* and *M. tuberculosis* are structurally similar. Interestingly, an *hmp* mutant of *E. coli*, unable to metabolize nitric oxide, exhibited very low NO uptake activity in the presence of *M. smegmatis* HbN as compared to HbN of *M. tuberculosis*. On the basis of cellular heme content, specific nitric oxide dioxygenase (NOD) activity of *M. smegmatis* HbN was nearly one-third of that from *M. tuberculosis*. Additionally, the *hmp* mutant of *E. coli*, carrying *M. smegmatis* HbN, exhibited nearly 10-fold lower cell survival under nitrosative stress and nitrite derived reactive nitrogen species as compared to the isogenic strain harboring HbN of *M. tuberculosis*. Taken together, these results suggest that NO metabolizing activity and protection provided by *M. smegmatis* HbN against toxicity of NO and reactive nitrogen is significantly lower than HbN of *M. tuberculosis*. The lower efficiency of *M. smegmatis* HbN for NO detoxification as compared to *M. tuberculosis* HbN might be related to different level of NO exposure and nitrosative stress faced by these mycobacteria during their cellular metabolism.**

Keywords: Hemoglobin; HbN; HbO; Nitric oxide; Acidified nitrite; Nitrosative stress; Oxygen uptake; *Mycobacterium smegmatis*

1. Introduction

Exploration of mycobacterial genome sequences in recent years has brought many novel insights into the biology of this fascinating group of organisms, leading to the identification of several previously unknown genes, including presence of genes for novel hemoglobins (Hbs) [1,2]. Two genes, *glb*N and *glb*O, encoding truncated hemoglobins (trHbs), HbN and HbO, respectively, have been first detected in *Mycobacterium tuberculosis* and, subsequent unraveling of mycobacterial genome data suggested that these Hbs may be ubiquitous in mycobacteria. Three distinct types of trHbs (HbN, HbO and HbP) have been identified within the mycobacterial genome [3]. The extent of amino acid identity between members of these three groups is less than 18% suggesting that these hemoglobins are distinct from each other and may be playing different function(s) in mycobacterial cellular metabolism. The opportunistic pathogen, *M. avium*, carries all three types of trHbs (HbN, HbO and HbP), whereas, intracellular pathogens like, *M. tuberculosis*, *M. bovis*, *M. marinum*, etc. carry two trHbs, HbN and HbO. Interestingly, the obligate intracellular pathogen, *M. leprae*, that has extensive reduction of its genome [2] and carries a minimum set of genes for its survival and pathogenicity, has retained at least one hemoglobin (HbO), suggesting that Hb-like proteins may be vital for the intracellular regime of pathogenic mycobacteria. Functions of these mycobacterial hemoglobins (Hbs) are not very well understood at present and may be diverse.

Studies on mycobacterial Hbs have been mainly concentrated on HbN and HbO of *M. tuberculosis* [4–8]. Physiological studies performed on *M. bovis* demonstrated that trHbO is expressed during all growth phases, whereas, HbN expression is induced only during stationary phase [4,6] indicating that these oxygen-binding proteins are required at different growth stages of mycobacteria. Although both HbN and HbO display characteristic 2-over-2 alpha-helical globin fold, there are distinct structural differences between these two trHbs. The three dimensional structure of *M. tuberculosis* HbN is characterized by the presence of an extra N-terminal pre-A helical region and extended apolar tunnel/cavity connecting the heme distal pocket to two distinct protein surface sites [9]. It has been speculated that these unique cavities present in HbN may provide an alternative port for the diffusion of ligands towards the distal site where solvent access through the classical E7 gate path is completely impaired due to orientation of E-helix and packing of the pocket by side chains of distal site residues [9,10]. Structural and biochemical characteristics of HbN of *M. tuberculosis* suggest that its oxygen binding stereochemistry, B10 hydrogen

*Corresponding author. Fax: +91 172 2690585.
E-mail addresses: kanak@imtech.res.in, kanak@imtech.ac.in (K.L. Dikshit).

0014-5793/$32.00 © 2006 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2006.06.037

bonding and unstrained heme-iron proximal coordination [11,12] may favor oxygen and nitric oxide (NO) interactions. Studies conducted in our laboratory have established that *M. tuberculosis* HbN has a potent oxygen dependent NO dioxygenase (NOD) activity and relieves nitrosative stress in heterologous hosts [7], very similar to flavohemoglobins [13,14]. Being single domain hemoglobin, how HbN is able to display an efficient NOD activity is not known at present. Integration of both globin and reductase domains together is essential for the NOD function of flavohemoglobin [15]. An electron donating partner protein, involved in modulating the function of *M. tuberculosis* HbN, has not been identified so far. However, potent NO-scavenging activity displayed by HbN of *M. tuberculosis* indicates that its primary function may be to protect the bacilli against toxic NO produced by the host macrophages during intracellular infection and latency that may be vital for the pathogenicity of *M. tuberculosis* [16].

Computational and sequence analysis of available mycobacterial genome data indicated the presence of Hb encoding genes in the non-pathogenic, fast growing, saprophytic *Mycobacterium smegmatis* as well. *M. smegmatis* neither enters epithelial cells nor persist in professional phagocytes although it has been known to cause soft tissue and bone infections in rare cases [17]. Hemoglobins of any fast-growing mycobacteria have not been studied so far. New insight into the functionality of trHbs can be gained if comparative data on functionality of this interesting group of small hemoglobins of slow- and fast-growing mycobacteria are available. In the present study, we have reported spectral and ligand-binding characteristics of truncated hemoglobin, HbN, of *M. smegmatis* and demonstrated that its functional properties are distinct from HbN of *M. tuberculosis*.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

E. coli JM109 and *E. coli* BL21DE3 strains were utilized for routine cloning and expression of recombinant genes. *E. coli* RB9060 (Δ*hmp*) strain was kindly provided by Prof. Ninfa (University of Michigan). Cultures of *E. coli* strains were grown in Luria-Bertani (LB) or Terrific Broth (containing 24 g of Yeast Extract, 12 g of Bacto-Tryptone, 12.3 g of K_2HPO_4, 2.3 g of KH_2PO_4) medium at 37 °C at 180 r.p.m. unless mentioned otherwise. *Mycobacterium smegmatis* mc_2 155 [18] was grown in Middlebrook 7H9 (Difco) supplemented with ADC (10% bovine serum albumin fraction V, dextrose and sodium chloride). When required, ampicillin and kanamycin (Sigma) were added at a concentration of 100 μg/ml and 30 μg/ml, respectively. Plasmids, pBluescript (Stratagene), pET9b (NEB) and pUC8:16 [19] were used for cloning and expression of recombinant genes as described earlier [7]. The oligonucleotides were custom synthesized by Integrated DNA Technologies Inc. Nitric oxide (NO; 98.5%) was obtained from Aldrich or saturated NO was prepared as described earlier [20] following the published procedure [21].

2.2. Cloning and expression of the *glbN* gene of M. smegmatis

The nucleotide sequence of *glb*N gene of *M. smegmatis* was retrieved after BLAST search from the unfinished genome sequence data of *M. smegmatis* available at TIGR site. The HbN encoding gene of *M. smegmatis* was expressed under the control of the *Vitreoscilla* hemoglobin (VHb) gene promoter following the strategy used earlier for the expression of HbN encoding gene of *M. tuberculosis* [7]. The forward (5′-GATCCTTAAGATGACGAGCATCTAAGAGCAGATCGGCGGC-3′) and reverse (5′-GAAGGGATCCTCACGACGTGCGGGCCGAGGCGATCTC-3′) primers were designed on the basis of gene sequence of HbN of *M. smegmatis*. The forward and reverse primers contained *Aff*II and *Bam*HI site, respectively. The HbN encoding gene was retrieved from the genomic DNA of *M. smegmatis* mc^2 155 after polymerase chain reaction (PCR) amplification and sequenced completely to verify its authenticity. The *vgb* gene promoter, carried on plasmid pUC8:16 [19], was selectively separated by isolating 2.8 kb *Aff*II–*Bam*HI fragment and ligated with the *Aff*II–*Bam*HI digested PCR product of *glb*N gene of *M. smegmatis*. The resulting HbN expressing plasmid was designated as pSGN. The HbN encoding genes were also overexpressed in mycobacteria under the constitutive promoter of 19 kD antigen of *M. tuberculosis* present on *E. coli*-mycobacteria shuttle vector, p19Kpro following the strategy described earlier [7]. Briefly, HbN encoding gene of *M. tuberculosis* and *M. smegmatis* were amplified by PCR using gene specific primers carrying *Bam*HI restriction site at the 5′ end and *Pst*I site at the 3′ end and cloned at *Bam*HI–*Pst*I site of p19Kpro. The resulting plasmids, carrying HbN gene of *M. tuberculosis* and *M. smegmatis*, were designated as pRPN-2 and pSGN-2, respectively.

2.3. Isolation, purification and characterization of HbN of M. smegmatis

For protein purification, cell culture of *E. coli* JM109 overexpressing *glb*N gene of *M. smegmatis*, was harvested by centrifugation at $14000 \times g$ for 10 min at 4 °C and resuspended in 10 mM Tris · Cl (pH 8.0) having 10 mM dithiothreitol, 1 mM EDTA, 45 μg/ml phenylmethylsulphonyl flouride (PMSF), 500 μg/ml RNase and 10 U of DNase I. Cells were lysed by sonication and subjected to ultracentrifugation at $170000 \times g$ at 4 °C for 2 h. The clear reddish brown cell lysate, thus, obtained was loaded on Ion-Exchange Column (DEAE-Sepharose CL4B, Pharmacia), equilibrated with 10 mM Tris · Cl (pH 8.0) and eluted using 0.12 M NaCl. It resulted in nearly 80% pure preparation of hemoglobin exhibiting distinct reddish brown color. This fraction was further purified by Gel filtration chromatography on to a Superdex 75 column equilibrated in 10 mM Tris · Cl (pH 8.0) and protein was eluted in 0.15 M NaCl in 10 mM Tris · Cl (pH 8.0). The protein and hemoglobin profile was monitored at 280 and 414 nm, respectively.

2.4. Spectral and ligand binding studies

Absorption spectra of whole cells or protein preparation were recorded using Shimadzu or Perkin Elmer Lambda 35 spectrophotometer. NO binding spectra were recorded by using anoxic preparation of HbN. Briefly, 6–8 μM solutions of different species of HbN in 0.1 M sodium phosphate buffer (pH 7.0) was placed in a rubber sealed cuvette and bubbled with nitrogen gas (99.9%, Sigma Gas Ltd.) for 10 min with gentle agitation to remove oxygen. Saturated solution of NO was then injected anoxically and spectra of NO-bound HbN were recorded. The oxygen affinity of HbN was checked using the tonometer as mentioned earlier [6] or Hemox Analyzer (TCS scientific Corp.).

2.5. Heme assays

Total cellular heme content was determined according to the method of Appleby [22]. Briefly, approximately $2–3 \times 10^9$ cells were washed by centrifugation with minimal salt medium (60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$ and 1.7 mM sodium citrate) and were suspended in 0.6 ml of alkaline pyridine reagent containing 2.2 M pyridine and 0.1 N NaOH and lysed with 30 s sonic burst with a microprobe sonicator. The resulting lysate was then clarified by centrifugation for 10 min at $25000 \times g$ to remove insoluble debris. Heme concentration was calculated from absorption difference at 556 and 539 nm for the dithionite-reduced and ferricyanide-oxidized sample.

2.6. Western blotting

For Western blotting, purified protein or cell extracts (10 to 15 μg of protein/slot) were resolved on 15% SDS–PAGE and transferred onto a nitrocellulose membrane (0.45 μM) in a mini trans-blot apparatus (Bio-Rad). Immobilized proteins were probed with primary (Polyclonal antisera raised against HbN of *M. tuberculosis*) and secondary (horseradish peroxidase-conjugated goat anti-rabbit IgG) antibodies and developed using diaminobenzidine and hydrogen peroxide.

2.7. NO consumption assay and determination of cell survival against NO donor and nitrosative stress

NO consumption activity of cells was monitored polarographically as described previously [20]. NO consumption buffer assay contained 60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.6 mM $(NH4)_2SO_4$, 1.7 mM so-

dium citrate, 10 mM glucose and 200 μg/ml chloramphenicol. Sodium nitroprusside (SNP) and acidified nitrite were used to generate nitrosative stress to check cell survival against these NO releasing agents. To check the response of *M. smegmatis* HbN against reactive nitrogen species, growth profile of *E. coli* expressing HbN of *M. smegmatis*, was determined at pH 6.0 and 7.5 in buffered LB medium supplemented with 10 and 30 mM sodium nitrite and compared with isogenic strain carrying HbN of *M. tuberculosis* by observing OD_{600} periodically. Survival of recombinant *E. coli* cells against SNP was determined as described previously [7]. Briefly, control and recombinant *E. coli* RB9060 cells, expressing hemoglobin, were grown aerobically in LB medium up to an optical density at 600 nm of 0.6. The cells were then treated with different concentrations of SNP for 60 min, serially diluted and plated to check the survival of cells. Results were expressed as the percentage of viable cells present in the control culture without any treatment.

3. Results

3.1. Structural features of M. smegmatis HbN and its comparison with other HbN type truncated hemoglobins

Truncated hemoglobin, HbN, homologs are less represented in bacterial genome sequences compared to its other molecular relative, HbO, that are present widely in bacterial and plant species. Exploration of genome data of *M. smegmatis* indicated that it might also carry HbN encoding gene very similar to the pathogenic strains of mycobacteria. Structure based sequence alignment of *M. smegmatis* along with other mycobacterial HbN (Fig. 1) revealed that it is relatively smaller in size than its counterpart present in *M. tuberculosis* and lacks 12-residue long positively charged N-terminal sequence motif constituting the pre-A region of HbN, although structural features, e.g., Tyr-B10, Phe-CD1, Leu-E7, Phe-E14, His-F8, three Gly motifs, crucial for attaining the trHb fold, and residues defining the protein matrix tunnel, are all conserved in this mycobacterial Hb. Preservation of trHbs signature sequences in *M. smegmatis* HbN indicates that it can attain two-over-two alpha-helical structure and may function as hemoglobin. The highly polar and charged N-terminal motif is present in all known mycobacterial HbN except *M. smegmatis*, however, its relevance in protein function is currently unknown. Detailed sequence comparison of HbN type [7] trHbs revealed that other HbN type trHbs do not carry such additional secondary structure at the N-terminus suggesting that it may not be crucial for the structural integrity of trHb fold and may be specific to certain mycobacterial HbN.

3.2. Expression and purification of recombinant trHbN of M. smegmatis

Our initial attempt to express *M. smegmatis* HbN encoding gene under T7 promoter did not result in good yield of soluble protein. Therefore, the *Vgb* gene promoter, which has been utilized earlier to express HbN encoding gene of *M. tuberculosis* [7], was used to clone and express HbN encoding gene of *M. smegmatis* in *E. coli*. It resulted in overexpression of *M. smegmatis* HbN as a soluble protein, constituting nearly 5–6% of total cellular protein that imparted a reddish brown tinge to the recombinant *E. coli* cells. SDS–PAGE analysis of these cells confirmed the presence of a 12.8 kD protein corresponding to the predicted size of HbN of *M. smegmatis* (Fig. 2A, Panel I) which is nearly 1kD shorter than HbN of

Fig. 1. Structure-based multiple sequence alignment of HbN of *M. smegmatis* with other mycobacterial HbN type trHbs. The globin fold topological positions are shown on the top of the aligned sequences. Important residues with respect to coordination of the heme and ligand binding properties are marked and conserved residues of trHbs family are highlighted on black box. Conserved and similar residues of HbN are shown in light shaded boxes and bold letters.

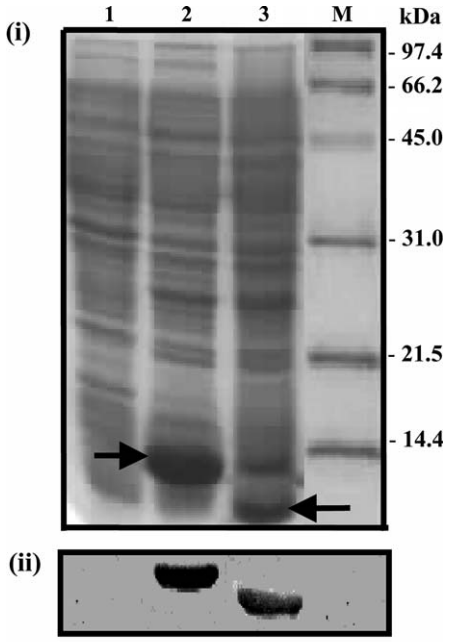

Fig. 2A. Expression of *M. smegmatis* HbN in *E. coli*. The total cellular protein content (10–15 μg) of recombinant cells expressing trHbN was resolved on 15% SDS–PAGE as described under Section 2 and protein bands were visualized after Coomassie blue staining and after Western blotting using HbN-specific polyclonal antibodies. Panel I. (1) *E. coli* JM109-pUC 18; (2) *E. coli* JM109 expressing HbN of *M. tuberculosis*; (3) *E. coli* JM109 expressing HbN of *M. smegmatis*; (4) Molecular weight marker. Panel II. Western blot analysis of HbN expressing cells of *E. coli*. Lanes 1–4 have the same samples as given in panel I.

M. tuberculosis. Recombinant *M. smegmatis* HbN was purified to near homogeneity using Ion Exchange, Phenyl Sepharose and Gel filtration chromatography (Fig. 2B) that revealed its molecular mass corresponding to 25 ± 1 kD, which is close to that expected for the dimeric form of 12.8 kD protein.

3.3. Spectral and functional properties of M. smegmatis HbN

The absolute absorption spectra of the *E. coli* cells, carrying *M. smegmatis* HbN, exhibited the presence of a Soret peak at 416 nm along with alpha and beta peaks at 580 and 543 nm (Fig. 3A) which is very similar to oxygenated form of *M. tuberculosis* HbN and other oxyhemoglobins and myoglobins, suggesting that predominant form of *M. smegmatis* HbN remains oxygenated *in vivo*. The deoxygenated ferrous derivative of *M. smegmatis* HbN shows a soret absorbance band at 430 nm and CO difference (reduced CO–reduced) spectra had maxima at 570, 533 and 419 nm, which is typical of hemoglobin exhibiting reversible ligand binding. Optical spectrum of the ferric species of *M. smegmatis* HbN at pH 7.5 exhibited absorption maxima at 406, 501 and 573 nm that is typical of hexa-coordinate high spin form of hemoglobin. The calculated oxygen concentration at 50% saturation of *M. smegmatis* HbN (Table 1) was found to be slightly higher (P_{50} = 0.081 ± 0.015 mm Hg) than that of HbN of *M. tuberculosis* (P_{50} = 0.013 mm Hg) but much lower than that of sperm whale Mb (P_{50} = 0.51 mm Hg). Upon standing at room temperature, oxygenated hemoglobins and myoglobins autooxidize to the met form. However, oxyform of *M. tuberculosis* HbN is highly stable and displays very slow rate of autooxidation [3]. In comparison, autooxidation rate of *M. smegmatis* HbN was nearly one and a half-fold higher (Table 1). Exposure of anoxic solution of both species of HbN to NO resulted in immediate reaction with these hemoproteins. Optical spectra of NO-bound proteins suggest that both HbN are able to interact with NO (Fig. 3B). *M. smegmatis* HbN exhibited strong cross reactivity (Fig. 2A, Panel II) with monospecific polyclonal antisera raised against HbN of *M. tuberculosis*, indicating serological similarity between these two HbN type mycobacterial trHbs.

3.4. CD spectra of HbN of M. smegmatis

Since HbN of *M. smegmatis* lacks pre-A, N-terminal region that is present in HbN of *M. tuberculosis*, we compared the CD spectra of these two HbN type hemoglobins to check if they have any structural differences. Near-UV CD spectra of both

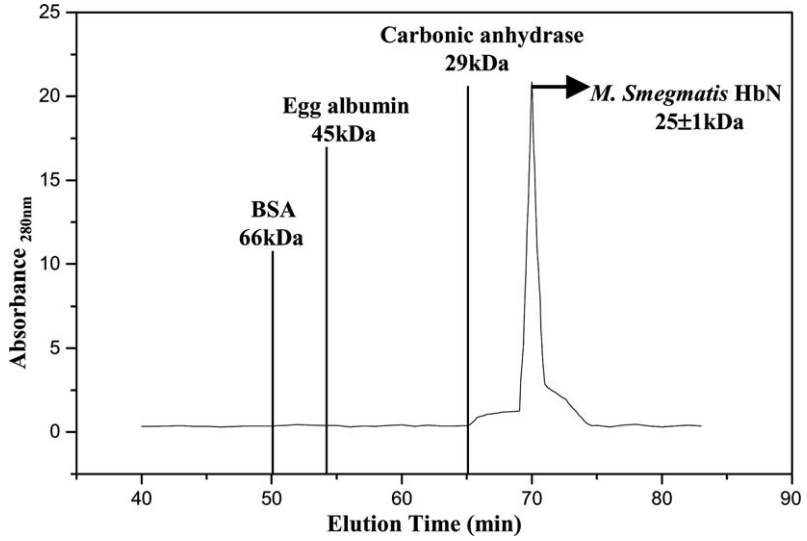

Fig. 2B. Size exclusion chromatography of trHbN of *M. smegmatis*. The elution profile of HbN and molecular weight standard are shown.

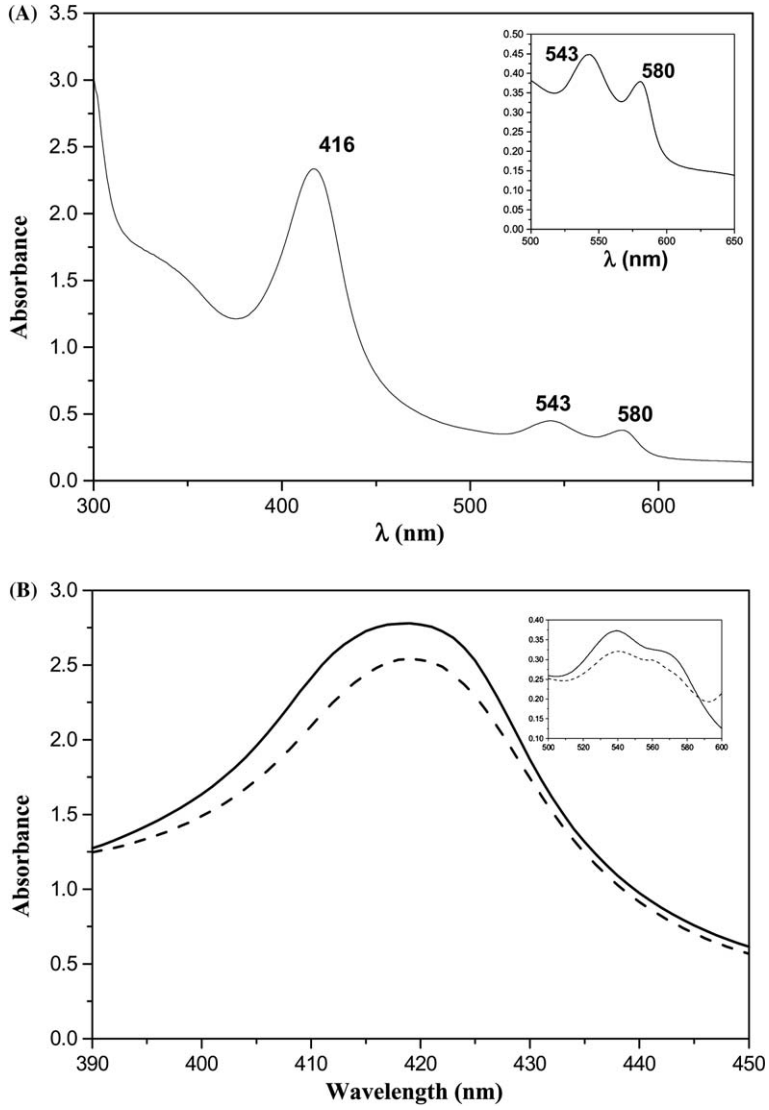

Fig. 3. Spectral characteristics of HbN of *M. smegmatis*. (A) Absolute absorption spectra of *E. coli* cell extract carrying HbN of *M. smegmatis*. (B) Absorption spectra of ferrous NO ligated species of HbN of *M. smegmatis* (dashed line) and *M. tuberculosis* (continuous line).

Table 1
Functional properties of truncated hemoglobin, HbN, of *M. smegmatis*

	Molecular weight (kDa)	Aggregation state	O_2 affinity (P_{50} mm/Hg)	Autooxidation ($t_{1/2}$ h)
HbN (*M. smegmatis*)	12.8	Homodimer	0.18	361
HbN[a] (*M. tuberculosis*)	14.4	Homodimer	0.013	537
Sperm whale myoglobin[b]	14	Monomer	0.51	13.8

[a]Couture et al. [4].
[b]Springer et al. [30] and Barantly et al. [31].

the trHbs (Fig. 4) displayed more or less similar profile exhibiting troughs at 208 and 224 nm. Such a profile has also been observed in HbN of *N. commune* [23]. The percentage of alpha helical content of both the proteins was more or less similar indicating that the deletion of pre-A region has not affected the overall folding of *M. smegmatis* HbN.

3.5. Growth properties and oxygen uptake of recombinant E. coli carrying HbN of M. smegmatis

To elucidate whether the functional expression of *M. smegmatis* HbN had any metabolic effect(s) on its host, we compared the growth and oxygen consumption properties of recombinant *E. coli*, carrying *M. smegmatis* HbN with the isogenic strain carrying HbN of *M. tuberculosis* along with the control cells having similar plasmid without any Hb encoding gene. Expression of HbN of *M. smegmatis* and *M. tuberculosis* under *vgb* gene promoter resulted in appearance of more or less similar level of both hemoglobins in recombinant *E. coli* and allowed us to compare their effect on physiology of their host. Under high aeration, there was no distinct difference in the growth characteristics of *E. coli* carrying any of these two mycobacterial HbN and none of these hemoglobins

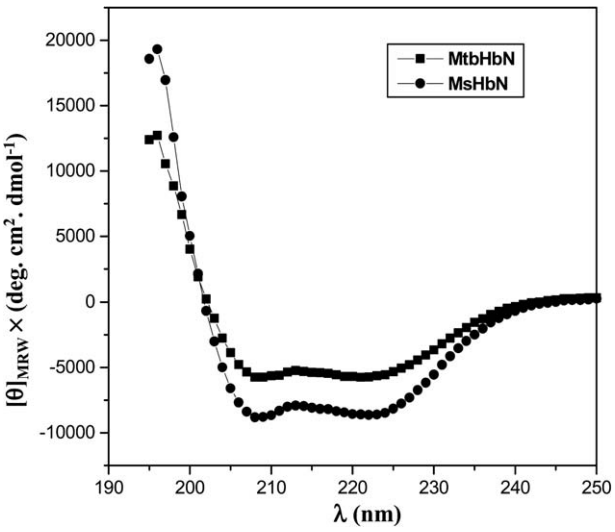

Fig. 4. The near-UV CD spectra of HbN of *M. smegmatis* and *M. tuberculosis*. *M. tuberculosis* and *M. smegmatis* hemoglobin has been designated as MtbHbN and MsHbN, respectively.

exerted any growth advantage over the control cells (Fig. 5A). However, under low oxygen condition, cells carrying *M. tuberculosis* HbN outgrew control and *M. smegmatis* HbN expressing cells at the late exponential stage and resulted in higher cell mass. In all cases, the correlation between optical density (OD_{600}), and the wet and dry cell mass was linear as determined by intermittent cell weight determination, thus, justifying the measure of growth. These results indicated that HbN of *M. smegmatis* might not be able to sustain growth and cell viability during hypoxic growth condition unlike HbN of *M. tuberculosis*. To determine the contribution of mycobacterial HbN to cellular respiration, oxygen uptake rates of recombinant *E. coli* expressing HbN of *M. tuberculosis* and *M. smegmatis*, were compared. Respiration rates of these strains remained more or less similar under aerobic condition when measured in the presence of endogenous substrates present in the cells or when stimulated by the addition of exogenous succinate (10 mM; data not shown).

3.6. *NO consumption activity of recombinant* E. coli *carrying HbN of* M. smegmatis

Earlier, we demonstrated that *M. tuberculosis* HbN possesses potent NO metabolizing activity and can protect its host from the toxicity of NO and its analogs [7]. To check whether HbN of *M. smegmatis* has similar NO scavenging activity, we compared the NO uptake activity of recombinant *E. coli* carrying these two mycobacterial HbN. For that purpose, we utilized a flavoHb (Hmp) deficient mutant of *E. coli* (RB9060) that does not show any measurable NO uptake activity [7]. In the presence of oxygen, expression of *M. tuberculosis* HbN conferred distinct NO consumption ability to the *hmp* mutant of *E. coli* (Table 2) that was significantly higher than the isogenic strain expressing HbN of *M. smegmatis*. On a per heme basis, specific NO uptake activity of *M. smegmatis* HbN was nearly one-third of *M. tuberculosis* HbN suggesting that NO scavenging by HbN of *M. smegmatis* is less efficient than HbN of *M. tuberculosis*. NO consumption activity of both HbN was sustained in *E. coli* with repetitive NO addition indicating the presence of an electron donating partner in *E. coli* that is compatible to carry out reduction of met HbN of *M. tuberculosis* as well as *M. smegmatis*.

3.7. *NO dioxygenase activity of HbN in* M. smegmatis

NO dioxygenase activity of bacterial hemoglobins requires presence of a compatible electron donating partner; therefore, we attempted to check the functional activity of *M. smegmatis* HbN in the cellular environment of its native host. Under normal growth conditions, *M. smegmatis* displays very low NO uptake activity [7]. Therefore, to compare NO metabolizing activity of *M. smegmatis* HbN with that of *M. tuberculosis*, we expressed both species of HbN in *M. smegmatis* under the constitutive promoter of 19 kD antigen of *M. tuberculosis*. Absolute spectra of cell lysate of *M. smegmatis*, expressing these hemoglobins, exhibited specific peak at 412 nm suggesting the presence of an oxygenated form of hemoglobin (data not shown). When HbN of *M. tuberculosis* was expressed in *M. smegmatis*, it elevated the level of NO uptake of these cells nearly 100-fold, whereas, *M. smegmatis* HbN expressing cells exhibited only 25- to 30-fold increase in NO uptake although cellular level of plasmid encoded Hb in both cases were more or less similar. On a per heme basis, specific NO consumption activity of *M. smegmatis*, expressing its native HbN, was nearly fourfold lower than that of expressing HbN of *M. tuberculosis* (Table 3). These results further substantiated that the NO scavenging abilities of these two trHbN are different.

3.8. *Potentiality of* M. smegmatis *HbN against nitrosative stress and reactive nitrogen species*

Since HbN of *M. smegmatis* exhibited lower NO uptake activity, we tested whether it will be effective in relieving toxicity against NO donors and reactive nitrogen species. No detectable growth differences between control and HbN carrying *hmp* strain of *E. coli* were observed under standard aerobic growth conditions. To test whether the presence of *M. smegmatis* HbN provide any protective effect against NO donor and nitrosative stress, we compared the growth profiles and survival pattern of *hmp* mutant of *E. coli* carrying *M. smegmatis* HbN with the isogenic strain having *M. tuberculosis* HbN (Fig. 6A and B) in the presence of different concentrations of the NO donor, sodium nitroprusside (SNP). Presence of *M. tuberculosis* HbN conferred distinct growth advantage to the *hmp* mutant of *E. coli* in the presence of 1 mM SNP, whereas, *M. smegmatis* HbN carrying cells grew slowly and only marginal growth increase over the control cells, growing under similar conditions, was observed (Fig. 6A). In the presence of different concentrations of SNP, survival of *M. smegmatis* HbN carrying cells of *E. coli* was 2 to 3-fold lower in comparison to the cells expressing *M. tuberculosis* HbN (Fig. 6B). These results clearly indicated difference in protective effect of HbN of *M. smegmatis* and *M. tuberculosis* against NO toxicity.

To further evaluate the potentiality of *M. smegmatis* HbN against other reactive nitrogen species, we tested tolerance of hemoglobin expressing cells towards acidified nitrite. Nitrite protonates to form HNO_2 that quickly dismutates to produce several species of nitrogen oxides including NO. There was no distinct difference in survival of control and HbN carrying cells in the presence of 10 mM sodium nitrite at pH 7.2. Increase in nitrite concentration to 30 mM resulted in nearly 70–75% drop

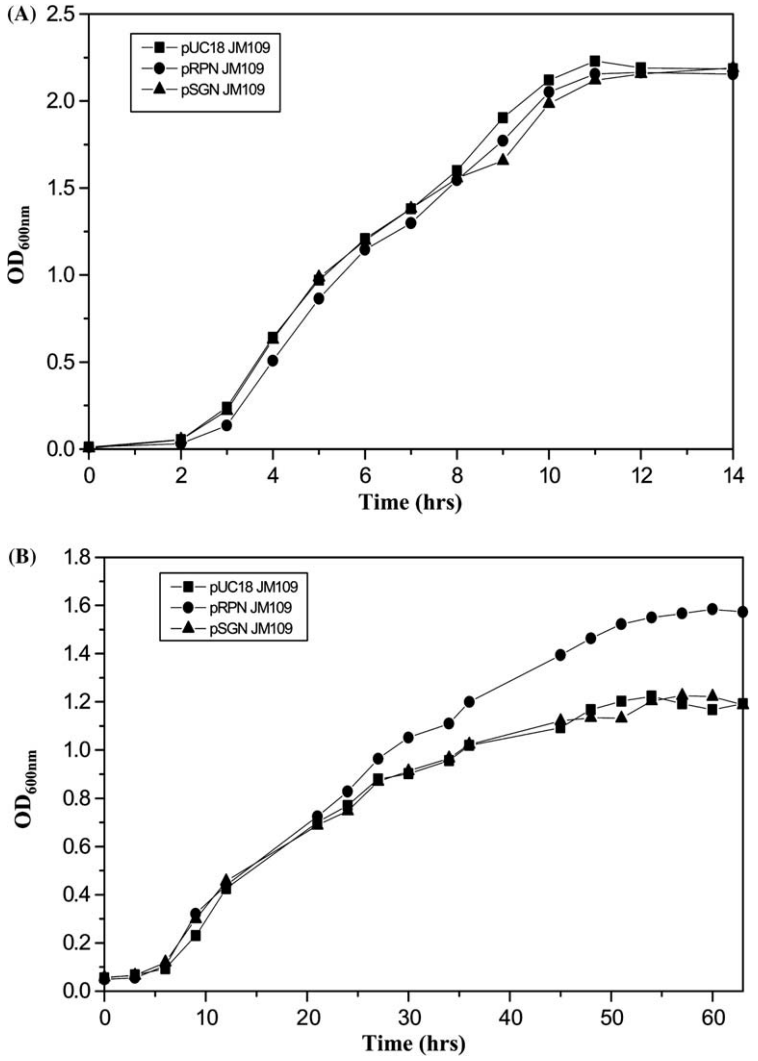

Fig. 5. Growth profile of recombinant *E. coli* expressing HbN of *M. smegmatis* and *M. tuberculosis*. (A) *E. coli* cells carrying pUC18 and recombinant plasmid expressing HbN of *M. tuberculosis* (pRPN) and *M. smegmatis* (pSGN) were grown in 100 ml baffled flask containing 25 ml of LB supplemented with 100 μg/ml ampicillin and incubated at 37 °C at 180 r.p.m. on a gyratory shaker and OD_{600} was monitored periodically. (B) *E. coli* cells carrying pUC18 and HbN expressing plasmids were grown in 100 ml sealed flasks containing 75 ml of LB supplemented with 100 μg/ml ampicillin and incubated at 37 °C at 100 r.p.m. and OD_{600} was monitored at different time intervals.

Table 2
Physiological properties of recombinant *E. coli* carrying mycobacterial hemoglobin HbN from *M. tuberculosis* and *M. smegmatis*

Strain	Heme content (pmol/10^8 cells)	NO uptake (nmol/NO/10^8 cells)	Oxygen uptake (μmole O_2/min/10^8 cells)
E. coli RB9060 (pUC18)	2.3 ± 0.02	0.01 ± 0.0	4.1 ± 0.28
E. coli RB9060 (pRPN)[a]	17.8 ± 0.5	9.8 ±0.4	5.1 ± 0.46
E. coli (pSGN)[b]	16.6 ± 0.2	2.7 ± 0.7	5.6 ± 0.51

[a]pRPN is an expression plasmid for *M. tuberculosis* HbN.
[b]pSGN is an expression plasmid for *M. smegmatis* HbN.

in cell survival in the case of control and cells expressing *M. smegmatis* HbN. Under similar conditions, cells expressing HbN of *M. tuberculosis* displayed only 30–35% decrease in cell survival as compared to the control cells not challenged with sodium nitrite (Fig. 7A). When the pH of the medium was lowered from 7.2 to 6 to promote nitrite protonation, cell survival dropped (80–85%) drastically in the presence of 10 mM sodium nitrite in the case of control and cells expressing *M. smegmatis* HbN, whereas, presence of *M. tuberculosis* HbN in isogenic *E. coli* strain allowed it to grow better resulting in nearly three-fold higher cell survival (Fig. 7B) as compared to control and *M. smegmatis* HbN expressing cells. Further increase in the nitrite concentration at pH 6 resulted in drastic drop in cell survival in all the cases.

Taken together, these results demonstrate that the potentiality of *M. smegmatis* HbN to cope with toxicity of NO and nitrosative stress is significantly lower than that of the HbN of *M. tuberculosis*.

Table 3
NO consumption activity of *M. smegmatis*, expressing HbN of *M. tuberculosis* and *M. smegmatis*

Strains	Heme content (pmol/10^8 cells)	NO consumption activity (nmole of NO $heme^{-1}$ s^{-1})
M. smegmatis (p19Kpro)	1.6 ± 0.5	0.09 ± 0.7
M. smegmatis (pRPN-2)	4.8 ± 0.9	18.6 ± 0.5
M. smegmatis (pSGN-2)	6.4 ± 0.1	5.1 ± 0.7

M. smegmatis cultures were grown in Middlebrook 7H9 broth (Difco) supplemented with ADC (10% bovine serum albumin fraction V, dextrose and sodium chloride), 2% glycerol, 0.05% Tween 80 and 50 μg/ml Hygromycin (wherever required) at 37 °C with vigorous shaking (250 r.p.m) for 10 h. Cells were then harvested by centrifugation (at 8000 × *g* for 30 min at 4 °C), washed with NO consumption assay buffer (see Section 2) and resuspended in the same buffer at a cell density of nearly 3×10^7 cells/μl and NO consumption activities of control *M. smegmatis* (p19Kpro) and that carrying HbN of *M. tuberculosis* (pRPN-2) and *M. smegmatis* (pSGN-2) were determined.

4. Discussion

Slow growing pathogenic mycobacteria, such as *M. tuberculosis*, *M. bovis*, *M. avium*, etc. carry genes encoding HbN and HbO type truncated hemoglobins that may play vital role in their cellular metabolism. Functional relevance of these truncated hemoglobins in mycobacterial cellular metabolism is not conclusively known. Biochemical and structural studies conducted on hemoglobins of *M. tuberculosis* in our laboratory and of others [4,5,7,8] suggest that HbN may be involved in oxygen-sustained detoxification of NO that may provide a defense mechanism to the tubercle bacillus against macrophage-generated reactive nitrogen species. In the present work, we have shown that truncated hemoglobin, HbN, of fast-growing, non-pathogenic mycobacterium, *M. smegmatis*, has functional characteristics that are distinct from HbN of *M. tuberculosis*.

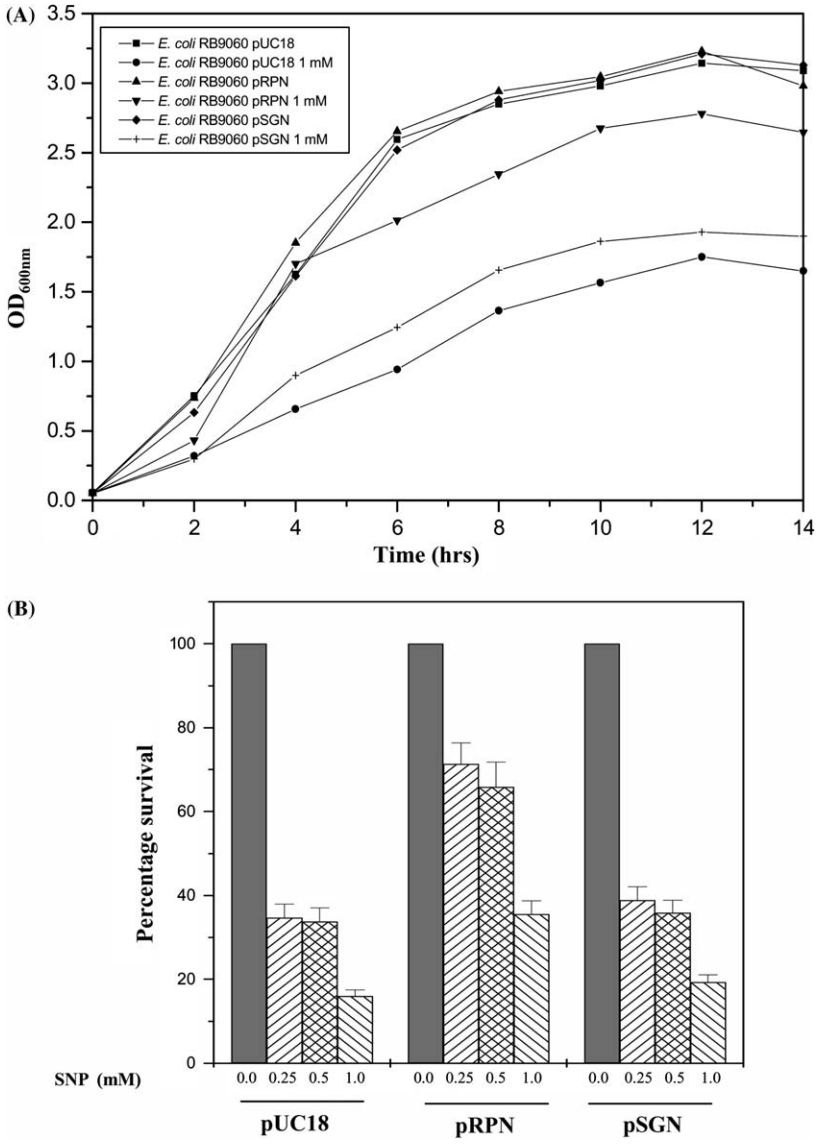

Fig. 6. Effect of sodium nitroprusside (SNP) on growth and survival of recombinant *E. coli* expressing HbN of *M. tuberculosis* and *M. smegmatis*. (A) Growth characteristics of *E. coli* expressing HbN of *M. tuberculosis* and *M. smegmatis* in the presence of SNP (1 mM). (B) Survival of HbN expressing *E. coli* in the presence of different concentrations of SNP. Survival is represented as percentage of the values for the untreated cells.

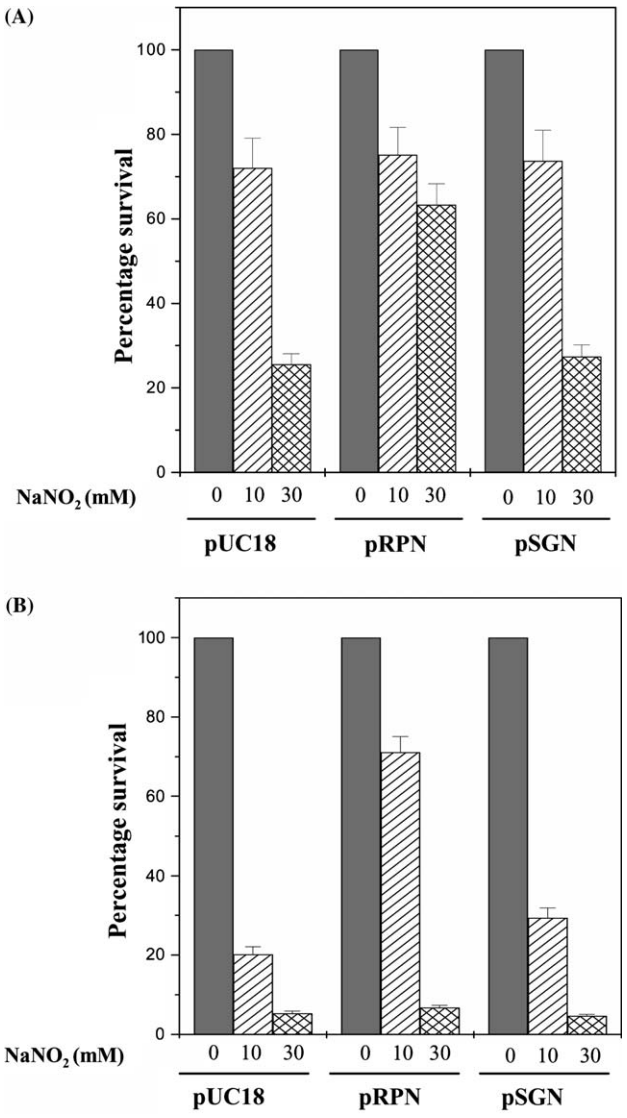

Fig. 7. Survival of HbN expressing recombinant *E. coli* in the presence of acidified nitrite. (A) Survival pattern of control and HbN expressing *E. coli* cells in the presence of different concentrations of sodium nitrite at pH 7.2. (B) Survival pattern of control and HbN expressing *E. coli* cells under different levels of sodium nitrite at pH 6.0. Survival is represented as the percentage value of the untreated cells.

M. smegmatis HbN is smaller in size than its counterpart present in other mycobacterial species and lacks N-terminal pre-A region, however, conservation of structural features crucial for the stabilization of the truncated globin fold suggests that *M. smegmatis* HbN may be able to function as hemoglobin. Near-UV CD spectra of HbN of *M. smegmatis* and *M. tuberculosis* is very similar indicating the presence of high degree of alpha-helical structure as has been observed in HbN of *N. commune* [23]. The spectral and biochemical characteristics show that *M. smegmatis* HbN is a cooperative homodimer and contains a five-coordinated ferrous heme group when the protein has no bound ligand and that CO and oxygen bind to the heme moiety in a reversible manner, very similar to other functional Hbs. Oxygen affinity of *M. smegmatis* HbN is relatively lower than HbN of *M. tuberculosis*, despite the fact that both display TyrB10 and GlnE7 distal residues and share a comparable distal site sequence composition. This suggests that other factors may play a role in modulating the overall ligand affinity of *M. smegmatis* HbN. Lack of pre-A region, that makes contact with the pre-F loop and G-helix in HbN of *M. tuberculosis*, may affect access and kinetics of ligand access through protein motion [9,10] resulting in differences in oxygen binding of *M. smegmatis* HbN. Autooxidation properties of HbN of *M. tuberculosis* and *M. smegmatis* further suggest that interactions of oxygen with these two trHbs may have some differences.

Although HbN of *M. smegmatis* and *M. tuberculosis* share close structural similarity, their ability to scavenge NO and provide protection from the toxicity of NO and nitrosative stress is quite different. *M. smegmatis* HbN exhibits lower NO metabolizing activity in heterologous host, *E. coli*, as well as in its native host as compared to HbN of *M. tuberculosis*. Molecular basis of this functional difference between these two mycobacterial HbN is currently unknown. Oxygen dependent NO consumption by single domain bacterial hemoglobin,VHb, has been demonstrated [20] and it has been suggested that structural features of VHb allows it to associate with a partner flavoreductase *in vivo* under certain physiological conditions to create a transient flavoHb like structure that may participate in NO metabolism. Optical spectra of NO-bound HbN of *M. tuberculosis* overlaps with that of *M. smegmatis* indicating that both species are able to bind NO. Thus, the distinct difference in NO metabolizing activity of these two hemoglobins may be due to their catalytic efficiency for NO dioxygenation. HbN exhibits potent NO metabolizing activity and protects heterologous hosts from the toxicity of NO and nitrosative stress, therefore, it is quite possible that a partner flavoreductase may associate with it *in vivo* to attain NOD function. NO dioxygenase activity of *M. smegmatis* HbN is significantly lower than HbN of *M. tuberculosis*. HbN of *M. smegmatis* has nearly 70% sequence similarity with HbN of *M. tuberculosis* and it does not carry any charged pre-A region, therefore, the coupling efficiency of these two hemoglobins with its electron-donating partner may differ resulting in differences in their ability to interact with NO and reactive nitrogen species. Difference in functionality of these two mycobacterial trHbN can be correlated with their functional relevance within the cellular environment of their native host. *M. tuberculosis* is an intracellular pathogen that resides and replicates within the hypoxic and NO-enriched environment of macrophages, thus, may require a strong NO detoxification system to cope with the toxic level of its intracellular environment. In comparison, *M. smegmatis* is not naturally exposed to such hazardous conditions and oxygen and NO binding properties of its HbN may provide sufficient protection from the level of stress faced by its host. Alternatively, some other NO-scavenging system may be operative in *M. smegmatis*. It is notable that *M. tuberculosis* genome carries gene encoding for a putative flavohemoglobin (flavoHb) and similar flavoHb homolog has also been identified within the genome sequence of *M. smegmatis* (unpublished observation). Role of flavoHbs in protection from the toxicity of NO and nitrosative stress has been well documented in the literature [24–26]. It is quite possible that *M. tuberculosis* requires multiple systems for relieving stress against high level of NO and reactive nitrogen species generated within the macrophages,

whereas, flavoHb may be sufficient to take care of the physiological stress encountered by *M. smegmatis*.

The pre-A region, carrying highly polar sequence motif, is conserved in HbN of other pathogenic mycobacteria; however, the relevance of this additional secondary structure in protein function is not known at present. HbN of *M. smegmatis* does not carry this polar motif at its N-terminus. It is quite possible that the presence of this additional secondary structure at the N-terminus of HbN may have some functional significance for pathogenic mycobacterium, which possibly may not be required for the function of HbN of *M. smegmatis*.

The data reported here provides first report on truncated hemoglobin, HbN, of a fast-growing mycobacterium, *M. smegmatis*, and demonstrates that its functional properties are significantly different from its counterpart present in pathogenic mycobacterium, *M. tuberculosis*. The major difference in functionality of these two HbN type truncated hemoglobins lies in their capability to metabolize NO and provide protection against NO and nitrosative stress. Physiological function(s) of *M. smegmatis* HbN is not very obvious at present. *M. smegmatis* HbN binds oxygen with reasonably high affinity, thus, there is a possibility that it may also participate in other metabolic reactions involving oxygen as has been envisaged for the protozoan and cyanobacterial HbN [27–29].

Acknowledgements: We are thankful to Prof. N. Ninfa for making available *E. coli* RB9060 strain for our work. Technical assistance provided by Mr. S. Muthukrishnan is gratefully acknowledged. This work has been supported by research grants provided by Council of Scientific and Industrial Research (SMM003) and the Department of Biotechnology, Govt. of India.

References

[1] Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., Mclean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutters, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. and Barrell, B.G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393, 537–544.

[2] Cole, S.T., Eiglmeier, K., Parkhill, J., James, K.D., Thomson, N.R., Wheeler, P.R., Honore, N., Garnier, T., Churcher, C., Harris, D., Mungall, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R.M., Devlin, K., Duthoy, S., Feltwell, T., Fraser, A., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Lacroix, C., Maclean, J., Moule, S., Murphy, I., Oliver, K., Quail, M.A., Rajandream, M.A., Rutherford, K.M., Rutter, S., Seeger, K., Simon, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Taylor, K., Whitehead, S., Woodward, J.R. and Barrel, B.G. (2001) Massive gene decay in the leprosy bacillus. Nature 409, 1007–1011.

[3] Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A. and Guertin, M. (2001) Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. J. Biol. Chem. 277, 871–874.

[4] Couture, M., Yeh, S.R., Wittenberg, B.A., Wittenberg, J.B., Ouellet, Y., Rousseau, D.L. and Guertin, M. (1999) A cooperative oxygen-binding hemoglobin from *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA 96, 11223–11228.

[5] Ouellet, H., Juszczak, L., Dantsker, D., Samuni, U., Ouellet, Y.H., Savard, P.Y., Wittenberg, J.B., Wittenberg, B.A., Friedman, J.M. and Guertin, M. (2003) Reaction of *Mycobacterium tuberculosis* truncated hemoglobin O with ligand reveal a novel ligand inclusive hydrogen bond network. Biochemistry 42, 5764–5774.

[6] Pathania, R., Navani, N.K., Rajamohan, G. and Dikshit, K.L. (2002) *Mycobacterium tuberculosis* Hemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant *E. coli*. J. Biol. Chem. 277, 15293–15302.

[7] Pathania, R., Navani, N.K., Gardner, A.M., Gardner, P.R. and Dikshit, K.L. (2002) Nitric oxide scavenging and detoxification by the *Mycobacterium tuberculosis* haemoglobin, HbN in *Escherichia coli*. Mol. Microbiol. 45, 1303–1314.

[8] Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J. and Guertin, M. (2002) Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. Proc. Natl. Acad. Sci. USA 99, 5902–5907.

[9] Milani, M., Pesce, A., Ouellet, Y., Ascenzi, P., Guertin, M. and Bolognesi, M. (2001) *Mycobacterium tuberculosis* hemoglobin N displays a protein tunnel suited for O_2 diffusion to the heme. EMBO J. 20, 3902–3909.

[10] Milani, M., Pesce, A., Ouellet, Y., Dewilde, S., Friedman, J., Ascenzi, P., Guertin, M. and Bolgnesi, M. (2004) Heme-ligand tunneling in group I truncated hemoglobins. J. Biol. Chem. 279, 21520–21525.

[11] Yeh, S.R., Couture, M., Ouellet, Y., Guertin, M. and Rousseau, D.L. (2000) A cooperative oxygen binding hemoglobin from *Mycobacterium tuberculosis*. Stabilization of heme ligands by a distal tyrosine residue. J. Biol. Chem. 275, 1679–1684.

[12] Samuni, U., Ouellet, Y., Guertin, M., Friedman, J.M. and Yeh, S.R. (2004) The absence of proximal strain in the truncated hemoglobins from *Mycobacterium tuberculosis*. J. Am. Chem. Soc. 126, 2682–2683.

[13] Poole, R.K. and Hughes, M.N. (2000) New functions for the ancient globin family,bacterial response to nitric oxide and nitrosative stress. Mol. Microbiol. 36, 775–783.

[14] Gardner, P.R. (2005) Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myogloin and their associated reductases. J. Inorg. Biochem. 99, 247–266.

[15] Hernandez-Urzua, E., Mills, C.E., White, G.P., Contreras-Zentella, M.L., Escamilla, E., Vasudevan, S.G., Membrillo-Hernandez, J. and Poole, R.K. (2003) Flavohemoglobin Hmp, but not its individual domains, confers protection from respiratory inhibition by nitric oxide in *Escherichia coli*. J. Biol. Chem. 278, 34975–34982.

[16] MacMicking, J.D., North, R.J., LaCourse, R., Mudgett, J.S., Shah, S.K. and Nathan, C.F. (1997) Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA 94, 5243–5248.

[17] Turenne, C.Y., Suchak, A.A., Wolfe, J.N., Kabani, A. and Nicolle, L.E. (2003) Soft tissue infection caused by a novel pigmented, rapidly growing mycobacterium species. J. Clin. Microbiol. 41, 2779–2782.

[18] Snapper, S.B., Melton, R.E., Mustafa, S., Kieser, T. and Jacobs Jr., W.R. (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. 4, 1911–1919.

[19] Dikshit, K.L. and Webster, D.A. (1988) Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene 70, 377–386.

[20] Kaur, R., Pathania, R., Sharma, V., Mande, S.C. and Dikshit, K.L. (2002) Chimeric *Vitreoscilla* hemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in *Escherichia coli*: new insight into the functional role of VHb. Appl. Environ. Microbiol. 68, 152–160.

[21] Poole, R.K., Anjum, M.F., Membrillo-Hernandez, J., Kim, S.O., Hughes, M.N. and Stewart, V. (1996) Nitric oxide, nitrite and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. 178, 5487–5492.

[22] Appleby, C.A. (1978) Purification of *Rhizobium* cytochromes P-450. Methods Enzymol. 52, 157–166.

[23] Thorsteinsson, M.V., Bevan, D.R., Ebel, R.E., Weber, R.E. and Potts, M. (1996) Spectroscopical and functional characterization of the hemoglobin of *Nostoc commune* UTEX 584 (Cyanobacteria). Biochim. Biophys. Acta 1292, 133–139.

[24] Hausladen, A., Gow, A.J. and Stamler, J.S. (1998) Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc. Natl. Acad. Sci. USA 95, 14100–14105.

[25] Liu, J., Zeng, M., Hausladen, A., Heitman, J. and Stamler, J.S. (2000) Protection from nitrosative stress by yeast flavohemoglobin. Proc. Natl. Acad. Sci. USA 97, 4672–4676.
[26] Stevanin, T.M., Ioannidis, N., Mills, C.E., Kim, S.O., Hughes, M.N. and Poole, R.K. (2000) Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes b*o*′ or b*d*, from nitric oxide. J. Biol. Chem. 275, 35868–35875.
[27] Yamauchi, K., Tada, H. and Usuki, I. (1995) Structure and evolution of *Paramecium* hemoglobin genes. Biochim. Biophys. Acta 1264, 53–62.
[28] Couture, M., Das, T.K., Lee, H.C., Peisach, J., Rousseau, D.L., Wittenberg, B.A., Wittenberg, J.B. and Guertin, M. (1999) *Chlamydomonas* chloroplast ferrous hemoglobin: heme pocket structure and reactions with ligands. J. Biol. Chem. 274, 6898–6910.
[29] Das, T.K., Weber, R.E., Dewilde, S., Wittenberg, J.B., Wittenberg, B.A., Yamauchi, K., VanHauwaert, M.L., Moens, L. and Rousseau, D.L. (2000) Ligand binding in the ferric and ferrous states of *Paramecium* hemoglobin. Biochemistry 39, 14330–14340.
[30] Springer, B.A., Egeberg, K.D., Sligar, S.G., Rohlfs, R.J., Mathews, A.J. and Olson, J.S. (1989) Discrimination between oxygen and carbon monoxide and inhibition of autooxidation by myoglobin. site directed mutagenesis of the distal histidine. J. Biol. Chem. 264, 3057–3060.
[31] Brantley Jr., R.E., Smerdon, S.J., Wilkinson, A.J., Singleton, E.W. and Olson, J.S. (1993) The mechanism of autooxidation of myoglobin. J. Biol. Chem. 268, 6995–7010.