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

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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

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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-