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A cooperative oxygen-binding hemoglobin from *Mycobacterium tuberculosis*

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ABSTRACT **Two putative hemoglobin genes, *glbN* and *glbO*, were recently discovered in the complete genome sequence of *Mycobacterium tuberculosis* H37Rv. Here, we show that the *glbN* gene encodes a dimeric hemoglobin (HbN) that binds oxygen cooperatively with very high affinity ($P_{50} = 0.013$ mmHg at 20°C) because of a fast combination (25 $\mu M^{-1} \cdot s^{-1}$) and a slow dissociation (0.2 s^{-1}) rate. Resonance Raman spectroscopy and ligand association/dissociation kinetic measurements, along with mutagenesis studies, reveal that the stabilization of the bound oxygen is achieved through a tyrosine at the B10 position in the distal pocket of the heme with a conformation that is unique among the globins. Physiological studies performed with *Mycobacterium bovis* bacillus Calmette–Guérin demonstrate that the expression of HbN is greatly enhanced during the stationary phase in aerobic cultures but not under conditions of limited oxygen availability. The results suggest that, physiologically, the primary role of HbN may be to protect the bacilli against reactive nitrogen species produced by the host macrophage.**

It is estimated that about one-third of the human population is latently infected by *Mycobacterium tuberculosis* (1). In most healthy individuals, the initial infection by the tubercule bacilli is contained by the immune system, which forces the bacteria to enter latency for decades with possible reactivation later in life. The initial event after infection involves multiplication of the bacillus inside the host macrophage (2, 3). Later, infected macrophages are isolated from the circulation by newly recruited macrophages to form the so-called caseous granuloma. Low O_2 levels, low pH, toxic oxygen species, and high CO_2 levels characterize the interior of the granuloma and appear to create an environment capable of restricting the growth of bacilli (4). How the bacilli enter and persist in the latent state is completely unknown.

Two genes, *glbN* and *glbO*, encoding hemoglobin-like proteins (HbN and HbO, respectively) were recently discovered in the complete genome sequence of the virulent *M. tuberculosis* H37Rv strain (5). Hemoglobins have been discovered in many unicellular organisms in the recent past, but their physiological role is controversial (6). The putative hemoglobins from *M. tuberculosis* are similar to those found in ciliated protozoa (7, 8), the cyanobacteria *Nostoc commune* (9) and *Synechocystis* (10), and the green alga *Chlamydomonas eugametos* (11). This family of hemoglobins is distinct from that comprising the dimeric hemoglobin of the bacterium *Vitreoscilla stercoraria* and the flavohemoglobins of bacteria and yeasts (12). In the cyanobacterium *N. commune*, the hemoglobin is localized along the cytosolic face of the cell membrane and is expressed under low oxygen tension, suggesting that it may be a component of a microaerobically induced terminal oxidase (13, 14). In the green eukaryotic alga *C. eugametos*, the chloroplast hemoglobin is expressed in response to activation of photosynthesis (11). Inside the chloroplast, the hemoglobin is localized, in part, in the same region as the thylakoid membranes, suggesting a possible involvement with the photosynthetic electron-transfer chains. Metabolic studies and gene disruption experiments suggest that hemoglobins belonging to the *V. stercoraria* family are involved in oxidative stress responses (15), metabolism of (16) and resistance to (17, 18) nitric oxide, oxygen diffusion to terminal respiratory oxidases (19), and oxygen sensing (20).

Crystallographic studies of the dimeric, cooperative hemoglobin of *V. stercoraria* (21) and the flavohemoglobin of *Alcaligenes eutrophus* (22) revealed that the characteristic globin fold of vertebrate proteins is retained in bacterial hemoglobins, despite the very low sequence similarity. However, marked differences were discovered in the distal heme pocket, which is very important in determining ligand-binding properties of hemoglobins (23). It suggests that the physiological functions of the hemoglobins from unicellular organisms may be very different from those of vertebrate hemoglobins. To assess their functional role, we studied one of the *M. tuberculosis* hemoglobins, HbN, in depth. The structural properties of HbN were characterized by the optical absorption and the resonance Raman spectra obtained under a variety of oxidation state and ligand-binding conditions; the functional properties were determined by measuring the ligand-binding constants; and the physiological role was addressed by following the expression of HbN during the cell growth cycle. Based on these results, we postulate that HbN may be involved in the protection of the bacilli against reactive nitrogen species produced by the host macrophage during the initial infection or during latency (24).

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) ATCC 35734 cells were grown at 37°C with constant shaking at 150 rpm in 300-ml Nephelo flasks containing 150 ml of 7H9 medium supplemented with 15 ml of Middlebrook ADC enrichment medium and 0.05% Tween 80. *Escherichia coli* cells were grown at 37°C in Luria–Bertani medium containing 200 μg/ml ampicillin.

Cloning, Expression, and Purification of Recombinant HbN. We used the PCR to amplify the coding region of the *glbN* gene from the Y48 cosmid (5). The DNA primers used were 5′-GCTGTTCCATATGGGACTACTGTCACGCTT-3′

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Abbreviation: BCG, bacillus Calmette–Guérin.

Data deposition: The *M. bovis* BCG *glbN* gene sequence has been deposited in the National Center for Biotechnology Information databank (accession no. AF130980).

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(upper primer) and 5′-CGGGATCCTCAGACTGGTGC-CGTGGT-3′ (lower primer). The *Nde*I/*Bam*HI-digested PCR fragment was cloned into the pET-3A prokaryotic expression vector, and the recombinant protein was expressed in freshly transformed *E. coli* BL21(DE3) cells. Recombinant HbN was purified by a four-step procedure (25). Protein purity was assessed by SDS/PAGE. The heme was identified and quantified by the pyridine–hemochrome method (26). The molecular weight of HbN under nondenaturing condition was determined at 4°C by using a Superdex75 16/60 gel filtration column (Pharmacia) equilibrated with 50 mM Tris-Cl (pH 7.5) buffer containing 150 mM NaCl and 50 μM EDTA. For anaerobic work, the buffer was equilibrated with nitrogen gas, and sodium dithionite was added to a final concentration of 1 mM.

Site-Directed Mutagenesis. The tyrosine residue found at position 33 in HbN (Fig. 1) was changed to either a leucine or a phenylalanine residue by the method previously described (25). The mutant hemoglobins were purified by the same method as described for the wild-type recombinant HbN.

Polyclonal Abs. Polyclonal Abs against HbN were obtained as described previously (11), except that Titermax (CytRx, Norcross, GA) was used in place of Freund's adjuvant. Western blot analysis was performed as previously described (25).

Cloning of *M. bovis* BCG *glbN* Gene. *M. bovis* BCG DNA (5 μg) was digested with *Bam*HI and cloned into *Bam*HI-digested and dephosphorylated pBluescript SK$^-$ plasmid DNA (Stratagene). Nine hundred clones were obtained after transformation of *E. coli* DH5α cells and were screened with the 411-bp, ^{32}P-labeled *M. tuberculosis glbN* probe. One positive clone, containing a DNA fragment of the expected size (4.7 kb), was purified, and the plasmid DNA was isolated. A 1.7-kbp region, containing the *glbN* gene, was sequenced on both strands.

Ligand-Binding Properties. The oxygen dissociation curve of HbN was obtained by the tonometer method (27). The rates of reactions of ferrous HbN with oxygen and carbon monoxide were measured by using a Hi-Tech (Salisbury, U.K.) model 61 stopped-flow apparatus, as described previously (28). The rate of autoxidation of the oxygenated form of HbN was determined in 50 mM Tris-Cl buffer (pH 7.5) containing 50 μM EDTA, as described previously (25).

Resonance Raman Spectroscopy. Resonance Raman measurements were made with previously described instrumentation (28). The output at 407 nm from a krypton laser (Spectra-Physics) was focused to a ≈30-μm spot on a rotating cell to avoid photodamage of the sample. The scattered light was collected and focused on the entrance slit of a 1.25-m polychromater (Spex Industries, Metuchen, NJ), where it was dispersed and then detected by a charge-coupled device camera (Princeton Instruments, Trenton, NJ).

RESULTS

Structural Features of the HbN Hemoglobin. To determine the functional properties of HbN, it is important to identify the residues located in the heme pocket. For this, we aligned the HbN sequence with those of other hemoglobins. As shown in Fig. 1, the alignment obtained suggests that HbN possesses the universally conserved proximal histidine at position F8 and, in the distal pocket, the phenylalanine at position CD1 (Fig. 1). The distal residue at the E7 position in vertebrate globins, which is very important in stabilizing the bound oxygen through hydrogen bonding, is histidine or glutamine (29). In HbN, the residue at the E7 position is a leucine, an amino acid not capable of hydrogen bonding. Another residue, at position B10, that often affects ligand-binding stability by hydrogen bonding in nonvertebrate hemoglobins (21, 28, 30–32) is assigned as a tyrosine in HbN. Pairwise comparisons indicated that the percentage amino acid identity between HbN and the different members ranges from 21% to 32%.

Biochemical Properties of Recombinant HbN. The recombinant HbN protein was expressed in *E. coli* from the cloned gene and purified to near homogeneity with a four-step procedure (25). The heme is noncovalently bound to the protein and is identified as an iron–protoporphyrin IX, as revealed by the oxidized-minus-reduced pyridine–hemochromogen spectrum, in which maxima at 525 and 556 nm and a minimum at 539 nm were detected (26). The purified apoprotein migrated with an apparent molecular mass of 14.3 kDa, as estimated by SDS/PAGE. This value is close to that predicted from sequence analysis of the 136-amino acid-long hydrophilic polypeptide (14.4 kDa).

The state of aggregation of the purified HbN was investigated with gel filtration. Ligand-free ferrous HbN was eluted with volumes corresponding to a molecular mass of about 29.8 kDa (result not shown), close to that expected for the dimeric protein with a theoretical molecular mass for the monomeric holoprotein of 15.1 kDa. As no cysteine residue is found in HbN, disulfide bonds are not involved in the dimer formation. Upon oxygenation, HbN elutes with a smaller apparent molecular mass, 21.1 kDa, which is not dependent on protein concentration from 10 to 500 μM. The origin of this ligand-dependent shift in molecular mass requires further investigation.

Optical Spectra. Optical spectra reveal that HbN can bind a variety of ligands. The optical spectrum of the ferric species of HbN at pH 7.5 is typical of a six-coordinate high-spin heme

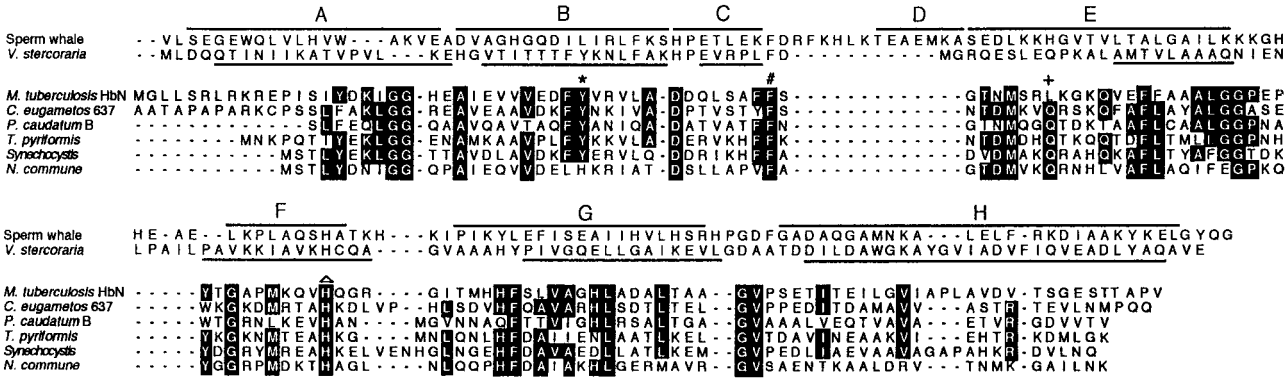

FIG. 1. Multiple sequence alignment of *M. tuberculosis* HbN and the hemoglobins found in ciliated protozoa, a green alga, and cyanobacteria. The amino acid sequences were aligned by using Clustal W. Identical residues are shown in reverse type if they occur in more than 60% of the sequences. The phenylalanine CD1 (#), the distal position at E7 (+), the proximal histidine at F8 (Δ), and the B10 position (*) are indicated. The positions of the helices, according to the structure of sperm whale myoglobin and *V. stercoraria* hemoglobin, are shown above the multiple alignment. The first 30 amino acids of the *C. eugametos* sequence were omitted as they are most likely part of a signal peptide.

with a water molecule as the sixth ligand (λ_{max} = 406, 503, and 624 nm; Fig. 2*A*). The wavelengths for the electronic transitions of the oxy (λ_{max} = 416, 545, and 581 nm; Fig. 2*A*) and carbon monoxide (λ_{max} = 420, 540, and 570 nm; Fig. 2*A*) derivatives are typical of globins. Examination of the optical spectrum of the ligand-free ferrous form at pH 7.5 (λ_{max} = 432 and 558 nm; Fig. 2*A*) indicates the presence of a five-coordinate high-spin heme.

Resonance Raman Spectra. The resonance Raman spectra show features that are unique compared with other globins. In the ligand-free ferrous form, a strong line is present at 226 cm^{-1}, which we assign as the iron–histidine stretching mode (Fig. 3), confirming the sequence alignment that indicates a histidine as the proximal ligand (Fig. 1). The frequency of the Fe–His stretching mode is significantly higher than that of the equilibrium form of most other globins, which are typically in the range of 200–220 cm^{-1}, but is similar to that of photodissociated globins prior to structural relaxation, which exhibit frequencies as high as 230 cm^{-1} (33). Thus, it is concluded that HbN has an unstrained histidine as the proximal heme ligand. As a consequence, a fast ligand on-rate is expected for HbN because the iron atom will be able to be brought to a fully planar conformation upon ligand binding without the constraint that is present in other globins. This is consistent with the observed fast ligand association rates for HbN (see below).

In the $^{16}O_2$ derivative of the ferrous hemoglobin, a line was detected in the resonance Raman spectrum at 560 cm^{-1}, which shifted to 542 cm^{-1} in $^{18}O_2$ (Fig. 3). It is assigned as the Fe–O_2 stretching mode in HbN as it is the expected shift for a Fe–O_2 diatomic oscillator. This frequency is lower than that for the Fe–O_2 stretching modes reported in most other globins. The lower frequency of the Fe–O_2 stretching mode is consistent with the presence of strong hydrogen bonding to the bound oxygen, such as that found in horseradish peroxidase in which the Fe–O_2 stretching mode is located at ≈560 cm^{-1} (34). We attribute the major residue responsible for the hydrogen bonding as the tyrosine at position B10. Site-directed mutagenesis of the B10 tyrosine to a leucine confirmed this proposal because the Fe–O_2 stretching mode shifted from 560 cm^{-1} to 570 cm^{-1} upon this mutation (result not shown), and the oxygen dissociation rate increased greatly as compared with wild-type HbN (Table 1).

Oxygen Binding at Equilibrium and Kinetics of Reactions with Ligands. The oxygen dissociation curve of HbN was determined at various temperatures. HbN shows a very high affinity for oxygen, with P_{50} values of 0.0085, 0.013, and 0.023 mmHg at 10, 20, and 37°C, respectively (Fig. 2*B* and Table 1). The sigmoidal shape of the curves indicates that the oxygen-binding process is cooperative. The Hill coefficient, *n*, that is used as a measure of heme–heme interactions is 2.0 ± 0.2, 2.0 ± 0.1, and 1.4 ± 0.2 at 10, 20, and 37°C, respectively. The very high ligand affinity of HbN was confirmed from the association and dissociation kinetics of ferrous HbN with oxygen and carbon monoxide. Monophasic kinetic patterns were observed for all combination and dissociation reactions independent of the concentrations examined. Plots of the combination rate against the ligand concentration were linear in the range examined, 21–35 μM and 25–100 μM, for oxygen and carbon monoxide, respectively. Second-order rate con-

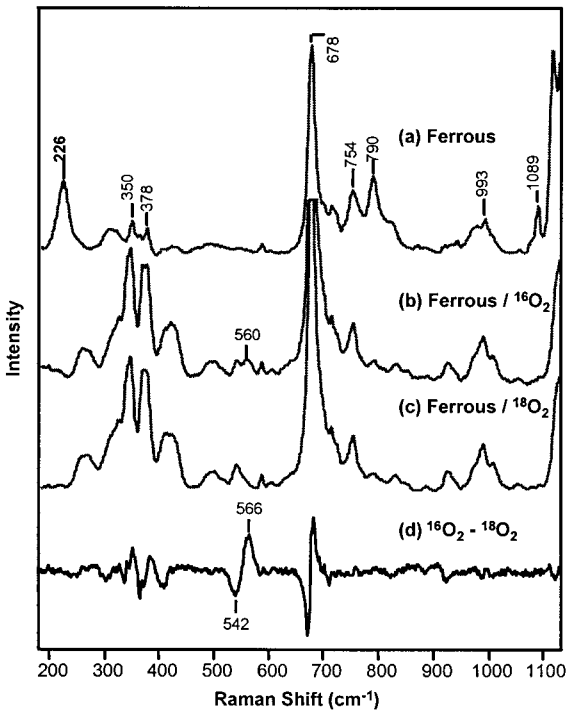

FIG. 3. Resonance Raman spectra of HbN. Trace a, deoxy; trace b, oxygenated with $^{16}O_2$; trace c, oxygenated with $^{18}O_2$; and trace d, $^{16}O_2$ minus $^{18}O_2$ difference spectrum.

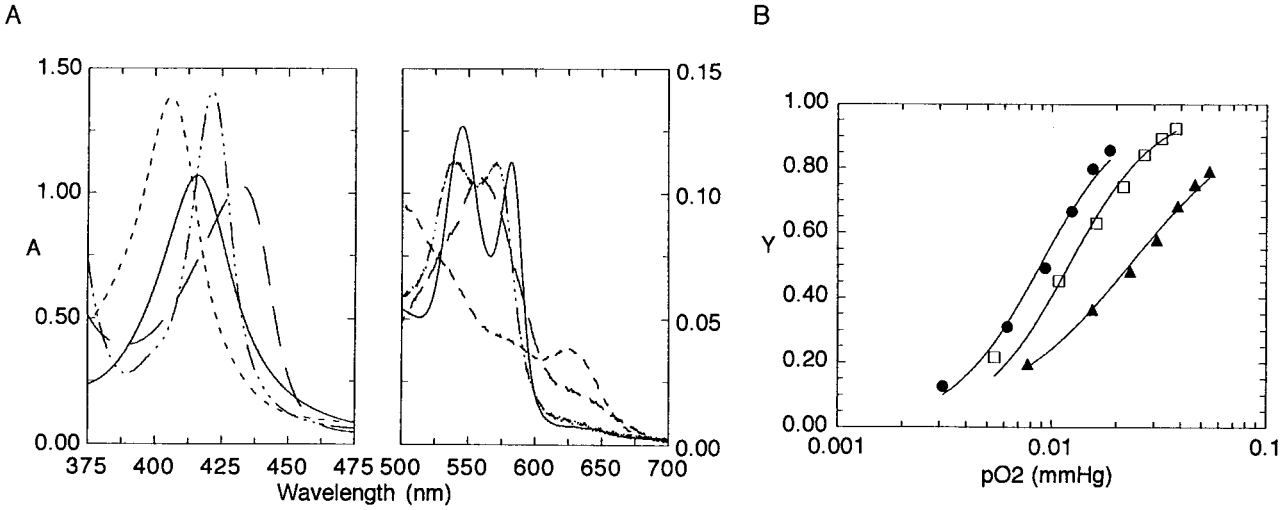

FIG. 2. Optical spectra of HbN and oxygen dissociation curve. (*A*) The optical spectra of the oxy (solid line), ferrous (dashed line), CO (dotted/dashed line), and ferric (short-dashed line) forms were recorded in 50 mM Tris-Cl (pH 7.5) containing 50 μM EDTA at 20°C. (*B*) Oxygen dissociation curve of HbN recorded at 10 (●), 20 (□), and 37°C (▲) in 50 mM Tris-Cl (pH 7.5) buffer containing 50 μM EDTA and 150 mM NaCl. *Y* is the fractional saturation with oxygen.

Table 1. Kinetics and equilibrium constants for the reactions of ferrous HbN with oxygen and carbon monoxide compared to those of other proteins

	Oxygen				Carbon Monoxide				
Protein	k'_{on}, $\mu M^{-1}\cdot s^{-1}$	k_{off}, s^{-1}	K_d (k_{off}/k'_{on}), nM	P_{50}, mmHg	l'_{on}, $\mu M^{-1}\cdot s^{-1}$	l_{off}, s^{-1}	L_d (l_{off}/l'_{on}), nM	M′ / K_d/L_d	Autoxidation $t_{1/2}$, h
HbN wild type	25	0.199	—	0.013	6.75	0.0051	—	7.44	537
HbN Y33L (B10)	—	45	—	—	—	—	—	—	—
HbN Y33F(B10)	—	30	—	—	—	—	—	—	—
C. eugametos wild type*	†	0.0141	—	—	†	0.0022	—	5.0	169
C. eugametos Y63L (B10)*	—	0.95	—	—	—	—	—	—	7.1
N. commune GibN‡	390	79	208	0.55	41	0.01	0.24	867	3.5
Barley Hb§	7.1	0.0272	3.82	—	0.57	0.0011	1.93	2.0	—
Ascaris Hb¶	1.5	0.0041	2.7	0.0038	17	0.018	1.1	2.5	—
Soybean leghemoglobin**	120	5.6	48	0.04	13	0.0078	0.62	78	3.5
Sperm whale Mb††	14	12	857	0.51	0.51	0.019	37	23	13.8

Kinetic constants for HbN represent the overall reactions and are not assigned to the binding or dissociation of the first or the second ligand molecule. *l*, analogous to *k*, is the rate constant for carbon monoxide. M′ for HbN, *Chlamydomonas,* and barley hemoglobins is expressed in molar terms, M′ = 1.34 × M, where M is the experimentally determined value expressed in terms of gas pressures. M′ for other proteins is given by the ratio K_d/L_d.

*Couture *et al.* (28), data obtained at pH 9.5.

†Combination rates of five-coordinate *Chlamydomonas* Hb with ligands are rate limited by the conversion of a six-coordinate species to a five-coordinate species prior to ligand binding (see details in ref. 28).

‡Thorsteinsson *et al.* (14).

§Duff *et al.* (35), k'_{on} is estimated from M′, k_{off}, and L_d.

¶Gibson and Smith 1965 (36); Antonini and Brunori, 1971 (37).

**Gibson *et al.* (38); Appleby (39).

††Springer *et al.* (40); Antonini and Brunori (37); Brantley *et al.* (41).

stants calculated from the slope of these relations were 25 $\mu M^{-1}\cdot s^{-1}$ for oxygen and 6.75 $\mu M^{-1}\cdot s^{-1}$ for carbon monoxide (Table 1). The overall dissociation rate measured is 0.2 s^{-1} for oxygen, which is very slow compared with that of other globins, and is 0.0051 s^{-1} for carbon monoxide (Table 1). When the tyrosine residue at position B10 is mutated to a leucine or a phenylalanine residue, the oxygen dissociation rate increases by 225- and 150-fold (Table 1), respectively, to values greater than those of vertebrate globins (Table 1). The fast oxygen dissociation rate measured for the phenlalanine B10 mutant, which misses only one hydroxyl on the phenyl ring, indicates the importance of the tyrosine hydroxyl for tight oxygen binding in the native HbN.

HbN Expression *in Vivo*. To elucidate the physiological function of HbN, we examined the temporal expression pattern of HbN in *M. bovis* BCG (ATCC 35734), which is widely used as a model system for *M. tuberculosis* (42). *M. bovis* causes tuberculosis in cattle and is highly virulent for man. When inhaled, *M. bovis* can cause pulmonary tuberculosis indistinguishable from that caused by *M. tuberculosis*, although the *M. bovis* BCG strain is nonpathogenic. To ensure that the BCG *glbN* gene is intact in the nonpathogenic strain, a 1.7-kb region hybridizing with the *glbN* probe was sequenced. It was confirmed that the genomic organization and the nucleotide sequence of the *glbN* gene in *M. bovis* BCG are identical to those of *M. tuberculosis.* This 1.7-kb DNA sequence has been deposited at National Center for Biotechnology Information databank (accession no. AF130980). Polyclonal Abs raised against HbN were used to detect the presence of this polypeptide in Western blot analysis. In aerobic cultures, a large increase in the level of HbN is detected only after cells have reached the stationary phase (Fig. 4). This result indicates that HbN expression is associated with the stationary phase. However, if cells enter the stationary phase because of oxygen depletion (43), they do not show this increase of HbN (result not shown).

DISCUSSION

We have shown that the *glbN* gene of *M. tuberculosis* encodes a functional hemoglobin related to those of the ciliated protozoa, a green alga, and cyanobacteria. Sequence comparison with sperm whale myoglobin and *V. stercoraria* hemoglobin suggests that, despite a high level of divergence, these hemoglobins have the universally conserved proximal histidine and CD1 phenylalanine (Fig. 1). All members, except HbN, are predicted to have glutamine in the distal position E7 instead of the more usual histidine. Most are predicted to have tyrosine at position B10 instead of the usual leucine. *N. commune* with histidine at position B10 is an exception.

Phylogenetic analysis, based on primary amino acid sequences, shows that the hemoglobins of ciliated protozoa, the green alga *C. eugametos*, and cyanobacteria form a natural group separate from that comprising the flavohemoglobins of eubacteria and yeast and the dimeric hemoglobin of *V. stercoraria* (12). The presence of HbN relatives in these distant organisms may indicate a widespread occurrence. However, the analysis of the currently available complete genome sequences of *E. coli* (44), *Saccharomyces cerevisae* (45), *Synechocystis* (10), and *M. tuberculosis* (5) indicates a scattered distri-

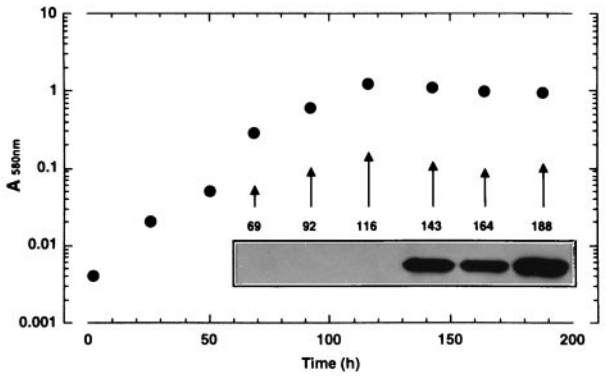

FIG. 4. HbN is expressed in resting cells of *M. bovis* BCG. *M. bovis* BCG cells (ATCC 35734) were grown at 37°C with constant shaking. Aliquots of suspension were taken at the indicated times for A_{580} determination and Western blot analysis of total proteins (arrows). The detection was performed by using polyclonal Abs raised against HbN. Each lane contains 5 μg of proteins.

bution. Indeed, *E. coli* and *S. cerevisae* possess only the flavohemoglobin gene, whereas *M. tuberculosis* and *Synechocystis* possess solely a gene related to *glbN*. Lateral gene transfer may explain this distribution.

Data on oxygen equilibria show that HbN is a cooperative homodimer that binds oxygen with high affinity (P_{50} = 0.013 mmHg). This is in contrast with *Paramecium caudatum* and *N. commune* hemoglobins, which show only moderate affinity for oxygen (P_{50} = 0.6 and 0.55 mmHg, respectively) and no cooperativity. The very large Hill coefficient of HbN will cause it to unload a majority of bound oxygen within a narrow range of pO_2 near its P_{50} value. These properties render HbN an interesting model for studying intersubunit communications. In this regard, studies of the homodimeric hemoglobins from the clams of the arcid family have revealed that the mechanisms of cooperativity can differ considerably from the mechanism proposed for mammalian hemoglobins (46, 47).

Our investigations revealed that HbN has a unique distal heme pocket structure, which allows strong stabilization of the oxygen molecule (k_{off} = 0.2 s^{-1}), largely by hydrogen bonding to the tyrosine residue at B10. In HbN, the distal group at the E7 position is not capable of donating a hydrogen bond to the oxygen ligand. In contrast, stabilization of the bound oxygen in the similar hemoglobin of *C. eugametos* involves both the tyrosine residue at B10 and the glutamine residue at E7, resulting in a very low dissociation rate (k_{off} = 0.014 s^{-1}) (28). In *N. commune* hemoglobin, a histidine residue should occupy the B10 position and a glutamine residue the E7 position. Interestingly, oxygen dissociation from *N. commune* hemoglobin is 400-fold faster than from HbN and 6000-fold faster than from *C. eugametos* hemoglobin (14). The above data emphasize the importance of the B10 tyrosine within this group of hemoglobins. They also indicate that different mechanisms have evolved for stabilizing bound oxygen, which probably reflect adaptation to the environments in which these organisms are found.

Owing to its high oxygen affinity, HbN will be oxygenated even at low O_2 tension. The low level of oxygen available through HbN may be important for the bacillus to survive in the hypoxic environment of the granuloma when the bacilli enter latency. However, most critically, a good defense system against cellular damage by reactive oxygen and nitrogen species produced by the host macrophages and phagocytes in the granuloma is indispensable. It is known that reactive oxygen species can be destroyed by a catalase–peroxidase system encoded by the *katG* gene in *M. tuberculosis* (48, 49). There is a great deal of evidence that nitric oxide generated by inducible nitric oxide synthase (NOSII) from macrophage controls the development of *M. tuberculosis* infection in its host (24, 50–52). Nitrogen intermediates are not only produced by NOSII in the macrophages during the initial infectious stage but also could be involved in restricting the bacteria in a latent state (24). The bacillus, in turn, has evolved a defense mechanism against reactive nitrogen intermediates. Recently, the expression of a set of proteins in response to the presence of reactive nitrogen intermediates was indeed reported in *M. tuberculosis* (53, 54), and a gene conferring resistance to these intermediates was cloned (55). That the oxygenated form of HbN is involved in the protection of the bacilli against reactive nitrogen species produced in the granuloma during latency is supported by the observation that the expression of HbN is elevated in the stationary phase during cell growth *in vitro*. The environment that the bacilli experience in the stationary phase *in vitro* is believed to be similar to that in the latent phase found *in vivo* (56, 57). The oxygenated HbN could destroy NO in a fashion similar to that observed in human oxyhemoglobin and flavohemoglobin of *E. coli* in which NO is converted to nitrate by the oxygenated heme (16). The high oxygen affinity and cooperativity of HbN ensure the availability of the active oxygenated heme.

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