The Truncated Oxygen-avid Hemoglobin from *Bacillus subtilis*

**X-RAY STRUCTURE AND LIGAND BINDING PROPERTIES**

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The group II truncated hemoglobin from *Bacillus subtilis* has been cloned, expressed, purified, and characterized. *B. subtilis* truncated hemoglobin is a monomeric protein endowed with an unusually high oxygen affinity (in the nanomolar range) such that the apparent thermodynamic binding constant for O₂ exceeds that for CO by 1 order of magnitude. The kinetic basis of the high oxygen affinity resides mainly in the very slow rate of ligand release. The extremely stable ferrous oxygenated adduct is resistant to oxidation, which can be achieved only with oxidant in large excess, e.g. ferricyanide in 50-fold molar excess. The three-dimensional crystal structure of the cyano-Met derivative was determined at 2.15 Å resolution. Although the overall fold resembles that of other truncated hemoglobins, the distal heme pocket displays a unique array of hydrophilic side chains in the topological positions that dominate the steric interaction with iron-bound ligands. In fact, the Tyr-B10, Thr-E7, and Gln-E11 oxygens on one side of the heme pocket and the Trp-G8 indole NE1 nitrogen on the other form a novel pattern of the “ligand-inclusive hydrogen bond network” described for mycobacterial HbO. On the proximal side, the histidine residue is in an unstrained conformation, and the iron-His bond is unusually short (1.91 Å).

Truncated hemoglobins (trHbs) are present in a wide variety of both Gram-positive and Gram-negative bacteria, as well as in some plants and fungi (1). TrHbs are 20–40 amino acids shorter than other globins, and the classical globin fold is not conserved as revealed by the crystal structures of the proteins from *Chlamydomonas eugametos, Paramecium caudatum*, and *Mycobacterium tuberculosis* (2–4). In contrast to vertebrate and other nonvertebrate hemoglobins, in which most heme pocket residues are conserved, bacterial hemoglobins and trHbs in particular are characterized by a remarkable variability in the nature of these residues, especially in the distal side of the heme pocket (1). Thus, whereas the proximal F8 histidine is invariant and the B9-B10 pair is commonly phenylalanine-tyrosine, other residues lining the heme pocket are not conserved.

A phylogenetic classification has suggested the partition of trHbs in three groups that share less than 30% sequence similarity with each other, and the sequence identity within the same group may be greater than 80% (1). The phylogenetic tree brings out distinct structural features of the active site that are characteristic of each group. In particular, group II and group III trHbs are characterized by the presence of a tryptophan residue at position G8, in the proximity of the heme group. Based on the crystal structure of *M. tuberculosis* HbO, the only available one for group II trHbs, such a large hydrophobic residue in the heme pocket is thought to hinder the escape of gaseous heme ligands and thereby contribute to the lower oxygen dissociation rate constants (around 10⁻³ s⁻¹) observed in group II trHbs (5, 6) with respect to the group I proteins (10⁻¹–10⁻² s⁻¹) (7–12). An additional contribution to the stabilization of heme ligands is provided by hydrogen bonding to Tyr-B10, a conserved residue in all trHbs. In some proteins carrying a polar residue at position E11, usually Gln, hydrogen bonding to the iron ligand may also involve this residue that is on the opposite side of the distal heme pocket relative to Tyr-B10. In HbO, even Tyr-CD1 has been shown to participate in ligand stabilization, thus contributing to the creation of an ensemble of polar residues in contact with the iron-bound ligand coordination shell that has been appropriately named a “ligand-inclusive hydrogen bond network” (6).

The high oxygen affinity displayed by most trHbs renders their role as oxygen transporters unlikely (1, 6). Other functions have been proposed, e.g. trHbs have been presumed to be terminal oxidases (13), to act as oxygen sensors (1), or to be involved in the response to oxidative and nitrosative stress (7, 15), but no final answer to this question has been given as yet. Some pathogenic bacteria, like *Mycobacterium avium* and *M. tuberculosis*, have more than one trHb belonging to different groups. In *M. tuberculosis*, group I HbN is expressed during the stationary phase and is thought to be involved in the defense against nitrosative stress, whereas no clear function has been attributed to group II HbO, which is expressed throughout the *Mycobacterium* growth phase (4). Such distinctive features have been correlated with the necessity of pathogenic bacteria to outlive the adverse conditions encountered during the infectious cycle (12, 16) and may hence pertain to other organisms. In any case, the large diffusion of trHbs also in bacteria with a severely reduced genome, like *Mycobacterium leprae*, is indicative of their importance for the survival of microorganisms.
The genes encoding for truncated hemoglobin genes have been identified in all species of the Bacillus genus, including Bacillus anthracis, Bacillus cereus, Bacillus halodurans, and Oceanobacillus iheyensis, as well as Bacillus subtilis. The proteins belong to group II trHBs, are closely related to each other (>60% sequence identity), and have the same heme pocket residues. Most interestingly, in all Bacillus species whose genomes have been sequenced, truncated hemoglobin genes coexist with true-type heme-containing chemotactic oxygen sensors and flavohemoglobins. However, in B. subtilis theglobin-like sensor domain of the HemAT oxygen sensor as well as the flavohemoglobin heme domain share little sequence similarity with the trHB. Most importantly, structural alignments reveal that key residues within the heme pocket are not conserved, suggesting different functional roles for the three proteins (17).

In this framework, unveiling the structure and ligand binding properties of Bacillus trHBs represents a key step toward the understanding of the physiological role of these proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tris, EDTA, NaCl, NaOH, HCl, and sodium dihydrogen phosphate were from Carlo Erba Reagents (Rodano, Italy); the other reagents were from Sigma, unless specified otherwise.

**Methods**

Cloning, Expression, and Purification of Recombinant B. subtilis trHB—The amplification of the yglb gene from B. subtilis genomic DNA was obtained through PCR. The primers 5′-GGA GTA GTC ACC ATG GGA CAA TC-3′ (forward) and 5′-TCA GAT AAA GGC TCA ACA AAC-3′ (reverse) were used. The DNA fragment was purified with the Qiagen GmbH (Hilden, Germany) kit, digested with restriction enzymes BamHI and NcoI (MBI Fermentas, Vilnius, Lithuania), and inserted into a pET28b (+) plasmid (Novagen, Darmstadt, Germany). Competent Escherichia coli BL21DE3 cells were transformed with the ligation mixture, and the colonies with the correct DNA insert were selected through PCR screening. E. coli cells were grown overnight in Luria-Bertani medium containing 0.017 mM kanamycin, and 0.3 mM 3-amino-5-methylindole (the heme precursor) and 0.2 mM isopropyl thiogalactopyranoside (the inducer) were added in the stationary phase. The cells were pelleted after 2–3 h, resuspended in a minimum volume of lysis buffer (50 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 0.5 mM dithiothreitol, 100 mM NaCl, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride), and sonicated until the supernatant was reddish and clear. After centrifugation, the supernatant was loaded on a DEAE-cellulose column (Whatman International Ltd., Maidstone, UK) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, and eluted with a NaCl step gradient (0–0.15 M). Further purification was obtained through gel filtration on a Sephadex G-75 column (Amersham Biosciences) equilibrated with 0.1 M sodium phosphate, pH 7.0. The protein obtained was >98% pure on SDS-PAGE. Both the plasmid and the protein N-terminal sequences were determined and found to correspond to the expected ones.

**Sequence Analysis**—The sequences were obtained from the NCBI protein sequence data base (www.ncbi.nlm.nih.gov/Entrez). The sequence alignment of group II trHBs (B. subtilis, accession number GI:16078821; Arabidopsis thaliana, GI:14165163; and M. tuberculosis HbO, GI:15699607) and group I trHBs (M. tuberculosis HbN, GI:15698680; Synechocystis sp., GI:27065788; Chlamydomonas eugametos, GI:1707907; and Paramecium cedonum, GI:10853566), whose three-dimensional structures and/or functional properties have been determined (Fig. 1), was performed with ClustalW (18) and refined, wherever possible, according to the structural alignment. The sequence of B. subtilis trHB (Bs-trHB) was used to search the NCBI nonredundant sequence data base (www.ncbi.nlm.nih.gov/) for sequence homologues using the programs PSI-BLAST (19) and SAM-T02 (20). A multiple sequence alignment of the collected homologues was generated using ClustalW and refined according to the structural alignment shown in Fig. 1. The resulting multiple sequence alignment (data not shown) was used to investigate sequence variability at specific positions.

**Protein Crystalization and Data Collection**—Attempts to crystallize the oxygenated derivative were unsuccessful. Crystallization of cyanomet Bs-trHB, obtained by addition of 2-fold molar excess KCN to the oxidized protein, was achieved at 293 K by the hanging drop vapor-diffusion technique. A 2-µL volume of protein at 20 mg/ml (ε280 = 115 mM−1 cm−1 for the cyanomet derivative) equilibrated versus 0.05 mM sodium phosphate buffer, pH 7.25, 2% KCN, was mixed with an equal amount of the reservoir solution containing (NH4)2SO4 in a range between 1.8 and 2.4 M, isopropl alcohol at 5% v/v, and 2 mM KCN. Crystals grew in 2 weeks and were very small. A preliminary data set was collected at 2.5 Å resolution using the MAR CCD detector on the x-ray beamline BW7A at DESY (Hamburg, Germany) at a wavelength of 0.926 Å. The deposited structure factors were calculated from a data set collected using the MAR CCD detector on the x-ray beamline XRD1 at ELETTRA, Basovizza (Trieste, Italy), at 2.15 Å resolution. The data were collected at 100 K using 26% glycerol as cryo-protectant. Intensities were integrated with DENOVO and scaled with SCALEPACK (21).

The data analysis indicates that the crystal is tetragonal (P41212) with unit cell dimensions of a = 41.193, b = 41.193, and c = 134.537 Å; the asymmetric unit contains one molecule. The data scaling gave an Rmerge of 9.8% and a χ2 of 1.2. The data set was 98.5% complete at 2.15 Å resolution.

**Structure Solution and Refinement**—The x-ray structure of Bs-trHB was solved by molecular replacement using the A subunit of M. tuberculosis HbO (Protein Data Bank entry IN6G) as search probe. The rotational and translational searches, performed with the program MOE (22), gave 100% in the resolution range of 10–3.0 Å, with 0% E-factor calculated as a measure of the quality of the model. The Ramachandran plot contains 95% of the residues in the favored region. The most important structural and refinement statistics are listed in Table I.

**Spectroscopic Characterization**—All spectra were measured on a Jasco V-570 spectrophotometer (Jasco Ltd., Japan).

**Association of the Oxygenated Derivative**—The state of association of the oxygenated derivative was determined through analytical ultracentrifugation. All experiments were conducted on a Beckman Optima XL-A ultracentrifuge equipped with an An60-Ti rotor at 20 °C in 0.1 M sodium phosphate buffer, pH 7.0. The scans were taken at an appropriate wavelength (500, 540, or 580 nm) for different protein concentrations, in the range 50 µM to 1.0 mM, with different protein concentrations, in the range 50 µM to 1.0 mM. Sedimentation velocity experiments were performed at 50,000 rpm, at an spacing of 0.05 cm/sec, with three averages. Scans were taken every 5 min. The data were analyzed with Sedfit 8.3 (26), and the sedimentation coefficient was reduced to s20,w by using standard procedures (27). Short column sedimentation equilibrium experiments were conducted at 30,000, 35,000, and 40,000 rpm. Scans were taken every 3 h at a spacing of 0.01 cm with 10 scans. Data were edited and analyzed in the program DASLIN (R. S. Naughton, University of Connecticut, Storrs, CT) both individually and globally. Control spectra were taken at the end of the experiment to check for the possible occurrence of degradation and/or oxidation.

**Kinetic Measurements**—The CO/O2 displacement kinetics was measured at 20 °C in an HP 3852 diode array spectrophotometer. The carbonmonoxo derivative was obtained by equilibrating the oxygenated protein with 1 atm CO gas over 24 h in a tonometer. The CO-bound protein was added to a 1-cm gas-tight quartz cuvette containing a measured amount of 0.1 M sodium phosphate buffer at different oxygen concentrations. The CO-O2 exchange was monitored through the decrease of absorbance at 421 nm, the Soret peak of carbonmonoxo-trHB. At the highest CO/O2 ratios analyzed, an alternative procedure was used. Concentrated samples of oxygenated trHB (1–10 µM at a protein concentration of 0.5–1.5 mM) were added to a 10-cm path length gas-tight cuvette (8 ml, total volume) containing buffer equilibrated with 1 mM CO. The O2-CO exchange was monitored through the increase of absorbance at 421 nm. Data points were taken every 12–36 s for 1–2 h. In all cases, the final trHB concentration was between 1 and 5 µM.

Rapid kinetic experiments for the measurements of the oxygen release reaction were conducted in a stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). The protein concentration was 5 µM in 0.2 M sodium phosphate buffer, pH 7.0. The rate constant of oxygen release was obtained by mixing a solution of oxy-trHB with CO-saturated (1 mM) buffer containing an excess of sodium dithionite (10–50 mM). Under these conditions, the CO binding reaction is fast, and oxygen release is the rate-limiting factor. The increase in absorbance at 421 nm, corresponding to the formation of carbonmonoxo-trHB, was
monitored. The dependence of the apparent rate of oxygen release, \( k_{\text{app}} \), upon dithionite concentration was investigated by mixing the oxygenated protein with increasing amounts of dithionite. A dithionite stock solution (98 mM) was prepared according to Lambeth and Palmer (14) and diluted to concentrations in the range 0.8–50 mM with degassed buffer. Absorbance changes at 434 nm, corresponding to the Soret absorption band of the deoxygenated derivative, and at 416 nm were followed. Control experiments were also carried out by mixing ferric horse Mb and ferric Bs-trHb with dithionite at varying concentrations in order to monitor the reduction kinetics of the ferric derivatives. In these experiments, the ferric protein solutions were degassed and equilibrated under nitrogen prior to mixing with the dithionite solutions.

The kinetics of CO binding to deoxy-trHb were measured by rapidly mixing a solution of deoxy-trHb with 0.2 mM phosphate buffer containing sodium dithionite (10–50 mM) and CO at concentrations ranging from 15 to 250 μM. The decrease in absorbance at 434 nm was followed.

The CO binding kinetics of \( B. \ subtilis \) trHb were measured also by laser photolysis. The protein concentration was 5 μM in 0.2 mM phosphate buffer, pH 7.0. The protein solution was degassed exhaustively in a tonometer equipped with a fluorescence quartz cell of 1 cm length and was equilibrated with CO gas at an equilibrium concentration of 1 or 0.2 mM. The measurements were carried out at 20 °C using as an optical pump the second harmonic from a Quanta System Nd-YAG laser (λ = 532 nm, frequency = 2 Hz with pulse width of 5 ns and pulse energy of ~80 mJ). The laser pulse flashes the sample orthogonally to the optical probe because of a 100-watt UV-visible source focused onto a monochromator SPEX 1681. Single wavelength measurements were acquired using a Hamamatsu photomultiplier tube H6870 as a detector. The time courses (average of 64 traces) were followed at the Soret peak of the deoxy derivative (434 nm) and were recorded using a Tektronix TDS 360 digital oscilloscope.

The CO dissociation kinetics were measured by mixing the trHb-CO adduct with solutions containing NO in excess. The trHb-CO derivative was prepared by equilibrating overnight a concentrated oxy-trHb sample (200 μM) with 1 atm CO. For the measurements, the CO derivative was diluted 80-fold with deoxygenated buffer containing a 2 mM NO solution in a sealed 1-cm path length cuvette. The signal decrease at 421 nm was followed over a period of 2 h.

Oxidation reactions were carried out by exposing oxygenated Bs-trHb in 0.1 mM phosphate buffer, pH 7.0, to H2O2 or potassium ferricyanide in the presence of a 50 mM excess of ferricyanide yields the fully oxidized protein. Excess oxidant and oxidation products were removed by passage through a G-25 column equilibrated with 0.1 mM phosphate buffer at pH 7.0 containing 0.5 M KCl.

EPR Spectra—Continuous wave EPR spectra were recorded using a Bruker ER 200D series X-band spectrometer equipped with a TE102 cavity and an Oxford Instruments ESR-900 helium flow cryostat. The spectra were measured on the ferric Bs-trHb adduct and on the ferrous oxygenated derivative at pH 7.0.

**RESULTS**

Sequence and Structure Analysis—The sequence alignment of representative group II and group I trHbs, selected among those whose structural and/or ligand binding properties have been determined, is presented in Fig. 1. The three glycine motifs, which characterize the compact 2-over-2 α-helical fold of trHbs, and relevant residues in the heme pocket are highlighted. In Bs-trHb residue B10 is Tyr as in many bacterial hemoglobins, whereas residue E7 is Thr, at variance with the other group II trHbs previously studied that have a hydrophobic Ala residue in this position (1). The B9 and CD1 positions, usually occupied by aromatic residues, are both Phe residues, and position E11 hosts the less conserved Gln.

Analysis of the three-dimensional structure (Fig. 2) brings out similarities and differences relative to \( M. \ tuberculosi s \) HbO, the only group II hemoglobin whose crystal structure has been determined (4). The overall fold and the relative position of the heme and of the heme contacting residues are similar in the two proteins. Thus, despite three deletions occurring in Bs-trHb relative to HbO (the first between helices A and B, the second between helices C and E, and the third between helices G and H, see Fig. 1), four α-helices (G, H, E, and B) are organized in a two-over-two α-helical fold. The structural comparison between the two proteins carried out by superimposing these four α-helices yields a root mean square deviation of 0.771 Å (Fig. 2). The F-helix is reduced to a one-turn helix placed in the middle of a flexible loop. Bs-trHb conserves the so-called α-helix (residues 62–65), a common characteristic of all group II trHbs.

The major differences between the two proteins are observed in the flexible regions (CD1 loop, pre-F loop, and the loop between helices G and H). In Bs-trHb the CD1 loop is less flexible than in HbO; in particular, the four residues 34–37 are organized in a one-turn helix. Moreover, in Bs-trHb the loop which precedes the small F-helix (residues 52–58) is more extended toward the solvent and appears to be less polar than in HbO (Pro\(^{60}\) substitutes an Arg residue in HbO). In addition, the loop between helices G and H, which is polar and extended toward the solvent in HbO, in Bs-trHb is shorter and apolar because of the deletion of three polar residues, namely Ser\(^{101}\) Glu\(^{102}\), and Thr\(^{103}\) of HbO, and to the substitution of Asp\(^{100}\) with a Gly residue (Gly\(^{101}\) in Bs-trHb).

Distal Heme Pocket—The distal heme pocket of Bs-trHb is characterized by the presence of an array of polar residues...
surrounding the bound ligand, namely Tyr25-B10, Thr45-E7, and Gln49-E11 (Fig. 3A). The cyanide ion is almost perpendicular to the heme plane, and the carbon atom is bound to the ferric heme iron at a distance of 1.92 Å. The cyanide ion is stabilized mostly by the Tyr25-B10 residue, which is hydrogen-bonded through the OH group directly to the cyanide nitrogen atom (distance \(H11005\) 2.54 Å). Trp89-G8 is buried in the distal pocket and is roughly parallel to the heme plane with the indole NE1 atom placed at a distance of 3.4 Å from the cyanide nitrogen atom. At the external edge of the distal site, a Lys residue (Lys48-E10) forms a salt bridge with the heme propionate D (distance O1D–NZ \(H11005\) 2.23 Å). This salt bridge is on the external surface of the protein and may hinder access to the distal pocket. Most interestingly, position E10 is occupied by a positively charged residue in all three groups of trHbs.

**Proximal Heme Pocket**—In Bs-trHb the heme is inserted into a crevice formed by the two antiparallel E- and G-helices and the short F-helix. The architecture of the heme pocket in the proximal region is dominated by Arg75-F7, His76-F8, Phe79, and Met121-H10 (see Fig. 3C) and differs from HbO where position H10 is occupied by a leucine residue (Fig. 3D). The position and orientation of the proximal histidine is typical of an unstrained imidazole ring that facilitates the heme in-plane location of the iron atom. Although the unconstrained histidine conformation appears to be a common feature of the trHbs investigated to date (28), the coordination bond formed by His76-F8 is stronger than in other hemoglobins as indicated by the unusually short iron-histidine distance (N-Fe(III) \(H11005\) 1.91 Å). It is of interest
that in Bs-trHb the proximal pocket appears to be directly exposed to solvent through a 55-Å² aperture situated in a shallow depression delimited by the phenyl ring of Phe79, the carbonyl backbone portion of Arg177-F7, and the heme propionates (Fig. 4A). The center of the depression is occupied by spurs formed by the positively charged residues Arg177-F7, whose NH2 is hydrogen-bonded to the main chain oxygen (distance = 2.84 Å) of His69, and Lys46-E10 that forms a salt bridge with the oxygen atom of the heme propionate D. In the HbO crystal, where Arg75 and Phe79 are conserved and an Arg residue substitutes Lys46-E10, a similar depression is observed with a similar, albeit smaller, opening (46 Å²) because of the ~1-Å displacement of the FG loop.

**Protein Cavities**—Two major cavities can be identified in Bs-trHb by means of an internal surface analysis performed using a 1.4-Å radius probe (program CAST P (29)). The inner cavity (see Fig. 4B) is lined by residues Val21 and Phe24 on the B-helix, Gln49 and Leu53 on the E-helix, and Trp89-G8 and Met93 on the G-helix, whereas the more external one is lined by residues Tyr56 and Leu57 on the C-terminal part of the G-helix and by Phe109, Leu110, and Arg113 on the H-helix. The passage between the two cavities is blocked by Tyr56, Leu53, and Leu114.

**Preparation and Spectral Properties of the Ferrous and Ferric Derivatives**—Bs-trHb is expressed in *E. coli* in the ferrous, oxygenated form. Because of the extremely high oxygen affinity, deoxygenation can be achieved only upon addition of sodium dithionite, as extensive degassing is ineffective. Addition of CO to deoxy-trHb yields the carbonmonoxy derivative. Unless otherwise specified, carbonmonoxy-trHb was obtained by this procedure. The spectra of ferrous oxy-, deoxy-, and carbonmonoxy-Bs-trHb are presented in Fig. 5. The EPR spectrum of the ferrous oxygenated adduct (Fig. 6) was measured at 10 K together with the spectrum of the ferric derivative (at the same protein concentration). It reveals the presence of a small amount (8%) of ferric high spin iron (g = 6) and trace amounts of a low spin ferric iron derivative (g values of 2.27 and 2.17). Full oxidation of the oxygenated adduct leads to the complete disappearance of the low spin features, a finding that argues against the low spin species being a hemichrome-like adduct.

Despite the presence of non-negligible amounts of ferric species in the EPR spectrum of the oxygenated protein, the conversion of the ferrous oxygenated Bs-trHb into the fully ferric adduct proved to be particularly difficult. Attempts to oxidize the oxygenated adduct at pH 7.0, in phosphate buffer, with stoichiometric amounts of potassium ferricyanide or hydrogen peroxide were unsuccessful. Hydrogen peroxide in 10 molar excess leads to slow heme degradation, as demonstrated by the steady decrease of the absorbance in the UV-visible region that takes place over 10s of minutes with no significant changes in the spectral line shape (data not shown). In turn, ferricyanide in at least 50 molar excess yields a fully oxidized protein. The spectra, measured after removing excess ferricyanide by G-25 gel filtration chromatography, display the typical charge transfer band at 625 nm and a peak in the Soret region at 410 nm and therefore can be assigned to a hexacoordinated high spin species (Fig. 5, C and D). This assignment was confirmed by the presence of a strong g = 6 signal, devoid of low spin contributions, in EPR measurements at 10 K on the fully oxidized protein (see Fig. 6). Moreover, the ferric protein undergoes an acid-alkaline transition at pH values higher than 10, consistent with the formation of an iron-hydroxyl low spin adduct (Fig. 5, C and D).

**State of Association**—Sedimentation velocity experiments conducted on oxygenated Bs-trHb yield an s20,w value around 1.8 S (data not shown), indicative of a monomeric species. The equilibrium sedimentation data confirm this finding and show unequivocally that the oxygenated derivative of *B. subtilis* trHb is monomeric and does not undergo association-dissociation reactions. Thus, the data fit to a single species with molecular mass of 14,000 ± 1000 Da (Fig. 7). The difference relative to the expected value (15,774 Da) could be because of a slight heterogeneity of the sample or to an otherwise undetectable amount of degraded protein. However, it should be stressed that the sample is stable during the course of the experiment, as the scans taken at equilibrium at 3-h intervals are all perfectly superimposable. Experiments on the deoxygenated derivative were unsuccessful because reoxygenation of the protein occurs despite the use of traces of dithionite to take care of oxygen leakage in the ultracentrifuge cell.

**Ligand Binding Properties**—The association and dissociation rate constants for oxygen and carbon monoxide were determined in flash photolysis, rapid mixing, and ligand displacement experiments. The values obtained are summarized in Table III. The rate of oxygen binding cannot be measured by laser photolysis, as no ligand dissociation is detected in the experiments conducted on the oxygenated derivative using the currently available experimental setup. Thus, the second-order oxygen binding rate was calculated from ligand displacement experiments (see below).

The rate of oxygen release was assessed in oxygen dissociation experiments with dithionite. In vertebrate Msbs and Hbs, the reaction of the oxygenated protein with excess dithionite

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**Fig. 2. Stereoview of the monomer fold of *B. subtilis* trHb.** The Cα carbon atoms are superimposed with those of *M. tuberculosis* HbO. The picture was generated with MOLSCRIPT (31).
FIG. 3. Stereo views of the heme environment of B. subtilis trHb and M. tuberculosis HbO. A and B, distal heme pockets of Bs-trHb and HbO, respectively. C and D, proximal heme pockets of Bs-trHb and HbO, respectively. Hydrogen bonds are indicated with dashed lines. The pictures were generated using BOBSCRIPT (32).
does not depend on dithionite concentration because the observed rate is limited by the rate of oxygen release from the heme iron. In contrast, in Bs-trHb the reaction of the oxygenated derivative with dithionite is strongly dependent on dithionite concentration over the concentration range 1–50 mM. The signal observed at 434 nm, which monitors formation of the deoxygenated derivative, was fitted to a monoexponential curve for every dithionite concentration. The rate constant thus obtained, \( k_{\text{obs}} \), depends linearly on the square root of dithionite concentration, rather than on dithionite concentration itself, with an overall rate of \( 4 \text{ M}^{-1/2} \text{s}^{-1} \), as estimated from the slope of the plot of Fig. 8A. This kinetic behavior indicates that the reduction process involves the \( \text{SO}_2^- \) anion, which is in dimerization equilibrium with dithionite, as a reducing species. It follows that, at variance with horse Mb, the reaction of oxy Bs-trHb with dithionite does not reflect simply oxygen scavenging by dithionite and cannot be used to calculate the value of the rate constant of oxygen release. Reduction experiments with dithionite, carried out on fully oxidized Bs-trHb in the absence of oxygen, yields a rate of \( 102 \text{ M}^{-1/2} \text{s}^{-1} \) (Fig. 8B). Ferric horse Mb displays a similar behavior and is reduced at a rate of \( 140 \text{ M}^{-1/2} \text{s}^{-1} \), in good agreement with the value reported by Lambeth and Palmer (14) (100 \( \text{M}^{-1/2} \text{s}^{-1} \) at pH 8.0).

Oxygen release experiments were also conducted by mixing a small volume of a concentrated oxy-trHb solution with buffer containing an excess of dithionite in the presence of 1 mM CO. Under these conditions, binding of CO to the heme iron is

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**FIG. 4.** *External surface and cavities of B. subtilis trHb.* A, external surface generated using GRASP (33); negatively and positively charged residues are shown in red and blue, respectively. B, cavities in the protein matrix. The surfaces, defined by a 1.4-Å probe, are shaded in purple. Tyr and Arg separating the cavities, and heme are depicted. The picture was obtained with INSIGHT II (34).

**FIG. 5.** *UV-visible spectra of ferrous and ferric B. subtilis trHb in the Soret and visible regions.* A and B, ferrous derivatives, oxy (solid line), carbonmonoxy (dashed line), and deoxy (dotted line). C and D, ferric derivative at pH 7.0 (solid line) and at pH 10.5 (dashed line). The protein concentration was 12 \( \mu \text{M} \). The deoxygenated derivative was obtained by addition of sodium dithionite to a degassed solution of oxy-trHb. The carbonmonoxy derivative was obtained by saturating a degassed solution of oxy-trHb with CO.
favored by the higher concentration of CO with respect to O₂, and oxygen dissociation is rate-limiting (see below and Table III). In these experiments, the value obtained, 4.8 ± 0.6 × 10⁻³ s⁻¹, was found to be independent of dithionite concentration over the range 10–50 mM.

The CO combination rate was determined in rapid mixing experiments (Fig. 9A). The observed pseudo first-order rates are relatively slow and provide an apparent second-order combination rate constant of about 0.35 μM⁻¹ s⁻¹, an intermediate value between those characteristic of group I (around 0.01 μM⁻¹ s⁻¹) and group II (around 1–10 μM⁻¹ s⁻¹) trHbs (5–7, 9–11). Laser photolysis experiments conducted on carbonmonoxy-trHb yield a similar value, 0.22 ± 0.04 μM⁻¹ s⁻¹, and in addition reveal that no geminate process takes place within the nanosecond time regime (Fig. 8B).

The rate of CO release was assessed in ligand displacement reactions by mixing the trHb-CO derivative with excess oxygen or excess NO and by following the decrease of the absorbance at 421 nm. The observed rate has a value of 4.6 ± 0.4 × 10⁻⁴ s⁻¹ (see Table III).

In order to obtain an independent estimate of the rate constants for oxygen and CO release and to calculate the apparent rate constant of oxygen binding, a complete set of CO/O₂ replacement reactions was carried out in the absence of dithionite. In the ligand displacement experiments, either a concentrated solution of carbonmonoxy-trHb was added to buffers containing decreasing amounts of dissolved oxygen or a concentrated solution of oxy-trHb was added to buffers containing decreasing amounts of dissolved CO. The reaction can be written (30) as indicated in Equation 1,

\[
\text{HbCO} \rightarrow \text{HbO}_2 \quad \text{(Eq. 1)}
\]

In the presence of high ligand concentrations, the amount of free Hb can be assumed to be small, and the rate of approach to equilibrium, \( R \), is defined as indicated in Equation 2,

\[
R = \frac{h}{k'} + \frac{k [O₂] + h [CO] + h k}{k' [O₂] + h [CO] + k} \quad \text{(Eq. 2)}
\]

A plot of \( R \) versus \( \log([CO]/[O₂]) \) yields the ligand displacement profile in which the lower asymptote corresponds to the CO dissociation rate constant and the higher asymptote to the oxygen dissociation rate constant. The solid line in Fig. 10 represents the least squares fit to the experimental data ob-
times lower than that for O₂, with an equilibrium constant of $10^{-9}$. Release measured in the presence of CO and dithionite (4.8 
fits to the experimental points (solid line) represents the best fit to the experimental data according to Equation 2. 
values of the fitting parameters are those listed in Table III. 
relative to other group II trHbs and M. tuberculosis HbO in particular, which add significant focus to the puzzling picture 
the structure of the Bs-trHb distal site is characterized by a set of residues that are conserved in all group II trHbs and 
the presence of unique amino acids. Thus, Phe²⁴-B₉, Tyr²⁵-B₁₀, Trp⁸⁹-G₈, and Phe⁵₂-E₁⁴ are conserved at positions that 
formation of a polar array running across the Bs-trHb distal pocket. Within this polar array, Tyr⁴⁵²-B₁₀ plays a pivotal 
the formation of a hydrogen bond between the phenolic oxygen and the cyanide nitrogen atom (2.54 Å distance). 
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ligated HbN and HbO indicating that, in contrast with mammalian hemoglobins, trHbs no ligand-triggered conformational transitions take place (28). Taken together, the available crystallographic and spectroscopic evidence on trHbs is highly suggestive of an extremely rigid active site conformation because of the presence of a 55-Å² opening, delimited on one side by the heme propionates and on the other by the proximal histidine. Inspection of the HbO three-dimensional structure reveals that the opening, although narrower, may provide direct access of solvent or other small molecules to the spatial relationships between the proximal histidine and the nearby Met residue (i.e., the 4-Å distance between the sulfur atom and the imidazole ring) and the syn conformation of the Met residue are also superimposable. At present, however, there is no evidence that Bs-trHb is endowed with peroxidase-like activity. In contrast, as discussed below, Bs-trHb appears to be unreactive toward hydrogen peroxide.

A last structural feature of Bs-trHb that deserves a comment is its existence as a monomeric species in solution. It should be pointed out that literature data on trHbs are scarce and heterogeneous with respect to both the techniques used and to the derivatives investigated. The only analytical ultracentrifugation data available to date were obtained on the ferric derivative of A. thaliana trHb, which is a dimer (5). Gel filtration experiments were conducted at an initial concentration of 0.15 mg/ml on M. tuberculosis HbO; the oxy, carbonmonoxy, and ferric derivatives are monomeric in the presence of 300 mM NaCl and associate partially into dimers at lower salt concentrations (6). Most intriguingly, the cyano-Met derivative of M. tuberculosis HbO crystallizes as a dodecamer because of the high concentration used in the crystallization experiments (13.5 mg/ml) and to the presence of sulfate anions that may bridge and hence neutralize positively charged patches of different subunits (4). Analysis of the intersubunit contacts indicated that only one of the various kinds of dimers within the dodecameric structure is likely to be stable in solution because it has a sufficiently large interface area (450 Å²) and is stabilized by two intermolecular salt bridges (4). Most of the residues at this dimer interface are solvent-accessible in B. subtilis trHb, but many of them are not conserved in type including those forming one of the two intermolecular salt bridges just mentioned. This difference might account for the different association state of the two proteins in solution.

The ligand binding properties of Bs-trHb provide a striking example of the ligand-inclusive hydrogen bond network that leads to stabilization of the heme ligands in group II trHbs. In this respect, it is of interest to note that the ligand-inclusive hydrogen bond network that characterizes the oxy, carbonmonoxy, and ferric derivatives is also present in the carbonmonoxy derivative of M. tuberculosis HbO and the oxy derivative of A. thaliana trHb (8).

### Table II

<table>
<thead>
<tr>
<th>Proximal and distal heme pocket distances in B. subtilis tr-Hb and in M. tuberculosis HbO</th>
<th>Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (CN)-O (Gln⁴⁵-E11)</td>
<td>3.3</td>
</tr>
<tr>
<td>N (CN)-OH (Tyr⁴⁵-B10)</td>
<td>2.6</td>
</tr>
<tr>
<td>N (CN)-N (Trp⁴⁸-G8)</td>
<td>3.4</td>
</tr>
<tr>
<td>O (CN)-Fe</td>
<td>1.9</td>
</tr>
<tr>
<td>OG1 (Thr⁴⁵-E7)-O2A (heme) (heme)</td>
<td>2.9</td>
</tr>
<tr>
<td>NZ (Lys⁴⁵-E10)-O1D (heme)</td>
<td>2.2</td>
</tr>
<tr>
<td>OE1 (Glu⁴⁵-E11)-OH (Tyr⁴⁵-B10)</td>
<td>3.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HbO distal heme pocket Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (CN)-OH (Tyr⁴⁵-CD1)</td>
</tr>
<tr>
<td>OH (Tyr⁴⁵-B10)-O2E (Tyr⁴⁵-CD1)</td>
</tr>
<tr>
<td>C (CN)-Fe</td>
</tr>
<tr>
<td>NH2 (Arg⁴⁵-E10)-O2A (heme)</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Ligand binding and dissociation constants of B. subtilis trHb and representative group II truncated hemoglobins</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis trHb</td>
</tr>
<tr>
<td>k_{on(O2)}</td>
</tr>
<tr>
<td>log M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>1.11</td>
</tr>
<tr>
<td>2.7</td>
</tr>
<tr>
<td>0.2</td>
</tr>
</tbody>
</table>

*This value was determined from O₂ displacement kinetics with CO in the presence of dithionite.*

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**Structure and Function of B. subtilis Truncated Hemoglobin**

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hydrogen-bonding potential because of the presence of Gln-E11. The polarity of the E7 residue in Bs-trHb (Thr instead of Ala) may account for the increased O₂ and CO affinity of this protein with respect to the other group II trHbs previously studied.

Although the structural basis of the slow rate of ligand release in Bs-trHb can be found in the hydrogen bonding potential of several donors within the distal pocket, the very high oxygen combination rate as calculated from the CO/O₂ ligand displacement experiments (see Table III) cannot be easily rationalized in molecular terms. It is in accordance with the in-plane heme location, favored by the unstrained proximal Fe-His bond. However, because these features are common to both Bs-trHb and HbO₂, the 2 orders of magnitude difference in the calculated second-order combination constant remains unexpected, just as the different ability to discriminate oxygen against CO within the second-order combination process.

It is tempting to speculate that the high stability of the oxygenated adduct and its resistance to oxidation are common to all group II trHbs because of the electron-rich Trp G8 in close contact with the heme moiety. The presence of a protein electron donor/acceptor species may also account for the complex spectrum of the oxygenated protein puts forward a slightly different interpretation that entails the occurrence of an inter-oxide before leaving the heme iron.

The presence of a small amount of ferric iron in the EPR spectrum of the oxygenated protein puts forward a slightly increased O₂ and CO affinity of this protein may account for the increased O₂ and CO affinity of this protein.

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The Truncated Oxygen-avid Hemoglobin from *Bacillus subtilis*: X-RAY STRUCTURE AND LIGAND BINDING PROPERTIES
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