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Electron transfer reactions, cyanide and O_2 binding of truncated hemoglobin from *Bacillus subtilis*

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ABSTRACT

The truncated hemoglobin from *Bacillus subtilis* (trHb-*Bs*) possesses a surprisingly high affinity for oxygen and resistance to (auto)oxidation; its physiological role in the bacterium is not understood and may be connected with its very special redox and ligand binding reactions. Electron transfer reactions of trHb-*Bs* were electrochemically studied in solution and at graphite electrodes. Spectrophotometrical potentiometric titration and direct electrochemical measurements gave a heme iron redox potential of -103 ± 4 mV and -108 ± 2 mV vs. NHE, at pH 7, respectively. The redox potential of the heme in trHb-*Bs* shifted -59 mV per pH unit at pH higher than 7, consistently with a $1e^-/1\,H^+$ – transfer reaction. The heterogeneous rate constant k_s for a quasi-reversible $1e^- - 1H^+$ – transfer reaction between graphite and trHb-*Bs* was $10.1 \pm 2.3\ s^{-1}$. Upon reversible cyanide binding the k_s doubled, while the redox potential of heme shifted 21 mV negatively, presumably reflecting changes in redox activity and in vivo signaling functions of trHb-*Bs* associated with ligand binding. Bioelectrocatalytic reduction of O_2 catalyzed by trHb-*Bs* was one of the most efficient hitherto reported for Hbs, with an apparent catalytic rate constant, k_{cat}, of $56 \pm 6\ s^{-1}$. The results obtained are of particular interest for applications of trHb in environmental biosensing and toxicity screening.

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1. Introduction

Globins comprise an ancient and diverse superfamily of heme *b*-containing cytosolic proteins [1]. In bacteria at least three types of globins are present; single domain globins, flavohemoglobins, and truncated hemoglobins (trHbs). The latter type, also found in plants and in unicellular eukaryotes, is the least well understood [2]. Discovered relatively recently, trHbs have attracted much attention with respect to their physiological role and ligand-recognition and binding properties [3–6].

TrHbs have a globin-like fold efficiently burying the heme prosthetic group; therewith, they display amino acid sequences 20–40 residues shorter than classical Hbs and have a novel globin fold comprised of a 2-on-2 α-helical sandwich secondary structure [2,4,6] (Fig. 1). The globin fold protects the heme iron from rapid oxidation and yet allows reversible dioxygen binding. The only invariant residue throughout all trHbs is the histidine at proximal F8 (helix F, position 8), which binds the heme to the protein by coordinating it in the iron's fifth coordinate position and leaves the iron's sixth coordinate site vacant for ligand binding [2,6]. The distal residues vary, but most bacterial trHb retain a tyrosine residue at the B10 position, presumably responsible for their high affinity for oxygen and for ligand stabilization [2,6,7]. Each of the oxidation states of the heme iron in trHbs (both ferric and ferrous) is known to be capable of binding different ligands; physiological ligands for the reduced ferrous form are O_2, CO, H_2S, nitric oxide (NO) and cyanide; NO also binds to the oxidized ferric heme [2,6,8].

Contrary to Hb, the physiological functions for which are the storage and transport of molecular oxygen in mammalian blood, the physiological role of trHb is still under discussion and may vary between different orthologs. Suggested functions include moderation of oxygen homoeostasis, sensing of diatomic gases, and redox sensing [2,4,6]. In the pathogen *Mycobacterium tuberculosis* trHb HbN may provide protection against NO [9].

Here, we have studied the redox chemistry of trHb from the Gram-positive soil bacterium *Bacillus subtilis* (trHb-*Bs*) and its variation upon ligand binding, in order to elucidate the functional mechanisms of trHbs and their possible applications in environmental biosensing. The heme iron of trHb-*Bs* is strongly coordinated axially to the imidazole nitrogen of the proximal histidine, H76,

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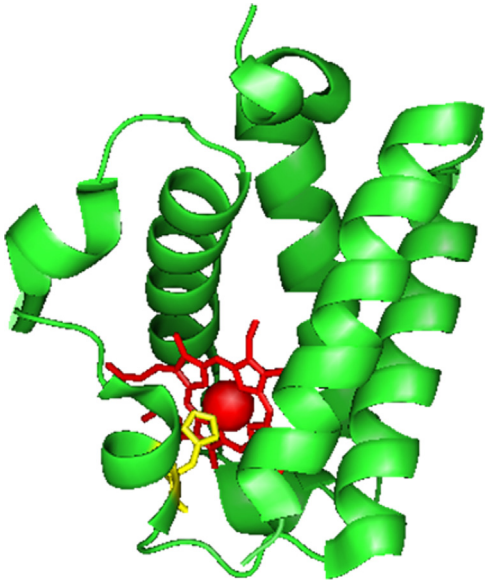

Fig. 1. Schematic representation of the *B. subtilis* trHb-*Bs* structure (PDB ID: 1ux8) [10], the heme is colored red, and a proximal F8 histidine residue in the binding site is colored yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with an unusually short ferric heme iron–histidine nitrogen distance of 1.91 Å [10]. In the native reduced state of the protein, an oxygen molecule is considered to occupy the sixth coordination position. In anaerobic conditions and/or in the oxidized ferric state, this coordination position is available for a water molecule or other ligands. To what extent ligation of the heme iron affects the electron transfer (ET) properties of the protein was of particular interest in view of the poorly understood physiological role of trHbs. To our knowledge this is the first report on the redox properties and the ET reactions of trHb with such diatomic molecules as cyanide anion and di-oxygen.

2. Experimental

2.1. General molecular techniques

Plasmid isolation, agarose gel electrophoresis, DNA restriction and modification, DNA ligation, PCR, and electroporation of *E. coli* cells were performed according to standard protocols [11]. Chromosomal DNA was prepared from *B. subtilis* strain 1A1 (*trpC2*) according to the method described by [12]. *E. coli* strains TOP10 (*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA1 nupG*) and TUNER(DE3) (F^- *ompT hsdSB gal dcm lacY1* (DE3)) were used for cloning and gene expression, respectively. L-broth or L-agar was used for growth of *E. coli* strains [11]. Fluorescent DNA sequencing was carried out on isolated plasmid DNA using the BigDye terminator v3.1 cycle sequencing reaction kit (Applied Biosystems) and an ABI prism 3100 DNA sequencer (PE Biosystems).

2.2. Cloning of the yjbI gene

The *yjbI* gene encoding trHb-*Bs* was amplified by PCR using oligonucleotides YjbI2 and YjbI3 and *B. subtilis* 1A1 chromosomal DNA template. The sequence of YjbI2 (5′-CCGGATCCAAGC TTGTCAAGATGATCGATCCTCCGC-3′) includes nucleotides complementary to the extreme 3′ end of the trHb-*Bs* coding sequence. The sequence of YjbI3 (5′-GGGCCATGGGACAATCGTTTAACGCACCTT-3′) includes nucleotides complementary to its extreme 5′ end. The PCR product was purified and digested with *Nco*I and *Hin*dIII. The cut PCR fragment was introduced into the corresponding sites of plasmid pET16b to yield plasmid pYjbI1. The integrity of the cloned *yjbI* gene was verified by DNA sequencing.

2.3. Expression and purification of trHbBs

E. coli TUNER (DE3) cells carrying the pYjbI1 expression plasmid were grown in (3 L, 3 × 1 L in 5 L baffled E-flasks) in TB medium [11] containing ampicillin (100 μg/mL) at 37 °C for 12 h with shaking (100 rpm). The cells were harvested by centrifugation (8000 rpm, 10 min), and the red-colored cell pellets were frozen (−20 °C, overnight). Thawed cells were resuspended in a minimal volume of buffer A (20 mM MOPS·HCl, pH 7.4) containing 1 mM EDTA. The cells were lysed by two passes through a French press operated at 16,000 psi. The red cell suspension was centrifuged at high speed (100,000 × *g*, 45 min, 4 °C) to pellet insoluble cellular debris. The supernatant containing trHb-*Bs* was loaded directly onto a Q-Sepharose High Performance column (2.6 cm × 10 cm) pre-equilibrated with buffer A. The column was washed with three column volumes of buffer A, prior to eluting trHb-*Bs* with a linear gradient (1 M NaCl in buffer A, 10 column volumes). Fractions were analyzed by UV/visible spectroscopy, and those containing trHb-*Bs* with an A_{416}/A_{280} ratio of 1 or higher were retained. The fractions were pooled and concentrated to 10 mL by ultrafiltration (Centriprep concentrators, 10 kDa cutoff, Millipore). To produce electrophoretically homogeneous trHb-*Bs*, and a final gel filtration step was done using Sephacryl S-100 HR resin (column dimensions 2.6 cm × 1 m) was used.

2.4. Potentiometric titrations

Redox titrations for trHb-*Bs* (in the range 10–40 μM protein) were carried out at 25 ± 2 °C in an anaerobic glove box (Belle Technology, Portesham, England) under a N_2 atmosphere with O_2 levels <5 ppm using the method of Dutton as described previously [13,14]. Redox titration buffer (50 mM potassium phosphate (PBS), pH 7.0) was deoxygenated by bubbling with Ar prior to transfer to the glove box. O_2 was removed from the trHb-*Bs* samples by passing concentrated stock solutions through a Bio-Rad Econo-pac 10DG gel filtration column in the glovebox, which had been pre-equilibrated with anaerobic redox titration buffer. Titrations were performed both in reductive and oxidative directions. Absorption changes during the titrations were monitored via a fiber optic absorption probe (Varian) immersed in the trHb-*Bs* solution in the anaerobic environment and connected to a Cary UV-50 Bio UV–vis spectrophotometer (Varian) outside the glovebox. Mediators were added to facilitate electrical communication between enzyme and electrode prior to titration: 2 μM phenazine methosulfate, 7 μM 2-hydroxy-1,4-naphthoquinone, 0.3 μM methyl viologen, and 1 μM benzyl viologen were included to mediate in the range between +100 and −480 mV. Data were analyzed by plotting the absorbance at an appropriate wavelength, corresponding to the maximal absorbance change between oxidized and reduced forms, against the potential. A one-electron Nernst function was then fitted to the data and the formal potential was calculated from these data fits.

2.5. Electrochemistry

Rods of solid spectroscopic graphite (SGL Carbon AG, Werk Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter) were cut; their disk surface was polished on emery paper (Tufbak Durite, P600), rinsed carefully with de-ionized water and fitted into Teflon holders. For adsorption, 6 μl of a 3.1 mg ml^{-1} trHb-*Bs* aqueous solution were placed onto the electrode surface and stored for 4 h at 4 °C under a plastic cover lid. Prior to experiments the modified

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