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Studies on the contributions of steric and polarity effects to the H_2S-binding properties of *Vitreoscilla* hemoglobin

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ABSTRACT

We have reported recently that *Vitreoscilla* hemoglobin (VHb) is a potential H_2S receptor and storage molecule in bacterial metabolism. In this study, molecular cloning and site-directed mutagenesis were employed to investigate the structural basis for H_2S binding. Association and dissociation rate constants (k_{on} and k_{off}) were determined using stopped-flow rapid-scanning spectrophotometry and compared with those for wild type VHb. Several unanticipated factors were found to govern H_2S binding properties, due to the distinct structure of VHb. The results presented in this paper show that: i) bulkier residues at positions E7 and E11 decrease H_2S binding accessibility, while the residue located at position B10 blocks bound H_2S from escaping. ii) hydroxyl sidechains within the distal heme pocket reduce H_2S reactivity to VHb; iii) Pro(E8) is involved in moving the E7-E10 loop region to trigger opening of the distal heme pocket to facilitate H_2S binding.

1. Introduction

Vitreoscilla, a gram-negative bacterium in the *Beggiatoa* family [1,2], inhabits freshwater sediments and cow dung, where oxygen availability is limited [1,3]. Under hypoxic conditions, *Vitreoscilla* synthesizes a soluble hemoglobin, VHb, the first bacterial Hb discovered [1]. In preliminary reports, VHb was found to be a homodimeric molecule consisting of two identical subunits of 15.7 kDa, each containing two protoheme IX complexes. This protein has been hypothesized to direct the energy metabolism of diverse organisms, including microorganisms, plants and mammalian cells [4,5]. In addition, Frey et al., 2002 and Kaur et al., 2002 reported that VHb is involved in protection of cells from nitrosation and oxidative stress. Related research has been conducted for approximately 30 years, but the exact role that this protein plays in the cellular metabolism of its host has not been fully elucidated.

Recently, we reported for the first time that VHb is one of the few known H_2S-reactive hemoglobins with a $K_d = 2.1$ nM, which is four orders of magnitude lower than values for vertebrate hemoglobins [6]. Interestingly, *Vitreoscilla* is always found in certain oxygen-limited, sulfide-rich environments [7] where environmental sulfide concentrations (0.54–1.13 μM) are much higher than the K_d between sulfide and VHb [8]. Accordingly, we have speculated that VHb may function in hydrogen sulfide receptor binding and storage in bacterial metabolism [6], even though hydrogen sulfide has been considered a toxic molecule for hundreds of years [9]. The primary mechanism for H_2S toxicity is its direct inhibition of cytochrome oxidase [10]. The discovery of endogenous hydrogen sulfide has subsequently spurred research to delineate its biological reactivity and potential physiological functions [11–14]. In fact, it has been suggested that H_2S can bind to hemeproteins to induce various responses that, in turn, modulate its cytotoxic and cytoprotective activities.

The interaction between H_2S and hemoglobin is a subject of a growing number of research studies. For instance, a typical H_2S-binding hemoglobin from the clam *Lucina pectinata* (HbI) has been studied for decades [15]. This protein is responsible for delivering H_2S to symbiotic bacteria [16]. Previous research has demonstrated that a Gln residue at the E7 positions of either VHb or HbI functions as an H-bond donor and Gln(E7) is responsible for high H_2S-binding affinities [16–19], playing a key role in modulating ligand binding properties. However, distinct from HbI, VHb exhibits an extraordinarily high affinity for both O_2 and H_2S. This feature plays a role in the evolutionary success of VHb under oxygen-limiting but sulfide-abundant conditions [20,21]. Two distinct structures of VHb are

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responsible for these ligand binding properties. First, the residues E7–E10 of VHb do not adopt an expected α-helical conformation, resulting in orientation of Gln(E7) in VHb towards solvent external to the heme pocket [22]. It seems that this orientation prohibits Gln(E7) from acting as an effective hydrogen donor in VHb. We have reported that substantial topological conformational changes could occur during entry of H_2S, which may cause E7 involvement in an H-bonding network [6]. Secondly, comparison of the structures of the two proteins using surface views reveals that the E helix in VHb is much closer to the heme plane (Fig. 1). Moreover, the particular residues present at positions B10 and E11 and their spatial orientations are markedly distinct from that of HbI. A previous study from our group reported that there are some unusual factors governing H_2S binding. For instance, the bulky residue Phe at position E11 in VHb serves to accelerate the escape of H_2S, while in the case of HbI, this residue functions as part of a cage to prevent H_2S from dissociating [23,24]. Overall, this work demonstrated that VHb serves as a new model for the exploration of an H_2S-binding hemoglobin that is distinct from HbI.

Site-directed mutagenesis of the distal amino acid residues of the heme pocket has helped elucidate the structural basis for differences in ligand affinity for this unusual hemoglobin. Research focusing on chemical interactions between VHb mutants and H_2S is an important approach to reveal the mechanisms behind H_2S entry and binding stabilization. This study is our first attempt to provide a molecular foundation for better characterization of the exact role of VHb involvement in H_2S receptor binding and storage in an organism-wide system.

2. Experimental section

2.1. Protein expression and purification

The plasmid pUC19-promoter-vgb was transformed into *E. coli* strain BL21(DE3)-pLysS. A His-tag was fused to the C-terminus of VHb to facilitate protein purification. The cells were cultured in 4 L LB medium containing 50 μg/mL ampicillin and 35 μg/mL chloramphenicol in 5 L flasks, with shaking at 170 rpm at 37 °C. Once cell density (OD_{600}) reached 0.6–0.8, synthesis of recombinant VHb was induced in an anaerobic environment at 25 °C, with shaking at 110 rpm for approximately 24 h [25]. Cell cultures were harvested by centrifugation at 10,000 × g. The bacterial pellet (~20 g) was suspended in 200 mL of 20 mM phosphate buffer (pH 7.4). The cells were disrupted using ultrasonication for 30 min. After centrifugation at 4 °C, 15,000 × g for 30 min, each supernatant was loaded onto a nickel affinity column then the column was washed with 20 mM PBS buffer (pH 7.4) and PBS buffer containing 5 mM imidazole until the OD_{280} of eluent returned to background level (0.02–0.05). VHb-His_6 was eluted with 20 mM PBS supplemented with 20 mM imidazole at a flow rate of 3.0 mL/min. A PD-10 column was used for desalting and buffer exchange. All mutants were purified using the same method above. A UV-2700 detection system (Shimadzu, Japan) was used to determine the "Reinheit Zahl" (Rz) value.

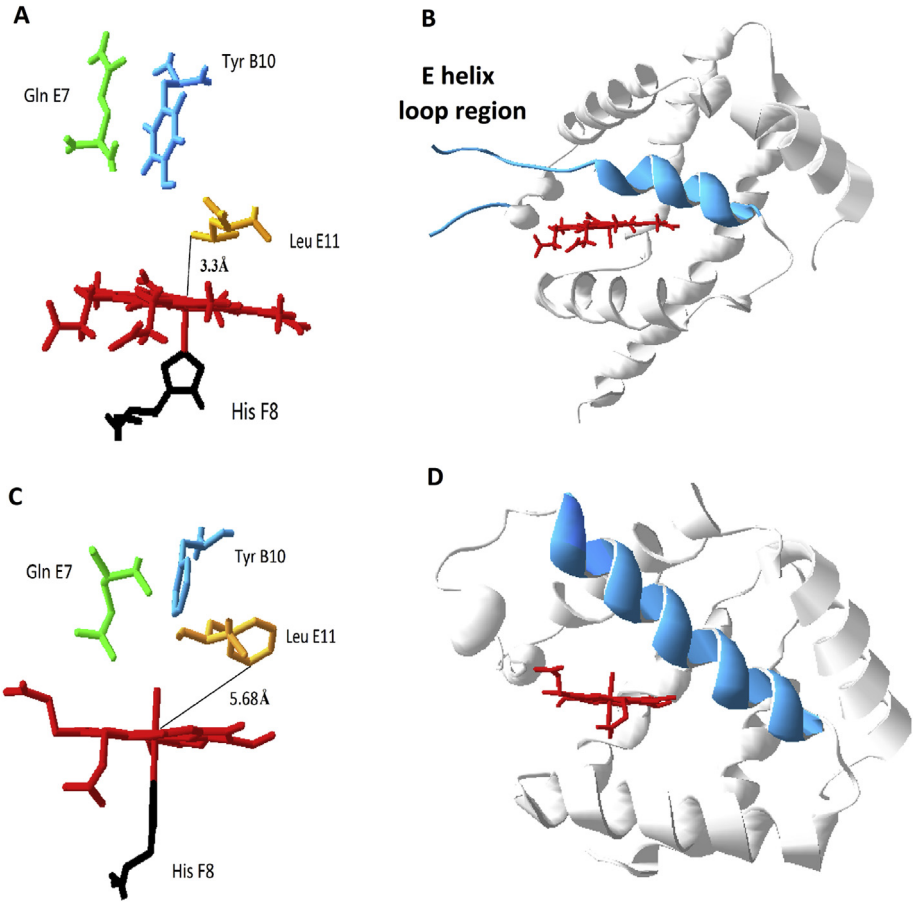

Fig. 1. (A) and (C) Representation of key residues in the heme pocket of VHb and HbI, respectively. (B) and (D) Superposition of structures of VHb [Protein Data Bank (PDB) entry 2VHB] and HbI [PDB entry 1MOH] conducted using PDB reviewer 4 (DeLano Scientific) [35]. The E helix is highlighted in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)