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H-bonding networks of the distal residues and water molecules in the active site of *Thermobifida fusca* hemoglobin ☆

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ABSTRACT

The ferric form of truncated hemoglobin II from *Thermobifida fusca* (Tf-trHb) and its triple mutant WG8F-YB10F-YCD1F at neutral and alkaline pH, and in the presence of CN^- have been characterized by resonance Raman spectroscopy, electron paramagnetic resonance spectroscopy, and molecular dynamics simulations. Tf-trHb contains three polar residues in the distal site, namely TrpG8, TyrCD1 and TyrB10. Whereas TrpG8 can act as a potential hydrogen-bond donor, the tyrosines can act as donors or acceptors. Ligand binding in heme-containing proteins is determined by a number of factors, including the nature and conformation of the distal residues and their capability to stabilize the heme-bound ligand via hydrogen-bonding and electrostatic interactions. Since both the RR Fe–OH^- and Fe–CN^- frequencies are very sensitive to the distal environment, detailed information on structural variations has been obtained. The hydroxyl ligand binds only the WT protein giving rise to two different conformers. In form 1 the anion is stabilized by H-bonds with TrpG8, TyrCD1 and a water molecule, in turn H-bonded to TyrB10. In form 2, H-bonding with TyrCD1 is mediated by a water molecule. Unlike the OH^- ligand, CN^- binds both WT and the triple mutant giving rise to two forms with similar spectroscopic characteristics. The overall results clearly indicate that H-bonding interactions both with distal residues and water molecules are important structural determinants in the active site of Tf-trHb. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Ligand binding in heme-containing proteins is determined by a number of factors, including the nature and conformation of the distal residues and their capability to stabilize the heme-bound ligand via hydrogen-bonding and electrostatic interactions. The truncated hemoglobin II from *Thermobifida fusca* (Tf-trHb) contains three polar residues in the distal site: TrpG8, TyrCD1 and TyrB10. Whereas TrpG8 can act as a potential hydrogen-bond donor, the tyrosines can act as donors or acceptors.

The ferric and ferrous derivatives of truncated hemoglobins, analogous to mammalian globins, bind a variety of small molecules, such as H_2O, NO, CN^-, F^-, and CO. Previous studies carried out on the CO [1], F^- [2,3] and HS^- [4] adducts formed with the native Tf-trHb and a combinatorial set of mutants, in which the three distal amino acids have been singly, doubly, or triply replaced by a Phe residue, revealed that all the ligands are stabilized by TrpG8 via a strong H-bond. TyrCD1 is able to interact with CO and fluoride, whereas TyrB10 is not directly involved in ligand stabilization and plays only a minor role.

In the present work we have extended the analysis to the ferric form, studying the behavior of the ferric native protein and its triple mutant WG8F-YB10F-YCD1F at neutral and alkaline pH, and in the presence of CN^-. Since both the RR Fe–OH^- and Fe–CN^- frequencies are very sensitive to the distal environment, detailed information on structural variations can be obtained. In particular, the comparison of the spectroscopic signature of the OH^- ligated proteins at alkaline pH with those of the cyanide adducts is expected to provide information

Abbreviations: trHb, truncated hemoglobin; Tf, *Thermobifida fusca*; Hb, hemoglobin; WT, wild type; ASV, acidic surface variant of Tf-trHb containing two single site mutations Phe107Glu and Arg91Glu; Mb, myoglobin; MES, 2-(N-morpholino)ethanesulfonic acid; MD, molecular dynamics; EPR, electron paramagnetic resonance; RR, resonance Raman; 5c, five-coordinate; 6c, six-coordinate; HS, high spin; LS, low spin

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on the effects of H-bond interactions between the ligand and the distal residues as well on the Fe–CN geometry.

2. Material and methods

2.1. Sample preparation

Wild type (WT) Tf-trHb was expressed as a recombinant protein in *Escherichia coli* cells and purified as described previously [5]. As previously reported [1] the acidic surface variant (ASV) of Tf-trHb differs from the WT protein by mutation of both Phe107 and Arg91 to glutamic acid, which increases protein solubility during recombinant expression, without affecting thermostability or ligand binding properties [1–3]. Therefore, ASV was taken as an engineered scaffold of the WT protein for subsequent site-directed mutagenesis studies on the relevant residues of the distal heme pocket.

Phosphate salts and glycine were obtained from Merck AG (Darmstadt, Germany). 2-[N-morpholino]ethanesulfonic acid (MES) was bought from Sigma-Aldrich (Steinheim, Germany). All chemicals were of analytical or reagent grade and were used without further purification. The cyanide complexes were prepared by adding a few microliters of a diluted solution of potassium cyanide to the ferric proteins (ASV and triple mutant). Buffered solutions (0.1 M) were used for experiments at pH 9.8–10.1 (glycine), 7.0 (phosphate), and 6.1 (MES).

Protein concentrations in the range 10–70 μM were used for the electronic absorption and RR samples. Sample concentration for low-temperature RR was between 30 and 100 μM. Sample concentrations for electron paramagnetic resonance (EPR) experiments were in the range 130–600 μM.

The protein concentration was determined on the CO derivative in the presence of 10–20 mM sodium dithionite by using an extinction coefficient of 174000 M^{-1} cm^{-1} for Tf-trHb.

2.2. Spectroscopic characterization

Electronic absorption spectra, measured with a double-beam spectrophotometer (Varian Cary 5), were recorded using a 1 cm quartz cuvette and a 600 nm/min scan rate. Absorption spectra (using a 5-mm NMR tube) were measured both prior to and after RR measurements to ensure that no degradation had taken place under the experimental conditions used. RR spectra were measured with excitation at 413.1 nm (Kr^+ laser, Coherent, Innova 300C) using a triple spectrometer (consisting of two Acton Research SpectraPro 2300i working in the subtractive mode, and a SpectraPro 2500i in the final stage with a 3600 grooves/mm grating), equipped with a liquid-nitrogen cooled CCD detector (Roper Scientific Princeton Instruments). RR spectra were calibrated with indene, n-pentane, and carbon tetrachloride as standards to an accuracy of 1 cm^{-1} for intense isolated bands.

The low-temperature experiments were carried out using an Air Products Displex closed-cycle He refrigerator with automatic temperature control. For the low-temperature RR measurements, 20 μL of the protein solution was deposited on the copper cold finger of the refrigerator at 90 K under a nitrogen flow. The temperature was then slowly decreased to 12 K under vacuum, and RR spectra were obtained at this temperature.

All RR measurements were repeated several times under the same conditions to ensure reproducibility. To improve the signal-to-noise ratio, a number of spectra were accumulated and summed only if no spectral differences were noted. All spectra were baseline corrected. To determine peak bandwidth and positions, a curve-fitting program (Lab Calc; Galactic) was used to simulate the spectra using a Lorentzian line shape. The frequencies of the bands were optimized with an accuracy of 1 cm^{-1}, and the bandwidths with an accuracy of 0.5 cm^{-1}. Bandwidths (full width at half-maximum) varied as follows: 12–14 cm^{-1} in the high frequency region; 9–12 cm^{-1} for the low frequency region.

EPR spectra were recorded as reported previously [6] and the g-values were determined by careful visual inspection of the spectra.

2.3. Molecular dynamics simulations

The simulations were performed starting from the crystal structure of WT Tf-trHb determined at 2.48 Å resolution, Protein Data Bank (PDB) entry 2BMM [5]. Two different systems of protein-ligand complexes, with a water or hydroxide ligand bound to the heme group, were built and simulated. The water molecule was added in the distal site bound to Fe(III) according to the equilibrium structure in an isolated model system QM calculation at the DFT level (with the PBE functional and 6–31 G** basis sets), taking into account the distal site environment. The charges and parameters for Fe(III) heme — water molecule were determined by a standard procedure: partial charges were computed using the restricted electrostatic potential (RESP) recipe and DFT electronic structure calculations with the PBE functional and 6–31 G** basis sets. The calculation has been performed in the high spin (HS) state. Equilibrium distances and angles, as well as force constants, were computed using the same methods and basis set used for computed charges. The same procedure was used for the hydroxide ligand. The parm99 force field implemented in AMBER was used to describe the protein [7]. The system was then immersed in a pre-equilibrated octahedral box of 4909 TIP3P water molecules using the tLEaP module of the AMBER package [7]. We used periodic boundary conditions with a 9 Å cutoff and Ewald sums for treating long-range electrostatic interactions. The SHAKE algorithm [8] was used to keep bonds involving H atoms at their equilibrium length, allowing us to use a 2 fs time step for the integration of Newton's equations. The histidine tautomeric state and protonation (Nε–H, Nδ–H, His^+) were carefully analyzed for each of the three His residues (namely His99, His122, and His135) and set to favor the hydrogen bond network suggested by the experimental crystal structure. Equilibration protocol consisted of (i) slowly heating the whole system from 0 to 300 K for 20 ps at constant volume, with harmonic restraints of 80 Kcal per mol A^2 for all Cα atoms; (ii) pressure equilibration of the entire system simulated for 1 ns at 300 K with the same restrained atoms. After these two steps unconstrained 30 ns of molecular dynamics (MD) simulations at constant temperature (300 K) were performed. To simulate the triple mutant (YB10F-YCD1F-WG8F) protein, we introduced in silico mutations by changing the corresponding amino acid in the original structure and allowing the system to equilibrate as mentioned earlier. All structures were found to be stable during the timescale of the simulations, as evidenced by the root mean square displacements (RMSD), depicted in Fig. S1, Supplementary data.

3. Results

3.1. Hydroxyl ligand

Fig. 1 shows the UV–vis titration and the RR spectra in the high frequency region of Tf-trHb between pH 6.1 and 10.1, together with the corresponding spectra of the triple mutant at neutral pH. In this pH range Tf-trHb undergoes coordination and spin state changes. At acidic pH the absorption spectrum shows a Soret band at 407 nm (409 nm at neutral pH), Q bands at 498, 541 and 577 nm, and a broad CT band centered at 634 nm. The corresponding RR spectrum clearly indicates that at low pH the protein is a mixture of three species, an aquo 6cHS (ν_3 at 1480 cm^{-1}, ν_2 at 1563 cm^{-1}), a 6cLS (ν_3 at 1503 cm^{-1}, ν_2 at 1579 cm^{-1}, ν_{10} at 1638 cm^{-1}) and a small amount of 5cHS form (ν_3 at 1489 cm^{-1} and ν_2 at 1570 cm^{-1}). At alkaline pH the 5cHS species disappears, and, similar to Hb and Mb [9], the absorption spectrum becomes typical of a hydroxo complex characterized by a mixture of 6cHS and 6cLS species with the Soret band at 413 nm, Q bands at 545 and 577 nm, and a shoulder at about 609 nm due to the CT1 band. Accordingly, at alkaline pH, in the high frequency RR region two sets of core size marker bands are found, corresponding to a

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