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Non-symbiotic hemoglobin conformational space dependence on the heme coordination using nESI-TIMS-TOF MS

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ABSTRACT

In this study, for the first time, the conformational space of the rice non-symbiotic hemoglobin type 1 (rHb1) was studied as a function of the starting solution pH using trapped ion mobility spectrometry coupled to mass spectrometry (TIMS-MS) and molecular dynamics. Comparison of the charge state distribution, apo to holo form ratio, and the collision cross section (Ω) profiles as a function of the solution pH showed higher stability of the rHb1 wild-type (WT) when compared with the H73L mutant at mildly acidic conditions. Comparison of the Ω profiles of the rHb1 WT and H73L holo and apo form showed that only the initial unfolding pathways involved the heme cavity, with and without a heme loss, followed by unfolding pathways not necessarily involving the environment of the heme prosthetic group. Candidate structures for the nine transitions observed in the Ω profiles were proposed using molecular dynamic simulations based on the Ω profiles, UV absorption spectroscopy and circular dichroism data as way to describe a potential unfolding pathway. The described unfolding pathway suggests that the rHb1 unfolding is driven by initial distancing of the A, B, and H helices, while the heme cavity and heme group remains intact, followed by the distancing of the E, F, and G helices and subsequent loss of the α-helical structure leading to a final random coil conformation.

1. Introduction

Non-symbiotic hemoglobins (nsHbs) are a class of heme proteins found in land plants which exhibit bis-histidyl coordination of the heme iron and can reversibly bind gaseous ligands, including carbon monoxide (CO), dioxygen (O_2), and nitric oxide (NO) [1]. NsHbs are localized in the cytoplasm of plant cells and share a 3-over-3 α-helical structure with a variety of other heme proteins. The heme B cofactor is found within a hydrophobic cavity in the protein referred to as the heme pocket [2]. NsHbs are closely related to symbiotic hemoglobins (sHbs), a class of heme proteins which participates in nitrogen fixation in the root nodules of leguminous plants through symbiotic interactions with *rhizobia* bacteria [3]. However, unlike sHbs, the functions of nsHbs *in vivo* are not fully understood. *In vitro*, nsHbs carry out an NO dioxygenase function, converting cytotoxic NO to nitrate (NO_3^-) by addition of O_2 [4]; this activity has led to the idea that detoxification of NO through NO dioxygenase activity is the primary function of nsHbs. NsHbs also typically exhibit a very high affinity for exogenous gaseous ligands; type 1 nsHb from rice (rHb1) has an affinity of ~1 nM for O_2 [1], showing stronger binding then that of the oxygen sensor protein FixL (~50 μM) suggesting that nsHbs may also act as oxygen sensors [5]. Expression of rHb1 is upregulated during seed germination and in differentiating plant tissues raising the possibility that nsHbs participate in developmental signaling by modulating levels of NO within the cell [6]. The rHb1 expression is also upregulated under conditions of etiolation (lack of sunlight) or hypoxia, suggesting that nsHbs are part of the cellular response to various stress conditions [7].

Previous studies using photoacoustic calorimetry (PAC) and transient absorption spectroscopy (TA) have shown the kinetics and energetics associated with CO photo dissociation from rHb1 WT and a distal histidine mutant, H73L, to be pH-dependent [8]. The use of mildly acidic conditions results in slower ligand migration from the protein matrix after photolysis of the iron-ligand bond. Classical molecular dynamics (cMD) simulations suggested that protonation of histidine residues within the protein leads to

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reorganization of the CD-loop and EF-helical regions and restructuring of hydrophobic cavities within the protein, which are critical for migration of gaseous ligands between the solvent and the heme active site. Therefore, the structural changes induced by histidine protonation may be a mechanism by which enzymatic activity of the protein is affected by modulating interactions of the protein with gaseous ligands. Histidine protonation could occur as a result of pH changes in the cytoplasm of plant cells directly caused by the etiolation, hypoxia, and other stress conditions [9].

With the recent introduction of trapped ion mobility spectrometry coupled to mass spectrometry (TIMS-MS) [10,11], several studies have taken advantage of the high mobility resolving power (R ~400) for the separation of complex mixtures and structural characterization of biomolecules [12–24]. Several studies have shown the advantages of TIMS-TOF MS for the characterization of kinetically trapped intermediates as a function of the starting solution (e.g., pH and organic content) and collision induced activation prior to TIMS-TOF MS analysis [25–27]. In particular, we have previously shown that TIMS-TOF MS when complemented with molecular dynamics is a powerful tool for the study of heme proteins (e.g., myoglobin and cytochrome c) [28,29].

In the present work, we explore for the first time the potential of nESI-TIMS-TOF MS to study the kinetically-trapped intermediates of rHb1 wild type (WT) and H73L mutant as a function of the solution pH, ranging from 4.9 to 8.0. Complementary molecular dynamic simulations were utilized to generate potential protein structures with collision cross sections (Ω) similar to those observed experimentally.

2. Methods and materials

2.1. RHb1 expression and purification

The rHb1 WT and H73L mutant with poly-histidine tags were expressed recombinantly in *E. coli* strain BL21 and purified using Ni^{2+} affinity chromatography [1]. The rHb1 WT and H73L amino acid sequences and molecular weight are contained in Table S1. Proteins were dialyzed extensively against 20 mM HEPES buffer at pH 7.0 and stored in 200 μL aliquots at −80 °C. Prior to nESI-TIMS-MS analysis, proteins were extensively dialyzed into 10 mM ammonium acetate using 3 kDa (Amicon Ultra) and/or 10 kDa (Microcon-10) MWCO centrifugal filter units as needed. The solution pH was adjusted to 4.9 and 8.0 using 0.001% v/v acetic acid (Fisher Scientific) and 0.006% v/v triethylamine (Sigma), respectively. All samples were studied within 24 h of preparation to avoid protein degradation.

2.2. Trapped ion mobility spectrometry – mass spectrometry analysis

Individual rHb1 proteins were analyzed by directly infusing the sample via nESI into the TIMS-TOF MS spectrometer. A detailed overview of the TIMS analyzer and its operation can be found elsewhere [10,30,31]. The nitrogen bath gas flow is defined by the pressure difference between entrance funnel (P_1 = 2.6 mbar) and the exit funnel (P_2 = 1.0 mbar) at *ca.* 300 K. The TIMS analyzer is comprised of three regions: an entrance funnel, analyzer tunnel (46 mm axial length), and exit funnel. A 880 kHz and 200 Vpp RF potential was applied to each section creating a dipolar field in the funnel regions and a quadrupolar field inside the tunnel. In TIMS operation, multiple ion species are trapped simultaneously at different E values resulting from a voltage gradient applied across the TIMS tunnel, typically V_{ramp} = −280–0 V for low resolution scans and ΔV_{ramp} = 40–80 V for high resolution scans. Deflector ($V_{deflector}$), capillary ($V_{capillary}$), entrance funnel (V_{funnel} in) and end of the tunnel regiont (V_{out}) were set to 60, 50, 0 and 60 V respectively to avoid ion heating prior to TIMS analysis. The mobility, K, of an ion in a TIMS cell is described by:

$$K = v_g/E = A/(V_{elution}\text{-}V_{out}) \quad (1)$$

where v_g and E are the gas velocity and applied electric field. $V_{elution}$ and V_{out} are the elution voltage and voltage at the end of the tunnel region. A is an instrumental parameter determined by calibration using standards of known mobility. After thermalization, species are eluted from the TIMS cell by decreasing the electric field in stepwise decrements (referred to as the "ramp") and can be described by a characteristic elution voltage ($V_{elution}$). Eluted ions are then mass analyzed and detected by a maXis impact Q-ToF MS (Bruker Daltonics Inc, Billerica, MA).

In a TIMS device, the total analysis time can be described as:

$$\begin{aligned} TotalIMStime &= t_{trap} + (V_{elution}/V_{ramp}) * t_{ramp} + TOF \\ &= t_o + (V_{elut}/V_{ramp}) * t_{ramp} \end{aligned} \quad (2)$$

where, t_{trap} is the thermalization/trapping time, *TOF* is the time after the mobility separation, and V_{ramp} and t_{ramp} are the voltage range and time required to vary the electric field, respectively. The elution voltage was experimentally determined by varying the ramp time (t_{ramp} = 100–500 ms) for a constant ramp voltage setting. This procedure also determines the time ions spend outside the separation region, t_o (e.g., ion trapping and time-of-flight). The TIMS cell was operated using a fill/trap/ramp/wait sequence of 10/10/100-500/50 ms. The ToF analyzer was operated at 10 kHz (*m*/*z* 50–3500). The data was summed over 100 analysis cycles yielding an analysis time of ~50 s for the largest trapping times (t_{ramp} = 500 ms). Mobility calibration was performed using the Tuning Mix calibration standard (G24221A, Agilent Technologies, Santa Clara, CA) in positive ion mode (e.g., *m*/*z* 322, $K_0 = 1.376\ cm^2\ V^{-1}\ s^{-1}$ and *m*/*z* 622, $K_0 = 1.013\ cm^2\ V^{-1}\ s^{-1}$) [31]. The TIMS operation was controlled using in-house software, written in National Instruments Lab VIEW, and synchronized with the maXis Impact Q-ToF acquisition program. A custom-built source using pulled capillary nESI emitters was utilized for all the experiments. Quartz glass capillaries (O.D.: 1.0 mm and I.D.: 0.70 mm) were pulled utilizing a P-2000 micropipette laser puller (Sutter Instruments, Novato, CA) and loaded with 10 μL aliquot of the sample solution. A typical nESI source voltage of +700–1500 V was applied between the pulled capillary tips and the TIMS-TOF MS instrument inlet. Ions were introduced via a stainless-steel inlet capillary (1/16 × 0.020″, IDEX Health Science, Oak Harbor, WA) held at room temperature into the TIMS cell.

Reduced mobility values (K_0) were correlated with collision cross section (Ω) using the equation:

$$\Omega = \frac{(18\pi)^{1/2}}{16}\frac{z}{(k_BT)^{1/2}}\left[\frac{1}{m_i}+\frac{1}{m_b}\right]^{1/2}\frac{1}{K_0}\frac{1}{N*} \quad (3)$$

where z is the charge of the ion, k_B is the Boltzmann constant, N^* is the number density of the bath gas and m_i and m_b refer to the masses of the ion and bath gas, respectively [32]. TIMS-MS spectra were analyzed using Compass Data Analysis 5.0 (Bruker Daltonik GmbH) and TIMS Data Viewer 1.4.0.31397 (Bruker Daltonik GmbH).

2.3. UV and CD measurements

The UV spectra of rHb1 WT and H73L were measured using a Biotek Synergy H1 hybrid multi-mode plate reader (Winooski, VT) using a Take3 micro volume plate from 2 μL volume of ~15 μM protein solution. The circular dichroism (CD) spectra of rHb1 WT and H73L were measured using a Jasco J-815 CD spectrophotometer (Oklahoma City, OK). The solution pH (5.0–8.0) was adjusted