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Direct electrochemistry and environmental sensing of rice hemoglobin immobilized at graphite electrodes

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ABSTRACT

Non-symbiotic hemoglobin from rice (*Oryza sativa* L.), *Os*Hb-1, with hexacoordinated rather than pentacoordinated heme and high affinity for oxygen, may have a particular role in O_2 and environmental sensing. Here, a 21 kDa monomer *Os*Hb-1 was electrochemically studied at graphite electrodes and further probed in analysis of environmental species such as hydrogen peroxide, cyanide, and superoxide. Redox potential of the *Os*Hb-1 heme iron was found to be −136 mV *vs.* SCE, at pH 6.5, while the rate constant k_s for the heterogeneous electron transfer (ET) between graphite and *Os*Hb-1 immobilized in the Nafion membrane at the carbon nanotubes-modified electrodes was below 0.2 s^{-1}. Despite sluggish ET, *Os*Hb-1 efficiently, with current densities exceeding 2 mA cm^{-2} at −0.3 V, electrocatalyzed reduction of O_2 starting from the potentials of *Os*Hb-1 heme. The bioelectrocatalytic reduction of O_2 was partially inhibited by CN^- thus enabling its sensing down to a 100 pM level. Peroxidase-like activity of *Os*Hb-1 and the reaction of the superoxide anion with the heme iron of *Os*Hb-1, in de-oxygenated solutions, were studied and analysed in terms of *Os*Hb-1 reactivity. The results obtained indicate *Os*Hb-1 is a sensitive tool for environmental biosensing and toxicity screening.

1. Introduction

Currently, heme proteins represent the largest group of proteins known to sense molecules that are either toxic or crucial to survival [1]. Of those, hemoglobins (Hbs), which are the heme-containing proteins possessing a well-known globin fold [2], with their unique diatomic gas ligand-binding properties are possibly the best candidates for the development of environmental analytical devices for sensitive analysis of oxygen and reactive oxygen and toxic species able to react with Hbs [2–5]. Hbs occur in most living organisms: animals, plants, fungi, and bacteria, and only in vertebrates the predominant Hb function is storage and facilitation of O_2 transportation in blood. In other species, depending on the affinities for diatomic ligands, Hbs can carry out a number of diverse, physiologically important functions: the above mentioned storage and transport of O_2, but also lipid peroxidation [6], involvement in the oxidative stress regulation [4,7,8], and environmental sensing and signal transduction [1,9], including regulation of carbon dioxide transport and regulation of blood pH [2].

Not surprisingly, redox reactions of the heme of Hb were intensively studied electrochemically and probed for electrochemical sensing of oxygen, hydrogen peroxide, trichloroacetic acid, nitrite and nitric oxide at Hb-modified electrodes [10,11]. The crucial step in the development of such electrochemical assays is the establishment of a direct electronic communication between the electrodes and the heme of Hb. Then any environmental reactions of Hb can be followed directly, through the variations in the electrochemical response from the heme [9]. However, possibly due to the complex oligomeric structure of the most of hitherto studied Hbs, pronounced direct electron transfer (ET) reactions between the electrodes and the heme of Hb were observed only when Hb was adsorbed on or entrapped in clay matrices [12–15] and surfactant and polymer films [16–19]. These matrices could produce disaggregation of the protein into the monomer components and provide the suitable conductivity properties, both facilitating ET communication between Hb and electrodes.

In this work we studied a novel type of Hb – a non-symbiotic hemoglobin from rice (*Os*Hb-1) that is genetically and structurally distinct from hitherto studied leghemoglobins. Like other Hbs, *Os*Hb-1 has a globin fold and contains a heme *b* prosthetic group with heme iron forming bonds between the distal and proximal histidines (Fig. 1) [20]. In contrast to the tetramer animal Hbs, whose evolutionary oligomerization allowed to acquire allosteric properties essential for oxygen transport [2], at physiologically relevant conditions *Os*Hb-1 may be considered a monomer, despite crystallographic structure showing a dimer [20]. The equilibrium dissociation constant for dimerization is 86 μM, indicating weak interactions between the monomers. Moreover, no cooperative ligand binding to *Os*Hb-1 has been observed [20]. Thus, *Os*Hb-1

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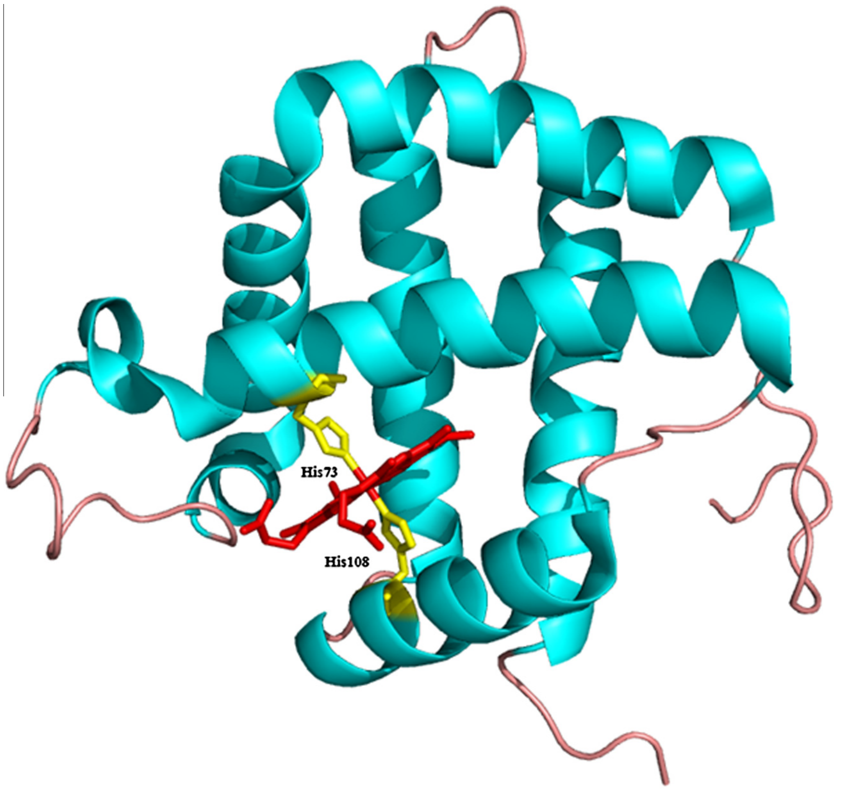

Fig. 1. Schematic representation of the *Os*Hb-1 structure (PDB ID: 1D8U), heme is indicated in red, and histidine residues in the binding site in yellow. The image was generated with PyMOL software [21]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protein dimers are most likely formed only at high concentrations as those used for crystallization, and at lower concentrations *Os*Hb-1 behaves as a monomer, which should facilitate its study at electrodes.

The important feature of *Os*Hb-1 is its hexacoordinated rather than pentacoordinated heme: the proximal and distal histidine side-chains are coordinated directly to the heme iron both in ferric and ferrous oxidation states [20]. Such coordination is unusual for the oxygen-binding protein. However, the distal His73 (Fig. 1) may rapidly dissociate from iron (dissociation rate constant 1900 s^{-1}) by displacement by the ferrous ligands [20]. The heme is then able to rapidly bind one oxygen molecule with extremely high affinity – the equilibrium constant for oxygen binding K_d was determined as less than 1 nM [3]. Such a high affinity of *Os*Hb-1 for O_2 excludes its natural O_2-transport or facilitated diffusion role, but along with substantial conformational changes that occur in response to ligand binding or release suggests its particular role as O_2-sensing protein [20]. These specific tertiary conformational changes accompanying reactions between bis-imidazole coordinated heme and ligands were discussed to possibly activate other signal-transduction proteins and enzymes with regulatory functions [20].

This work is the first electrochemical study of the electron transfer reactions between *Os*Hb-1 and electrodes to find out if sensing properties of *Os*Hb-1 can be used for electroanalysis of environmental species such as oxygen, cyanide and reactive oxygen species.

2. Experimental

2.1. Materials

Rice (*Oryza sativa* L.) hemoglobin, *Os*Hb-1, was cloned from vector pEMBL-19-*OsHbI* construct [22]. *Os*Hb-1 was expressed and purified as described in Supporting information and prepared as a 3 mg ml^{-1} stock solution in 40 mM Tris–HCl; it was stored at −18 °C when not in use. Nafion (5% solution in a mixture of lower aliphatic alcohols and water) was from Fluka, USA, potassium cyanide (puriss.) was from Riedel-de Haën, Germany, potassium superoxide, KO_2 (powder), 30% H_2O_2 and other reagents of analytical grade and components of buffer solutions were from Sigma–Aldrich, Germany. Thin multiwall carbon nanotubes (MWCNT), type NC3100, with the average diameter of 9.5 nm and length of 1.5 μm, <5% metal oxide content and >95% carbon purity, were supplied by the Nanocyl, Belgium, and used as received. All solutions were prepared with de-ionized Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Electrode modification

For *Os*Hb-1 immobilization, 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 deionized water and fitted into Teflon holders. For the adsorption of the protein, 3 μl of a 3 mg ml^{-1} *Os*Hb-1 in 40 mM Tris–HCl buffer (pH 7.8) aqueous solution were placed on the electrode surface and stored for 1.5 h at room temperature under a plastic cap. For modification with MWCNT, a 3 μl drop of 2 mg ml^{-1} MWCNT suspension (prepared by ultrasonication of MWCNT in dimethylformamide (DMF)) was placed onto the polished graphite electrode surface. After solvent evaporation, 3 μl of a 3 mg ml^{-1} of aqueous solution of *Os*Hb-1 were placed on the top of the MWCNT-modified graphite electrodes and left for another 1.5 h or mixed with added 3 μl of 0.1% Nafion solution in ethanol. Prior to the experiments the modified electrodes were thoroughly washed with 50 mM sodium phosphate buffer (PBS), pH 6.5, and mounted in the cell.