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Preliminary electrochemical studies of the flavohaemoprotein from *Ralstonia eutropha* entrapped in a film of methyl cellulose: Activation of the reduction of dioxygen

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Abstract

A flavohaemoprotein (FHP) from *Ralstonia eutropha*, obtained in a pure and active form, has been entrapped in a film of methyl cellulose on the electrode surface and gives a stable and reproducible electrochemical response at pH 7.00 when subject to cyclic voltammetry using a glassy carbon electrode. To our knowledge, no previous direct electrochemistry had been achieved with a bacterial flavohaemoglobin, which possess both a FAD and a haem. A single couple is observed which is assigned to the haem moiety of the protein, since the same result is obtained with a semi-apo form of the protein deprived of FAD (semi-apo FHP). The data collected were further confirmed by potentiometry with a platinum electrode, and the homogeneous electron transfer rate estimated by double potential step chronocoulometry at a bare glassy carbon electrode in the presence of methyl viologen (MV). The presence of FAD in the holoprotein is easily confirmed by UV–Vis spectrophotometry, but its expected electron relay role remains elusive. The protein activates the reduction of dioxygen by about 400 mV, the reduction current being proportional to the concentration of dioxygen up to 10% in volume in the gas mixture.

Keywords: Flavohaemoprotein; Cyclic voltammetry; Methyl cellulose; Spectroelectrochemistry; Chronocoulometry; Dioxygen reduction activation

1. Introduction

The flavohaemoprotein (FHP) from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) [1,2] is a 43 kDa bacterial haemoglobin whose existence was first mentioned in the 1970s [3,4]. Like all the haemoglobins found in Nature, it possesses a haem moiety that reversibly binds dioxygen (O_2) when in its reduced state (i.e. when the haem iron is in the +2 oxidation state) [2,4]. This may easily be checked by UV–Vis spectrophotometry, since the oxidised haem Soret band at 395 nm shifts to 436 nm upon reduction, and is finally found at 414 nm and more intense when reduced haem binds dioxygen [4]. The UV–Vis spectrum of the oxidised FHP has several bands: apart from the Soret band at 395 nm, the ferric haem group gives rise to two more bands, indicative of a high spin iron, at 486 and 645 nm [4]. The band at 456 nm has been assigned to flavine adenine dinucleotide (FAD), which is noncovalently bound to the protein [4]. Finally, there is a band at 277 nm, which is characteristic of the aromatic amino acid residues of any protein, particularly tryptophan and tyrosine [5].

The existence of a haem iron that interconverts between the oxidation states +2 and +3 has several implications regarding the binding affinity of the FHP towards ligands such as nitric oxide ($NO^{\bullet}$), dioxygen (O_2) and carbon monoxide (CO). Indeed, while nitric oxide coordinates to haem irrespective of the oxidation state of the iron, dioxygen and carbon monoxide bind just ferrous haem [6].

The determination of the primary sequence [7], composed of 403 residues, and then of the crystal structure of the protein at 1.75 Å resolution confirmed the presence of a haem (axially coordinated to the imidazole of His-85) and of a FAD per

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molecule [8] (Fig. 1). The former is embedded in a hydrophobic crevice, while the latter is partially exposed to the solvent [8]. The shortest distance between the haem and the FAD is 6.3 Å, allowing direct electron transfer between the two sites [8–10]. Apart from these two prosthetic groups, the FHP has been shown to possess a phospholipid adjacent to the haem, and therefore susceptible of modulating the haem's biochemical properties [11].

Even if the biological function of bacterial haemoglobins has not been unambiguously established, they are thought to be $NO^{\bullet}$ dioxygenases (NOD), which render the bacteria resistant to $NO^{\bullet}$ toxicity by converting it into nitrate (NO_3^-) in the presence of O_2 as co-substrate and with the participation of reduced nicotinamide adenine dinucleotide (NADH), according to the following equation [6]:

$$2NO^{\bullet} + 2O_2 + NADH \rightarrow 2NO_3^- + NAD^+ + H^+ \quad (1)$$

In Eq. (1), the natural reductant, NADH, may in principle be replaced by another source of electrons, which may be another chemical species or an electrode. In the latter case, the biochemical reaction catalysed by the FHP will be modulated by the potential at which the electrode will be poised, provided no complications arise (e.g. electrode fouling, protein denaturation, non-effective electron transfer pathways) [12–15].

Successful protein electrochemistry has been achieved by confining the biomolecule to a space delimited by the electrode surface and a biocompatible film that retains the protein in its native, active state. Films may be made of lipids [16–18], cellulose derivatives [19–22], silica sol–gels [23], collagen [24], surfactants [25–28], TiO_2 and SnO_2 nanoparticles [29,30], polyelectrolytes [31–35], polystyrene latex beads [36], Eastman AQ® [37] and Nafion® [37,38].

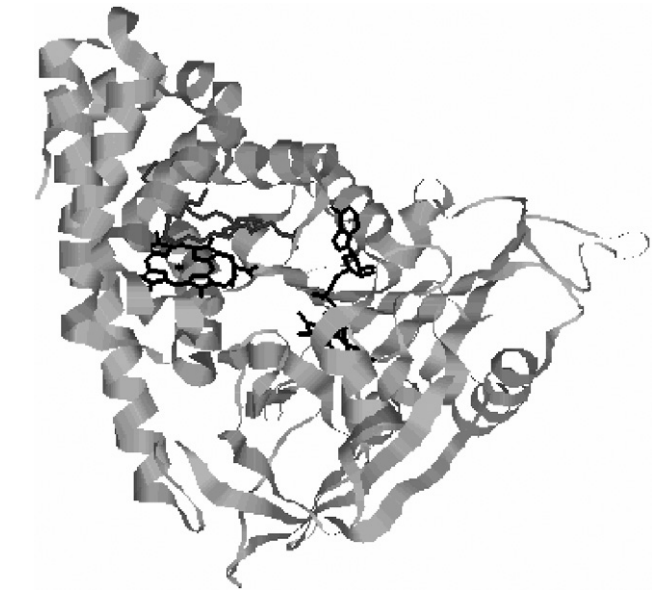

Fig. 1. 3D structure of the FHP from *R. eutropha* (PDB entry code: 1CQX): the haem is shown in black on the left, with the lipid above it in grey, and the FAD, which partially protrudes from the surface, appears towards the upper centre, in black.

In this work, a methyl cellulose film was used to confine the protein to the vicinity of the electrode surface, and the FHP/methyl cellulose modified electrode was studied by cyclic voltammetry. Potentiometry and double potential step chronocoulometry data were also gathered from experiments involving the FHP.

2. Experimental

2.1. FHP purification

The purity of the protein sample is an essential requirement for any attempt of obtaining meaningful data from electrochemical experiments, and quite often the sole way to aim at getting a good reproducibility. A slightly improved purification method, partially influenced by previous contributions [4,39], is now well established, giving several millilitres of pure samples whose concentration in FHP can reach a few hundreds of micromolars. The purity of each sample was checked by SDS-PAGE, which revealed a single band corresponding to the expected molecular weight of the FHP, and further confirmed by UV–Vis spectrophotometry [4]. The FHP solutions used were prepared by buffer exchange with PD-10 columns filled with Sephadex G-25 M (Amersham Pharmacia).

2.2. Materials

Potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, sodium dithionite, safranine, phenazine methosulfate (PMS) and methyl viologen (MV) were from Acros Organics; potassium 1,4-naphtoquinone-2-sulfonate (NQSP) was from Eastman; sodium anthraquinone-2,6-disulfonate (AQDS) was from Ega Chemie; potassium ferricyanide and potassium chloride were from Prolabo; dimethylformamide (DMF) was from Aldrich; methyl cellulose was from Sigma; 99.995% argon and the mixture 80% dinitrogen/20% dioxygen were from Air Liquide. The reagents were used without further purification.

All experiments were done in 50 mM potassium phosphate buffer, 100 mM potassium chloride, pH 7.00. All solutions were prepared with pure water obtained from a Milli-RiOs 8 unit followed by a Milli-Q academic purification set (water resistivity: 18.2 MΩ.cm), and were either thoroughly deoxygenated with argon or set at a desired dioxygen concentration by sparging for 30 min with a convenient mixture of pure argon and 80% dinitrogen/20% dioxygen prior to the experiments.

2.3. Electrode modification, electrochemistry and UV–Vis spectrophotometry

Glassy carbon electrodes were conveniently polished [40] on polishing cloths of different roughness with diamond paste (Struers), and thoroughly cleaned with a wet tissue. Cyclic voltammetry and double potential step chronocoulometry were carried out in a three-electrode cell comprising a glassy carbon working electrode, a platinum counter electrode and a saturated