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Hydrogen bioelectrooxidation in ionic liquids: From cytochrome c_3 redox behavior to hydrogenase activity

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ABSTRACT

Electrochemistry of polyheme bacterial cytochrome c_3 and catalytic oxidation of hydrogen by two different bacterial [NiFe] hydrogenases were investigated for the first time in pure room-temperature ionic liquids (RTILs) as electrolyte. Direct electrochemical response of *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 (DvH cytc$_3$) adsorbed at a pyrolytic graphite (PG) electrode was observed in the RTILs used in this work: 1-butyl-3-methylimidazolium tetrafluoroborate (BmimBF4), 1-ethyl-3-methylimidazolium tetrafluoroborate (EmimBF4) and 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (EmimNTf2). The electrochemical signal differed however from that obtained in aqueous buffer, and depended on the type of RTIL. UV–vis measurements as well as transfer experiments from aqueous buffer to RTILs or RTILs to aqueous buffer strongly suggested that the protein was not denatured in the presence of RTILs. EmimNTf2, as a hydrophobic non-water-miscible RTIL, was demonstrated to stabilize the native form of DvH cytc$_3$. Moreover it allowed an amount of electroactive DvH cytc$_3$ 30-fold higher than observed in aqueous buffer. Catalytic oxidation of H_2 via *Desulfovibrio fructosovorans* [NiFe] hydrogenase (Df Hase) mediated by DvH cytc$_3$ failed however. Further investigation suggested that Df Hase could be inhibited in the presence of RTILs. Reasons for such an inhibition were explored, including the blocking up of the substrate channels. By using hyperthermophilic [NiFe] membrane-bound hydrogenase from *Aquifex aeolicus* (Aa Hase) an efficient direct catalytic oxidation process was obtained in mixed aqueous buffer/RTILs electrolytes, although direct H_2 oxidation was not observed in pure RTIL. Chronoamperometric experiments showed that Aa Hase could afford 80% RTILs in aqueous buffer, thus giving the opportunity of future electrolytes with uncommon and variable properties for biofuel cell design.

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1. Introduction

Efficient electron transfer coupling between a protein and an electrode can help in the understanding of energetic metabolisms in biological system. Furthermore, the so-obtained bioelectrodes open large avenue for biotechnological devices in various fields, including biosensors or biofuel cells [1–3]. Bioelectrochemical devices more often proceed in aqueous media. However, the advantages of the use of non-aqueous media have been exploited, such as the use of substrates sparingly soluble in water, the efficient immobilization of a water soluble enzyme onto porous supports, or the increase in the thermal stability of enzymes [4]. Among the non-aqueous media, room-temperature ionic liquids (RTILs) (a class of solvents composed of ions liquid at room temperature) present highly attractive properties in view of their use in bioelectrochemistry. In particular, they present a wide electrochemical window, a good conductivity, a high chemical and thermal stability, and a negligible vapor pressure. Furthermore, the large diversity in RTILs composition allows varying their solvent properties [5,6]. Many reports demonstrated the increase in activity and stability of various enzymes in RTILs compared to organic solvents [6 and references herein]. Redox proteins have received much less attention than lipases and hydrolases; however, two main avenues were explored in bioelectrochemistry. The first one used RTILs as a component for electrode modification. This field has received much attention during the recent years, and many various architectures have been built including RTILs with as examples gold nanoparticles [7], carbon nanotubes [8], fullerenes [9], or carbon nanofibers [10]. In the second one, RTILs were the electrolyte in which the bioelectrochemical reactions were directly studied [11]. The electrochemistry of hemin [12], microperoxidase [13], hemoglobin [14], catalase [15], or laccase [16] was evaluated in RTILs with the molecules immobilized onto the electrode. Depending on the type of RTILs, peroxidasic activity, enzymatic oxidation of phenol, or reduction of trichloroacetic acid were successfully achieved. On the other hand, loss of Fe(III)/Fe(II) redox signal was reported for cytochrome c in RTILs [17]. No reliable

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relationship between the capability of biocatalysis in the presence of RTILs and the class of biocatalysts can be inferred up to now.

Our group has been focusing for several years on sulfate reducing bacteria metabolism [18], and particularly on two intrinsic enzymatic reactions which could be interestingly exploited for bioanalytical applications. Sulfate-reducing bacteria have been shown to develop a metal reductase activity under anaerobiosis [19]. This reductive precipitation process allows the conception of bioreactors for heavy metals polluted environments. Desulfovibrio species metabolism involves hydrogenases, the enzyme that catalyzes the hydrogen conversion according to $H_2 \leftrightarrow 2H^+ + 2e^-$ [20]. Also the use of hydrogenases emerges in a future "green" hydrogen economy, in replacement of chemical catalysts in fuel cells [21,22]. The current advantages of enzymatic biofuel cells reside on the availability, and biodegradability of the biocatalyst, coupled to high power densities, comparable to platinum catalysts [23,24]. Hydrogenases have the added advantage of high specificity towards H_2, which can eliminate the need for a membrane separator. These two enzymatic reactions are mediated by Desulfovibrio tetraheme low-potential cytochrome c_3. *In vitro*, it has been demonstrated to efficiently reduce metals such as Fe(III), Cr(VI) or U(VI) [25]. *In vivo*, Desulfovibrio cytochrome c_3 acts as an electron shuttle between hydrogenase, and an electron transfer chain which uses oxidized sulfur compounds as terminal acceptors.

Otherwise, during the last decade, numbers of "exotic" microbial species have been discovered. These organisms are source of extremely stable enzymes at extreme temperatures, pressure or pH. In most of them, hydrogenases have been detected. Among these organisms, *Aquifex aeolicus* (Aa) is a hydrogen-oxidizing, microaerophilic bacterium growing at 85 °C [26]. A membrane-bound [NiFe] hydrogenase (Aa Hase) has been identified in Aa, that is involved in a complex with a diheme cytochrome b anchored in the membrane [27]. Previous studies have shown that Aa Hase was stable at elevated temperatures, and exhibited tolerance to oxygen and CO that makes it a good candidate for biotechnological uses such as biofuel cells [28–30]. Very recently, it was otherwise reported that the hydrogenase from *Thiocapsa roseopersicina* was stimulated by water-miscible organic solvents [31]. In addition to the high operating temperature of Aa Hase for efficient catalysis, this result prompted us to investigate the use of RTILs instead of aqueous buffer for biohydrogen technology.

In this work we aim to enlarge the knowledge of protein electrochemistry in RTILs, while exploring the possibility of enhancement of hydrogenase activity. Three different RTILs were chosen. 1-Butyl-3-methylimidazolium tetrafluoroborate (BmimBF4) and 1-ethyl-3-methylimidazolium tetrafluoroborate (EmimBF4) are water-miscible RTILs, which differ on the cation size. 1-Ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (EmimNTf2) presents a different anion and is water-immiscible. These RTILs were chosen because of their good conductivity and low viscosity. We report on the electrochemistry of hemin, then *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 (DvH cytc_3) immobilized onto a graphite electrode in these RTILs. We show that a redox signal relative to the Fe(III)/Fe(II) transition is maintained depending on the type of RTILs, though different from the native redox signal for the polyheme protein. We take benefit of this electroactivity to investigate the capability of RTILs as electrolyte for catalytic oxidation of H_2 by hydrogenases. In a first step coimmobilisation of *Desulfovibrio fructosovorans* [NiFe] hydrogenase (Df Hase) with DvH cytc_3 at the graphite electrode is performed. Then, H_2 oxidation by adsorbed Aa Hase onto PG electrode is studied and shown to be highly dependant on the RTILs water content. Reasons for the inhibition of hydrogenase activity in pure RTILs are finally discussed.

2. Experimental

2.1. Reagents and instruments

BmimBF4 was from Acros Organic (Fischer Scientific, France). EmimBF4 and EmimNTf2 were from Solvionic (Toulouse, France). Their conductivity and viscosity at 25 °C have been reported in [5]. The ionic liquids were stored under N_2 in a glove box.

Glucose oxidase (Gox) from *Aspergillus niger* was purchased from Sigma. DvH cytc_3 [32] and Df Hase [33] were prepared and purified in our laboratory as previously described. Aa Hase was purified as described in Luo et al. [28], except that Amicon PM 100 was used for the concentration step. The MW and p*I* are (63 kDa, 4.9), (12 kDa, 10.2), (87 kDa, 7), and (103 kDa, 7) for Gox, DvH cytc_3, Df Hase, and Aa Hase, respectively. Iron(III) protoporphyrin(IX) chloride (hemin), was purchased from Sigma. 1 mM solutions of hemin were freshly prepared before experiment by dissolving in a 30% ethanolic aqueous solution containing 0.025 M sodium borate, pH 10, as previously described [34]. All other chemicals were reagent grade. All solutions were prepared with distilled, deionized water. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (0.05 M HEPES, pH 7.2) served as the supporting electrolyte for comparison with electrochemistry in ionic liquids. The concentrations of the biological stock solutions were 1 μM and 400 μM for hydrogenases and DvH cytc_3 respectively.

Cyclic voltammetry (CV) and chronoamperometry were done using a PARSTAT 2273 potentiostat from Princeton Applied Research (PAR). A conventional three-electrode system was used with a Ag/AgCl/NaCl (sat.) reference electrode, and a gold wire as auxiliary electrode. All potentials were quoted against the Ag/AgCl reference. Chronamperometric experiments in mixed HEPES/RTILs electrolyte were realized by successive addition of aliquots of RTILs in 50 mM HEPES buffer under stirring. The CV curves and reported amperometric current are the average from triplicate experiments. Prior to each experiment, the solutions were deoxygenated by bubbling with high-purity nitrogen or hydrogen. Hemin and DvH cytc_3 studies were performed at room temperature. Df Hase and Aa Hase experiments were performed at 40 °C and 60 °C, respectively, unless otherwise specified. Temperature of the electrochemical cell was regulated using a water bath. The reference electrode was separated from the warmed electrolyte using a side junction maintained at room temperature.

UV–vis spectra were measured with a Cary 50 Bio Varian. Dried films of DvH cytc_3-graphite powder, and DvH cytc_3-graphite powder-RTIL were prepared under N_2 in a glove box by entrapping a volume of the mixtures between two quartz planar windows.

2.2. Fabrication of the working electrode

The working electrode was constructed from 3 mm rods of pyrolytic graphite (PG) inserted in Peek polymer casings from Bio-Logic SAS. The PG electrode was carefully polished on wet fine emery papers (PRESI, P800 then P1200). After polishing, the PG electrode was washed thoroughly with water, followed by sonication and rinsed again with water. 2 μl of the protein samples were deposited onto the surface of the PG electrode, and gently dried under N_2.

2.3. Structure modeling and analysis

The structural model of Aa Hase was built as previously described [28]. Channels were computed by Caver 2.0 [35]. The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC, was used for visualization and to compute surface electrostatic potential distribution.

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