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Direct electrochemistry and spectroelectrochemistry of osmium substituted horseradish peroxidase

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ABSTRACT

In this contribution the substitution of the central protoporphyrin IX iron complex of horseradish peroxidase by the respective osmium porphyrin complex is described. The direct electrochemical reduction of the Os containing horseradish peroxidase (OsHRP) was achieved at ITO and modified glassy carbon electrodes and in combination with spectroscopy revealed the three redox couples Os^{III}HRP/Os^{IV}HRP, Os^{IV}HRP/Os^{V}HRP and Os^{V}HRP/Os^{VI}HRP. The midpoint potentials differ dependent on the electrode material used with $E_{1/2}$ ($Os^{III/IV}$) of −0.4 V (ITO) and −0.25 V (GC), $E_{1/2}$ ($Os^{IV/V}$) of −0.16 V (ITO) and +0.10 V (GC), and $E_{1/2}$ ($Os^{V/VI}$)of +0.18 V (ITO), respectively. Moreover, with immobilised OsHRP the direct electrocatalytic reduction of hydrogen peroxide and tert-butyl hydroperoxide was observed. In comparison to electrodes modified with native HRP the sensitivity of the OsHRP-electrode for tert-butyl hydroperoxide is higher.

1. Introduction

In order to elucidate the role of the central metal atom in the formation of the different redox states of heme proteins during the catalytic cycle, different metals were inserted in the porphyrin [1–3]. For example, it has been shown that manganese substituted horseradish peroxidase (MnHRP) forms with H_2O_2 an intermediate peroxo compound, which reacts slowly with reducing agents like ferrocytochrome *c*, ferrocyanide and ascorbic acid [2,3]. It was found that MnHRP compound I (MnHRP-I) and compound I of the native HRP (HRP-I) differ in the chemical structure. The MnHRP-I forms a radical cation distributed on the protein scaffold, whereas HRP-I forms a porphyrin based π-cation radical [1,4].

Substitutions of the iron by metals from the same period were performed to elucidate the oxidation chemistry of the heme proteins myoglobin (Mb), hemoglobin (Hb), HRP, and cytochrome c (Cyt *c*) [3,5–10]. Isoelectronic metals from the same group, like ruthenium or osmium, were only used for the substitution of iron in Mb [11–13]. Here, ruthenium (II) substituted Mb (Ru^{II}Mb) binds carbon monoxide and can be oxidized to Ru^{III}Mb by molecular oxygen [11].

Osmium porphyrins were synthesized and characterized as mimicks for cytochrome b_5, Cyt *c* and Cyt P450 [14,20–23]. In the biomimetic models the osmium porphyrins have similar axial ligands as in the cytochromes, but have imidazole instead of histidine and thioether ligands instead of methionine. The UV/VIS spectra of the osmium porphyrins are comparable to those of the respective iron porphyrins [15,20]. Furthermore, the difference in the redox potentials for symmetrical (N/N) and asymmetrical (N/S) axial ligands are similar [14,15]. Compared to iron porphyrins, the metal-ligand bond in osmium porphyrins is much stronger [14,15]. Furthermore, osmium is a high-valent 5d metal and can achieve stable oxidative states from +2 to +6 [14–19]. The (5,10,15,20-tetramesitylporphyrinate) osmium(II) carbonyl complex can catalyze the oxidation of unactivated C–H bonds in hydrocarbons similar to Cyt P450 [22,23]. Ruthenium and manganese porphyrins have been reported to catalyse the aziridination of alkenes and amidation of saturated C–H bonds [24]. Direct protein electrochemistry is a powerful tool to elucidate the redox properties of heme proteins such as peroxidases and evaluate their potential for practical application [25].

This paper presents for the first time a horseradish peroxidase, where iron was substituted by osmium (OsHRP). OsHRP was synthesized with protoporphyrin IX dimethyl ester and $Os_3(CO_{12})$ as starting material and subsequently reconstituted from apoHRP. The resulting OsHRP is characterized electrochemically in absence and presence of H_2O_2 and $^tBuO_2H$.

2. Experimental

2.1. Materials

Protoporphyrin IX dimethyl ester, tert-butylhydroperoxide ($^tBuO_2H$), HRP (type VI), FeHRP, sodium dithionite, sodium dodecylsulphate (SDS) and didodecyldimethylammonium bromide (ddab) were obtained from

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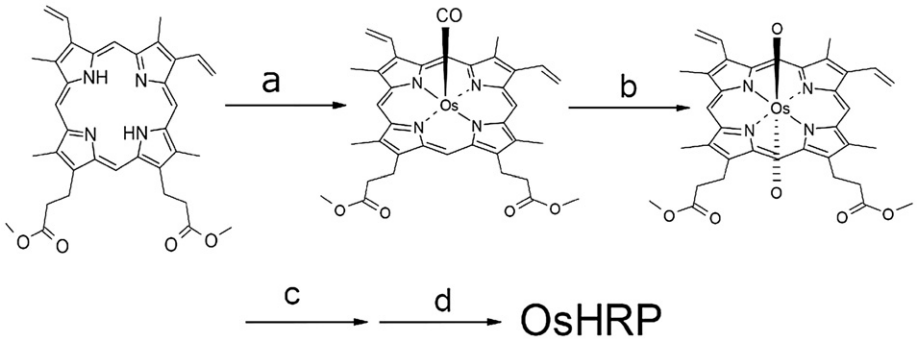

Fig. 1. Preparation of the semisynthetic osmium protoporphyrin IX and OsHRP. a: Os_3CO_{12}, 2-methoxyethanol, 130 °C, 12 h; b: $^tBuO_2H$, CH_2Cl_2/THF, RT, 5 h; c: KOH, H_2O/THF, RT, 12 h; d: $S_2O_4^{2-}$, apo-HRP, Na-buffer, pH 8.0 (adapted from [31,27]).

Sigma-Aldrich (Germany). $Os_3(CO_{12})$ was purchased from ABCR (Germany). Hydrogen peroxide was from Merck (Germany) and isopropanol from (Roth, Germany). All chemicals were of analytical grade.

2.2. Preparation of OsHRP

Osmium porphyrin was synthesised by a combination of the method reported by Che et al. [18] and that of Buchler [21]. Osmium was inserted into protoporphyrin IX dimethyl ester from $Os_3(CO)_{12}$ and purified on a silica gel column [18]. The carbonyl was removed by oxidation with $^tBuO_2H$ [21]. After basic cleavage of the dimethyl ester the osmium protoporphyrin IX, the complex was inserted into apoHRP under reducing conditions (Fig. 1).

The apoHRP was prepared by a modification of Teale's acid-butanone method [37]. An ice-cold solution of 3–4 mg/ml HRP in 100 mM Na-phosphate was mixed with an equal volume of ice-cold 2-butanone and allowed to separate. The aqueous phase containing the apoHRP was washed several times with 2-butanone until the organic phase became colourless. The aqueous phase was dialysed against 10 mM $NaHCO_3$, water and finally 10 mM tris/borate buffer containing 1 mM $CaCl_2$ (pH 8.0).

For the insertion into apoHRP, the osmium protoporphyrin IX dissolved in DMSO and 10 mM tris/borate buffer containing 1 mM $CaCl_2$ (pH 8.0) was added along with a 20 times excess of sodium dithionite. The mixture was stirred at room temperature under nitrogen atmosphere for a few hours before adding apoHRP. After stirring for another 15 min the solvent was evaporated in a vacuum centrifuge.

The OsHRP was purified on a size-exclusion Sephadex G-25 column. The OsHRP was eluted with phosphate buffer (3 mM KH_2PO_4, 10 mM Na_2HPO_4, 20 mM KCl, 1 mM EDTA, pH 7.0), dried in a vacuum centrifuge and stored at −20 °C.

2.3. Spectroscopic measurements

For the spectroscopic measurements quartz cuvettes and a Shimadzu UV-2501 photometer were used.

2.4. Spectroelectrochemical measurements

For the spectroelectrochemical measurements a homemade flow-through cell with an Ag/AgCl reference, a steel case as counter electrode, a glass slide covered with 200 nm indium tin oxide (ITO), a PGSTAT30 potentiostat (Autolab, EcoChemie, Utrecht, Netherlands) and a StellarNet (Tampa, USA) light source and detector were used. The ATR (attenuated total reflection) principle was used for the spectroscopic measurements. The ITO was pre-treated over night in cold saturated KOH solution in 2-propanol. Prior to mounting into the measuring cell it was further treated by sonicating for 10 min each in 0.1% SDS, ethanol and twice in water. 10 mM Na-phosphate buffer with 20 mM KCl, 1 mM EDTA, pH 9.0 with 0.1–1 mg/ml OsHRP were used for the measurements. Potentials were applied for 10–30 min before the absorption spectra were recorded.

2.5. Electrochemical measurements

For the electrochemical measurements a homemade 1 ml batch cell with a magnetic stirrer, an Ag/AgCl reference and a Pt wire counter electrode and a CHI 750B electrochemical workstation (CH Instruments Inc., Austin, USA) was used. To remove oxygen, the solution was purged with nitrogen. The glassy carbon (GC) electrodes were modified with an OsHRP/ddab film by dropping 10 µl of a sonicated 1:1 mixture of 10 mM ddab with 1 mg/ml OsHRP on the electrode surface, covering it and drying it at 4 °C over night. Subsequently the electrode was rinsed with phosphate buffer (3 mM KH_2PO_4, 10 mM Na_2HPO_4, 20 mM KCl, 1 mM EDTA, pH 7.0) and stored until use at 4 °C. The same buffer was used for the measurements.

3. Results and discussion

In initial experiments the optical properties of OsHRP and the osmium protoporphyrin IX were compared with those of the native HRP (FeHRP) and apoHRP using UV/VIS spectroscopy. Unlike the native HRP, which is stable in its ground state (Fe^{III}HRP), both Os^{III}HRP and the free Os^{III} protoporphyrin IX are oxidised to the corresponding Os^{VI} complex in the presence of oxygen. The maxima of the Soret bands of the Os^{III} protoprophyrin IX complex are located at 390 nm

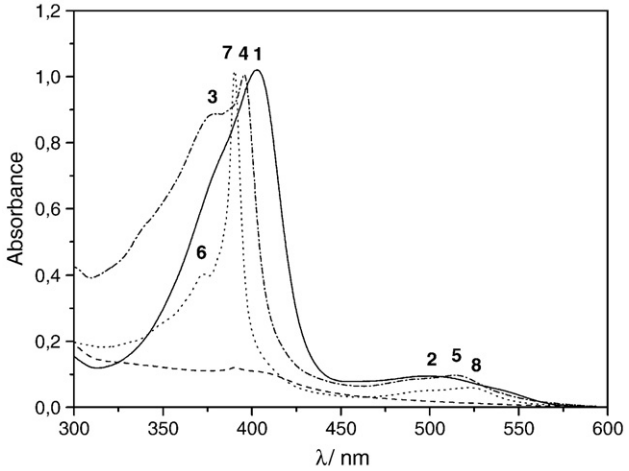

Fig. 2. UV/VIS spectra of (—) FeHRP [(1) 403 nm (2) 499 nm], (-·-·-) OsHRP [(3) 377 nm (4) 395 nm (5) 516 nm], (- - -) apoHRP and (· · · ·) Os protoporphyrin IX [(6) 373 nm (7) 390 nm (8) 523 nm] in Na-phosphate buffer, pH 7.0.