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X-ray absorption spectroscopy and electrochemistry on biological samples

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In order to study metalloproteins, which change the metal oxidation states during the catalytic cycle, we have developed an electrochemical cell for *in situ* XAS measurement on biological samples. To be able to use proteins and mutants that are usually available in small quantities the cell was designed to minimise: a) cavity of RVC working electrode and b) cavities for electric contact between RVC working electrode and the other electrodes (counter and reference). The sample volume of 0.4 ml is sufficient for measurements at several applied potentials. We have investigated the reduction of (a) the hydroxocobalamin (from Co(III) to Co(I)) and (b) microperoxidase (from Fe(III) to Fe(II)). We have then determined the correct energy shift of XANES in the two systems. In the case of hydroxocobalamin, reduction from Co(III) to Co(II) produces the most significant structural changes (Giorgetti et al. 1997) The reduction from Co(II) to Co(I) produces mainly electronic effects with no apparent change of the coordination number. Microperoxidase XANES spectrum shifts by 1 eV ± 0.5 eV upon oxidation.

Keywords: hydroxocobalamin; electrochemical cell; microperoxidase; redox centre; XANES.

1. Introduction

Electron transfer proteins are involved in redox processes having a fundamental function in biological systems. XAS is able to give an important contribution to the understanding of the electron transfer mechanism, as it is sensitive to both electronic and structural changes occurring in redox reactions. For these studies, the last generation storage rings present consistent advantages: the intense, well-focused monochromatic radiation available allows to minimize metalloprotein concentration and sample volumes. Nevertheless, there is an important increase of photon density on the sample and, consequently, a significant amount of free oxygen radicals is produced in solution. Oxygen radicals can photoreduce the metal center during sample X-ray irradiation. This effect is critical for the oxygen binding proteins in which the oxidation state have to be determined. Combination of electrochemical methods and XAS allows the study of the redox processes *in situ* without any chemical pollution. Therefore species otherwise unstable can be isolated. Moreover the electrode, under electrostatic control, acts as a scavenger avoiding damages to the protein structure. We have developed a three-electrode spectroelectrochemical cell (figure 1) based on the model proposed by Dewald et al. (1986). The main modification is that the total volume of the sample is considerably reduced (0.4 ml).

The small amount of sample used permits also to minimize the whole faradic current, reducing the time to reach redox equilibrium.

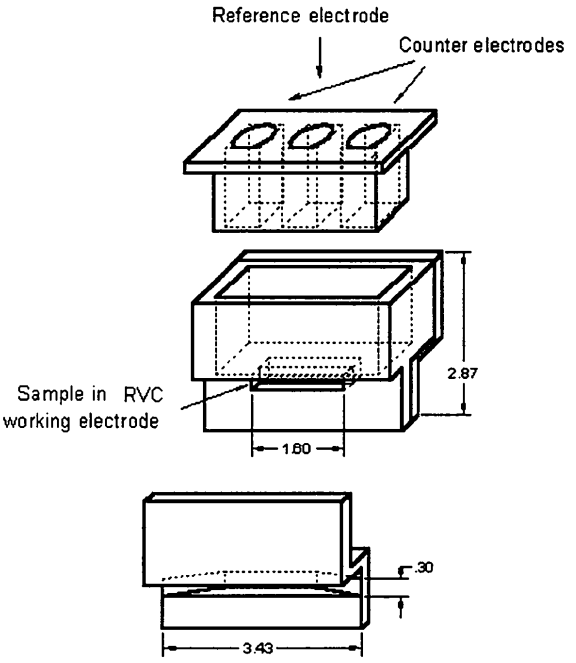

Figure 1
Schematic view of the spectroelectrochemical cell used for experiments. This cell operate using a RVC (reticulated vitrous carbon) working electrode (thin slice connected by platinum wire to a potentiostat apparatus to ensure electrochemical control; Ag/AgCl saturated reference electrode (BASMF-2020) connected to the cell through two salt bridges was used. This apparatus was assembled with de-oxygenated solution to avoid any contamination by oxygen during the experiment.

Experimental

X-ray absorption data were collected several times on samples that have been characterized by optical spectroscopy. The electrochemical method was used to control *in situ* the hydroxocobalamin and the microperoxidase oxidation state in solution while taking XAS data at room temperature. The reduction procedure was previously tested coupling UV-

visible spectroscopy performed using AMEL mod. 5000 electrochemical station and a HP 8452 diode array spectrometer.

2. Results and Discussion

2.1 *Cell Description*

The experimental cell used is schematically shown in Fig. 1, it can be used for measurements, both in transmission or in fluorescence mode. The cell has been machined out of a KELEF block and designed in such a way to minimize the amount of solution necessary to achieve a rapid equilibration of the solution with the applied potential. In practice the solution to be electrolyzed is trapped inside the honeycomb structure of a piece of RVC (reticulated vitreous carbon) pressed into the cavity (16 x 3 x 3 mm^3). The volume of the working electrode, containing the sample, is really very small (0.14 ml) compared to the Dewald cell (1.19 ml). Moreover looking at the scheme of the Dewald cell, it is evident (even if dimensions are not available) that reference and auxiliary compartments increase the total volume necessary for measurement. In the cell, herein described, the amount of solution necessary to fill the electrode cavity and to assure the electrical contact with the reference and the salt bridges is only 0.4 ml.

RVC is an inert form of carbon with a porous structure having a free volume (depending of the density) as high as 97% associated with a very high specific area (66 cm^2/cm^3 for the 100 ppi grade). As shown in a previous study, (Zamponi S. *et al.* 1990) bare or gold-plated RVC electrodes have been successfully employed in the electrochemistry of cytochrome c, native or chemically modified and of microperoxidase. The cavity containing the RVC electrode is sealed with two Kapton windows; the electrical contact is ensured by Pt wire pressed onto the material at the bottom of the cavity, out of the area covered by beam.

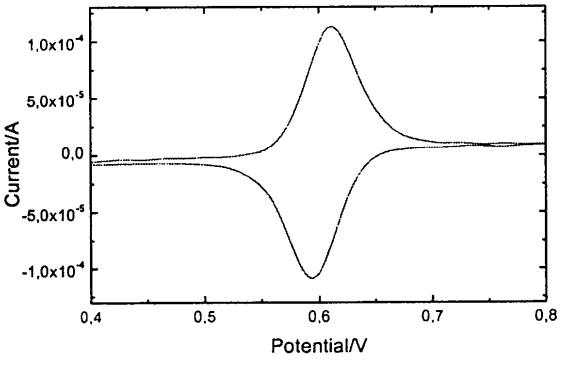

Figure 2 Ciclyc voltammetry of 7.25 10^{-4} M *o*-toluidine in CH3COOH 0.5 M and $HClO_4$ M. Scan limits: +450 mV to +800 mV.

The cell is equipped with two salt bridges (KCl satured) connecting the main body to two counter electrodes and with an Ag/AgCl reference electrode. The two counter electrodes, located at opposite side of RVC slide, assure a current density as uniform as possible. The cell may be filled with airtight deoxygenated solution.

On the typical time-scale of thin-layer electrochemistry (mV/s), the cell behaves as a thin-layer electrode as only the solution trapped inside the porous structure of the RVC, is electrolyzed. This is demonstrated by the thin-layer voltammogram of *o*-toluidine shown in Fig. 2.

The peak separation is characteristic of thin-layer voltammogram with a rather high uncompensated IR drop. The diffusional tail is indicative of diffusion regime intermediate between the one expected for thin-layer and semi-infinite linear-diffusion. This is expected for RVC at 0.5 mV/sec (Zamponi et al., 1990). Nevertheless complete electrolysis of solution may be achieved rather rapidly (figure 3). The volume of *o*-toluidine electrolyzed is 9.1x 10^{-5} l.

At the concentration levels required for XAS measurements at DCI (millimolar) it is almost impossible to obtain a reasonable cyclic voltammogram. The cell may be, however, used as a coulombometric cell, by staying at each potential value a sufficiently long time to completely electrolyzed the entire solution and/or to compensate for any "sluggish" kinetics (Dewald et al, 1986).

In situ XAS has been used to follow the electronic and the structural changes that occur during the reduction reaction of hydroxocobalamin and microperoxidase.

No appreciable differences, either in intensity or in energy position, have been found in the XANES spectra at the end of a complete redox cycle.

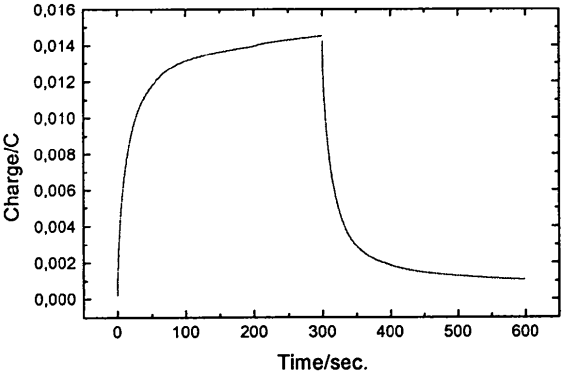

Figure 3 Cronocoulometry (Q/t) of 7.25 10^{-4} M *o*-toluidine in CH_3COOH 0.5 M and $HClO_4$ M.). The volume of *o*-toluidine electrolyzed is 9.1x 10^{-5} l.

The new experimental set-up has allowed to study cobalt environment in hydroxocobalamin for three electronic states, Co(III), Co(II) and Co(I). In previous works (M. D. Wirt et al.,

1992; I. Sagi et al. 1990) the reduced species have been produced either chemically or photochemically. This means that data relative to the different oxidation states have sometimes been obtained in different samples and, in most cases, the real oxidation state was difficult to control. Conventional reducing methods produce easily five to ten percent of an unwanted cobalt oxidation state. For example, some Co(III) or Co(I) complex could be present in the Co(II) hydroxocobalamin sample depending on the reduction process. This contamination affects the intensity of the peaks in the pre-edge and edge region of XANES spectra. This spectral domain contains important information on the electronic structure of the complex.

Moreover with conventional reducing methods, the energy shift of XANES (which is normally of the order of 1-2 eV, or less) is measured with a systematic error.

For these reasons XANES data relative to redox processes cannot be properly investigated without an appropriate electrochemical set-up.

The reduction from Co(III) to Co(II) produces the most significant structural changes: cobalt coordination number decreases from six to five and the edge position shifts of 2.4±0.3 eV. This value is more accurate of the one obtained by (Giorgetti et al., 1997) as the integration was increased and the energy step is smaller.

We have also investigated the reduction from Co(II) to Co(I) which produces mainly electronic effects (figure 4) with no apparent change of the coordination number (manuscript in preparation).

Microperoxidase XANES spectrum presents a small but reproducible shift of 1 eV ±0.5 eV upon reduction, the general shape, of the oxidized and reduced forms, is identical showing that iron ligands are the same and the coordination geometry is unchanged.

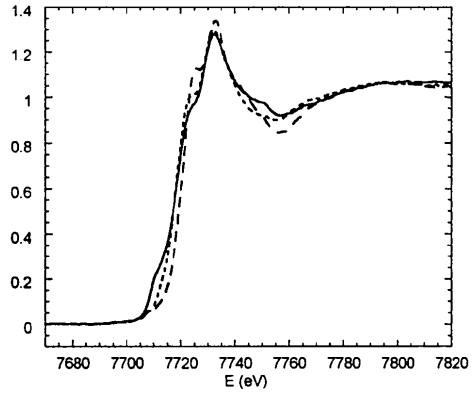

Figure 4 XANES spectra of hydroxocobalamin samples at selected potential: —— -930, ····· -650 mV, - - - - - OVC

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