Cytochrome C reductase immobilized on carbon paste electrode and its electrocatalytic effect on the reduction of cytochrome C

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In memorian of Dr. Guido S. Canessa C.

ABSTRACT

Carbon paste electrodes were modified with cytochrome c reductase enzyme. The behavior of the electrodes was characterized by cyclic voltammetry. The cytochrome c reduction was investigated both on the enzyme modified carbon paste electrode and in a homogeneous system. In the first case, a significant electrocatalytic effect on the reduction of cytochrome c was found. The effect is supported by an increase of 4.3 fold of the peak current and a shift of the cathodic peak potential toward less cathodic values. Also, apparent Michaelis constant values were calculated.

Results from this work show that carbon paste electrode modified by the cytochrome c reductase represents a new interface that will permit the study of electron transfer reactions involved in biodegradation processes.

Key Words: Cytocrome c reductase, electrocatalysis, modified carbon paste electrode.

RESUMEN

El electrodo de pasta de carbono fue modificado con la enzima Citocromo C reductasa. El comportamiento de los electrodos fue caracterizado por voltametría cíclica. La reducción de citocromo C fue investigada tanto sobre la enzima como en un sistema homogéneo. En el primer caso, un significante efecto electrocatalítico sobre la reducción de Citocromo C fue observado. El efecto concuerda con un aumento de 4.3 veces de la corriente de pico y por un desplazamiento del potencial de pico a valores menos catódicos. También, valores para la constante de Michaelis aparente fueron calculados.

Los resultados de este trabajo muestran que el electrodo de pasta de carbono modificado por la enzima Citocromo C reductasa representa una nueva interfase que permitirá el estudio de reacciones de transferencia electrónica involucradas en procesos de biodegradación.

Palabras claves: Citocromo C reductasa, electrocatálisis, electrodo de pasta de carbono modificado.

INTRODUCTION

During the last years, modified electrodes based on the incorporation of biological entities within carbon pastes are gaining considerable attention [1-7]. Such a versatile strategy allows the co-immobilization of the enzyme, its mediator or cofactor, and another enzyme or stabilizer, as needed for a reagentless biosensing device. Short response times accrue from the absence of supporting membranes and the close proximity of the biocatalytic and graphite sites. The bulk of the paste serves as a source of the biocatalytic activity, and fresh biosurfaces can easily be obtained by renewing the surface. The fact that many enzymes retain their activity within the hydrophobic paste environment is not surprising considering the known activity of the enzymes in organic media. This avenue is not limited to enzyme electrodes [5].

Flavin is the trivial name of the redox-active prosthetic group of a class of respiratory enzymes, which occur widely in animals and plants.

Flavoenzymes mediate electrons transfers processes (e.g., in photosynthesis and oxidative phosphorylation) and are involved in the regulation of neurotransmitters and the detoxification of xenobiotics [8].

Mammalian cytochrome c reductase (CCR) is a flavoprotein of this class. This enzyme has a specific activity of 190 and it is electrophoretically homogeneous. From physicochemical measurements, the molecular weight was calculated to be 75.000 to 80.000; from flavin content a value of 78.000 was found, assuming one flavin per molecule; and from iron content, assuming four iron atoms per enzyme molecule, the molecular weight was determined as 80.000 [9-11]. The enzyme contains a flavin adenine nucleotide not identical with FAD but giving the correct analysis for FAD (or a mixture of FMN and AMP). Iron is also part of the prosthetic group [9].

In 1984, it was demostrated that this enzyme isolated from Trypanosoma cruzy epimastigotes [12] corresponds to a protein of molecular weight of 100.000 and it was composed of two identical subunits of molecular weight of 52.000. The enzyme contains FAD in the ratio of 1mol FAD per mole of enzyme subunit [13]. Also, it had been purified and characterized an NADPH-dependent cytochrome c reductase from human neutrophil membranes with a molecular weight of 68.000 (FMN/FAD aprox. 1) [14].

In this study, we have used cytochrome c reductase enzyme of porcine heart fixed on the carbon paste electrode together NADH, in order to facilitate the electron transfer between the electrode surface and cytochrome c. We also explore the applicability of a modified electrode containing this enzyme as a new model for biotransformation studies.

EXPERIMENTAL

Cytochrome c (Merck, 124804), NADH (Merck, 24644.), were used as received. NADH-cytochrome c reductase of porcine heart (Sigma EC 1.6.99.3) was purified as described in literature [9]. Electrophoresis of this enzyme was homogeneous and showed one band corresponding to 78.000 according to the literature [9]. Protein was determined by the Bradford method.

0.3 M phosphate buffer at pH 7.4 was used in spectrophotometric and electrochemical experiments.

Spectrophotometric measurements were carried out with an UNICAM UV-Visible UV3 spectrophotometer connected to a DTK computer.

Electrochemical experiments were carried out with a CV-50 W BAS potentiostat connected to a GATEWAY 2000 computer. A two-compartment cell and a three-electrode system were used as the electrochemical cell. The working electrode was a carbon paste with geometric area of 0.04 cm2 (Metrohm carbon paste 6.2801.000) or CCR-carbon paste modified electrode with the same geometric area. Platinum wire was used as a counter electrode and the reference electrode was a SCE. All potentials were referred to the SCE electrode. All solutions were purged with nitrogen by 10 min. before each run to eliminate oxygen.

Modified electrodes were prepared by mixing 4 mg of the enzyme with 20 mg of carbon paste. Furthermore, 4 mg of the enzyme was mixed with 4 mg of NADH and 16 mg of carbon paste in order to make an enzyme-cofactor-carbon paste modified electrode. Then, modified electrodes were washed with buffer before the electrochemical measurements. After each measurement the working electrode was cleaned by the application of a pulse of 0 V for 5 s.

Electrochemical Assay for CCR activity- To compare the enzyme activities in both types of interfaces, the current was measured according to the electron transfer in the cytochrome c reduction having a) all the species in solution, and b) the enzyme and NADH immobilized in a carbon paste electrode (CCR-NADH-CPE) and the substrate cytochrome c in solution. The current due to the background (electrode, enzyme and NADH) was discounted from the total current obtained in the presence of cytochrome c.

RESULTS AND DISCUSSION

The cyclic voltammogram of the enzyme modified carbon paste electrode (CCR-NADH-CP) with the enzyme without purification and with the enzyme after purification are shown in Fig. 1a) and 1b), respectively. In the last figure, the voltammogram has been obtained by the substraction of the background current. As can be seen from this figures, only the current values were decreased after the purification procedure, but the peak potential values remain practically inaltered. Also, to achieve a stable response was necessary at least 60 minutes of incubation in the buffer solution at 37ºC (Fig. 2a). Moreover, if a continuous cycling of potentials, i.e. 10 cycles, a reproducible electrochemical response is obtained (Fig.2b). Reproducibility of the voltammetric response at 37ºC after 10 cycles of potential sweep is shown in table 1. Results of this study show that in these experimental conditions, a good reproducibility both in current and potential are found.

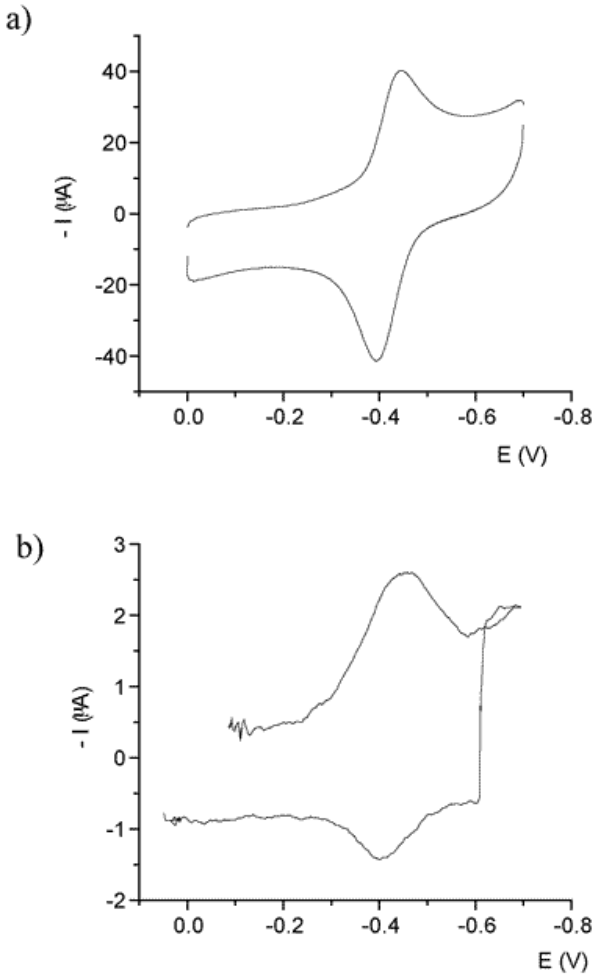

Fig 1. Cyclic voltammogram of CCR-NADH modified carbon paste electrode in 0.1 M phosphate buffer pH 7.4 al 37ºC, Scan rate 0.1 V/s. a) Commercial enzyme and b) enzyme after purification

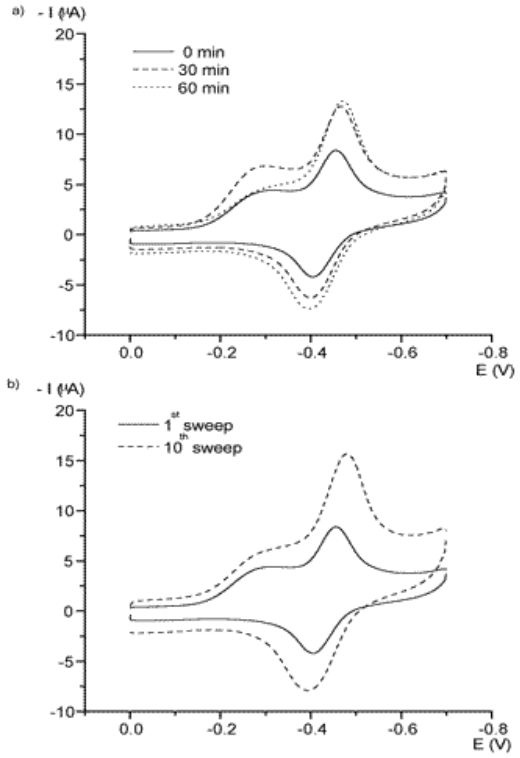

Fig. 2 Cyclic voltammogram of CCR modified carbon paste electrode in 0.1 M phosphate buffer pH 7.4 at 37ºC. Scan rate 0.1 V/s Stable response obtained by a) incubation time of 60 minutes and b) continuos cycling potential sweep.

Then, a cathodic peak potential ($E_{p,c}$) of -0.45 V and an anodic peak potential ($E_{p,a}$) of -0.39 V (vs Ag/AgCl) were obtained after of continuous cycling. The formal potential $E^o = (E_{p,a}+E_{p,c})/2$ was -0.42 V. Exchange of the enzyme modified carbon paste electrode by a bare carbon paste electrode resulted in a complete disappearance of the redox wave of the enzyme. The scan rate variation studies showed that both peaks shift towards negative and positive values of potential, respectively. There was also an increase in the peak current as a function of the scan rate. Both, the peak separation (DEp) of 60 mV and the variation of ΔE_p with sweep rate were indicative of a quasi-reversible one-electron transfer process.

When the CCR-NADH-CP electrode was exposed to a cytochrome c solution, a voltammetric response was obtained (Fig. 3a). As a control experiment, the contribution in current and potential of NADH was determined. Results of these experiments permit us to conclude that NADH does not exhibits any signal in the potential range in which the present study was performed. Then, the complete modified system CCR-NADH-CP electrode and the substrate cytochrome c in solution was assessed. In Fig. 3a) cyclic voltammograms corresponding to the modified carbon paste electrode including the enzyme and its cofactor (dashed line) and after the addition of cytochrome c (full line) at 37ºC are displayed. By subtraction of dashed from full lines, a cathodic current of 13.5 μA at a potential of -530 mV was obtained. Also, the homogeneous system (CCR, NADH and cytochrome c in solution) was investigated. In Fig. 3b), the voltammetric response of the enzyme and NADH in solution (dashed line) and the respective response after the addition of cytochrome c (full line) at 37°C are shown. Under these experimental conditions the variation of the cathodic peak current values was 3.1 μA at a potential of -580 mV.

Tabla I. Reproductibility test of the voltametric response of CCR-NADH-CPEs at 37ºC afte 10 cycles.

run	Ip,c (μA)	-Ep,c(mV)	ΔEp(mV)
1	-15.23	454	51
2	-15.39	458	54
3	-15.77	456	52
4	-15.11	458	55
5	-15.11	458	55
Mean±sd	-15.52±1.30	457±2	53.4±1.8

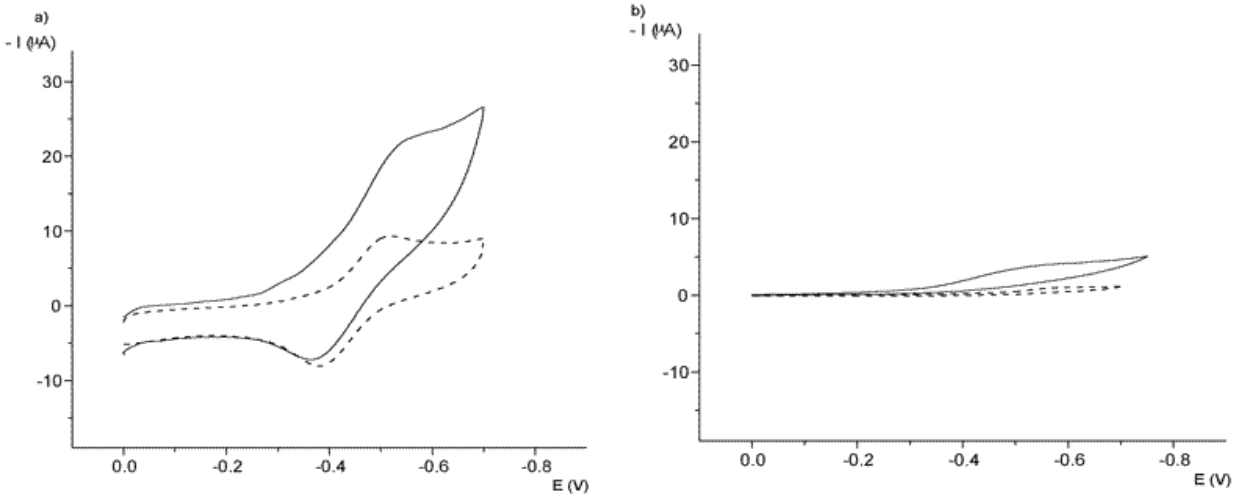

Fig 3. Cyclic voltammogram of 0.5 mM cytochrome c (full line) and the background current in abscence of cytpchrome (dashed line). (0.1 M phosphate buffer pH 7.4, scan rate 0.1 V/s at 37ºC).
a) on CP electrode in presence of CCR (4 mg/ml) and NADH (5mM) in solution, and
b) on CCR-NADH-CP modified electrode.

Furthermore, if the cyclic voltammogram of cytochrome c on CCR-NADH-CP electrode is compared with the cyclic voltammogram obtained on a bare carbon paste electrode (Fig. 4), a significant increase of the cathodic current and a shifting of the reduction potential are observed. On CCR-NADH-CP electrode, the cytochrome c reduction shown a cathodic peak potential ($E_{p,c}$) of -0.53 V and an anodic peak potential ($E_{p,a}$) of -0.37 V (vs Ag/AgCl). Thus, there is a clear catalytic effect when the enzyme is immobilized on a carbon paste electrode.

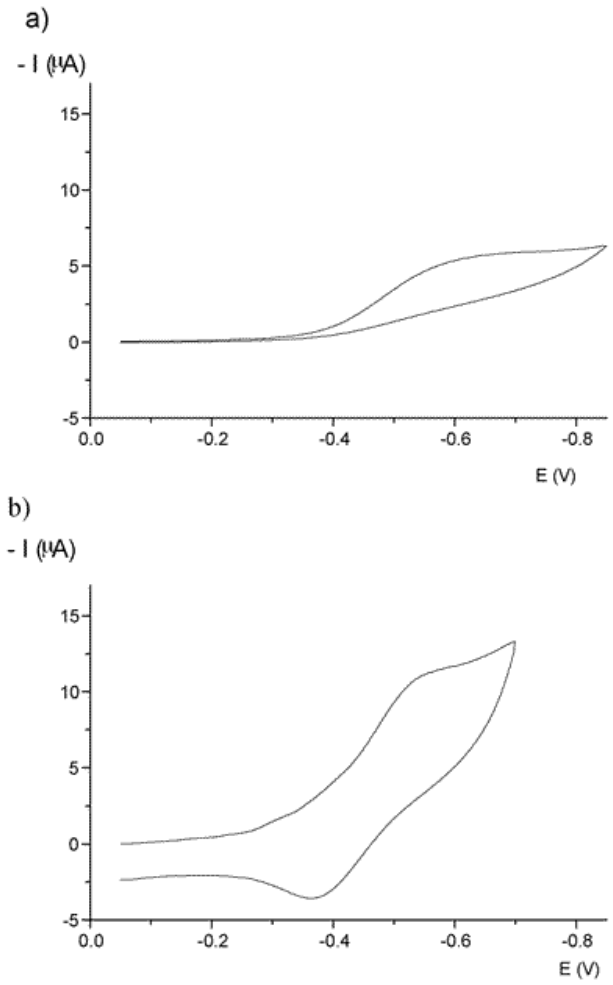

Fig 4. Cyclic voltammogram of 0.5 mM cytochrome c. (0.1 M phosphate buffer pH 7.4 scan rate 0.1 V/s at 37ºC). a) on CP electrode; and b) on CCR-NADH-CP modified electrode

On the other hand, if the rate of the enzymatic reaction is catalitycally controlled, an apparent Michaelis constant value K_M^{app}, can be amperometrically determined (peak current obtained from the voltammetric curves) using the following "Lineweaver-Burk" type equation:

$$\frac{1}{i_p} = \left(\frac{K_M^{app}}{i_{pmax}}\right)\left(\frac{1}{C}\right) + \frac{1}{i_{pmax}}$$

Equation represents a rearrangement of Equation 24b from Shu and Wilson [15], where i_{pmax} and *ip* are the currents measured for enzymatic product detection under conditions of substrate saturation and steady state, respectively, for a given substrate concentration, *C*. A plot of $1/i_p$ vs. $1/C$ will give a straight line with the slope equal to K_M^{app} / i_{pmax} and intercept equal to $1/i_{pmax}$.

Then, the K_M^{app} values were calculated againts cytochrome c reduction in both systems, i.e. in solution and on the enzyme immobilized carbon paste electrode. The concentration of the second substrate (NADH) was always in excess. The results are shown in table II and clearly indicate the best catalytic effect of the modified electrode when compared with the same system in solution.

Table II. Kinetic parameters for CCR enzyme for reduction of (0.4 - 1mM) cytochrome c, both in solution and on immobilized enzyme carbon paste electrode.

	K_M^{app} (mM)*	i_{pmax} (μA)	Catalytic capacity**
In solution	18.82	13.16	0.69
Inmobilized on CPE	8.25	625.00	75.76

* reaction concentrations of the homogeneous system were 5 mM NADH and mg/ml CCR. The modofied carbon paste electrodes were prepared with 4 mg of CCR and 4 mg of NADH.

** Catalytic capacity indicate $/i_{pmax}/\ K_M^{app}$

CONCLUSIONS

The system containing the enzyme cytochrome c reductase and its cofactor NADH have been immobilized on a carbon paste electrode. Reproducibility of this modified electrode is good when at least 10 cycles of potential are applied and the temperature is kept constant at 37°C (temperature for a maximum activity of the enzyme).

The inclusion of the CCR and its cofactor NADH into a carbon paste electrode produces a significant electrocatalytic effect for the reduction of cytochrome c when compared with the same system in solution. Thus, this effect is supported by an increase of 4.3 fold in the cathodic current and the reduction occurring at less cathodic potential, with a shifting of potential of 70 mV. Furthermore, $K^{app}{}_M$ value for the immobilized enzyme was significantly lower than the $K^{app}{}_M$ of the enzyme in solution.

Finally, the present results permit us to propose that the modified electrode here studied will be a good interface to study electron transfer processes for a great number of compounds of pharmacological relevance to elucidate their biotransformation reactions.

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