

Preliminary note

## Direct electrical communication of cytochrome *c* and cytochrome *b<sub>5</sub>* at basal plane graphite electrodes modified with lauric acid or laurylamine

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### INTRODUCTION

Because many biological processes involve [1] electron transfer reactions through membranes, much work has been devoted to the study of similar processes at membrane–solution interfaces. Langumir–Blodgett films [2], adsorbed thiols [3,4] and bilayer lipid membranes [5] have been the object of electrochemical investigation and some theoretical models of their function have been developed [6]. Electrode surfaces, modified with components of biological membranes, were used to carry out these electrochemical studies. For example, Nelson et al. [7,8] have investigated the formation and properties of stable phospholipid monolayers on a mercury electrode.

Pyrolytic graphite electrodes [9–18] have been used successfully in bioelectrochemical studies. Direct (*unmediated*) [19] electron transfer between “edge plane” graphite (EPG) electrodes and several redox proteins has been demonstrated [20–24]. Unmediated electron transfer of proteins on EPG was achieved by controlling the electrostatic nature of the electrode surface using multivalent cations as promoters. On the other hand, in general, proteins show only poor electrochemistry on “basal plane” graphite (BPG).

Herein we report two examples of the direct electrochemistry of cytochrome *c* and cytochrome *b<sub>5</sub>* to BPG electrodes, modified with lauric acid and laurylamine, respectively. Presumably the hydrophobic chain of modifier is bound to the graphite electrode, allowing the hydrophilic groups to interact with the charged residues carried by cytochrome *c* or cytochrome *b<sub>5</sub>*. Indeed, this modification forms a

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lipophilic film on the electrode surface that mimics a synthetic membrane. In addition, it gives rise to a charged electrode-solution interphase that promotes a transient interaction between the redox protein and the electrode, followed by electron transfer.

#### EXPERIMENTAL

Yeast iso-1-cytochrome *c* (*Saccharomyces cerevisiae*) was extracted and purified from bakers' yeast according to published procedures [25,26]. Further purification was performed by ion-exchange chromatography using an FPLC system (Pharmacia) on a cation exchange column (Mono S HR 10/10, Pharmacia). Cytochrome *b<sub>5</sub>* was isolated as the trypsin-cleaved soluble form from chicken livers by the method of Reid and Mauk [27]; deamidated forms, then, were removed by FPLC on an anion exchange column (Mono Q HR 515, Pharmacia). Protein solutions were concentrated and desalted by ultrafiltration using a diafiltration unit (Amicon, Lexington, MA, USA) and then stored as pellets under liquid nitrogen. Potassium chloride, lauric acid (both Analar, BDH), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, Sigma), laurylamine (Aldrich) were used without further purification. Other chemicals were either of Analar or Aristar grades. Water was purified by reverse osmosis (Millipore RO-6), followed by ion-exchange (Millipore, Milli-Q).

Electrochemical experiments were carried out using an Oxford Electrodes (now Usar Scientific Instruments) potentiostat coupled to a X-Y chart recorder (Gould 60000 series A3). The electrochemical cells used (300  $\mu$ l volume) incorporated a conventional three electrode system with two electrolyte compartments: one housed the working and counter electrodes while the other (connected to the working compartment through a Luggin capillary) contained the reference electrode. The counter electrode was made of platinum gauze, placed around the Luggin tip, while the reference electrode used was saturated calomel (SCE 244 mV vs. SHE at 25°C) (Radiometer type K401). Disk working electrodes (BPG) were constructed from standard pyrolytic graphite (Le Carbone Lorraine, Paris) cut with the a-b plane parallel to the disk face and housed in Teflon sheaths. Two BPG electrodes were employed; electrochemical determination of their areas gave values of 0.126 and 0.196 cm<sup>2</sup>.

BPG electrodes were modified with laurylamine or lauric acid by dipping the electrode surface in ethanolic solution (1 g l<sup>-1</sup>) of the modifier, followed by thorough washing with water. Prior to each modification, a fresh basal plane surface was prepared by cleaving the electrode parallel to its base with a sharp cutting edge followed by sonication in water. Cyclic voltammogram experiments were performed at room temperature and under a humidified argon atmosphere.

#### RESULTS AND DISCUSSION

##### *Electrochemistry of cytochrome *b<sub>5</sub>* at laurylamine modified BPG electrode*

Figure 1a-c shows background cyclic voltammograms of bare (a) and laurylamine-modified (b) BPG electrodes. The broad peaks around 0 V of Fig. 1a are

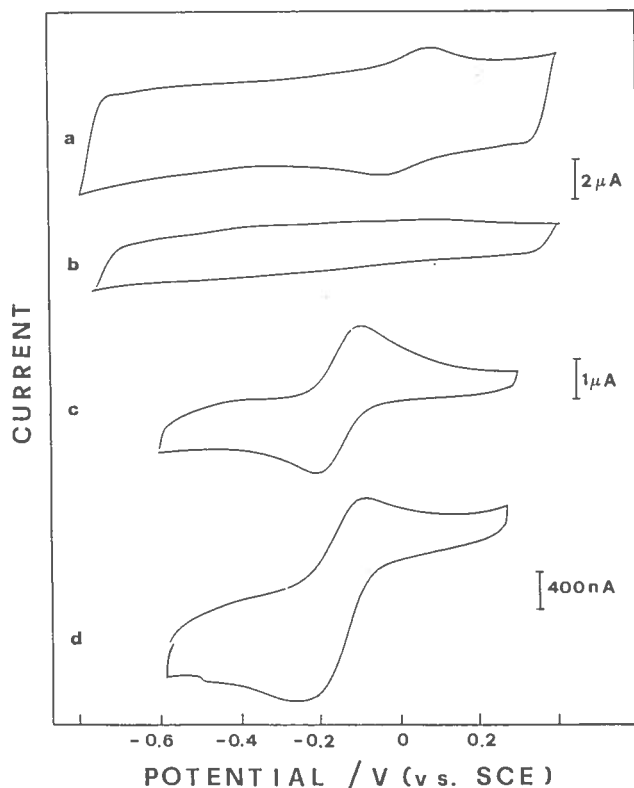


Fig. 1. Typical background cyclic voltammograms of a basal plane graphite (BPG) electrode unmodified (a) and modified with laurylamine (b). Electrode dipping time 2 min in ethanolic solution of laurylamine ( $1 \text{ g l}^{-1}$ ). Voltammograms were obtained in 10 mM HEPES + 100 mM KCl, pH 7 and  $25^\circ \text{C}$ ; scan rate  $200 \text{ mV s}^{-1}$ . (c), (d) Steady-state cyclic voltammograms of soluble cytochrome  $b_5$  ( $90 \text{ } \mu\text{M}$ ) at modified electrode with laurylamine: scan rates 50 and  $20 \text{ mV s}^{-1}$ , respectively.

probably due to impurities adsorbed on the surface and/or C–O groups present on the graphite electrode [11]. After modification with laurylamine, lower background currents were observed (Fig. 1b), indicating a large extent of laurylamine adsorption. In the presence of soluble cytochrome  $b_5$ , a well-defined cyclic voltammogram was obtained (Fig. 1c). The anodic and cathodic peaks showed a time-dependent response; they decreased with the number of potential sweep, reaching a steady-state after a few cycles.

Figure 2 presents the dependence of the ratios  $i_{pa}/i_{pc}$  and  $i_{pc}/v^{1/2}$  on the scan rate. For values larger than  $50 \text{ mV s}^{-1}$ , the system presented a quasi-reversible electron transfer, i.e. nearly unity anodic/cathodic peak current ratio, an increase of peak current and of  $\Delta E_p$  with increasing scan rate. The formal potential ( $E^{0'}$ ), evaluated as the mean of  $E_{pa}$  and  $E_{pc}$ , was  $-0.170 \text{ V (vs. SCE)}$ . At scan rates of  $20 \text{ mV s}^{-1}$  and lower, voltammograms similar to that in Fig. 1d were recorded. Under

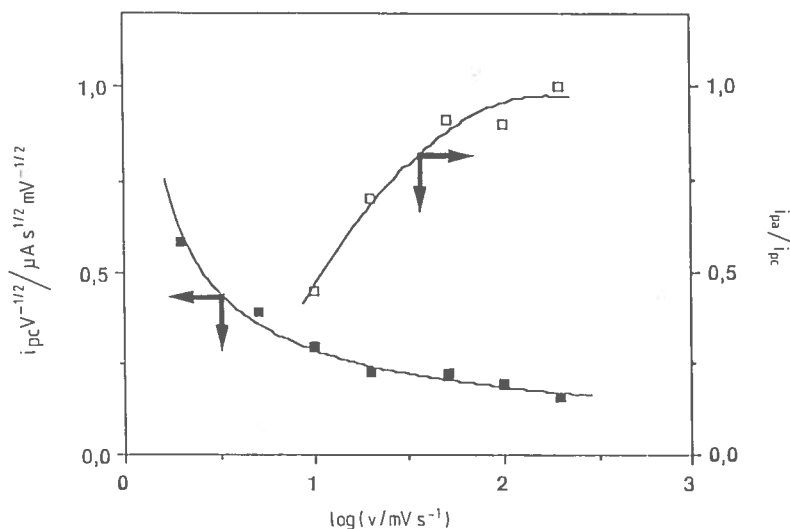


Fig. 2. Plots of the ratios  $i_{pa}/i_{pc}$  and  $i_{pc}/v^{1/2}$  vs. logarithm of scan rate. Solution of soluble cytochrome  $b_5$  (90  $\mu$ M) in buffer solution as in Fig. 1.

these conditions, the cathodic peak current reached a limiting value higher than that expected from the Randles–Sevcik equation [28] while its peak potential was shifted towards negative values. As far we know [29], the electrochemistry of soluble cytochrome  $b_5$ , at different modified electrode surfaces, does not show coupled chemical reactions (e.g. chemical oxidation) even in the presence of oxygen. The propionate side chains of cytochrome  $b_5$  heme are not bound to the protein [30]: one of them seems to be involved in the stabilization of the protein in oxidized form. When the two propionate side chains are esterified, the redox potential becomes more positive, i.e.  $-175$  mV compared to  $-239$  mV (vs. SCE) for the native protein [27,31].

Laurylamine-modified BPG electrodes were also employed to investigate the electrochemistry of redox proteins having a positive charge distribution around the heme crevice, e.g. cytochrome  $c$ . In this case, no electrochemical response has been observed.

These results show how it is possible to modify basal plane graphite electrodes through the spontaneous adsorption of laurylamine. The latter forms an amphiphilic film capable of selective promotion of the electrochemistry of a negatively charged protein like cytochrome  $b_5$ . Although no direct information about laurylamine attachment and orientation on the electrode surface is available, it is proposed that the aliphatic chains are adsorbed on the surface with the ionized amine groups (laurylamine  $pK_a = 10.63$  [32]) oriented towards the solution as has been done for phospholipids adsorbed on mercury electrodes [7]. The opposite electrochemical behaviour of cytochromes  $b_5$  and  $c$  seems to confirm our suggestion.

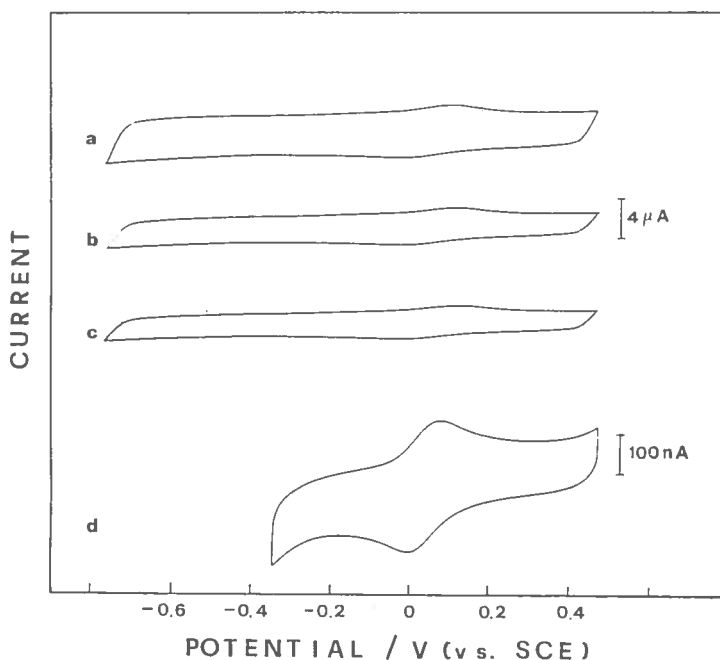


Fig. 3. Background cyclic voltammograms of lauric acid modified BPG electrodes. Dipping time (a) 1 min, (b) 6 min and (c) 70 min in ethanolic solution of lauric acid ( $1 \text{ g l}^{-1}$ ). The voltammograms were obtained in 10 mM HEPES+100 mM KCl, pH 7 and  $21^\circ\text{C}$ ; scan rate  $200 \text{ mV s}^{-1}$ . (d) Cyclic voltammogram of yeast cytochrome *c* ( $118 \text{ } \mu\text{M}$ ) on BPG electrode modified with lauric acid. Dipping time 70 min, scan rate  $10 \text{ mV s}^{-1}$ .

#### *Electrochemistry of cytochrome c at a lauric acid modified BPG electrode*

Figure 3a–c shows typical background cyclic voltammograms of the BPG electrodes, modified with lauric acid. By increasing the electrode dipping time, the background current decreased and for dipping times greater than 1 h, no further change was observed.

After addition of yeast cytochrome *c*, a quasi-reversible cyclic voltammogram was obtained (Fig. 3d). At high scan rate values, the anodic/cathodic peak current ratio is less than one, indicating a transfer coefficient slightly different from 0.5. A plot of cathodic peak current vs. square root of scan rate was linear up to  $200 \text{ mV s}^{-1}$  (Fig. 4) and gave a diffusion coefficient of  $2.6 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ . The formal potential ( $E^{0'}$ ), evaluated at low scan rates, was 32 mV (vs. SCE). Similarly  $\Delta E_p$  ( $75 \text{ mV}$  at  $10 \text{ mV s}^{-1}$ ) increases on increasing the scan rate indicating a quasi-reversible electrode process. At the same modified electrode, preliminary experiments with soluble cytochrome *b*<sub>5</sub> did not show any electrochemical response. Therefore the modification with lauric acid might involve formation of a charged electrode–solution interphase, where the negatively charged carboxylic groups of modifier interact favourably with lysine residues of cytochrome *c* (but not with cytochrome *b*<sub>5</sub>)

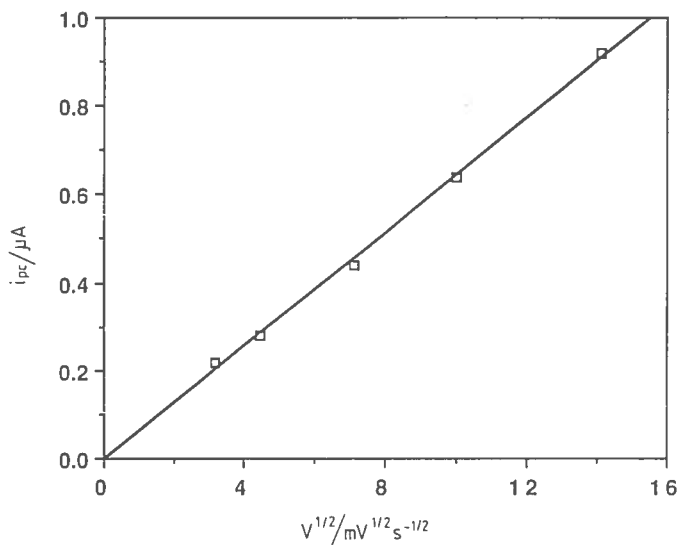


Fig. 4. Plot of the cathodic peak current vs. square root of the scan rate. Solution of yeast cytochrome *c* (118  $\mu\text{M}$ ). Other experimental conditions as in Fig. 3.

allowing a quasi-reversible electron transfer. An electrode surface composed of many pinholes has been invoked [2], to explain the electrochemical behaviour of the ion complex  $\text{Mo}(\text{CN})_8^{-4}$  on the Langmuir–Blodgett monolayer of stearic acid on a platinum electrode. The presence of pinholes could also in our case explain both the capability of the redox proteins to permeate the modified layer and the observed electrochemistry.

#### CONCLUSIONS

It is possible to obtain selective and direct electrical communication between redox proteins and modified electrodes by using amphiphilic compounds as modifiers. Laurylamine and lauric acid can be used to promote, on basal plane graphite electrodes, direct electrochemistry of cytochrome *b<sub>5</sub>* and cytochrome *c*, respectively. The modifiers investigated can be also used to form lipid-like layers on the electrode surface. From an analytical point of view, such layers can be exploited advantageously to minimize undesired electrochemical reactions in analytical devices using redox proteins (e.g. biosensors) and to extend electroanalytical techniques such as adsorptive stripping voltammetry to biological compounds that bind naturally to membranes.

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