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# Electrochemical studies of cytochrome *c* disulfide at gold electrodes

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**Abstract**

The electrochemistry of disulfide in cytochrome *c* on gold electrodes was reported. The observed electrochemical response was used to explain why the electrochemical reaction of cytochrome *c* is irreversible at gold electrodes. Disulfide bonds in cytochrome *c* were strongly adsorbed onto the surface of gold electrodes and caused slow rate of electron transfer of the heme group. It was found that the presence of disulfides in cytochrome *c* was responsible for the lack of electrochemical response of the heme group on a gold electrode. The mechanisms for this effect were studied using electrochemistry and photoelectron spectroscopy. 

*Keywords:* Disulfide bond; Cytochrome *c*; Electrochemistry; XPS; ESCA

## 1. Introduction

Probably the most thoroughly studied redox protein in bioelectrochemistry is cytochrome *c* [1]. Since the biological function of cytochrome *c* is to carry out oxidation–reduction reactions, it follows that measurements of the oxidation–reduction potentials and associated electron transfer kinetics of cytochrome *c* are central to understand this protein. Generally, cytochrome *c* does not give a voltammetric response at a simple (untreated) electrode due to reactant adsorption–desorption (vide infra) [1]. One reason for this is that cytochrome *c* molecules in the concentrated solution would exist as oligomers which are electrochemically inactive [2,3]. Over the last few years, major efforts were devoted to understand the factors which led to direct voltammetric response for proteins so that electrodes could be designed for protein electrochemistry [4]. Many publications address the subject of developing electrode modification protocols to facilitate the specific electrochemistry of cytochrome *c*. A major aspect of the surface modification is to prevent irreversible adventitious adsorption, including hydrophobic adsorption of the protein itself.

The key to solve the redox properties would be

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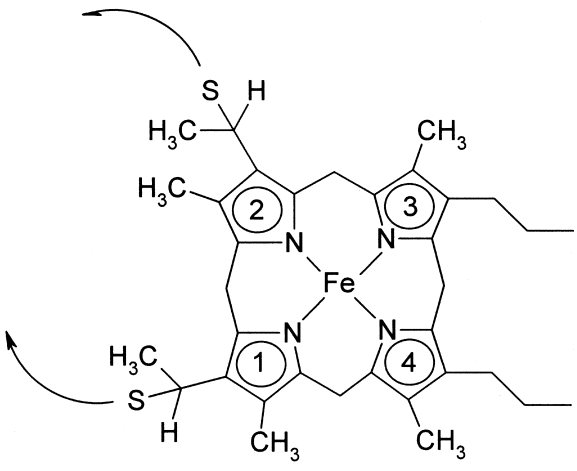
specific adsorption of cytochrome *c* on the surfaces of metal electrodes, especially at gold electrode and then denaturation [5]. The main purpose of our present work is to study in detail the adsorption (and denaturation) of cytochrome *c* at gold electrodes, as understanding the reasons would be extremely useful for improving electrode design.

The redox potentials of cytochrome *c* are related to the microenvironment of the heme group [6,7], i.e. to the conformation of cytochrome *c* molecules. In cytochrome *c*, the heme group is buried in hydrophobic protein except for one edge, which is near the surface [8]. As shown in Scheme 1, Rings 2 and 3 are on this edge, and ring 2 is bound to a sulfur-containing group. Since sulfur compounds such as thiols, disulfides, sulfides and their related moieties have been known to adsorb strongly on coinage metals, particularly on gold, as well as on platinum and mercury [9], detailed knowledge of the sulfur-containing groups will provide important information about the electrochemistry of cytochrome *c*.

In our previous work, we reported an enhanced redox activity of disulfide present in cytochrome *c* at electropolymerized polyaniline electrodes [10], which was attributed to the formation of adducts between the disulfide group in cytochrome *c* and the nitrogen sites in the polyaniline moieties. It was concluded that the disulfide group in cytochrome *c* might be able to attach on the gold electrode, causing slow electron kinetics or reactant adsorption–desorption. In this report the redox process of disulfide present in cytochrome *c* on gold electrodes is discussed in order to explain the adsorption of cytochrome *c* on gold electrode. To our knowledge, this is the first work reporting the importance of disulfide groups in cytochrome *c* electrochemistry and their possible role in the lack of electrochemical response of heme groups on gold electrodes.




Scheme 1. Structural formula of heme *c*, illustrating the interaction of the group with the gold electrode at rings 1 and 2.

## 2. Experimental

Horse heart muscle cytochrome *c* was purchased from Aldrich Chemical Co. and was further purified by passing it through a Sephadex G-25 column to remove protein impurities and metal ions. All other chemicals were of reagent grade. All the solutions were prepared with triply distilled water and were deoxygenated before use through nitrogen bubbling.

The electrochemical experiments were performed with a Princeton Applied Research (PAR) Model 273 Potentiostat/Galvanostat (EG&G). A conventional three-electrode electrochemical cell was used. The working electrode was constructed from a gold rod, which was sealed into poly(tetrafluoroethylene) tubing. The exposed area was approximately 0.20 $mm^2$. A Pt wire was utilized as the auxiliary electrode and a saturated calomel electrode (SCE) served as the reference electrode. The working electrode was sequentially polished with 5, 0.3 and 0.05 $\mu$m alumina slurries until a shiny mirror-like finish was obtained. The polishing procedure was repeated before each experiment. The electrode was then sonicated in de-ionized water and washed thoroughly. In the electrochemical measurements, cytochrome *c* was dissolved in a solution containing 0.025 mol/l phosphate buffer (pH 7.0) and 0.1 mol/l sodium perchlorate to yield a concentration of 0.1 mmol/l.

X-ray photoelectron spectroscopy (XPS) of the electrode surface was carried out using a Model ESCAL-AB-MK (II) (VG, England). The spectra calibration was made with respect to the $C_{1s}$ spectral line at 284.6 eV.

## 3. Results and discussion

### 3.1. Electrochemistry of cytochrome c disulfide at gold electrodes

Electrochemical techniques were used to investigate the redox activity of cytochrome *c*, i.e. electron transfer processes between the disulfide group in cytochrome *c* and the electrode interface. The cyclic voltammogram in curve 2 of Fig. 1 shows electrochemistry of the protein. At a switching potential of 0.75 V, cytochrome *c* exhibited a quasi-reversible voltammetric response with a formal potential of 0.308 V vs. NHE, which is 0.29 V more positive than the reported value of native cytochrome *c* [11]. A peak potential ~122 mV indicates that a kinetic effect was associated with the heterogeneous electron transfer. As the gold electrode was re-immersed into phosphate buffer, the peaks appeared once again (Fig. 1, curve 3) demonstrating that cytochrome *c* was adsorbed irreversibly during the first electrochemical measurement. When extending the potential range up to −0.6 V, as shown in Fig. 2, there was no reduction peak of the heme in adsorbed cytochrome *c*.

### 3.2. Electrochemistry of cytochrome c disulfide at platinum, glass carbon, graphite and antimony electrodes

Specific adsorption of the disulfide can be seen best in the cyclic voltammograms recorded using platinum, glassy carbon, graphite and antimony electrodes (Fig. 3). It is worth noting that, none of the glassy carbon, graphite and antimony electrodes exhibited any voltammetric responses attributed to the redox reaction of cytochrome *c* in this potential range, except for similar but much smaller peaks at the platinum electrode. The platinum electrode gave some response at similar potentials but the peak intensity was much smaller than that for the gold electrode (Figs. 1 and 2). As stated in Section 1, gold and platinum metals have high affinities towards adsorption of sulfur-containing groups, explaining the observed effect [9].

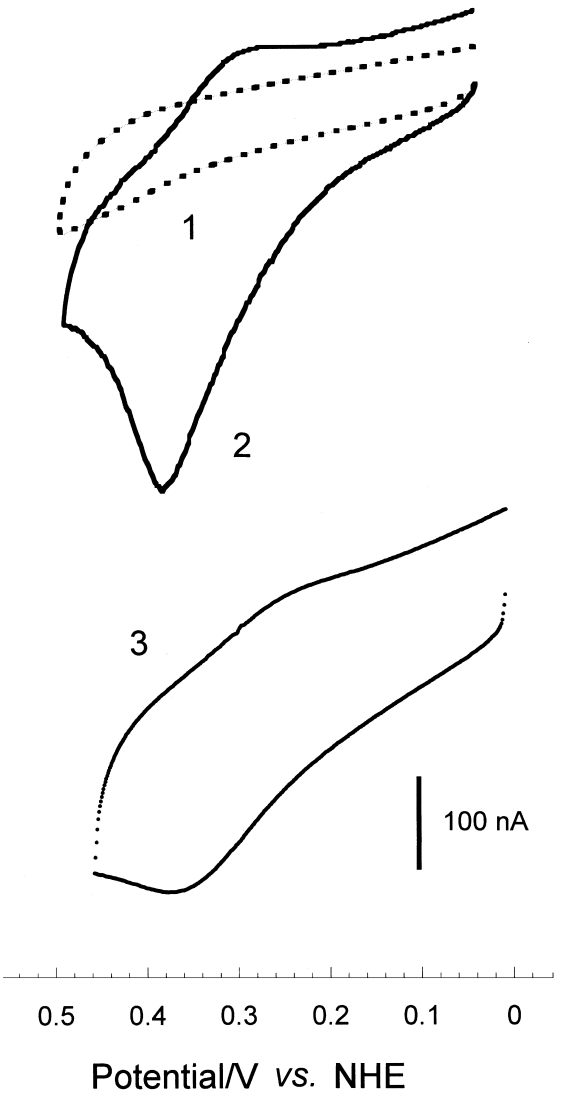



Fig. 1. Typical cyclic voltammograms of cytochrome *c* at a freshly polished gold electrode in a 0.025 mol/l phosphate buffer (pH 7.0) with 0.1 mol/l $NaClO_4$ at a scan rate of 50 mV/s. Curve 1: a background voltammogram; curve 2: a voltammogram for 0.1 mmol/l cytochrome *c*; curve 3: a voltammogram for the buffer after the experiment for the curve 2.

A linear relationship between the cathodic peak current and scan rate was observed at a constant cytochrome *c* concentration, as expected for an electrode reaction of adsorbed species (Fig. 4). These results are in good agreement with that of results in Fig. 1, curve 3.

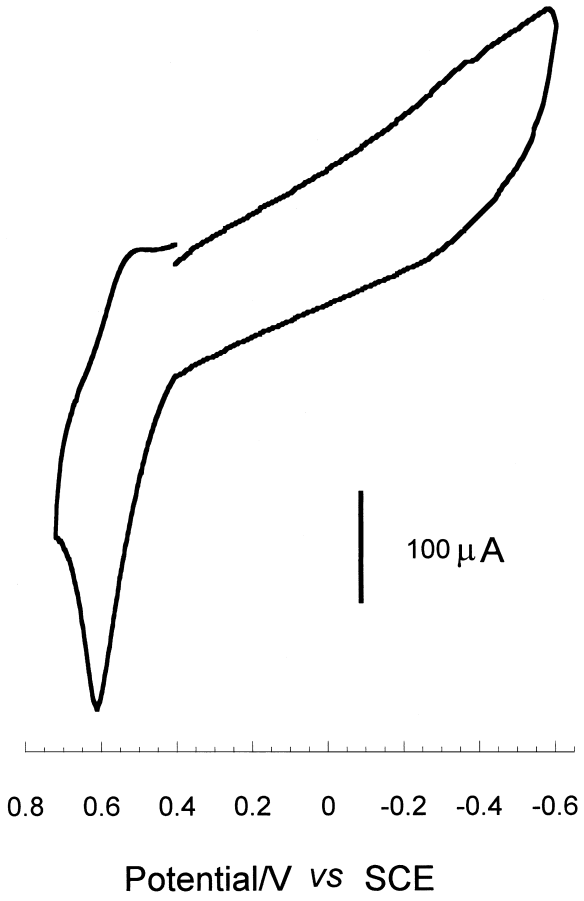



Fig. 2. Cyclic voltammograms of cytochrome *c* at a freshly polished gold electrode, other condition as in Fig. 1.

### *3.3. Sulfur-containing amino acids in cytochrome c at gold electrode*

In horse heart ferricytochrome *c* [Fe(III)], a heme *c* group is bound to the polypeptide chain via covalent sulfur linkage at Cys-17 and Cys-14, as well as by the axial iron ligands His-18 imidazole and Met-80 sulfur [8]. In cyclic voltammetric measurements, as shown in Fig. 5, L-cystine and oxidized glutathione at a gold electrode shown as formal potentials of 0.48 V and 0.558 V, respectively. However, methionine, cysteine and reduced glutathione did not exhibit any electrochemical responses. These results indicated

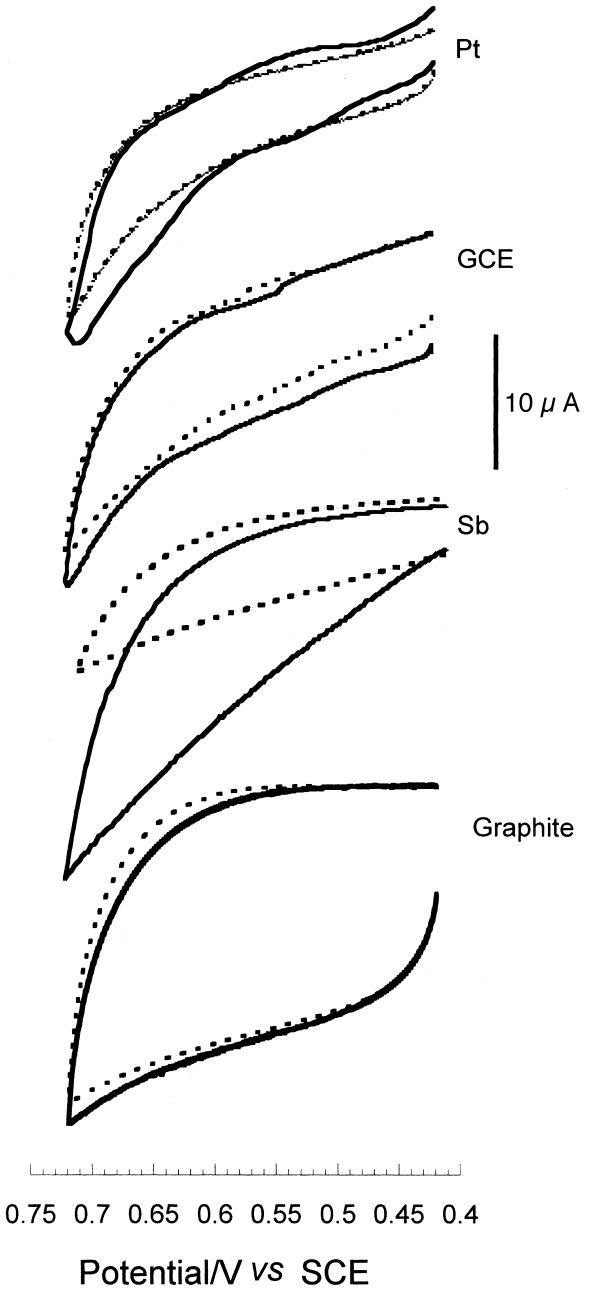



Fig. 3. Cyclic voltammograms of 0.1 mmol/l cytochrome *c* at platinum, glassy carbon, graphite and antimony electrodes in phosphate buffer at 10 mV/s; solid line (———): in the presence of 0.1 mmol/l cytochrome *c*, dash line (··· ···): background. The experimental conditions were the same as those in Fig. 1.

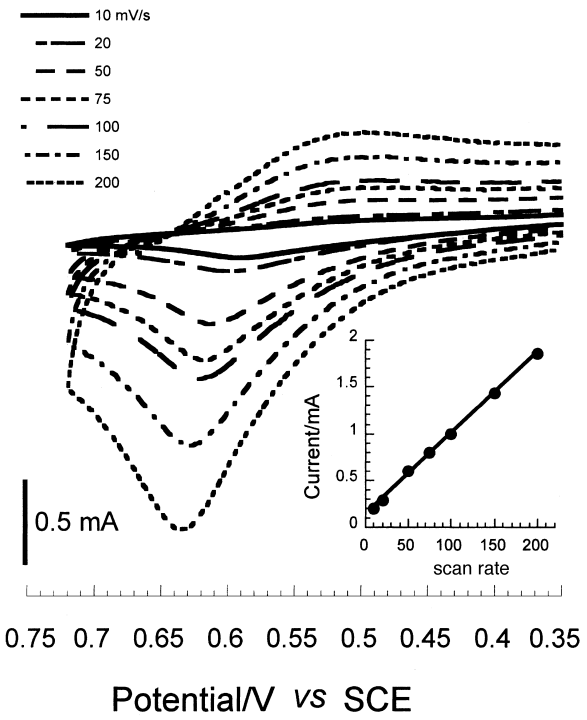



Fig. 4. The relationship between peak current of cytochrome *c* and the scan rate. The experimental conditions were the same as those in Fig. 1.

that the peaks in Fig. 1 were the response of disulfide in cytochrome, not −S− (as Met-80), nor any other sulfur containing groups in cytochrome *c*.

### *3.4. XPS spectroscopy of cytochrome c on a gold electrode*

XPS spectra of cytochrome *c* on the gold were recorded to confirm the adsorption of disulfide and analyzed to obtain structural information from the surface.

#### *3.4.1. Analysis based on the $S_{2p}$ peak*

Fig. 6 shows the $S_{2p}$ signals for the different oxidation states of cytochrome *c*. As generally observed for Au–S environments, the $S_{2p}$ peak for the oxidized disulfide (curve 3) was shifted to higher binding energy, 169 eV, which was approximately 6.4 eV more positive than the binding energy of the free species (162.6 eV) and approximately 6.2 eV more positive than the reduced form on the gold surface (162.8 eV). The same behavior has been observed for disulfide (169 eV) [12]. It is worth noting that the reduced form still represented a small peak at 162.6 eV, which may be due to unoxidized disulfide. However, the actual structure surrounding the disulfide would be complex, so other sulfur compounds such as $SO_4^-$ cannot be excluded as the reason for the appearance of this small peak. It can be concluded from the above evidence that when a potential of 0.75 V is applied, the binding energy of electroactive disulfide in cytochrome *c* shifted to the more positive direction compared to the reduced state due to the oxidation of disulfide bonds in cytochrome *c*.

#### *3.4.2. Analysis of the $Fe_{2p}$ signals*

To confirm the presence of the disulfide in cytochrome *c* adsorbed on the gold surface, we examined $Fe_{2p}^{3/2}$ spectra for the different redox states of cytochrome *c*. As shown in Fig. 7, the peak positions of the 2p doublets with the usual peak separation of 11.5 eV virtually did not change in the oxidized and reduced forms, demonstrating that the peaks in Fig. 1 do not arise from the redox reaction of heme Fe. These results are well supported by the electrochemical results in Fig. 2. Consequently, it can be concluded that the voltammetric peaks that appear at 0.308 V vs. NHE were due to the disulfide in cytochrome *c* and not the heme group.

Disulfide-containing proteins exhibit a voltammetric reduction peak on mercury electrodes at approximately −0.6 V vs. SCE at pH 7.0 [13–16]. Not all disulfide bonds in proteins are electroactive, and it has been proposed by Kuznetsov et al. [17] that it is the disulfide bonds in hydrophobic region of the proteins are electroactive. The disulfide bonds in hydrophobic region may be in direct contact with the electrode surface and located approximately 1.2–1.5 nm from the protein surface [11]. Consequently, the electrochemical response of the cytochrome *c* adsorbed on gold electrodes using a potential range of 0.25–0.7 V may be due to the denaturation of cytochrome *c*, although how the disulfide could be exposed from within the heme crevice and how the orbital over-

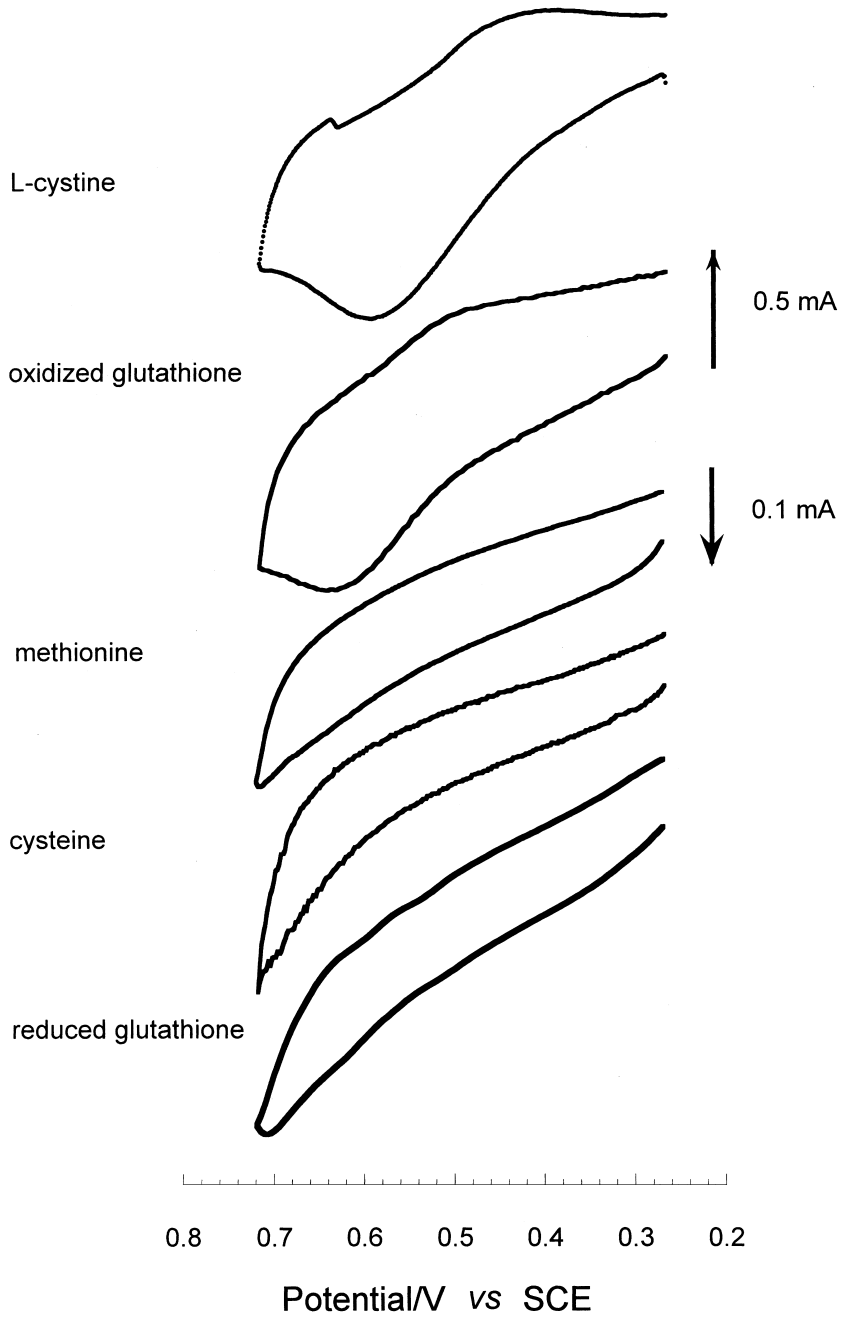



Fig. 5. Cyclic voltammograms of 0.5 mmol/l L-cystine, oxidized glutathione, methionine, cysteine and reduced glutathione at a gold electrode in phosphate buffer at 50 mV/s.

lap between the disulfide and gold surface could occur is difficult to explain. For cytochrome *c*, the electroactive disulfide is buried within the hydrophobic interior and when cytochrome *c* diffuses and associates with an electrode surface, it orients to gain a suitable direction for the subse-

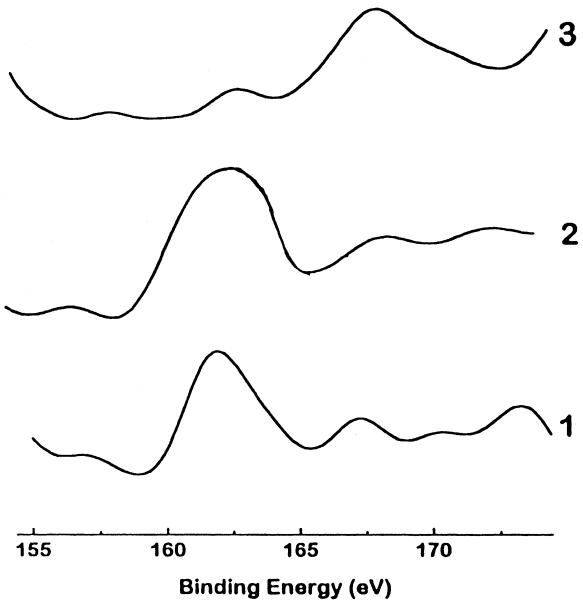



Fig. 6. XPS spectra of $S_{2p}$ for cytochrome *c* sample (curve 1), cytochrome *c* reduced at 0.3 V on the gold surface (curve 2) and cytochrome *c* (curve 3) oxidized at 0.7 V on the gold surface.

quent electron-transfer at the heme group, but not for the disulfide. However, the situation would be quite different on gold electrodes, and since compounds containing disulfide have strong interactions with the gold surface thus would denature the cytochrome *c*. In addition, the unfavorable orientation of the heme for electron-transfer as a result of adsorption would make the voltammetric peaks irreversible and undetectable. These may explain why we were unable to obtain redox peaks for the disulfide using other electrodes. However, small redox peaks were observed using the platinum electrode, since it is a slightly less acidic metal than gold [18] (Fig. 3).

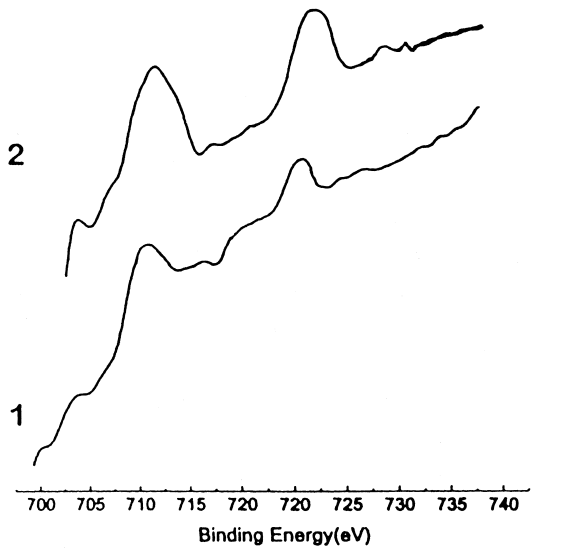



Fig. 7. XPS spectra of $Fe_{2p}$ $^{3/2}$ for cytochrome *c* at 0.3 V (curve 1) and at 0.7 V (curve 2) on the gold surface.

Of all the methods used to investigate the redox properties of metalloproteins, electrochemical methods seem to provide the most valuable and direct evidence. However, adsorption of proteins and slow electron transfer at the electrodes have raised many difficulties for effective use of these techniques. It has been observed that the electrochemical response of cytochrome *c* could be destroyed by aggregation of cytochrome *c* molecules [2,3]. In addition, we have proposed another possible explanation in involving disulfide electrochemistry.

## 4. Conclusions

Disulfide in cytochrome *c* can be strongly adsorbed on the gold surface, causing denaturation and slow electron transfer with the heme group.

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