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Electrochemical behaviour of cytochrome *c* at electrically heated microelectrodes

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Abstract

The structural changes in cytochrome *c* with temperature have been been followed using a recently developed electrically-heated microelectrode sensor. Differential pulse voltammetry was used to perform electrochemical measurements of cytochrome *c* oxidation at different temperatures at heated bare gold electrodes contained in phosphate-buffered cytochrome *c* solution at room temperature. The voltammetric response shows the onset of unfolding and a marked dependence of the signal on electrode temperature. This augurs well for applications of heated electrodes as local probes in the study of the temperature dependence of electron transfer processes of other redox proteins, avoiding problems of bulk deterioration.

Keywords: Electrochemistry of cytochrome *c*; Hot-wire electrodes; Protein unfolding

1. Introduction

The metalloprotein cytochrome *c*, a small ($M_r = 13000$) relatively abundant electron-transferring protein which occurs in all animals, plants and aerobic microorganisms, has been the object of a large number of electrochemical studies, following the demonstration of its voltammetric response corresponding to the process

$$\text{cyt.Fe(II)}c \rightarrow \text{cyt.Fe(III)}c + e^-$$

at tin-doped indium oxide electrodes [1] and at gold electrodes modified with 4,4′-bipyridyl [2] at around zero volts versus SCE. It was assumed for many years that the function of the modifier was to increase the rate of the electron-transfer and permit an observable response, usually obtained through cyclic voltammetry. This led to much research activity for developing the most effective promoter. However, in the 1980s, there were reports that electrochemistry could be observed at unmodified electrodes [3,4]. This has been more recently confirmed [5–7]. Two important aspects have come out of this work. First, that the prior surface treatment of the electrode is extremely important and secondly the importance of the identity of the electrolyte solution, and even its concentration. In [3] a tris/cacodylate buffer was employed, in [5,6] phosphate buffer and in [7]

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potassium chloride, which led to some differences in response. In all cases, substantial blocking of the electrode surface occurs, which can be explained by a self-blocking mechanism, and which reduces the electrode response to close to zero very quickly.

Consequently, an alternative way to approach the problem of obtaining measurable signals due to oxidation of cytochrome *c* would be thought to be to increase the temperature. This has to be done carefully since the rate of protein adsorption will also be increased, so that the time window during which measurements can be performed before blocking will also be reduced. More important than this, unfolding and denaturing of the protein will begin to occur above a certain temperature. The way in which this influences the voltammetric response depends on the stereochemistry of interaction and the conformation necessary for electron transfer to take place. Some investigations on this theme have been undertaken [8–14]. In particular, it has been stated that conformation and effective charge can be influenced by the temperature [8–10]; it has also been found that deterioration of cytochrome *c* is faster the higher the temperature [6]. Nevertheless, the influence of temperature on the formal potential has been shown to be small [8]. Adsorption and redox thermodynamics have been investigated using electrochemical methods [11–13] and protein folding triggered by electron transfer using photochemical electron injection [14].

In fact, the biological activity of most protein molecules occurs only over a very limited range of temperature and pH. Exposing soluble or globular proteins to high temperatures for even short periods causes them to undergo the physical change known as denaturation, the unfolding of the characteristic native folded structure of the polypeptide chain of globular protein molecules. When thermal agitation causes the native folded structure to uncoil or unwind into a randomly looped chain, with freedom of rotation about bonds in both the polypeptide backbone and the side chains, the protein loses its biological activity. Since no covalent bonds in the backbone of the polypeptide chain are broken during this relatively mild treatment, the primary structure remains intact. Most globular proteins undergo denaturation when heated above 40–70°C, depending on the pH of the medium. To follow the change with temperature as the transition between the native and unfolded conformation occurs would be of interest.

An experimental strategy which should permit such observations at higher temperatures whilst minimising the problems of bulk deterioration, and not only for cytochrome *c*, is that of heating the electrode rather than the solution, producing a localised increase in temperature whilst the bulk solution remains at ambient temperature. Such an electrically-heated electrochemical sensor has been recently developed using Joule heating at wire electrodes [15,16]. In the set-up most commonly used, a 25 μm wire is heated using a 100 kHz alternating current. Alternatively, thin metallic layers on an inert substrate can be heated. In this paper, a gold layer on a ceramic chip was used. It was prepared by a combination of screen printing and a special technology known in microelectronics as the LTCC (Low Temperature Cofired Ceramics) procedure (Du Pont®).

In this technique, a thin heated layer near to the electrode surface is formed. The temperature of this layer is well-known and well controlled; it can be varied arbitrarily over a wide range by adjusting the heating current amplitude. The electrode surface temperature in such experiments was measured in different ways. One way was to follow the open circuit potential variations of a well investigated redox couple, i.e. ferricyanide/ferrocyanide. The temperature coefficient of this couple is well known and allows the calculation of the temperature changes of the electrode during heating or cooling periods. Temperature gradients spread into solution in a highly reproducible way that can be precisely calculated numerically. After some heating time, density gradients cause convection that leads to a steady state with constant surface temperature and linear temperature profile in a layer of constant thickness. This state is fully established after maximum 0.2 s and forms the basis of all experiments described here. In other types of experiment, short heating pulses are applied in the period before the effect of convection becomes perceptible. This way, temperature values above the boiling point are accessible.

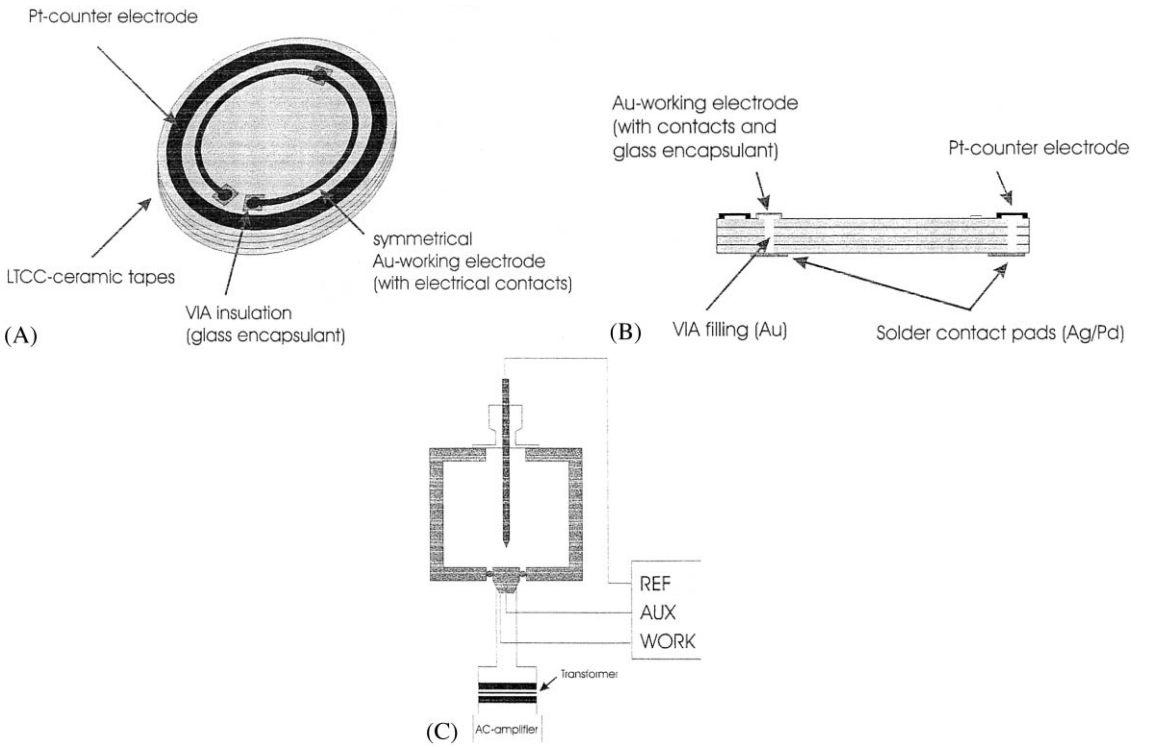

Fig. 1. Electrically-heated microelectrodes: (A) design of the electrically-heated gold sensor; (B) cross-section of the hot-layer-sensor; (C) schematic view of the cell.

The objective of this paper is to demonstrate that these localised electrode heating techniques can be employed to probe the changes in structure, denaturation and unfolding processes, occurring with biological molecules through application to cytochrome *c* at bare gold electrodes. Differential pulse voltammetry has been employed and the results show a marked dependence of the signal on temperature.

2. Experimental

Cytochrome *c* (horse-heart), was obtained from Sigma (St Louis, MO) and was used as received. Phosphate buffer solutions of ionic strength 0.2 at pH 7.0 were used in all experiments and were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (resistivity ≥ 18 MΩ cm). All solutions were at room temperature during experiments.

Experiments were carried out with an electrochemical sensor for hot-layer-electrochemistry. This sensor was constructed at the University of Rostock, Germany. Working and counter electrodes were incorporated directly on the sensor chip, see Fig. 1A, B. The working electrode, which can be electrically heated, was a gold-layer circular arc-shaped electrode, 4.4 mm diameter and 130 μm width, and the counter electrode was a platinum-layer circular ring concentric with the working electrode. The reference electrode used was a saturated calomel electrode (Radiometer). A schematic view of the apparatus is shown in Fig. 1C. Electrodes were tested at different temperatures in blank phosphate buffer electrolyte solution prior to use in cytochrome *c* containing buffer electrolyte.

Voltammograms were recorded using a Autolab potentiostat/galvanostat (PGSTAT20) running with GPES version 3 software, from Eco-Chemie, Utrecht, Netherlands. Differential pulse voltam-

metry conditions were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s^{-1}.

3. Results and discussion

The difficulty of obtaining a voltammetric response for cytochrome *c*, that is the oxidation of Fe(II) to Fe(III) complexed in an iron protoporphyrin (heme) group at ~ 0.05 V versus SCE, was already alluded to above. Cytochrome *c* is the only common heme protein in which the heme is bound to the protein by a covalent linkage. In the cytochrome *c* three-dimensional structure, the hydrophobic aminoacids cluster about the heme on the inside of the molecule and the hydrophilic residues tend to lie on the surface of the molecule. It is therefore not surprising, due to the conformation of the molecule, that direct electron transfer at a bare electrode surface is difficult; in principle, it can only occur through approximately 0.6% of the protein surface where the edge of the heme is exposed.

One of the important factors in obtaining a response is the initial state of the electrode surface. In this work, preparation of the electrode surface was done by polishing before each experiment with diamond suspensions of decreasing particle size (6, 3 and 1 μm), i.e. without the elaborate cleaning procedures mentioned in some publications, e.g. [7] involving cycling in sulphuric acid, dipping in concentrated nitric acid and ethanol, polishing and sonicating. Additionally, most of the publications on cytochrome *c* use cyclic voltammetry to probe the oxidation process; however, with the heated electrodes, after preliminary cyclic voltammetry experiments, differential pulse voltammetry was used which has a higher sensitivity than cyclic voltammetry and was found to permit easier observation of the voltammetric peaks.

In Fig. 2 the differential pulse response is shown at ambient temperature and at a raised temperature of 75°C. Whereas in the former case there is no signal, in the latter there is a large peak of 20 nA, corresponding to a current density of 1.9 μA cm^{-2}. The peak half-width can be estimated as ~ 90 mV which implies reversible electron-transfer kinetics under such conditions [17]. The electrically-heated gold film electrode appears to probe the protein at the electrode surface through measuring the current corresponding to iron (II) oxidation; this transfer occurs more easily from the unfolded cytochrome-*c* molecule.

Further evidence that the signal is due to changes in structure is given in Fig. 3, where the effect of temperature on the signal at a number of different temperatures is shown. Between each of these recordings, the electrode surface was freshly polished in the way described above. A signal at ~ 0.05V versus SCE only begins to appear above 37°C and the current increases until 78°C, the maximum temperature employed. Small peaks can be attributed to impurities in the cytochrome *c* reagent, which was used as received. Future work using recommmended purification methods, e.g. [18], is planned.

The change in current with temperature observed parallels that of the change of formal potential with temperature in [8,12]. There tends to be a biphasic behaviour: a linear variation of formal potential up to a temperature between 30 and 50°C (depending on the type of cytochrome *c*), then another linear variation of different slope at higher temperatures. The explanation for this has been the progressive opening of the heme

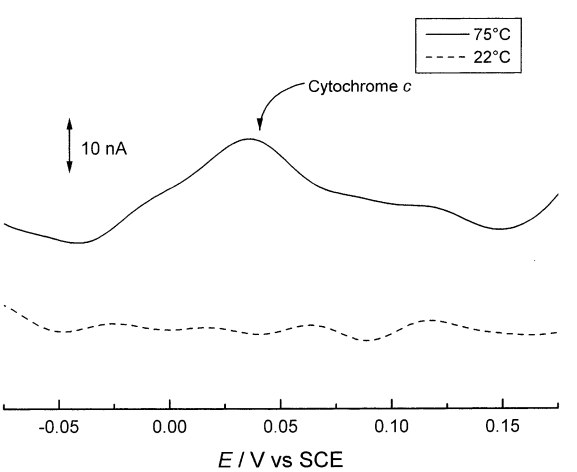

Fig. 2. Differential pulse voltammograms of 0.2 mM cytochrome *c*. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1} Supporting electrolyte 0.2 M phosphate buffer pH 7.0.

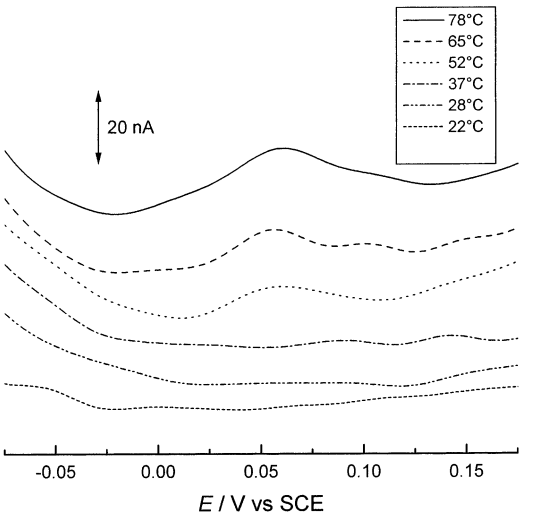

Fig. 3. Differential pulse voltammograms of 0.2 mM cytochrome *c* at temperatures 22, 28, 37, 52, 65 and 78°C. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1} Supporting electrolyte 0.2 M phosphate buffer pH 6.8. Electrode cleaned between scans.

crevice with increasing temperature, stabilising the oxidised form and making the formal potential more negative [12]; at the break temperature there is a conformational transition.

It can be noticed that the peak potentials in Fig. 2 and Fig. 3 differ. Whilst a full explanation for this difference will require further work, it is probably due to different adsorption orientations of cytochrome-*c* on the electrode surface, thus altering the energy required for electron transfer. The degree of opening of the heme crevice will be influential in this regard. Indeed, some scatter in the formal potential values obtained has always been noted, e.g. [8].

The high temperature, which leads to opening and unfolding apparently permits the electron transfer to take place more easily. Fig. 4 shows a plot of peak current with the data from Fig. 3 as a function of working electrode temperature. First, it can be seen that the current begins to increase from zero at a temperature of around 40°C and continues increasing. This is the transition temperature mentioned above. Other evidence of the importance of this temperature comes from calorimetric [19] and heat capacity [20] studies of cytochrome *c*. In [19] the free energy difference between denatured and native states of cytochrome *c* was deduced from calorimetric measurements; the relative stability of the folded state begins to decline from 33 kJ mol^{-1} just before 40°C and crosses zero at $\sim$80°C. The heat capacity change upon protein unfolding begins to decline more rapidly above 40°C [20].

The importance of adsorption on the gold surface is demonstrated in Fig. 5. This shows that without convenient treatment and cleaning of the electrode surface prior to each experiment, even if a signal can be registered at 37°C, at higher temperatures no peak is recorded without cleaning the surface. This surface blocking is an important process which has to be taken into account.

Previous electrochemical studies at gold electrodes have focussed on adsorption of cytochrome *c*, the conformation of the adsorbed species and electrode blocking. Additionally, it has also been found that, although it is very difficult to obtain any response at bare electrodes without the extremely elaborate surface preparation procedures indicated above, this is not necessary if the electrolyte concentration is sufficiently low. In fact, the effective charge of the cytochrome *c* molecule is high and it has a large dipole ($\sim$300 Debye), so that strong interactions with electrolyte ions can be expected. In the particular case of phosphate buffer, however, it was found [7], by varying the pH, that any interaction which does occur is non-specific with regard to $H_2PO_4^-$ and HPO_4^{2-}. In principle, the electron transfer results from adsorbed molecules so long as they have the correct conformation. Given the high charge, there is also the possibility that the molecules join to form oligomeric species, either in solution (at high concentration) or on the electrode surface. The latter would make the parts of the electrode covered by the oligomers inert to electrochemical activity and constitutes a self-blocking mechanism. Direct evidence that this, or some equivalent process, occurs was obtained by us. After prolonged experimentation at high temperature the gold electrode was covered with a polymer-like substance and the voltammetric response disappeared.

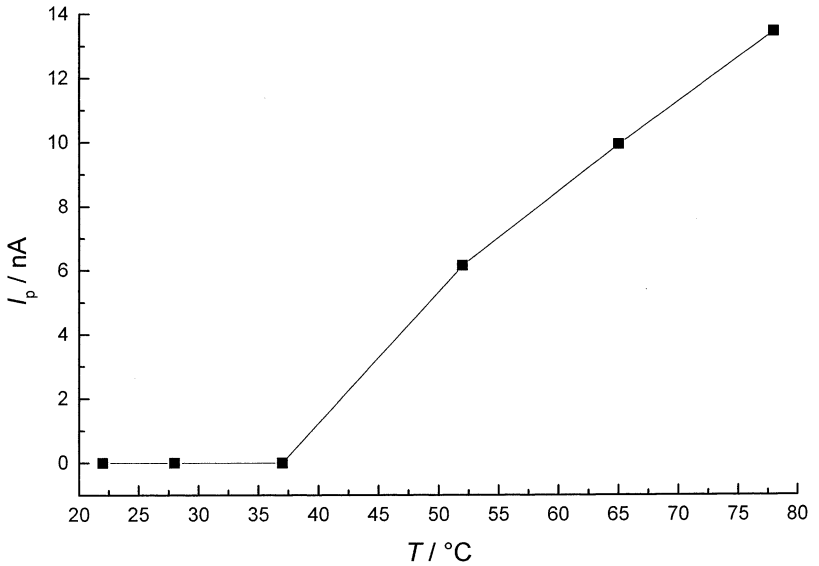

Fig. 4. Variation of cytochrome *c* oxidation peak current with sensor temperature from data in Fig. 3.

There is some evidence of the beginning of a levelling off of the response in Fig. 4. The zero-crossing point in [18] of 80°C suggests that complete unfolding of all molecules will not yet have happened. A maximum peak current corresponding to all molecules being unfolded at the electrode surface would occur if higher temperatures could be reached. This is not viable with a constantly-heated sensor owing to the solution boiling point being reached and would have to be done by pulsed heating. This is an objective of future work. Nevertheless, the results obtained have demonstrated the potentialities of the heated-electrode approach.

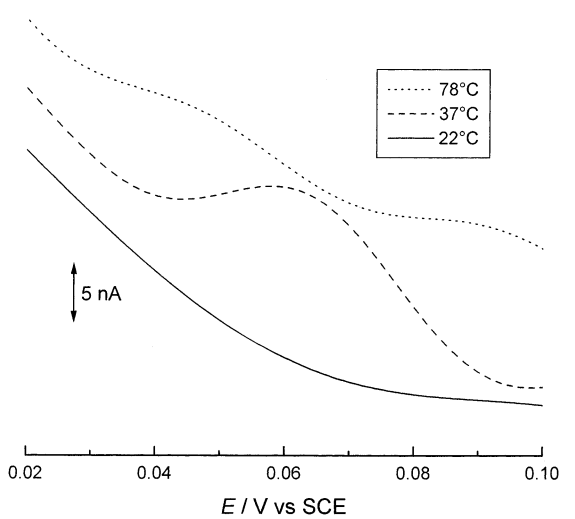

Fig. 5. Successive differential pulse voltammograms of 0.2 mM cytochrome *c* at temperatures 22, 37 and 78°C. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1} Supporting electrolyte 0.2 M phosphate buffer pH 7.0.

4. Conclusions

An electrically-heated electrochemical sensor, in combination with differential pulse voltammetry, has been shown to be extremely useful for probing the conformational changes and unfolding process of cytochrome *c* with increased temperature at bare gold electrodes. The merit of the technique is that it enables high temperatures to be used at the electrode whilst the protein is at ambient temperature in bulk solution. Further work will address more detailed aspects of this transition and employ pulsed heating techniques to reach higher temperatures. Applications to other redox proteins can be anticipated.

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