Direct electrochemical identification of an activated intermediate formed by cytochrome C with hydrogen peroxide

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An activated intermediate formed from H_2O_2 and cytochrome C is identified by direct electrochemical measurements.

Hydrogen peroxide (H_2O_2) is a normal byproduct of mitochondrial and cellular metabolism[1] and is toxic to cells at elevated concentrations.[2] Vandewalle *et al.*[3] indicated that H_2O_2 formed directly or as a product of superoxide dismutation can oxidize ferrocyt C at rates comparable to those at which ferricyt C is reduced by superoxide. Furthermore, Xu[4] suggested that an 'electron leak' perhaps exists on the cyt C side, in which the electron that transfers in the respiratory chain may leak out from cyt C to H_2O_2 resulting from the ubiquinone side[5] (H_2O_2 is reduced to H_2O). However, Turrens *et al.*[6] demonstrated that the build up in concentration of H_2O_2 during most reactions in which superoxide anion is being produced is not enought to affect the rate of ferricyt C reduction. Thus, for the interaction of cyt C with H_2O_2, many challenging problems remain obscure. Due to its location on the surface of the inner mitochondrial membrane, cyt C is considered to be a typical extrinsic membrane protein.[7] In a series of elegant experiments, Hill and coworkers[8] have demonstrated that electron transfer of cyt C can be achieved using suitably modified gold electrodes. Most recently, a variety of self-assembled alkanethiol monolayers (SAMs) on gold electrodes with a net negative charge have been prepared for the study of the direct electrochemistry of adsorbed or bound cyt C at the modified electrode.[9–11]

We have investigated the interaction of bound cyt C (horse heart) with H_2O_2, and as a first step in such studies, found that the interaction can be observed using direct electrochemical techniques.

The gold electrode was modified by immersion in a 4 mmol dm^{-3} aqueous solution of 3-sulfanylpropionic acid (SPA) (Aldrich) for 24 h, and was then washed with distilled water. Horse heart cyt C (Type IV, Sigma) was coupling with SPA immobilized at the modified electrode using the water-soluble condensing reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma). The SPA modified gold electrode was placed in a solution of 0.6 g dm^{-3} cyt C with 1 g dm^{-3} EDC in 5 mmol dm^{-3} sodium phosphate buffer (pH 6.4) at 4 °C for 24 h. The electrode was finally washed exhaustively with 5 mmol dm^{-3} sodium phosphate buffer (pH 6.4). As a result, cyt C was bound covalently to a self-assembled SPA monolayer on the gold electrode, which was represented as a bound cyt C/SPA/Au system. Electrochemical measurements were performed at 26 °C in a glass microelectrochemical cell equipped with a reference Ag/AgCl electrode (saturated KCl), a platinum plate as the counter electrode and a 1 mm diameter gold disc as the working electrode.

Both Figs. 1(*a*) and 2(*a*) show typical DC cyclic voltammogram (CV) and AC capacitive–potential (C–E) curves at 100 Hz for the bound cyt C/SPA/Au system in 5 mmol dm^{-3} sodium phosphate buffer (pH 6.4). The anodic and cathodic peaks observed at 0.14 V and 0.09 V, respectively, are well defined, corresponding to the formal potential $E^{\circ\prime}$ of 0.115 V (*i.e.* 0.31 V *vs.* normal hydrogen electrode, NHE). There is evidence to show that the redox peaks are related to the bound cyt C molecules. The redox peaks did not appear when an SPA/Au electrode was used in the blank buffer, as shown in Fig. 1(*c*). This value (0.31 V *vs.* NHE) was 30 mV more positive than that of cyt C in solution (our result of 0.280 V *vs.* NHE) at the SPA/Au electrode, and 24 mV more positive than that of cyt C in solution (0.286 V *vs.* NHE) at the 4*S*,4*S*′-bipyridine modified gold electrode.[12] Our observation of a positive shift of 24 mV is less than the positive shift of *ca.* 74 mV (between 0.286[12] and 0.36 V *vs.* NHE[13]) which has been observed for cyt C covalently attached to an activated glass carbon electrode.[13] The results of Song *et al.*[10] showed that when cyt C was adsorbed on a HS–$(CH_2)_n$–CO_2H monolayer/Au electrode the redox potential of cyt C shifted cathodically by −45 mV from its solution value, and the peak splitting approached zero at n = 5 and 10. In our experiment, the thinner HS–$(CH_2)_2$–CO_2H film was used, and its carboxy groups were activated by using the water-soluble condensing reagent EDC. The bound contact of the protein molecule with the thinner CO_2H-terminated monolayer/Au electrode surface may cause some changes in its conformation and increase the energy of the conformational transition from the oxidized to the reduced form.[13] This phenomenon results in a shift of the redox potential up to 0.31 V *vs.* NHE. The activated carboxy groups on the glassy carbon electrode lead to a more positive shift of the redox potential for the bound cyt C up to 0.36 V *vs.* NHE.[13] Our CV responses are stable, persisting in a more or less unperturbed form for hours under experimental conditions, and the electrochemical behaviour is observed to be essentially reversible even at scan rates of 1 V s^{-1}. This system displays diffusionless[10] electron transfer. The C–E curve [Fig. 2(*a*)] shows that the cyt C molecules remain in the bound state over the whole potential range studied.

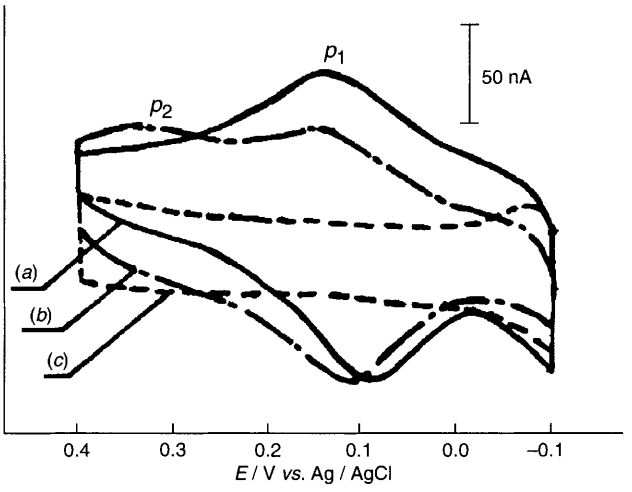

Fig. 1 DC cyclic voltammograms of (*a*) the bound cyt C/3-sulfanylpropionic acid (SPA)/Au system in 5 mmol dm^{-3} sodium phosphate buffer (pH 6.4), (*b*) the bound cyt C/SPA/Au system in the above buffer containing 6.7 μmol dm^{-3} H_2O_2 and (*c*) the SPA/Au system in the blank buffer. Scan rate: 50 mV s^{-1}.

When the potential is kept at −0.1 V and 6.7 μmol dm^{-3} H_2O_2 is added to the bulk solution, we observed that the presence of H_2O_2 alters the CV and C–E curve of the bound cyt C/SPA/Au system, as shown in Figs. 1(*b*) and 2(*b*). The anodic peak p_1 at 0.14 V is obviously decreased, and a new anodic peak p_2 appears at 0.34 V. When the concentration of H_2O_2 is changed from 1.3 to 25 μmol dm^{-3}, the peak p_1 is gradually decreased, and the peak p_2 is slightly increased. The cathodic peak potential is altered from 0.09 to 0.11 V, but the change of peak current is much less than that of the anodic peak. When the concentration of H_2O_2 is beyond 25 μmol dm^{-3}, there are no clearly identifiable CV peaks, but the C–E curve is still effective for identifying the peak p_2 as shown in Fig. 2(*c*). Meanwhile, a new peak p_3 can be observed at −0.05 V with the disappearance of the peak p_1. In order to stress the main points on the peak p_2, we do not discuss the peak p_3 in this paper (peak p_3 may be related to a bleaching reaction of cyt C in higher concentrations of H_2O_2).

Because the cathodic peak in Fig. (1*b*) does not exhibit electrocatalytical behaviour,[14] it can be ruled out that the ferrocyt C may be chemically oxidized to ferricyt C by H_2O_2. Turrens *et al.*[6] showed that the oxidation of ferrocyt C in solution caused by H_2O_2 will be insignificant at concentrations of H_2O_2 below 0.1 mmol dm^{-3}, due to an oxidation rate of *ca.* 0.01% min^{-1} per μmol dm^{-3} H_2O_2. In addition, peak p_1 obviously corresponds to the electrochemical oxidation of the bound cyt C, while peak p_2 is related to effect of H_2O_2 added. If the peak p_2 resulted from the electrochemical oxidation of H_2O_2 and its derivatives, it would not be reasonable that the anodic peak p_1 of cyt C decreased and the cathodic peak of cyt C was not obviously changed. If the SPA/Au electrode is put in a solution the same as that in Fig. 1(*b*), no peak p_2 appears. This indicates the cyt C bound on SPA/Au is necesssary to show peak p_2. Combining the facts of an effectively constant cathodic peak by CV, necessary bound cyt C and the effect of H_2O_2, it may be suggested that an activated intermediate is formed from H_2O_2 and the bound cyt C. Peak p_2 is primarily attributed to the activated intermediate. The activated intermediate is not very stable, and it may be destabilized with potentials sweeping towards negative. Thus, the cathodic peak in the CV is essentially constant. When the bound cyt C/SPA/Au system mentioned above is washed with phosphate buffer and is then placed in 5 mmol dm^{-3} sodium phosphate buffer, the same CV and C–E curve as that in Figs. 1(*a*) and 2(*a*) is again observed. This shows that the intermediate can not be electrochemically observed unless the bulk solution contains a fixed concentration of H_2O_2 (1.3 to 25 μmol dm^{-3}). For the adsorbed cyt C/SPA/Au system (cyt C is immobilized on SPA/Au by physical adsorption), peak p_2 is not observed for buffer solutions containing H_2O_2. The peak p_2 is also not observed at the SPA/Au electrode if cyt C is added to the solution. In both these cases, only a decrease of the poor redox peaks of cyt C can be observed. However, on a successful 4*S*,4*S*′-bipyridine/Au electrode[15] for cyt C electrochemistry, we can observe the peak p_2 in a solution containing cyt C. This suggests that better electrochemical responses are necessary for observation of the peak p_2.

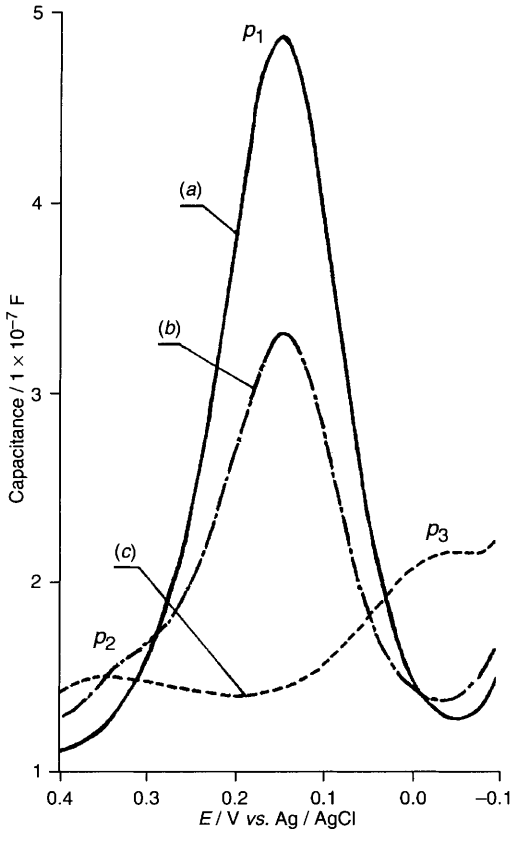

Fig 2 AC capacitance–potential (C–E) curves for the bound cyt C/SPA/Au system, with (*a*) and (*b*) the same as in Fig. 1, and (*c*) in a buffer containing beyond 25 μmol dm^{-3} H_2O_2. Frequency used = 100 Hz.

The hydroxylation of 4-nitrophenol by cyt C plus H_2O_2 has been reported by Florence.[16] By using this method[16] for hydroxylation of 4-nitrophenol to 4-nitrocatechol, we had observed that formation of the ferryl species is in good agreement with the inhibitiory effects of uric acid on substrate hydroxylation since uric acid is an excellent scavenger of the oxo-heme species.[17,18] The 4-nitrophenol hydroxylation had also been investigated by using the DC CV method. The results showed that peak p_2 in Figs. 1 and 2 may be attributed to formation of the ferryl species due to the inhibitory effects of uric acid on the peak p_2. The cathodic peak in Fig. 1(*b*) may be formed by the reduction of ferricyt C (producing from peak p_1) and ferrylcyt C (producing from peak p_2).

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