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948 — ELECTROCHEMICAL BEHAVIOUR OF CYTOCHROME *c* AT LOW POTENTIALS *

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SUMMARY

d.c. polarograms of bovine heart cytochrome *c* show reduction currents at low potentials. This is observed in buffer solutions with pH values between 1 and 10.5. These currents are attributed to catalytic hydrogen formation (pre-sodium currents). After succinylation of the protein, the current in glycine–NaOH buffer of pH 10.5 disappears almost completely, whereas that in acetate buffer of pH 4.5 is affected only slightly. It is concluded that different groups are responsible for the currents observed in these two buffers.

INTRODUCTION

The polarographic behaviour of beef-heart cytochrome *c* has been investigated extensively [1–3]. The reduction of the ferriheme prosthetic group ($U_0' = +0.26$ V (*versus* s.h.e.), for native protein) usually occurs with an overpotential, the magnitude of which depends on the concentration of cytochrome *c* and on the medium.

Betso *et al.* [1] observed, in addition to the heme reduction wave, a wave with $U_{1/2} = -1.38$ V (s.h.e.) in a citrate buffer of pH 2.5. The limiting current of this second wave was much larger than that of the heme reduction wave.

The present work is aimed at elucidating the process and groups responsible for the wave at low potentials.

EXPERIMENTAL

A P.A.R. 174A polarographic analyzer was used. The capillary characteristics, at open circuit, were $m = 1.996$ mg s^{-1} and $t = 4.96$ s. The reference electrode was an

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s.c.e. Potentials are given against the s.h.e. The cell was thermostatically controlled at 25 °C. The buffers were flushed for 13 min with argon; then a concentrated solution of cytochrome *c* was added, after which flushing was continued for another 2 min.

Beef-heart cytochrome *c* (Sigma Type VI) was purified further by cation-exchange chromatography on Amberlite CG 50 [4], followed by gel filtration on Sephadex G25. Succinylation of the protein was performed by treatment with succinic anhydride, as described in Ref. 5, followed by gel filtration on Sephadex G25.

RESULTS AND DISCUSSION

Figure 1 shows *d.c.* polarograms of cytochrome *c* at low potentials. Cytochrome *c*, when dissolved in 0.1 *M* glycine–NaOH buffer of pH 10.5 (Fig. 1A), produces a large current before the point where the discharge of the buffer alone occurs. No limiting current is obtained. Most probably, the current is due to catalytic hydrogen formation (cf. Refs. 6, 7).

When cytochrome *c* is dissolved in 0.1 *M* sodium acetate buffer of pH 4.5 (Fig. 1B), a current is also observed before the discharge of the buffer. In this case, however, a limiting current is obtained. After correction for the residual current, the limiting current is about thirty times higher than expected on the basis of the Ilkovic equation, assuming $n = 1$ and a diffusion coefficient of 1.1×10^{-6} cm^2 s^{-1} for native cytochrome *c* [8]. (At more extreme pH values the diffusion coefficient could be lower due to unfolding of the apoprotein.) A similar high wave was obtained in citrate buffer of pH 2.5, in agreement with the results of Betso *et al.* [1]. At pH 1, in 0.1 *M* HCl, not a plateau, but a peak is found in the *d.c.* polarogram at −1.34 V.

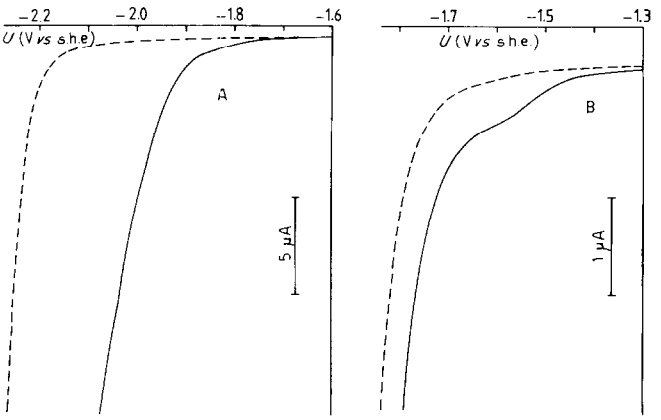

Fig. 1. *d.c.* polarograms of solutions without (— — —) and with (———) 12.1 μ*M* cytochrome *c*. (A) In 0.1 *M* glycine-NaOH, pH 10.5; (B) in 0.1 *M* sodium acetate, pH 4.5.

Betso *et al.* [1] suggested that the wave at low potential might be due to a multi-electron reduction of some amino-acid moiety. However, amino acids are not reducible at the d.m.e. with the exception of the disulfide bridge of cystine [9,10]. As the latter is not present in cytochrome *c*, we investigated if the porphyrin group might be reducible. The porphyrin group of ferrocytochrome *c* is known to be reducible by hydrated electrons [11]. The product obtained, in that case, undergoes a rapid intramolecular redox reaction by which a thiol group is formed:

$$\text{Fe(II)porph}^{\cdot}\text{–S–protein} \xrightarrow{k = 10^5\ \text{s}^{-1}} \text{Fe(III)porph} + \text{HS–protein} \qquad (1)$$

To investigate whether thiol groups are also formed at a mercury cathode, a controlled-potential electrolysis was performed. Ferricytochrome *c* was dissolved in a medium containing 0.1 *M* potassium phosphate buffer (pH 7.0) and 0.1 *M* KCl. Electrolysis was performed with a mercury pool as cathode, held at either −1.80 V, which is well on the polarographic plateau in this medium, or at −2.04 V. After exhaustive electrolysis at −1.80 V, the absorption spectrum in the visible range was practically that of normal ferrocytochrome *c*, whereas by electrolysis at −2.04 V a product was formed with an absorption peak at 610 nm in the reduced form. The latter product could be oxidized reversibly by ferricyanide and reduced by ascorbate. However, thiol groups were not detected after either of these electrolyses, using a spectrophotometric assay with *p*-chloromercuribenzoate [11]. Apparently, the course taken by the reaction at a mercury electrode at −2.04 V differs from that due to the one-electron reduction by hydrated electrons.

As to the polarographic wave found in media of low pH, several lines of evidence suggest that it may also be due to catalytic hydrogen formation. Firstly, as mentioned, the current is much higher than expected for a diffusion-controlled reaction. Secondly, the current is not proportional to the protein concentration, but saturation sets in above 5–10 μM (Fig. 2B, see also Ref. 1). Thirdly, at high protein concentrations, the current was found to be independent of the height of the mercury column.

The attribution of the wave of low pH values to catalytic hydrogen formation is in contradiction with a rather generally held view, that this catalysis by proteins can be observed only in buffers above neutral pH, but becomes masked by hydrogen evolution in buffers below neutral pH values [6,12,13].

Betso *et al.* [1] found the wave in a buffer of pH 2.5, but not in a cacodylate buffer of pH 6.0. In phosphate buffer of the same pH value, however, a wave can clearly be observed.

Several studies have been aimed at identifying the protein groups responsible for the catalytic currents that occur in buffers above neutral pH [7,14]. On the basis of the effects of chemical modification of groups with ketene [14], formaldehyde [7,14] and monoiodoacetate [7], it has been concluded that mainly nitrogenous groups are responsible. However, these reagents are rather unspecific. It is known that succinylation of proteins, when performed in aqueous medium, specifically modifies primary and secondary amino groups [5]. The imidazole ring of the histidine residue does not become modified by this treatment.

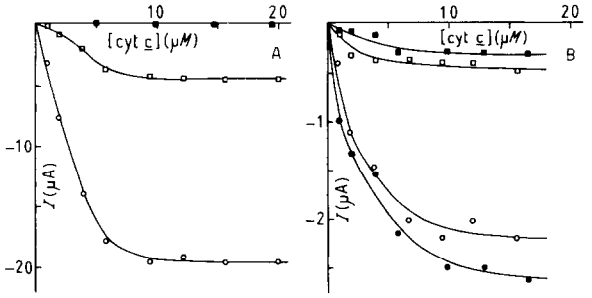

Fig. 2. The effect of succinylation of cytochrome *c* on currents at low fixed potential. The currents are corrected for the residual currents of the buffer solution. (A) In 0.1 *M* glycine–NaOH, pH 10.5. (○———○) Cyt. *c*, *I* at −2.09 V; (●———●) succinylated cyt *c*, *I* at −2.09 V; (□———□) cyt *c*, *I* at −1.965 V, (■———■) succinylated cyt *c*, *I* at −1.965 V. (B) In 0.1 *M* sodium acetate, pH 4.5. (○———○) Cyt *c*, *I* at −1.79 V; (●———●) succinylated cyt *c*, *I* at −1.79 V; (□———□) cyt *c*, *I* at −1.59 V; (■———■) succinylated cyt *c*, *I* at −1.59 V.

Figure 2A shows that the catalytic currents of cytochrome *c*, as measured at two arbitrary potentials, in a buffer of pH 10.5, disappear almost completely after succinylation. It is, therefore, concluded that these currents are almost exclusively due to amino groups.

In contrast, the currents at pH 4.5 are affected much less by succinylation (Fig. 2B). Therefore, these currents at low pH value must be due largely to groups which remain unaffected by this treatment. Responsible groups might then be the imidazole group of histidine residues or the guanidino group of arginine residues. Both groups are protonable nitrogenous groups. It is also possible that the heme group is, somehow, involved. This group has been found to be involved in catalytic hydrogen formation by cytochrome *c* in the presence of cobalt salts [15].

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