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STUDY OF CYTOCHROMES c_3 FROM *DESULFOVIBRIO VULGARIS* (HILDENBOROUGH) AND *DESULFOVIBRIO DESULFURICANS* (NORWAY) BY DIFFERENTIAL PULSE POLAROGRAPHY AND SPECTROELECTROCHEMICAL METHOD

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Summary

(1) Cytochromes c_3 from *Desulfovibrio vulgaris* (Hildenborough strain) and *Desulfovibrio desulfuricans* (Norway strain) have been studied by differential pulse polarography and spectroelectrochemical method. Both cytochromes exhibit two reduction potential values $E_h \approx -0.25$ and -0.34 V (*D. vulgaris*), $E_h \approx -0.16$ and -0.34 V (*D. desulfuricans*).

(2) Titrations by dithionite and controlled potential electrolysis coupled with polarography and spectrophotometry suggest that in both cases electronic exchanges are rapid.

Introduction

Polarographic techniques applied to biopolymers [1—4] may yield useful data on the transport of electroactive species to and from an electrode surface as a way of approach the mechanism of electron transfer.

A number of electrochemical studies on hemoproteins, and in particular on mammalian cytochrome *c*, have been recently reported [5,6] and attention has been payed to electrode interaction phenomena [7]. Though adsorption of proteins has a great influence, no inhibition is caused. It has even been shown by cyclic voltammetry that cytochrome c_3 from *Desulfovibrio vulgaris* (Miyazaki strain) [8] should exchange electrons rapidly.

Cytochromes c_3 are a class of hemoproteins containing four hemes per molecule and having negative redox potentials at pH 7 [9]. A few values of potentials have been measured using redox titrations by strong reducers such as dithionite: for *D. vulgaris* (Hildenborough strain) cytochrome c_3, potentials of about -0.21 V (vs. normal hydrogen electrode) have been initially reported

[10,11]. By NMR reduction-titration it has been indicated that this protein should exist in at least three redox states [12]. Recently, four different mid-point potentials of the hemes have been determined in the range —0.284 to —0.324 V (vs. normal hydrogen electrode) by EPR spectroscopy coupled with potentiometry (Der Vartanian, D.V., Xavier, A.V. and Le Gall, J., unpublished).

It has been reported [13] that spectral properties of *Desulfovibrio desulfuricans* (Norway strain) cytochrome c_3 are very similar to those of the other cytochromes c_3 of *Desulfovibiro* species. After preliminary experiments, it is very likely that this cytochrome should have also a very low redox potential.

The present paper reports a study of the cytochrome c_3 from *D. vulgaris* (Hildenborough strain) and *D. desulfuricans* (Norway strain) using chiefly differential pulse polarography and electrolysis at controlled potential in connection with spectrophotometry, with the view of a better knowledge of the electron transfer in these biological systems.

Experimental

Materials. Cytochromes c_3 from *D. vulgaris* (Hildenborough) and *D. desulfuricans* (Norway) were prepared and purified in the Laboratoire de Chimie Bactérienne du C.N.R.S., Marseille [9,13] and were supplied freshly prepared either lyophilized or in 0.01 M Tris-HCl buffer (pH 7.6). All experiments were performed in this medium which served also as supporting electrolyte.

For purity controls, the purity coefficient defined as (A_{553} [red.] $- A_{570}$ [red.])/A_{280} [ox.] [13] was determinated before and after each experiment. Its initial value of 3.1 was unchanged after polarographic analysis.

Concentrations of solutions were calculated after measuring A_{553} [red.]: $\epsilon = 159\,500\ M^{-1} \cdot cm^{-1}$ for *D. vulgaris* cytochrome c_3 and $\epsilon = 130\,000\ M^{-1} \cdot cm^{-1}$ for *D. desulfuricans* cytochrome c_3 (Le Gall, J., Personal communication).

Methods. Polarograms were recorded on a Sefram XY recorder coupled with a PAR 174 A polarographic analyzer equipped with an M 174/10 drop timer. The dropping mercury electrode was used as an indicator electrode. The counter-electrode was a platinum one, the reference electrode was a Metrohm silver-silver chloride (saturated NaCl solution) electrode (symbolized by Ag/AgCl).

Differential pulse polarograms were made at 23°C, at a scan rate of 2 mV/s using a pulse amplitude of 50 mV, a drop time of 0.5 s and a mercury flow of about 1 mg/s. The peak currents i_p were measured after correction of the blank relative to the supporting electrolyte. Oxygen was purged from sample solutions (5 ml) by bubbling nitrogen for 30 min before recording.

For preparative electrolysis, the cell consisted of a stirred mercury pool working electrode of about 7 cm^2, a dip-type Ag/AgCl reference electrode and a second Ag/AgCl electrode used as an auxiliary. Buffer solutions (5 ml) were first deaerated with nitrogen for 25 min, then preelectrolyzed at —0.800 V (vs. Ag/AgCl) for about 2 h under nitrogen. A known volume of cytochrome solution was added to the cell and the electrolysis was performed for 5—6 h at selected potentials (—0.400 and —0.800 V vs. Ag/AgCl). At the end of the electrolysis, aliquots were removed under anaerobic conditions and spectra were immediately recorded. Control polarograms were made after each electrolysis.

In order to check that the observed peaks (or waves) were indeed due to cytochromes, the absence of inorganic impurities was investigated after mineralisation of samples as previously described [14].

Optical adsorption spectra were recorded on a Cary 14 spectrophotometer with HELLMA quartz cells of 1—10 mm path length.

Results

Differential pulse polarograms of *D. vulgaris* and *D. desulfuricans* cytochromes c_3 are shown in Figs. 1 and 2 at various concentrations. In the same figures are also given direct current polarograms relative to the most concentrated solutions.

In both cases, three peaks can be observed but some differences appear: for *D. desulfuricans* cytochrome, the most positive peak at about $E_p \approx -0.08$ V (vs. Ag/AgCl) is not well defined, whereas the peaks at $E_p \approx -0.33$ and -0.51 V are well formed and separated; for *D. vulgaris* cytochrome, the most positive peak at about $E_p \approx -0.17$ V is better defined, but the two other peaks at $E_p \approx -0.42$ and -0.51 V, which are well separated up to a concentration of

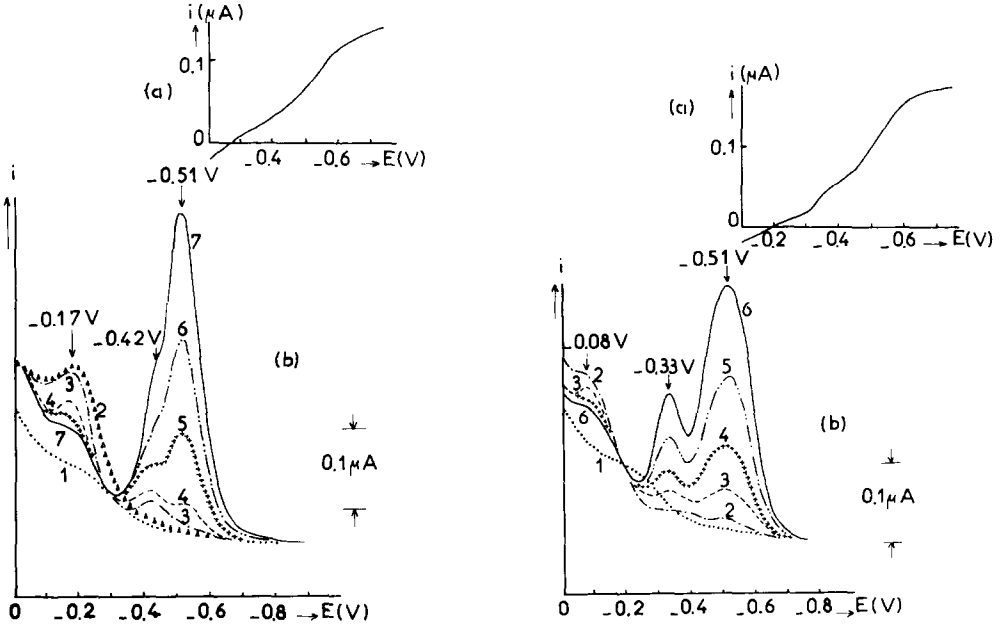

Fig. 1. (a) Direct current polarogram of *D. vulgaris* cytochrome c_3 (c = 23.1 μM) in 0.01 M Tris-HCl (pH 7.6). (b) Differential pulse polarograms of *D. vulgaris* cytochrome c_3 at various concentrations in 0.01 M Tris-HCl (pH 7.6). 1, supporting electrolyte; 2, 3.7 μM; 3, 6.5 μM; 4, 8.6 μM; 5, 12.5 μM; 6, 16.6 μM; 7, 23.1 μM; Pulse amplitude, 50 mV; drop time, 0.5 s; scan rate, 2 mV/s; reference electrode, Ag/AgCl (satd. NaCl).

Fig. 2. (a) Direct current polarogram of *D. desulfuricans* cytochrome c_3 (c = 33.6 μM) in 0.01 M Tris-HCl (pH 7.6). (b) Differential pulse polarograms of *D. desulfuricans* cytochrome c_3 at various concentrations in 0.01 M Tris-HCl (pH 7.6). 1, supporting electrolyte; 2, 9.4 μM; 3, 13.2 μM; 4, 17.8 μM; 5, 24.7 μM; 6, 33.6 μM. Pulse amplitude, 50 mV; drop time, 0.5 s; scan rate, 2 mV/s; reference electrode, Ag/AgCl (satd. NaCl).

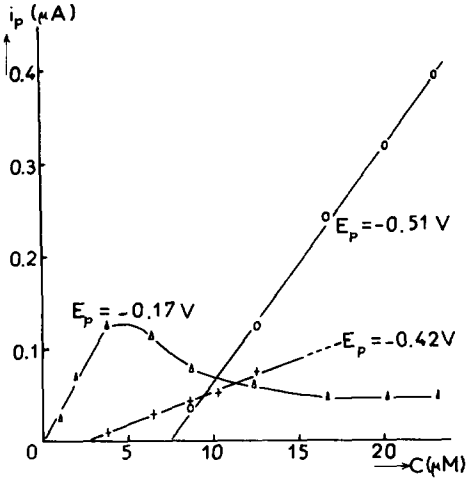

Fig. 3. Plot of peaks currents (i_p) versus concentration of *D. vulgaris* cytochrome c_3.

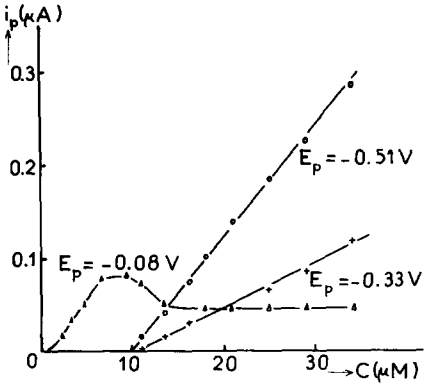

Fig. 4. Plot of peaks currents (i_p) versus concentration of *D. desulfuricans* cytochrome c_3.

approx. 13 μM tend to be confused by increasing concentration. It should be noticed that direct current polarograms do not supply so many informations as differential pulse polarograms: the waves corresponding to the most positive peaks are not observed and only one wave appears on the direct current polarogram of the *D. vulgaris* cytochrome c_3.

Dependences of the peak heights i_p on concentration are given in Figs. 3 and 4. For both cytochromes, the height of the most positive peak is found to increase linearly with the concentration until a limit is reached at approximately 4 μM (*D. vulgaris*) and 7 μM (*D. desulfuricans*).

Above these concentrations heights remain constant, then decrease slightly, and above $c \approx 13$ μM remain approximately constant in the range of concentrations investigated. This behaviour denotes the existence of adsorption phenomena, which is also suggested by the lowering of the electrocapillary curve in the presence of cytochrome c_3, as shown by complementary experiments. The second and third peaks appear simultaneously above a concentration of about

TABLE I

VALUES OF PEAK POTENTIALS E_p AND HALF-WAVE POTENTIALS E_h

D. vulgaris cytochrome c_3		*D. desulfuricans* cytochrome c_3	
E_p (V) (vs. Ag/AgCl)	E_h (V) *	E_p (V) (vs. Ag/AgCl)	E_h (V) *
−0.17 ± 0.02	0.00	−0.08 ± 0.02	+0.09
−0.42 ± 0.005	−0.25	−0.33 ± 0.005	−0.16
−0.51 ± 0.005	−0.34	−0.51 ± 0.005	−0.34

* The application of the Parry and Osteryoung relationship [15] $E_p = E_{1/2} - \Delta E/2$ relating the peak potential E_p to the half-wave potential $E_{1/2}$ for a given amplitude ΔE and the change of reference [16] lead to half-wave potentials E_h (vs. normal hydrogen electrode).

10 μM for the *D. desulfuricans* cytochrome c_3 and separately above 3 and 7 μM for the *D. vulgaris* cytochrome c_3.

The heights vary linearly with the bulk protein concentration. E_p values are independent of concentration for each peak in the range studied here. In Table I, E_p values (vs. Ag/AgCl) are given with corresponding E_h values (vs. normal hydrogen electrode).

In spite of the somewhat poor height of the direct current polarographic waves, their diffusion-controlled nature appears to be demonstrated by plotting the logarithm of the limiting current vs. the logarithm of the mercury column height which gives slopes near 0.5. Moreover, additional peaks at very negative potentials (—1.2 to —1.5 V) are observed, with intensities i_p considerably higher than those reported above. Since these phenomena, probably relative to the catalytic reduction of hydrogen [17], are of peripheral interest to the understanding of the electron transfer properties of cytochromes, no more detailed experiments were performed to elucidate the corresponding mechanism.

Study of the rapidity of electronic transfer

The question of rate of the electrode process is of great importance for the interpretation of the polarographic data. The classical criterion in direct current polarography based on the replacement of a cathodic wave by an anodic one of same half-wave potential after reduction [18] is difficult to use in the present case on account of the bad definition of diffusion current as compared to residual current. Other techniques such as cyclic voltammetry may be used [8], but in the present investigation, other ways have been carried out.

(a) Titration by dithionite. If the electrode reaction is reversible, no variation of the peak location should be observed, since a cathodic or anodic wave of the same half-wave potential corresponds in the same way to a similar peak. When an excess of dithionite is present, the peak corresponding to dithionite oxidation begins to appear ($E_p \approx -0.42$ V vs. Ag/AgCl) and increases with increasing dithionite concentration.

(b) Controlled potential electrolysis. If the electrode reaction is reversible, the same result should be obtained before and after electrolysis, as previously. In addition, this technique could offer the advantage of obtaining the reduced form with no need of a foreign reducing agent such as dithionite.

In the case of the cytochromes c_3 studied in the present paper, it has been found that, by adding aliquots of dithionite, no variation of the peaks is observed until the appearance of the peak corresponding to an excess dithionite. Controlled potential electrolysis with a stirring mercury-pool electrode lead to the same result.

Electrolysis combined with spectrophotometry

Electrolysis combined with spectrophotometry was used for both cytochromes c_3 at a controlled potential of —0.800 V (vs. Ag/AgCl). With *D. desulfuricans* cytochrome c_3, an additional electrolysis was performed at —0.400 V, a value which corresponds to the plateau of the first direct current polarographic wave. Samples were removed for spectrophotometric analysis before and after each electrolysis. The observal spectral changes relative to *D. desulfuricans* cytochrome c_3 before and after electrolysis are shown in Fig. 5:

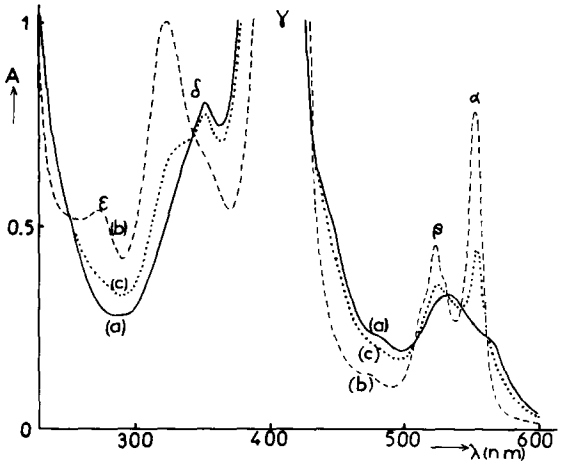

Fig. 5. Absorption spectra of *D. desulfuricans* cytochrome c_3 (c = 36.0 μM) in 0.01 M Tris-HCl (pH 7.6). (a), before electrolysis; (b), after electrolysis at —0.800 V for 6 h; (c), after electrolysis at —0.400 V for 8 h.

(a) before electrolysis, (b) after electrolysis at —0.800 V (77% of reduced form) and (c) after electrolysis at —0.400 V (25% of reduced form).

These spectra are quite similar to those obtained by Le Gall et al. [19] after reduction of cytochrome c_3 by sodium borohydride (which has no absorption band in the wavelength range) and show the presence of ϵ and δ bands (277—325 nm). These bands disappear on reoxidation. It is thus suggested that the reduced form is prepared during electrolysis, but it is possible that proteins undergo a partial degradation as indicated by a slight lowering of the purity coefficient (3.0) after electrolysis.

Ageing of the solution

Differential pulse polarograms and adsorption spectra of *D. vulgaris* cytochrome c_3 have been recorded as a function of time. No change has been found either in polarograms or in purity coefficient with a 12 μM solution kept at room temperature for 3 weeks, which is in agreement with the great stability of this cytochrome, as previously noticed [20]. With *D. desulfuricans* cytochrome c_3 small shifts of the E_p potential and of the purity coefficent values are observed after 3 or 4 days.

Discussion

The electrolysis results suggest that the electrode reactions observed in the range of potentials 0 to —0.6 V (vs. Ag/AgCl) correspond to the reduction of the prosthetic group. Moreover, the occurrence of adsorption phenomena on the mercury surface is also indicated: (a) the electrocapillary curve in presence of cytochrome is lower than the solvent curve; (b) the most positive peaks (at approx. —0.08 and —0.17 V) observed with both cytochromes have the characteristics of an adsorption 'pre-peak', the height of which increases up to a limit value; (c) the slight lowering of this limit value, which remains approxi-

mately constant in a large range of concentration above $c \approx 13$ μM could be explained by a modification of the adsorbed layer. Nonetheless, the overall electrode reaction results in the conversion of diffusing oxidized to reduced form. This study confirms that the reduction proceeds in at least two steps separated by about 0.09 V (*D. vulgaris*) and 0.18 V (*D. desulfuricans*).

The values of potentials E_h = —0.25 V and —0.34 V obtained for *D. vulgaris* cytochrome c_3 (Table I) are not far from potentials (—0.284, —0.310, —0.319, —0.324 V) obtained by Der Vartanian, Xavier and Le Gall (unpublished) in spite of differences between experimental conditions. Differential pulse polarography is unable to separate too close peaks, which explains that only two values are obtained by this technique. The two observed peaks should result from an overlapping of the individual peaks relative to each heme, and so it is rather difficult to quantitate n-values for each reduction step.

The question of why there are differences in potentials for the hemes of cytochromes c_3 studied in the present paper may correlate with the biological importance of oxidation-reduction intermediates, as reported in other works [9,12,21]. It is suggested that the reduction level of the hemes is very dependent on the biological reduction in which cytochromes c_3 are involved.

An important conclusion from this study is the likely rapidity of the electrochemical systems, as reported previously in the case of the protoporphyrin IX by Bednarski and Jordan [22] and very recently by Niki et al. [8] for cytochrome c_3 from *D. vulgaris* (Miyazaki strain). By comparison with a monohemic protein such as cytochrome *c* (a slow redox system) the fast electronic transfer in cytochromes c_3 may be due to the multiple heme system of this type of protein which intensifies electronic exchange. Another reason may be that the hemes are relatively exposed at the surface of the protein [23]. A knowledge of structures would be very useful to provide more information on the structure-function relationship and mechanism of electron transfer. It should explain also the role of each heme and the electrochemical behaviour of both cytochromes c_3 studied in the present paper.

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