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Kinetic studies of the electron exchange reaction between the octaheme cytochrome c_3 (Mr 26000) and the hydrogenase from *Desulfovibrio desulfuricans* Norway

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The octaheme cytochrome c_3 (Mr 26000) from *Desulfovibrio desulfuricans* Norway was studied using cyclic voltammetry at the pyrolytic graphite electrode. The kinetics of reduction of the octaheme cytochrome c_3 (Mr 26000) from *D. desulfuricans* Norway by the Ni-Fe-Se hydrogenase purified from the same organism was investigated by an electrochemical method. From cyclic voltammetry experiments a value of $8.10^8 M^{-1}s^{-1}$ was obtained for the second order homogenous rate constant of the electron transfer between the two proteins. Results are compared with similar experiments performed on the electron exchange between the tetrahemic cytochrome c_3 (Mr 13000) and hydrogenase.

Cytochrome c_3 is a periplasmic electron carrier protein which holds a key position in the electron transport chain regulating the metabolism of the anaerobic sulfate reducing bacteria belonging to the genus *Desulfovibrio* . The electron transfer proteins from *Desulfovibrio* appear to be very diverse and contain at least four different c type cytochromes, the monohemic cytochrome c_{553} (Mr 9000)(1), the tetrahemic cytochrome c_3 (Mr 13000)(2), the octahemic cytochrome c_3 (Mr 26000) (3-5) and a high molecular weight cytochrome c (Mr 65000) called Hmc containing sixteen hemes (6). A common pattern of the cytochrome c_3 superfamily is that they contain c-type hemes having low redox potentials within the approximate range -120 to -400 mV (7) . Histidine residues are both the fifth and sixth heme iron axial ligands. Both primary and tertiary structure of cytochrome c_3 (Mr 13000) have been determined (2,8,9).
Desulfovibrio desulfuricans Norway cytochrome c_3 (Mr 26000) has been described as a dimer of a tetraheme cytochrome c_3 different from cytochrome c_3

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(Mr 13000) isolated from the same organism (4,10). Removing the hemes from this cytochrome gives evidence, after SDS polyacrylamide gel electrophoresis, that it is a dimer consisting of two identical subunits of Mr 13500. Crystals of the homologous octaheme *D. gigas* cytochrome c_3 (Mr 26000) have been obtained and X-ray structure is in progress (11).
Tetraheme and octaheme cytochromes c_3 are redox partners for *Desulfovibrio* hydrogenases, which are enzymes that catalyse the reversible activation of the simplest molecule: molecular hydrogen (12). Three classes of hydrogenases have been described namely Fe-hydrogenases, Ni-Fe-hydrogenases and Ni-Fe-Se-hydrogenases (13). *D. desulfuricans* Norway hydrogenase is a Ni-Fe-Se-hydrogenase of Mr 88000 consisting of two distinct subunits of Mr 26000 and 62000 (14).

In previous works, the electrochemical approach was used to study the interactions between *D. desulfuricans* Norway (15) or *D. gigas* (16) cytochrome c_3 (Mr 13000) and the hydrogenase isolated from the same organism respectively, and to determine the second order rate constants of electron transfer for both systems. In order to compare the electron transfer mechanisms between hydrogenase and two of its redox partners cytochrome c_3 (Mr 13000) and cytochrome c_3 (Mr 26000) (17) we have undertaken the kinetic study of the reduction of cytochrome c_3 through coupling to the Ni-Fe-Se hydrogenase, both isolated from *D. desulfuricans* Norway under H_2 atmosphere.

MATERIALS AND METHODS

Cytochrome c_3 (Mr 26000) and hydrogenase were extracted from *D. desulfuricans* Norway strain and purified as previously described (4,14). All chemicals were of reagent grade.

Experiments were performed in Tris-HCl buffer at pH 7.6 which also served as the supporting electrolyte. Oxygen was purged from the solutions by bubbling with U-grade nitrogen before the experiments. When studying the interaction between cytochrome and hydrogenase, U-grade hydrogen was used and served as the substrate of the enzymatic reaction.

Cyclic voltammetry experiments were performed with a three-electrode cell, using a basal-plane pyrolytic graphite electrode (Le Carbone Lorraine, Paris) as the working electrode. Prior each experiment, the carbon electrode was polished using 0.05 μm alumina slurry. The reference electrode was a Metrohm silver-silver chloride (saturated NaCl) electrode but all potentials are given vs the normal hydrogen electrode (NHE).

Cyclic voltammograms were obtained by using an EG&G PAR 273 potentiostat modulated by an IBM XT microcomputer with an EG&G PAR M270 software and coupled to a Sefram X-Y chart recorder. Experiments were performed at 23 $\pm$1°C.

RESULTS AND DISCUSSION

Effect of the ionic strength on the electrochemistry of cytochrome c_3 (Mr 26000)

Cyclic voltammograms relative to a 3 μM cytochrome c_3 (Mr 26000) solution at increasing Tris-HCl concentration are shown in Fig. 1. In 10 mM Tris-HCl buffer, virtually no response is detected except one cathodic peak at approx. -0.10 V. This peak which does not disappear neither after prolonged nitrogen bubbling nor repetitive scanning is ascribed to the reduction of residual oxygen present at the graphite electrode surface (see under). When the Tris-HCl concentration rises to 50 mM, the height of the cathodic peak strongly decreases and a pair of peaks (Ic-Ia) is detected at E_{pIc} -0.35 V, E_{pIa} -0.25 V. This couple of peaks corresponds to the reduction-reoxidation of cytochrome c_3 (Mr 26000). When the Tris-HCl concentration is increased up to 500 mM, an improvement in the electrochemical response is observed without any contribution of the cathodic peak corresponding to the oxygen reduction. In fact, it appears that peaks (Ic) and (Ia) are composite and result from the overlapping of close redox processes in accord with our previous results obtained at the mercury electrode (redox potential values in 500 mM Tris-HCl buffer for the four pairs of hemes : - 0.210, - 0.270, -0.325, -0.365 V [5]).

Such a result can be explained by the existence of an electrostatic repulsion between the graphite electrode and the protein molecules. The graphite surface is negatively charged and cytochrome c_3 (Mr 26000) which has a low isoelectric point (4.8, see [5]) possesses also an excess of negative charges. By increasing the Tris-HCl concentration, positive charges progressively accumulate at the electrode/solution interface and contribute to diminish the repulsive effect which impedes the approach of protein molecules towards the electrode surface. This interpretation is consistent with the fact that enhanced currents for peaks (Ic) and (Ia) are also observed by adding electrolytes as sodium or baryum chloride to the 10 mM Tris-HCl solution. Such a phenomenon (i.e. cyclic voltammograms virtually unchanged in the 10-500 mM Tris-HCl concentration range) does not occur for cytochrome c_3 (Mr 13000) which possesses a higher isoelectric point

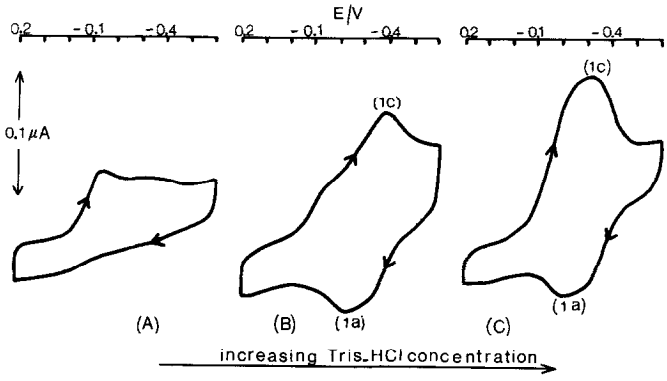

Fig.1. Cyclic voltammograms at the pyrolytic graphite electrode of 3 μM *D. desulfuricans* Norway cytochrome c_3 (Mr 26000).(A) 10 mM, (B) 50 mM, (C) 500 mM Tris-HCl buffer (pH 7.6).Scan rate: 5 mV/s.

(7.0, see [5]), but was observed previously for other negatively charged proteins [18].

The peak at -0.10 V in Fig. 1A which is absent on the curve relative to the supporting electrolyte is attributed to a catalytic reaction since it is known that the heme can catalyze the reduction of oxygen [19] . The disappearance of the peak at -0.10 V in Fig. 1A, when the ionic strength is increased, could result from the reduction of the residual oxygen by reduced cytochrome c_3 (Mr 26000) electrochemically formed.

Kinetic study of the cytochrome c_3 (Mr 26000) reduction by hydrogenase

In the presence of substrate (here hydrogen) the cytochrome enzymatically reduced by hydrogenase from the same strain is electrochemically re-oxidated at the graphite electrode and an enhanced anodic current Δi is detected as shown in Fig. 2A by recording the current-time curve at E = 0.2 V. As in a previous work on cytochrome c_3 (Mr 13000) [15], cyclic voltammetry was used to measure the homogeneous rate constant of the reaction between cytochrome c_3 (Mr 26000) and hydrogenase. Quantitative data were obtained according to the method of Nicholson and Shain [20] for analysing the following catalytic reactions :

$$1/2\ H_2 + Hase_{ox} \longrightarrow H^+ + Hase_{red}$$

$$Hase_{red} + Cyt\ c_{3ox} \xrightarrow{k} Hase_{ox} + Cyt\ c_{3red}$$

$$Cyt\ c_{3red} \rightleftharpoons Cyt\ c_{3ox} + 8\ e^-$$

(where hydrogenase is abbreviated as Hase).

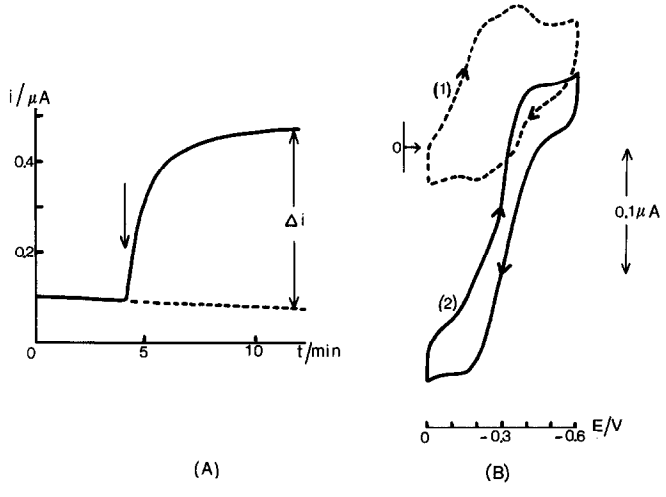

Fig.2. (A) Current-time curve at the controlled-potential value of 0.2 V relative to 4 μM cytochrome c_3 (Mr 26000) + 100 nM hydrogenase in 50 mM Tris-HCl buffer at pH 7.6 : (1) under nitrogen, (2) under hydrogen atmosphere. The arrow indicates the exchange of gas.
(B) Cyclic voltammograms at the pyrolytic graphite electrode of 3 μM *D. desulfuricans* Norway cytochrome c_3 (Mr 26000) in 100 mM Tris-HCl (pH 7.6), (1) without hydrogenase, (2) after the addition of 13.6 nM *D. desulfuricans* Norway hydrogenase. Scan rate: 5 mV/s. The cross indicates the point of zero intensity.

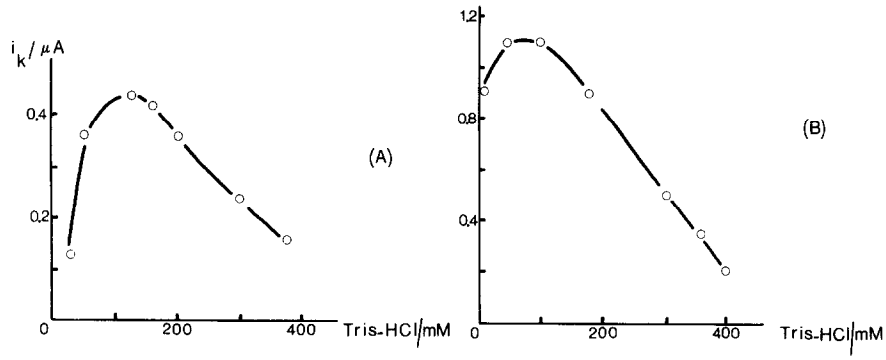

Fig.3. Dependence of the catalytic current i_k on the concentration of Tris-HCl buffer (pH 7.6).
(A) Concentration of cytochrome c_3 (Mr 26000): 4.7 μM, concentration of hydrogenase: 0.2 μM.
(B) Concentration of cytochrome c_3 (Mr 13 000) : 10 μM, concentration of hydrogenase : 0.2 μM.

In Fig. 2B, curve (1) is relative to the cytochrome c_3 (Mr 26000) solution without hydrogenase, curve (2) is obtained after the addition of hydrogenase and an anodic catalytic current i_k is measured. The dependence of i_k on the Tris-HCl concentration is given in Figs. 3A and 3B for cytochrome c_3 (Mr 26000) and (Mr 13000) respectively. A maximum value is observed around 100 mM for both cytochromes. The low catalytic currents observed at lower Tris-HCl concentrations, though it was more marked in the case of cytochrome c_3 (Mr 26000) is also developed in the case of cytochrome c_3 (Mr 13000). It can be assumed that a precursor complex formation occurs, followed by a rapid intramolecular electron exchange. Hazzard et al (21) have proposed that at low ionic strength the complex could represent a species which is not structurally optimized for electron transfer. Thus such an unproductive complex can dissociate or rearrange to form an "active" complex at increasing Tris-HCl concentrations. Moreover, in the case of cytochrome c_3 (Mr 26000) it has been shown in the foregoing experiments that the protein becomes electrochemically inactive at the lowest Tris-HCl concentrations and cannot transfer electrons between hydrogenase and the electrode.

When the Tris-HCl concentration is increased (above 100 mM, the cytochrome-hydrogenase association necessary for a productive electron-exchange is less and less favored, decreasing values of the catalytic current are observed.

The second order homogeneous rate constant k was determined in 100 mM Tris-HCl which corresponds to maximum i_k values. The value of (8 ± 3). $10^8 M^{-1}s^{-1}$ obtained for the cytochrome c_3 (Mr 26000)-hydrogenase system compares advantageously with the value of (6 ± 3) $10^7 M^{-1}s^{-1}$ measured for the cytochrome c_3 (Mr 13000) hydrogenase system in the same medium (15).

The foregoing results would indicate that the ionic strenght has a determinant role especially in the reactivity of cytochrome c_3 (Mr 26000) either at the electrode or with hydrogenase. In both cases, it is pertinent to take into account the marked acidity of cytochrome c_3 (Mr 26000). Considering that *D.*

desulfuricans Norway cytochrome c_3 (Mr 26000) and hydrogenase are quite acidic proteins (pI 4.8 and 6.0 respectively) a protein-protein electrostatic recognition process would necessitate either very localized charged interacting sites or an important role of intermediate ions. So further structural studies of the hydrogenase-cytochrome c_3 (Mr 26000) complex is of highly importance for the understanding of the electron exchange mechanism.

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