Direct electron transfer between a chemically, viologen-modified glassy-carbon electrode and ferredoxins from spinach and *Megasphaera elsdenii*

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1. INTRODUCTION

Direct reversible electron transfer between an electrode and ferredoxins has been described by several authors [1–8]. Recently, we have shown that at the dropping mercury electrode the efficiency of reduction of both 4Fe–4FS and 2Fe–2S ferredoxins can be enhanced drastically [8]. This increase in reduction efficiency was obtained by addition of a positively charged polymer or surfactant to the negatively charged ferredoxins, indicating that electrostatic effects play an important, if not a predominating role. Authors in [5,6] have reported on the reduction and oxidation of spinach ferredoxin at a gold electrode on which a polymeric form of methyl viologen was adsorbed. Authors in [7] have described the direct reversible electrochemical reduction of *Clostridium pasteurianum* ferredoxin at a pyrrolytic graphite electrode in the presence of bivalent metal ions. They suggested that these ions promote electron transfer by forming a bridge between the negatively charged ferredoxin and carboxylate groups on the graphite surface. They also reported [9]on the oxidation of this ferredoxin; bivalent metal ions not being obligatory, because the graphite electrode is positively charged at the potential area of interest. The results in [7,9] confirm the importance of electrostatic interactions.

For this reason we have bound covalently, by means of a peptide linkage, a monolayer of a viologen at the surface of a glassy-carbon electrode. Due to this modification, the surface of the electrode is always more positively charged than an unmodified electrode. Furthermore viologens are artificial electron donors/acceptors for ferredoxins.

Abbreviations: DAPV; *N,N'*-di(γ-aminopropyl)dipyridinium bromide hydrobromide; MAPV, *N*-mono-(γ-aminopropyl)dipyridinium bromide hydrobromide; i_{pc}, i_{pa}, cathodic, anodic peak current, respectively; $E_{1/2}$, half-wave potential; k_s, standard heterogeneous rate constant

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2. MATERIALS AND METHODS

Spinach ferredoxin [8] and *M. elsdenii* ferredoxin [10] were purified according to published methods. Concentrations were determined at 420 nm ($E_{420} = 9.7\ mM^{-1} \cdot cm^{-1}$) and 390 nm ($E_{390} = 30$

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$mM^{-1} \cdot cm^{-1}$) respectively. The purity indices of these ferredoxins were 0.43 (A(420/280)) and 0.80 (A(390/280)) respectively. After anaerobic dialysis against 25 mM Tris (pH 7.0) the ferredoxins were stored in liquid nitrogen.

2.1. *Synthesis*

DAPV and MPV were synthesized as in [11]. The first half-wave potentials of DAPV and MAPV are at -600 and -960 mV, respectively *vs* SCE.

2.2. *Electrochemistry*

Cyclic linear sweep voltammetry was performed with a Wenking Potentioscan POS 73 from Bruker Elektronik, connected to a Kipp BD-30 XY recorder using a conventional 3-electrode system. The working electrode consisted of a (chemically modified) glassy-carbon electrode (Tokai Electrode, Tokio), 0.6 mm in diameter, sealed in a glass tube. The reference (SCE) and counter (platinum) electrodes were placed in two separate arms with electrical conductivity to the main compartment (0.5 ml total volume) via a Luggin-Haber capillary and an agar salt bridge, respectively. This latter was done to avoid obligatory oxygen evolution at the counter electrode in the main compartment. The oxygen evolved could damage iron-sulfur clusters, especially those of *M. elsdenii* ferredoxin. The electrochemical measurements were performed as in [8], however, no glucose–glucose oxidase was used as oxygen scavenging system. All potentials are given *vs* SCE ($+240$ mV *vs* NHE). The temperature was 20 ± 2°C. If not stated otherwise the buffer used was 50 mM Bis-tris (pH 7.0) plus 100 mM NaCl.

2.3. *Chemical modification of electrode surfaces*

The glassy carbon electrodes were polished on cotton wool using an alumina/water slurry with decreasing particle size of the alumina. After polishing the electrodes were heated in a blue flame for about 10 s to oxidize the electrode surface. Next the electrodes were refluxed for 2 h in a 40:60 (v/v) mixture of a hydrogen peroxide solution (30%) and acetic acid to complete the oxidation of the surface carbon-oxide groups into carboxylic groups. After intensive washing with water the carboxylic groups were activated with 10 mM *N*-cyclohexyl-*N'*-[β-(*N*-methylmorpholino)ethyl]carbodiimide *p*-toluolsulfonate at pH 4.5 for 2 h. After washing the electrodes with water, DAPV (10 mM) or MAPV (10 mM) were allowed to react with the activated electrode surface at pH 7.0 for another 2 h. Due to this procedure we expect that a monolayer of DAPV or MAPV is bound to the electrode surface.

After being carefully washed the electrodes were stored in 20 mM phosphate buffer (pH 7.0). Prior to use the electrodes were scanned anaerobically between -250 and -900 mV for the DAPV modified electrode and between -600 and -1200 mV for the MAPV-modified electrode at 100 $mV \cdot s^{-1}$ until the cyclovoltammograms were constant. During the first few scans the electrochemical response decreased after which it becomes stable. This indicates that in addition to binding part of the viologens are adsorbed. This latter fraction goes into solution upon scanning.

3. RESULTS AND DISCUSSION

In fig.1 cyclic voltammograms of the DAPV-modified electrode are shown in the absence of ferredoxin. In accordance with the theory [12] both the cathodic peak current, i_{pc} (see inset,fig.1) and the anodic peak current i_{pa} are proportional to the scan rate. Values for i_{pc} and i_{pa} are obtained from the cyclic voltammograms, corrected for the background currents. The amount of DAPV bound to the electrode surface was calculated from the plot of i_{pc} *vs* scan rate, substituting the geometrical surface area of the electrode. The surface concentration of bound DAPV was on average $(1.2 \pm 0.3) \times 10^{-10}$ $mol \cdot cm^{-2}$. Furthermore, for the DAPV-modified electrode a peak separation $\Delta E_p \sim 20$ mV was observed, independent of the scan rate up to 0.5 $V \cdot s^{-1}$. The half-wave potential, $E_{½}$, of the bound DAPV was -635 mV. A shift of -35 mV as compared to $E_{½}$ of DAPV in solution may be due to mutual interactions, as described in [13], or to the chemical binding of the viologen at the electrode surface.

In fig.2 cyclic voltammograms of spinach ferredoxin at the DAPV-modified electrode are shown. The half-wave potential of spinach ferredoxin at this modified electrode is -640 mV, in accordance with results in [8]. At the scan rates tested, up to 0.5 $V \cdot s^{-1}$ we found that ΔE_p was 75 mV. However, for a diffusion-controlled reversi-

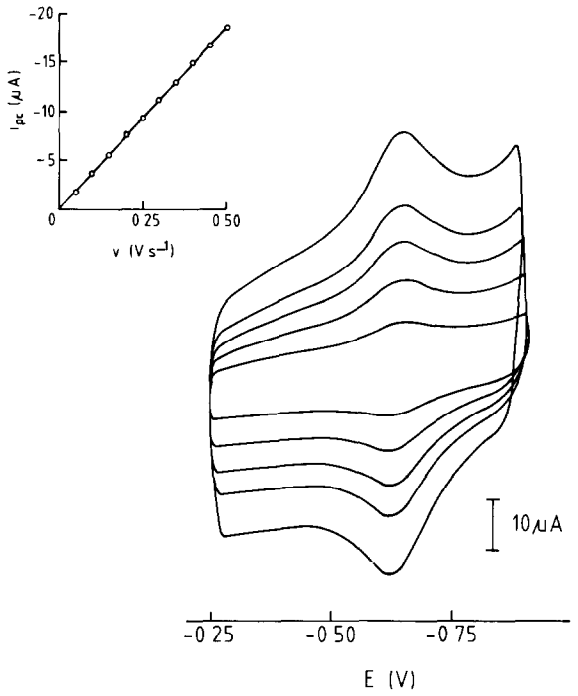

Fig. 1. Cyclic voltammograms of a DAPV-modified glassy-carbon electrode. Scan rates; 0.05, 0.10, 0.15, 0.20 and 0.30 $V \cdot s^{-1}$.

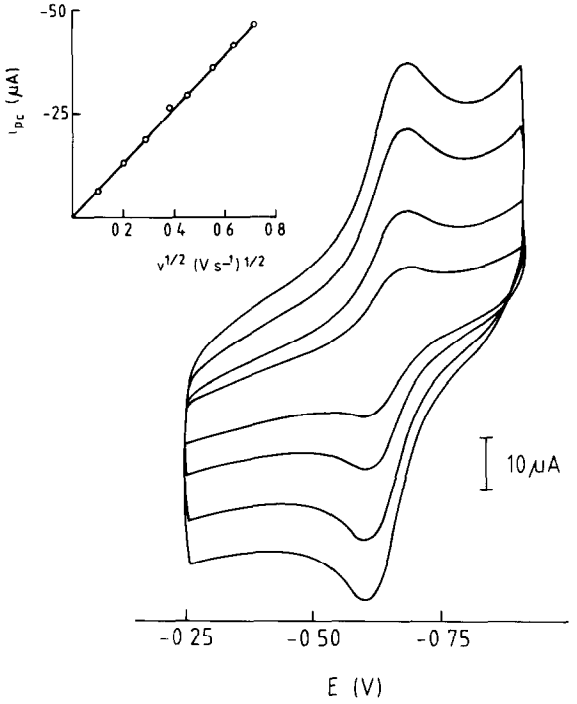

Fig. 2. Cyclic voltammograms of spinach ferrodoxin at a DAPV-modified glassy-carbon electrode. The concentration of spinach ferrodoxin was 0.80 mM. Scan rates: 0.02, 0.05, 0.10 and 0.15 $V \cdot ^{-1}$.

ble one-electron redox process the theory predicts $\Delta E_p \sim 60$ mV. The reason for the larger value of ΔE_p, independent of the scan rate, is unknown. In accordance with the theory [12] i_{pc} (see inset fig.2) and i_{pa} are proportional to the square root of the scan rate.

From the tangent of the slope of i_{pc} *vs* the square root of the scan rate, the reduction efficiency, as defined in [8], was calculated to be 1.0. This clearly shows that we are dealing with a diffusion-controlled reduction and oxidation process of spinach ferredoxin at the modified electrode. Recently, authors in [6] determined the value for the heterogeneous electron transfer rate constant, k_f, of spinach ferredoxin at a methyl viologen-modified gold minigrid electrode to be 6.5×10^{-5} $cm \cdot s^{-1}$. At the half-wave potential $k_f = k_s$ (k_s, standard heterogeneous rate constant) and by using this value for k_s in the theory derived in [14] one should expect that ΔE_p depends on the scan rate. However, for the DAPV-modified electrode in the presence of spinach ferredoxin no effect of the scan rate on ΔE_p was observed. Therefore, under our conditions k_s will be some orders of magnitude larger than the value determined in [6]. We estimate k_s to be larger than 10^{-2} $cm \cdot s^{-1}$.

The cyclic voltammograms of *M. elsdenii* ferredoxin at the DAPV-modified electrode are shown in fig.3. The half-wave potential of *M. elsdenii* ferredoxin is at -615 mV in accordance with results in [8]. In contrast to spinach ferredoxin ΔE_p is dependent on the scan rate indicating that the standard heterogeneous rate constant for *M. elsdenii* ferredoxin will be smaller than the rate constant for spinach ferredoxin. At scan rates up to about 0.15 $V \cdot s^{-1}$, i_{pc} (see inset, fig.3) and i_{pa} are proportional to the square root of the scan rate. The reduction efficiency derived from the apparent linear part of the plot shown in the inset of fig.3 was also found to be 1.0. In the calculations the concentration of ferredoxin was doubled to take into account that each molecule contains two [4Fe-4S] clusters. This indicates a diffusion-controlled redox process of this protein at the

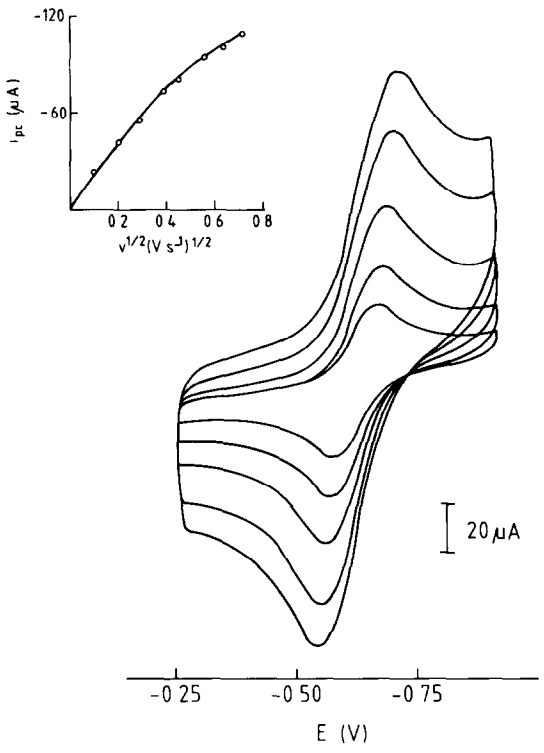

Fig. 3. Cyclic voltammograms of *M. elsdenii* ferredoxin at a DAPV-modified glassy-carbon electrode. The concentration of *M. elsdenii* ferredoxin was 0.99 mM. Scan rates: 0.02, 0.05, 0.10, 0.20 and 0.30 $V \cdot s^{-1}$.

modified electrode. It was observed that the value for k_s of *M. elsdenii* ferredoxin depends on the ionic strength. At 0.1, 0.25 and 0.5 M NaCl these values are $(1.6 \pm 0.1) \times 10^{-3}$, $(3.6 \pm 0.2) \times 10^{-3}$ and $(4.0 \pm 0.2) \times 10^{-3}$ $cm \cdot s^{-1}$, respectively. These values for k_s were derived from ΔE_p as function of the scan rate as in [14]. Previously we found for *M. elsdenii* ferredoxin that the two clusters are either identical, or non-identical but interacting [8]. Since the intramolecular electron exchange is very fast (10^6–10^8 s^{-1}; submitted) the electron exchange between the clusters will not be rate limiting. Neither ferredoxins showed any electrochemical response at the MAPV-modified electrode. Possible arguments which may explain this are;

(i) The difference in valency between DAPV ($Z = 3+$) and MAPV ($Z = 1+$) bound to the electrode;

(ii) Quaternization of both nitrogen atoms of bipyridyl is necessary to obtain electron transfer;

(iii) In contrast to cytochrome *c* the compound bound/adsorbed to the electrode surface has to show a redox behaviour in the potential area where both ferredoxins are redox active [15].

Also no redox current was observed for the unmodified electrodes in the presence of *M. elsdenii* ferredoxin. However, spinach ferredoxin showed a slight response at the unmodified electrode. The reduction efficiency was about 10–20%. As shown in fig.1 the DAPV bound to the electrode is redox active. However, in the presence of ferredoxin the total observed peak currents do not contain a contribution of bound DAPV. If so, a plot of i_{pc} *vs* the square root of the scan rate would be a concave upward curve instead of the observed straight line for spinach ferredoxin (see inset, fig.2). Since for *M. elsdenii* ferredoxin the plot of i_{pc} *vs* the square root of the scan rate is concave downward we suggest that the same argument holds for this ferredoxin. This suggests that in the presence of the ferredoxins DAPV is necessary for electron transfer but its redox currents are somehow not observable.

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