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539—ELECTROCHEMICAL BEHAVIOUR OF LOW-POTENTIAL ELECTRON-TRANSFERRING PROTEINS AT THE MERCURY ELECTRODE

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SUMMARY

This paper reports on a pulse polarographic study of low-potential electron-transferring proteins at the mercury electrode *. The proteins studied were the negatively charged iron–sulphur cluster containing ferredoxins from spinach, and from *Megasphaera elsdenii*, the iron-containing rubredoxin and the FMN-containing flavodoxin both from *M. elsdenii*. Furthermore, the positively charged, four haem-containing cytochrome c_3 from *Desulfovibrio vulgaris* strain Hildenborough was studied. It was observed that the electrode reaction of these proteins could be made much more efficient when a polymer or surfactant was added, with a charge opposite to the protein. The reduction efficiency of these proteins reaches an optimum when the net charge of the protein times its concentration is about equal to the same amount of opposite charges, which was added as a polymer or surfactant.

INTRODUCTION

During the past decade increased attention has been paid to the electrochemical behaviour of proteins which function in electron-transfer reactions. The proteins studied were the positively charged, haem-containing cytochromes [1–24] and the negatively charged, iron–sulphur cluster containing ferredoxins [25–34].

Much effort has been spent in studying the elecrochemical behaviour of cyt c_3 from *Desulfovibrio* species. This cytochrome is a low-potential, four haem-containing

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** List of abbreviations: cyt, cytochrome; CTAB, cetyltrimethyl ammonium bromide; DTAC, dodecyltrimethylammonium chloride; EDTA, ethylene diamine tetra acetate; I_{obs}, I_{calc}, observed and calculated current; i, ionic strength; MES, 2-(*N*-morpholino)ethanesulphonic acid; z.c.p. zero charge point; s.c.e., saturated calomel electrode; SDS, sodium dodecyl sulfate; $W_{1/2}$, width of differential pulse peak at half the peak height.

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protein located in the periplasmic space of this group of bacteria. *In vivo* it functions as electron carrier for hydrogenase. The formal potential of this protein is at about -0.55 V *versus* s.c.e. [1–8]. In contrast to cyt *c*, well-developed reversible redox waves are obtained from which diffusion coefficients can be derived which are not anomalously low as is the case with cyt *c*. Recently, Eddowes and Hill have extensively studied the reaction of horse-heart cyt *c* at a gold electrode on which bipyridyl was adsorbed. With this surface-modified electrode, correct values are obtained for the diffusion coefficient and the formal potential (0 V *versus* s.c.e.) of this cytochrome, even at high protein concentrations [17–21]. It is interesting to note that both types of cytochromes are, under physiological conditions, also subjected to large electric fields, due to the surface of the membrane [35], which can reach values of 10^7–10^8 V m^{-1}. Similar fields also exist at electrode surfaces.

Another group of electron-transferring proteins is that of the iron and acid-labile sulphur-containing ferredoxins. Bacterial-type ferredoxins, which contain one or two $(4Fe–4S)^{1+,2+}$ iron–sulphur clusters, have also been studied by electrochemical methods [25–34]. These studies report on badly resolved redox waves, adsorption phenomena and destruction of the cluster and formation of apo-ferredoxin. Different values for the half-wave potential have been reported, and there is disagreement about the number of electrons involved in the redox reaction of the cluster ($n = 1$ or $n = 2$). The formal potential of the bacterial-type ferredoxins ranges from -0.6 to -0.7 *versus* s.c.e. [36]. Plant-type ferredoxins, which contain one (2 Fe–2 S) cluster function in the last part of photosynthesis to reduce $NADP^+$. The formal potential of this type of cluster is about -0.65 V *versus* s.c.e. Rubredoxin contains one iron, which is bound to the protein by four cysteines. The physiological role of rubredoxin is unknown. Despite the fact that rubredoxins do not contain acid-labile sulphur, they are supposed to belong to the group of ferredoxins because of the way in which the iron is liganded to the protein.

Flavodoxins are also electron-transferring proteins, which contain FMN as a prosthetic redox group. Neither polarographic nor voltammetric data have been reported for flavodoxin, plant-type ferredoxin and rubredoxin. However, for flavodoxin a coulometric study, in which methyl viologen was used as mediator, has been reported [37]. For more detailed information about ferredoxins and flavodoxins, see respectively Refs. 36 and 38.

MATERIALS AND METHODS

Purification of electron-transferring proteins

Flavodoxin and ferredoxin of *Megasphaera elsdenii*, strain LC_1 of Elsden et al. [39], were purified as described previously [40]. Rubredoxin from *M. elsdenii* was purified from a side fraction of the purification of flavodoxin and ferredoxin. Cytochrome c_3 from *Desulfovibrio vulgaris* strain Hildenborough was purified as described by van Leeuwen et al. [41].

Spinach ferredoxin was purified by a method similar to that of *M. elsdenii*

ferredoxin. Concentrations of *M. elsdenii* flavodoxin, ferredoxin and rubredoxin were determined from absorption measurements at respectively 445 nm ($\epsilon_{445} = 10.2$ mM^{-1} cm^{-1}), 390 nm ($\epsilon_{390} = 30$ mM^{-1} cm^{-1}) and 420 nm ($\epsilon_{420} = 8.8$ mM^{-1} cm^{-1}). Concentrations of plant-type ferredoxin, horse-heart cytochrome *c* and *D. vulgaris* cytochrome c_3 were respectively determined at 420 nm ($\epsilon_{420} = 9.7$ mM^{-1} cm^{-1}), 552 nm ($\epsilon^{red}_{552} = 27.7$ mM^{-1} cm^{-1}) and 532 nm ($\epsilon^{ox}_{532} = 59$ mM^{-1} cm^{-1}).

Electrochemistry

The polarograph was a P.A.R. 174A polarographic analyser, modified as described by Abel et al. [42], equipped with a P.A.R. 174/70 drop timer and connected to a Philips XY recorder. If not stated otherwise, the P.A.R. 174A was used in its differential pulse mode (d.p.p.), the drop time was 0.5 s, the mass of a mercury drop 0.74 mg, the column height 75 cm, the pulse width 11.8 ms, the sampling width 4.9 ms and the memory time constant 2.8 ms. The scan rate varied from 1 to 10 mV s^{-1}. It was controlled that high scan rates did not affect the polarogram. The temperature was 20°C ± 1°C and all buffers used were at pH 7.4. All potentials are ±5 mV and are given *versus* s.c.e., all other values are ±5%. In the d.p.p. mode the theoretical peak current, I_{calc}, is proportional to the bulk concentration of the redox component, the square root of its diffusion coefficient and *n* (number of electrons involved in the redox process; see further [43]). For nernstian redox behaviour the width at half the peak height, $W_{1/2}$, gives a value for *n*. This width at 20°C is (88.9/*n*) mV [43]. For the standard potential, derived from the peak potential and for the value of *n*, derived from $W_{1/2}$, the effect of the pulse amplitude has been taken into account [43]. For cyt c_3 and *M. elsdenii* ferredoxin the concentrations used to calculate the theoretical peak current were respectively four and two times the concentration of protein added, since cyt c_3 contains four and *M. elsdenii* ferredoxin two redox groups. The four formal potentials of the haems of cyt c_3 are probably not identical [41]. Because of this, $W_{1/2}$ is expected to be larger than 90 mV; this corresponds to $n < 1$. For a multi-redox protein with non-identical redox sites, n_{app} is defined as 88.9 mV divided by the observed width at half the peak height. It can easily be seen that the smaller the value for n_{app}, the larger the redox gap between the individual haems. When the redox gap between two successive haems is larger than about 80 mV it can be calculated that two distinct peaks will be observable in the d.p. polarogram. To calculate the theoretical current, I_{calc}, for the reduction of cyt c_3 we used the measured value for n_{app}, as defined above.

The diffusion coefficients of the proteins studied used to calculate I_{calc} are listed in Table 1. Since the observed peak current, I_{obs}, will be smaller than or equal to the calculated peak current, I_{calc}, the effectiveness of the electrode reaction will be denoted as the reduction efficiency. The reduction efficiency is defined as $(I_{obs}/I_{calc}) \times 100\%$.

Normal pulse polarography (n.p.p.) and reversed pulse polarography (r.p.p.) can be used to detect firstly, whether irreversible adsorption phenomena are important [46–48] and secondly whether reduction is reversible [49]. As can be derived from

TABLE 1

Diffusion coefficients of several low-potential electron-transferring proteins

Protein	Molecular weight $\times 10^{-3}$ kdalton	Diffusion coefficient $\times 10^{7}$ cm^2 s^{-1}	Ref.
M. elsdenii flavodoxin	16	11.4	44
M. elsdenii ferredoxin	6	15.4	36 [a]
M. elsdenii rubredoxin	6	15.0	36 [a]
Spinach ferredoxin	11	10.4	45
D. vulgaris cytochrome c_3	13.5	11.4	56 [b]

[a] These values are estimated from the data for *Clostridium pasteurianum* ferredoxin and rubredoxin, whose physico-chemical properties are very similar to those of *M. elsdenii* ferredoxin and rubredoxin respectively.

[b] As derived from cyt *c* from bovine heart.

the work of Flanagan et al. [47,48], the peak potential in the d.p.p. mode (after correction for the pulse amplitude) is only identical to the half-wave potential in the n.p.p. mode if no irreversible adsorption of reactant and/or product takes place. In cases where reversibility of the redox reaction could not be tested by means of n.p.p. and r.p.p. (see Results section), d.p.p. was used with positive pulses.

Preparation and chemicals

Before performing the electrochemical measurement the appropriate buffer was transferred into the electrochemical cell of 2 cm^3 volume. The buffer was deaerated by bubbling purified argon through the cell for 5 min. This gas was purified of oxygen by passing it through a column of BASF catalyst at about 120°C followed by passage through a wash bottle that contained a solution of photo-reduced methyl viologen semiquinone. Next glucose (final concentration 5 m*M*) and glucose oxidase (final concentration 0.1 mg cm^{-3}; 2 units) were added in order to remove the last traces of oxygen. After addition of this oxygen-scavenging system the argon was passed over the solution in order to prevent inactivation of the protein due to foam formation. A bottle equipped with a septum, containing the protein to be studied, was also purified of oxygen by several cycles of evacuating and filled with argon. The protein was transferred by syringe from the bottle into the cell. It was controlled that the glucose–glucose oxidase system did not affect the background current. The electrochemical behaviour of the proteins was also studied in the presence of polymers or surfactants with charge opposite to the protein (see Results section). In order to prevent precipitation of the protein with the surfactant or polymer the electrochemical measurements were performed, if not stated otherwise, in 25 m*M* Tris buffer pH 7.4 plus 0.4 *M* KCl.

Poly-L-lysine (average polymerization degree of 60), poly-L-aspartic acid (average polymerization degree of 150), horse-heart cyt *c* and glucose oxidase (grade III) were

from Sigma. Sodium dodecyl sulphate (SDS) was from BDH, dodecyltrimethylammonium chloride (DTAC) and cetyltrimethylammonium bromide (CTAB) from Serva.

RESULTS

Cytochrome c_3 from Desulfovibrio vulgaris strain Hildenborough

Figure 1A shows the d.p. polarogram for cyt c_3 in Tris buffer pH 7.4. The peak potential is at -586 mV, the reduction efficiency is 70% and $n_{app} = 0.72$. As judged by n.p.p. and r.p.p. neither irreversible adsorption nor irreversibility in the redox

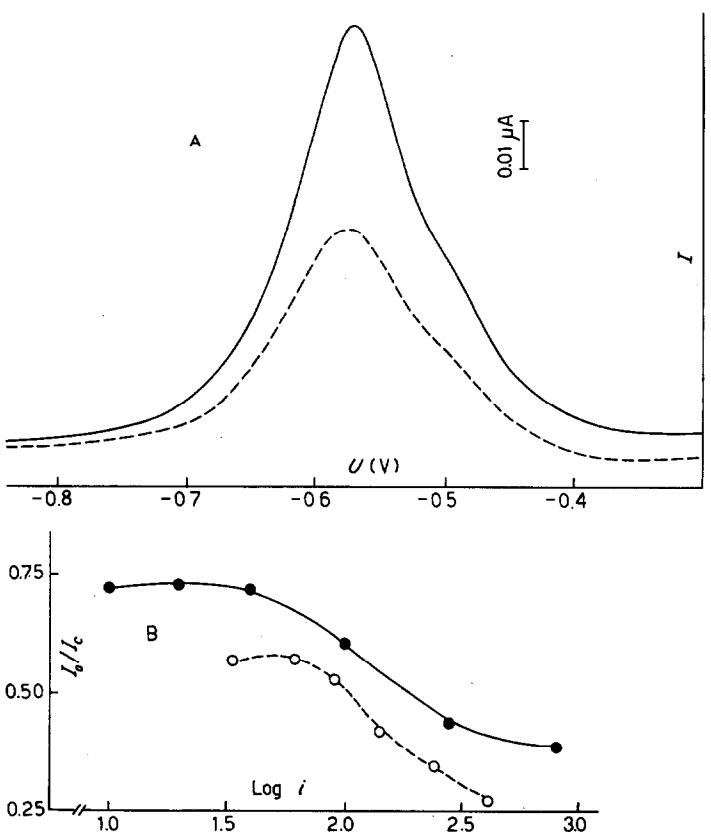

Fig. 1A. Differential pulse polarograms of cytochrome c_3 of *Desulfovibrio vulgaris* strain Hildenborough; (- - - - - -) in 25 m*M* Tris–HCl pH 7.4 in the absence of SDS; (———) in 25 m*M* Tris–HCl pH 7.4 in the presence of 0.2 m*M* SDS. The concentration of cyt c_3 is 28 μM; pulse amplitude, -25 mV. (B) Effect of ionic strength, i, on the reduction efficiency, I_o/I_c, of cytochrome c_3. (●------●) chloride anions; (O------O) phosphate anions; i in m*M*.

reaction was observed. Similar results were obtained with cyt c_3 from *D. vulgaris* Miyazaki [1,4–6]. For this cytochrome the peak potential is at −528 mV, $n_{app} = 0.74$ and the reduction efficiency is 79% [1]. As shown by Niki et al. [6], increasing concentrations of horse-heart cyt *c* to Miyazaki cyt c_3 (both are positively charged proteins) progressively decrease the limiting current for the reduction of this latter protein without altering the half-wave potential. This decrease in current was shown to be due to surface coverage of the electrode by cyt *c*. As a result, the electron transfer from the mercury electrode to the Miyazaki cyt c_3 was inhibited. With horse-heart cyt *c* Eddowes and Hill [19] found that addition of poly-L-lysine also resulted in a decrease in the reduction current of this protein at a gold electrode on which bipyridyl was adsorbed. With cyt c_3 of *D. vulgaris* Hildenborough it was observed that increasing concentrations of both poly-L-lysine and horse-heart cyt *c*, progressively decrease the peak current for the reduction of this cyt c_3. These results are given in Table 2.

Serre et al. [16] have reported on the effect of the medium on the electrode response of cyt *c*. They reported that the peak potential is dependent on the type of buffer and on the cyt *c* concentration. Under our standard conditions we did not observe a dependence of the peak potential on the cyt c_3 concentration, in agreeement with Bianco and Haladjian [15]. However, as is shown in Fig. 1B, it appeared that the peak current, thus the reduction efficiency, is dependent on the type of

TABLE 2

Effect of poly-L-lysine, cytochrome *c* and SDS on the electrochemical behaviour of cytochrome c_3. All experiments were performed in 25 m*M* Tris–HCl buffer pH 7.4. Similar results were obtained in phosphate buffer pH 7

[Cyt c_3] (μM)	Effector	[Effector] (μM)	n_{app}	I_o/I_c [a]	Peak potential U_p [b] (mV)
115		None	0.72	0.70	−586
115	pLl [c]	6.6	0.57	0.30	−591
111		25.4	0.39	0.29	−593
106		48.5	0.33	0.29	−590
28	cyt *c*	6.0	0.70	0.45	−588
28		12	0.69	0.28	−592
28		27	0.67	0.17	−596
28	SDS	25	0.74	0.65	−580
28		50	0.75	0.69	−582
28		100	0.78	0.78	−582
28		150	0.82	0.83	−583
28		200	0.85	1.0	−585
28		500	0.85	1.0	−585
28		1000	0.84	0.86	−585
28		2000	0.84	0.44	−597

[a] I_o and I_c are respectively the observed and calculated values for the peak current.
[b] U_p is the peak potential under conditions where I_o/I_c is optimum.
[c] Poly-L-lysine.

buffer and on the ionic strength. For example, in phosphate bufferpH 7.4 (13 mM) the peak potential is also at -586 mV, $n_{app} = 0.79$ and the reduction efficiency is 57%. Furthermore, from this figure it appears that the effect of the ionic strength on the reduction efficiency becomes apparent above $i = 30$ mM for Cl^- and $i = 50$ mM for phosphate anion. At $i = 600$ mM the peak potential in the presence of Cl^- is not affected. However, in phosphate buffer the peak potential has shifted by -35 mV. In both cases the value for n_{app} decreases slightly by 0.15. This indicates that upon increasing the ionic strength the formal potentials of the four individual haems are affected. However, the effect of chloride anions differs from that of phosphate anions. Furthermore, an increase in ionic strength probably affects the reduction efficiency due to its influence on both the cytochrome c_3 itself and the double-layer structure and thus the interaction between the cytochrome and the mercury electrode.

Since positively charged polymers like poly-L-lysine and cytochrome *c* inhibit the electrode reaction of cytochrome c_3 (see Table 2), the effect of a negatively charged polymer, like poly-L-aspartic acid, was investigated. Addition of this polymer to cyt c_3, up to concentrations of 50 μM, only affects the value of n_{app} to increase to 0.84, but affects neither the peak potential nor the reduction efficiency of cyt c_3. Addition of SDS, which is negatively charged, at a final concentration of 0.2 mM results in an increase in the peak current; the reduction efficiency becomes 100% and $n_{app} = 0.85$. The results described here were similar for Tris and for phosphate buffer (see Table 2). It should be noted that in the experiments described above no KCl was added, since cyt c_3 did not precipitate with SDS and poly-L-lysine at the concentrations of polymer and surfactant used. Addition of SDS (0.2 mM) to cyt c_3 (28 μM) at high ionic strength ($i = 400$ mM) completely reverses the effect of salt (KCl or phosphate) and gives the polarograms observed at low ionic strength in the presence of SDS. Thus, the reduction efficiency was 100% and $n_{app} = 0.85$. Apparently, SDS completely counteracts the effect of the ionic strength. Furthermore, SDS also counteracts the effect induced by poly-L-lysine (see Table 2). In the presence of 28 μM cyt c_3 and 48 μM poly-L-lysine the optimum concentration was 4 mM. In this case the reduction efficiency is 100% and $n_{app} = 0.83$. It must be pointed out that at this concentration SDS is above its critical micelle concentration. However, SDS could not counteract the inhibition caused by cyt *c*, which suggests different types of inhibition induced by the synthetic and natural polymer.

In Tris buffer pH 9, similar results were obtained with SDS as in Tris buffer pH 7.4. However, in MES and phosphate buffer pH 5.5 the peak potential shifts by $+0.25$ V to -0.33 V. This suggests a pH dependence of the protein below pH 7 with $\Delta U^\circ/\Delta\text{pH} \sim 170$ mV, which is unusually large. This pH effect was studied further by addition of small increments of diluted hydrochloric acid to phosphate buffer with an initial pH of 7. At pH 7.0, 6.6, 6.0 and 5.4 the values for the reduction efficiency of the peak at -0.58 V are respectively 57%, 44%, 28% and 0%. The peak current at -0.33 V steadily increases. Addition of 0.6 mM SDS at pH 5.4 results in a d.p. polarogram of cyt c_3 (final concentration of 28 μM) characterized by a reduction efficiency of 100% and $n_{app} = 0.85$. However, the peak potential shifts by

+50 mV to −535 mV. At low pH (pH 5.4) the ratio of SDS over cyt c_3 to obtain an optimum reduction is three times that obtained with pH 7. As judged by n.p.p. and r.p.p., only the peak observed at −0.33 V shows irreversible adsorption. Under none of the other conditions tested could irreversible adsorption be observed. In all these cases the peak potential (d.p.p. mode) was the same as the half-wave potential (n.p.p. mode), indicating no irreversible adsorption and reversible redox behaviour of cyt c_3.

Ferredoxins and rubredoxins

In Fig. 2A the d.p. polarogram for *M. elsdenii* ferredoxin is given, which shows a $n = 2$ peak at −0.58 V and a shoulder at about −0.65 V. It is assumed that this shoulder is caused by reduction of the iron–sulphur clusters of ferredoxin. The peak current of the $n = 2$ peak at −0.58 V is dependent on the ferredoxin preparation used. The intensity of this peak can be enlarged in several ways, as will be shown below. Furthermore, a peak was often observed at −0.2 V. It can be assumed that, since the iron–sulphur clusters of *M. elsdenii* ferredoxin are sensitive to oxygen damage, the breakdown products are iron, sulphide and cystine/cysteine. d.p. polarograms at pH 7.4 of an iron–EDTA complex and of Na_2S show peaks with peak potentials at −0.20 V ($n = 1$) and −0.58 V ($n = 2$) respectively. Addition of iron–EDTA or Na_2S to *M. elsdenii* ferredoxin increases the peak currents at −0.20 V and −0.58 V respectively. Addition of a mixture of iron, EDTA and Na_2S (molar ratios 1 : 2 : 1) to the ferredoxin gives identical results compared with the individual components of the mixture. EDTA is necessary to chelate the iron in order to prevent it precipitating with S^{2-}. It is noticeable that in the presence of EDTA the formal potential of the ferric/ferrous redox couple shifts by −0.7 V. Apparently, extraneous iron present in ferredoxin preparations is liganded to the protein in a way similar to that with EDTA, as was observed previously [28]. Furthermore, n.p.p. clearly shows that S^{2-} strongly adsorbs to the mercury electrode.

For the positively charged cytochrome c_3 it was observed that poly-L-lysine inhibits the electrode reaction of this cytochrome. This effect is reversed by addition of SDS, which is negatively charged. This result indicates that the reduction efficiency can be enhanced by addition of a negatively charged surfactant to a positively charged redox protein, and is probably due to counteraction of electrostatic effects. In the case of cyt c_3, this effect could not be induced by merely increasing the ionic strength.

Since ferredoxins are negatively charged proteins it can be expected that, in analogy with the effect of SDS on the electrochemical response of cyt c_3, addition of a positively charged surfactant or polymer could favour the electrode reaction of ferredoxins. For *M. elsdenii* ferredoxin, the reduction efficiency is about 20% (Fig. 2A). Addition of KCl (final concentration 0.4 *M*) increases, in contrast to cyt c_3, this efficiency to about 40%, while the peak potential increases by 10 mV. Addition of the positively charged poly-L-lysine or DTAC increases the reduction efficiency to

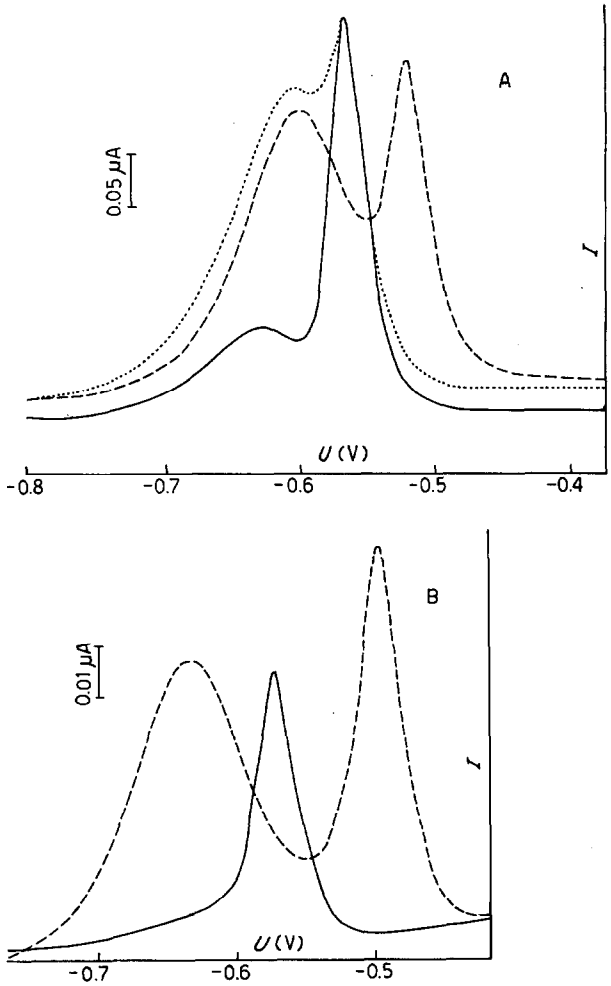

Fig. 2. Differential pulse polarograms of (A) *Megasphaera elsdenii* ferredoxin and (B) spinach ferredoxin: (A) (———) d.p. polarogram of *M. elsdenii* ferredoxin in 25 m*M* Tris–HCl pH 7.4; (· · · · · ·) d.p. polarogram in 25 m*M* Tris pH 7.4, 0.4 *M* KCl and 14.4 μ*M* poly-L-lysine; (- - - - - -) d.p. polarogram in 0.1 *M* phosphate buffer pH 6 in the presence of 14.4 μ*M* poly-L-lysine. (B) (———) d.p. polarogram of spinach ferredoxin in 25 m*M* Tris–HCl pH 7.4 in the absence and the presence of 0.4 *M* KCl; (- - - - - -) d.p. polarogram at pH 5.9 in the presence of 2.5 m*M* DTAC. Concentrations of *M. elsdenii* ferredoxin and spinach ferredoxin are respectively 117 and 119 μ*M*; pulse amplitude, −25 mV.

100% (see Table 3). The peak potential increases by another 25 mV to −617 mV. If the observed peak current has reached the theoretical peak current a further increase in the concentration of poly-L-lysine or DTAC does not affect the effectiveness of the electrode reaction of *M. elsdenii* ferredoxin. Next KH_2PO_4 was added until the pH was 5.9. At this pH the $n = 2$ redox peak shifts to more positive potentials by

TABLE 3

Effect of charge compensation on the reduction efficiency of electron-transferring proteins

Protein	Concentration μM	Effector (μM)			z^-/z^+ [a]	I_o/I_c	U_p [b] mV
		SDS	DTAC	pLl [c]			
Cyt c_3	28	200	—	—	1.2	1	−585
M. elsdenii ferredoxin	117	—	—	14.4	1.6	1	−617
	81	—	1250	—	0.8	1	−612
Spinach ferredoxin	74	—	—	18.6	1.4	0.4	−646
	119	—	2500	—	1.0	0.5	−635
M. elsdenii rubredoxin	122	—	—	12.4	1.0	0.4	−245
M. elsdenii flavodoxin	140	—	—	12.1	0.25	0.14	−357 −635

[a] Here, z^- and z^+ were calculated from the net charge of the protein, respectively polymer or surfactant times its concentration. For poly-L-lysine (polymerization degree of 60), the net charge used to calculate z^+ was +60. The net charge for cyt. c_3 is from ref. 41, for *M. elsdenii* flavodoxin from ref. 44 and for *M. elsdenii* ferredoxin and rubredoxin, and plant-type ferredoxin from ref. 36.

[b] U_p is the peak potential observed under conditions where I_o/I_c is optimum.

[c] Poly-L-lysine.

about 90 mV, while the $n = 1$ peak only becomes more positive by 10 mV. At this pH the two peaks are well separated (Fig. 2A). $W_{1/2}$ at this pH is 90 mV for the $n = 1$ peak, indicating that a one-electron process is involved in the redox reaction of *M. elsdenii* ferredoxin.

For spinach ferredoxin similar results were obtained compared with *M. elsdenii* ferredoxin (Fig. 2B). No reduction of the plant-type ferredoxin is observed in the absence or presence of 0.4 *M* KCl, while a $n = 2$ peak is observable at −0.58 V. Addition of poly-L-lysine or DTAC increases the intensity of a $n = 1$ peak which we ascribe to the reduction of the iron–sulphur cluster. At the highest concentration of poly-L-lysine tested the reduction efficiency is 40%. A comparable value was found for DTAC—a reduction efficiency of 50% at a concentration of 2.5 m*M* DTAC (see Fig. 2B and Table 3). At higher concentration of the surfactant the peak current of the $n = 1$ peak decreases, while the intensity of the $n = 2$ peak dramatically increases. Apparently, at DTAC concentrations greater than about 3 m*M* the iron–sulphur cluster is destroyed, liberating S^{2-} and cystine/cysteine. At the lowest poly-L-lysine concentration tested at which reduction of the spinach ferredoxin can be observed, the peak potential is at −665 mV. At the highest concentration of the polymer tested, the peak potential is at −646 mV. At the optimum DTAC concentration this potential is at −635 mV (see Table 3). In some experiments CTAB was used instead of DTAC. No difference in electrochemical response could be observed between these two surfactants.

In the n.p.p. mode no well-developed reduction waves could be observed for the

reduction of the bacterial and plant-type ferredoxins. This is due to the S^{2-} present in the preparations which shows an irreversible adsorption behaviour and thus contaminates the polarograms for both the ferredoxins. The r.p.p. mode also could not be used to test the reversibility of the redox reaction of *M. elsdenii* and spinach ferredoxin due to adsorption phenomena caused by S^{2-}. However, d.p.p. with positive pulses (ΔU ranges from +5 mV to +100 mV) showed that the peak potentials can be seen at the same potentials as those observed in the d.p.p. mode with negative pulses. This suggests that the redox reactions of both ferredoxins are reversible.

For *M. elsdenii* rubredoxin a similar behaviour is observed to that of *M. elsdenii* and spinach ferredoxin. In the absence of poly-L-lysine at 0.4 *M* KCl only the $n = 2$ peak is seen (Fig. 3). After addition of poly-L-lysine a rather broad peak is observed, of which the peak potential and width at half the peak height are dependent on the amount of polymer added. At the optimum polymer concentration the reduction efficiency is 40% with a peak potential at −245 mV. The reason for the rather broad peak in case of rubredoxin is not known, since the two types of ferredoxins show an ideal behaviour with respect to the width at half the peak height, −90 mV for both ferredoxins. Another contrast to ferredoxins is that addition of DTAC does not favour the reduction of the rubredoxin. At increasing concentrations of DTAC a peak develops at −0.2 V. This peak is observed at the same potential as the peak of the iron–EDTA complex (see above). This suggests that rubredoxin is denaturated by this surfactant and iron becomes liberated upon addition of DTAC.

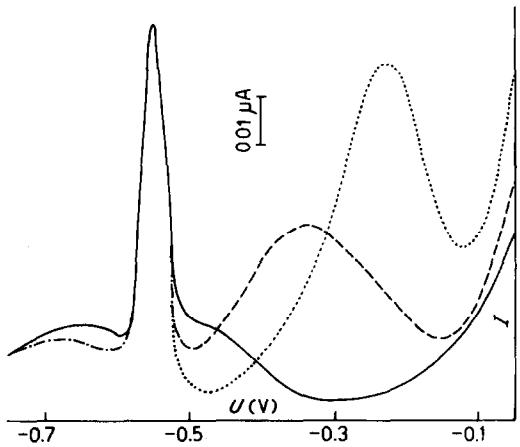

Fig. 3. Differential pulse polarograms of *Megasphaera elsdenii* rubredoxin: (———) d.p. polarogram in 25 m*M* Tris pH 7.4 in the presence of 0.4 KCl; (· · · · · ·) d.p. polarogram after addition of 6.5 μ*M* poly-L-lysine; (- - - - - -) d.p. polarogram after addition of 12.2 μ*M* poly-L-lysine. Concentration of rubredoxin is 122 μ*M*; pulse amplitude, −25 mV.

Flavodoxin

As is the case for spinach ferredoxin and *M. elsdenii* rubredoxin, no reduction of the prosthetic group is observed with flavodoxin alone. Addition of KCl (final concentration 0.4 *M*) also does not favour the reduction of the flavin moiety within the protein. To obtain reduction of this protein poly-L-lysine is necessary (Fig. 4). At the greatest polymer concentration tested, two redox peaks can be observed. The peak potentials equal the published potentials of the two redox couples of respectively the oxidized/semiquinone and semiquinone/hydroquinone redox couples at −357 and −635 mV. However, the latter potential is about 20 mV more negative as compared with the data of Mayhew et al. [50]. Under the conditions described above, the efficiency of the reduction is very low, about 14%. The peak currents of the peaks at −357 and −635 mV are almost the same. This suggests that, once the flavodoxin is in its semiquinone state the fully reduced state is effectively formed from the semiquinone. An increase in concentration of the artificial polymer results in the formation of free FMN and apoflavodoxin, as is the case for DTAC: one peak is observed at the potential where FMN can be detected. The release of free FMN was also confirmed by means of fluorescence (data not shown). N.p. polarography showed that flavodoxin is adsorbed on the mercury electrode. As a result, the value of 14% for the reduction efficiency might not be correct. Furthermore, the shift of −20 mV for the semiquinone/hydroquinone redox couple might also be caused by

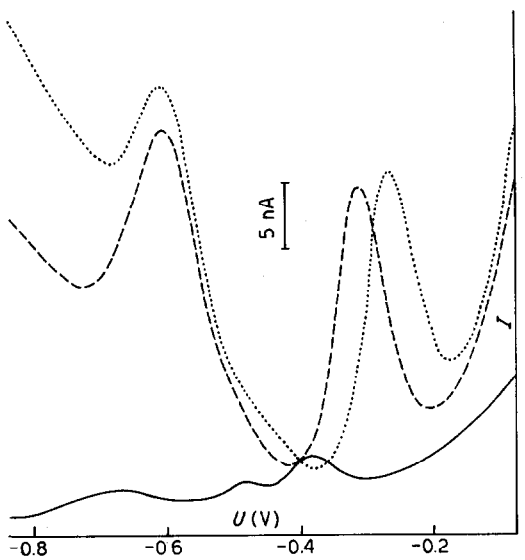

Fig. 4. Differential pulse polarogram of flavodoxin of *Megasphaera elsdenii*; (———) in 25 m*M* Tris–HCl pH 7.4 in the absence and the presence of 0.4 *M* KCl; (- - - - - -) in the presence of 12.1 μ*M* poly-L-lysine; (· · · · · ·) in the presence of 12.1 μ*M* poly-L-lysine at pH 6. Concentration of flavodoxin is 140 μ*M*; pulse amplitude, −25 mV.

adsorption phenomena (see Ref. 48). However, d.p.p. with positive pulses showed that both redox reactions are reversible.

DISCUSSION

In reports on the electrochemical behaviour of electron-transferring proteins [1–34], electrostatic effects have never been taken into consideration. This paper clearly shows that they play an important role in the electrochemistry of this type of proteins. By taking only electrostatic effects into account, which of course is an oversimplification of the redox systems studied, it can be argued that it will be difficult to reduce positively charged redox proteins at potentials more positive than the zero charge point (z.c.p.) of the working electrode. Similar difficulties in reduction can be expected upon reduction of negatively charged proteins at potentials more negative than the z.c.p. Indications that electrostatic effects play a role in the reduction of electron-transferring redox proteins are:

(1) Cytochrome c_3, which is positively charged, is quite efficiently reduced at the mercury electrode. The peak potential of this multi-haem cytochrome is such that the mercury electrode is almost uncharged (z.c.p. is about -500 mV ± 50 mV [49]). Thus, electrostatic effects will not be as important as compared with cytochrome *c* (see below).

(2) Bacterial and plant-type ferredoxins and flavodoxin are negatively charged. The formal potential of these proteins (semiquinone/hydroquinone couple for flavodoxin) is such that the mercury electrode has the same charge as these proteins. Very poorly developed redox waves or even no reduction of the prosthetic group is observed.

(3) The positively charged cyt *c* is hardly reduced at the mercury electrode, which is also positively charged at the formal potential of this protein [51].

A possible explanation why cyt *c* gives such a low reduction efficiency is the orientation of its dipole-moment relative to the haem; according to Koppenol and Margoliash [52] its angle is 33°. This means that on approach of the protein to a positively charged surface the back side of the protein is preferentially orientated to the surface, which may prevent an efficient reduction.

Rubredoxin and flavodoxin (oxidized/semiquinone redox couple), which are both negatively charged proteins, have formal potentials such that the mercury electrode is positively charged. However, no reduction of the proteins takes place at all. A possible explanation might be, in analogy with cyt *c*, the orientation of the dipole moment of these two proteins relative to the redox sites, causing a low reduction efficiency.

Another argument to explain the difference in reduction efficiency of the proteins studied may be the diffusion-controlled collision efficiency. The collision efficiency accounts for the fact that the surface of the redox site is much smaller than the total surface of the protein. For example, in the absence of an effector, the reduction efficiency of cyt c_3 is 70%, of *M. elsdenii* ferredoxin 20%, and of spinach ferredoxin 0%. These proteins respectively contain four, two and one redox sites. This collision

efficiency may explain why addition of *n*-propanol (0.1 *M*), which results in a very broad trajectory of zero charge of the mercury electrode, ranging from −0.4 V to −0.8 V [49], does not affect the reduction efficiency of flavodoxin and ferredoxins. As is indicated above, electrostatic effects may play an important role in the reduction of the group of proteins studied. If these effects play a predominant role one would expect the reduction efficiency to increase at high ionic strength due to electrostatic shielding. An increase in reduction efficiency was observed for *M. elsdenii* ferredoxin, while the opposite holds for cyt c_3. No effect of salt was observed for the reduction of spinach ferredoxin, rubredoxin and flavodoxin. As is shown in Table 2, poly-L-lysine inhibits the electrode reaction of cyt c_3, but favours the electrode reaction of the negatively charged redox proteins. We therefore suggest that poly-L-lysine adsorbs to the electrode surface, creating a local environment at the surface which causes inhibition of the reduction of cyt c_3 and favours the reduction of the negatively charged redox proteins. Similar arguments may hold for the surfactants used. As can be derived from Table 3, optimum reduction is observed if charge compensation has taken place. This means that the sum of the charges of the particular protein times its concentration has to be compensated by the same amount of opposite charges, introduced by a polymer or a surfactant. Despite the fact that the ionic strength is rather high (0.4 *M* KCl), resulting in an effective shielding of charges by counter-ions, the effect of charge compensation, as shown in Table 3, is striking. For cyt c_3 this charge compensation effect is observed at both low and high ionic strength.

In our opinion, all this together suggests that the local environment at the surface of the electrode has to be altered by means of polymers or surfactants with charge opposite to the particular electron-transferring protein. Because of this, optimal conditions are created for the reduction of this type of protein. Furthermore, this effect is independent of the charge of the mercury electrode; reduction of rubredoxin is obtained above the z.c.p., reduction of ferredoxins below the z.c.p. Recently Albery et al. [21] have studied the kinetics of reduction and oxidation of horse-heart cyt *c* at a gold electrode on which bipyridyl was adsorbed. From their measurements they concluded that rapid adsorption and desorption of cyt *c* is necessary for fast electron transfer. However, the reason why adsorbed bipyridyl is essential to obtain fast electron transfer is not clear. We assume that in our study, because of the polymers and surfactants used, optimal conditions at the electrode surface are created to obtain reversible reduction. The reduction efficiency is optimal when charges have been compensated.

As shown in Fig. 1B, the reduction efficiency of cyt c_3 in the presence of phosphate anions is, at the same ionic strength, always less than in the presence of chloride anions. This might be due to the difference in valency between these two anions, or to preferential binding of phosphate to the protein [53]. The effect of charge compensation upon the electrochemical behaviour of cytochrome c_3 results in an increase in the value of n_{app} from 0.72 to 0.85 without altering the peak potential. This increase in value of n_{app} indicates that the differences between the values of the formal potential of the four individual haems become less. Recently van Leeuwen et

al. [41] have characterized, by means of pulse radiolysis, the redox behaviour of this cytochrome. At low ionic strength ($i = 3$ mM) they calculated values for n_{app} of 0.64 and of -535 mV for the averaged sum of the formal potentials of the four haems. At low ionic strength due to electrostatic interactions, an interaction energy of 32 mV was calculated. These electrostatic interactions are progressively abolished at increased ionic strength. At $i = 50$ mM it is calculated that the value for n_{app} increases to about 0.7. This latter value is in good agreement with the value n_{app} determined in this study. However, the difference of 50 mV between the peak potential and the averaged sum of the potentials found for the four haems is striking. It is noticeable that the peak potential of cyt c_3 at pH 5.4 in the presence of SDS is also at -535 mV.

It is intriguing to speculate that the heterogeneous reduction of cyt c_3 by means of the mercury electrode represents a more *physiological* situation for this protein compared with homogeneous reduction [41], since this protein is associated with membrane of the bacterium, where it is subjected to the surface potential. It can be argued that the effects of SDS and poly-L-aspartic acid, which result in an increase in the value of n_{app}, do not represent physiological conditions. However, it should be kept in mind that under physiological conditions cytochrome c_3 transfers electrons from hydrogenase to ferredoxin *vice versa*, the latter two proteins being negatively charged polypeptides. Our data suggest that due to the interaction of cyt c_3 with ferredoxin and hydrogenase the formal potentials of the four individual haems become more or less equivalent. However, after dissociation from the iron–sulphur proteins the difference in the formal potentials of the haems becomes larger. This effect might exhibit a regulatory role at the level of the redox sites of cyt c_3 due to electrostatic interactions.

For rubredoxin and the two types of ferredoxin the optimum conditions for electrochemical reduction are also obtained in the case where the charges of the proteins are compensated (see Table 3). The value of the peak potential of spinach ferredoxin at pH 7.4 at optimum reduction efficiency (-647 mV) is in very good agreement with the value determined by Tagawa and Arron [54]. This suggests that correct values for the formal potentials are obtained if charge compensation has taken place. Since no data are available on the formal potentials of *M. elsdenii* ferredoxin and rubredoxin it is assumed that, in analogy with spinach ferredoxin, the formal potentials are those obtained at optimal reduction efficiency; -612 mV and -250 mV respectively. Recently Armstrong et al. [55] have determined the formal potential of *Clostridium pasteurianum* ferredoxin by means of cyclovoltammetry. They found a value of -614 mV, which is identical to the value we determined for *M. elsdenii* ferredoxin. For the latter ferredoxin which contains two structural identical redox sites, we found that $n = 1$. To explain this we assume that the ferredoxin contains two identical, non-interacting redox sites. For *C. pasteurianum* ferredoxin it has been confirmed by means of *e.p.r.* [57] and pulse-radiolysis [58] that the two clusters of this protein do not show strong cooperative interaction (less than 5 mV). In their study, Ikeda et al. [31] showed the formation of apo-ferredoxin at the mercury electrode surface. If formation of apo-ferredoxin, during the pre-pulse

period, is the case for *M. elsdenii* ferredoxin, one would never expect a reduction efficiency of 100% since part of the redox centres of ferredoxin would be destroyed. Since the reduction efficiency for spinach ferredoxin and rubredoxin is lower than 100%, destruction of the redox centre might be an explanation in these cases. *Megasphaera elsdenii* flavodoxin showed an optimal reduction efficiency of 14%. The peak potentials observed for both redox peaks are in close agreement with the published data [48]. However, the potential for the second peak is about 20 mV more negative, which might be explained by adsorption phenomena.

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