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Electrochemical behaviour of human adrenodoxin on a pyrolytic graphite electrode

Daniel Johnson[a], Shona Norman[a], Robert C. Tuckey[b], Lisandra L. Martin[a,*]

[a] Department of Chemistry, Flinders University, G.P.O. Box 2100, Adelaide, SA 5001, Australia
[b] Department of Biochemistry, University of Western Australia, Nedlands, WA 6009, Australia

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Abstract

Adrenodoxin (Adx) functions as a redox protein in the delivery of electrons to all mitochondrial cytochromes P450. In order to further characterize the human form of this protein, direct electrochemistry of human adrenodoxin (Hadx) has been observed for the first time on a pyrolytic graphite electrode (PGE) modified with poly-L-lysine. A single well-defined redox wave was observed with a midpoint potential of -448 ± 3 mV vs. Ag/AgCl (sat. KCl) at a scan rate of 10 mV/s and over the pH range 4.0–8.0. At slow scan rates, the reduction process was close to being electrochemically reversible whereas, at faster scan rates, only quasi-reversibility was observed. A correlation was observed between the peak separation (ΔE) for the cyclic voltammograms and pH over a wide range of scan rates. The variation of ΔE with pH was at a minimum (optimum reversibility) at pH 7.0 for all scan rates tested. This correlation may suggest that the direct electrochemistry method could possibly provide a means for determining protein or enzyme activity. The electron transfer rate constant, k_s, was determined to be 0.28 s^{-1} at pH 7.0 and a small pH dependence was observed. The results obtained in this study demonstrate the facile nature of direct electron transfer for human adrenodoxin, and provide an estimate of the midpoint reduction potential at a pyrolytic graphite electrode via electrostatic immobilisation.

Keywords: Human; Adrenodoxin; Cyclic voltammetry; Pyrolytic graphite; [2Fe2S] centres; Metalloprotein

1. Introduction

Adrenodoxin (Adx) is a [2Fe2S]-containing protein that functions as a mobile one-electron carrier in the electron transport chain for all mitochondrial cytochromes P450. Adrenodoxin receives one electron from adrenodoxin reductase (AR) and delivers it to cyctochrome P450. Adrenodoxin must bind to cytochrome P450 twice in the catalytic cycle since the P450 requires two electrons to activate molecular oxygen to achieve substrate oxidation, most commonly hydroxylation [1]. Current evidence strongly supports Adx acting as a shuttle between AR and the P450, forming binary complexes with each [1–4]. Mitochondrial cytochromes P450 play an essential role in the synthesis of steroid hormones with cytochrome P450scc (CYP11A1) catalysing the initial cleavage of the side chain of cholesterol to produce pregnenolone, a step common to the synthesis of all steroids [2,5]. Another mitochondrial cytochrome, $P450_{11\beta}$ (CYP11B1) is required for both cortisol and aldosterone synthesis while cytochrome $P450_{18}$ (CYP11B2) catalyses the last two steps of aldosterone synthesis [6]. Mitochondrial cytochromes P450 also play an essential role in converting vitamin D to its active form and participate in bile acid synthesis [2]. Studies on yeast proteins homologous to mammalian AR and Adx suggest that these proteins may also play an important role in the assembly of iron sulphur centres [7].

In the cholesterol side-chain cleavage reaction, adrenodoxin appears to act as both an electron donor and effector for P450scc. The oxyferro complex of bovine cytochrome P450scc can be generated by addition of oxygen to chemically reduced cytochrome but reduced adrenodoxin is required for donation of the second electron [8–10]. The oxidized adrenodoxin, present in the human placenta during P450scc catalysis due to limiting AR, acts as a competitive inhibitor of cytochrome P450scc, decreasing electron deliv-

* Corresponding author. Tel.: +61-8-8201-2465; fax: +61-8-8201-2905.
E-mail address: Lisa.Martin@Flinders.edu.au (L.L. Martin).

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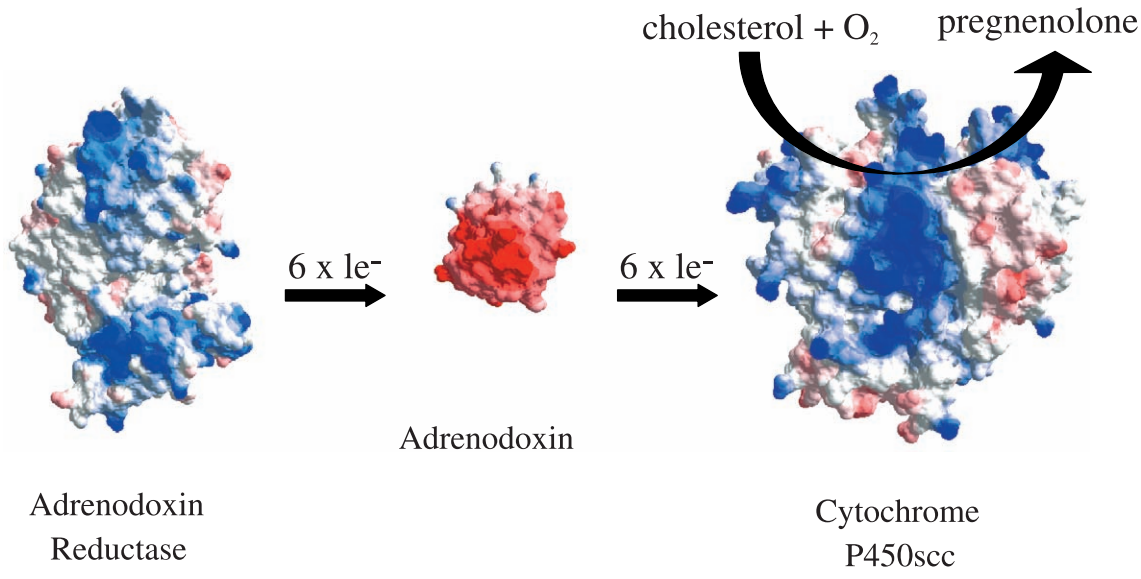

Fig. 1. Surface representation of electron transfer proteins involved in the conversion of cholesterol to pregnenolone by cytochrome P450scc. The red- and blue-coloured molecular surfaces indicate regions of negative and positive electrostatic potential, respectively. Protein images were prepared using Swiss PDB Viewer. Adrenodoxin reductase (1CJC.PDB) and adrenodoxin (1AYF.PDB) crystal structure coordinates were obtained from the Brookhaven Protein Database and cytochrome P450scc is an unpublished model structure from our laboratory.

ery to the P450scc by binding in preference to reduced Adx [11,12]. In bacteria, putidaredoxin plays a similar role to adrenodoxin in the catalytic cycle of P450cam (CYP101) acting as both an electron donor and effector for the cytochrome [13].

Direct electrochemistry has proved to be an invaluable probe for the investigation of FeS-containing ferredoxins [14–17]. For these small metalloproteins (~ 10,000 Da) containing FeS redox centres, a sustained redox signal is typically achieved by electrostatic adsorption of the protein onto an electrode surface. Cyclic voltammetry is the electrochemical technique most often employed as it exploits the ability to vary both the applied potential (voltage) and time domains for the redox process. Valuable thermodynamic and kinetic information is therefore obtained for the protein.

Electrochemical methods have been used to examine the redox properties of both putidaredoxin and bovine adrenodoxin and in each case the electrode surface was extensively modified. The bovine adrenodoxin study used a multilayer gold electrode modified with mercaptoundeconoic acid and polyallylamine [18] whereas the putidaredoxin electrochemistry employed a mercaptoethylamine pretreatment of gold by soaking overnight [19]. Here, we present our results with human adrenodoxin (Hadx) by cyclic voltammetry over a wide range of pH and scan rates using an edge-oriented, pyrolytic graphite electrode (PGE) pretreated with poly-L-lysine as previously reported in our laboratory [20]. This electrostatic method of adrenodoxin immobilisation is advantageous as it provides a similar environment to that in which the metalloprotein functions *in vivo* in the human adrenal cortex.

2. Experimental details

2.1. Protein purification

The mature form of Hadx was expressed in *Escherichia coli* and purified as described previously [21]. The concentration of Hadx was determined from its absorption at 414 nm, using $\varepsilon = 11{,}000\ M^{-1}cm^{-1}$ [22] and sample concentrations used for electrochemistry were 319 μM unless otherwise noted. The Hadx was stored at −80 °C in 50 mM Hepes at pH 7.4 prior to use.

2.2. Electrochemistry

Electrochemical measurements were obtained using cyclic voltammetry on either a BAS 100B Electrochemical Analyser or a BAS CV-27 interfaced with a MacLab four-channel device and the E'chem software program. Working electrodes were prepared using "edge-oriented" pyrolytic graphite sealed in a Teflon sheath with the available potential assessed in aqueous solution. The reference electrode was Ag/AgCl (sat. KCl), fitted with a vycor tip and the reference potential at 21 °C was 0.197 V vs. NHE. All electrochemical data reported here were measured against this reference electrode unless otherwise stated and, before and after each series of measurements, the potential and reversibility of the $[Fe(CN)_6]^{3-/4-}$ couple were measured ($E_{mid}\{[Fe(CN)_6]^{3-/4-}\} = 260 \pm 4$ mV vs. Ag/AgCl) to check for instrumental artefacts and found to be invariant during all the experimental procedures. A platinum wire was used throughout as an auxiliary electrode. Solvated nitrogen gas was used to purge the cell prior to each measurement

and a blanket of nitrogen was maintained over the cell between measurements. Midpoint potentials (E_{mid}) are reported in mV and were calculated as the average of anodic and cathodic peak potentials, $E_m = 1/2(E_{pa} + E_{pc})$. All measurements were undertaken at room temperature, 21 ± 1 °C. Prior to each measurement, the PGE electrode was polished using Al_2O_3 powders starting at 1 μm and decreasing to 0.3 and 0.05 μm. Between each polishing step, the electrode was sonicated in MilliQ water.

All measurements were undertaken in a glass cell, which was soaked in H_2SO_4 between experiments. A "mixed buffer electrolyte" solution was prepared using 5 mM each of Mes, Hepes, Taps, AcO^- (a total of 20 mM) and 0.1 mM EGTA in 0.1 M NaCl. The pH of this buffer solution was adjusted to the required pH prior to the electrochemical measurement. MilliQ water was used throughout all the experiments. In a typical experiment, the working electrode was pretreated with 3 μl of a 0.1% poly-L-lysine solution and allowed to dry. A film of protein was then placed onto the poly-L-lysine coat and also allowed to dry. The electrode was then placed into the electrochemical cell containing 5 ml of mixed buffer-electrolyte (described above) and measurements commenced. Evidence for the formation of a stable protein-poly-L-lysine film was assessed using the

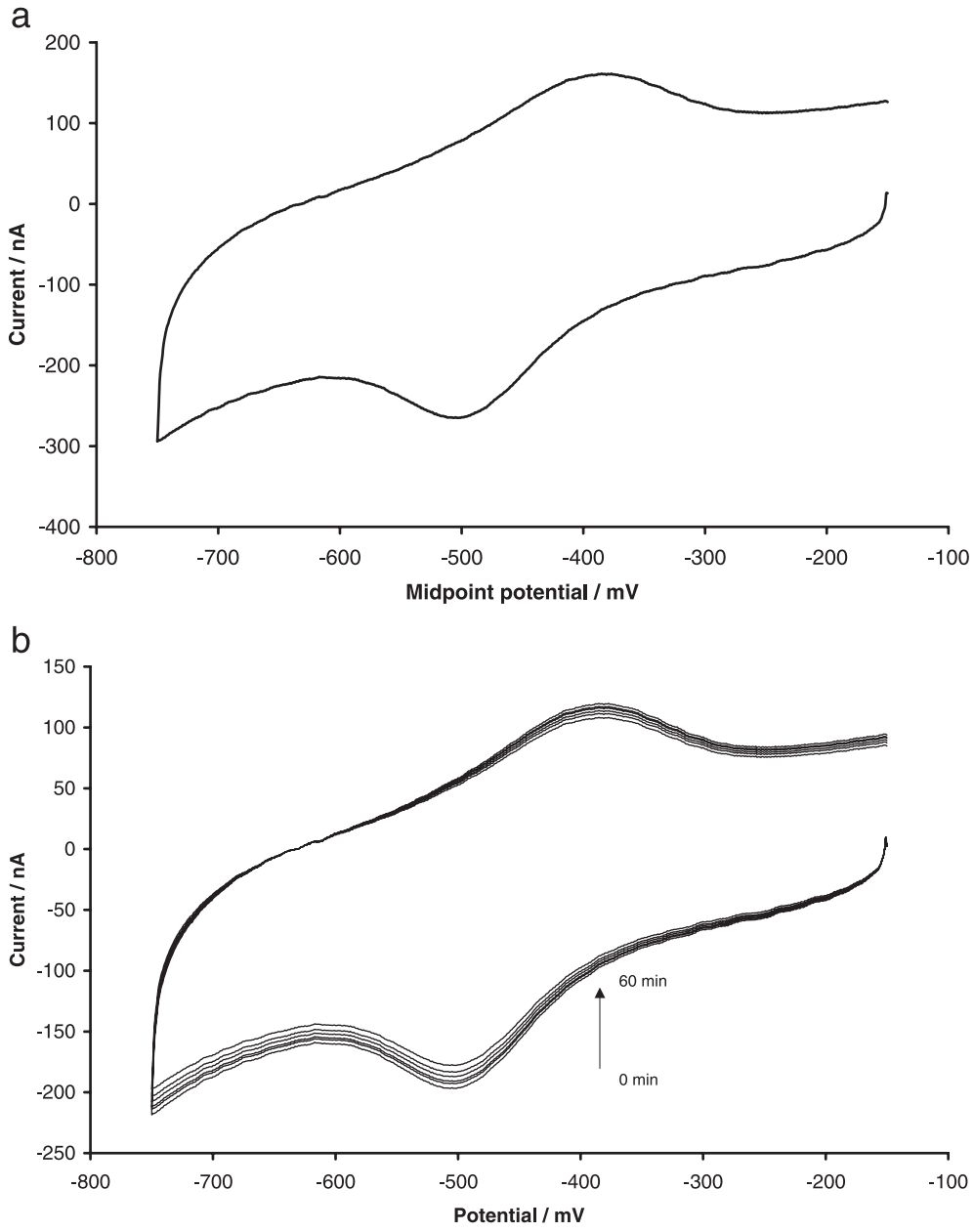

Fig. 2. (a) A cyclic voltammogram of human adrenodoxin adsorbed onto poly-L-lysine on an edge-oriented pyrolytic graphite electrode at pH 7.0. Scan rate of 20 mV/s. (b) Cyclic voltammogram of a film of Hadx on a poly-L-lysine pretreated PGE showing the effect of time on the current response. (c) Dependence of i_{pc} on Hadx concentration and scan rate. The concentrations of Hadx used were 319 and 260 μM.

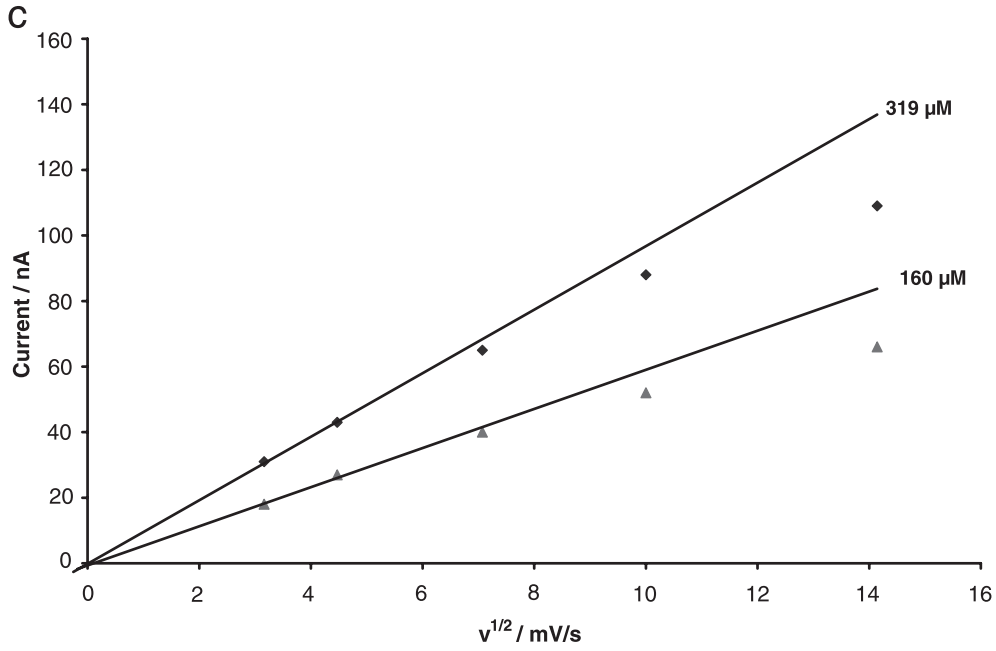

Fig. 2 (*continued*).

current response over time, which remained unchanged over a 1-h period (Fig. 2b). In addition, the PGE was unable to sustain an electrochemical response without a film of poly-L-lysine. The thickness of the poly-L-lysine film was estimated using Atomic Force Microscopy (AFM). The film prior to attachment of the Hadx has a depth of 6.5 nm whereas the film, after washing with MilliQ water, has a depth of 5.5 nm. Data were collected on at least three different occasions and with different equipment to ensure reproducibility; no smoothing or baseline subtraction techniques were applied.

3. Results

A film of the Hadx was adsorbed directly onto an edge-oriented pyrolytic electrode (PGE) pretreated with a film of poly-L-lysine. The p*I* for Hadx calculated from the primary amino acid sequence of the mature form is 4.4 [23]. Accordingly, Hadx is expected to be negatively charged at pH values above this value. Cyclic voltammograms of Hadx displayed excellent signal-to-noise responses and the measured currents typically fell in the range of several hundred nA to several µA. A typical example of the cyclic voltammetric response obtained at pH = 7.0 is illustrated in Fig. 2a. In each experiment, the initial potential was 0 V and the voltage sweep was in the cathodic (reduction) direction until the switching potential was reached at −0.8 V. The scan direction was then reversed to sweep anodically until 0 V. No redox response was observed over the same potential range with just a film of poly-L-lysine attached to the PGE working electrode. The strong adsorption of the adrenodoxin to the electrode was verified (Fig. 2b) over an hour with very little change in peak currents. Thus, it was thought that the protein-poly-L-lysine-PGE composite maintained integrity during all experiments. Also, as the initial reduction of surface oxides on the PGE were always present on the first cathodic scan, all the electrochemical data recorded in this study used the second or third scan cycles to derive the redox parameters of interest.

A summary of the observed redox response of Hadx for a range of pH values and scan rates is found in Table 1. A one-electron transfer process was always observed and the midpoint potential was observed at $E_{mid} = -448 \pm 3$ mV for

Table 1
Variation of electrochemical parameters with pH and scan rate for Hadx immobilised on PGE using poly-L-lysine

pH	Scan rate, mV/s	E_{mid}, mV	ΔE, mV	i_{pc}/i_{pa}
4.0	10	−449	117	1.22
	20	−448	153	1.17
	50	*−446*	*363*	*1.20*
5.5	10	−451	106	1.27
	20	−444	149	1.36
	50	−448	262	1.35
	100	*−457*	*349*	*1.33*
7.0	10	−445	82	1.29
	20	−444	101	1.03
	50	−449	144	0.99
	100	−452	210	1.13
	200	−458	245	1.15
	500	*−468*	*360*	*1.19*
8.0	10	−447	89	1.20
	20	−451	141	1.14
	50	−458	191	1.18
	100	−457	284	1.00
	200	*−462*	*352*	*1.09*

Data in italics refers to values obtained with poor reproducibility.

all pH values and at a scan rate of 10 mV/s. At higher scan rates, E_{mid} did not vary for pH 4.0 and 5.5; however, at pH 7.0 and 8.0, a cathodic shift by 15 mV was evident at the fastest scan rates used here. Despite these small variations in E_{mid}, the ratio of charge transferred in both cathodic and anodic processes (i_{pc}/i_{pa}) were close to unity for all scan rates 10–500 mV/s and pH values. At each pH value, the redox response for Hadx was observed to be quasi-reversible as assessed by the separation between cathodic and anodic waves, ΔE. Values of ΔE were close to reversible at pH 7.0 and 8.0 with values of 82 and 89 mV, respectively, at 10 mV/s. Interestingly, at this slow scan rate, the value of ΔE was greatest at pH 4.0 ($\Delta E = 117$ mV), where the pH of the buffer lies below the pI of the Hadx. At all pH values, ΔE was found to increase with scan rate and at the highest scan rate used the redox process was electrochemically irreversible with $\Delta E \sim 350$ mV. These data are represented graphically in Fig. 3, where the error bars refer to separate experiments for each scan rate and pH value used here. The separation between cathodic and anodic waves at pH 7.0 and 10 mV/s was 64 mV, thus, close to the Nernstian value of 59 mV. The apparent electron transfer rate constant, k_s, for the immobilised adrenodoxin was calculated using the method of Laviron [24] for several pH conditions. Data used to calculate k_s were only at scan rates that resulted in ΔE values <200 mV. At pH 7.0, k_s was 0.28(7) s^{-1} while calculated values at lower and higher pH were 0.13 s^{-1} at pH 4.0 and 0.15 s^{-1} at pH 8.0.

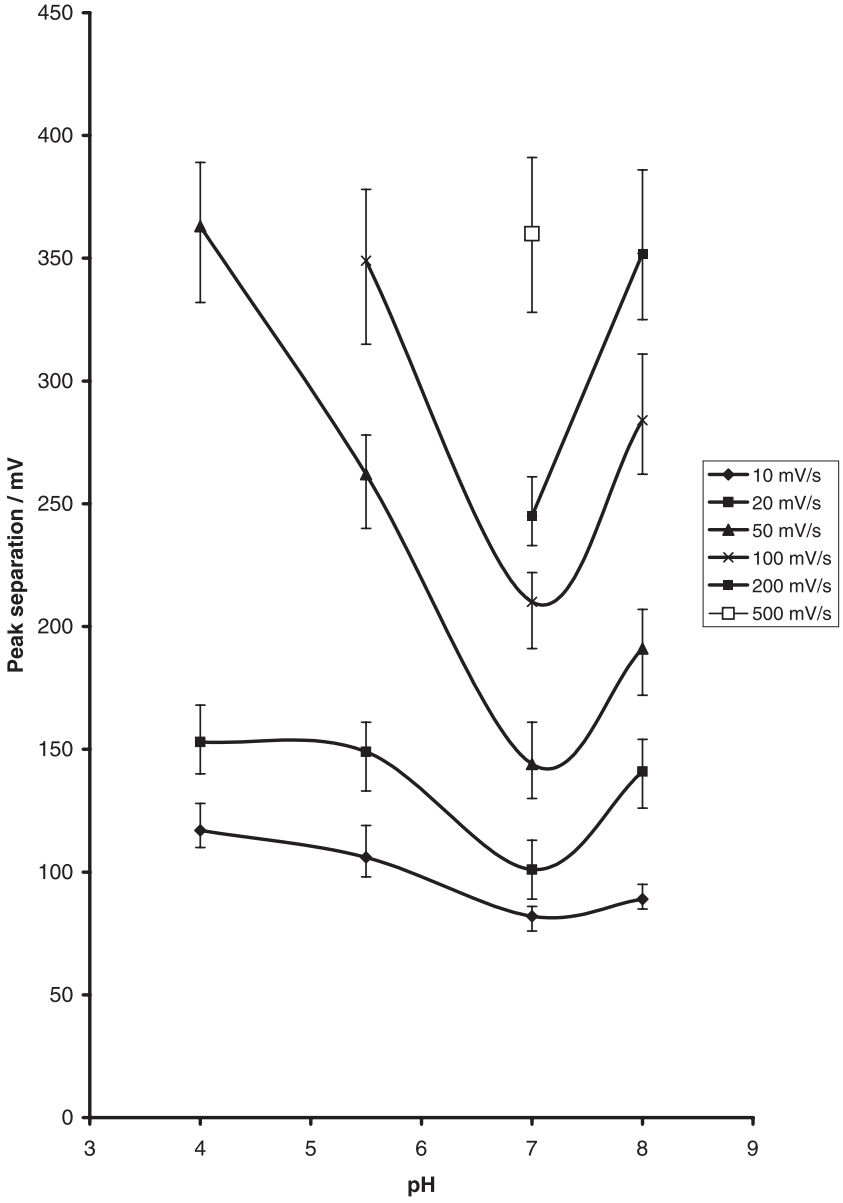

Fig. 3. Scan rate and pH dependence of the peak separation, ΔE, for Hadx at the PGE immobilised by poly-L-lysine. Data were obtained in three separate experiments and each point represents an average value with the error bars indicating the standard error of the mean.

4. Discussion

The edge-oriented pyrolytic graphite (PGE) electrode has been used by us [16,17,20] and others [25] to reproduce the redox processes for low molecular weight electron transfer metalloproteins, including ferredoxins. The nature of both the promoter and electrode must be considered in the interpretation of these data, particularly in light of *in vivo* correlations [26]. Ferredoxins containing overall negative charges can be electrostatically adsorbed onto PGE using a range of polyamines including aminoglycosides as coadsorbates with the protein to achieve good surface adsorption and electrochemical response. We have shown that poly-L-lysine coadsorbs onto PGE and that the positively charged surface is able to reduce the negative surface charge of the PGE and encourage strong adsorption of a small negatively charged protein on the graphite electrode surface. In the present study, several aminoglycosides were examined to determine the efficacy of Hadx adsorption to PGE (data not shown). These included neomycin, tobramycin, kanamycin, and polymyxin, and although a weak redox response obtained was observed with all these coadsorbants, the signal-to-noise ratio was always poor. However, the electrochemical response with poly-L-lysine as a coadsorbant gave an immediate and well-sustained redox response for Hadx with an excellent signal-to-noise ratio (illustrated in Fig. 2).

The orientation of the Hadx on the poly-L-lysine is assumed to occur by electrostatic interactions between the region of high negative charge on the protein associated with residues Asp72, Glu73, Asp76, and Asp79. These amino acid residues have been implicated in the recognition of Adx by its electron donor (AR) and acceptor (P450) for the electron transfer event [2,26,27]. Proteins immobilised electrostatically are believed to be immobilised as a monolayer on the electrode surface. However, in all our experiments, the peak separation was greater than 0 mV expected for a strongly adsorbed molecule, hence, greater than the Nernstian value of 59 mV/*n* expected for diffusion-controlled electrochemistry. This may be suggesting that either some diffusion-controlled electrochemistry is occurring by some loss of the protein from the electrode interface or that the rate of electron transfer measured in this study was always slower than the scan rate used in these experiments.

The midpoint redox potential, E_{mid}, for Hadx was found to be invariant to scan rate over a range of pH values (4.0–8.0) and in good agreement with those observed for bovine adrenodoxin and bacterial putidaredoxin (Table 2). As seen in Table 1, at the slowest scan rate used, 10 mV/s, the observed midpoint potential was -448 ± 3 mV. For any scan rate below 10 mV/s, a stable film of protein on the electrode could not be maintained. Relatively, large peak separations were observed throughout this study and were valuable to determine the electron transfer rate constant, k_s, also giving values that compare well with the bovine adrenodoxin and bacterial putidaredoxin as shown in Table 2.

Table 2
A comparison of some electrochemical parameters for [$2Fe_2S$]-containing human, bovine adrenodoxins and bacterial putidaredoxin

	E_{mid}, mV	ΔE, mV	k_s, s^{-1}	Measurement conditions	Reference
Human adrenodoxin	−449	144	0.28	Direct e'chem @ PGE pH 7.0	this work
Bovine adrenodoxin	−460	200	0.13	Direct e'chem @ SAM modified Au, pH 7.2	[18]
Bacterial putidaredoxin	−450	160	4.5[a]	Direct e'chem, @ modified Au, pH ~ 7.5	[19]

Electrochemical data are measured vs. Ag/AgCl (sat. KCl).
[a] This rate constant was obtained by analysis of square wave data under different conditions to the other two studies [30].

The Hadx sample appears to be *more reversible* at pH 7.0 compared with data collected at other pH values and this led us to present these results graphically in Fig. 3 with ΔE scrutinised as a function of pH and scan rate. Fig. 3 illustrates an unexpected result for protein electrochemistry. Notably, the redox waves at the slower scan rates, particularly 10 mV/s, were close to reversible over a wide range of pH values (4.0–8.0). Also, for each scan rate measured, the graphical dependence of peak separation on pH indicates that the minimum separation occurs at pH 7.0. We suggest here that this type of graphical representation may be providing a rapid assessment of optimum biological activity or function for the Hadx. The redox function of Hadx is widely accepted as providing a mobile shuttle between its natural redox partners, AR and P450scc. Thus, the redox state of the adrenodoxin during binding and subsequent electron transfer between its redox partners is thought to be extremely important [1,28,29]. Supportive data of the optimum pH for Hadx activity using spectrophotometric methods would provide an interesting comparison for these data. To the best of our knowledge, there is no data available on the pH dependence of Hadx. However, Lambeth and Kamin [28] did examine the pH dependence of the bovine adrenodoxin reductase–adrenodoxin complex by potentiometric titration, although they were unable to unequivocally assign an observed pH dependence to the adrenodoxin. Our electrochemical data do not support a pH dependence for Hadx, although it does appear that there is a pH dependence in the reversibility of the redox couple, as assessed by plots of ΔE vs. pH shown in Fig. 3. Our interpretation of Fig. 3 is that the electron transfer reaction is *most efficient* at pH 7.0 compared to that observed at pH values higher and lower and that this effect was enhanced at higher scan rates. The possibility that there is a correlation between measured ΔE for Hadx and biological activity is beguiling. If such a profile could be confirmed for other electron transfer metalloproteins, this may lead to a rapid determination of optimum protein activity *in vitro,* which may also relate to *in vivo* activity or function.

The well-defined electrochemical redox responses we observed suggest that the combination of soft carbon surface together with an electrostatic immobilisation using a biogenic poly-amino acid is suitable for similar metalloproteins such as bovine adrenodoxin or putidaredoxin. We are continuing to explore the interactions between the Hadx with P450scc using various modified methods for immobilisation of the redox donor–acceptor complex.

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