Direct Electrochemistry of Redox Proteins at Pyrolytic Graphite Electrodes

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Abstract: The direct (unmediated) electrochemistry of several redox proteins at pyrolytic graphite electrodes has been studied by square-wave voltammetry. For rubredoxin and the 2[4Fe-4S] ferredoxin from *Clostridium pasteurianum* and flavodoxin from *Megasphaera elsdenii* rapid heterogeneous electron transfer is promoted by multivalent cations such as Mg^{2+} and $Cr(NH_3)_6^{3+}$. These proteins carry an excess of negatively charged residues, and it is suggested that their approach to the electrode surface is assisted by the high positive charge density in or near the outer Helmholtz plane that may be generated by multivalent cations. In addition, specific protein–cation association may be important. For azurin from *Pseudomonas aerguginosa*, direct electrochemistry is observable without the requirement for cation promoters. At a square-wave frequency of 31 Hz, net current peak potentials for each protein are in good agreement with published potentiometrically determined values.

Among current approaches to the study of electron transfer in biological systems the quest for conditions conducive to the observation of rapid heterogeneous electron transfer between redox proteins and electrode surfaces attracts considerable interest.[1-5] In addition, since many physiological electron-transfer processes are "heterogeneous" in kind, it is possible that electrochemical studies may allow comment on aspects of in vivo behavior that elude more conventional approaches.

Osteryoung and associates[6,7] have recently reappraised the use of square-wave voltammetry (SWV) and discussed its application to systems of kinetic complexity.[8] We have found SWV to be a sensitive and informative technique for the study of the direct (unmediated) electrochemistry of redox proteins. Here we report the electrochemistry of four proteins at a pyrolytic graphite electrode and illustrate, for three examples, the profound ability of multivalent cations, such as Mg^{2+} and $Cr(NH_3)_6^{3+}$, to promote heterogeneous electron transfer.

Experimental Section

Rubredoxin and the 2[4Fe-4S] ferredoxin from *Clostridium pasteurianum*[9] and azurin from *Pseudomonas aeruginosa*[10] were isolated and purified according to literature procedures. Flavodoxin (*Megasphaera elsdenii*)[11] was a gift of Prof. Cees Veeger, University of Wageningen. Hexaamminechromium(III) chloride [$(Cr(NH_3)_6]Cl_3$) was prepared as described in the literature.[12] All other reagents were of Analar or Aristar grade. Electrochemical experimwnts were carried out at 25 °C. The glass electrochemical cell (ca. 300-μL capacity) incorporated a conventional three-electrode configuration. The working electrode, a 5-mm diameter disc of pyrolytic graphite (General Electric Co., Detroit) was housed in a sheath of epoxy ressn with the surface of deposition (a–b plane) parallel to the solution/electrode interface. Prior to each experiment the electrode was polished using an alumina (particle size 0.3 μm)–water slurry and thoroughly rinsed with distilled water. The counter electrode consisted of a semicylindrical piece of platinum gauze, and the reference electrode was saturated calomel (SCE), $E = +244$ mV vs. NHE at 25 °C. Anaerobicity of samples was achieved by passing a slow stream of humidified Oxygen-free argon across the surface. Solutions were typically 0.2 mM in protein and contained 0.10 M NaCl and 20 mM acetate or Tricine (*N*-tris[hydroxymethyl]methylglycine) as the support medium. Aliquots of 3.0 M $MgCl_2$ or 0.3 M $Cr(NH_3)_6Cl_3$ stock solutions were added as required via a 25-μL Hamilton syringe. The flavodoxin semiquinone radical form was generated in situ by titration with NADPH in the presence of a catalytic amount of ferredoxin–NADP reductase (EC 1.18.1.2).

Symmetric SWV ($\rho = \sigma = 0.5$)[6] was carried out using microprocessor-based instrumentation. A Research Machines 380-Z microcomputer was interfaced to a rack system containing digital-to-analog and analog-to-digital converters. The potentiostat used was constructed in this laboratory. Parameter input and data manipulation were carried out in BASIC while experimental control software was written as assembler language routines which are CALLed from BASIC.

Results and Discussion

Square-wave voltammograms[6] for each protein are shown in Figure 1. As described in ref 3 for the 2[4Fe-4S] ferredoxin, we were able to complement our observations in each case by the use of DC cyclic voltammetry. In a previous report[3] we drew attention to the promotion of direct electrochemistry of *C. pasteurianum* 2[4Fe-4S] ferredoxin by divalent metal ions, their presence making possible rapid and reversible bulk electrolysis. Thus in the absence of, e.g., $MgCl_2$, i.e., with 0.1 M NaCl, 20 mM Tricine as suppporting electrolyte, no faradaic electrochemistry of the protein was detectable. We have now found that the direct electrochemistry of rubredoxin and flavodoxin (semiquinone–hydroquinone) is promoted in a similar manner. Once again, without $MgCl_2$ in the supporting electrolyte, no redox process of any significance corresponding to the electrochemistry of the protein was observed. Background SW and cyclic voltammograms measured without protein were essentially identical in the absence or presence of 60 mM $MgCl_2$. Maximum peak net currents for these proteins were obtained at Mg^{2+} concentrations ranging between 40 and 80 mM. Limited investigations carried out with $Cr(NH_3)_6^{3+}$, which is redox inactive over the potential range of interest, showed this also to be an effective promoter. With the trivalent cation, optimal concentrations were between 5 and 10 mM, i.e., an order of magnitude lower than the requirement for Mg^{2+}. In the case of azurin, where direct electrochemistry is easily observable *without* the requirement for divalent cation promoters, addition of 100-mM $MgCl_2$ produces no meaningful change in current although there is a *ca.* 10-mV positive shift in redox potential.

Clearly, well-defined forward and reverse current contributions to the net current are observable for each protein. For a reversible

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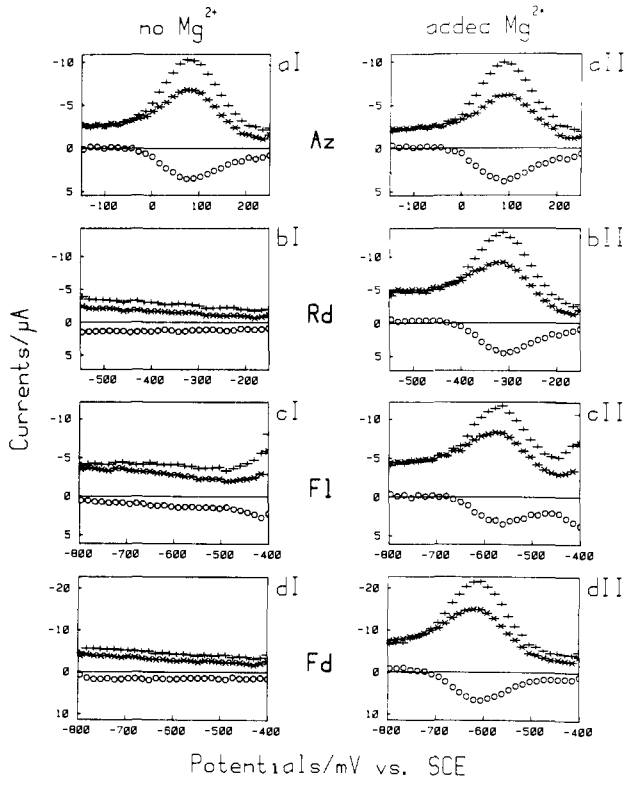

Figure 1. Square-wave voltammograms showing forward (*) and reverse (O) contributions to the net current (+). (a) Azurin: (I) 0.2 mM in 20 mM acetate, 100 mM NaCl, pH 5.2; (II) as I, with addition of 100 mM $MgCl_2$. (b) Rubredoxin: (I) 0.2 mM in 20 mM Tricine, 100 mM NaCl, pH 8.0; (II) as I, with addition of 65 mM $MgCl_2$. (c) Flavodoxin (semiquinone–hydroquinone): (I) 0.2 mM in 20 mM acetate, 100 mM NaCl, pH 5.0; (II) as I, with addition of 32 mM $MgCl_2$. (d) 2[4Fe–4S] ferredoxin: (I) 0.2 mM in 20 mM Tricine, 100 mM NaCl, pH 8.0; (II) as I, with addition of 60 mM $MgCl_2$. Voltammograms were obtained using parameters of square-wave frequency 31 Hz, amplitude (half peak to peak) 50 mV, background potential step size −1 mV, and are reproduced showing 1 in 15 data points for clarity.

electrochemical reaction, the net current peak potential corresponds[6] to the redox potential of the couple. At sufficiently low frequencies quasi-reversible systems also give[8] SW voltammograms approaching this reversible limit. We have measured the potentials of peak net current at 31 Hz for the four proteins studied. The results, as shown in Table I, are in satisfactory agreement with published potentiometric values.

The current responses at less negative potentials in Figure 1c are due to trace amounts of FMN released from the flavoprotein.[13] Experiments conducted with fully oxidized flavodoxin at pH 5.0 and 8.0 showed negligible electroactivity[14] at redox potentials corresponding to one-electron reduction of the quinone form (E_2 vs. SCE; pH 5.0, −241 mV; pH 8.0, −418 mV[15]). At pH 5.0 the physiologically relevant semiquinone–hydroquinone redox potential E_1[15] is well separated from that due to free FMN, and while cation-promoted direct electrochemistry corresponding to this redox couple is discernible using solutions of the fully oxidized

Table I. Comparison of Electrochemical and Potentiometrically Determined Redox Potentials for Small Redox Proteins

protein	square-wave net current peak potential/mV vs. NHE (corrected)	potentiometric redox potential/ mV vs. NHE
azurin	323 (pH 5.2)[a]	360,[b] 330[c]
rubredoxin	−74 (pH 8.0)[d]	−57[e]
flavodoxin (semiquinone–hydroquinone)	−318 (pH 5.0)[d]	−328[f]
2[4Fe-4S] ferredoxin	−369 (pH 8.0)[d]	−403,[g] −371[h]

[a] 0.2 mM in 20 mM acetate, 100 mM NaCl. [b] pH 5.0; calculated from kinetic data: Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* 1979, *101*, 2302. [c] pH 7.0: Sailasuta, N.; Anson, F. C.; Gray, H. B. *J. Am. Chem. Soc.* 1979, *101*, 455. [d] Concentration of Mg^{2+} as given in legend to Figure 1. [e] pH 7.0: Lovenberg, W.; Sobel, B. E. *Proc. Natl. Acad. Sci.* U.S.A. 1965, *54*, 193. [f] Calculated for pH 5.0 from data given in: Mayhew, S. G.; Foust, G. P.; Massey, V. *J. Biol. Chem.* 1969, *244*, 803. [g] pH 7.0: Stombaugh, N. A.; Sundquist, J. E.; Burris, R. H.; Orme-Johnson, W. H. *Biochemistry* 1976, *15*, 2633. [h] Calculated limiting redox potential from pH dependence data, $pK_{ox} = 7.4$, $pK_{red} = 8.9$: Magliozzo, R. S.; McIntosh, B. A.; Sweeney, W. V. *J. Biol. Chem.* 1982, *257*, 3506.

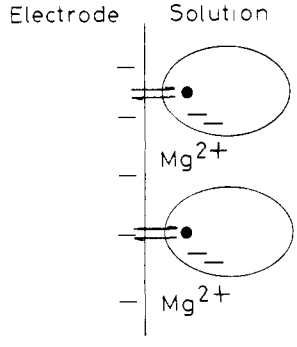

Figure 2. Schematic representation illustrating how multivalent cations can promote protein–electrode interaction in the electrical double layer.

protein, the response is enhanced following prior generation of the semiquinone form.

Cyclic voltammetry carried out with azurin, rubredoxin, and ferredoxin showed behavior consistent with quasi-reversible electrochemistry. Typical peak–current potential separations ΔE_p (mV) at scan rates of 10 (and 50) mV s^{-1} were, for azurin, 87 (122), rubredoxin (100 mM Mg^{2+}), 70 (80), ferredoxin (40 mM Mg^{2+}), 85 (107). Plots of peak current vs. square root of scan rate (v) were linear for rubredoxin up to at least $v = 100$ mV s^{-1}, for azurin and ferredoxin small deviations from linearity became apparent above 20 mV s^{-1}.

All the proteins exhibiting cation-promoted direct electrochemistry carry an excess of negatively charged residues[16,17] and have redox potentials (see Table I) that lie in a domain where the graphite surface is expected to have a net negative capacitative charge.[18,19] Oxidized-carbon functional groups located at the edges of extended aromatic regions may be important in providing *discrete* areas of negative charge.[20-22] The promotion by multivalent cations suggests a striking analogy between our observations and reported cation-sensitive photosynthetic phenomena including thylakoid stacking, electron transfer, and chlorophyll

(13) We found the faradaic responses for FMN were variable. Additions of Mg^{2+} produced no significant changes in this response although there was a marked increase as a function of the electrode–solution contact time. This is likely to be due to progressive adsorption of FMN on the graphite electrode. See, for example: Gorton, L.; Johansson, G. *J. Electroanal. Chem.* **1980**, *113*, 151.

(14) One explanation for the absence of a faradaic response corresponding to the quinone–semiquinone redox couple may be in terms of slower electron-transfer rates for this physiologically less relevant process. Previous kinetic studies on the reduction of flavodoxin by dithionite have indicated that the rate-limiting step may be one-electron reduction of the fully oxidized form. See, for example: Mayhew, S. G.; Massey, V. *Biochim. Biophys. Acta* **1973**, *315*, 181.

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fluorescence.[23] There the effects of divalent cations have been attributed to their ability to "screen" negatively charged membrane surfaces. In our electrochemical studies the high positive charge density with solutions containing multivalent cations[24] that is likely to be concentrated in and near the outer Helmholtz plane is expected to stabilize the otherwise repulsive electrostatic interactions between electrode and protein. Additionally, intermolecular electrostatic repulsions between adjacent protein molecules near the electrode surface may be greatly diminished. Alternatively protein–cation association, which might result in a significant decrease in the effective negative charge of the protein may be important. Rates of reaction of such complexes, as, for example, reported for chloroplast and bacterial ferredoxins,[25] may differ considerably from those of the native protein. Consideration of the various interaction possibilities leads us to propose the simple model illustrated schematically in Figure 2.

The importance of protein–electrode binding as a prerequisite for electron transfer has been discussed previously.[2] For asymmetric protein molecules, binding in an orientation conducive to rapid heterogeneous electron transfer may depend upon *specific* interactions with the electrode surface. Though the 2[4Fe-4S] ferredoxin displays a remarkable 2-fold symmetry axis,[26] we note that rubredoxin,[27] flavodoxin,[28] and azurin[29] possess quite pronounced asymmetry both in terms of the location of redox centres and (to varying degrees) the distribution of charged and hydrophobic surface residues. The absence of cation sensitivity of azurin is consistent with the three-dimensional structure which shows[29] extensive *charge pairing* among side chains. In all these proteins the redox centers are contained within regions of invariant or semiconserved hydrophobic residues. Kinetic studies with flavodoxins have moreover implicated[30] the exposed dimethylbenzene ring of the flavin as "point of entry and exit of the electron". It is possible that the hydrophobic environment of the active-site regions of all four proteins may interact favorably[31] with extended aromatic arrays on the graphite basal plane[20] once the electrostatic repulsion is overcome.

In conclusion, we have demonstrated the direct electrochemistry of four different redox proteins and illustrated an interesting aspect of the influence of multivalent cations on heterogeneous electron transfer. Quantitative studies are currently in progress, which may allow comparison between the electrode reactions and those relevant to the biological functions of the proteins.

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State-Selective Photochemistry from the Higher Excited States of Methylbenzaldehydes: Intermolecular vs. Intramolecular Hydrogen Abstraction

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Abstract: We have found that the hydrogen abstraction reactions of 2,4,5-trimethylbenzaldehyde and 2,4-dimethylbenzaldehyde, each isolated in durene single crystals, do not occur via either of the lowest two triplet states but take place primarily through excited states above the singlet origins. The reactions can be directed to give different products by changing the wavelength of the photolyzing light.

Since Hammond and co-workers demonstrated the triplet nature of the photoreactive state responsible for the hydrogen abstraction reactions of aromatic carbonyl molecules,[1] much work has been done correlating the orbital nature (e.g., $n\pi^*$, $\pi\pi^*$) of the lowest triplet state of these molecules with their photoreactivity.[2] It is now generally believed that the reactions proceed via the lowest triplet state, T_1, and that $n\pi^*$ states are far more reactive than $\pi\pi^*$ states since the electron density at the carbonyl oxygen is decreased when an $\pi^* \leftarrow n$ transition occurs.[2a] The photoreactivity of aromatic carbonyls having a $T_1(\pi\pi^*)$ state has been attributed to configurational mixing[2b] with, or thermal population[3] of, a close lying second triplet state, T_2, which is predominantly $n\pi^*$ in character. In some systems, the extent of configurational mixing is not sufficient to explain the photoreactivity.[3] 2,4,5-Tri-

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