Protein film cryovoltammetry: demonstrations with a 7Fe ([3Fe–4S] + [4Fe–4S]) ferredoxin

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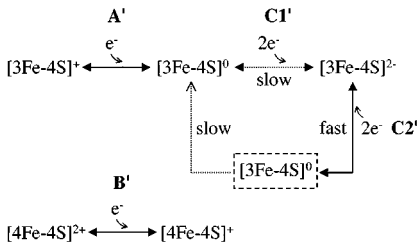
Low-temperature protein film voltammetry, demonstrated here by studies of Fe–S clusters in the 7Fe ferredoxin from *Thermoplasma acidophilum*, offers a novel way to investigate active site redox reactions, allowing resolution of complex transformations and transient states, and providing an immediate and sensitive gauge of solvent effects.

Protein film voltammetry (PFV) is proving to be a powerful method of investigating biological redox-active sites.[1,2] The protein sample is adsorbed onto a suitable electrode surface as an electroactive film, and its electron transfer and coupled processes are probed as functions of potential and time. Signals obtained from a minuscule quantity of sample (monolayer coverage or less, equating to pmol cm^{-2}) lead to the detection and characterization of labile active sites and deconvolution of complex reaction sequences. The potential may be cycled at rates > 100 V s^{-1}, accessing coupled reactions occurring on the sub-millisecond time scale. Here we report an important extension of PFV in which the sample is easily and reversibly transferred to a cryosolvent. Details of redox chemistry are revealed across a wide sub-zero temperature range, and transient states can be trapped at slow (< 100 mV s^{-1}) scan rates. The approach draws together several areas, notably cryoenzymology,[3] enzymes in organic solvents[4] and cryovoltammetry.[5]

We demonstrate the concept with experiments on a 7Fe ferredoxin (Fd). These proteins contain a [3Fe–4S] and a [4Fe–4S] cluster, and have been extensively characterized. They display distinctive voltammetry with a unique combination of characteristics, both simple and complex, that can be identified and correlated with behavior established under more conventional conditions.[6–10] The ferredoxin from *Thermoplasma acidophilum* (*Ta*),[11] MW *ca.* 16 kDa, which also contains a single Zn atom, is related structurally to the previously studied ferredoxin isolated from *Sulfolobus acidocaldarius*.[9,10,12,13]

The redox reactions of interest are shown in Scheme 1. The [4Fe–4S] cluster undergoes only one redox transition (2+/+), referred to in PFV as signal B′, while the [3Fe–4S] cluster exhibits more complex behavior. The '+' and '0' oxidation levels (whose interconversion gives rise to signal A′) are well characterized[8,9,14] and a further two electrons are taken up in a chemically reversible process (signal C1′) to produce an unusual all-Fe(II) cluster.[10] It has been shown[13] that this hyper-reduced state is capable of undergoing a rapid and reversible two-electron–two-proton oxidation (signal C2′), the immediate oxidized product of which reverts to the normal '0' state within a second at 273 K unless re-reduced within this time. The C′ signals are due to *cooperative* two-electron transfers (as revealed by the narrow voltammetric peak shapes),[15] making it likely that this novel cluster reaction involves disulfide coupling instead of Fe-based redox transitions.



Scheme 1 Redox transitions of the [3Fe–4S] and [4Fe–4S] clusters in *Thermoplasma acidophilum* ferredoxin. The transition between alternative states of $[3Fe–4S]^0$ is also shown, with the metastable form capable of very rapid $2e^-$–$2H^+$ transfer indicated by the dashed box. Proton transfers are not included.

Fig. 1 shows voltammograms, scan rate 20 mV s^{-1}, obtained from a film of *Ta* Fd formed on a pyrolytic graphite edge (PGE) electrode in aqueous solution[16] and then transferred to a cell containing 70% v/v methanol–water (pH* = 7.4 at 273 K[17]), the temperature of which could be varied down to 188 K.[3a] The potential range engages only the $[3Fe–4S]^{+/0}$ couple, to provide the simplest test of the system. At 273 K the oxidation and reduction peaks of signal A′ are nearly symmetrical and the reduction potential $E^{\circ\prime}$ is −297 ± 10 mV, compared with −250 mV obtained under the same conditions in aqueous electrolyte. Experiments with 60% v/v ethane-1,2 diol–water gave similar results with $E^{\circ\prime}$ = −276 mV. The electroactive coverage, *ca.* 55 × 10^{-12} mol cm^{-2} based on geometric electrode area, is as expected for a very densely packed monolayer on an ideal flat surface.[1b] As the temperature is lowered, $E^{\circ\prime}$ increases (linearly with temperature, as expected), the peak separation increases to reflect slower interfacial electron transfer, and the peaks broaden slightly. In the limit of a Nernstian system, half-height peak widths should decrease from 83 mV at 273 K to 60 mV at 198 K.[15] However, the expected sharpening was not observed even if a very slow scan rate (2 mV s^{-1}) was used; reductive and oxidative peak widths at 273 K were 86 and 90 mV respectively, with peak separation 7 mV, whereas at 198 K these broadened

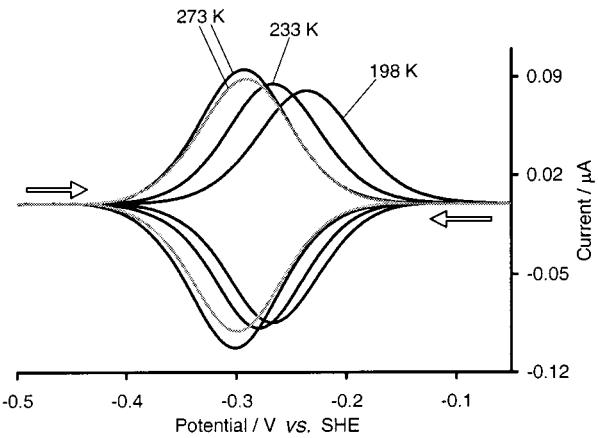


Fig. 1 Background-subtracted cyclic voltammograms obtained from a single film of *Thermoplasma acidophilum* ferredoxin adsorbed on a PGE electrode (coated from ice-cold aqueous solution) and transferred to a 70 : 30 methanol–water electrolyte, showing the temperature dependence of the $[3Fe–4S]^{+/0}$ couple. Each scan was conducted at 20 mV s^{-1} and started from the oxidative limit. The film was cooled from 273 to 233 and then to 198 K, before re-warming to 273 K (final voltammogram shown in grey). The cell solution (pH* = 7.4 as measured at 273 K) contained 0.1 M NaCl, 20 mM HEPES buffer and 200 μg ml^{-1} polymyxin B sulfate. Chunky arrows indicate the direction of scanning.

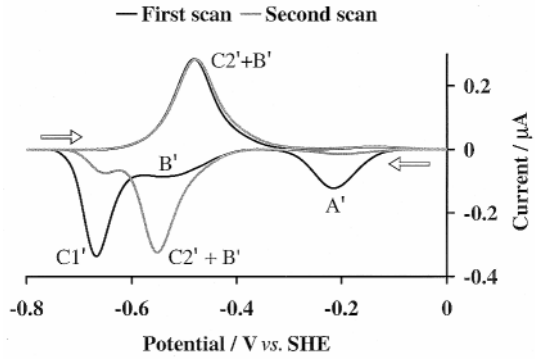


Fig. 2 Background-subtracted cyclic voltammograms showing the first cycle (black) and the second (grey) obtained from a film of *Thermoplasma acidophilum* ferredoxin (coated from aqueous solution) adsorbed on a PGE electrode in 70:30 methanol–water electrolyte at 198 K. The scan was conducted at 40 mV s^{-1} and started from an oxidative poise. The cell solution, pH* = 5.0 as measured at 273 K, contained 0.1 M NaCl, 20 mM acetate buffer and 200 μg ml^{-1} polymyxin B sulfate. Arrows indicate direction of scanning.

slightly to 90 and 96 mV, with a modest 23 mV peak separation.[18] Warming to 273 K almost completely restores the voltammetry measured at the start of the experiment (*ca.* 1 h previously).[19]

Fig. 2 shows the voltammetry obtained across a wide potential range, engaging all the redox couples shown in Scheme 1 to provide a more demanding test. The solution acidity (pH* = 5.0 at 273 K) parallels conditions used to reveal the novel cooperative two-electron couple at ambient temperatures in aqueous electrolyte.[13] In the first cycle, started from an oxidative poise, the reduction peaks correspond to $[3Fe–4S]^{+/0}$ (A′ is at a higher potential than in Fig. 1 because pH* is below p*K*[16]), $[4Fe–4S]^{2+/+}$ (B′, somewhat broader than A′), and $[3Fe–4S]^{0/2-}$ (C1′, narrow and intense). The A′:B′:C1′ peak area ratio is 1:1.1:1.8, close to that expected. The subsequent oxidative scan is dominated by one large signal in the position expected for a superposition of B′ and C′. Notably, the oxidation peak of signal A′ has almost vanished; the second reductive scan also reveals very little A′ and is dominated instead by the new couple C2′. As reported recently, the immediate product of two-electron oxidation of $[3Fe–4S]^{2-}$ is an alternative '0' state which cannot be oxidized rapidly to $[3Fe–4S]^{+}$. Poising for sufficient time at potentials higher than the $[3Fe–4S]^{+/0}$ couple restores almost completely the voltammetry of the first cycle. At 245 K this requires *ca.* 3 min, whereas at 273 K the metastable '0' species is too short-lived to be detected below 1 V s^{-1}.[13]

The behavior of this system therefore mimics that observed in fast-scan experiments at ambient temperatures in pure aqueous media. We conclude that extremely small samples of protein, immobilized on an electrode, can be introduced conveniently and reversibly into cryosolvents—in effect, an instantaneous microscopic dialysis—and studied at low temperatures. Convoluted and multi-step redox reactions are retarded to allow deconvolution and detection of intermediates at conventional scan rates. Other proteins respond similarly well to this technique, and we are currently extending its application to enzymes.

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16 The methodology was based on that described in ref. 6. Polymyxin B sulfate (200 μg ml^{-1}) was present as co-adsorbate in both the aqueous electrode-coating solution and the cryosolvent cell solution (usually 70% v/v methanol; Prolabo > 99%) with 0.1 M NaCl buffered by HEPES at pH* 7.4 or acetate at pH* 5.0. The aqueous saturated calomel reference electrode (SCE) with internal salt bridge was held in a Luggin side arm filled with cryosolvent but maintained at room temperature with a water jacket. Values of pH and pH* [see: ref. 3(*a*), and R. G. Bates, *Determination of pH: Theory and Practice*, Wiley, New York, 1973] were measured with glass pH electrode at 273 K, without corrections. Cell anaerobicity was maintained by purging with Ar. Temperatures were controlled by immersing the cell compartment in a stirred ethanol bath, to which was added dry ice or frozen ethanol, and measured (±1 K) with a thermometer after 10 min equilibration. Background currents were subtracted using an in-house analysis program (Dr H. A. Heering).

17 Measurements of the $[3Fe–4S]^{+/0}$ couple between pH* 4 and 9 (recorded at 273 K) showed that, over the full temperature range studied, the p*K* for protonation of $[3Fe–4S]^{0}$ remains significantly lower than the pH* used in measuring the voltammetry for Fig. 1, thus ruling out the possibility that $E^{\circ\prime}$ varies trivially as a result of pH* changes. Values obtained were: at 273 K (aqueous) p*K* = 5.6, at 273 K (70% v/v methanol) p*K* = 6.7, at 198 K (70% v/v methanol) p*K* = 6.8 (an apparent value since pH* is measured at 273 K).

18 The peak broadening observed as the temperature is lowered is not due to increased uncompensated resistance (*iR*); like irreversible electrode kinetics, *iR* effects are associated with large increasaes in peak separation. See: L. Roullier and E. Laviron, *J. Electroanal. Interfacial Electrochem.*, 1983, **157**, 193.

19 Peak potentials were completely restored although peak areas were *ca.* 10% smaller than measured at the start of the experiment.

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