Very Rapid, Cooperative Two-Electron/Two-Proton Redox Reactions of [3Fe−4S] Clusters: Detection and Analysis by Protein-Film Voltammetry

Judy Hirst, Guy N. L. Jameson, James W. A. Allen, and Fraser A. Armstrong*

Contribution from the Inorganic Chemistry Laboratory, Oxford University, South Parks Road, Oxford OX1 3QR, England

Received May 18, 1998

Abstract: The "hyper-reduced" $[3Fe-4S]^{2-}$ cluster (consisting formally of three Fe(II) atoms and four sulfides (S^{2-})) possesses a remarkable capability for very rapid and reversible two-electron/two-proton oxidation ($E^{o'} > -650$ mV below pH 7) that is strongly suggestive of disulfide-based (as opposed to Fe-based) redox chemistry. This otherwise elusive reactivity is most readily revealed by performing fast-scan protein-film voltammetry on ferredoxins that contain a [3Fe−4S] cluster using an electrolyte composed of D_2O. Fast, cooperative two-electron/two-proton transfer is observed after first generating the fully reduced $[3Fe-4S]^{2-}$ state and then cycling rapidly to more oxidizing potentials. The unusual voltammetric characteristics can be modeled by using a coupled electron-transfer scheme involving multiple states of both the $[3Fe-4S]^0$ and $[3Fe-4S]^{2-}$ forms. Rapid two-electron/two-proton oxidation produces an unstable species, most likely a disulfide, which may either be rapidly re-reduced or undergo an internal redox reaction to produce the normal $[3Fe-4S]^0$ form, which formally comprises two Fe(III) and one Fe(II). Relaxation to the normal "0" form is a factor of 4 faster when the experiment is conducted in H_2O, thus making the fast couple more difficult to observe—the retardation observed in D_2O may be attributed to the need to rearrange the hydrogen-bonding interactions in the cluster binding domain.

Introduction

Simple iron−sulfur centers in proteins are not normally associated with two-electron reactions,[1−4] so it is interesting that the $[3Fe-4S]^0$ cluster (the normal reduced form, which has been extensively characterized[5−9]) undergoes a further *cooperative two-electron* reduction to the "hyper" reduced (2−) level[8−12] (by cooperative, we mean that transfer of the second electron occurs spontaneously following transfer of the first). This novel two-electron reaction is usually observed by protein-film voltammetry, a technique which probes the properties of a mono-/submonolayer of protein molecules adsorbed on a "friendly" electrode surface and provides exquisite capabilities for manipulating and characterizing complex redox chemistry that may be difficult to define by conventional methods.[13−15] The progress of reactions may be monitored simultaneously in both the thermodynamic and kinetic domains, and systems of

(1) By "simple" we refer to the small and extensively characterized [2Fe−2S], [3Fe−4S], and [4Fe−4S] clusters. By contrast, the [8Fe−7S] supercluster ("P-cluster") of nitrogenase (ref 2) is known to undergo a cooperative two-electron reaction (see ref 3 and Note Added in Proof).

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10.1021/ja981693a CCC: $15.00
Published on Web 11/10/1998

interest have included those in which electron transfer is coupled to proton transfer.[6−8] We have made extensive studies of the electrochemical properties of various Fe−S proteins adsorbed at a pyrolytic graphite "edge" (PGE) electrode and have found the appearance of a sharp, two-electron, chemically reversible signal at low potential to be the typical hallmark of a [3Fe−4S] cluster.[8−12] However, this 0/2- redox transformation *appears* slow, because the voltammetric oxidation and reduction peaks become widely separated even at modest scan rates (up to 1 V s^{-1}). For *Sulfolobus acidocaldarius* 7Fe ferredoxin, the fully reduced product has been generated in bulk and partially characterized by MCD and EPR as an all-Fe(II) species.[10] The strong pH dependence of reduction potentials for the $[3Fe-4S]^{0/2-}$ couple shows that two protons are taken up and bound, at or very close to the cluster (n.b., a maximum of three are taken up with respect to the fully oxidized $[3Fe-4S]^{+}$ level), although the $[3Fe-4S]^{2-}$ species does not produce H_2 at any significant rate.[10−12] The sites of protonation and the reason for the cooperativity remain unclear. There is no evidence for a stable one-electron (1−) species, as appears at very negative potential for a nonprotein [3Fe−4S] analogue in aprotic solvents.[16]

In this article, we report our discovery that the three-electron reduced (all-Fe(II)) cluster, $[3Fe-4S]^{2-}$, is capable of undergoing a *very rapid* and reversible two-electron/two-proton oxidation that is much more characteristic of S-based (disulfide coupling) than Fe-based chemistry. The results may have important implications for the role of Fe−S clusters in enzymes such as hydrogenase and nitrogenase, where the exact nature of the multi-electron/proton transfers remain obscure.[2,3,17]

Experimental Section

Protein-film voltammetry was carried out as previously described.[7,13,14] Cell and protein solutions contained a 20 mM mixed buffer system comprising 5 mM in each of acetate, MES, HEPES, and TAPS, with 0.1 M NaCl as background electrolyte and 200 μg ml^{-1} polymyxin B sulfate as coadsorbate. For experiments in D_2O, the correction [pD = pH(measured) + 0.4] was applied.[18] The pH (pD) of each cell solution was checked immediately after each experiment. The cell was thermostated at 0/2 °C, and anaerobicity was maintained by purging thoroughly with high purity argon throughout the experiments. *S. acidocaldarius* 7Fe ferredoxin,[9] freshly purified by fast protein liquid chromatography (FPLC, Pharmacia, Mono Q column) was applied to the surface of a PGE electrode (polished using 1 μm alumina and washed thoroughly with Millipore water, resistivity 18 MΩ.cm) which was immediately introduced into the cell. To ascertain that no 6Fe ferredoxin was present,[19] voltammograms were scanned up to a switching potential of 200 mV, whereupon no signal due to the second $[3Fe-4S]^{+/0}$ cluster was observed (in the 6Fe ferredoxin, this appears at approximately 0 mV, and the relatively broad signal due to $[4Fe-4S]^{2+/+}$ is completely absent[20]).

Fast-scan analogue cyclic voltammetry[7,21a] was carried out using an Autolab electrochemical analyzer (Eco-Chemie, Utrecht, The Netherlands) equipped with a PGSTAT 20 module and fast analogue scan generator, in combination with a fast AD converter (ADC750). To reduce the effects of uncompensated resistance, the area of the working electrode was minimized (to ca. 0.01 cm^2) and it was positioned reproducibly close (ca. 0.15 cm) and normal to the Luggin tip of the SCE side arm. The remaining resistance (500−1000 Ω) was compensated for by positive feedback, set at a value immediately below the onset of current oscillations.[21] To reduce electrical noise, the cell was enclosed in a Faraday cage, and where possible, any remaining noise was removed by Fourier transformation. Background currents, due to the electrode, were subtracted using an in-house program (Dr. H. A. Heering) which fits a cubic spline function to the baseline on each side of the peak.[22]

Numerical modeling was carried out using finite difference procedures.[23] Small steps in potential, progressing across the experimental potential range, were simulated, and changes in the populations of all species were calculated accordingly. Simulations were assumed to have converged once decreasing the potential step size produced no further change in the response.

Results and Discussion

Figure 1 shows the voltammetry obtained when a film of *S. acidocaldarius* 7Fe ferredoxin, in contact with D_2O buffer−electrolyte at pD 5.3, is cycled twice at 2 V s^{-1} commencing from an oxidative poise (duration 30 s at −0.3 V). The potential region which is scanned (−0.30 to −0.87 V) engages two redox couples which have been assigned previously.[9,10] On the basis of bulk solution electrochemistry and spectroscopic methods, these are $[4Fe-4S]^{2+/+}$ (the voltammetric response of which is referred to as signal B′) and $[3Fe-4S]^{0/2-}$ (signal C′). Signal B′, with a reduction potential of −580 mV, has broad oxidation and reduction peaks[9,10] which are effectively obscured at this pH by the prominent peaks of signal C′, upon which we will now focus attention. These peaks are narrow, although the oxidative peak is somewhat broader than its reductive counterpart, and their areas correspond to two electrons, as referenced to signal A′ (the well-established one-electron $[3Fe-4S]^{+/0}$ couple) which can be observed on the same film at higher potential. The electroactive coverage is accordingly estimated at 6 pM cm^{-1}, i.e., approximating to an electroactive monolayer

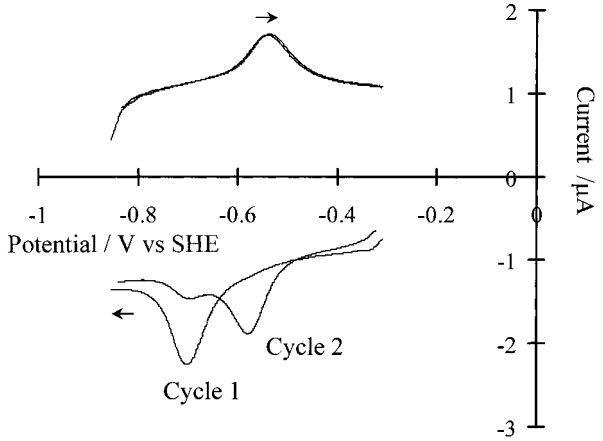

Figure 1. Cyclic voltammetry recorded in D_2O, for a film of *S. acidocaldarius* Fd formed at a PGE electrode, started from a 30 s oxidative poise, and conducted at a scan rate of 2 V s^{-1}. On the first cycle, a single reductive peak is observed at negative potential, and the separation relative to the subsequent oxidation peak is large. On the second cycle, the low-potential reduction peak is largely replaced by one at higher potential. The new redox couple shows much faster electron-transfer kinetics, as evidenced immediately by the greatly decreased peak separation. Conditions: 20 mM mixed buffer, pD 5.32, with 0.1 M NaCl and 200 μg ml^{-1} polymyxin B sulfate, temp 2 °C.

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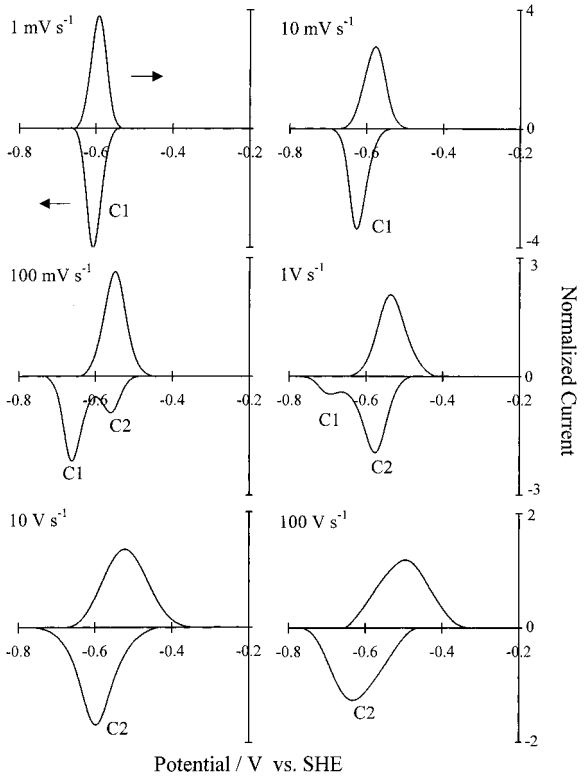

Figure 2. Background subtracted voltammograms recorded over a wide range of scan rate for a film of *S. acidocaldarius* Fd adsorbed at a PGE electrode, with D_2O electrolyte. In each case, the potential was poised at the reductive scan limit (sufficiently negative of the redox activity) for 30 s prior to scanning. Results are for scan rates from 0.001 to 100 V s^{-1} and show how peak C1 is replaced by peak C2 as the scan rate is increased. Conditions: pD 5.32, temp 2 °C, 20 mM mixed buffer with 0.1 M NaCl amd 200 μg ml^{-1} polymyxin B sulfate. Arrows shown in the upper plates indicate direction of scanning. The voltammetry has been corrected for background (electrode) currents and capacitance by baseline subtraction,[7,21a,22] and the peaks have been normalized to the current expected for a perfect Nernstian system, which displays a maximum current of 4 for a cooperative two-electron transfer.

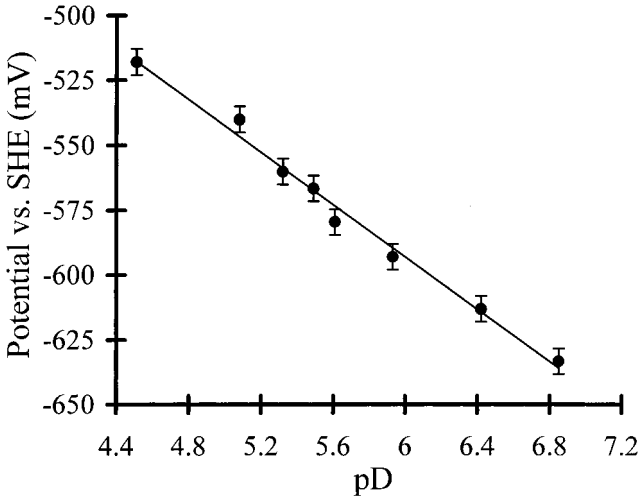

Figure 3. The pD dependence of the reduction potential of the fast redox couple (having reduction peak C2) observed for a film of *S. acidocaldarius* Fd. Conditions: 20 mM mixed buffer with 0.1 M NaCl and 200 μg ml^{-1} polymyxin B sulfate, temp 2 °C. The reduction potential was measured at 1 V s^{-1} in each case. The slope is −52 mV/pH which corresponds to 0.95 $H(D)^+$ per electron.

upon an idealized flat surface. The important observation is that on the second cycle the greater part of the signal C′

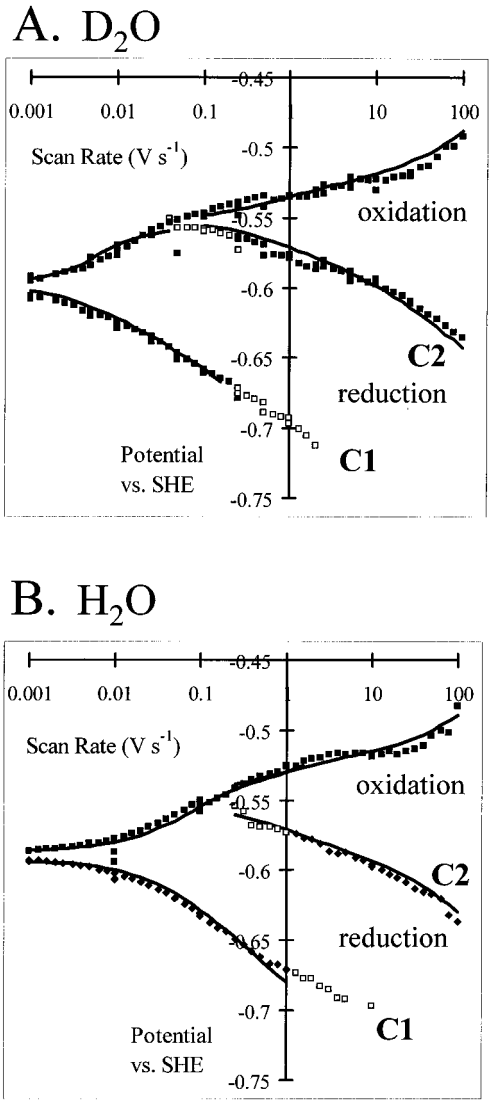

Figure 4. Voltammetric peak positions (oxidation and reduction components C1 and C2) as a function of scan rate, following a reductive poise in (A) D_2O and (B) H_2O, according to the data presented in Figures 2 and 5 (pH(D) = 5.3) and displayed in Scheme 1. Open squares indicate the positions of reductive peaks having the smaller amplitude. Errors in peak positions range from ±5 mV at low scan rate to ±10 mV at high scan rate. Also shown (as lines) are the predictions of the model using the parameters shown in Scheme 1.

reduction peak is shifted markedly to more positive potential, whereas the oxidation peak remains a single (but broader) entity. A second, faster redox couple has thus appeared. Excursions to higher potential showed that the A′ couple disappears concurrently with the appearance of the new peak, consistent with formation of a different and unusual (0) state unable to be oxidized directly to $[3Fe-4S]^+$.

Figure 2 shows background-corrected and normalized voltammograms obtained over a range of scan rates in D_2O electrolyte commencing instead from a reductive poise, which prearranges the [3Fe−4S] cluster to be in the all-Fe(II) "2−" level.[10] At low scan rates the voltammetry appears simple and reversible, but as the scan rate is increased, the reductive peak separates into two components, C1 and C2, ultimately transforming completely to the high-potential form, C2. As discussed below, this observation reflects the decreasing ability of the cluster to relax from its more active state during the increasingly shorter excursions to high potentials. Although broadening occurs with both peaks as the scan rate is increased, the narrow

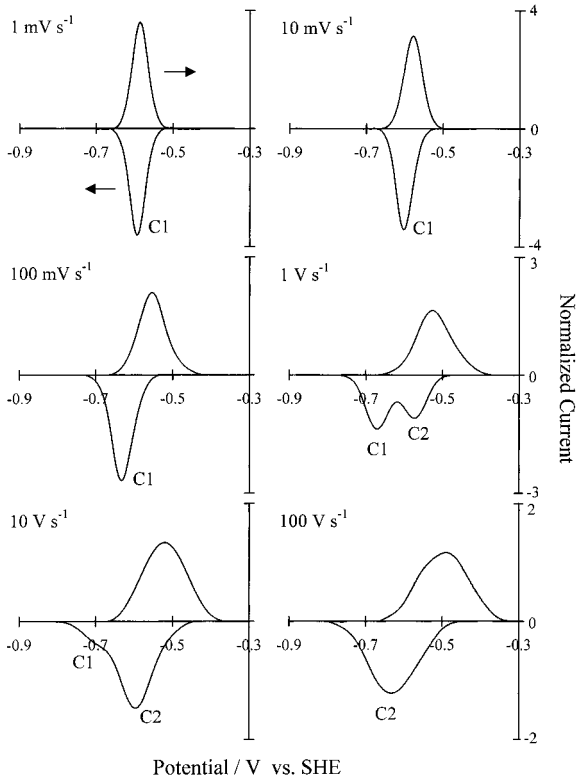

Figure 5. Background-subtracted voltammograms recorded for a film of *S. acidocaldarius* Fd at a PGE electrode, in H_2O. In each case, the potential was poised at the reductive scan limit (sufficiently negative of the redox activity) for 30 s prior to scanning. Results are for scan rates from 0.001 to 100 V s^{-1} and show how peak C1 is replaced by peak C2 as the scan rate is increased. Conditions: pH 5.32, temp 2 °C, 20 mM mixed buffer with 0.1 M NaCl amd 200 μg ml^{-1} polymyxin B sulfate. Arrows shown in the upper plates indicate direction of scanning. The voltammetry has been corrected for background (electrode) currents and capacitance by baseline subtraction,[7,21a,22] and the peaks have been normalized to the current expected for a perfect Nernstian system, which displays a maximum current of 4 for a cooperative two-electron transfer. Note that C2 does not appear until 1 V s^{-1}, where it contributes only ca. 50% of the current, whereas in D_2O, it is virtually the sole component at this scan rate.

half-height widths (45 mV at 1 mV s^{-1} and still <85 mV at 1 V s^{-1}) show that the two electrons are transferred cooperatively, i.e., addition of the second electron proceeds spontaneously.[24] As shown in Figure 3, the reduction potential of the new couple varies linearly with pD over the range 4.5−6.8, with the slope of −52 mV corresponding closely to a 1:1 H^+/e^- ratio, i.e., two protons transfer along with the two electrons. Significantly and in contrast with the initial formation of $[3Fe-4S]^{2-}$ from $[3Fe-4S]^0$, the kinetics are extremely fast. Figure 4A shows how peak positions (in D_2O) vary with scan rate, where the transition from "slow" (C1) to "fast" (C2) kinetics is clearly visible. At 100 V s^{-1}, the peak separation remains <150 mV.

Figure 5 shows voltammograms from the analogous experiment conducted in H_2O instead of D_2O, where it is clear that the new reduction peak (C2) becomes visible only at much higher scan rates (ca. 1 V s^{-1} instead of 0.1 V s^{-1}). The corresponding variation of peak positions with scan rate is presented in Figure 4B. Preliminary observations showed that a similar result is also obtained for *Azotobacter vinelandii* Fd I, which has little homology with *S. acidocaldarius* Fd.[25] In both systems, the new redox couple can be maintained

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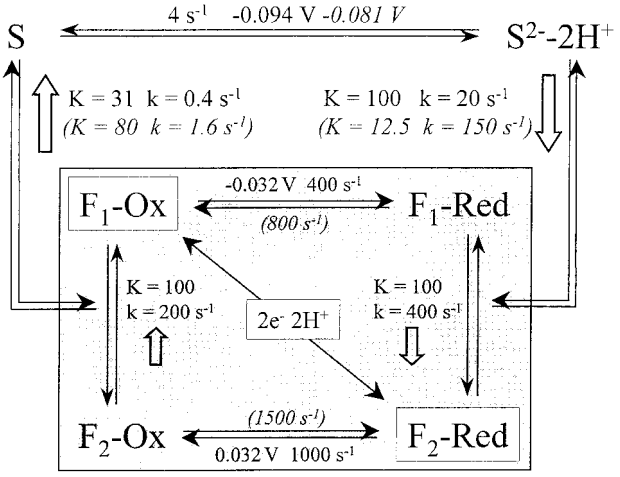
Scheme 1. Redox Reactions Occurring within the Two-Electron Transformation between "0" and "2−" Levels of the [3Fe−4S] Cluster[a]



[a] Slow and fast electron transferring species are represented by S and F forms, respectively. "S" is the well-characterized "0" level, formally comprising 2 Fe(III) and 1 Fe(II). The model requires that the 2-electron/2-proton reduction of S to give S^{2-}-$2H^+$ is slow, but that S^{2-}-$2H^+$ reverts to a more stable species, F-red, which can undergo a very fast 2-electron/2-proton process. To improve the fit, F-red and F-ox are each assumed to exist in two states (denoted 1 and 2, and perhaps reflecting some heterogeneity among adsorbed protein molecules) that have slightly different standard electrochemical rate constants. The fast redox couple is thus displayed as a "square scheme" instead of a simple linear redox process (see ref 7). F-ox relaxes back to the "resting state", S, if it is not rereduced rapidly (note retardation in D_2O). Values shown are potentials, equilibrium constants (*K*), and rate constants (*k*) used to give the best fit to the data (parentheses and italics indicating values for H_2O) with relevant directions being denoted by boxed arrows.

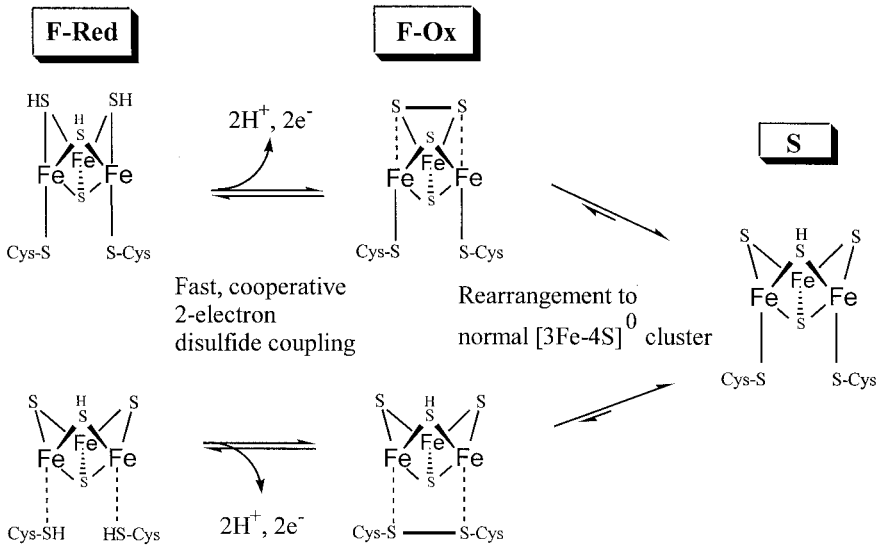
indefinitely at sufficiently fast scan rates, whereas slowing down the scan rate causes reappearance of C1.

From a kinetic standpoint, the results require that the $[3Fe-4S]^{2-}$ cluster (formally 3 Fe(II) and 4 S^{2-}) exists predominantly in a state poised to undergo a fast and cooperative two-electron/two-proton oxidation, with the product being a metastable "intermediate" which reverts to the normal form of $[3Fe-4S]^0$ unless rereduced rapidly by the electrode. The reactions are depicted empirically in Scheme 1, where "F" denotes fast- and "S" slow-reacting species (S being the normal form of $[3Fe-4S]^0$). Digital simulation[23] exploited the large difference in time scales between slow and fast processes. At slow scan rates, voltammetry was fitted using the major square (Scheme 1) to describe the trapping and escape of the cluster from the kinetically fast forms. At fast scan rates, use of the second square to model the fast $2e^-/2H^+$ transfer gave a superior fit to that of a simple linear reaction F-ox ↔ F-red. In all cases, the parameters obtained were a unique solution; note also the thermodynamic balance in Scheme 1, as required.

Figure 4 includes the fits obtained using the reduction potentials, equilibrium and rate constants, and standard (first-order) electrochemical Butler−Volmer rate constants (the rate of electron exchange when the electrode potential is held at the formal reduction potential) given in Scheme 1 (D_2O data are shown in normal type, and H_2O data are in italics). Reduction potentials shown in Scheme 1 are referenced to the value of the F_1-Ox ↔ F_2-Red diagonal transformation at a pD (pH) of

(25) As expected, the D15N mutant of *Av* Fd I, in which proton transfer to the [3Fe−4S] cluster is blocked (refs 6 and 7), shows no C′ signals at high scan rates.

Scheme 2. Two Options for Disulfide Coupling Reactions Involving Reduced (0) and Hyper-Reduced (2−) Levels of the [3Fe−4S] Cluster



5.3. Considering the large dynamic range; i.e., *5 orders of magnitude* in time scale, the resulting fits (particularly that obtained in D_2O) are excellent, with the main effect of H_2O being to shift the plot in the direction of higher scan rate.

The facts thus far established are that (a) the [3Fe−4S] cluster, once fully reduced (i.e., at the all-Fe(II) 2− level), is capable of undergoing an extremely rapid and cooperative two-electron/two-proton oxidation process; (b) the product of this oxidation is an unstable species which reverts to a stable form that displays only slow reduction; and (c) this reversion is retarded significantly in D_2O. Such facile two-electron/two-proton activity is not consistent with simple Fe(III,II)-based redox chemistry and leads us instead to propose disulfide coupling as the only reasonable explanation. Two options are shown in Scheme 2. These are that (a) two of the cluster μ_2-hydrosulfides deprotonate and undergo oxidative coupling to give a bridging disulfide S_2^{2-} species;[26] and (b) the Fe(II) subsites are ligated only weakly by cysteine, and two thiol-S atoms may approach each other sufficiently closely to give facile oxidative coupling.[27] Cross coupling between cysteine and sulfide is a third possibility. At this point, we stress that forms F_1 and F_2 are merely states suggested by the data to be distinguishable kinetically (at a marginal level); the difference between them may be due only to orientation, and no association with the differences between options (a) and (b) is implied.

Option (a) is particularly attractive as it accounts for the (thus far) unique ability of the [3Fe−4S] cluster to display this reactivity, whereas option (b) ought to be more generally observed among Fe−S clusters and other active sites that possess multicysteine ligation. Many examples of S_2^{2-} complexes are known, and of particular note are those in which the ligand is coordinated in a metal cluster.[26] Typical −S−S− bond distances in these (μ-S_2^{2-}) complexes lie in the region of 201−209 pm, whereas the center-to-center distance between μ_2-S atoms in the fully oxidized $[3Fe-4S]^+$ cluster ranges between 367 and 382 pm, as gauged from the recent high-resolution structure of *A. vinelandii* Fd I.[28] However, we need only invoke the expected pliability of the *nido*-type [3Fe−4S] cluster,[29] in which case it is likely that the necessary modification may be particularly easy to accommodate in the electron-rich 2− level which has not so far been structurally characterized.[10]

In each case, formation of a −S−S− bridge within a preorganized assembly would have interesting consequences: (a) the coupling process is entropically constrained and should thus have a negative reduction potential[27] and (b) electron transfer should be extremely fast. Both properties are characteristic of this system; indeed, the apparent standard electrochemical rate constant for the $2H^+(D^+)/2e^-$ reaction equates to a half-life ≤1 ms *even at zero electronic driving force.* Notably, the major consequence of using D_2O in place of H_2O is to retard rearrangement (internal electron transfer) which restores the [2Fe(III) + Fe(II)] "resting state" (the normal $[3Fe-4S]^0$ cluster). A likely explanation for this kinetic isotope effect, which equates to a factor of approximately 4, is that N−H(D)···S hydrogen-bonding contacts must be ruptured to accommodate the rearrangement.[30] As far as we could judge from the simulation, rates of the fast $2H(D)^+/2e^-$ transfer itself are marginally slower in D_2O versus H_2O (up to 2-fold) and thus proton-transfer kinetics are unlikely to be rate-determining.

Is there a biological role for this chemistry? The inescapable conclusion is that Fe−S clusters are capable of executing very

(26) For examples of metal cluster complexes with coordinated S_2^{2-}, see: Wei, C. H.; Dahl, L. F. *Inorg. Chem.* **1979**, *18*, 3060−3064. Young, C. G.; Kocaba, T. O.; Yan, X. F.; Tiekink, E. R. T.; Wei, L.; Murray, H. H., III; Coyle, C. L.; Stiefel, E. I. *Inorg. Chem.* **1994**, *33*, 6252−6260. Coyle Lee, C.; Halbert, T. R.; Pan, W.-H.; Harmer, M. A.; Wei, L.; Leonowicz, M. E.; Dim, C. O. B.; Miller, K. F.; Bruce, A. E.; McKenna, S.; Corbin, J. L.; Wherland, S.; Stiefel, E. I. *Inorg. Chim. Acta* **1996**, *243*, 147−160. Sokolov, M. N.; Hernandez-Molina, R.; Elsegood, M. R. J.; Heath, S. L.; Clegg, W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1997**, 2059−2065 and references therein.

(27) See: Gilbert, H. F. In *Bioelectrochemistry of Biomacromolecules* (Vol. 5 of *Bioelectrochemistry of Macromolecules*); Lenaz, G., Milazzo, G., Eds.; Birkhauser Verlag: Basel, 1997; pp 256−324. Reduction potentials for cysteine disulfide transformations in proteins are generally calculated from the equilibrium constant observed with the glutathione/glutathiol redox system ($E^0 = -0.25$ V at pH 7). The lowest value reported in this work is −0.46 V ($K_{ox} = 1.1 \times 10^7$ M) for a cysteine pair in bovine trypsin inhibitor. More negative potentials (larger K_{ox}) are expected for even more preorganized (entropically constrained) disulfide systems.

(28) Stout, C. D.; Stura, E. A.; McRee, D. A. *J. Mol. Biol.* **1998**, *278*, 629−639.

(29) By describing the [3Fe−4S] structure as *nido* (borane cluster classification) we allude to the greater pliability and reactivity gained by loss of one vertex, with respect to the corresponding *closo* species (e.g., [4Fe−4S]) See, for example: Greenwood, N. N.; Earnshaw, A. In *Chemistry of the Elements*; Pergamon Press, 2nd Ed. 1997.

(30) Hydrogen bonding between N−H and S-atoms is believed to play an important role in determining the properties of Fe−S clusters in proteins. See, for example: Carter, C. W., Jr. In *Iron−Sulfur Proteins*; (Lovenberg, W., Ed.; Academic Press: New York; Vol. 3, pp 157−204.

rapid, coupled transport of two electrons and two protons at potentials above −650 mV at pH 7 (and at considerably more positive potentials at lower pH). The idea that iron−sulfur clusters can function in this way and might, as we have proposed, carry out exquisitely optimized −S−S− /−SHHS− transformations may have wider relevance.[31] Indeed, superclusters in enzymes such as nitrogenase are implicated in coupled proton/electron transfer, yet few mechanistic details are established.[2,3] Protein-film voltammetry is a powerful method for accessing such species, enabling the generation and interrogation of short-lived intermediates[7,14,21a] which are difficult to obtain in any form appropriate for spectroscopic analysis. Our experiments confirm that oxidation of the hyper-reduced cluster $[3Fe-4S]^{2-}$ is extremely facile, a problem we encountered when isolating samples for EPR and MCD.[10] The short-lived oxidized species F-ox, possibly bearing a disulfide group as proposed, will clearly be even more difficult to characterize spectroscopically, but efforts are now under way. In this respect, the use of D_2O to prolong the lifetime of a transient species offers a lesson that may prove useful in studies of other systems where extensive hydrogen bonding controls reactivity of the active site.

(31) Other reports of direct participation of S-atoms in Fe−S cluster redox chemistry concern one-electron oxidations of cysteine to give S-based radicals. See, for example: Hu, Z. G.; Jollie, D.; Burgess, B. K.; Stephens, P. J.; Münck, E. *Biochemistry* **1994**, *33*, 14475−14485. Staples, C. R.; Gaymard, E.; Stritt-Etter, A.-L.; Telser, J.; Hoffman, B. M.; Schürmann, P.; Knaff, D. B.; Johnson, M. K. *Biochemistry* **1998**, *37*, 4612−4620.

Acknowledgment. This research was supported by the United Kingdom EPSRC (grant GR/J84809). We thank Dr. Dirk Heering for helpful discussions and Professor Barbara Burgess (University of California, Irvine) for making available a sample of *A. vinelandii* ferredoxin I.

Note Added in Proof. During processing of this manuscript, a paper appeared showing that the one-electron oxidized form of the nitrogenase [8Fe−7S] cluster (P^{1+}) is stabilized with respect to P^{2+} and P^0 as the pH is lowered to 6 (Lanzilotta, W. N.; Christianson, J.; Dean, D. R.; Seefeldt, L. C. *Biochemistry* **1998**, *37*, 11376−11384).

JA981693A