Properties of Novel Iron–Sulphur Proteins formed by the Introduction of a Synthetic Iron–Sulphur Cluster into Bovine Serum Albumin and Bovine Insulin

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The interactions of the proteins bovine insulin and bovine serum albumin with the water-soluble cluster $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ (1) are described. The proteins were found to stabilise the cluster to air. Changes in the visible absorption and circular dichroism spectra indicated that inclusion of the cluster into the protein had occurred. Anodic shifts of redox potential between 170 and 220 mV were observed for the included clusters using cyclic voltammetry. The redox potentials of the bovine serum albumin iron–sulphur cluster exhibited a pH dependence of −63 mV per pH unit (one proton dependence) in the pH range 5—8. Beyond pH 8, a pH dependence of up to −235 mV per pH unit was observed in accordance with four reversible thiolate–hydroxide species exchange reactions.

Attempts to establish efficient catalytic cycles utilising synthetic iron–sulphur clusters have been unsuccessful because of the inherent instability of the clusters to oxidation.[1,2] The process whereby dioxygen reacts with synthetic and natural iron–sulphur proteins is not fully understood. Solvent–ligand exchange to give unstable solvent-susbstituted products [3] or direct oxidation of the core units followed by oxidation of the mercaptide ligands to give disulphides are mechanisms that have been proposed.[3] A variety of compounds [*e.g.* 2,2′-bipyridyl, ethylenediaminetetra-acetic acid (H_4edta), diethylaminoethylcellulose, 5-sulphosalicylic acid, and bovine serum albumin] [4,5] appear to protect hydrogenase from dioxygen inactivation, but the mechanism is not known. To our knowledge, there are no reports of synthetic iron–sulphur clusters that are stable to dioxygen.

We report some preliminary results on the stabilisation of the water-soluble cluster $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ (1) to mild aerobic oxidation by use of aqueous protein solutions. We chose to examine the species (1) because of its solubility in aqueous solution [6,7] and its similar redox properties [$E_{\frac{1}{2}}$ − 0.827 V *vs.* saturated calomel electrode (s.c.e.), pH 10.1] compared with those of natural ferredoxin iron–sulphur proteins [$E_{\frac{1}{2}}$ *ca.* −0.645 V *vs.* s.c.e.].[6] The proteins bovine insulin (chain A and B, molecular weight *ca.* 5 800) and bovine serum albumin (bsa) (molecular weight *ca.* 69 000) were chosen as both contain disulphide moieties (three and 18 disulphide linkages, respectively) that may provide sulphydryl groups on reduction. The proteins should then be capable of undergoing ligand exchange with the synthetic cluster (1) to form a protein-co-ordinated iron–sulphur cluster. The effect of proteins in stabilising clusters against oxidation may originate from their action as a kinetic barrier to dioxygen diffusion or alternatively *via* NH · · · S hydrogen bond formation.[8] We also wished to compare our results with those of Lovenburg and McCarthy,[9] who reported the preparation of an iron-containing protein from the addition of $(NH_4)_2SO_4{\cdot}FeSO_4{\cdot}6H_2O$ and Na_2S to bsa–2-mercaptoethanol mixtures. Although only characterised by visible absorption and circular dichroism spectroscopy, these proteins have been utilised in hydrogenase modelling experiments [10-12] with limited success. Our initial objective was to stabilise synthetic clusters to dioxygen. During the course of this study, we have, however, found that the disulphide-containing proteins can undergo ligand exchange with the synthetic cluster (1). The resulting clusters exhibit redox potentials that are shifted anodically by as much as 220 mV compared with (1) at pH 9.2. Reduction potentials of bsa solutions of (1) were found to vary by over 500 mV in the pH range 5—9.75, thus implicating the involvement of protons in electron transfer. It is proposed that two protons are associated with the reduced cluster in bsa solutions.

Experimental

General.—Bovine serum albumin was obtained from Sigma Chemical Co. It was purified by treatment with $Na_2[H_2edta]$ (0.09 mmol bsa : 2.0 mmol $Na_2[H_2edta]$ in 3 cm^3 H_2O) to remove trace metals, and eluted through a Sephadex (G25) column [2.5 cm (diameter) × 35 cm (length)] equilibrated in mercaptide buffer (0.1 mol dm^{-3} $HSCH_2CH_2CO_2H$ titrated with 0.1 mol dm^{-3} KOH to pH 9.2). The resulting bsa solution was dialysed *vs.* mercaptide buffer (pH 9.2) and clarified by centrifuging at 30 000 r.p.m. for 30 min and then concentrated using an Amicon ultrafiltration cell (PM 10 membrane) to give 0.02 mol of bsa in mercaptide buffer solution (pH 9.2). The bovine insulin (zinc content *ca.* 0.5%) was also purchased from Sigma Chemical Co. and used without further purification.

The iron–sulphur complex $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ was prepared as a salt with six cations comprising $Na_5[NBu_4]$ and five molecules of *N*-methylpyrrolidin-2-one (C_5H_9NO) of crystallisation by the procedure described by Carrell and co-workers.[6] The purity of the compound was established by comparison of the electronic spectrum: $\lambda_{max.}$ 300 (lit.,[6] 300 nm), ε 2 × 10^4 (lit.,[6] 1.98 × 10^4 dm^3 mol^{-1} cm^{-1}); λ_{sh} 400 (lit.,[6] 400 nm), ε 1.6 × 10^4 (lit.,[6] 1.63 × 10^4 dm^3 mol^{-1} cm^{-1}) and from cyclic voltammetry data * ($E_{pc}^{6-/7-}$ −0.7, $E_{pa}^{7-/6-}$ −0.36, $E_{pc}^{7-/8-}$ −1.1, and $E_{pa}^{8-/7-}$ −0.7 V). The mercaptide buffer was used to suppress aquation of the co-ordinated mercaptopropionate ligand. The ^1H n.m.r. spectrum of $Na_5[NBu_4]$-$[Fe_4S_4(SCH_2CH_2CO_2)_4]$ recorded at 360 MHz contained isotropically shifted SCH_2- proton resonances, $\delta(^1H)$ −11.86 p.p.m. [$CH_2CO_2^-$ protons are thought to be located in the range $\delta(^1H)$ 0—10 p.p.m. but were obscured by solvent ($HSCH_2CH_2CO_2^-$, H_2O, and *N*-methylpyrrolidin-2-one) and cation peaks (NBu_4^+)]. Initially all preparations were made up under rigorously air-free conditions, using syringe and standard Schlenk techniques.

Interactions of Protein Solutions with $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$.—(1) *Bovine serum albumin.* (*a*) *Untreated protein.* Bovine serum albumin purified as described in the preceding section (0.5 g, 0.007 mmol) dissolved in mercaptide buffer

* Cyclic voltammetry was performed at a platinum-wire working electrode with $LiClO_4$ (0.05 mol dm^{-3}) electrolyte in mercaptide buffer at pH 9.2. Potentials are recorded *vs.* s.c.e.

Table. Cyclic voltammetry data for the $2^-/3^-$ and $3^-/4^-$ redox couples of Fe_4S_4 clusters in mercaptide buffer (0.1 mol dm^{-3}, pH 9.2) [a]

Complex	E_{pc} (V) $2^-/3^-$	E_{pa} (V) $3^-/2^-$	$E_{pc} - E_{pa}$ (mV) $2^-/3^-$	E_{pc} (V) $3^-/4^-$	E_{pa} (V) $4^-/3^-$	$E_{pc} - E_{pa}$ (mV) $3^-/4^-$
$[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$	−0.7	−0.38	320	−1.1	−0.78	320
$[Fe_4S_4(SCH_2CH_2OH)_4]^{2-}$ [b]	−0.78	−0.65	130	−0.78	−0.65	130
$[Fe_4S_4]^{2-}$–bsa	−0.53	−0.35	180	−0.8		
$[Fe_4S_4]^{2-}$–bsa–$NaBH_4$	−0.48	−0.27	210	−0.8		
$[Fe_4S_4]^{2-}$–bsa–$Na_2S_2O_4$	−0.6	−0.38	220	−0.875	−0.8	75
$[Fe_4S_4]^{2-}$–insulin	−0.5	−0.325	175			
C. pasteurianum Fd_{ox} [c,d]	−0.67					
Desulphovibrio desulphuricans Fd_{ox} [c,e]	−0.572					
Bacillus stearothermophillus Fd_{ox} [c,f]	−0.522					

[a] Cyclic voltammograms were carried out using a platinum-wire working electrode and E_{pa}, E_{pc} values were relative to a saturated calomel electrode (0.05 mol dm^{-3} $LiClO_4$ supporting electrolyte), platinum wire counter electrode, scan rate 50 mV s^{-1}. [b] Cyclic voltammogram carried out using 40 mmol dm^{-3} $HSCH_2CH_2OH$. [c] C. L. Hill, R. H. Holm, L. E. Mortenson, and J. Renaud, *J. Am. Chem. Soc.*, 1977, **99**, 2549. [d] $E_{\frac{1}{2}}$ value relative to s.c.e., pH 8.4. [e] $E_{\frac{1}{2}}$ value relative to s.c.e., pH 7.0. [f] $E_{\frac{1}{2}}$ value relative to s.c.e., pH 8.0.

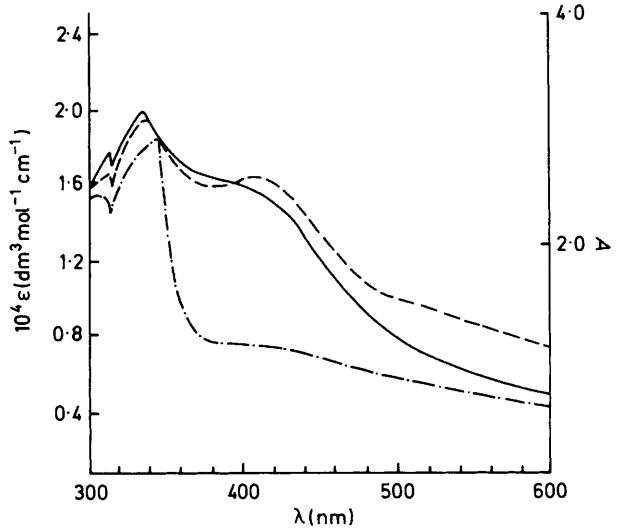


Figure 1. Electronic spectra of Fe_4S_4–protein solutions: Fe_4S_4–bsa in Ar (——), F_4S_4–bsa in O_2 (16 h) (— — —), and Fe_4S_4–bsa–$Na_2S_2O_4$ in Ar (— · — · —)

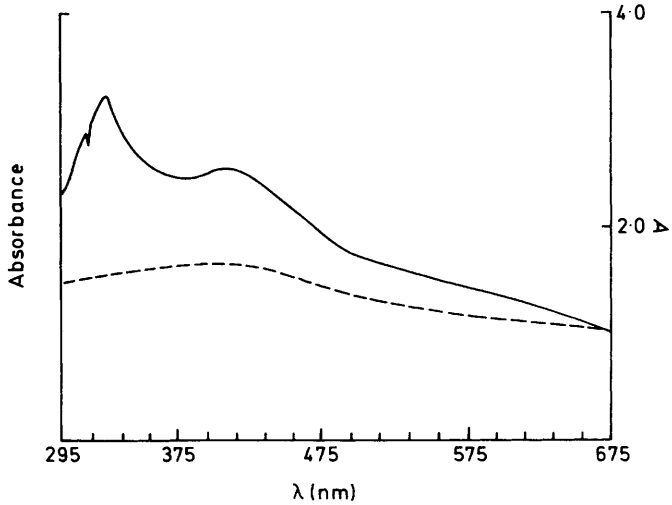


Figure 2. Electronic spectra of Fe_4S_4–bsa–$NaBH_4$ solution under Ar (— — —) and exposed to O_2 (——) (4 h)

(10 cm^3, pH 9.2) was added to a solution of $Na_5[NBu_4][Fe_4S_4(SCH_2CH_2CO_2)_4]$ (0.113 g, 0.07 mmol) in mercaptide buffer (2 cm^3, pH 9.2) to give a brown-black solution. A portion (2 cm^3) of the mixture was separated by anaerobic gel filtration on a column [2.5 cm (diameter) × 35 cm (length)] of Sephadex G25 (superfine), to separate protein from free iron–sulphur cluster and the eluted brown solution was concentrated by ultra-filtration as before to give the Fe_4S_4–bsa complex. Iron was analysed by the method of Zabinski and co-workers [13] as modified by Crutcher and Geary [14] yielding a cluster : protein ratio of 1 : 8. The visible absorption spectrum displayed $\lambda_{max.}$ at 320 nm (ε 2.12 × 10^4 dm^3 mol^{-1} cm^{-1}) and λ_{sh} at 410 nm (ε 1.6 × 10^4 dm^3 mol^{-1} cm^{-1}) as shown in Figure 1. The stability of the Fe_4S_4–bsa cluster * on exposure to air was monitored by visible absorption spectroscopy and cyclic voltammetry at regular time intervals over a period of seven days.

(*b*) *Treatment with sodium dithionite.* In a typical experiment, bsa (0.5 g, 0.007 mmol) in mercaptide buffer (10 cm^2, pH 9.2) was treated with an excess of $Na_2S_2O_4$ (0.138 g, 0.07 mmol) in 5 cm^3 of mercaptide buffer and the mixture was stirred for 1 h under argon. A solution containing $Na_5[NBu_4][Fe_4S_4(SCH_2CH_2CO_2)_4]$ (0.115 g, 0.076 mmol) in mercaptide buffer (2 cm^3, pH 9.2) was then added with stirring to the bsa–$Na_2S_2O_4$ mixture to give a black mixture. Following elution through the Sephadex (G25 superfine) column previously described, with mercaptide buffer containing $Na_2S_2O_4$ (0.077 mmol), the remaining black solution was centrifuged (20 000 r.p.m., 30 min) to remove traces of a fine black precipitate. The visible absorption spectrum of the mixture (λ_{sh} 410 nm, ε 7 980 dm^3 mol^{-1} cm^{-1}) is shown in Figure 1 and the redox potentials are listed in the Table.

(*c*) *Treatment with sodium tetrahydroborate.* Bovine serum albumin (0.35 g, 0.005 mmol) in mercaptide buffer (10 cm^3, pH 9.2) was treated with $NaBH_4$ solution (0.002 g, 0.05 mmol in 1 cm^3 of mercaptide buffer) which resulted in some foaming of the protein component. After stirring for 1 h, the mixture was treated with $Na_5[NBu_4][Fe_4S_4(SCH_2CH_2CO_2)_4]$ (0.77 g, 0.51 mmol) in mercaptide buffer (2 cm^3) to give a black solution which was eluted from Sephadex G35 (superfine) as before and centrifuged (20 000 r.p.m., 30 min). The visible absorption spectrum after exposure to air is shown in Figure 2 and the redox potentials are listed in the Table.

(2) *Bovine insulin.* Bovine insulin (chain A and B) from bovine pancreas (0.2 g, 0.0345 mmol) was dissolved in mercaptide buffer (5 cm^3, pH 9.2) and treated with $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ (0.052 g, 0.034 mmol) in mercaptide buffer

* In this context, the notations Fe_4S_4–bsa and Fe_4S_4–insulin refer to compound (1) in which one or more ligands have been exchanged with cysteine residues of the protein.

(5 cm^3, pH 9.2) to give a dark brown solution. After stirring under argon for 1 h the mixture was eluted from Sephadex G25 (superfine) as before and centrifuged (20 000 r.p.m., 30 min) to give a brown solution; $\lambda_{max.}$ at 330.0 nm (ε 2.28 × 10^4 dm^3 mol^{-1} cm^{-1}) and λ_{sh} at 410.0 nm (ε 1.645 × 10^4 dm^3 mol^{-1} cm^{-1}). The visible absorption spectrum (Figure 3) and cyclic voltammetry data are shown in the Table.

(3) *Ligand exchange with thiophenol; core extrusion experiments.* Extrusion of the Fe_4S_4–protein complexes after exposure to air (2 d) was performed by the method of Que and co-workers.[15] In a typical experiment, an eluted solution of Fe_4S_4–insulin (pH 9.2) was adjusted to pH 8.6 by addition of 2-amino-2-(hydroxymethyl)propane-1,3-diol buffer (20 mmol dm^{-3}) and HCl (0.1 mol dm^{-3}). Dimethyl sulphoxide (dmso) was then added to a final concentration of 80% (v/v) and the visible absorption spectrum was recorded under argon. The spectrum was typical of $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ ($\lambda_{max.}$ 300 nm, ε 2.0 × 10^4 dm^3 mol^{-1} cm^{-1}; λ_{sh} 400 nm, ε 1.63 × 10^4 dm^3 mol^{-1} cm^{-1}). An excess of thiophenol (0.4 μl of a stock solution containing 0.4 cm^3 of thiophenol in 10 cm^3 of mercaptide buffer) was added to the anaerobic Fe_4S_4–insulin–dmso solution and the colour of the resulting solution changed from brown to burgundy giving an absorption maximum typical for $[Fe_4S_4(SPh)_4]^{2-}$ ($\lambda_{max.}$ 447.5 nm, ε 1.7 × 10^4 dm^3 mol^{-1} cm^{-1}).

Physical Measurements.—The stability of protein-bound cluster to dioxygen was monitored by spectral changes in the visible absorption spectra ($\lambda_{max.}$ 405—415 nm) on a Beckman DU8 spectrometer. Circular dichroism (c.d.) spectra were recorded on a Cary model 61 spectrometer. ^1H N.m.r. spectra were recorded at 30 °C in D_2O at 360 MHz using a Bruker WH360 spectrospin spectrometer and chemical shifts were determined relative to $SiMe_4$ internal standard.

Electrochemical measurements were carried out by cyclic voltammetry using a PAR model 173 potentiostat in conjunction with a model 175 universal programmer equipped with a model 379 coulometer. Results were recorded using a Houston Omnigraphic 2 000 *x,y*-plotter. The cell was equipped with a side arm for evacuation and contained a working electrode (glassy carbon, platinum wire or tin oxide coated glass), an aqueous saturated calomel reference electrode and a platinum counter electrode. The dependence of reduction potential upon pH was monitored at 25 °C by cyclic voltammetry using $LiClO_4$ (0.05 mol dm^{-3}) as supporting electrolyte. The pH was adjusted with small additions of a mixture of KOH (0.1 mol dm^{-3}) and $HSCH_2CH_2CO_2H$ (0.1 mol dm^{-3}) and added separately.

Ultracentrifuge analyses were performed using a Beckman model 'E' analytical ultracentrifuge. The sedimentation-velocity method was used, movement of the boundary being recorded by schlieren optics at 56 000 r.p.m. and 20 °C in thiolate buffer.

Results and Discussion

The visible absorption spectra of chromatographed Fe_4S_4–protein solutions were found after exposure to air to be similar to those of *C. pasteurianum* ferredoxin (oxidised) (Fd_{ox}) and the bsa–iron–sulphide complex of Lovenburg and McCarthy [9] (Figures 1 and 3). The change in the visible absorption spectrum on exposure to air was more rapid for Fe_4S_4–insulin than for Fe_4S_4–bsa solutions. Since the visible absorption spectra of iron–sulphur proteins are rather insensitive to the iron–sulphur protein environment, the circular dichroism (c.d.) spectra were compared. Examination of the c.d. spectra (Figure 4) of solutions of bsa and insulin iron–sulphur cluster complexes clearly indicates that the

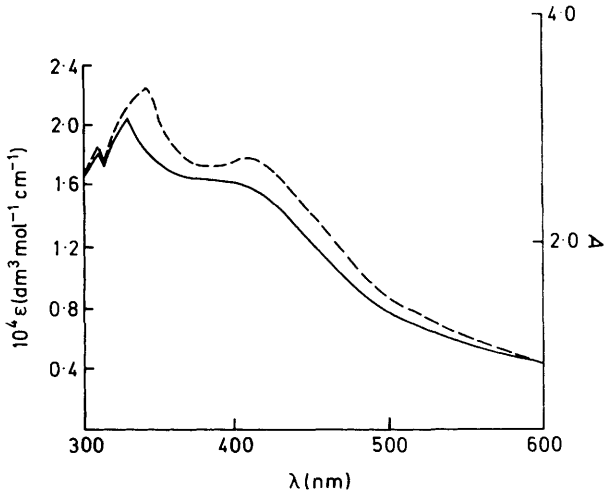


Figure 3. Electronic spectra of Fe_4S_4–insulin under Ar (——) and exposed to O_2 (8 h) (— — —)

Fe_4S_4 core unit is bound to the protein since (1) alone does not exhibit a c.d. spectrum and bsa only exhibits c.d. absorption in the region below 300 nm. The results indicate different environments for the cluster in both bsa and insulin solutions. Qualitatively the spectra of Fe_4S_4–bsa solution and that reported for the bsa–iron–sulphur complex [9] are identical, showing comparably, intense and detailed c.d. spectra (positive maxima at 308, 385, 425 nm, a positive minimum at 400, and negative maxima at 345 and 475 nm). It is noteworthy that the c.d. spectrum of Fe_4S_4 solutions containing bsa and $NaBH_4$ (as reductant) that have been exposed to dioxygen showed qualitative similarities in peak maxima and minima to oxidised bacterial ferrodoxin (*C. pasteurianum*).

When the Fe_4S_4–bsa solution was treated with dmso, under argon, the visible absorption spectrum resembled that of (1), the iron–sulphur starting material, whilst the c.d. spectrum was indicative of the mononuclear complex, bsa–Fe^{II} [9] made from the addition of $[NH_4)_2SO_4{\cdot}FeSO_4{\cdot}6H_2O$ to bsa–2-mercaptoethanol. This observation suggests that the addition of dmso gives rise to denaturation of the Fe_4S_4–bsa complex to give a mixture of $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ (c.d. inactive) and a protein-bound mononuclear Fe^{II} species (c.d. active) which may be formed during dissociation of the Fe_4S_4–bsa complex and subsequent disruption of part of the unbound cluster. Subsequent addition of thiophenol to the reaction mixture yielded the characteristic u.v.–visible spectrum for $[Fe_4S_4(SPh)_4]^{2-}$ (λ_{sh} 447.5 nm, ε 17 700 dm^3 mol^{-1} cm^{-1}),[15] thus indicating that some core extrusion had taken place. During these studies, it was found that chain B insulin (two cysteine groups) failed to co-ordinate to the iron–sulphur complex, as evidenced by the structureless c.d. spectrum, by the fact that the visible absorption spectrum was unchanged for $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$, and by the comparative instability of the insulin chain B complex to dioxygen.

Stabilisation of a Water-soluble Cluster to Dioxygen.—The inclusion of preformed iron–sulphur clusters in disulphide containing proteins may be explained by the random reduction of S–S linkages by the mercaptide buffer (pH 9.2) on standing to give free sulphydryl groups which then undergo ligand exchange with the cluster SR groups. It has been suggested [9] that only a certain percentage (*i.e.* 44%) of the disulphide residues contained by higher molecular weight proteins, such as bsa, are accessible to cleavage and that the

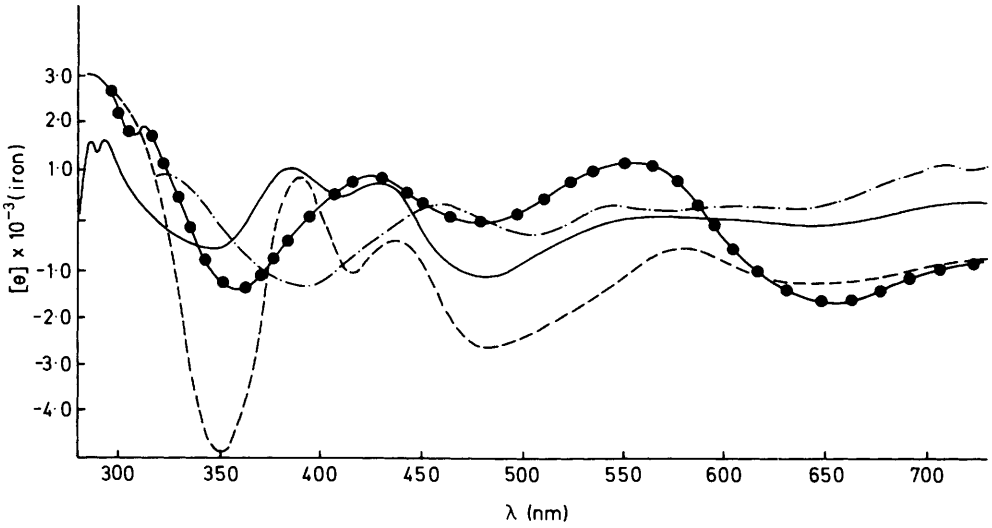


Figure 4. Circular dichroism spectra of Fe_4S_4–protein solutions: Fe_4S_4–bsa solution in O_2 (24 h) (——), Fe_4S_4–bsa in 80% dmso (v/v) (— — —), Fe_4S_4–insulin in O_2 (8 h) (— · — · —), and Fe_4S_4–bsa–$NaBH_4$ in O_2 (4 h) (—●—●—). [θ] is the molar ellipticity (° cm^{-2} dmol iron); [θ] = $\theta M/10\,lc$, where M = gram weight of iron, c = concentration of iron (g cm^{-3}), and l = pathlength (dm)

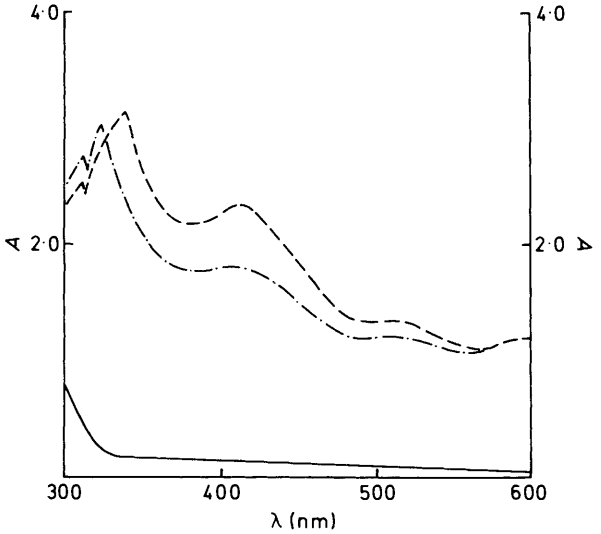


Figure 5. Electronic spectra of Fe_4S_4–protein solutions exposed to O_2 for 7 d in mercaptopropionate buffer (pH 9.2): $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ in O_2 (——), Fe_4S_4–bsa in O_2 (— · — · —), and Fe_4S_4–insulin in O_2 (— — —)

rest remain buried within hydrophobic regions of the protein. Individual molecules may also undergo different degrees of reduction producing binding sites suitable for inclusion of clusters on only a small proportion of molecules. The limited availability of accessible sulphydryl groups on bsa may explain why the iron analysis indicates only one cluster bound per eight protein molecules.

The air stability of the included clusters was monitored by investigating the loss of absorbance in the region 405—415 nm at pH 9.2 (Figure 5) since this broad peak is characteristic of the Fe_4S_4 chromophore (a peak at 320—330 nm was noted for both the mononuclear bsa–Fe^{II} complex [9] and the Fe_4S_4–bsa species). As shown in Figure 6, both bsa and insulin (chains A and B) considerably stabilise the cluster to aerobic decomposition. For example, extrapolation of the curves in Figure 6 gives half-lives ($t_{\frac{1}{2}}$) in the range 9—28 d for Fe_4S_4–protein complexes compared with the $t_{\frac{1}{2}}$ of 21 h for (1) in the absence of protein. Similar decomposition effects have been reported for the stabilisation of hydrogenase to dioxygen by diethylaminoethylcellulose, H_4edta, and bsa.[4,5] The cyclic voltammograms and c.d. spectra of the protein solutions were unchanged even after a 48 h exposure to dioxygen at 20 °C.* This result is contrary to the work of Lovenberg and McCarthy [9] who claimed that both bsa–Fe^{II} and bsa–iron–sulphide chromophores decomposed steadily at room temperature, but were stable for up to 1 h at 2 °C. However Okura and co-workers [12] found that a bsa–iron–sulphide complex was still intact after a 44 h reaction to evolve hydrogen anaerobically.

The protective influence of these proteins on irreversible FeS cluster oxidation may be considered to arise from the incorporation of the cluster within the microenvironment of the protein *via* both ligand exchange with cysteine residues of the protein and strong adsorption of the cluster to the polyionic surfaces of the proteins by electrostatic attractive interactions. It is also possible for the cluster anion to interact appreciably with protein amino-groups by NH · · · S hydrogen bond formation, thus altering the stabilisation energy of the cluster to oxidation *via* mercaptide disulphide formation.[16] It is significant that *X*-ray structural studies of FeS proteins indicate that they contain several NH · · · S hydrogen bonds, and molecular orbital calculations have shown that hydrogen bonding of this nature can have a substantial effect on the stabilisation of negative charge on the iron–sulphur core unit.[16] The redox properties of the Fe_4S_4–protein solutions support the view that interactions of the type discussed may be operative.

Influence of the Polypeptide Chain on the Redox Properties of Iron–Sulphur Clusters.—The cyclic voltammograms of Fe_4S_4–bsa † and Fe_4S_4–insulin complexes are shown in Figures 7

* Beyond 48 h, microbial activity is thought to take place. If solutions were too concentrated they formed gels (on cooling to −5 °C) into which the iron–sulphur clusters were homogeneously incorporated. The original solutions could not be obtained on rewarming.

† The nomenclature used for the protein associated clusters is the same as that described for synthetic clusters; $[Fe_4S_4]^{2-}$, $[Fe_4S_4]^{3-}$, and $[Fe_4S_4]^{4-}$. In the IUB–IUPAC nomenclature these are equivalent to the protein oxidation states $[4Fe^{-}4S]^{2+}$, $[4Fe^{-}4S]^{1+}$, and $[4Fe^{-}4S]^{0}$ respectively.

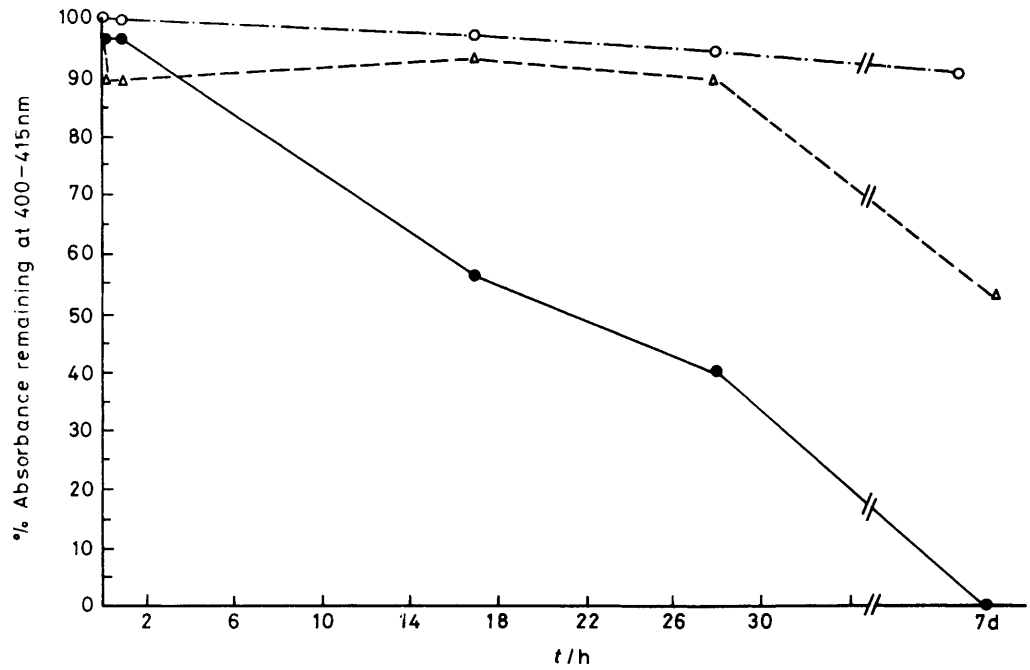


Figure 6. Change in absorbance for Fe_4S_4–protein exposed to dioxygen at 20 °C: Fe_4S_4–bsa (10 : 1) (— — —), Fe_4S_4 (——), and Fe_4S_4–insulin (1 : 1) (— · — · —)

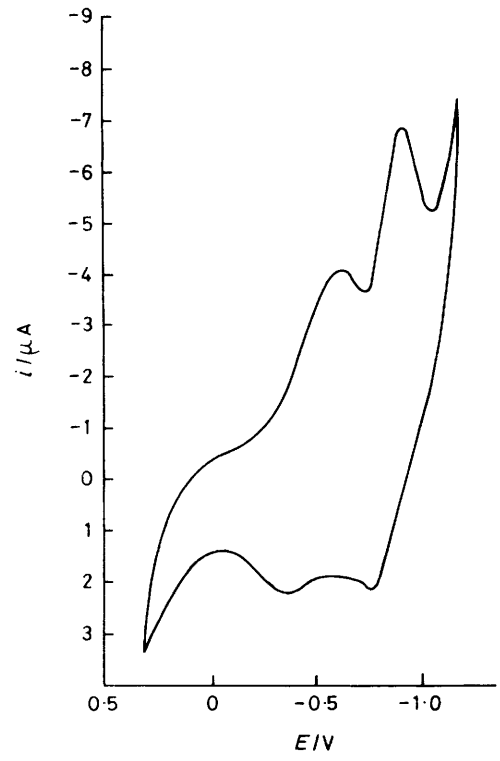


Figure 7. Cyclic voltammogram of a solution containing 5×10^{-3} mol dm^{-3} $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ in 5×10^{-4} mol dm^{-3} bsa in mercaptide buffer $[HSCH_2CH_2CO_2H$–KOH (0.1 mol dm^{-3}), pH 9.2] after elution through a Sephadex (G25) column using a platinum-wire working electrode, s.c.e., and a platinum counter electrode at a scan rate of 50 mV s^{-1} using $LiClO_4$ (0.05 mol dm^{-3}) as supporting electrolyte

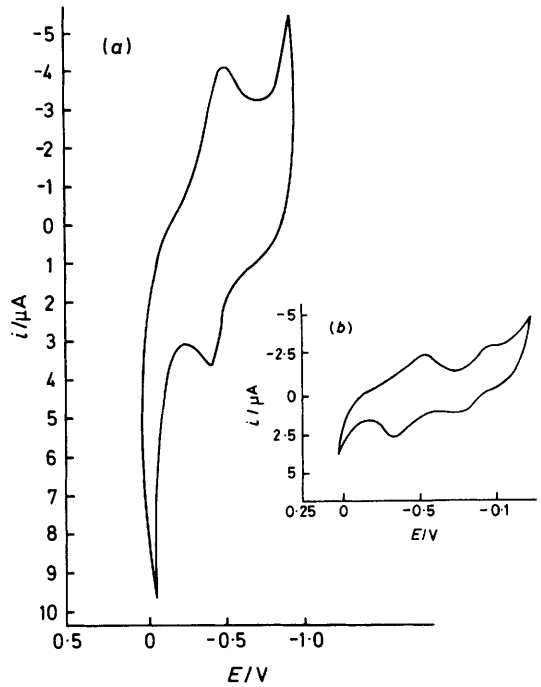


Figure 8. Cyclic voltammogram of a solution containing 5×10^{-3} mol dm^{-3} $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ and 5×10^{-3} mol dm^{-3} insulin in mercaptide buffer (pH 9.2, 0.1 mol dm^{-3}) after elution through a Sephadex (G25) column: (*a*) using a platinum-wire working electrode at scan rate 100 mV s^{-1} and (*b*) using a tin oxide glass electrode at scan rate of 100 mV s^{-1}. Both (*a*) and (*b*) were run using $LiClO_4$ (0.05 mol dm^{-3}) as supporting electrolyte

and 8 and the redox potentials are summarised in the Table. The first reduction peak (at pH 9.2) is thought to correspond to a quasi-reversible process involving $[Fe_4S_4]^{2-}/[Fe_4S_4]^{3-}$

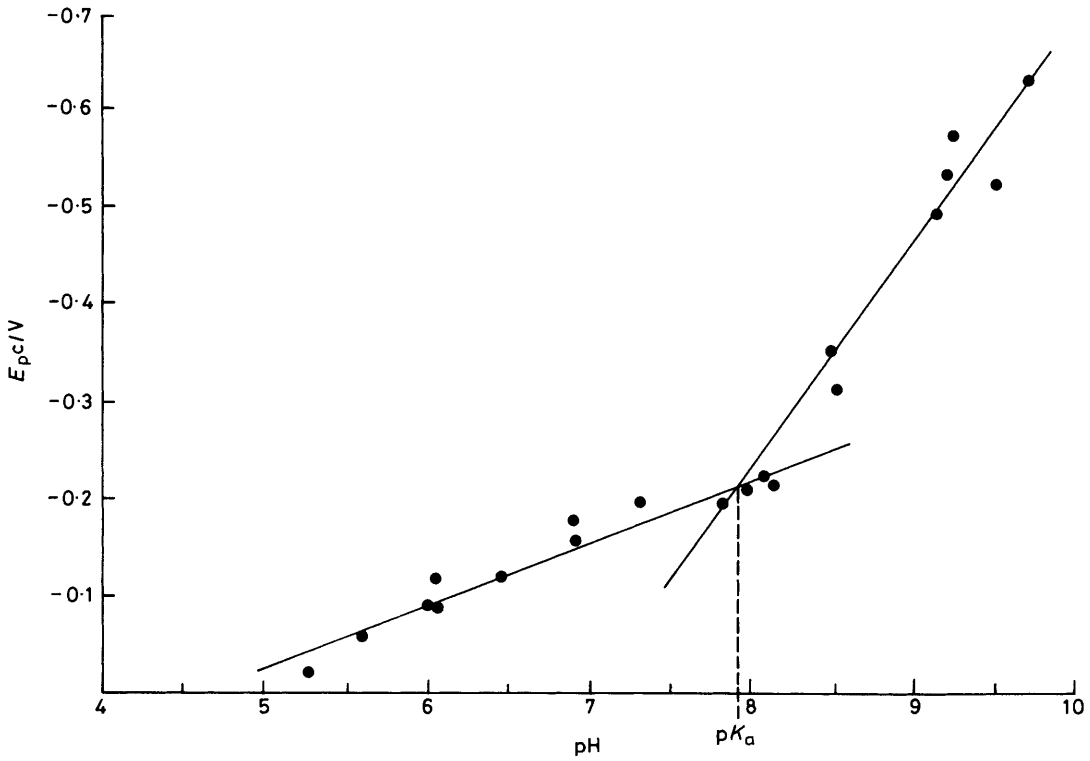


Figure 9. Dependence of reduction potential of $[Fe_4S_4]^{2-}$–bsa on pH

species, and a second more reversible wave corresponds to the $[Fe_4S_4]^{3-}/[Fe_4S_4]^{4-}$ species. As shown in the Table, the redox potentials of Fe_4S_4–protein solutions were shifted anodically (by 170—220 mV) compared with the starting material $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ and thus are similar in value to 4- and 8-iron ferredoxins (*ca.* −0.645 V *vs.* s.c.e.).[17] The similarity in redox potential may be due to the local protein environment of the clusters, the involvement of hydrogen-bond formation between protein back-bond amide hydrogens and thiolate or bridging sulphide ligands.

Chemical reduction of Fe_4S_4–protein solutions was examined, since previous reports of reduced water-soluble synthetic Fe_4S_4 complexes have been limited to highly unstable transient species characterised by stopped-flow spectrophotometric methods [18] or e.s.r. spectroscopy using rapid freezing techniques.[19] Sodium dithionite reduction of Fe_4S_4–bsa gave a black solution, which remained unchanged after elution and centrifugation. The visible absorption spectrum (Figure 1) was consistent with that of the reduced $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{7-}$ species obtained by stopped-flow spectrophotometric methods.[18,*]

Attempts were made to perform sodium dithionate reduction on insulin solutions, but immediate turbidity of the protein solution precluded further examination. An alternative reductant, sodium tetrahydroborate, was employed with bsa solutions. This was found to be a much more powerful reductant resulting in foaming accompanied by possible denaturation of the bsa. Solutions containing Fe_4S_4–bsa–$NaBH_4$ were sufficiently stable to allow elution through a Sephadex column and characterisation by u.v.–visible and c.d. spectroscopy, and cyclic voltammetry (Figure 2 and Table). It is thought that a reduced protein-bound cluster of the type $[Fe_4S_4]^{3-}$ is formed under argon but on exposure to air an oxidised ferredoxin-type species $[Fe_4S_4]^{2-}$ then results. Ultracentrifuge analysis showed little change in the molecular weight of bsa treated with $NaBH_4$ and it is suggested that the more powerful reducing agent yields an increased number of –SH groups suitable for complex formation, certain of which lie in hydrophobic regions of the protein. The cluster environment may thus be more like that in the natural ferredoxin.

Initial cyclic voltammetry studies employing sodium dithionite solutions of the Fe_4S_4–bsa cluster indicated a substantial shift in redox potential (from E_p^c −0.53 to E_p^c −0.2 V, shift of 330 mV) on addition of reductant. Part of this shift was thought to be due to a change in pH of the solution on adding the reductant. The redox potentials of the Fe_4S_4–bsa cluster were found to be dependent on pH (Figure 9), being shifted by −63 mV per pH unit (one proton bound per electron) between pH 4.75 to 8.0 and by −235 mV per pH (formally equivalent to the binding of four protons per electron) above pH 8. The involvement of protons in redox reactions has been reported for N-2 iron–sulphur centres in mitochondrial nadh (reduced form of nicotinamide adenine dinucleotide) dehydrogenase (redox potential pH dependence of −60 mV per pH unit) and in the mitochondrial ' Rieske ' iron–sulphur centre.[22,23]

Synthetic clusters in the oxidised state, $[Fe_4S_4(SR)_4]^{2-}$, behave as the bases of weak acids with pK_a values ranging from 3.92 for $[Fe_4S_4(SBu^t)_4]^{2-}$ (ref. 24) to 7.4 for $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$ (ref. 7). A pK_a value of 7.4 for the equivalent cluster in *Clostridium pasteurianum* has also been reported.[25] If we

* E.s.r. spectra were recorded in the presence of an excess of $Na_2S_2O_4$ and showed more than one component, although a rhombic-type signal with $g_{av.} > 2$ ($g_x = 2.07$, $g_y = 2.03$, and $g_z = 2.006$) was clearly evident. The e.s.r. signal was similar to HiPIP.[20] The formation of an oxidised cluster from $Na_2S_2O_4$ addition may possibly have arisen as a result of employing an excess of reagent which has been reported to cause oxidation of low-potential carriers in a number of cases.[21]

assume that the oxidised form of the Fe_4S_4–bsa cluster has a similar pK_a value, then it will be protonated in the pH range over which a −63 mV per pH dependency is observed. Since this pH dependency indicates that one proton is bound on reduction, the reduced form of the cluster must be doubly protonated [equation (1)]. The pH could be cycled from 4.75

$$[Fe_4S_4(SR)_4]^{2-} \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} [Fe_4S_4(SR)_4H]^- \underset{-H^+/-e^-}{\overset{H^+/e^-}{\rightleftharpoons}} [Fe_4S_4(SR)_4H_2]^- \quad (1)$$

to 9.75 repeatedly and cluster precipitation occurred only at pH 4.75 owing to precipitation of the protein. Similar redox potential pH dependence was demonstrated for the synthetic cluster (1) alone in the presence of high concentration of mercaptide (>1 mol dm^{-3}) indicating that the protein was not causing this effect. The complex (1), however, was only stable for up to 1 h at high mercaptide concentrations, and thereafter decomposition gave electrochemically inactive products (within the potential range studied).

Above pH 8.0, the cluster is thought to be unprotonated prior to reduction ($E_p{}^c$ −0.6 V, pH 9.55). If the pK_a's of the reduced form are greater than 8, we might expect two protons to be bound on reduction, which would lead to a −120 mV per pH dependency on reduction potential [equation (2)].

$$[Fe_4S_4(SR)_4]^{2-} \underset{-2H^+/-e^-}{\overset{2H^+/e^-}{\rightleftharpoons}} [Fe_4S_4(SR)_4H_2]^- \quad (2)$$

This is, however, insufficient to explain the observed pH dependence. A possible mechanism to accommodate the result involves the release of hydroxide ions, rather than protonation, upon reduction of the dianion [equation (3)], where n = 1—4,

$$[Fe_4S_4(SR)_{4-n}(OH)_n]^{2-} \underset{}{\overset{e^-/nRS^-}{\rightleftharpoons}} [Fe_4S_4(SR)_4]^{3-} + nOH^- \quad (3)$$

RS^- = thiolate or cysteinate nucleophiles.

Ligand-exchange reactions[7,24,26] have been reported for both ferredoxins and the model complex (1) using spectrophotometric methods.

The appearance of a second more cathodic peak in the cyclic voltammograms above pH 7.8, which is pH independent ($E_p{}^c$ −0.82 V), may reflect further reduction of the $[Fe_4S_4(SR)_4]^{3-}$ cluster [equation (4)]. A second reduction peak

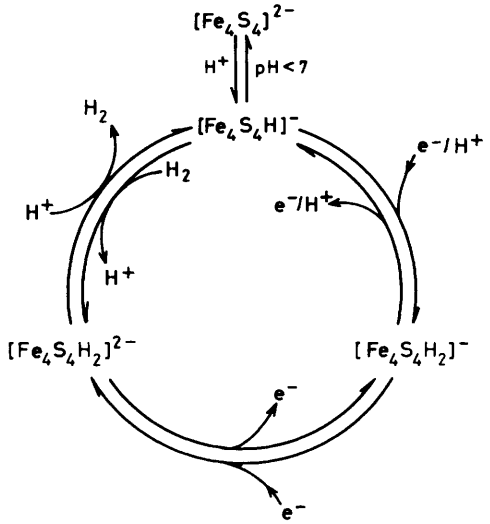
$$[Fe_4S_4(SR)_4]^{3-} \underset{-e^-}{\overset{e^-}{\rightleftharpoons}} [Fe_4S_4(SR)_4]^{4-} \quad (4)$$

should also be observed in the cyclic voltammograms at pH values below 7.8, but may be obscured due to overlapping with the first reduction peak.

This explanation of the electrochemical data assumes that: (*a*) the ligand-exchange reactions occur at a rate comparable with the reduction rate, and (*b*) the RS^- ligands have a greater affinity for the reduced cluster than for the oxidised cluster.

Conclusions

The literature presents a diverse view of the dependence of iron–sulphur protein electrode potentials on pH. Some investigators have found virtually no pH dependence [27] (−2 to −24 mV per pH), whereas others have reported a pH dependence of −60 mV per pH unit.[22] An attempt was made to place some literature values for the midpoint potentials of iron–sulphur proteins at specified pH values on our plot of reduction potential *vs.* pH and a number of natural iron–sulphur proteins were found to lie on the correlation (*e.g.* mitochondrial



Scheme. Proposed mechanism for hydrogenase activity (at pH < 7)

4-Fe centre N-2,[22] *Veillonella alcalescens* and photosynthetic *Chlorobium* [8]). Iron–sulphur proteins with much smaller pH dependencies [*e.g.* various spinach ferredoxin, *Desulphovibrio desulphuricans* Fd and *Chromatium* high-potential iron–sulphur protein (HiPIP)] and synthetic clusters in dmso–H_2O mixtures {*e.g.* $[Fe_4S_4(SAcCysNHMe)_4]^{2-}$, $[Fe_4S_4(SPh)_4]^{2-}$ *etc.*} did not conform to the correlation. A dependence of electrode potential upon pH may, therefore, be exhibited only by iron–sulphur clusters that can allow complete accessibility to water. Examples that show no correlation may contain bulky protein substituents that hinder proton association or bind their cluster in a hydrophilic environment. In the Fe_4S_4–bsa solution, we have shown that redox potentials can vary between −0.64 and −0.02 V (*vs.* s.c.e.) depending on the pH of the solution. The protein solution serves to protect the cluster against irreversible hydrolysis and oxidation, which rapidly takes place in the absence of protein.

Our electrochemical results indicate the direct involvement of protons as well as electrons in redox reactions. A mechanism for hydrogenase activity is proposed in the Scheme which is consistent with our findings and with reports of maximum rates of hydrogen production under acidic conditions.[28] The effective acidity can be governed by the local protein environment. It is likely that clusters in the $[Fe_4S_4]^{2-}$ state are singly protonated at pH < 7 to $[Fe_4S_4H]^-$ and in the presence of an electron donor (*e.g.* methyl viologen and ferredoxins) undergo facile reduction and protonation at a higher potential than that of the unprotonated form, giving a diprotonated complex $[Fe_4S_4H_2]^-$. Protonation can be considered to involve bridging hydride species [24,29] or alternatively, an intermediate formed from the cleavage of one Fe–S bond to form a terminal Fe–H species (*i.e.* Fe–H + H–S). An electron is accepted to give $[Fe_4S_4H_2]^{2-}$ which then reacts with a proton to give dihydrogen and regeneration of the singly protonated cluster.

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