Electron-Paramagnetic-Resonance Studies on the Redox Properties of the Molybdenum–Iron Protein of Nitrogenase between +50 and –450 mV

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The midpoint potentials, E_m , for the oxidation of the characteristic e.p.r. signal, with g values near 4.3, 3.7 and 2.01, of the nitrogenase Mo–Fe proteins from a number of bacteria were measured. They were 0 mV for *Clostridium pasteurianum*, –42 mV for *Azotobacter chroococcum* and *Azotobacter vinelandii*, –95 mV for *Bacillus polymyxa* and –180 mV for *Klebsiella pneumoniae* Mo–Fe proteins at pH 7.9. The oxidations were thermodynamically reversible for the proteins from *A. chroococcum*, *A. vinelandii* and *K. pneumoniae* and the E_m was independent of protein activity for this last protein. The protein from *C. pasteurianum* required a lower potential for reduction than for oxidation, and the oxidation of the protein from *B. polymyxa* was only 70% reversible. The apparent E_m of the latter protein was decreased by 40 mV in the presence of 60 mM-MgCl₂. The pH-dependence of the E_m of the protein from *K. pneumoniae* was interpreted in terms of a single ionization, not directly associated with the e.p.r.-active centre, with a pK_a of 7.0 in the oxidized form of the protein and a pH-independent region at low pH ($E_m = -118 \pm 6.3$ mV). Approx. 20% increase in activity after oxidation was observed for the proteins from *B. polymyxa*, *A. chroococcum* and *K. pneumoniae*. The significance of the above results and their relationship to other published data are discussed.

Nitrogenase (Eady & Postgate, 1974; Zumft & Mortenson, 1975) is the enzyme responsible for dinitrogen reduction to ammonia. It will also reduce a number of small triple-bonded substances, such as acetylene, the reduction of which to ethylene forms the basis of a widely used assay for nitrogenase activity. Nitrogenase from all sources so far investigated consists of two proteins usually referred to as the Fe protein and the Mo–Fe protein. Both proteins are irreversibly damaged by oxygen. For activity, both proteins, ATP (which is hydrolysed to ADP and P_i), a bivalent cation (usually Mg²⁺) and a source of electrons (Na₂S₂O₄ *in vitro*) are required.

The nomenclature system (Eady *et al.*, 1972) used here for distinguishing between the nitrogenase proteins from different organisms represents the genus by a capital letter, the species by a lower-case letter, the Mo–Fe protein by the number 1 and the Fe protein by the number 2. Hence the Mo–Fe proteins from *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Bacillus polymyxa*, *Chromatium vinosum*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* become Acl, Avl, Bpl, Cv1, Cpl and Kpl proteins respectively.

The data presented in this paper relate exclusively to the larger Mo–Fe protein. This protein has a mol.wt. of around 220 000 and is a tetramer consisting of two of each of two different subunits (Eady *et al.*,

1972; Kennedy *et al.*, 1976). The purest preparations of Kpl contain 2 g-atoms of Mo and 32.5 ± 3 g-atoms of Fe per mol, but metal contents lower than this are usually obtained (Smith *et al.*, 1976). The most active preparations of the Mo–Fe protein from other sources are also reported to contain 2 g-atoms of Mo per mol, with Fe contents of 24–33 g-atoms per mol. It is not clear whether the Fe content and acid-labile sulphur content are equivalent (Huang *et al.*, 1972; Yates & Planqué, 1975; Shah & Brill, 1977).

Mössbauer studies (Smith & Lang, 1974; Munck *et al.*, 1975) showed that there were three or four types of Fe species in the Mo–Fe protein. One of these species had magnetic character and could be correlated with the characteristic e.p.r. signal from the Na₂S₂O₄-reduced Mo–Fe protein. The Mössbauer parameters of this species were very similar (Smith & Lang, 1974) to those of oxidized HiIP (high-potential iron protein) from *Chromatium* (Evans *et al.*, 1970). The e.p.r. spectrum of the Mo–Fe protein (Fig. 1) is most unusual, with g values near 4.3, 3.7 and 2.01. Isotope studies showed this signal to be associated with the Fe atoms in the protein (Palmer *et al.*, 1972; Orme-Johnson *et al.*, 1972; Smith *et al.*, 1973).

During turnover, electrons are transferred from the MgATP-activated Fe protein to the Mo–Fe protein, which becomes 'super-reduced' (Scheme 1, step 1),

i.e. reduced further than is possible with $\text{Na}_2\text{S}_2\text{O}_4$ alone. Spectroscopic investigations showed that the e.p.r. signal was lost during 'super-reduction' (Orme-Johnson *et al.*, 1972; Smith *et al.*, 1972) and the Mössbauer parameters of the species responsible for this signal became similar to those of reduced HiPIP (Smith & Lang, 1974). These observations show that this species is very important in enzyme turnover.

Mild oxidation with dyes also results in loss of the e.p.r. signal (Scheme 1, step 2) (Palmer *et al.*, 1972; Orme-Johnson *et al.*, 1972; Smith *et al.*, 1972) without concomitant loss of activity and thus at least three stable oxidation levels of the Mo-Fe protein can be defined (Scheme 1).

In view of the importance of the e.p.r.-active Fe species in the Mo-Fe protein, a number of attempts have been made to measure its redox properties, in particular those of redox step 2, Scheme 1 (Albrecht & Evans, 1973; Walker & Mortenson, 1973; Zumft *et al.*, 1974; Watt & Bulen, 1976).

The published data differ considerably (see the Discussion section). Therefore we have further investigated this redox step with potentiometric measurements on AcI, AvI, BpI, CpI and KpI proteins.

Materials and Methods

Chemicals

Benzyl Viologen, phenosafranine, anthraquinone-2-sulphonate, Indigo Carmine, Methylene Blue,

Fig. 1. E.p.r. spectrum of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 protein in 50 mM-Bes, pH 6.8

At this pH only the low-pH form can be seen (Smith *et al.*, 1973) with g values of $g_1 = 4.32$, $g_2 = 3.63$ and $g_3 = 2.009$. The spectrum was recorded at 12K with a microwave frequency of 9.16 GHz and microwave power of 150 mW using 1.6 mT field modulation at 100 kHz. The protein concentration was 12.03 mg/ml.

thionine and potassium ferricyanide were obtained from BDH Chemicals, Poole, Dorset, U.K. Methyl Viologen was from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and 2,5-dihydroxy-*p*-benzoquinone was from Eastman Organic Chemicals, Eastman Kodak Co., Rochester, NY 14560, U.S.A. Cresyl Fast Violet and the buffers Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid} and Mes (4-morpholine-ethanesulphonic acid) were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., and Tris and Ada {[carbamoyl-methyl]imino]diacetic acid} buffers from Sigma Chemical Co., London S.W.6, U.K. Indigo tetrasulphonate was a gift from Dr. R. Cammack, Kings College, London.

Proteins

Klebsiella pneumoniae was grown as previously described and KpI protein was isolated and purified by the usual methods (Eady *et al.*, 1972; Smith *et al.*, 1976). *Bacillus polymyxa* was grown on nitrogen-free Winogradsky medium (Hino & Wilson, 1958) and BpI protein isolated and purified by methods similar to those used for KpI protein (A. E. Funnel, J. Galazin & B. E. Smith, unpublished work). AcI protein was a gift from Dr. M. G. Yates, AvI protein was a gift from Dr. R. R. Eady and CpI protein was a gift from Professor L. E. Mortenson.

Redox measurements (versus hydrogen electrode)

Potentiometric titrations were carried out essentially by the method of Dutton (1970) at $20 \pm 1^\circ\text{C}$ in an anaerobic glass vessel fitted with a miniature reference calomel electrode (Radiometer K401) and a miniature Pt electrode (Radiometer P101). Electron equilibration between the protein and the Pt electrode was achieved by using various dye substances as mediators. These included Methyl Viologen, Benzyl Viologen, phenosafranine, anthraquinone-2-sulphonate, Cresyl Fast Violet, Indigo Carmine, 2,5-dihydroxy-*p*-benzoquinone, Indigo tetrasulphonate, Methylene Blue and thionine. The potential of the system was poised by addition of either $\text{K}_3\text{Fe}(\text{CN})_6$ or $\text{Na}_2\text{S}_2\text{O}_4$ dissolved in the appropriate buffer.

Because of the O_2 sensitivity of the protein, for each experiment, oxidized dyes were added anaerobically to the cell and then fully reduced with $\text{Na}_2\text{S}_2\text{O}_4$. $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Mo-Fe protein was then added and an oxidative titration carried out. Although for each titration, protein, mediators, $\text{Na}_2\text{S}_2\text{O}_4$ and $\text{K}_3\text{Fe}(\text{CN})_6$ were all prepared in the same buffer, it

Scheme 1. Some oxidation levels of the Mo-Fe proteins

was observed that the pH always decreased during the titration. This decrease was due to the oxidation products of $\text{Na}_2\text{S}_2\text{O}_4$ and was proportional to the amount of oxidized $\text{Na}_2\text{S}_2\text{O}_4$ present. The amount of $\text{Na}_2\text{S}_2\text{O}_4$ required for initial reduction varied, presumably owing to varying amounts of residual O_2 , and so the change in pH was never constant. However, a calibration curve was constructed relating the change in pH of a particular buffer at a particular pH to the amount of $\text{Na}_2\text{S}_2\text{O}_4$ added, and a pH corresponding to 50% oxidized protein was calculated. In some cases this calculation was checked by measuring pH values during the titration. It was correct to ± 0.05 pH unit.

After an equilibration period of 10 min at each potential, a sample was removed through a Suba-Seal (Griffin and George, Wembley, Middx., U.K.) closure by means of a gas-tight Hamilton syringe fitted with a 30 cm long no. 21 stainless-steel needle. The syringe was flushed with the solution several times, a sample was transferred to an e.p.r. tube, frozen in liquid N_2 and its spectrum recorded.

Electron-paramagnetic-resonance spectroscopy

Samples for e.p.r. measurement were prepared in 3 mm internal diameter quartz tubes which were degassed and flushed with scrubbed N_2 gas before addition of the sample as described above. E.p.r. spectra were measured with a Varian E9 spectrometer fitted with a variable temperature probe (Lowe *et al.*, 1972). Conditions were as described in the legends to the Figures.

Preparation of $^2\text{H}_2\text{O}$ samples

Mes (50 mM, pH 5.9) was freeze-dried and the residue was redissolved in an equivalent volume of $^2\text{H}_2\text{O}$. A sample was removed and its pH measured as 6.05. The pH-meter was standardized against aqueous pH buffers, but using the simple relationship between pD and the meter reading (Glasoe & Long, 1960) that:

$$\text{pD} = \text{meter reading} + 0.40$$

a pD of 6.45 was calculated. Two samples of the same Kp1 protein were prepared in 50 mM-Mes, one at pD 6.45 and one at pH 6.45 by eluting the Kp1 protein from a Sephadex G-25 column equilibrated in the appropriate buffer.

Assays of samples from e.p.r. tubes

After recording the e.p.r. spectra, the samples in the e.p.r. tubes were evacuated on a vacuum line and flushed with scrubbed N_2 three times before thawing. Then samples of the Mo-Fe proteins from the tubes were transferred under a flow of N_2 (using a gas-tight Hamilton syringe, previously soaked in $\text{Na}_2\text{S}_2\text{O}_4$) to bottles containing all assay components apart from Kp2 protein. Assays were started by addition of a

20-fold molar excess of Kp2 protein, and acetylene-reduction activity (Eady *et al.*, 1972) was determined.

Results

Potentiometric titrations

Midpoint potentials for redox step 2 of Scheme 1 were determined by plotting the intensity of the line near $g = 3.7$ of the e.p.r. spectrum as a function of the applied potential. Fig. 2(b) shows the measured midpoint potentials of Ac1, Av1 and Kp1 proteins in 50 mM-Hepes, pH 7.90. Two separate titrations on Ac1 protein gave midpoint potentials of -40 and -52 mV and a single titration on Av1 protein gave a midpoint potential of -48 mV. Since these values were within the experimental error of ± 10 mV, an identical redox potential of -42 mV was assigned to Ac1 and Av1 proteins. Two separate titrations were carried out on Kp1 protein and gave midpoint potentials of -175 and -180 mV. The last of these was carried out in 50 mM-Tris/HCl, pH 7.90, thus indicating that there was no buffer effect on redox step 2 in Kp1 protein. The data of Fig. 2(b) show only one redox step between -450 and $+50$ mV for these proteins and the curves themselves are the theoretical curves for a one-electron redox process. The data of Fig. 2(b) were obtained from oxidative and reductive titrations; the intensity of the $g = 3.7$ line was fully restored on re-reduction, thus showing that redox

Fig. 2. Potentiometric titrations of Ac1, Av1 and Kp1 proteins

(a) Acetylene-reducing activities of Kp1 protein taken from the e.p.r. tubes corresponding to the potentials indicated in the potentiometric titration. (b) Potentiometric titrations of Ac1 (●), Av1 (▲) and Kp1 (■) in 50 mM-Hepes, pH 7.90. E.p.r. conditions were as described for Fig. 1. (c) Acetylene-reducing activities of Ac1 protein taken from the e.p.r. tubes corresponding to the potential indicated in the potentiometric titration.

step 2 for Ac1, Av1 and Kp1 proteins was fully reversible.

Fig. 3. shows the redox behaviour of Bp1 and Cp1 proteins. Non-ideal behaviour was observed. The oxidations of Bp1 and Cp1 proteins gave midpoint potentials of -95 and 0 mV respectively in 50 mM-Hepes, pH 7.90. The value for Cp1 protein is based on one titration only as the supply of this protein was limited. Four titrations on Bp1 protein gave midpoint potentials of -94 , -95 , -95 and -98 mV. The last of these titrations, as with Kp1 protein, was carried out in 50 mM-Tris/HCl, pH 7.86, and thus indicates no apparent buffer effect for redox step 2 with this protein.

Redox step 2 for Cp1 protein was not thermodynamically reversible, since it required a lower potential for re-reduction than for oxidation. However, reduction eventually fully restored the intensity of the $g = 3.7$ line. With Bp1 protein, full signal intensity was not restored. In three separate experiments re-reduction gave recoveries of 80, 70 and 65% of the intensity of the $g = 3.7$ line.

Effect of Mg^{2+} on the oxidation wave of Bp1 protein

All the titrations so far described were performed in the absence of Mg^{2+} . In two experiments in which Bp1 protein was titrated in the presence of Mg^{2+} at final salt concentrations of 70 mM- and 60 mM- $MgCl_2$ in 50 mM-Hepes, pH 7.95, oxidation-wave midpoint potentials of -138 and -132 mV were measured. These data are shown in Fig. 4, where a single line with a midpoint potential of -135 mV is drawn. Thus the presence of Mg^{2+} produced a difference in midpoint potential of about 40 mV.

Effect of pH on the redox behaviour of Kp1 protein

Redox titrations on Kp1 protein were performed at different pH values, and Fig. 5 shows the relationship between midpoint potential and pH. Different

Fig. 3. Potentiometric titrations of Bp1 and Cp1 proteins in 50 mM-Hepes, pH 7.90

■ and □ respectively represent oxidation and re-reduction of Bp1 protein, whereas ● and ○ respectively represent oxidation and re-reduction of Cp1 protein. E.p.r. conditions were as described for Fig. 1.

Fig. 4. Potentiometric titration and activities of Bp1 protein in the presence of Mg^{2+}

(a) Acetylene-reducing activities of Bp1 protein taken from the e.p.r. tubes corresponding to the potentials indicated in the potentiometric titration. (b) Potentiometric titration of Bp1 protein in 50 mM-Hepes, pH 7.95, plus 60 mM- and 70 mM- $MgCl_2$. E.p.r. conditions were as described for Fig. 1.

Fig. 5. Variation of the midpoint potential (E_m) of Kp1 protein with pH

Conditions were as described in the Results section.

buffer systems were necessary to cover the range of pH values studied. Redox titrations in different buffers at the same pH showed that there were no buffer effects on the midpoint potential.

Titration were performed in 50 mM-Tris/HCl at pH 8.70, 8.50 and 7.90, in 50 mM-Hepes at pH 7.97 and 7.30, in 50 mM-Bes at pH 6.93, and in a mixture of 50 mM-Hepes plus 50 mM-Ada at pH 7.30, 7.05, 6.75, 6.40 and 6.30. It was observed that at lower pH values longer equilibration periods were required to obtain good experimental fits to the theoretical one-electron curve.

Over the pH range examined, the data can be related by the simple equation (Clark, 1960):

$$E_m = E_x + \frac{RT}{nF} \ln \left(\frac{H^+}{H^+ + K} \right) \quad (1)$$

which describes a single ionization affecting the oxidation-reduction potential. E_m is the midpoint potential, E_x is the potential where the slope of the

curve is zero and K is an apparent equilibrium constant for the redox-linked dissociation of a proton affecting this particular Fe-S centre. From the data of Fig. 5 a pK of 7.0 with $E_x = -118$ mV was found. The line drawn through the data is the theoretical line calculated by using these values in eqn. (1).

The root-mean-square value of the deviation of the points about the calculated line was 6.3 mV. We have used this value as an estimate of the error in E_x .

Effect of 2H_2O on the e.p.r. spectrum of Kp1

Since the redox potential of the e.p.r.-active centre was affected by protonation, we investigated the possibility that the centre itself was being protonated. Samples of pD and pH 6.45 were prepared and the widths at half height of the $g = 2.01$ lines of the e.p.r. spectra compared. The linewidths were the same to within 0.1 mT, indicating that direct protonation of this centre had not occurred, if it had a narrower line would have been observed for the sample prepared in 2H_2O .

Effect of protein damage on the redox behaviour of Kp1 protein

Albrecht & Evans (1973) found that oxidation with $K_3Fe(CN)_6$ damaged Cvl protein and altered its redox behaviour. Therefore we have investigated the effect of oxidative damage on the redox behaviour of Kp1 protein. Kp1 protein was placed in the potentiometric vessel with the appropriate mediators. The protein was then damaged by maintaining the system at a potential of +350 mV for 10 min. The inactivation was arrested by fully reducing the system with $Na_2S_2O_4$ and then a potentiometric titration was performed. At pH 8.7 in 50 mM-Tris/HCl, a single midpoint potential of -222 mV was measured, similar to that already measured for fully active protein (Fig. 5). Assay of this Kp1 protein before and after the experiment revealed that the specific activity fell from 1200 to 850 nmol of ethylene produced/min per mg.

Effect of potentiometric titration on protein activity

For one of each of the potentiometric titrations on Ac1, Bp1 and Kp1 protein, the contents of the e.p.r. tubes themselves were assayed for acetylene reduction, and activity was plotted as a function of potential. Figs. 2(a) and 2(c) show the results for Kp1 and Ac1 proteins respectively and Fig. 4(a) shows the results for Bp1 protein. For Ac1 and Kp1 proteins there is no loss of activity over the range covered. On oxidation, Bp1 protein activity fell in those samples with potentials greater than -75 mV (indicating oxidative degradation) and continued to fall until re-reduced to a similar potential, whereupon the activity remained constant at 70% its original value. This 30% loss of activity was the same as the percentage diminution in the e.p.r. line intensity, confirming the relationship between this e.p.r.-active

centre and active protein.

An interesting feature of Figs. 2(c) and 4(a) is that Ac1 and Bp1 proteins show a significant increase in acetylene-reduction activity after exposure to potentials of -60 and -200 mV respectively. For Kp1 protein similar increases have been observed on oxidation with dyes such as thionine.

Discussion

Comparison with the results of other workers

In this paper we have presented results on the oxidation (Scheme 1, step 2) of the iron-sulphur centre giving rise to the e.p.r. signal with g values at 4.3, 3.7 and 2.01 in the Mo-Fe proteins from a number of bacteria. In all cases a single one-electron curve was observed. However, Watt & Bulen (1976) with Av1 protein and Albrecht & Evans (1973) with Cvl protein both observed two redox steps.

The data of Watt & Bulen (1976) are in complete contrast with our own observations with Av1 protein. They reported midpoint potentials at -450 and -320 mV at pH 8.0, whereas we found a value of -42 mV at pH 7.90. Furthermore, their lower potential corresponded to a two-electron process. Watt & Bulen's (1976) data were obtained solely from amperometric measurements on the Methylene Blue-oxidized protein. They did not monitor any parameter of the protein itself and apparently only used Methyl Viologen ($E_m = -440$ mV) as a mediator. Orme-Johnson *et al.* (1977) have reported parallel e.p.r. and Mössbauer studies on the oxidation of Av1 protein by thionine. They found that the oxidation was biphasic with some of the iron centres being oxidized before the e.p.r.-active iron centre.

In the light of these observations we propose that our measurement of -42 mV corresponds to the true redox potential of the e.p.r.-active centre of Av1 protein. This proposal is supported by our observation of an identical potential for the same process in the closely related Ac1 protein. We suggest that the -320 mV reduction wave of Watt & Bulen (1976) corresponds to the same redox process with $E_m = -42$ mV but that their measured potential is low owing to the inability of the mediator used to transfer electrons to Av1 protein at higher potentials. The effectiveness of Methyl Viologen is considerably diminished at higher potentials (at -320 mV only 1% of the dye is reduced) and thus the rate of reduction of the Mo-Fe protein would be expected to be low. This suggestion is partially supported by the shape of the redox wave. It is noticeably sharper than the two-electron wave at -450 mV, implying that more than two electrons were transferred at a time. This departure from a more usual one-electron reduction process would be expected if our explanation is correct. We further suggest that the second reduction wave at -450 mV observed by Watt & Bulen (1976) corresponds to reduction of the other, e.p.r.-inactive

iron centres which Orme-Johnson *et al.* (1977) found were more easily oxidized than the e.p.r.-active centre. This hypothesis is clearly amenable to experiment. It is still surprising that this second redox wave is a two-electron process, since as yet no iron-sulphur centre has been observed to transfer two electrons simultaneously.

We cannot rationalize the observations of Albrecht & Evans (1973) in the same way. They observed two one-electron redox waves (presumably associated with different centres) with Cv1 protein, one with an E_m of -260 mV and the other of -60 mV at pH 7.5, but both associated with the same e.p.r. signal. The two centres appeared to make an equal contribution to the e.p.r. signal and the authors suggested that both centres were present in each molecule, but had different midpoint potentials because they had different environments. Our results certainly indicate that the environment of the e.p.r.-active centre can affect its redox potential, but would favour the view that with Cv1 protein the two types of centre are in two different forms of the protein, each exhibiting a single redox wave.

Zumft *et al.* (1974) found only one oxidation wave for Cp1 protein with a midpoint potential of -20 mV at pH 7.5 and corresponding to a one-electron process. Only oxidative titrations were performed as they reported that re-reduction of Cp1 protein with $\text{Na}_2\text{S}_2\text{O}_4$ was a very slow process. Their results are in good agreement with our own (Fig. 3), but we have found re-reduction of Cp1 protein at a potential of -150 mV and below, suggesting that perhaps re-reduction is brought about by specific mediators only active at a potential of around -150 mV . It is not clear if this re-reduction gives a characteristic redox curve.

Medium effects

(a) *pH.* Clark (1960) analysed the relationship between pH and midpoint potential in terms of ionizations of the oxidized and reduced species. A change in the slope of a graph of pH against E_m to more negative values indicates a pK in the oxidized form, whereas a change to more positive values indicates a pK in the reduced form. Hence, Fig. 5 can be regarded as involving one ionization of the oxidized form of the protein with a pK_a of 7.0. A similar redox-linked ionization was not observed with the reduced form of the protein and our results suggest that the reduced form of the protein was protonated throughout the pH range investigated.

Our e.p.r. experiments using $^2\text{H}_2\text{O}$ suggest that direct protonation of the Fe-S centre was not involved. A remote ionizing group might affect the Fe-S centre indirectly through a conformational change in the protein. Such a change could alter the position of a charged or hydrophobic group in the vicinity of the cluster or involve extensive rearrange-

ment of the peptide chain with resultant changes in dielectric constant.

A second means whereby an ionization remote from the cluster might influence its environment is through a hydrogen-bonding network of the type proposed by Salemm *et al.* (1973) for *Rhodospirillum rubrum* cytochrome c_2 . In this case the ferricytochrome was stabilized by the net negative charge of an internal tyrosine residue which was part of a hydrogen-bond network involving a tyrosine and serine residue. Protonation of the serine would be expected to destabilize the oxidized state and result in a higher redox potential. It is not known if any such system operates in Kp1 protein.

The primary structure is not known for Kp1 protein, but the amino acid composition has been determined (Eady *et al.*, 1972). There are many more acidic than basic amino acids. Cysteine and histidine are obvious examples of residues which ionize at neutral pH values and Kp1 protein contains about 40 and 50 of these residues respectively.

Similar behaviour with the other proteins was not investigated, but there are some indications that the redox potential of Bp1 protein may be dependent on pH.

(b) *Salt concentrations.* The effect of Mg^{2+} on the oxidation-wave midpoint potential of Bp1 protein is an interesting observation. It has been reported that MgATP^{2-} affects the redox potential of Cp2 protein causing a negative shift of 110 mV from -294 to $-404 \pm 20\text{ mV}$ (Zumft *et al.*, 1974). The significance of this is obscure, since the product MgADP^- apparently causes a similar effect. However, the fact that the midpoint potential of Bp1 protein is shifted by -40 mV indicates that Mg^{2+} alters in some way the environment of the Fe-S centre, either directly or by a protein conformation change as a result of Mg^{2+} binding. Although the Mg^{2+} concentrations used have no physiological significance, the effect does illustrate the sensitivity of this important centre to protein-conformation changes brought about by the environment.

Relationship between protein activity and potentiometric titration

Partial inactivation of Kp1 protein decreased the e.p.r.-signal intensity, but did not produce different redox behaviour from that of fully active protein over the range of potential investigated, so we feel confident that our measurements relate only to active Kp1 protein. Albrecht & Evans (1973) found that the lower of their two redox curves observed with Cv1 protein was eliminated when the protein was damaged by oxidation with $\text{K}_3\text{Fe}(\text{CN})_6$. We have not made any similar observations.

Perhaps the most interesting observations in this context are the increases in activity of Ac1 and Bp1 proteins shown in Figs. 2(c) and 4(a). Similar increases

have been observed for Kp1 protein on treatment with dyes such as thionine. There does not appear to be a relationship between these increases and the E_m , or apparent E_m , of the e.p.r.-active centre and their significance is not clear.

All of the Mo-Fe proteins were isolated in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ and dithiothreitol, and it is possible that during isolation they became partially inactivated owing to reduction of some centre not reduced in the native proteins. Careful oxidation could reverse this process and thus re-activate the proteins. Reduction of the Fe-S centres by $\text{Na}_2\text{S}_2\text{O}_4$ is known to be slow (Smith & Lang, 1974). If oxidation of one of them is associated with a conformational change resulting in the activation, the Mo-Fe proteins could be trapped in this conformation by complex-formation with the Fe protein in the assay, and thus the activation retained. The observation of slight activation on oxidation in three different proteins makes it appear that the process may be generally applicable to Mo-Fe proteins.

Significance of the potentials

As described in the introduction, the e.p.r.-active centre of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Mo-Fe proteins is known to become further reduced during enzyme turnover (Scheme 1, step 1) and to be involved in electron transfer to the enzyme-active site. It is generally considered that step 2 of Scheme 1 is not involved in enzyme turnover. Our results provide further evidence for this hypothesis, since, under the same conditions, a 180 mV range in midpoint potential for step 2 was observed for proteins from different sources. It seems improbable that such a wide range in potential would be observed for a physiologically significant process. Although we have observed pH and Mg^{2+} ion effects on the step 2 midpoint potential, these are not large enough to allow the potentials to converge. Very high Mg^{2+} concentrations were necessary to decrease the Bp1 protein potential by 40 mV and the pH-dependence of the Kp1 protein redox potential did not bring it close to the Acl, Av1 or Cp1 potentials in the physiological pH range.

In contrast, the small amount of data available on the potential required for enzymic activity indicates that it is highly conserved at -460 ± 5 mV (Evans & Albrecht, 1974; Watt & Bulen, 1976).

A similar pattern has been observed for the flavodoxins. With these proteins the physiologically significant semiquinone-hydroquinone redox equilibrium (Yates, 1972), with potentials varying between -372 and -450 mV, is more highly conserved than the quinone-semiquinone redox equilibrium, where the potentials vary from -50 to -221 mV at pH 7.0 (Mayhew & Ludwig, 1975).

We are unable to present a completely satisfying explanation for the observed species variation in midpoint potential of step 2, Scheme 1 for the Mo-Fe

proteins. *A. chroococcum* and *A. vinelandii* are strict aerobes, *C. pasteurianum* is a strict anaerobe and *K. pneumoniae* and *B. polymyxa* are facultative anaerobes which only synthesize nitrogenase in the absence of O_2 . One possible rationalization of the observed midpoint potentials might stem from the consideration that if step 2 is not of physiological significance, then it would be a waste of reducing equivalents for the resting Mo-Fe protein to become oxidized. Evolutionary pressure might then select for mutations that maintained the potential required for step 2 greater than that of the surrounding medium under conditions of physiological stress, e.g. when electron carriers in the enzyme environment are oxidized. More detailed knowledge of the physiology of the bacteria studied might allow this hypothesis to be tested.

One further inference can be drawn from our data. Smith & Lang (1974) noted that the Mössbauer spectrum of the e.p.r.-active centre in $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 protein was very similar to that of oxidized HiPIP. Super-reduction of Kp1 protein resulted in a change of Mössbauer parameters to those resembling reduced HiPIP, i.e. super-reduction was thought to be equivalent to $[\text{4Fe-4S}]^- \rightleftharpoons [\text{4Fe-4S}]^{2-}$, although the e.p.r. signals of oxidized HiPIP and $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Mo-Fe proteins differed. Thomson (1975) has shown that this difference can be rationalized in terms of different spin states of the $[\text{4Fe-4S}]^-$ cluster within his molecular-orbital description. Our data show that the oxidized form of this centre is relatively stable, i.e. $[\text{4Fe-4S}]^0$ in Kp1 protein is stable. If this analysis is true, it is of some significance for Fe-S cluster chemistry generally. No stable analogue at this oxidation level of the $[\text{4Fe-4S}]$ clusters has yet been prepared and HiPIP itself has not been oxidized past the $[\text{4Fe-4S}]^-$ level. Thus more detailed studies on the oxidized cluster may further advance our understanding of these important iron-sulphur clusters in biological molecules.

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