Applications of e.p.r. to molecular enzymology

E.p.r. spectroscopy is reasonably sensitive for biochemical purposes, typically requiring concentrations of paramagnetic species in the 10–100 μM range. Because the paramagnetic centres in one sample can be distinguished by their different spectroscopic parameters (g-values, temperature dependence, etc.), the method can be applied to complex multi-enzyme systems. Moreover, since e.p.r. is selective, and unaffected by diamagnetic centres, the samples need not necessarily be pure. A considerable amount of work has been done by e.p.r. on membrane-bound systems and intact organelles, exemplified by the articles by Beinert, Ohnishi and Evans in this Colloquium. To carry the investigations further and elucidate detailed enzyme mechanisms, it is necessary to work with purified proteins, which are at least free of other paramagnetic impurities.

The spectra of transition metal complexes must normally be measured at cryogenic temperatures. Iron–sulphur proteins are typically observed at temperatures below 77 K, because at higher temperatures the spectra are broadened by rapid electron–spin relaxation, and because excited electron states, which are undetectable by e.p.r., become populated. The use of low temperatures produces an increase in sensitivity, because the signal strength has a dependence on 1/Τ. However, it means that the kinetics of the reaction cannot be followed directly by e.p.r. Often a considerable amount of information about the intermediate steps in a reaction mechanism can be obtained from samples of enzyme frozen under appropriate conditions, such as the presence of substrates. To study short-lived intermediates it is possible to apply rapid-freeze techniques (see the article by Bray in this Colloquium).

A useful feature of e.p.r. is that it is quantitative. For an S = 1/2 system, regardless of its chemical form, the doubly integrated intensity of the spectrum is proportional to spin concentration (provided that it is measured under non-saturating conditions). Measurement of the spin concentration is normally done by comparison with a standard such as a copper(II) sample, and requires careful attention to the temperature, sample geometry, and instrument settings (Randolph, 1972). Double integration is readily performed by a computer system, which is an almost indispensable part of the spectrometer system.

Abbreviations used: TDP, thiamin diphosphate; DCIP, 2,6-dichlorophenolindophenol.

E.p.r. spectroscopy generally only detects paramagnetic centres with an odd number of electrons. Therefore it will detect either the oxidized or reduced state of a redox centre, but not both. This feature is inconvenient when the even-spin state is much more interesting (in haemoglobin, for example). However, use can be made of it to follow redox reactions and estimate redox potentials of centres.

Iron–sulphur proteins

Several different arrangements of iron and sulphur atoms, designated the [2Fe–2S], [3Fe–4S] and [4Fe–4S] clusters, have been found by X-ray crystallography to occur in the simplest iron–sulphur proteins, the ferredoxins. The clusters have different redox potentials and spectroscopic properties (Table 1). It is believed that similar clusters, in various combinations, occur in the iron–sulphur enzymes. Unlike the cytochromes, the iron–sulphur clusters have optical absorption spectra that are relatively featureless and are often obscured by the spectra of other prosthetic groups such as haem. E.s.r has been invaluable in studying them, though it should be emphasized that additional and often complementary information can be provided by other spectroscopic techniques such as Mössbauer spectroscopy and magnetic circular dichroism. Within complex iron–sulphur proteins, for which X-ray crystallographic data are not available, e.p.r. can often be used to determine which types of iron–sulphur clusters are involved. Examples of spectra of the different types of clusters are given in Fig. 1. The characteristic features are (a) whether the e.p.r. signal occurs in the oxidized or reduced state; (b) the temperature dependence of the signal (this is not an infallible guide, but [2Fe–2S] clusters are often detectable at higher temperatures); and (c) g-values and lineshape, which can differentiate individual clusters.

The sole function of most of the iron–sulphur clusters that occur in enzymes appears to be to transfer electrons (exceptions are aconitase, and possibly certain hydrogenses). Therefore other groups are required for interacting with substrates and extracting reducing equivalents from them. These other groups such as haem or flavin, by the nature of their functions, usually undergo one-electron transfers, and are e.p.r.-detectable. When two centres within an enzyme become paramagnetic, the e.p.r. spectra are affected by spin–spin interactions, which therefore can provide information about interactions between paramagnetic centres, and in principle their relative orientations and the distances between them. The electron has a large magnetic moment (considerably greater than that of the nucleus), and the interactions can be observed over distances of about 1.5 nm. It is over distances of this order that electrons can be transferred by tunnelling mechanisms, between or within proteins.

Electron spin–spin interactions between paramagnetic centres occur by two mechanisms: (a) the exchange interaction, which is a consequence of the Pauli exclusion principle, and can be considered as essentially electrostatic; and (b) the dipolar interaction, which is a magnetic effect. The magnitudes of both interactions are strongly dependent on the distance between the centres. They cause a number of effects on the e.p.r. spectrum. We will consider these effects in turn, starting first with those that occur at shortest distances.

Antiferromagnetic coupling can occur when two paramagnetic centres are connected through an intervening ligand. The two centres, coupled by the exchange interaction, behave as a single spin system. The iron–sulphur
clusters are themselves antiferromagnetically coupled systems of high-spin iron atoms; the coupling of the \( S = S/2 \) and \( S = 2 \) states produces ground states of \( S = 0 \) and \( S = 1/2 \). The effect of antiferromagnetic coupling is most noticeable when two centres with an odd number of spins, which would be expected to be detectable by e.p.r., couple to produce a system with even or zero spin and hence become undetectable. On the basis of such 'missing' e.p.r. spectra, antiferromagnetic coupling has been proposed to occur between the haem \( a_3 \) and copper(II) in cytochrome oxidase (Griffith, 1971), and between the sirohaem and \( [4 \text{Fe}-4 \text{S}] \) cluster in sulphite reductase (Janick & Siegel, 1982).

Interactions between the spins, particularly dipolar, will lead to splittings of the spectrum, which may become very complex if the g-tensors are anisotropic. There is also likely to be a signal at half-field due to a \( \Delta M_s = 2 \) transition, which occurs when the microwave quantum causes both spins to 'flip' simultaneously. This signal occurs at half the expected value of the \( H_g \) field, corresponding to twice the expected g-value, because the transition requires twice as much energy. As the distance between the interacting spins increases, the probability of the half-field transition becomes very low. At greater distances too the splittings may not be resolved, but may be detected by line broadening.

When the centres are separated by longer distances, approx. 1.5–2 nm, where the splitting is so small as to be hidden in the spectral linewidth, spin–spin interactions may manifest themselves by an increase in the spin–lattice relaxation rates. This may be detected, for example, by relief from microwave power saturation (Beinert & Orme-Johnson, 1967; Rupp et al., 1978).

From the magnitude of the dipolar coupling it is possible in principle to calculate the distance between the paramagnetic centres, because the dipolar interaction varies as \( r^{-3} \). In practice this is subject to uncertainty, because of the problem of assessing the relative extent of dipolar and exchange interactions, and because dipolar couplings depend on the angles between the g-tensor axes of the two centres. An empirical calibration of the distances in terms of the magnitude of these effects can be obtained for the few cases where the structure of the proteins has been determined by X-ray crystallography. An example is the \( 2 [4 \text{Fe}-4 \text{S}] \) ferredoxin from \textit{Peptococcus aerogenes}. The centre-to-centre distance between the clusters is 1.2 nm (Adman et al., 1973). The spectrum of the reduced protein and others of this type (Mathews et al., 1974) shows considerable distortion, with additional broad outer lines (compare Figs. 1a and 1c). The \( \Delta M_s = 2 \) transition is detectable but very weak.

It should be noted that if a change occurs in the line-shape of the spectrum of one centre when the other one is reduced, this need not necessarily be by spin spin interactions; the change may be transmitted through the protein by conformational changes. The g-values, and the extent of g-strain broadening, of some iron–sulphur clusters are sensitive to subtle changes in protein conformation (Cammack, 1975). Therefore the reduction of one centre may cause a change in the other conformation of the protein, and thus alter the spectrum of the other. It is possible that changes in the line-shape of the 'Rieske' iron–sulphur protein in the mitochondria during reduction of cytochrome b (Orme-Johnson et al., 1974) may occur in this way.

We will describe two case histories where e.p.r. has contributed to the elucidation of reaction mechanisms of iron–sulphur enzymes. In each case, stable derivatives of the enzymes were found, in which the iron sulphur clusters were involved in a spin–spin interaction with another paramagnetic species. It is proposed that these species represent intermediates in the reaction cycles.

\textit{2-Oxo acid ferredoxin oxidoreductases} - These enzymes, found principally in anaerobic bacteria, are significantly different from the NAD-linked oxo acid dehydrogenases of eukaryotes. They catalyse reactions of the type:

\[ \text{R.CO.CO}_2 + 2 \text{Fd}_{ox} + \text{CoA} = \text{R.CO.COA} + 2 \text{Fd}_{red} + \text{CO}_2 \]
The oxo acid:ferredoxin oxidoreductases of the extreme halophile *Halobacterium halobium* were isolated by Kerscher & Oesterhelt (1981a) as electron donors to its unusual [2 Fe–2 S] ferredoxin. The enzymes were found to be similar in composition and catalytic ability to those of the anaerobic fermentative bacteria, which use 2 [4 Fe–4 S] ferredoxins as acceptors. However, the halobacterial enzymes had the advantage of being relatively stable in air, the only requirement for stability being the presence of high concentrations of salts.

*H. halobium* contains at least two oxo acid:ferredoxin oxidoreductases, one specific for 2-oxoglutarate, the other with activity towards a number of oxo acids including pyruvate. Each enzyme has a total molecular mass of about 250 kDa, within which there are two subunits of approx. 85 kDa, two of approx. 40 kDa, two molecules of thiamin diphosphate (TDP) and two iron–sulphur clusters.

**E.p.r. spectra observed in oxo acid:ferredoxin oxidoreductases.** We began to examine the oxo acid:ferredoxin oxidoreductases by e.p.r., with the intention of studying the iron–sulphur clusters. However, we were surprised to observe a stable free radical in the enzymes as isolated (Cammack *et al.*, 1980). The radical signals disappeared if CoA was added, but were enhanced by addition of oxo acid substrates instead. The e.p.r. signals of the radicals were broad, and close examination of them revealed a specific hyperfine structure which depended both on the particular reductase and the oxo acid used (Fig. 2). The most likely assignment is that they represent acyl-TDP radicals (Kerscher & Oesterhelt, 1981b).

The iron–sulphur clusters were observed in the reduced state in the oxo acid:ferredoxin oxidoreductases after addition of dithionite. They have unusually anisotropic g-values (Fig. 3c). From the temperature dependence of the spectra, and the effects on the spectrum of 80% (v/v) dimethylsulphoxide (Cammack, 1975; Kerscher & Oesterhelt, 1981b), the clusters were identified as being of the [4 Fe–4 S] type. The midpoint potentials were found to be extremely negative. Oxidation–reduction titrations monitored by e.p.r. gave an estimate for the midpoint potential in pyruvate:ferredoxin oxidoreductase of −570 mV at pH 9.

When the enzymes were treated with excess oxo acid plus CoA (Fig. 3b), a more complex spectrum was observed. The spectrum is interpreted as due to two superimposed components: the reduced [4 Fe–4 S] cluster, arising from

![Fig. 1. E.p.r. spectra of iron–sulphur clusters in ferredoxins](image)

![Fig. 2. E.p.r. spectra of radicals from *H. halobium* oxo acid:ferredoxin oxidoreductases](image)
step B of the reaction cycle (Fig. 4), and a complex signal due to an interaction between the radical and a reduced [4 Fe-4 S] cluster in the same enzyme molecule.

The spectrum of the interacting spin system contains some broad outer lines but the central region resembles a radical spectrum split by approx. 4.5 mT. This spectrum can be compared with that observed in the interaction between a reduced [4 Fe-4 S] cluster and a flavin semiquinone radical in trimethylamine dehydrogenase (Steenkamp et al., 1978). In the latter case, the broad outer lines were stronger, and an intense \( \Delta M_s = 2 \) transition was observed. The distance between the centre of the flavin isoalloxazine ring and the [4 Fe-4 S] cluster in trimethylamine dehydrogenase has recently been determined by X-ray crystallography to be 1.2 nm (Mathews et al., 1984). The distance of closest approach, between the 8-methyl group of the flavin and the nearest iron atom, is 0.6 nm. In oxo acid:ferredoxin oxidoreductase, no \( \Delta M_s = 2 \) transition was detected at the sample concentrations used. We would therefore expect the distance between the TDP and [4 Fe-4 S] cluster in oxo acid:ferredoxin oxidoreductase to be of the order of 1–1.5 nm.

Elucidation of the reaction cycle of oxo acid:ferredoxin oxidoreductase. By observing spectra of the enzyme frozen under different reaction conditions, e.p.r. spectroscopy was of considerable help in suggesting possible reaction mechanisms for the enzyme. However, it should be emphasized that in these, as in many other investigations, it became more powerful when combined with other spectroscopic and biochemical techniques. Spectrophotometry provided additional information about the kinetics of the reaction, because the occurrence of the radical was found to be associated with a charge-transfer absorption band at 360 nm (Kerscher & Oesterhelt, 1981b). The optical absorption by the iron sulphur clusters could also be followed.

Radioactive labelling demonstrated that, as with the enzyme from *C. acidi-urici*, the oxo acid was decarboxylated before being attached to the enzyme. The resulting complex is proposed to represent the free-radical species. Such one-electron transfers are logical for an enzyme that uses the one-electron acceptor ferredoxin rather than the two-electron acceptor NAD. The number of electrons transferred as substrates were added could be measured spectrophotometrically by using cytochrome c as electron acceptor. The complete reaction cycle, as proposed by Kerscher & Oesterhelt (1981b) on the basis of these experiments, is illustrated in Fig. 4. Two molecules of cytochrome c are reduced per molecule of oxo acid assimilated. During the formation of the enzyme-bound radical, one electron is transferred to the iron–sulphur cluster and then to ferredoxin. After addition of CoA, acyl-CoA is released and a further electron transferred.

This mechanism is fully in agreement with the e.p.r. spectra of samples frozen in the presence of substrates (Fig. 3). It should be noted that these samples were not obtained by rapid-freezing and therefore represent the final outcome of the reactions of the enzyme with the substrates added. In particular, if other electron acceptors are unavailable the enzymes are autoxidized by air.

1. When isolated, the oxo acid:ferredoxin oxidoreductase is in the oxidized state, partly in the free form (form A), which is e.p.r.-silent, and partly complexed with substrate from the cell (form C), giving the stable free-radical signal (Fig. 2).

2. When an oxo acid substrate is added it will combine with the enzyme to produce form B. The iron sulphur cluster will rapidly be oxidized by traces of oxygen to produce form C. Hence the spectrum observed is that of the free radical (Fig. 2 and Fig. 3a).

3. When both oxo acid and CoA are added, the reaction can proceed, consuming the oxygen in the solution until it is exhausted. The enzyme will stop at those points where the electron transfers are

Fig. 3. E.p.r. spectra of *H. halobium* pyruvate:ferredoxin oxidoreductase
Spectra were recorded at the temperatures shown, with microwave frequency 9.26 GHz.

Fig. 4. Proposed mechanism of *H. halobium* pyruvate:ferredoxin oxidoreductase
Fig. 4. Proposed mechanism of *H. halobium* pyruvate:ferredoxin oxidoreductase
needed, namely forms B and D. The spectrum observed (Fig. 3b) is a superimposition of spectra of the two species. In form B, both the substrate—TDP radical and the reduced [4Fe−4S] cluster are paramagnetic and the spectrum is that of the interaction between them. In form D, only the [4Fe−4S] cluster is reduced.

**Hydrogenase from Desulfovibrio gigas**

Hydrogenases are diverse and widely distributed enzymes that catalyse the production or consumption of hydrogen gas (Adams et al., 1981). All of those that have been isolated are iron–sulphur proteins, and most (though not all) contain nickel (Cammack et al., 1984).

The hydrogenase of *Desulfovibrio gigas* catalyses the reduction of cytochrome *c*₃ by hydrogen. The enzyme contains 11–12 iron and sulphide atoms, probably in the form of two [4Fe−4S] and one [3Fe−xS] cluster (Teixeira et al., 1983), and approx. one nickel atom in a molecule of 89 kDa (Hatchikian et al., 1978).

In common with many hydrogenases (Cammack et al., 1982a), the [3Fe−xS] cluster in the enzyme from *D. gigas* gives an e.p.r. signal in the oxidized state at *g* = 2.01, which is detectable at temperatures below 30 K. No signals have been reported from the [4Fe−4S] clusters. It was assumed that they are rendered undetectable by e.p.r., either by spin-coupling, or by having a spin state greater than *S* = 1/2. However, we have previously noted that in the soluble and membrane-bound hydrogenases of *D. desulfuricans*, Norway strain (Lalla-Maharajh et al., 1983; Bell et al., 1984), the [4Fe−4S] clusters gave rise to spectra in the reduced state that were unusually broad (Bell et al., 1984; R. Cammack & K. K. Rao, unpublished work). In Figs. 5 a and c they are compared with the spectrum of a reduced [4Fe−4S] ferredoxin (Fig. 5a).

In reduced *D. gigas* ferredoxin we observed a spectrum which was extremely broad (Fig. 5d). Nevertheless the integrated intensity of this signal, assuming it to be an *S* = 1/2 species, was approximately twice that of the nickel signal. It is possible therefore that it represents the missing spin intensity of the reduced [4Fe−4S] clusters. However, the reason for such a broad signal is obscure.

The hydrogenase in the form as isolated gives a signal with narrow linewidths and *g*-values 2.32, 2.23, 2.01 (Fig. 6a), which has been demonstrated to be due to nickel by substitution with ⁶¹Ni (Moura et al., 1982) like several similar hydrogenases. The signal disappeared on reduction, and was attributed to low-spin Ni(II) (Cammack et al., 1982b; Lancaster, 1982; Le Gall et al., 1982).

A second type of signal, also due to nickel (Moura et al., 1982), at *g* = 2.19, 2.16, 2.01, appeared when the enzyme was treated with its substrate hydrogen, or with strong reducing agents (Fig. 6b). However, this experiment is the converse of rapid-reaction, taking 4 h for the signal to reach a maximum. The slowness of this reaction is a consequence of the activation/deactivation processes that this enzyme undergoes. The hydrogenase, as isolated, is in a relatively inactive form, and the slow step is the conversion to the active form by reducing agents (Fernandez et al., 1984; Lissolo et al., 1984). Activation could also...
be brought about by the milder reductant dithiothreitol, with the dye mediator indigotetrasulphonate, under which conditions the \( g = 2.19, 2.16, 2.01 \) signals could be induced almost immediately by reduction with dithionite or hydrogen. These results show that the signal represents a reduced species, presumably Ni(I). Control experiments indicated that activation was unlikely to be due to cleavage of an accessible disulphide bridge or conversion of a [3 Fe-xS] to a [4 Fe-4S] cluster. Instead, the activation of the enzyme corresponds to a change in the coordination state of the nickel. The change occurred in parallel with the activation of the enzyme.

The Ni(I) signal differed from the other nickel signals in that it showed extremely rapid electron-spin relaxation. As the temperature of measurement was decreased the spectrum began to show a pronounced splitting (Fig. 7). At similar temperatures the broad signal began to appear. We interpret these results as a spin–spin interaction between the Ni(I) and the reduced [4 Fe-4S] clusters.

A splitting of a nickel signal by spin–spin interaction with a [4 Fe-4S] cluster has been observed in the hydrogenase from *Chromatium vinosum* (Albracht et al., 1984). However, in that case the nickel was of the Ni(III) type, and the iron–sulphur cluster was assigned to the [4 Fe-4S]\(^{3+}\) state. The behaviour of the two enzymes appears to be rather different. In *C. vinosum* hydrogenase the [3 Fe–xS] cluster appears to be caused by oxidative damage to the [4 Fe–4S] cluster and to be closely associated with the nickel centre (Albracht et al., 1983), whereas in *D. gigas* hydrogenase there appears to be no association of the nickel with the [3 Fe–xS] cluster (Cammack et al., 1982).

A third type of nickel e.p.r. spectrum was obtained by oxidizing the activated *D. gigas* hydrogenase anaerobically with 2,6-dichlorophenolindophenol (DCIP). This signal had \( g \) values at 2.34, 2.16, 2.01 (Fig. 6c). It was in fact present as a minor component in the spectra of all samples of the hydrogenase when oxidized, and may represent the oxidized state of the active enzyme. It was rapidly activated by reducing agents, in contrast to the enzyme as isolated, which was only slowly activated. Therefore there are at least three oxidation states of the nickel in the active form, and two in the inactive form:

**Enzyme before activation:**

<table>
<thead>
<tr>
<th>Ni(III)</th>
<th>( g = 2.32, 2.23, 2.01 )</th>
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<tbody>
<tr>
<td>Ni(II)</td>
<td>No e.p.r. signal</td>
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**Activated enzyme:**

<table>
<thead>
<tr>
<th>Ni(III)</th>
<th>( g = 2.34, 2.16, 2.01 )</th>
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<tbody>
<tr>
<td>Ni(II)</td>
<td>No e.p.r. signal</td>
</tr>
<tr>
<td>Ni(I)</td>
<td>( g = 2.19, 2.15, 2.01 )</td>
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The Ni–B signal also disappeared on reduction to very low redox potentials. It seems unlikely that this would represent further reduction to the next oxidation state, Ni(0). One possible explanation for this effect is the reduction of another centre (for example an iron–sulphur cluster) to a paramagnetic state, which would couple with the nickel to produce an e.p.r.-silent state. However, the redox behaviour of the signals attributed to the [4 Fe–4S] clusters is not consistent with this proposal. Another possibility is the production of a nickel hydride, Ni(H), which would be undetectable by e.p.r. Such a species, for which there are models in certain inorganic catalysts (Tolman, 1971), could well participate in the catalytic cycle of the enzyme.
transfer to the rest of the enzyme. This conclusion implies that there is a different mechanism for those hydrogenases that do not contain nickel (Adams & Mortenson, 1984; Huynh et al., 1984).

Appendix

Biochemists are often confused by the fact that two terms, e.s.r. (electron spin resonance) and e.p.r. (electron paramagnetic resonance), are used for this technique, although both are measured by the same instrument. A rather arbitrary distinction has been drawn between them. E.s.r. tends to be used for systems in which the spin–orbit coupling is small, and hence g is close to the free electron value of 2.0023193044, such as organic free radicals [presumably Mn(II) should also fall into this category]. In medical circles, e.s.r. also stands for 'erythrocyte sedimentation rate'. E.p.r. is applied to systems where g is significantly different from 2, such as transition metal complexes, where, strictly speaking, there is no free electron spin. The resonance phenomenon is due to interaction of the paramagnetism of the centre with the applied static and microwave fields. Since all paramagnetism is due to electrons the phrase is a tautology, and therefore perhaps a compromise, such as 'electron magnetic resonance', by analogy with nuclear magnetic resonance, would have been better.

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Palmer, G. (1975) Enzymes. 3rd edn. 11, 1–56