Review Letter

ELECTRON PARAMAGNETIC RESONANCE IN BIOCHEMISTRY

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1. Introduction

Electron paramagnetic resonance* (EPR) is a method for studying unpaired electrons. In matter generally the large majority of electrons are paired. Unpaired electrons occur, however, and are present mainly in organic free radicals and in compounds of transition metal ions in their paramagnetic oxidation states. In each paramagnetic centre, the unpaired electron (or electrons) is influenced only by atoms in its immediate environment, so that EPR provides a tool for picking out and studying these centres, even when they are present at fairly high dilutions (e.g. 10^{-5} M). It is this specificity which makes the method of interest in biological work, and EPR continues to provide useful if limited application in diverse fields of biology.

The mathematical background required for a full understanding of EPR theory (see for example Bersohn and Baird, Introduction to Electron Paramagnetic Resonance, Benjamin, New York, 1966; McGarvey in Transition Metal Chemistry, ed. Carlin, Arnold, London 3 (1966) 89) may seem rather frightening to a biochemist. However, we shall concentrate here on more practical aspects (cf. Beinert and Palmer, Adv. Enzymology 27 (1965) 105). As in other forms of spectroscopy, much useful information may be obtained by simple inspection and comparison of EPR spectra. This information is of various types. At its lowest level EPR may be used analytically, simply to decide whether free radicals or transition metals are present in samples. At a slightly higher level, frequently the metal ion responsible for the signal may be identified, its valency established and its approximate concentration determined. It is also possible sometimes, having decided a given transition metal is present in a given valency, to find out whether the metal occurs in the specimen in more than one type of site and to obtain information about the symmetry of bonding of the metal within such sites. It is, however, when hyperfine interactions with neighbouring atoms are studied that the method begins to show its real power. From studies of these it is possible in principle to decide not only with which atoms the unpaired electron is interacting but also the extent to which the electron is delocalized onto these atoms. Finally, it must be added that in certain cases kinetic EPR studies employing reasonably fast time-resolutions, are possible. This has added considerably to the usefulness of the method, particularly in the enzyme field.

Papers on EPR started to appear in the biochemical literature around 1956 and specific applications of the technique have by now covered the entire range from simple diatomic free radical species up to whole living tissues. While in some areas EPR has opened up whole new fields of research, in others it has been distinctly less successful. This review will emphasize some more recent developments within the enzyme and protein field and touch on several other topics.

2. Free radicals

2.1. *Tissues and flavins*

Indications of the importance of free radicals in biological processes were provided from the earliest

* The alternative name, electron spin resonance (ESR) is frequently used, particularly in Britain. However, EPR is to be preferred, since in transition metal work not only the spin of the electron but also its orbital motion is of interest.

days of studies in the field by the observation of EPR signals from whole animal tissues. However, organic free radicals are all characterized by *g*-values very near to the free electron value of 2.0023 and hyperfine structure tends to be lost when radicals are bound to macromolecules. In general, then, tissue EPR signals tend to be uninformative. Furthermore, it has been pointed out that free radical levels in different tissues seem to correlate with so simple a parameter as their contents of mitochondria (Wyard, Proc. Roy. Soc. A302 (1968) 355). This seems to detract seriously from the value of tissue EPR work. However, in mitochondria free radical signals presumably come from flavoproteins and within the flavoprotein field EPR does continue to make quite significant contributions. One interesting recent structural application of the method concerns the old question of how flavin is linked covalently to protein in succinic dehydrogenase. The semiquinone free radical EPR spectrum from the half-reduced enzyme is of course uninformative. However, a flavin peptide may be obtained from the enzyme by proteolytic digestion and this yields a good EPR signal with well-resolved hyperfine structure. Analysis of the structure and comparison with model compounds has led to the conclusion that the peptide is linked to the riboflavin nucleus via the methyl group in position-8 (Hemmerich et al., FEBS Letters 3 (1969) 37). Another interesting point of flavoprotein chemistry (which may not be unexpected on chemical grounds), is the large variation which has come to light among different flavins of the extent to which the free radical is formed on half-reduction. Though many flavoproteins, like riboflavin itself, give only a few percent of the semiquinone, so that there may be room for reasonable doubt as to whether this intermediate is of significance in their enzymic reactions, nevertheless a number of other flavoproteins yield virtually 100% semiquinone. For these proteins there can be little doubt of the importance of the radical (Shethna et al., Biochem. Biophys. Acta 113 (1966) 225; Huang et al., Biochem. Biophys. Res. Commun. 34 (1969) 48; see also Mayhew et al., J. Biol. Chem. 244 (1969) 803).

2.2. *Substrate radicals*

EPR ought in principle to be a good tool for detecting low molecular weight free radicals derived from substrate molecules which may occur as intermediates in enzymic reactions. Though progress in this field has been slow, it would be premature to generalize that free radicals are unimportant in such reactions. In pioneering experiments on the mechanism of action of peroxidase Mason and co-workers (Yamazaki et al., J. Biol. Chem. 235 (1960) 2444) used EPR to look at substrate radicals and were able to identify those from, e.g. ascorbate and hydroquinone. From kinetic studies they came to the conclusion that the enzyme catalyses substrate oxidation only to the semiquinone free radical stage, further reaction stages taking place non-enzymically. More recently this work has been amplified and extended considerably and important quantitative data has been obtained directly by EPR on the formation and reactions of substrate radicals generated not only by peroxidase but also by ascorbate oxidase and by the microsomal flavin enzymes, cytochrome-b_5 reductase and NADPH-cytochrome-c reductase (Ohnishi et al., Biochim. Biophys. Acta 172 (1969) 357; Iyanagi and Yamazaki, Biochim. Biophys. Acta 172 (1969) 370). Some stable substrate radicals have also been studied by Caldwell and Steelink (Biochim. Biophys. Acta 184 (1969) 420). Also recently EPR has confirmed that a free radical derived from oxygen, the superoxide ion, O_2^-, is a real entity in certain biochemical systems. At relatively high pH-values O_2^- turns out to be much more stable than had been thought, with a lifetime of the order of a few hundred millisec at pH 10 and 20°. It is produced by reduction of oxygen in the catalytic cycle of xanthine oxidase, apparently by reduced enzyme-bound flavin (Knowles et al., Biochem. J. 111 (1969) 53; see also Komai et al., J. Biol. Chem. 244 (1969) 1692). O_2^- is also produced by certain other systems (Nilsson, Pick and Bray, Biochim. Biophys. Acta 192 (1969) 145). Bound, as opposed to free, O_2^- has been proposed by Blumberg and co-workers (Wittenberg et al., J. Biol. Chem. 243 (1968) 1863; Peisach et al., J. Biol. Chem. 243 (1968) 1871) in the structure of the oxidation product ("enzyme-substrate complex") obtained on treating cytochrome-c peroxidase with hydrogen peroxide. In this species, they formulate O_2^- as a ligand for haem iron with the metal in the diamagnetic low spin ferrous form. The compound has EPR parameters at helium temperatures distinctly similar to those for the free O_2^- radical produced by xanthine oxidase. However, susceptibility and Mössbauer data (Iizuka et al., Biochim. Biophys. Acta 167 (1968) 257) may complicate this simple picture somewhat.

2.3. *Other biological systems*

Turning to another branch of biology, free radicals were long ago implicated in photosynthetic processes, but only comparatively recently has it become reasonably clear what is the nature of the species involved and what are the functions they perform in the overall processes. The field has been reviewed by Weaver (Ann. Rev. Plant Physiol. 19 (1968) 283). Two light-induced free radicals have been distinguished. One is due to the oxidized form of the photoreactive centre, "P700", and the other is a semiquinone, perhaps from plastoquinone.

In cancer research the possibility that free radicals derived from carcinogenic compounds may be important in their biological action continues to receive attention. An example of such work is reported by Nagata et al. (Gann 59 (1968) 289). 3,4-Benzpyrene is readily converted by rat liver homogenates to a free radical identified, as the 5-phenoxy radical of the carcinogen. Though the 5-hydroxy compound has not so far been shown to be carcinogenic, possible importance of the radical in the carcinogenic processes is not at present excluded.

2.4. *Spin labelling*

Finally the field of "spin labelling" must be mentioned. This technique was introduced by McConnell and co-workers (Stone et al., Proc. Nat. Acad. Sci. 54 (1965) 1010). The principle is simply that a stable organic free radical, usually one containing a nitroxide group, is attached by suitable chemical means to a specific site in a biological macromolecule. The EPR spectrum of the product then gives information regarding the extent to which the label is free to move in the product and hence about the conformation of the macromolecule at and around the binding site. Although the method is a most ingenious and interesting one, up to the present time it seems to have yielded relatively little information in the enzyme field which is not available from other techniques. Apart from problems concerned with obtaining truly specific labelling of a given site, the technique as generally used has two fundamental limitations. The first is that for chemical reasons the unpaired electron located on the nitroxide group is always rather a long way (several bond distances) away from the original group of the macromolecule which was labelled. The second is that the only information obtainable from the spectrum is a semi-quantitative indication of the extent to which the spin is free to tumble in the labelled product.

As an example of spin labelling we may cite work on haemoglobin. Extensive studies have been carried out on the molecule labelled via the thiol group in position 93 of the β-chain. Striking changes take place reversibly in the EPR spectrum when the labelled molecule is oxygenated and de-oxygenated. It is, of course, well known from X-ray data that oxyhaemoglobin has a different conformation from the de-oxy form. However, information on the conformation of partially oxygenated intermediates is of particular interest in understanding the mechanism of the change. Evidence has been presented that in the labelled haemoglobin molecule oxygenation of the haems of the α-chains affects the spin labels on the β-chains (Ogawa et al., Proc. Nat. Acad. Sci. 61 (1968) 401; McConnell et al., Nature 220 (1968) 787). This is in agreement with independent conclusions based on X-ray work.

Other recent interesting applications of the labelling technique include studies in which a spin-labelled NAD analogue was used to replace the co-enzyme of alcohol dehydrogenase (Weiner, Biochemistry 8 (1969) 526). The analogue was bound reversibly by the enzyme, presumably in a manner analogous to that of the co-enzyme. The effect of the presence or absence of the zinc of the enzyme and of the substrate on the spin was studied but NMR relaxation experiments proved more revealing than did EPR (Mildvan and Weiner, Biochemistry 8 (1969) 552).

3. Transition metals in proteins ($S=\frac{1}{2}$)

It is perhaps in the field of transition metal-containing proteins in which the metal has a single unpaired electron that EPR has made some of its most unique contributions to biochemistry. The function of bound transition metals in proteins and especially in enzymes, is difficult to study by many techniques and EPR has provided a welcome adjunct to other methods. If a spin-label represents an artificial and somewhat unsatisfactory probe with which to look at an enzyme, then a paramagnetic metal ion placed by nature right in the active centre and functioning in the catalytic cycle is a much more attractive one. However, the number of enzymes amenable to study in this way is very limited.

Interesting work has been carried out on Mo(V) enzymes, on Cu(II) enzymes and on iron-sulphur proteins. Examples relating to each of these groups will be discussed in turn.

3.1. *Mo(V) systems*

Milk xanthine oxidase has been extensively studied by EPR and since the work illustrates many of the presently realized applications of EPR in the metal enzyme active centre field it may be appropriate to review it in some detail. The enzyme contains 2 Mo, 8 Fe and 2 FAD per mole and prior to EPR work little was known of their function in the catalytic reaction. The first EPR experiments (Bray et al., Biochem. J. 73 (1959) 193) showed that Mo(V) signals appeared on treatment with substrates, indicating that reduction from Mo(VI) was taking place. To show that the signals appeared and disappeared at catalytically significant rates, a special fast reaction method, the "rapid freezing technique" was developed (Bray, Biochem. J. 81 (1961) 189). Results made it clear that not only Mo(V) but also iron and free radical signals appeared and disappeared within the turnover time of the enzyme. The time courses for the development of the various signals differed, giving indications of intramolecular redox reactions going on among the different constituents of the enzyme molecule as part of the turnover processes (Bray et al., J. Biol. Chem. 239 (1964) 2667). Most important, so far as the function of molybdenum is concerned, was the finding that two distinct Mo(V) signals appeared and followed differing kinetics. The change from one signal to another was taken to represent a change in the bonding of the molybdenum in the active site occurring as part of the catalytic cycle.

More recently, the range of Mo(V) signals obtainable from xanthine oxidase has been extended considerably and it is now recognised that there are four main signal types (Bray and Vänngård, Biochem. J. 114 (1969) 725). While some appear within the turnover time, others take very much longer to develop and are not thought to be of catalytic significance. Their general EPR features are all tolerably well understood and satisfactory computer simulations of some of them have been obtained. Each signal must represent a different conformation around molybdenum at the active site of the enzyme and no doubt changes in ligand and perhaps in symmetry type are involved in transitions from one signal type to another, though these changes are not yet fully understood. The signals have also provided information on differences between the two molybdenum atoms in the enzyme molecule and on complex formation between reduced enzyme and substrate molecules (Pick and Bray, Biochem. J., 114 (1969) 735).

An unexpected finding which has turned out to be of great significance in understanding the catalytic reaction of xanthine oxidase is that three of the four signal types show coupling of Mo(V) to protons. This indicates the unique nature of the bonding of the metal in the enzyme active centre, since Mo(V)-proton coupling does not seem to have been observed in low molecular weight complexes of the metal. The detection of EPR signals showing interaction with protons and appearing at rates faster than the turnover, might invite speculation on the origin of these protons and on the possibility that they might be derived from substrates and transferred to the enzyme in the catalytic reaction. Using specifically deuterated substrates it has been demonstrated that this is indeed the case. EPR signals at the shortest reaction times with several deuterated substrates gave clear evidence for Mo-D rather than Mo-H interaction. At slightly longer times, still within the turnover, the deuterium exchanged out again from the enzyme into the solvent. On the basis of these results a tentative reaction mechanism for the enzyme has been proposed (Bray et al., 5th FEBS Symposium, Academic Press, New York 16 (1969) 267).

3.2. *Cu(II) systems*

A number of enzymes contain Cu(II) which is firmly bound and essential for catalytic activity. Of these, one of the most extensively studied is fungal laccase. This enzyme contains four atoms of copper per mole. EPR has shown that the copper atoms in the molecule are not equivalent to one another. Only two are detectable by EPR and are therefore definitely Cu(II). There is still some uncertainty as to the valency of the other two. Though they were originally assumed to be Cu(I) more recent work makes it more probable that a pair of interacting Cu(II) atoms is involved (Malkin et al., European J. Biochem., in press).

The two EPR-detectable copper atoms are quite different from one another, as judged from their spectra and apparently play quite different, though equally essential, roles in the catalytic reaction (Malmström et al., Biochim. Biophys. Acta 156 (1968) 67). One atom (Type 1 Cu(II)) is associated with the intense blue colour of the enzyme and is very rapidly reduced by substrates (Malmström et al., European J. Biochem. 9 (1969) 383). It has rather unusual EPR parameters, showing an abnormally small hyperfine splitting and a significant distortion of the square planar symmetry generally found in model Cu(II) complexes. Substrates are thought to interact with the enzyme via this copper atom. The other detectable copper (Type 2) has much more normal EPR parameters but is also required for catalysis (Malkin et al., European J. Biochem. 7 (1969) 253). It has been shown to be the binding site for anionic inhibitors such as fluoride and cyanide (Malkin et al., FEBS Letters 1 (1968) 50). Titration studies on the enzyme have shown that three to four reducing equivalents must be taken up before Type 1 Cu(II) is fully reduced (Fee et al., J. Biol. Chem. 244 (1969) 4200). However, for the fluoride-treated enzyme, which shows the same Type 1 signal as the native enzyme, the titrations were more revealing. Here, the first equivalent of reducing agent added reacts specifically to reduce the Type 1 copper and additional equivalents produce changes in the optical properties of the molecule believed to be related to reduction of the two copper atoms not detectable by EPR (Malkin et al., European J. Biochem., in press). The fact that laccase takes up four electrons seems distinctly relevant to enzymic reduction of oxygen to water, apparently in a single 4-electron step, though the nature of the oxygen binding site is still unknown. Work on the enzyme is complicated by the presence of significant amounts of irreversibly bound fluoride which can be detected by EPR in most preparations (Fee et al., J. Biol. Chem. 244 (1969) 4200.

3.3. *Iron-sulphur proteins*

This class of proteins, containing non-haem iron and liberating H_2S on mild acidification, is characterized under reducing conditions by rather broad asymmetric EPR signals in the region of g 1.94. The signals have somewhat unusual temperature dependence, frequently becoming undetectable at temperatures only a little above 77°K but sharpening considerably at helium temperatures. They were originally observed in various flavoproteins and attributed to iron first by Beinert and Sands (Biochem. Biophys. Res. Commun. 3 (1960) 41, 47) working with mitochondrial systems and shortly afterwards by Bray et al. (Biochem. J. 81 (1961) 178) working with xanthine oxidase. Detection of the signals represented a landmark in studies on the function of non-haem iron in mitochondrial oxidation processes. Up till then there had been no satisfactory method of studying it and the metal had frequently been regarded as non-functional in mitochondrial systems. The discovery that an EPR signal presumably related to iron appeared on reduction and disappeared on re-oxidation, clearly implied a redox function for the metal in the enzymes. In keeping with a redox role it was shown for xanthine oxidase that appearance and disappearance of the signal takes place at catalytically significant rates (Bray, Biochem. J. 89 (1961) 196; Bray et al., J. Biol. Chem. 239 (1964) 2667). The possible relevance of mitochondrial iron to oxidative phosphorylation processes has received attention in recent years (e.g. Sharp et al., Arch. Biochem. Biophys. 122 (1967) 810; Light et al. FEBS Letters 1 (1968) 4).

The scope of EPR work on iron sulphur proteins widened considerably following the observation that ferredoxins give signals similar to the flavoproteins (Palmer and Sands, J. Biol. Chem. 241 (1966) 253; Hall et al., Biochem. Biophys. Res. Commun. 23 (1966) 81). Their structure and the state of the iron are still not finally established. EPR work involving isotopic and chemical substitution has recently provided some very valuable information. The molecule of putidarexodin contains two atoms of iron and takes up one electron on reduction with dithionite. EPR signals from the protein enriched with ^{57}Fe showed very clearly that this electron is delocalized onto both iron atoms (Tsibris et al., Proc. Nat. Acad. Sci. 59 (1968) 959). Later, replacement of the labile sulphur with ^{77}Se, which can be done without loss of biological activity, made it clear that the electron must be delocalized onto both labile sulphur atoms also (Orme-Johnson et al., Proc. Nat. Acad. Sci. 60 (1968) 368).

4. Transition metal proteins ($S > \frac{1}{2}$)

The basic EPR theory applicable to metal ions having more than one unpaired electron is more complicated and less well understood than is that for $S = \frac{1}{2}$ systems (see for example Dowsing and Gibson, J. Chem. Phys. 50 (1969) 294). However, this has not prevented the EPR method from making significant contributions of interest to biochemists in this field also. The two main areas of interest are those haem compounds containing high spin ferric iron and the so-called $g = 4.3$ iron compounds. This latter group includes proteins such as transferrin (Aasa and Aisen, J. Biol. Chem. 243 (1968) 2399), rubredoxin (Bachmayer et al., Proc. Nat. Acad. Sci. 57 (1967) 122) and pyrocatechase (Nakazawa et al., J. Biol. Chem. 244 (1969) 119). Recently, studies have been carried out by Aisen, Aasa and Redfield on transferrin in which the iron is replaced by chromium (J. Biol. Chem., in press). They found the EPR spectra more revealing than those of the natural iron-containing protein and concluded that the two-metal-binding sites of the molecule are not exactly equivalent.

In the haem field no review would be complete without mention of the now classical work on haemoglobin by Ingram and co-workers (Bennett et al., Proc. Roy. Soc. A. 240 (1957) 67). From studies on single crystals they were able to deduce the orientations of the planes of the four haem groups relative to the crystallographic axes. This work was done before X-ray results were available and was later fully substantiated by them. More recent work on haem proteins has been mainly carried out on frozen solutions. Ehrenberg obtained EPR evidence for the existence in certain haem compounds of temperature-dependent equilibria between the high and low spin forms (Arkiv. für Kemi 19 (1962) 119). Blumberg and co-workers have studied horseradish and cytochrome-c peroxidases and their compounds and have come to the conclusion that all the properties of these can be explained in terms of ferrous and ferric iron and that higher oxidation states of the metal need not be invoked (Peisach et al., J. Biol. Chem. 243 (1968) 1871). Finally, work on abnormal haemoglobins, variant M, must be mentioned. These haemoglobins occur with much of the iron in the ferric state and are difficult to reduce to ferrous. EPR signals may be observed simply by putting untreated blood samples from patients into the EPR apparatus and show deviations from the axial symmetry shown by other ferric haemoglobins. Apparently tyrosine in the variant causes the ligand field of the iron to become rhombic, this being related to the unusual properties of this haemoglobin (Watari et al., Biochem. Biophys. Acta 120 (1966) 131).

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