Long-distance spin–spin interactions with iron-sulphur clusters as observed by e.p.r. spectroscopy

Richard Cammack*†, Ruth Williams*, Bruno Guigliarelli†, Claude Moret and Patrick Bertrand†

*Centre for the Study of Metals in Biology and Medicine, King’s College, London W8 7AH, U.K. and †Laboratoire d’Electronique des Milieux Condensés, Université de Provence, Centre de Saint-Jérôme, Marseille, Cedex 13, France

Introduction

E.p.r. spectroscopy is the method of choice for observing, identifying and characterizing paramagnetic centres in complex electron-transfer systems of bioenergetic systems [1]. Redox proteins and membrane-bound complexes provide an environment which is unusual in chemistry, in which paramagnetic centres are held at fixed, rather long distances, and at fixed orientations relative to each other. Electron spin-spin interactions between adjacent centres will affect the e.p.r. spectrum [2–5]. These interactions are analogous to the nuclear spin–spin interactions that are observed in n.m.r., but are observable over longer spin–spin distances because the magnetic moment of the electron is much greater than the magnetic moment of any nucleus.

The effects of spin–spin interactions on the e.p.r. spectrum may be interpreted to yield structural information. With membrane-bound electron-transfer proteins it may be possible to orient the paramagnetic samples about one axis, by using stacked membrane preparations. The study of e.p.r. spectra, obtained by varying the membrane plane relative to the magnetic field, can then be combined with the analysis of spin–spin interactions, making it possible to determine the organization of centres within the membrane (Figure 1).

Physical basis for the spin–spin interactions

Let us consider the interaction between two paramagnetic centres, A and B. Systems with more than two centres are much more complex and it is simplest to consider pairwise interactions between the most strongly interacting species. Two types of interactions contribute to the spin–spin effects on the e.p.r. spectrum: exchange and magnetic dipolar interactions. Exchange interactions are essentially electrostatic, and take place through chemical bonds. Dipolar interactions are magnetic in character, and occur through space. In general, the strength of interaction decreases with increasing distance.

The type and extent of interactions affecting the e.p.r. spectrum depend on the relative electron-spin relaxation rates of the two centres. Since electron-spin relaxation rates of iron-sulphur clusters are strongly temperature-dependent, static splittings are often seen at low temperatures. As the temperature is raised, the splittings in the spectrum collapse, and dynamic electron-spin relaxation effects are observed. These take the form of either broadening of the spectrum of the slower-relaxing centre (due to a shorter T2), or faster spin relaxation of the spectrum, with an unchanged lineshape. In the extreme case of very short T1 of the faster-relaxing centre, no interaction is detectable.

Static effects

These occur when the electron-spin relaxation of both the centres is slow, which means that the relaxation rate (expressed in frequency units, such as MHz) is considerably less than the coupling (also in frequency units). In this case the spectrum shows a splitting or broadening. An example of a spectrum showing static splittings is shown in Figure 2. In some cases, a signal at half the field of the normal resonance, due to the ‘double spin-flip’, or \( \Delta m = 2 \) transition, is observed. These effects on the spectrum are capable of providing detailed information about the strength and type of interaction between the centres, and also the relative orientation of the \( g \) tensor principal axes of the two centres (Figure 1). This information is provided by simulation of the spectrum.

Dynamic effects

Interaction with a faster-relaxing spin can enhance the relaxation of a slower-relaxing spin. As with static splittings, this interaction has contributions from dipolar and exchange interactions, and the dipolar term can, in principle, provide distance information. An example would be the interaction of a flavin radical with an iron-sulphur cluster. Enhancement of the electron-spin relaxation may be observed as an increase in linewidths due to shortening of T2. If the lineshape is already broadened by other effects such as hyperfine splittings and \( g \) strain, this effect may not be detectable in

†To whom correspondence should be addressed.
Figure 1
Illustration of a protein in a membrane, containing two paramagnetic centres, A and B

The $g$ matrix axis systems, $g_A^x$, $g_A^y$, and $g_A^z$, represent local molecular axes for the two centres. The protein is generally randomly oriented relative to the applied magnetic field $B_0$, although orientation about the normal to the membrane plane is possible.

continuous-wave e.p.r., but the enhancement of relaxation may still be observed as a decreased tendency to microwave power saturation. Once again this effect can provide information about the magnitudes of the exchange and dipolar interactions.

Information derived from spin–spin interactions
The exchange interaction between two spins $S_1$ and $S_2$ is written

$$-J S_1 \cdot S_2$$

where the exchange coupling constant $J$ may be either positive (ferromagnetic coupling) or negative (antiferromagnetic coupling). The magnitude of $J$ is related to the covalently bonded pathway between centres A and B. Hence a relationship would be expected to the probability of electron transfer between the centres. In some cases, the exchange parameter $J$ is related to the electronic matrix element $T_{ab}$ given by electron-transfer theories [6–8]:

$$J = \frac{T_{ab}'}{U}$$

The involvement of the energy term, $U$, means that prediction of the rates from e.p.r. data depends on a number of approximations which are difficult to verify.

Dipolar interactions can provide information about distances between the electron spins. For two point dipoles, the effect of the dipolar interaction in splitting the e.p.r. spectrum is proportional to $(1 - 3 \cos^2 \theta)/r^3$, where $\theta$ is the angle between the interspin vector and the applied magnetic field $B_0$, and $r$ the interspin distance (Figure 1). The effects on electron spin relaxation rates are even more sensitive to distances. They depend on several terms, the dominant one varying as $(1 - 3 \cos^2 \theta)/r^6$.

Experimental methods
Microwave power saturation measurements may be used to estimate the relaxation times $T_1$ and $T_2$, by the method of Castner [9]. This depends on getting a sufficient range of microwave power so as to cover the range from non-saturation to significant saturation. This range may be adjusted by judicious choice of measurement temperature.

More accurate estimates of relaxation rates may be obtained by pulsed e.p.r. techniques [10]. These include Hahn-echo measurements for $T_2$, and saturation recovery for $T_1$ (inversion recovery
Figure 2
X-band e.p.r. spectra of D. gigas hydrogenase

(a) Spectrum of the nickel centre in the Ni-C state, recorded at a temperature of 15 K, microwave power 0.1 mW. (b) Spectrum of Ni-C and reduced [4Fe-4S] clusters, recorded at 42 K and 1 mW microwave power, showing the effects of static splittings. Other conditions of measurement: microwave frequency, 9.378 GHz; modulation amplitude, 1 mT. (c) Numerical simulation of the split Ni-C spectrum, using the following parameters: $J = 0.008 \text{ cm}^{-1}$, $r = 0.86 \text{ nm}$.

may also be used, but is less effective for anisotropic spectra). The limitations of these measurements are set by the time resolution of the instruments and usually restrict measurements to relaxation times greater than 100 ns. Hirsh et al. [11,12] have used measurements of relaxation rates by saturation-recovery e.p.r. to estimate distances between the manganese cluster and the tyrosyl radical in photosystem II, and the exchange coupling between the dinuclear iron centre and tyrosyl radical in ribonucleotide reductase.

Static splittings and broadenings of the e.p.r. spectrum may be observed by comparison of the spectrum of the two paramagnetic species together with those of the isolated components. The latter may often be obtained by selective oxidation or reduction of the samples. When the spectrum of one centre is altered by the oxidation or reduction of the other, the effects of spin–spin interactions must be distinguished from alterations due to changes in protein conformation. The presence of electron spin–spin interactions may be confirmed by a spectral shape which varies with microwave frequency. Although the splittings or broadening are constant when expressed in magnetic field units, they vary proportionally to the inverse of the microwave frequency when spectra are represented on a common $g$ scale. For example the splittings are expected to be more prominent at S-band (4 GHz), and less at Q-band (35 GHz). By contrast, on such a $g$ scale, changes in the spectrum due to shifts in $g$ factors, for example, are frequency-independent.

A quantitative analysis of static effects due to magnetic interactions should be based on the numerical simulation of the e.p.r. spectrum [13]. The parameters used in this calculation include the values of $r$ and $J$, the $g$ factors of the two centres, and the angles between the $g$ tensor principal axes and the interspin vector. These values are adjusted to give the best fit to the experimental spectra. The analysis of the spectra must take into account the fact that the protein molecules themselves, usually in frozen solution, are randomly oriented, so an average of all orientations is taken. However, if the shape has a sufficient number of characteristic features, an unambiguous assignment may be made, giving an estimate of the distance $r$ between the two centres. Moreover, the same set of parameters should be used to simulate spectra recorded at different microwave frequencies. This may be very time-consuming unless some values of the parameters may be estimated first. One way to do this is to estimate $J$ from the positions of broad outer lines [13].

Biochemical examples
The first example identified of spin–spin interaction between iron-sulphur clusters was in the [2Fe-4S] ferredoxin of Clostridium pasteurianum [14]. The two clusters in the ferredoxins of this type are separated by a distance of $\sim 1.2 \text{ nm}$ [15]. A recent simulation of the spectrum has yielded an estimate of the intercluster distance of 0.8 nm [16]. The shorter distance is expected, because the model used assumed that the clusters are point dipoles, whereas the spin density on an iron-sulphur cluster is strongly delocalized. Since the dipolar interaction varies as $1/r^3$, spin density at one end of a cluster which is closest to the other, will disproportionately
Table I

Examples of proteins in which the effects of weak spin–spin interactions have been observed in the e.p.r. spectrum.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Interacting centres</th>
<th>Effects on spectrum</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium</em> ferrodoxin</td>
<td>[4Fe-4S]⁺</td>
<td>R, S, H</td>
<td>[14]</td>
</tr>
<tr>
<td><em>Thermus</em> ferrodoxin</td>
<td>[4Fe-4S]⁺</td>
<td>S</td>
<td>[24]</td>
</tr>
<tr>
<td>High-potential iron-sulphur protein</td>
<td>[4Fe-4S]⁺</td>
<td>S</td>
<td>[25]</td>
</tr>
<tr>
<td>Succinate dehydrogenase/fumarate reductase</td>
<td>[4Fe-4S]⁺</td>
<td>[2Fe-2S]⁺⁺</td>
<td>R</td>
</tr>
<tr>
<td>Complex II</td>
<td>Semiquinone</td>
<td>Semiquinone</td>
<td>R, S</td>
</tr>
<tr>
<td>Trimethylamine dehydrogenase</td>
<td>Flavin radical</td>
<td>[4Fe-4S]⁺</td>
<td>R, S, H</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Mo(V)</td>
<td>[2Fe-2S]⁺⁺</td>
<td>R</td>
</tr>
<tr>
<td>Dimethyl sulphoxide reductase</td>
<td>Mo(V)</td>
<td>[4Fe-4S]⁺</td>
<td>R</td>
</tr>
</tbody>
</table>

contribute to the interaction. A more refined analysis, in which the electron spin distribution over the clusters is taken into account, is required. Such an analysis, based on a local spin description, has been performed for the interactions between a mononuclear and a dinuclear centre and between two dinuclear centres [17].

The first example of a static spin–spin splitting in a conjugated iron-sulphur protein was the splitting of the spectrum of MoV by interaction with a [2Fe-2S] cluster in xanthine oxidase [18, 19]. The splitting is small but detectable because of the narrow linewidth of the molybdenum signal. A detailed analysis, based on a local spin model, indicates a distance between the cluster and the molybdenum centre of 1.9 nm [17].

The interaction between the nickel centre and a [4Fe-4S] cluster in the nickel-containing hydrogenase of *Desulfovibrio gigas* provides an interesting case, where the effects of temperature on relaxation rates are dramatically illustrated [20, 21]. The nickel, in the oxidation state known as Ni-C, shows a static splitting at 4.2 K, but broadens out above 8 K. The spectrum of the [4Fe-4S] cluster is itself broadened out, possibly due to interaction with the reduced [3Fe-4S] cluster and a second [4Fe-4S] cluster. Above 30 K, the e.p.r. spectrum of the [4Fe-4S] cluster broadens out completely, and the spectrum of the nickel sharpens up to resemble the 'unsplit' spectrum of isolated Ni-C. At this temperature only dynamic relaxation effects are observed. Simulation of the 'split' spectrum of Ni-C at three microwave frequencies has given an estimate of 0.85 nm for the distance between the centres (B. Guigliarelli, C. More, A. Fournel, M. Asso, E. C. Hatchikian, R. Williams, R. Cammack and P. Bertrand, unpublished work). The simulation at X-band frequency is illustrated in Figure 2.

The spectrum of the [4Fe-4S] clusters A and B in photosystem I was known to be due to spin–spin interactions [22]. The shape of the spectrum was difficult to simulate, until it was found that the spectrum of the *Synechocystis* 6803 photosystem I shows broad outer lines, which permitted an estimate of the exchange coupling between centres A and B. From this, and simulation of the spectra at X-band and Q-band, the interspin distance was estimated [13]. Furthermore, by means of spectra of oriented membrane preparations, it was possible to estimate the orientation of the A–B axis relative to the membrane plane. The result shows reasonable agreement with the latest estimates from a low-resolution X-ray structure [23]. This result suggests that e.p.r. measurements may be of wider use where crystallographic data are not available.

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Metal-Redox Centre Interactions

Electron transfer reactions of cellobiose oxidase
Michael T. Wilson* and Bing-Lan Liu

Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, U.K.

Introduction

Cellobiose oxidase (CBO) is a flavo-haem b enzyme secreted by the white rot fungus Phanerochaete chrysosporium and is thought to play a part in the ligno-cellulose degradation system of this microorganism [1-4]. The enzyme (M_r ~ 89,000) [5], similar to yeast flavocytochrome b, comprises two separable domains, a haem protein (M_r of 31,000) and a larger flavin-bearing protein synonymous with cellobiose:quinone oxidoreductase [6]. The haem b group is hexaco-ordinate and low-spin in both redox states and spectral analysis indicates that the protein donates histidine and methionine ligands to the iron as in the class I c-type cytochromes. Previous kinetic investigations have shown that the substrate, cellobiose, rapidly donates


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Abbreviations used: CBO, cellobiose oxidase; SVD, singular value decomposition.
*To whom correspondence should be addressed.