Simulation of the Electron-Paramagnetic-Resonance Spectrum of the Iron-Protein of Nitrogenase

A PREDICTION OF THE EXISTENCE OF A SECOND PARAMAGNETIC CENTRE

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The e.p.r. spectra of the Fe-proteins of nitrogenase from all sources studied have unusual features in that they have very anisotropic linewidths and low integrated intensities. These characteristics can be explained by assuming that one of the two electrons accepted by these proteins is located at a rapidly relaxing paramagnetic centre that is unobservable by e.p.r., but causes anisotropic broadening of the e.p.r. signal of the other electron. Complex-formation between Fe-proteins and MgATP is described in terms of a 50–60° rotation of the e.p.r.-observable centre.

Nitrogenase from all sources so far studied consists of two proteins: a tetrameric protein (mol.wt. approx. 230000), containing 1–2 Mo atoms and 24–32 non-haem Fe atoms, is believed to contain the site of substrate reduction, and a dimeric protein (mol.wt. approx. 65000), containing 4 non-haem Fe atoms, transfers electrons to the Mo-Fe-protein during the catalytic cycle (Eady & Smith, 1978). In addition to the two proteins, MgATP and a source of reducing equivalents (dithionite *in vitro*) are required for catalytic activity. The Fe-proteins from every organism examined have similar e.p.r. spectra, which have been attributed to an Fe-S cluster. This e.p.r. spectrum has certain unusual characteristics; in particular the linewidths are very anisotropic, and they also appear to have a low integrated intensity [Cp2 protein: 0.2 electron/molecule (Palmer *et al.*, 1972; Zumft *et al.*, 1973; Orme-Johnson *et al.*, 1977); Kp2 protein: 0.45 electron/molecule (Smith *et al.*, 1973); Ac2 protein: 0.24 electron/molecule (Yates *et al.*, 1975)]. The low integrated intensity is particularly surprising, since one molecule of the Fe-protein Ac2 protein has been shown to accept 2 electrons from dithionite (Thorneley *et al.*, 1976).

Materials and Methods

The Fe-protein was purified from *Klebsiella pneumoniae* as described by Eady *et al.* (1972). All

Abbreviations and nomenclature: the component proteins of nitrogenase from various organisms are designated by a capital letter for the genus and a lower-case letter for the species, the number 1 for the Mo-Fe-protein and 2 for the Fe-protein. Thus in this paper the Fe-protein from *Klebsiella pneumoniae* is Kp2, that from *Clostridium pasteurianum* is Cp2, and that from *Azotobacter chroococcum* is Ac2.

biochemicals were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K., and all salts from British Drug Houses, Poole, Dorset, U.K. E.p.r. spectroscopy and data handling were carried out as described by Lowe *et al.* (1972) and Bray *et al.* (1978). Computer simulation of spectra was carried out on a CDC 7600 computer at the University of Manchester Regional Computer Centre and on a PDP 11/10 computer (Digital Equipment International, Galway, Eire); the algorithms used were based on the BASIC program described by Lowe (1978) and programs were written in FORTRAN IV (CDC 7600) and BASIC (PDP 11/10). Lineshapes were taken as Lorentzian. Integrations were carried out over one hemisphere of the *g*-value tensor.

Results and Discussion

Since Thorneley *et al.* (1976) have shown that the Fe-protein is capable of accepting two electrons, it becomes of interest to ask (Orme-Johnson *et al.*, 1977) why only 0.2–0.45 electron can be observed in the integrated e.p.r. spectra. The possibilities are that either 1.55–1.8 electrons go to a site where they are spin-paired and therefore diamagnetic, or that in some way they are e.p.r.-invisible, although still paramagnetic. The present paper shows that the second of these assumptions is capable of predicting the somewhat unusual properties of the Fe-protein's e.p.r. signal. In the model to be considered one electron is placed on a conventional Fe-S centre, and the other on a centre that has such a short transverse relaxation time, T_2 , that it is relaxing too quickly to be observed by e.p.r. The theory of this situation has been discussed by Leigh (1970) when the

correlation time between the two electrons is less than the T_2 of the observed spin and greater than the reciprocal of the angular frequency of the microwaves. He shows that the spin with the short T_2 (and longitudinal relaxation time, T_1) produces a relaxing field at the e.p.r.-observable centre, and that the effect of this field is to increase the linewidth of the observed spectrum in some orientations, but not in others. This is the observed effect in the Fe-proteins of nitrogenase. It was therefore decided to attempt to simulate its e.p.r. spectrum by adding a term C , as described by Leigh (1970), to the linewidth expression in the spectral simulation program. The simulation that fits the experimental spectrum of Fig. 1(a) best is shown in Fig. 1(a'); this simulation assumes that the second centre is isotropic. The spectrum that the cluster would have in the absence of the rapidly relaxing centre is shown in Fig. 1(c). It should be noted that as well as the fit shown, the integrated intensity of the spectrum of Fig. 1(a') is considerably decreased if the very long tails are not fully included; this is because parts of the spectrum have become so severely broadened as to become almost unobservable. The parameters that were allowed to vary in producing Fig. 1(a') were the three principal g values of the observed spin, its intrinsic linewidth (assumed to be isotropic), the interaction parameter C (cf. Leigh, 1970), and the two angles θ_R and ϕ_R defining the direction to the rapidly relaxing centre with respect to the principal axes of the g -tensor of the observed centre. The final values are given in Table 1. Improved simulations may be obtained if heterogeneities such as some of the protein not containing the 'invisible' centres are allowed, or if the invisible centre is allowed to become anisotropic. Detailed studies of these effects, however, produce many ambiguities, and so have not been described in detail.

Fig. 1(b') shows a simulation of the e.p.r. spectrum of Kp2 protein complexed with MgATP; Fig. 1(b) shows the experimental spectrum. As shown in Table 1, the only difference between the parameters of the simulations of the spectra of protein with and without MgATP is that the angles relating the two centres have changed. The model would predict that the effect of ATP is not to change the symmetry at the e.p.r.-active centre, as has been suggested previously (Orme-Johnson & Davis, 1977; Eady & Smith, 1978), but to cause a gross conformational change in the protein, which rotates the centre giving rise to the e.p.r. signal through 50 – 60° relative to the line joining it to the second centre. It is also possible to produce the effects of MgADP on the e.p.r. spectrum of Kp2 protein by introducing smaller rotations. In general the g_x and g_z lines may be broadened and moved towards the g_y feature at certain angles and the g_y line itself may be severely broadened.

Fig. 1. Experimental and interacting simulated e.p.r. spectra of Kp2 protein

Kp2 protein (activity 700 nmol of ethylene produced/min per mg of protein) at 11 mg/ml was prepared in the presence of (a) 50 mM-MgCl₂ alone and (b) additional 1.9 mM-ATP. The buffer was 25 mM-Tris/HCl, pH 8.1 (at 0°C), with 1 mM-Na₂S₂O₄. Spectra were recorded at about 18 K at a microwave frequency of 9.1 GHz and 20 mW microwave power. They are the spectra of Figs. 3(a) and 3(b) respectively of Smith *et al.* (1973). (a') and (b') are simulations of (a) and (b) by using the parameters given in Table 1. (c) is a simulated spectrum with no interaction present. Addition of further ATP to the sample of (b) has no significant effect on the spectrum.

The shape of the simulated spectrum is highly dependent on the angles used, but much less so on the size of the interaction parameter, C ; this mainly changes the amplitude of the signal, so that differences in integrated intensity between species or on complexing with MgATP are probably

Table 1. *Parameters used in simulations of Kp2-protein e.p.r. spectra of Fig. 1*

C is the interaction parameter between the two centres; see Leigh (1970). ϕ_R and θ_R are the equatorial and azimuthal angles (degrees) specifying the direction to the rapidly relaxing centre relative to the principal g -value tensor of the observable spin; $\phi_R = 0$, $\theta_R = 90^\circ$ corresponds to the x -axis and $\theta_R = 0$ corresponds to the z -axis.

Figure	Spectrum type	g_x	g_y	g_z	Linewidth(mT)	θ_R	ϕ_R	C (mT)
1(a')	Na ₂ S ₂ O ₄ -reduced, uncomplexed	1.85	1.942	2.05	1.0	25	63	3.5
1(b')	Na ₂ S ₂ O ₄ -reduced, MgATP-complexed	1.85	1.942	2.05	1.0	67	4	3.5
1(c)	No interaction	1.85	1.942	2.05	1.0	—	—	0

due to changes in C. Leigh (1970) gives C as being proportional to the correlation time, τ , and inversely proportional to r^6 (where r is the distance between the centres). Hence, if we can estimate τ , we can obtain a value for r . Taking τ as 10^{-9} s and assuming that the second centre has a spin of $\frac{1}{2}$ we get a value for r of the order of 1 nm, which is a reasonable value, but relies entirely on the value of τ taken; this is very difficult to estimate. One important consequence of the model is that if r becomes smaller (or C increases for any other reason), the apparent intensity of the e.p.r. signal also falls. This may explain the apparently small e.p.r. signal of the Fe-protein during catalytic activity; this has been interpreted as implying that the Fe-protein is largely oxidized in this state (Thorneley *et al.*, 1976; Orme-Johnson *et al.*, 1977). It may be, however, that r is lower in the active complex of both proteins. This affords greater consistency between the e.p.r. measurements and the stopped-flow spectrophotometric studies of Thorneley (1975), which imply a rapid reduction of Kp2 protein by sodium dithionite and a slow oxidation of Kp2 protein by Kp1 protein. In the full fixing system, if the kinetics are simple, this predicts a high concentration of reduced Kp2 protein; the model described in this paper casts doubt on the use of e.p.r. intensities as a probe of the extent of reduction of Fe-proteins when their conformations may change. In this context it is interesting to note that Zumft *et al.* (1973) observed a large decrease in the intensity of the Cp2-protein e.p.r. spectrum in the presence of the protein denaturant urea (0.5M).

It is interesting to speculate on the nature of the proposed second centre. Since it has such a short T_2 , it is presumably a metal, and the most likely candidate would seem to be iron. Unfortunately Orme-Johnson *et al.* (1977) and Gillum *et al.* (1977) have shown that a 4Fe-4S cluster can be extruded from Cp2 protein, and this accounts for 94% of the iron atoms. It should be noted, however, that the studies of Smith & Lang (1974) show slight heterogeneities in the 77 K Mössbauer spectra; this may imply the existence of two kinds of Fe in the protein, and the Mössbauer

results do not preclude the existence of a 4Fe-4S cluster plus one additional magnetic Fe atom (B. E. Smith, personal communication). If the model is correct, very-low-temperature e.p.r. spectroscopy should reveal the spectrum of the second centre.

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