Chloroplast Rieske Center

EPR STUDY ON ITS SPECTRAL CHARACTERISTICS, RELAXATION AND ORIENTATION PROPERTIES*

(Received for publication, January 22, 1991)

Astrid Riedel†, A. William Rutherford‡§, Günter Hauska∥, Adolf Müller‡, and Wolfgang Nitschke‡**

From the Institut für Biophysik und Physikalische Biochemie and the ||Institut für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 8400 Regensburg, Federal Republic of Germany, the §§Section de Bioénergétique, Département de Biologie Cellulaire et Moléculaire, CE Saclay, 91 191 Gif sur Yvette Cedex, France

The crucial role of a relatively small membrane-bound protein in the enzyme-catalyzed oxidation of ubiquinol had already been recognized (Trumpower, 1976) a long time before the general protein composition and mode of functioning of the ubiquinolcytochrome c oxidoreductases (the so-called cytochrome bc complexes) was elucidated. This protein, initially termed "oxidation-factor," was subsequently shown to contain the ubiquinol:cytochrome bc complex from spinach and bearden, A. J. (1980) Biochim. Biophys. Acta 592, 323–337.

The unusual anisotropic saturation behavior of the EPR spectrum was characterized in detail, whereby hitherto controversial results on the spectral parameters of the chloroplast Rieske center could be rationalized.

The observed phenomena are discussed on the basis of three tentative models which are able to qualitatively explain the effects.

The spectral parameters, the orientation of the g tensor in two-dimensionally ordered multilayers and the relaxation properties of the Rieske center in purified cytochrome bc complex from spinach were studied by EPR spectroscopy.

A trough at \( g = 1.76–1.74 \) was unambiguously identified as the \( g_e \) signal on the basis of the sensitivity of its field position to inhibitors and oxidized plastocyanine.

In contrast to previous reports, the orientation of the chloroplast Rieske center was found to be identical to that of its cytochrome bc, counterpart, provided that non-saturating EPR conditions were applied. Upon onset of microwave saturation, however, the orientation with respect to the ordered multilayers where the \( g_e \) signal (\( g = 1.9 \)) was maximal, changed drastically and was then similar to the orientation reported previously (Prince R. C., Crowder, M. S., and Bearden, A. J. (1980) Biochim. Biophys. Acta 592, 323–337).

The unusually anisotropic saturation behavior of the EPR spectrum was characterized in detail, whereby hitherto controversial results on the spectral parameters of the chloroplast Rieske center could be rationalized.

The observed phenomena are discussed on the basis of three tentative models which are able to qualitatively explain the effects.

The crucial role of a relatively small membrane-bound protein in the enzyme-catalyzed oxidation of ubiquinol had already been recognized (Trumpower, 1976) a long time before the general protein composition and mode of functioning of the ubiquinolcytochrome c oxidoreductases (the so-called cytochrome bc complexes) was elucidated. This protein, initially termed "oxidation-factor," was subsequently shown to contain a \( 2Fe2S \) cluster (Trumpower and Edwards, 1979; Slater and de Vries, 1980) and to be identical to the Rieske protein first described in beef heart mitochondria (Rieske et al., 1964).

The presence of a Rieske protein has since then been demonstrated in quinolcytochrome c/plastocyanine oxidoreductases from many different organisms (Trumpower, 1981). The Rieske FeS centers from all these sources share a set of unique properties, which clearly distinguish them from most other \( 2Fe2S \) centers (e.g. \( 2Fe2S \) ferredoxins). (a) Their average \( g \) factor (\( g_e \)) of 1.90 is much lower than in ferredoxins (\( g_e = 1.96 \)). (b) Their redox midpoint potentials are unusually high. Whereas ferredoxins have \( E_{\text{redox}} \) values of about \(-400 \, \text{mV} \), most Rieske centers titrate around \(+300 \, \text{mV} \). At present only four members of the group of Rieske proteins are known to have a considerably lower \( E_{\text{redox}} \), i.e. the Rieske centers from Chlorobium (Knaff and Malkin, 1976), Bacillus alcalophilus (Lewis et al., 1981), Chloroflexus aurantiacus (Zannoni and Ingledew, 1985), and Heliochromatium chlorum (Liebl et al., 1990). These four centers titrate at about \(+100 \, \text{mV} \) and are thus still much more oxidizing than the ferredoxins.

Despite these common features of all Rieske-type \( 2Fe2S \) clusters possible differences between the chloroplast and the cyanobacterial Rieske centers (present in cytochrome bs complexes) on one hand and the Rieske centers found in cytochrome bc complexes on the other hand, have been the subject of recurring controversy (Prince et al., 1980; Hootkins and Bearden, 1983; Salerno et al., 1983; Hauska et al., 1983; Malkin, 1986; Nitschke et al., 1989). In most of these cases, however, the chloroplast Rieske center eventually was demonstrated to be a typical representative of the group of Rieske proteins.

Partial non-cysteine ligation had been invoked to explain the unusually high midpoint potentials of the Rieske clusters (Blumberg and Peisach, 1974; Fee et al., 1984). On the basis of strong nitrogen couplings seen by electron nuclear double resonance spectroscopy in samples containing bacterial and mitochondrial Rieske centers (Cline et al., 1985; Telser et al., 1987), histidines were proposed to play the role of these non-cysteine ligands. Such strong nitrogen couplings, however, could not be seen in electron nuclear double resonance spectra on the chloroplast Rieske center (Nitschke and Hauska, 1987a), again providing an apparent major difference between the Rieske centers from cytochrome bc complexes and those from cytochrome bc complexes. In fact, the strong couplings turned out to be artefactual (Gurbiel et al., 1989), thereby solving this apparent controversy. It is of note, however, that weaker couplings were detected and unambiguously attributed to interactions with nitrogen nuclei (Gurbiel et al., 1989), therefore, still leaving room for the idea that histidines are involved in liganding the cluster.

A recent report on electron spin echo envelope modulation spectra of Rieske proteins from cytochrome bc complexes again showed no major differences between the two types of enzymes (Britt et al., 1991).

The abbreviations used are: \( E_{\text{redox}} \), redox midpoint potential at pH 7; DBMBI, 2,5-dibromo-3-methyl-6-isopropyl-benzo-quinone; \( Q_\text{b} \), the quinol-oxidizing site of cytochrome bc complexes; UHDBT, 5-(\( n \)-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; mW, milliwatt.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Centre National de la Recherche Scientifique Grant URA 1290.

∥ Supported by the Deutsche Forschungsgemeinschaft. To whom correspondence should be addressed.
An as yet unexplained discrepancy, however, becomes obvious on comparing the orientations of the g tensors of the various Rieske centers with respect to the membrane. These orientations have been determined in partially ordered samples from many different systems, such as mitochondria (Salerno et al., 1979), purple bacteria (Prince, 1983), green sulfur bacteria, and heliobacteria (Liebl et al., 1990). In all these systems the high field g, trough was found to be maximal when the magnetic field was perpendicular to the membrane plane, whereas both g, and g, were oriented parallel to the membrane. By contrast, the Rieske center in cytochrome bf complex behaved differently: its g, peak was maximal, when the magnetic field was perpendicular to the membrane plane in spinach chloroplasts (Prince et al., 1980) or showed no orientation dependence in samples from the green halophilic alga Dunaliella parva (Hootkins and Bearden, 1983). A g, trough was not assigned in these works, and it was suggested that the cytochrome bf Rieske center might have an axial spectrum. Salerno et al. (1983), however, claimed that a trough at g = 1.77 attributable to the Rieske g, signal can be seen in spectra of isolated cytochrome bf complex. It is of note that the strikingly different orientation of the g, peak in cytochrome bf complex is not due to an artifact caused by the orientation procedure, as the same orientation is found by using magnetic orientation instead of partial dehydration.

In view of the otherwise far-reaching similarities the data arguing for differing orientations of the clusters are puzzling. Therefore, we set out to reexamine the spectral parameters and the orientation of the chloroplast Rieske cluster.

**EXPERIMENTAL PROCEDURES**

Cytochrome b/f complex was prepared from spinach chloroplasts using the detergent nonanoyl-N-methylglucamide (MEGA-9) instead of octyl glucoside (Hauska, 1986). To obtain higher concentrations, the final sucrose density gradient was replaced by a second ammonium sulfate precipitation in the presence of 0.25% cholate (Rich et al., 1987).

Oriented samples were obtained by partial dehydration according to Blasie et al. (1978). The detergent-solubilized cytochrome b/f complex was diluted 100-fold in unbuffered H₂O and pelleted by ultracentrifugation (15 h, 300,000 × g). The wet pellet was applied to the Mylar sheets and dried for approximately 48 h in darkness at 4 °C. Various redox states were achieved by applying solutions of sodium ascorbate or sodium dithionite to the dehydrated multilayers followed by a renewed drying under a stream of argon or nitrogen gas.

In the case of the inhibitor-treated samples, it was necessary to add the inhibitor to the concentrated cytochrome b/f sample immediately before it was applied to the Mylar sheets. Only DBMIB was found to be able to bind to the Q₀ site, when applied to an already dried sample.

Cytochrome bc₁ complex was prepared from beef heart mitochondria, as described by Ljungdahl et al. (1987). 2Fe2S ferredoxin from spinach was obtained from Fluka, Federal Republic of Germany (F. R. G.), DBMIB was obtained from Sigma, UHDBT from Dr. B. L. Trumpower, Hanover. Stigmatellin was a kind gift from Dr. G. Hofs, Braunschweig.

EPR spectra were recorded on Bruker ER200 and ER300 spectrometers, equipped with an Oxford Instruments helium cryostat and temperature control system.

The power saturation data were treated as described by Sahlin et al. (1986). The first derivative Y* of the EPR absorption amplitude was converted into a normalized form according to X* = (Y*/Po)/[(Y*/Po)²], where Y* was obtained at a given power Po under non-saturating conditions.

---

**RESULTS AND DISCUSSION**

**Spectral Parameters of the Chloroplast Rieske Center**

The EPR spectra of highly concentrated, reduced samples from purified cytochrome b/f complex are shown in Fig. 1a. A peak at 2.03, a derivative-shaped signal at 1.90, and a broad trough at 1.75 were seen together with the signal of a radical at g = 2.00, the intensity of which varied between samples. Identical spectra were found in ascorbate- and dithionite-reduced samples, demonstrating that none of the three main features arose from the spectrum of one of the cytochromes (cytochrome f and cytochrome b, are reduced in the presence of dithionite). The spectrum of Fig. 1a was recorded on a complex as isolated, i.e. without further treatment besides reduction. However, if an excess of oxidized plastoquinone was added to the sample, the g, trough shifted toward higher field to a g value of 1.74, both in the ascorbate- and in the dithionite-reduced samples (Fig. 1b). A slightly different behavior was reported for the Rieske center of cytochrome bc₁ complex (de Vries et al., 1979), where in the presence of oxidized ubiquinone the g, is 1.8 is "sharp," but broadens considerably and shifts to g, = 1.78, when ubiquinone goes reduced or is extracted from the complex.

When the inhibitors UHDBT or stigmatellin, known to bind at the Q₀ site, i.e. in close vicinity of the Rieske center, were added, again an upfield shift of the trough signal was seen (Fig. 1, c and d), together with smaller shifts on both the peak- and the derivative-shaped signals. An analogous action was observed for the Rieske center in cytochrome bc₁ complex is well-known (von Jagow and Ohnishi, 1985; Matsuura et al., 1983).

This sensitivity of the g, trough on the presence of Q₀ site inhibitors and oxidized quinone clearly demonstrates that the spectrum of the Rieske center in the cytochrome b/f complex is rhombic with g values of g, = 2.03, g, = 1.9, and g, = 1.76–1.74 (depending of the redox state of the quinone pool), in

---

*W. Nitschke and A. W. Rutherford, unpublished result.
J. Bergström, personal communication.
agreement with the result presented by Salerno et al. (1983) and contrary to what has been suggested by Prince et al. (1980) and Hootkins and Bearden (1983).

Orientation of the g Tensor with Respect to the Membrane

A signal at \( g = 1.74 \) was also reported in EPR studies on oriented thylakoid membranes from spinach (Prince et al., 1980) and the green alga \( D. parva \) (Hootkins and Bearden, 1983). This signal was found to be maximal when the magnetic field was perpendicular to the membrane plane, just as was the case for the derivative-shaped signal at \( g = 1.9 \), which was attributed to the Rieske center. These two directions (both perpendicular to the membrane), together with the \( g_z \) direction, which was determined to be parallel to the membrane plane, do not form an orthonormal set. Thus, the possibility that the signal at \( g = 1.74 \) might be part of the Rieske spectrum was excluded by these authors.

Our data, however, showed that the \( g = 1.74 \) was indeed the \( g_z \) signal. Thus, we reexamined the orientation data using oriented isolated cytochrome \( b_f \) complex. This offered the advantage of both much better signal-to-noise and the lack of overlap by other FeS centers. However, it was not obvious that the isolated complex would orient the same way as the membranes do. Therefore, we checked the orientations of all paramagnetic species seen in the oriented samples from purified cytochrome \( b_f \) complex. Fig. 2 shows spectra taken perpendicular and parallel to the mylar sheets in fully oxidized (Fig. 2a) and ascorbate reduced samples (Fig. 2b) at 15 K. The fully oxidized sample showed the presence of the \( g \) peaks of cytochrome \( f \) (at \( g \approx 3.5 \)) and cytochrome \( b_6 \) (at \( g \approx 3.6 \)).

Spectra recorded at intermediate angles (not shown) demonstrated that the \( g \) direction of cytochrome \( b_6 \) was oriented parallel to the plane of the Mylar sheets, whereas the \( g \) direction of cytochrome \( f \) formed an angle of about 60° with the Mylar sheets. As the \( g \) directions in low spin hemes are usually close to the direction perpendicular to the heme plane (Taylor, 1977), this suggested that cytochrome \( b_6 \) was oriented perpendicular to the Mylar sheets, whereas cytochrome \( f \) lies at an angle of about 30°. An identical orientation for cytochrome \( f \) had been shown in magnetically oriented thylakoid membranes of spinach chloroplasts by Bergström and Vännägard (1982). Crowder et al. (1982) determined an orientation of about 50° for the two high spin signals at \( g = 6 \), which they attributed to cytochrome \( b_6 \). From these data they deduced an orientation of 50° for the heme plane with respect to the membrane plane. However, as the two signals around \( g = 6 \) in a rhombic high spin spectrum belong to directions which are both in the plane of the heme and mutually perpendicular, this assignment must be wrong. The data rather argue for a heme orientation perpendicular to the membrane, having \( g_z \) and \( g_x \) (around \( g = 6 \)) both at 45° to the membrane plane.

Yet, the high spin signals are most probably due to denatured cytochrome \( b_6 \) (Bergström, 1985; Nitschke and Hauska, 1987b). By contrast, the \( g \) peak of the intact cytochrome \( b_6 \) at \( g = 3.6 \) was found in membranes and was determined to be maximal when the magnetic field was parallel to the membrane plane (Bergström, 1985).

A perpendicular orientation of the cytochrome \( b_6 \) hemes with respect to the membrane has furthermore been shown for the cytochrome \( b_c \) complex (Ereçinska and Wilson, 1979).

It is of note, that only a signal at \( g = 3.6 \) for cytochrome \( b_6 \) was seen in our spectra. The signal of the low potential component of cytochrome \( b_6 \), previously described in highly active cytochrome \( b_f \) preparations (Nitschke and Hauska, 1987c) could not be detected, which was most probably due to inactivation of the enzyme during the drying process. As will be detailed below, the orientation of the Rieske center (in the reduced sample) was indistinguishable from the orientations published on oriented membranes (Prince et al., 1980; Hootkins and Bearden, 1983), provided that the same experimental conditions were employed. Thus, cytochrome \( b_6 \), cytochrome \( f \), and the Rieske center were oriented in our samples exactly the same way with respect to the Mylar sheets as they are in intact systems with respect to the native membrane. Therefore, we concluded, that using the purified complex does not introduce complications for comparisons with the native system.

Fig. 3 shows the orientation dependence of the \( g_z \) and \( g_x \) signal amplitudes of the Rieske center recorded at 10 K (broken lines). The orientation dependence of the \( g_z \) peak was omitted from the figure to avoid confusion, but it was oriented as reported in membranes (Prince et al., 1980). \( g_z \), was nicely oriented perpendicular to the membrane, whereas \( g_x \) appeared to be almost isotropic with a slight maximum at the same orientation as found for \( g_z \). However, if the temperature was raised to 50 K a different behavior was seen. \( g_x \) was still far from being well-oriented, but nevertheless a clear maximum is seen at orientations parallel to the Mylar sheets (continuous lines). The orientation of \( g_x \), however, was not affected by the
change in temperature. This temperature effect indicated, that saturation phenomena might be involved in the reported peculiar orientation properties of the chloroplast Rieske center.

**Relaxation Properties**

(a) *g* Factor Dependence of the Relaxation Properties—The comparison of spectra taken under non-saturating conditions (Fig. 1a) and under highly saturating conditions (Fig. 1c) in a non-oriented sample already showed that the relaxation properties of the center were strongly *g* factor dependent. The *g*<sub>1</sub> trough saturated much more readily than the remaining spectrum. This effect is quantitatively demonstrated by the saturation curves measured on the three principal *g* tensor axes (Fig. 4). Whereas the saturation curves of the *g*<sub>1</sub> and *g*<sub>2</sub> signals almost overlap, the curve characterizing the saturation behavior of the *g*<sub>1</sub> trough is clearly different. The same general behavior was observed when the sample was reduced by dithionite.

(b) Orientation Dependence of the Relaxation Properties in the *g*<sub>2</sub> Region—If the saturation experiments were performed on oriented samples, the relaxation behavior of the spectrum could be examined with respect to additional parameters, i.e. the orientation of the Mylar sheets. In the *g*<sub>2</sub> region, the signal was below detection, when measuring with the magnetic field parallel to the Mylar sheets. The *g*<sub>2</sub> peak could be seen in all orientations, however, its saturation properties were essentially not affected by the orientation. By contrast, the measurements on the *g*<sub>2</sub> signal yielded rather different saturation curves when measured at different orientations (Fig. 5). The signal saturated more readily, when the magnetic field was parallel to the Mylar sheets, i.e. at the orientation where the "true" *g*<sub>2</sub> axis, orthonormal to the determined *g*<sub>1</sub> and *g*<sub>2</sub> directions had to be expected. The *inset* of Fig. 5 shows the angular dependence of the ratio between signal sizes measured under non-saturating (0.63 mW) and under saturating conditions (126 mW). The signal saturated most readily close to the plane of the Mylar sheets (±20°) and least readily perpendicular to the plane (Fig. 5, *inset*).

Orientation-dependent saturation changes could in principle also be artifactually produced by sample sizes exceeding the region of approximate cylindrical symmetry of the microwave mode in the cavity. In such a case, turning the sample would result in variations of the actual microwave power applied to the sample. However, that such problems occur in our case could be ruled out by the observation that the saturation curve of the *g*<sub>2</sub> peak did not show an equivalent behavior. Thus, the observed orientation dependence of the saturation behavior of the *g*<sub>2</sub> signal was a true property of the Rieske center.

**Effect of Inhibitors on the Orientation Dependence of the *g*<sub>2</sub> Peak**

As shown above (Fig. 1, c and d), both UHDBT and stigmatellin altered the shape of the Rieske EPR spectrum. This went along with marked changes in the orientation dependence of the *g*<sub>2</sub> peak. The *inset* of Fig. 6 shows that after addition of stigmatellin the *g*<sub>2</sub> signal had a distinct maximum when the magnetic field was parallel to the Mylar sheets, even under conditions where the control sample showed the maximum for the *g*<sub>2</sub> signal at the perpendicular orientation. An equivalent behavior was found after addition of UHDBT. This seemed to suggest that the inhibitors might enhance the relaxation rates of the paramagnetic center in such a way that the conditions, which were previously saturating for parts of the spectrum, no longer induce saturation. However, this was not true. The saturation curves measured on the inhibitor-treated samples showed the same general behavior as those taken on the control sample. Especially, the slower relaxation of the *g*<sub>2</sub> region of the spectrum was fully conserved (Fig. 6 for stigmatellin).

**The Anisotropic Relaxation Is a General Property of the Rieske Centers and Can Even Be Observed in 2Fe2S Ferredoxins**

It was suggested (Prince et al., 1980; Hootkins and Bearden, 1983) that the cytochrome *b*<sub>5</sub> Rieske center might be different
from its cytochrome \( bc_1 \)-type counterpart, concerning the orientation of the \( g \) tensor and the absence/presence of a \( g \) trough. Since we showed above that these apparent differences did not occur if measurements were performed under non-saturating conditions, it was of interest to compare the saturation properties of the cytochrome \( bc_1 \)-type clusters. For this purpose, we repeated the above measurements on cytochrome \( bc_1 \) complex from beef heart mitochondria (using the purified enzyme) (Fig. 7a) and from the purple bacterium \( \text{Rhodospirillum viridis} \) (not shown). Qualitatively, the same results as for the chloroplast Rieske center were found, although less pronounced. The \( g \) trough saturated more readily than the \( g_x \) and \( g_z \) signals (Fig. 7a). The anisotropic ratio of the \( g \) signal became smaller when the temperature was lowered and/or the microwave power was increased (not shown). As the effect was less marked than in the chloroplast Rieske center, a "wrong" orientation could not be induced under usual measuring conditions. This is probably the reason why the effect remained unreported so far.

An equivalent \( g \) factor-dependent relaxation behavior was present in the 2Fe2S ferredoxin from spinach, an iron-sulfur protein belonging to the \( g_{av} = 1.96 \) class (Fig. 7b). While the effect was even less pronounced than in the cytochrome \( bc_1 \) Rieske centers, it was still clearly observable. Since the ferredoxins are soluble proteins, they cannot be oriented two-dimensionally by our method. Thus, the orientation effects seen in the Rieske centers could not be studied in ferredoxins.

This prevented us from obtaining experimental evidence pertinent to the question whether the two properties, 1) anisotropic relaxation and 2) anomalous orientation, are actually manifestations of a single phenomenon or are unrelated effects.

Three tentative models, which could explain the observed phenomena, will be given in the following section.

(a) The Dimer Model—In this model, the described effects are due to the presence of two distinguishable Rieske clusters, which have different orientations with respect to the Mylar sheets and which relax differently. A situation with two distinguishable Rieske centers has been proposed already some time ago in the model of the dimeric Q-cycle (de Vries et al., 1983). To account for the observed effects, the two clusters should have their \( g \) directions collinear, their \( g \) directions mutually perpendicular, and the faster relaxing cluster should furthermore have an axial spectrum or its \( g \) trough should be broadened below detection. Such a situation could explain the observed effects perfectly well.

A lot of evidence argues against an indispensable role of a dimeric state of the cytochrome \( bc \) complexes during catalysis (Hurt and Hauska, 1981; Nalecz and Azzi, 1985; Nugent and Bendall, 1987). However, even if the cytochrome \( bc \) complexes should turn out to be a functional or structural dimer, it seems unlikely that the same protein would be inserted in the membrane in two different ways and could show two different EPR spectra. As there is no evidence neither on the level of molecular biology nor biochemistry that two different Rieske proteins are present in cytochrome \( bc \) complexes, we consider this model as the least likely explanation.

(b) Saturation-induced Deformation of the Spectrum—The \( g \) tensor of the chloroplast Rieske center measured under non-saturating conditions was correctly oriented, i.e. its principal axes formed an orthonormal set of directions. As shown above, the relaxation behavior of the paramagnetic center was different for all three axes. Thus, spectra observed under saturating conditions in oriented samples do not represent proper integrals over all possible orientations. Rather, the contributions of orientations, which saturate easily, are suppressed and underestimated in the integral. This effect, however, can manifest itself only on \( g \), since only for this intermediate \( g \) value orientations can be overemphasized which differ considerably from the principal axis. According to our data (see above), contributions from orientations close to the principal \( y \) axis were diminished by saturation. Therefore, other \( g \) directions could in principle become dominant in the spatial integral.

In order to assay the validity of these considerations, we tried to simulate our data based on this model. For this purpose, we used the method proposed by Salerno et al. (1977) for the simulation of two-dimensionally ordered systems. To account for the anisotropic relaxation properties, a tensorial "saturation factor" was included. These simulations produced a slight decrease of the anisotropy of the \( g \) signal under saturating conditions. However, to achieve simulations in line with the experimental data, the contributions from the principal \( g \) axis had to be almost completely suppressed. This is in disagreement with the observed, rather moderate diminishing of the \( g \) peak as measured parallel to the Mylar sheets, i.e. parallel to the principal \( g \) axis. Furthermore, a simultaneous fit for all \( g \) factors could not be obtained. Thus, the effects described above for the uninhibited cluster could in principle result in the observed phenomena. However, for a satisfactory simulation of the experimental data, additional parameters have to be introduced, which at present cannot be justified. Moreover, this model does not provide an explanation for the anisotropic relaxation properties of the cluster.

Furthermore, an equivalent wrong orientation should be expected for the inhibitor-treated samples, since the same \( g \) factor-dependent saturation behavior as in the control sample was seen. This was not the case, as evidenced by the polar plot shown in the inset of Fig. 6 again arguing against this model.

(c) Conformational Substates—Bertrand and Gayda (1979) proposed a model to explain the \( g \) values of 2Fe2S centers from the ferredoxin (\( g_{av} = 1.96 \) class). This model was subsequently expanded to the Rieske-type clusters (\( g_{av} = 1.91 \)) (Bertrand et al., 1985). On the basis of their model, these authors were able to simulate the full \( g \) strained spectrum of

![Fig. 7. Power saturation curves taken on the three \( g \) values of the Rieske center of isolated cytochrome \( bc_1 \) complex from beef heart mitochondria (a) and the 2Fe2S ferredoxin of spinach chloroplasts (b) at 15 K. EPR conditions were as in Fig. 1.](image-url)
ferredoxins without having to invoke field-dependent linewidths (More et al., 1987). The key idea in these simulations was that the 2Fe2S clusters exist in a range of conformational substates (in the frozen state), which can be characterized by a single variable, called $\Theta$. The three principal $g$ values of the spectrum depend on $\Theta$, as detailed in Bertrand and Gayda (1979). Upon allowing for a Gaussian distribution of conformational substates, described by a distribution of $\Theta$ values about a mean $\Theta_m$, the experimental $g$-strained spectra could be reproduced.

If one applies these simulations to the Rieske-type centers, a peculiar situation arises. Bertrand et al. (1985) estimated a value of $-24$ to $-30^\circ$ for the $\Theta$ parameter of the Rieske-type clusters. This comes rather close to the critical value of $-20$ to $-22^\circ$ (depending on the exact choice of the energy parameters, see Bertrand et al., 1985), i.e., the intersection point of the curves representing the dependence of $g_\perp$ and $g_\parallel$ values on the $\Theta$ parameter (for a graphical representation see More et al., 1987). Below this critical value, $g_\perp$ becomes $g_\parallel$, and vice versa.

We applied the method proposed by More et al. (1987) to the case of the chloroplast Rieske center. The experimental linewidths on the three principal $g$ values could be simulated according to this method, if a width of 1.5 to 2.0$^\circ$ for the Gaussian distribution of the $\Theta$ parameter was used. In this case, 10–15% of the integral of the distribution function lie beyond the intersection point, which is equivalent to the fact that 10–15% of the centers are in conformational substates where $g_\perp$ and $g_\parallel$ are swapped. If these “swapped” substates were to saturate less readily than the remaining substates, the data could be explained. There are several observations which are in line with this model. (a) On going toward saturating conditions, both the $g_\perp$ and $g_\parallel$ values increase slightly (not shown). From a plot of the three $g$ values for the Rieske center (analogous to that shown in More et al., 1987 for the 2Fe2S ferredoxins), it is predicted that this shift occurs beyond the intersection point. (b) Both stigmatellin and UHDBT drastically change the orientation behavior of the $g$ signal together with modified principal $g$ values and decreased linewidths. In the plot mentioned above, this is equivalent to a displacement of $\Theta_0$ and a decrease of $\Delta \Theta$. This results in smaller contributions of the swapped conformations to the total spectrum, hence enhancing the “correct” orientation.

It might seem unlikely that only 10–15% of the centers with the swapped orientation could result in such pronounced orientation effects. However, one must keep in mind, that a $g$ direction which is perpendicular to the plane of the twodimensional orientation represents a special case in that only for this orientation all molecules in the sample contribute to the EPR signal (as there is only one perpendicular orientation). By contrast, if this $g$ direction lies in the plane of the twodimensionally ordered layers, only the fraction of molecules, for which this direction is parallel to the magnetic field, gives rise to an EPR signal. Thus, the contribution of the 10–15% of the centers, where $g_\perp$ is perpendicular to the plane, is drastically enhanced over that of the majority of the centers, where $g_\parallel$ is parallel to this plane.

(d) Basic Assumptions within the Three Models—It is of note, that the observed effects arise from rather different physical origins according to the choice of model. For model b, a real anisotropy of the relaxation rates must be inferred. Such anisotropy could straightforwardly be expected from an anisotropic interaction with a neighboring spin. However, the presence of the relaxation effects even in fully reduced samples (i.e., when the cytochromes have $S = 0$) together with the absence of detectable changes in the peculiar relaxation properties upon reduction by ascorbate and by dithionite (see above) excludes an involvement of the three hemes in the complex (i.e., hemes $b_a$, $b_c$, and $f$). Furthermore, no change could be observed after quinone depletion of the cytochrome $b$-complex (not shown). Thus, no obvious candidate for such an anisotropic interaction could be found.

Models a and c, by contrast, do not rely on such anisotropy of relaxation rates. Instead, the anisotropic saturation results from the superposition of spectra with isotropic relaxation while differing slightly from each other with respect to principal $g$ values (especially $g_\parallel$) and relaxation rates. Differing $g$ anisotropies and concomitantly different relaxation rates have been reported for a number of binuclear iron-sulfur clusters (Salerno et al., 1977). Equivalent phenomena can be expected to exist within a given set of conformational substates as inferred to account for the observed $g$-strained spectra (More et al., 1987).

A more profound discussion of the mechanistic origins for the proposed models has to await a better understanding of the 2Fe2S clusters in general.

CONCLUSIONS

As demonstrated above, the reported apparently unique features of the chloroplast Rieske cluster compared to other Rieske centers result from the application of measurement conditions inducing microwave saturation. When the center was examined under non-saturating conditions, it showed all the usual features known for Rieske clusters, i.e., the rhombic spectrum, the orientation of the $g$ tensor with respect to the Mylar sheets, and the sensitivity of the $g$ values on $Q_0$ site inhibitors. Therefore, we conclude that the chloroplast Rieske center is a typical representative of Rieske-type 2Fe2S centers.

Furthermore, the peculiar saturation behavior of the chloroplast Rieske center was not confined to this cluster but was found to be a general phenomenon of 2Fe2S centers. Only the extent to which these phenomena become apparent varies depending on the rhombicity of the spectrum.

As detailed above, several models can account for the observed effects. These comprise biological (i.e., the dimer model) as well as physical explanations. In view of the fact that even ferredoxin, which is clearly a monomer, exhibits the $g$ factor-dependent saturation, together with the arguments given above, we consider the dimer model to be unlikely.

The second model cannot reproduce the experimental data satisfactorily without further assumptions. Only the model of Bertrand and Gayda (1979) taken together with the concept of the conformational substates (More et al., 1987) is able to account for the orientation phenomena in a consistent way. We therefore consider it as the most appropriate description of the physical basis of the reported effects.

Acknowledgments—We would like to thank H. Beinert (Milwaukee) and J. Bergström (Göteborg) for communicating results prior to publication, P. Bertrand (Marseille), W. R. Hagen (Wageningen), E. Lang (Reesensburg), J. C. Salerno (Troy), C. Veegar (Wageningen), and J.-L. Zimmermann (Saclay) for stimulating discussions.

REFERENCES

Bergström, J. (1980) FEBS Lett. 133, 87–90
Bertrand, P., and Gayda, J.-P. (1979) Biochim. Biophys. Acta 579, 107–121
Bertrand, P., Guigliarelli, B., Gayda, J.-P., Beardwood, P., and Gibson, J. F. (1985) Biochim. Biophys. Acta 831, 261–266
Blasie, J. K., Erečinska, M. Samuels, S., and Leigh, J. S. (1978) Biochim. Biophys. Acta 501, 33–52
