Biochimica et Biophysica Acta, 723 (1983) 133–138
Elsevier

BBA 41287

THE LOCATION, ORIENTATION AND STOICHIOMETRY OF THE RIESKE IRON-SULFUR CLUSTER IN MEMBRANES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

ROGER C. PRINCE

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)

(Received November 3rd, 1982)

Key words: Membrane asymmetry; Ubiquinol-cytochrome c oxidoreductase; Bacteral photosynthesis; Iron-sulfur cluster; (Rps. sphaeroides)

Neutral and negatively charged dysprosium complexes are able to enhance the spin relaxation rate of the Rieske iron-sulfur cluster only when added from the cytochrome c_2 side of the photosynthetic membrane, indicating that the Rieske cluster is asymmetrically placed in the membrane, nearer the cytochrome c_2 side. The g_z-axis of the Rieske cluster, taken to be the iron-iron axis of this binuclear cluster, lies in the membrane plane, as does the g_y-axis. Appropriately, the g_x-axis is orthogonal to the membrane plane. A comparison with a mammalian mitochondrial standard indicates that there are 0.65 ± 0.1 Rieske clusters per reaction center. This is in excellent agreement with previously determined estimates of the number of antimycin-binding sites, and binding sites for what is known phenomenologically as Q_Z, suggesting that there is one of each per ubiquinol-cytochrome c_2 oxidoreductase.

Introduction

The ubiquinol-cytochrome *c* oxidoreductases of mitochondria and many bacteria, and the plastoquinol-cytochrome *f* oxidoreductase of chloroplasts, contain a distinctive iron-sulfur cluster, first reported by Rieske et al. [1], which has become known as the Rieske cluster. Apparently functioning as the oxidant of a quinol and the reductant of a bound *c*-type cytochrome, the Rieske cluster plays an essential role in electron transfer in these oxidoreductases [2].

The Rieske iron-sulfur cluster of the photosynthetic bacterium *Rhodopseudomonas sphaeroides* has already been extensively characterized and studied [3–6]. It has an oxidation-reduction midpoint potential of +280 mV at pH 7, and exhibits a pK on the oxidized form at pH 8 [3,4]. Its oxidation by cytochrome c_1 is rapid ($t_{1/2} \approx 200$ μs), and is inhibited by quinone analogs such as UHDBT [5] and UHNQ [6]. This paper addresses the location and orientation of the Rieske cluster within the photosynthetic membrane, and assesses the concentration of the cluster in the ubiquinol-cytochrome *c* oxidoreductase.

A powerful approach to determining the location of a paramagnetic center such as the Rieske cluster is to attempt to relieve the cluster's inherent microwave saturation properties by adding paramagnetic rare earth ions [7–9]. Dy^{3+} is a good extrinsic probe because of its short spin-lattice relaxation time (T_1) and large magnetic anisotropy [10]. Several groups [8,11,12] have found that the microwave power needed for half saturation of a biological paramagnetic center (see Ref. 13) varies proportionately with the concentration of relaxing extrinsic paramagnetic probe, and inversely proportionately with the sixth power of the distance of closest approach of the relaxing probe. In this

Abbreviations: HEDTA, hydroxyethylethylenedinitrilotriacetic acid; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Mops, 3-(*N*-morpholino)propanesulfonic acid; UHNQ, 2-(n-undecyl)-3-hydroxy-1,4-naphthoquinone.

work the distance of closest approach is assessed when the dysprosium is added to the outside of chromatophores or spheroplast-derived vesicles, which have opposite polarity.

Oriented membrane vesicles have been widely studied in recent years because they approximate one-dimensional crystals oriented relative to the membrane normal, but with no preferential orientation in the plane of the membrane. The highest 'dichroic ratios' are obtained using partial dehydration in a 90% relative humidity chamber [14–18]. Blum et al. [17] have shown that although the principal g values of a crystalline paramagnetic center vary as the angle of the applied magnetic field is varied, even a small amount of disorder, or 'mosaic spread', about the predominant orientation leaves the g values approximately constant. However, under such conditions the amplitudes of the individual g values show an orientation dependence, being maximal when the magnetic field is parallel to the g-axis of the paramagnetic center.

The results indicate that the Rieske iron-sulfur cluster, which seems to be a [2Fe-2S] cluster, lies near the cytochrome c_2 side of the membrane, with the iron-iron axis in the membrane plane. There seems to be a single Rieske center per ubiquinol-cytochrome c_2 oxidoreductase, and 0.65 ± 0.1 of these complexes per reaction center in the chromatophore membrane.

Materials and Methods

Rps. sphaeroides Ga was grown anaerobically in the light on a medium containing succinate as the sole carbon source [19]. Chromatophores were prepared using a French pressure cell [19] and spheroplast-derived vesicles were prepared by the method of Takemoto and Bachmann [20]. The concentrations of reaction centers in the chromatophores and spheroplast-derived vesicles were assayed by monitoring the 605 nm absorbance band of the reduced bacteriochlorophyll 'dimer' in a Johnson Foundation rapidly responding dual-wavelength spectrophotometer following flash activation [19]. Chromatophores contain cytochrome c_2 inside the vesicles [21] and less than 5% of the reaction centers were accessible to externally added mammalian cytochrome c. In contrast, the spheroplast-derived vesicles are essentially devoid of cytochrome c_2, and more than 90% of their reaction centers were accessible to externally added mammalian cytochrome c.

$DyCl_3$, $LaCl_3$ and $Dy(NO_3)_3$ were obtained from Alfa Chemicals, Danvers, MA. The chlorides were chelated 1 : 1 with EDTA (sodium salt) or HEDTA (sodium salt). All were prepared as 100 mM stock solutions at pH 6.8, and were added immediately prior to freezing the samples, after poising the ambient redox potential, to minimize the possibility that they might permeate the membrane.

Chromatophores were oriented by layering a concentrated suspension onto mylar and partially dehydrating the sample in a 90% relative humidity atmosphere for 36 hours at 4°C.

Results

The location of the Rieske cluster in the photosynthetic membrane

The derivative EPR spectrum of the Rieske cluster of *Rps. sphaeroides* is well known, with $g_x = 1.81$, $g_y = 1.90$ and $g_z = 2.03$ [3–6]. Fig. 1 shows saturation profiles of the $g_y = 1.90$ signal in chromatophores and spheroplast-derived vesicles supplemented with dysprosium and lanthanum complexes. LaEDTA is used as a diamagnetic control. The Rieske cluster is not very sensitive to paramagnetic relaxation from the added Dy^{3+}, indicating that the cluster is well buried in the membrane. Nevertheless, both DyEDTA (a negatively charged complex) and DyHEDTA (a charged complex with no net charge) consistently affected the Rieske cluster when added to spheroplast-derived vesicles, but not to chromatophores. The effects of the two complexes were similar, and linearly dependent on their concentration (data not shown). At 13 K the change in microwave power for half-saturation was 0.7 (± 0.1) mW/mM dysprosium complex.

The orientation of the Rieske cluster in the membrane

Fig. 2 shows spectra taken at representative angles of oriented multilayers of chromatophore membranes; the magnetic field and the membrane plane are parallel at 0°C. Circular coordinate plots for the three g values are shown in Fig. 3; clearly,

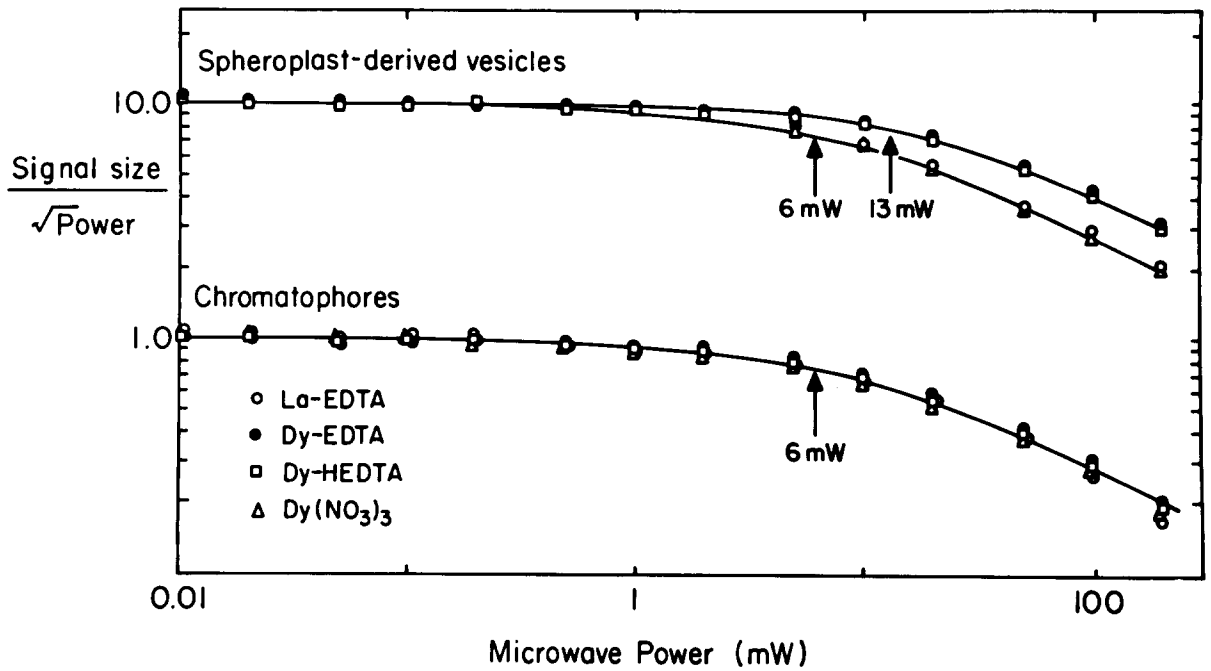

Fig. 1. The effect of dysprosium complexes on the microwave power saturation properties of the Rieske iron-sulfur cluster of *Rps. sphaeroides*. Chromatophores (1.5 mM bacteriochlorophyll) or spheroplast-derived vesicles (0.7 mM bacteriochlorophyll) suspended in 20 mM Mops, 100 mM KCl were reduced with a few crystals of solid sodium ascorbate plus 10 μM *N*-methylphenazonium methosulfate. This brings the E_h to approx. +150 mV. Immediately prior to freezing in ligroin cooled to near 77 K with liquid nitrogen, 10 mM of the indicated lanthanum or dysprosium complex was added. The magnitude of the $g_y = 1.90$ signal was measured at 13 K and the data normalized for easy visual inspection. The curves were fitted from Fig. 5 of Ref. 33. The arrows indicate the power required for half-saturation.

the g_x-axis is normal to the membrane plane, with g_y- and g_z-axes in the membrane plane.

The addition of inhibitors such as UHDBT or UHNQ shifts the g values of the Rieske cluster to $g_z = 2.04$, $g_y = 1.89$ and $g_x = 1.79$ [5,6]. Samples oriented in the presence of these inhibitors showed that the g-axes retained the same orientation shown in Figs. 2 and 3.

Upon reduction of the quinone pool of *Rps. sphaeroides*, the g_x band of the Rieske clusters shifts from $g = 1.81$ to $g = 1.79$. It has proven impossible to produce oriented multilayers with the Q pool reduced by the addition of reductants prior to partial dehydration, but dehydrated samples treated with reductant after orientation do reveal the $g = 1.79$ signal at the same orientation as when the signal was at $g = 1.81$.

De Vries et al. [23] have concluded that there are two equal populations of slightly different Rieske clusters in mammalian mitochondria, with slightly different line shapes. No clear evidence for such a dichotomy can be seen in Fig. 2, but if there are two centers, their orientation seems to be very similar.

The concentration of the Rieske cluster in the photosynthetic membrane

Attempts to integrate the derivative EPR spectrum of the Rieske cluster have proven difficult because of the ubiquitous presence of a variety of free radical signals in the chromatophore membrane, which sometimes overlap the g_z signal of the Rieske cluster. However, the Rieske cluster of *Rps. sphaeroides* seems to be so similar to that of mammalian mitochondria that comparison with the concentration of the Rieske cluster in the mitochondrial ubiquinol-cytochrome *c* oxidoreductase (Complex III) may yield a reasonable estimate of its concentration. There is a large amount of evidence that mitochondrial 'complex

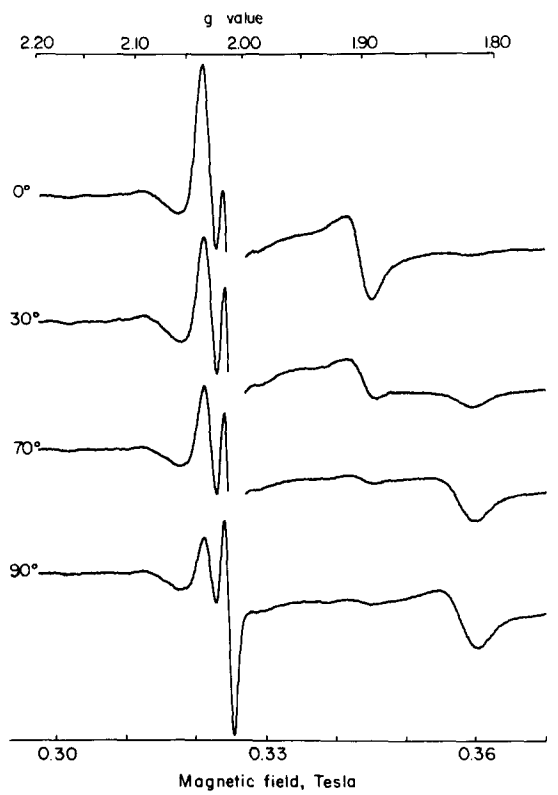

Fig. 2. The orientation of the Rieske iron-sulfur cluster in chromatophores of *Rps. sphaeroides*. Partially dehydrated samples were measured at 10° intervals, and representative spectra are shown. At 0° the membrane plane of the dehydrated sample is parallel with the applied magnetic field. The temperature was 19 K, with 2 mW of microwave power and 1.6 mT modulation.

III' contains one Rieske cluster per cytochrome c_1 [22–24] although De Vries et al. [23] have concluded that there are in fact two slightly different forms of the Rieske cluster, each present at a stoichiometry of one-half per cytochrome c_1. Assuming that the mitochondrial cytochrome b-c_1 complex contains one equivalent of the Rieske cluster per cytochrome c_1 (measured spectrophotometrically as described by Rieske [22]), and measuring the height of the $g_y = 1.90$ signal in both the mitochondrial and chromatophore systems under identical, nonsaturating conditions (see Fig. 1), a relative concentration of the Rieske cluster in the chromatophore membrane could be obtained. This in turn could be related to the concentration of photochemical reaction centers in the chromatophore membranes, determined spectrophotometrically as described by Dutton et al. [19]. Since the concentration of chromatophores was so different in these two assays, they were normalized using the total amount of bacteriochlorophyll in the sample, measured as described in Ref. 19. In such comparisons ten separate preparations of chromatophores compared to two separate preparations of the mitochondrial cytochrome b-c_1 complex yield a stoichiometry of 0.65 ± 0.1 clusters per reaction center.

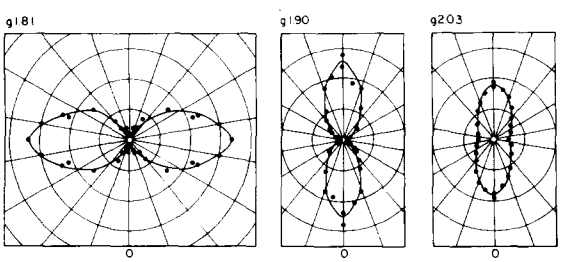

Fig. 3. The orientation of the Rieske iron-sulfur center in chromatophores of *Rps. sphaeroides*. This figure plots the amplitudes of the three g values seen in Fig. 2. At 0° the membrane plane is parallel with the applied magnetic field.

Discussion

The location of the Rieske cluster in the photosynthetic membrane

Added dysprosium complexes of either negative or neutral charge are able to relieve the inherent microwave saturation properties of the Rieske cluster of *Rps. sphaeroides* only when added from the cytochrome c_2 side of the membrane, indicating that the Rieske cluster is asymmetrically disposed in the membrane. Estimates of the distance from the Rieske cluster to the membrane surface are at best only very crude, but using the relationship between the change in power for half-saturation ($\Delta P_{1/2}$) and distance (r) found by Blum et al. [12] for the high-potential tetranuclear cluster from *Rps. gelatinosa*:

$$\Delta P_{1/2}\,(\mathrm{mW/mM}) = 4.12 \cdot 10^{8} \cdot r^{-6} \exp(-12.5/T)$$

yields a value for r of 25 Å. This must be decreased by 5 Å because the dysprosium is at the center of the chelator, yielding a distance of 20 Å. Furthermore, since the dysprosium complexes are

not symmetrically disposed about the Rieske cluster, but are restricted to only the membrane surface this must be reduced by about 30% [25], to yield an estimate of 14 Å from the aqueous/membrane interface. Probably the major errors in this calculation are the assumptions that the membrane is 'flat' and that the dysprosium complex can only approach this membrane surface. Nevertheless, the estimate is reasonable, it agrees fairly well with mammalian mitochondrial estimates (17–20 Å from the cytochrome *c* side, [25]), and is in accord with the finding that electron flow from the Rieske cluster, probably via cytochrome c_1, to cytochrome c_2 is not accompanied by a carotenoid band shift [5], indicating that if the reaction is indeed an electron transfer, as the equilibrium properties would suggest, it does not occur through the region of low dielectric constant which is usually believed to be the middle two-thirds of the membrane.

Orientation of the Rieske cluster

The mammalian mitochondrial Rieske cluster is a [2Fe-2S] cluster [26,27] and the *g* values of the bacterial cluster suggest that it is very similar. As such, the model of Gibson et al. [28] predicts that the g_z-axis is the iron-iron axis of the cluster, and the data presented in Figs. 2 and 3 indicate that this lies in the membrane plane. This is identical to the orientation of the Rieske cluster in both animal [29] and plant (Bonner, W.D. and Prince, R.C., unpublished observations) mitochondria and of the binuclear centers S-1 and N-1 of animal mitochondria [29]. It is also similar to the orientation of the Rieske cluster in chloroplasts [18], but in this case the $g = 1.90$ axis is orthogonal to the membrane plane (cf. Figs. 2 and 3). Furthermore, DBMIB shifts the $g = 1.90$ axis of the chloroplast cluster to $g = 1.95$, in the process changing the orientation so that this new *g* value lies in the membrane plane [18]. DBMIB had no effect on the Rieske cluster of *Rps. sphaeroides*, and while UHNQ and UHDBT shift all three *g* values slightly [5,6] they do not alter the orientation of the axes in the membrane plane.

The concentration of the Rieske cluster in the photosynthetic membrane

The stoichiometry measured here, 0.65 ± 0.1 Rieske clusters per reaction center, is in excellent agreement with other estimates of the number of ubiquinol-cytochrome c_2 oxidoreductase (or cytochrome bc_1 complexes) in the photosynthetic membrane. Thus, there are 0.7 ± 0.1 antimycin-binding sites per reaction center [30], and $0.8 \pm 0,.1$ special quinone-binding sites (for the so-called Q_Z [31]) per reaction center, suggesting that there is one Rieske cluster, one Q_Z-binding site and one antimycin-binding site per oxidoreductase. These are a somewhat unsatisfactory group of numbers, because they lie between a clearcut 1 or 2 reaction centers per oxidoreductase, but it should be borne in mind that *Rps. sphaeroides* is capable of aerobic metabolism, and some oxidoreductases may be in parts of the membrane that lack reaction centers and some reaction centers are known to lack oxidoreductases (e.g., see Refs. 30–32).

The relatively low concentration of the Rieske clusters, Q_Z-binding sites and antimycin-binding sites is in contrast to the amount of *b*-type cytochromes present. Chromatophores contain an apparent 4-fold excess of cytochrome *b*-560 over reaction centers [32]. A role for this apparent excess has not been suggested.

Acknowledgment

This work was supported by a grant from the National Science Foundation, PCM 7919598.

References

1 Rieske, J.S., Hansen, R.E. and Zaugg, W.S. (1964) J. Biol. Chem. 239, 3017–3022
2 Trumpower, B.L. and Katki, A.G. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R.A., ed.), pp. 89–200, Marcel Dekker, New York
3 Prince, R.C., Lindsay, J.G. and Dutton, P.L. (1975) FEBS Lett. 51, 108–111
4 Prince, R.C. and Dutton, P.L. (1976) FEBS Lett. 65, 117–119
5 Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochem. Biophys. Acta 592, 445–460
6 Matsuura, K., Bowyer, J.R., Ohnishi, T. and Dutton, P.L. (1983) J. Biol. Chem., 258, 1571–1579
7 Sarna, T., Hyde, J.S. and Swartz, H.M. (1976) Science 192, 1132–1134
8 Case, G.D. and Leigh, J.S. (1976) Biochem. J. 160, 769–783
9 Blum, H., Leigh, J.S., Salerno, J.C. and Ohnishi, T. (1978) Arch. Biochem. Biophys. 187, 153–157
10 Nieboer, E. (1975) Struct. Bonding 22, 1–47

11 Hyde, J.S. and Rao, K.V.S. (1978) J. Magn. Resonance 29, 509–516
12 Blum, H., Cusanovich, M.A., Sweeney, W.V. and Ohnishi, T. (1981) J. Biol. Chem. 256, 2199–2206
13 Portis, A.M. (1953) Phys. Rev. 91, 1071–1078
14 Blasie, J.K., Erecinska, M., Samuels, S. and Leigh, J.S. (1978) Biochim. Biophys. Acta 501, 33–52
15 Blum, H., Harmon, H.J., Leigh, J.S., Salerno, J.C. and Chance, B. (1978) Biochim. Biophys. Acta 502, 1–10
16 Tiede, D.M., Leigh, J.S. and Dutton, P.L. (1978) Biochim. Biophys. Acta 503, 524–544
17 Blum, H., Salerno, J.C. and Leigh, J.S. (1978) J. Magn. Resonance 30, 385–391
18 Prince, R.C., Crowder, M.S. and Bearden, A.J. (1980) Biochim. Biophys. Acta 592, 323–337
19 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morse, S.D. (1975) Biochim. Biophys. Acta 387, 536–556
20 Takemoto, J. and Bachmann, R.C. (1979) Arch. Biochem. Biophys. 195, 526–534
21 Prince, R.C., Baccarini-Melandri, A., Hauska, G.A., Melandri, B.A. and Crofts, A.R. (1975) Biochim. Biophys. Acta 387, 212–227
22 Rieske, J.S. (1967) Methods Enzymol. 10, 239–245
23 De Vries, S., Albracht, S.P.J. and Leeuwerik, F.J. (1979) Biochim. Biophys. Acta 546, 316–333
24 Bowyer, J.R., Edwards, C.A., Ohnishi, T. and Trumpower, B.L. (1982) J. Biol. Chem. 257, 8321–8330
25 Ohnishi, T., Blum, H., Harmon, H.J. and Hompo, T. (1982) in Electron Transport and Oxygen Utilization (Ho, C., ed.), pp. 387–393, Elsevier Amsterdam
26 Rieske, J.S., Zaugg, W.S. and Hansen, R.E. (1964) J. Biol. Chem. 239, 3023–3030
27 Albracht, S.P. J. and Subramanian, J. (1977) Biochim. Biophys. Acta 462, 36–48
28 Gibson, J.F., Hall, D.O., Thornley, J.H.M. and Whatley, F.R. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 987–990
29 Salerno, J.C., Blum, H. and Ohnishi, T. (1979) Biochim. Biophys. Acta 547, 270–281
30 Van den Berg, W.H., Prince, R.C., Bashford, C.L., Takamiya, K., Bonner, W.D. and Dutton, P.L. (1979) J. Biol. Chem. 254, 8594–8604
31 Prince, R.C., Bashford, C.L., Takamiya, K., Van den Berg, W.H. and Dutton, P.L. (1978) J. Biol. Chem. 253, 4137–4142
32 O'Keefe, D.P. and Dutton, P.L. (1981) Biochim. Biophys. Acta 635, 149–166
33 Blum, H. and Ohnishi, T. (1979) Biochim. Biophys. Acta 621, 9–18