

EPR, Electron Spin Echo Envelope Modulation, and Electron Nuclear Double Resonance Studies of the 2Fe2S Centers of the 2-Halobenzoate 1,2-Dioxygenase from *Burkholderia (Pseudomonas) cepacia* 2CBS*

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The 2-halobenzoate 1,2-dioxygenase from *Burkholderia (Pseudomonas) cepacia* 2CBS (Fetzner, S., Müller, R., and Lingens, F. (1992) *J. Bacteriol.* 174, 279–290) contains both a ferredoxin-type and a Rieske-type 2Fe2S center. These two significantly different 2Fe2S clusters were characterized with respect to their EPR spectra, electrochemical properties (Rieske-type cluster with $g_z = 2.025$, $g_y = 1.91$, $g_x = 1.79$, $g_{av} = 1.91$, $E_m = -125 \pm 10$ mV; ferredoxin-type center with $g_z = 2.05$, $g_y = 1.96$, $g_x = 1.89$, $g_{av} = 1.97$, $E_m = -200 \pm 10$ mV) and pH dependence thereof. X band electron spin echo envelope modulation and electron nuclear double resonance spectroscopy was applied to study the interaction of the Rieske-type center of the 2-halobenzoate 1,2-dioxygenase with ¹⁴N and ¹H nuclei in the vicinity of the 2Fe2S cluster. The results are compared to those obtained on the Rieske protein of the cytochrome *b₅f* complex ($E_m = +320$ mV) and the water-soluble ferredoxin ($E_m = -430$ mV) of spinach chloroplasts, as typical representatives of the $g_{av} = 1.91$ and $g_{av} = 1.96$ class of 2Fe2S centers. Properties common to all Rieske-type clusters and those restricted to the respective centers in bacterial oxygenases are discussed.

2Fe2S clusters are usually divided into two distinct groups on the basis of their EPR spectra. The ferredoxin-type 2Fe2S centers are characterized by an average g-value of $g_{av} = 1.96$, whereas the so-called Rieske-type 2Fe2S centers show a pronounced g-factor anisotropy with $g_{av} = 1.91$.

For a long time, the membership in either of the two classes was considered to be correlated with significantly different redox midpoint potentials, *i.e.* with an $E_m^1 < -400$ mV for the ferredoxins and an $E_m > +260$ mV for the Rieske centers of the

cytochrome *bc* complexes, the enzymes in which such a 2Fe2S center had been discovered (Rieske *et al.*, 1964). The midpoint potential of the Rieske centers was supposed to arise from a different coordination of the cluster compared to ferredoxin-type centers.

To date, crystal structures of six 2Fe2S ferredoxins are available (Tsukihara *et al.*, 1981; Tsutsui *et al.*, 1983; Rypniewski *et al.*, 1991; Sussman *et al.*, 1989; Correll *et al.*, 1992; Jacobson *et al.*, 1993) showing that the iron atoms are coordinated by four cysteines and are bridged by a pair of acid-labile sulfur atoms. For the Rieske centers, no x-ray structure has been solved yet. The model of the cluster suggesting a coordination via two cysteines and two histidines is supported by ¹⁵N Q band ENDOR experiments on the terminal oxygenase of the phthalate dioxygenase from *Pseudomonas cepacia* (Gurbiel *et al.*, 1989). Resonances in the frequency range below 10 MHz were attributed to two slightly inequivalent nitrogen nuclei from histidines interacting with one of the iron ions. The hyperfine coupling constants were claimed to be too high for only dipolar interaction postulating a covalent binding between the iron atom and the histidines. Further ENDOR, ESEEM, and extended x-ray absorption fine structure studies as well as results obtained from site-specific mutagenesis were interpreted in favor of this model (Gurbiel *et al.*, 1991; Britt *et al.*, 1991; Shergill and Cammack, 1994a; Powers *et al.*, 1989; Tsang *et al.*, 1989; Davidson *et al.*, 1992).

During recent years, the discovery of several "odd" systems weakened the clearcut E_m distinction between the $g_{av} = 1.91$ and $g_{av} = 1.96$ clusters. (a) 2Fe2S clusters contained in cytochrome *bc* complexes involved in oxidation of menaquinol have been characterized (Liebl *et al.*, 1992; Riedel *et al.*, 1993). These clusters show all characteristic features of the Rieske centers of cytochrome *bc* complexes except that their redox midpoint potential is about 150 mV lower ranging between +100 and +165 mV. (b) In bacterial oxygenases, 2Fe2S centers with $g_{av} = 1.91$ (the "Rieske-type" centers) have been reported having midpoint potentials as low as -155 mV (Geary *et al.* 1984; Rosche *et al.* 1995b). (c) Ferredoxin-type $g_{av} = 1.96$ clusters were found titrating as high as -174 mV in bacterial dioxygenases (Correll *et al.*, 1992) or even at -7 mV for the center S1 in the mitochondrial succinate dehydrogenase (Cammack and Palmer, 1977; Shergill and Cammack, 1994b).

Even if the generally higher E_m of the Rieske-type centers is due to a histidine ligation, other factors have to be considered to explain the wide range of redox midpoint potentials such as a varying number of coordinated histidines, electrostatic effects

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¹ The abbreviations used are: E_m , redox midpoint potential; g_{av} , average value of the g tensor, $g_{av} = 1/3(g_z + g_y + g_x)$; MOPS, 3'-(N-morpholino)propanesulfonic acid; ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance.

of the surrounding amino acids or differences in the hydrogen bonds to the cluster. In this work, the EPR spectroscopic, electrochemical, ^{14}N ESEEM, and ^1H ENDOR characteristics of the $g_{\text{av}} = 1.91$ and $g_{\text{av}} = 1.96$ 2Fe2S centers in 2-halobenzoate 1,2-dioxygenase from *Burkholderia cepacia* 2CBS (Fetzner *et al.*, 1992) are described and compared to other examples of these two classes of 2Fe2S centers, such as the Rieske center of the cytochrome b_6f complex and the water-soluble ferredoxin from spinach.

MATERIALS AND METHODS

B. (Pseudomonas) cepacia 2CBS (DSM 9959) was grown in a chloride-free mineral salts-medium containing 2-chlorobenzoate as the sole source of carbon and energy (Fetzner *et al.*, 1989).

The two components of the 2-halobenzoate 1,2-dioxygenase were purified as described in Fetzner *et al.* (1992). However, for the gel filtration step of the oxygenase component, the Sephadex G-150 column was replaced by a Superdex 200 HiLoad 16/60 column (Fast Protein Liquid Chromatography (FPLC), Pharmacia Biotech, Freiburg, Germany).

Cytochrome b_6f complex was isolated from spinach chloroplasts modifying the preparation of Hauska (1986) according to Rich *et al.* (1987). 2Fe2S ferredoxin from spinach was obtained from Fluka (Germany).

Redox titrations were carried out at pH 7 (50 mM MOPS) and pH 10.4 (50 mM glycine) according to Dutton (1971) using sodium dithionite for the reductive and porphyraxide for the oxidative titrations. The following mediators were used: methyl viologen, benzyl viologen, neutral red, safranin T, anthraquinone-2-sulfonate, anthraquinone-1,5-disulfonate, 2-hydroxy-1,4-naphthoquinone, 2,5-dihydroxy-*p*-benzoquinone, indigotetrasulfonate, pyocyanine, menadione, duroquinone, toluidine blue, phenazine ethosulfate, phenazine methosulfate, toluene blue, variamine blue, 2,5-dimethyl-*p*-benzoquinone, each at a concentration of 50 μM .

EPR and ENDOR spectra were taken on a Bruker X band ER200 spectrometer with an Oxford helium cryostat and temperature control system. For the ENDOR experiments, the Bruker ENDOR unit UN810 (cavity, synthesizer, amplifier) was used. The ENDOR spectra were recorded at 6 K, and microwave and radio frequency power levels of 12–20 milliwatts and 150 watts, respectively, were applied. The ESEEM data were collected on a BRUKER ER300 spectrometer equipped with the ESP380/HR300 FT unit and a helium bath cryostat. The measurements were performed at 4.2 K according to the 3 pulse-stimulated echo procedure ($90^\circ\text{-}\tau\text{-}90^\circ\text{-}\tau\text{-}90^\circ$). FT transformation was carried out by using a modified version of the dead time reconstruction method of Mims (1984).

RESULTS

EPR and Electrochemical Properties of the 2Fe2S Centers of the 2-Halobenzoate 1,2-Dioxygenase—Fig. 1a shows the EPR spectra of the 2Fe2S clusters of the 2-halobenzoate 1,2-dioxygenase from *Burkholderia cepacia* 2CBS after reduction with dithionite at pH 7.0. The 2Fe2S cluster in the reductase component shows a ferredoxin-type spectrum (continuous line in Fig. 1a) with $g_z = 2.05$, $g_y = 1.96$, and $g_x = 1.89$ ($g_{\text{av}} = 1.966$), whereas the oxygenase component has the typical Rieske-type spectrum (marked with dots in Fig. 1a) with $g_z = 2.025$, $g_y = 1.91$, and $g_x = 1.79$ resulting in the lower g_{av} of 1.91.

The results of the redox titration performed on the reductase and the oxygenase components at pH 7 monitored by EPR spectroscopy are shown in Fig. 1b. The redox midpoint potential of the ferredoxin center was determined to be -200 ± 10 mV, and the Rieske-type cluster in the terminal oxygenase titrated with an E_m of -125 ± 10 mV. A titration at pH 10.4 of the $g_{\text{av}} = 1.91$ cluster yielded a redox potential of around -90 ± 30 mV. The instability of the dioxygenase at high pH² resulted in higher experimental errors for the E_m value measured at pH 10.4.

^{14}N ESEEM Spectra—The FT ESEEM spectrum obtained on the $g_{\text{av}} = 1.91$ center from the 2-halobenzoate 1,2-dioxygenase is shown in Fig. 2a. The spectrum taken on the g_y signal of the

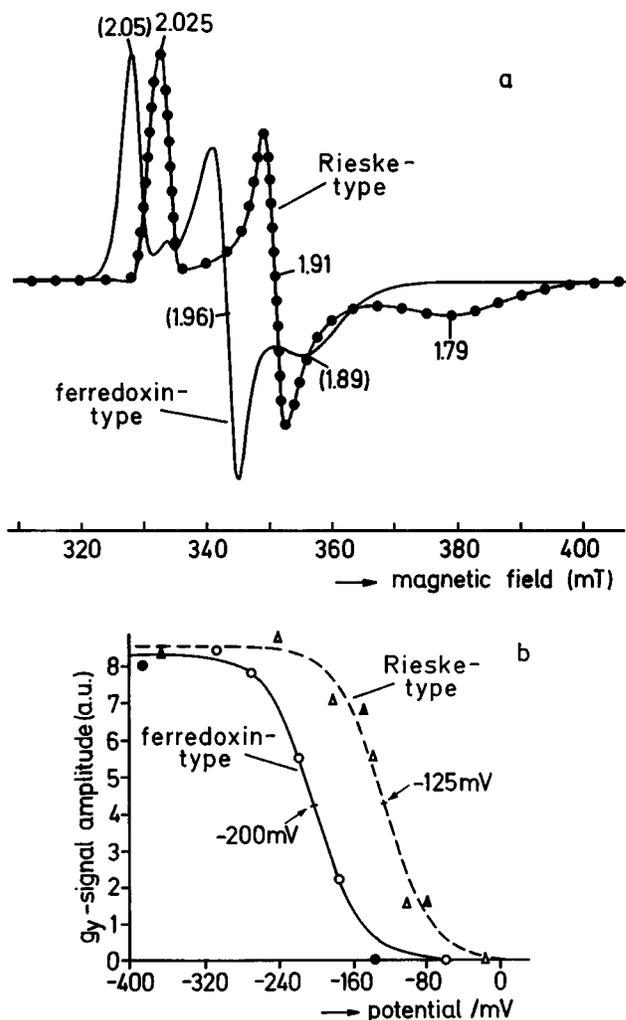


FIG. 1. EPR spectra of the 2Fe2S centers of the 2-halobenzoate 1,2-dioxygenase from *B. (Pseudomonas) cepacia* 2CBS (a) and determination of the redox midpoint potentials (b). a, the spectra were taken on the purified enzyme components upon addition of dithionite ($g_{\text{av}} = 1.91$ center, dotted line; $g_{\text{av}} = 1.96$ center, continuous line). Instrument settings: temperature, 15 K; microwave frequency, 9.39 GHz; modulation amplitude, 1.6 mT; microwave power, 6.3 milliwatts. b, the signal amplitudes of the g_y signals of the ferredoxin-type (circles) and Rieske-type (triangles) centers are plotted as a function of the ambient potential. Open and filled symbols denote data points obtained while titrating toward lower and higher potentials, respectively. The curves represent a fit of the Nernst equation ($n = 1$) to the data points.

EPR absorption is characterized by modulations in the frequency range from 1–8 MHz with pronounced resonances at 2.3, 4.3, 6.2, and 7.8 MHz. Fig. 2, b and c, represents the respective FT ESEEM spectra obtained on the Rieske 2Fe2S cluster of purified cytochrome b_6f complex and from spinach ferredoxin, respectively.

The signals of the Rieske centers in the frequency range between 6 and 8 MHz have been attributed to double quantum transitions ν_{dq}^+ due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_1 = 3.8$ MHz and $A_2 = 4.6$ MHz and quadrupolar couplings of $e^2qQ = 2.5\text{--}2.9$ MHz³ for both nitrogen nuclei (Britt *et al.*, 1991). The $g_{\text{av}} = 1.91$ 2Fe2S center from the 2-halobenzoate 1,2-dioxygenase also shows signals in this frequency range. Quantitative analysis of our data following Britt *et al.* (1991) yields hyperfine couplings with $A_1 = 3.7$ MHz (and $e^2qQ =$

² S. Fetzner, unpublished results.

³ For the calculation of the quadrupole coupling constant, the asymmetry parameter η was varied between 0 and 1.

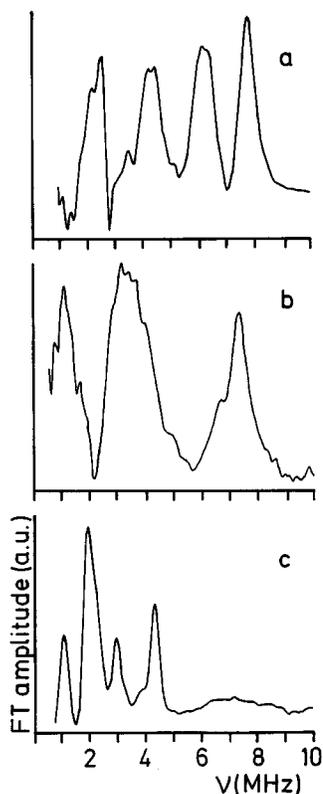


FIG. 2. 3-pulse FT ESEEM spectra of $g_{av} = 1.91$ and $g_{av} = 1.96$ $2Fe_2S$ centers. ^{14}N FT ESEEM results are shown for the Rieske-type center of the 2-halobenzoate 1,2-dioxygenase with $E_m = -125$ mV (a), the Rieske center from isolated cytochrome $b_6 f$ complex from spinach with $E_m = +320$ mV (b), and the water-soluble ferredoxin from spinach with an $E_m = -430$ mV (c). The samples in a and c were reduced with dithionite, the sample in b with ascorbate. All spectra were taken on the g_y signal of the EPR absorption. Instrument settings: temperature, 4.2 K; microwave frequency, 9.77 GHz; pulse power, 1 kilowatt; (a) τ , 128 ns; magnetic field H_0 , 366.7 mT; (b) τ , 144 ns; H_0 , 368.2 mT. Spectrum c is taken from Britt *et al.* (1991).

2.0–2.3 MHz) and $A_2 = 4.7$ MHz (with $e^2qQ = 3.6$ –4.1 MHz)³ rather similar to the results obtained for the spinach Rieske center. By contrast, it has been reported that no distinct modulations can be detected on the plant-type ferredoxin in this range of frequencies deriving a hyperfine constant of $A = 1.1$ MHz for remote nitrogens (Fig. 2c, taken from Britt *et al.* (1991)).

1H ENDOR Spectra—Fig. 3 shows a comparison of the 1H ENDOR spectra obtained on the g_y peak of the Rieske-type center of the 2-halobenzoate 1,2-dioxygenase (Fig. 3a) with the respective spectra recorded on the Rieske protein of purified cytochrome $b_6 f$ complex (Fig. 3b) and on spinach ferredoxin (Fig. 3c). The spectra show symmetrical hyperfine-split doublets centered about the Larmor frequency ν_{H} , as expected for hyperfine interaction with protons ($I = 1/2$). For the Rieske centers, we have detected up to 8 pairs of lines with hyperfine values between 0.5 and 10 MHz. Because of the low signal to noise ratio of the outer resonances, however, only the inner lines <7 MHz can be unambiguously assigned (Table I). For the spinach ferredoxin, 9 pairs could be identified with coupling constants ranging between 0.1 and 11 MHz. The spectra shown in Fig. 3 are recorded with a relatively low modulation amplitude of 100 kHz in order to resolve narrow lines. The complete set of hyperfine couplings summarized in Table I, *i.e.* the additional broader lines at the edges were taken from spectra measured with modulation amplitudes of 250 kHz (not shown).

We observed symmetrical 1H ENDOR spectra in all $2Fe_2S$ clusters examined so far. The hyperfine coupling constants,

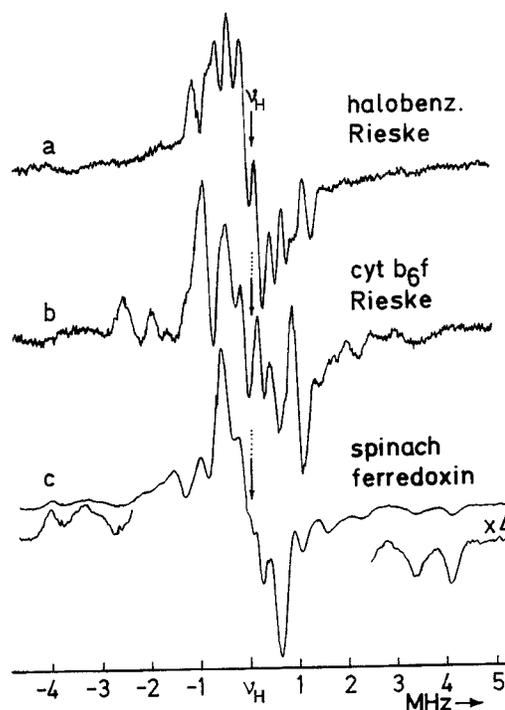


FIG. 3. Proton ENDOR spectra of Rieske- and ferredoxin-type $2Fe_2S$ centers. The measurements were performed on the same samples as described in Fig. 2. The spectra were recorded on the g_y signal of the EPR absorption. Instrument settings: temperature, 6 K; microwave frequency, 9.46 GHz; microwave power, 12.6 milliwatts (a and b) and 20 milliwatts (c); radiofrequency power, 150 watts; magnetic field H_0 , 354.6 mT (a), 356.4 mT (b), 344.5 mT (c).

TABLE I
Proton hyperfine couplings derived from X band ENDOR spectra for $g_{av} = 1.91$ and 1.96 $2Fe_2S$ centers

Hyperfine couplings A in MHz for $2Fe_2S$ centers from		
2-Halobenzoate 1,2-dioxygenase ($E_m = -125$ mV)	Cytochrome $b_6 f$ complex ($E_m = +320$ mV)	Spinach ferredoxin ($E_m = -430$ mV)
		0.1
0.5	0.5	0.5
0.9	1.1	1.1
1.4		
1.7	2.0	2.0
2.4	2.7	
3.2	3.4	3.1
3.6		
	4.1	4.3
	5.3	
6.2	6.7	6.6
		8
	9.5	11

however, vary considerably between these centers (Table I). Since the ENDOR experiments are performed on frozen solutions, these differences could be due to differing numbers and/or strength of proton couplings or to differing dihedral angles between the respective hyperfine tensors and g tensors of the clusters. To address this question, a detailed study of the 1H ENDOR resonances throughout the EPR spectrum is in progress both for the cytochrome $b_6 f$ complex and the spinach ferredoxin.

DISCUSSION

The $g_{av} = 1.91$ $2Fe_2S$ Center of the 2-Halobenzoate 1,2-Dioxygenase

The Rieske-type $2Fe_2S$ cluster of the 2-halobenzoate 1,2-dioxygenase from *B. cepacia* 2CBS has been studied with respect to the EPR properties, the electrochemical potential, and

pH dependence thereof, as well as with respect to ^{14}N and ^1H nuclei interacting with the cluster.

The midpoint potential of -125 mV (at pH 7) for the $g_{\text{av}} = 1.91$ 2Fe2S cluster from the 2-halobenzoate 1,2-dioxygenase lies well in the range of potentials obtained so far for analogous clusters (Rosche *et al.*, 1995b). Thus, the difference in redox midpoint potential of ferredoxin-type and Rieske-type 2Fe2S clusters can become rather small. The Rieske-type center of the 2-halobenzoate 1,2-dioxygenase from *B. cepacia* 2CBS was found to be only 75 mV more positive than the ferredoxin-type cluster in the same enzyme system.

Similar to what was found for the phthalate dioxygenase (Kuila and Fee, 1986) and the 2-oxo-1,2-dihydroquinoline 8-monooxygenase (Rosche *et al.*, 1995a, 1995b),⁴ the redox midpoint potential of the Rieske-type center in the 2-halobenzoate 1,2-dioxygenase does not depend on pH within the experimental errors between pH 6 and pH 10.4. This behavior is significantly different from what is found for the centers from cytochrome *bc* complexes (see below).

*The $g_{\text{av}} = 1.91$ 2Fe2S Centers from Bacterial Oxygenases and Cytochrome *bc* Complexes*

Redox Midpoint Potentials— $g_{\text{av}} = 1.91$ 2Fe2S clusters were found with redox midpoint potentials at (a) $+400$ mV in plasma membranes of the archaeon *Sulfolobus acidocaldarius* (Anemüller *et al.*, 1993, 1994), (b) at around $+300$ mV in cytochrome bc_1 and b_6f complexes with ubiquinol and plastoquinol as electron donors to the complex (Ding *et al.*, 1992; Nitschke *et al.*, 1992), (c) at around $+130$ mV in the menaquinol-oxidizing cytochrome *bc* complexes (Liebl *et al.*, 1992; Riedel *et al.*, 1993), and (d) at around -100 mV in bacterial oxygenases (Mason and Cammack, 1992; Rosche *et al.*, 1995b). The class of these 2Fe2S centers thus covers a range of more than 500 mV.

The high redox midpoint potential of the $g_{\text{av}} = 1.91$ class as compared to the ferredoxins was proposed to be due to non-sulfur and less-electron donating ligands to the cluster. In fact, hyperfine couplings from nitrogen nuclei on two inequivalent histidine residues were detected and the respective histidines were proposed to be ligands to the cluster (Gurbiel *et al.*, 1989, 1991; Britt *et al.*, 1991; Shergill and Cammack, 1994a). Since the range of redox potentials determined in the $g_{\text{av}} = 1.91$ clusters (>500 mV) is significantly larger than the smallest difference between a $g_{\text{av}} = 1.91$ and a $g_{\text{av}} = 1.96$ center (75 mV, see above), one could speculate that the number of these crucial histidines might vary thereby tuning the electrochemical potentials. This is clearly not the case. The presence of hyperfine couplings arising from two inequivalent nitrogen nuclei seems to be a common feature of the $g_{\text{av}} = 1.91$ 2Fe2S centers found in the bacterial oxygenases as well as in cytochrome *bc* complexes (Fig. 2). According to the interpretation of Gurbiel *et al.* (1989), this indicates that in all these systems one of the two iron atoms is coordinated by two histidines instead of cysteines. In this context, it is worth mentioning that all Rieske centers examined so far contain (at least) four cysteines and two histidines in their amino acid sequence (for cytochrome *bc* complexes, see Hauska *et al.* (1988) and Schütz *et al.* (1994); for the 2-halobenzoate 1,2-dioxygenase, see Haak *et al.* (1995)).

While the presence of strongly coupled histidines may be linked to the on average higher E_m value of the $g_{\text{av}} = 1.91$ clusters, additional effects must play important roles in modulating the redox midpoint potentials. As a possible candidate, we propose the structure of the network of hydrogen bonds

toward the cluster (see also Backes *et al.* (1991)). In the work of Cline *et al.* (1985) and Telser *et al.* (1987), ^1H ENDOR spectra of the Rieske center in the phthalate dioxygenase, in *Thermus thermophilus*, and the mitochondrial complex III have been reported. The respective spectra, however, were absorption spectra and significantly less well-resolved than those of Fig. 3. Furthermore, the interpretation of the data was later disclaimed by the authors themselves (Gurbiel *et al.*, 1989). As can be seen from our data (Fig. 3) and the work of Shergill and Cammack (1994a) on the Rieske center from bovine heart mitochondria, the pattern of proton couplings varies significantly on going from the Rieske centers from cytochrome *bc* complexes to those of the bacterial oxygenases. As mentioned above, however, a more detailed comparison of the strengths, numbers, and spatial distribution of ^1H couplings to the clusters has to await determination of complete ENDOR data sets and, if possible, the collection of data on site-directed mutants of the respective proteins.

pH Dependence of the Electrochemical Potentials—Despite their midpoint potential differing by ~ 150 mV, all Rieske centers from cytochrome *bc* complexes share the same pH dependence, *i.e.* constant E_m below pH 8 and decreasing E_m with a slope > -60 mV per pH unit above pH 8.0 (Prince and Dutton, 1976; Riedel *et al.*, 1993). In these centers, the involvement of two deprotonatable groups having distinct pK values has been proposed (Liebl *et al.*, 1992; Nitschke *et al.*, 1992; Link *et al.*, 1992; Link, 1994).

In the $g_{\text{av}} = 1.91$ center found in the archaeon *S. acidocaldarius*, however, the midpoint potential becomes pH-dependent already above pH 6.0. In this system, the presence of a second deprotonatable group on the oxidized cluster could be unambiguously observed. The pK values were determined to be at 6.2 and 8.5 (Anemüller *et al.*, 1994).

By contrast, the E_m of the Rieske-type clusters of the bacterial oxygenases was seen to be independent of pH between pH 7 and 10.4. If the Rieske centers of the oxygenases should behave similarly to the above described systems, then their first pK must be above pH 10.4.

Previously, it was proposed for the Rieske centers in cytochrome *bc* complexes that the pH dependence of their E_m values arises from the protonation/deprotonation equilibria on the N_ϵ nitrogen atom of the two putative histidine ligands (Kuila and Fee, 1986; Liebl *et al.*, 1992; Link *et al.*, 1992; Link, 1994). However, the results obtained on the bacterial oxygenases (with a possible first pK not lower than pH 10.4) and those recently reported for the $g_{\text{av}} = 1.91$ cluster in *S. acidocaldarius* (first pK at 6.2) suggest some caution concerning this interpretation. Shifts of the pK values of histidines (pK ~ 6.0 for free histidine) by four pH units are at the limit of what can be expected to be induced by the protein environment of these residues.

Moreover, the E_m versus pH curves determined for the ferredoxin-type 2Fe2S clusters showing a decrease of the E_m value by -30 mV/pH unit for putidaredoxin (Wilson *et al.*, 1973) and -60 mV/pH for center N1 of beef heart mitochondria (Ingledew and Ohnishi, 1980) indicate that a significant pH dependence of the E_m can be induced by the protein environment of the clusters without having to invoke deprotonation of ligands. Therefore, we suggest that the interpretation of the pH dependence of the midpoint potential of the $g_{\text{av}} = 1.91$ 2Fe2S centers might be more complicated than previously proposed (see, for example, Link (1994)). It does not seem unreasonable to us that the pH dependence arises from other amino acid residues than the two histidines hyperfine-coupled to the cluster. Site-specific mutagenesis directed to possible hydrogen bond-forming residues in the cluster's binding region of the sequence or even a

⁴ B. Rosche and A. Riedel, unpublished results.

re-examination of already existing mutants might yield crucial information pertinent to this question.

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