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 \(g_\text{av} = 2.025\), \(g_x = 1.91\), \(g_y = 1.79\), \(g_{\text{av}} = 1.91\), \(E_m = -125 \pm 10\) mV; ferredoxin-type center with \(g_\text{av} = 2.05\), \(g_x = 1.96\), \(g_y = 1.89\), \(g_{\text{av}} = 1.97\), \(E_m = -200 \pm 10\) 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 \(^{14}\)N and \(^{2}\)H nuclei in the vicinity of the 2Fe2S cluster. The results are compared to those obtained on the Rieske protein of the cytochrome \(b_6f\) complex (\(E_m = +320\) mV) and the water-soluble ferredoxin (\(E_m = -430\) mV) of spinach chloroplasts, as typical representatives of the \(g_{\text{av}}\) = 1.91 and \(g_{\text{av}}\) = 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 \(g_{\text{av}} = 1.96\), whereas the so-called Rieske-type 2Fe2S centers show a pronounced \(g\)-factor anisotropy with \(g_{\text{av}} = 1.91\).

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 \(E_m < -400\) 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 \(^{15}\)N 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 \(g_{\text{av}}\) = 1.91 and \(g_{\text{av}}\) = 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 \(g_{\text{av}} = 1.91\) (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 \(g_{\text{av}} = 1.96\) 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...
of the surrounding amino acids or differences in the hydrogen bonds to the cluster. In this work, the EPR spectroscopic, electrochemical, $^{15}$N ESEEM, and $^1$H ENDOR characteristics of the $g_{x\perp}=1.91$ and $g_{y\perp}=1.96$ 2Fe2S centers in 2-halobenzoate 1,2-dioxygenase from Burkholderia (Pseudomonas) 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 $b_{6}f$ complex and the water-soluble ferrodoxin from spinach.

MATERIALS AND METHODS

B. (Pseudomonas) cepacia 2CBS (DSM 9995) 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 $b_{6}f$ complex was isolated from spinach chloroplasts modifying the preparation of Hauksa (1986) according to Rich et al. (1987). 2Fe2S ferrodoxin 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 porphyrinexide for the oxidative titrations. The following mediators were used: methyl viologen, benzyl viologen, 2,5-dimethylbenzimidazolium, neutral red, safrole, T$_{2}$ anthraquinone-2-sulfonate, T$_{2}$ 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, varianline blue, 2,5-dimethyl-p-benzoquinone, each at a concentration of 50 $\mu$M.

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 350 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°-T$_{-}$90°). 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 $g_{x}=2.05$, $g_{y}=1.96$, and $g_{z}=1.89$ ($g_{av}=1.966$), whereas the oxygenase component has the typical Rieske-type spectrum (dotted line in Fig. 1a) with $g_{x}=2.025$, $g_{y}=1.91$, and $g_{z}=1.79$ 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 ferrodoxin center was determined to be $-200 \pm 10$ mV, and the Rieske-type cluster in the terminal oxygenase titrated with an $E_m$ of $-125 \pm 10$ mV. A titration at pH 10.4 of the $g_{av}=1.91$ cluster yielded a redox potential of around $-90 \pm 30$ mV. The instability of the dioxygenase at high pH resulted in higher experimental errors for the $E_m$ value measured at pH 10.4.

$^{15}$N ESEEM Spectra—The FT ESEEM spectrum obtained on the $g_{av}=1.91$ center from the 2-halobenzoate 1,2-dioxygenase is shown in Fig. 2a. The spectrum taken on the $g_{y}$ signal of the 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 $A_{x}=3.7$ MHz (and $e^2Q=3$ MHz) due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_{x}=3.8$ MHz and $A_{y}=4.6$ MHz and quadrupolar couplings of $e^2Q=2.5-2.9$ MHz for both nitrogen nuclei (Britt et al., 1991). The $g_{av}=1.91$ 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 $A_{x}=3.7$ MHz (and $e^2Q=3$ MHz) due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_{x}=3.8$ MHz and $A_{y}=4.6$ MHz and quadrupolar couplings of $e^2Q=2.5-2.9$ MHz for both nitrogen nuclei (Britt et al., 1991). The $g_{av}=1.91$ 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 $A_{x}=3.7$ MHz (and $e^2Q=3$ MHz) due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_{x}=3.8$ MHz and $A_{y}=4.6$ MHz and quadrupolar couplings of $e^2Q=2.5-2.9$ MHz for both nitrogen nuclei (Britt et al., 1991). The $g_{av}=1.91$ 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 $A_{x}=3.7$ MHz (and $e^2Q=3$ MHz) due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_{x}=3.8$ MHz and $A_{y}=4.6$ MHz and quadrupolar couplings of $e^2Q=2.5-2.9$ MHz for both nitrogen nuclei (Britt et al., 1991). The $g_{av}=1.91$ 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 $A_{x}=3.7$ MHz (and $e^2Q=3$ MHz) due to the magnetic interaction of the cluster with two inequivalent nitrogen nuclei with coupling constants of $A_{x}=3.8$ MHz and $A_{y}=4.6$ MHz and quadrupolar couplings of $e^2Q=2.5-2.9$ MHz for both nitrogen nuclei (Britt et al., 1991).
2.0–2.3 MHz) and A2 = 4.7 MHz (with \( e^2gQ = 3.6–4.1 \text{ MHz} \)) 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. 2 c, taken from Britt et al. (1991)).

\[ \text{FIG. 2.} \quad 3\text{-pulse FT ESEEM spectra of } g_{av} = 1.91 \text{ and } g_{av} = 1.96 \text{ 2Fe2S centers.} \quad ^{14}N \text{ FT ESEEM results are shown for the Rieske-type center of the 2-halobenzoate 1,2-dioxygenase with } E_m = -125 \text{ mV (a), the Rieske center from isolated cytochrome } b_6f \text{ complex from spinach with } E_m = +320 \text{ mV (b), and the water-soluble ferredoxin from spinach with } E_m = -430 \text{ mV (c). The samples in a and c were reduced with dithionite, the sample in b with ascorbate. All spectra were taken on the } g_\parallel \text{ signal of the EPR absorption. Instrument settings: temperature, } 4.2 \text{ K; microwave frequency, } 9.77 \text{ GHz; pulse power, 1 kilowatt; (a) } \tau, 128 \text{ ns; magnetic field } H_0, 366.7 \text{ mT; (b) } \tau, 144 \text{ ns; } H_0, 368.2 \text{ mT. Spectrum c is taken from Britt et al. (1991).} \]

\[ \text{FIG. 3.} \quad \text{Proton ENDOR spectra of Rieske- and ferredoxin-type 2Fe2S centers. The measurements were performed on the same samples as described in Fig. 2. The spectra were recorded on the } g_\parallel \text{ signal of the EPR absorption. Instrument settings: temperature, } 6 \text{ K; microwave frequency, } 9.46 \text{ GHz; microwave power, } 12.6 \text{ milliwatts (a and b) and } 20 \text{ milliwatts (c); radiofrequency power, } 150 \text{ watts; magnetic field } H_0, 354.6 \text{ mT (a), 356.4 mT (b), 344.5 mT (c).} \]

\[ \text{TABLE I} \quad \text{Proton hyperfine couplings derived from X band ENDOR spectra for } g_{av} = 1.91 \text{ and } 1.96 \text{ 2Fe2S centers} \]

<table>
<thead>
<tr>
<th>2-Halobenzoate 1,2-dioxygenase ((E_m = -125 \text{ mV}))</th>
<th>Cytochrome (b_6f) complex ((E_m = +320 \text{ mV}))</th>
<th>Spinach ferredoxin ((E_m = -430 \text{ mV}))</th>
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<tr>
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\[ \text{FIG. 3.} \quad \text{Proton ENDOR spectra of Rieske- and ferredoxin-type 2Fe2S centers.} \]

Proton hyperfine couplings A in MHz for 2Fe2S centers from

\[ \text{DISCUSSION} \]

The \( g_{av} = 1.91 \text{ 2Fe2S Center of the 2-Halobenzoate 1,2-Dioxygenase} \]

The Rieske-type 2Fe2S 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 $^1$H nuclei interacting with the cluster.

The midpoint potential of $-125$ mV (at pH 7) for the $g_{av} = 1.91$ 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. capsici 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-monoxygenase (Rosche et al., 1995a, 1995b), 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 $g_{av} = 1.91$ 2Fe2S Centers from Bacterial Oxogenases and Cytochrome bc Complexes

Redox Midpoint Potentials—$g_{av} = 1.91$ 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$_2$ and bc$_1$ complexes with ubiquinoidal 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 $g_{av} = 1.91$ 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; Brits et al., 1991; Shergill and Cammack, 1994a). Since the range of redox potentials determined in the $g_{av} = 1.91$ clusters ($>500$ mV) is significantly larger than the smallest difference between a $g_{av} = 1.91$ and a $g_{av} = 1.96$ 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 $g_{av} = 1.91$ 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 $g_{av} = 1.91$ 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 Teller et al. (1987), $^1$H 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 $^1$H 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.

The $g_{av} = 1.91$ 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 $pK_v$ values were determined to be at 6.2 and 8.5 (Anemüller et al., 1994). By contrast, the $E_{m}$ of the Rieske-type centers 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 $pK_v$ 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 $N_7$ 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 $pK_v$ not lower than pH 10.4) and those recently reported for the $g_{av} = 1.91$ duster in S. acidocaldarius (first $pK_v$ at 6.2) suggest some caution concerning this interpretation. Shifts of the $pK_v$ values of histidines ($pK_v = -6.0$ 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 $g_{av} = 1.91$ 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

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4 B. Rosche and A. Riedel, unpublished results.
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re-examination of already existing mutants might yield crucial information pertinent to this question.

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REFERENCES


