Comparison of the "Rieske" [2Fe-2S] Center in the bc 1 Complex and in Bacterial Dioxygenases by Circular Dichroism Spectroscopy and Cyclic Voltammetry †

ARTICLE *in* BIOCHEMISTRY · JUNE 1996

Impact Factor: 3.02 · DOI: 10.1021/bi960004+ · Source: PubMed

6 AUTHORS, INCLUDING:

Richard Cammack
King's College London
345 PUBLICATIONS **8,642** CITATIONS

SEE PROFILE

Comparison of the "Rieske" [2Fe-2S] Center in the bc_1 Complex and in Bacterial Dioxygenases by Circular Dichroism Spectroscopy and Cyclic Voltammetry†

Thomas A. Link,*,‡ Oliver M. Hatzfeld,‡ Piyush Unalkat,§ Jasvinder K. Shergill,§ Richard Cammack,§ and Jeremy R. Mason§

Therapeutische Biochemie, Universitätsklinikum Frankfurt, ZBC, D-60590 Frankfurt/Main, Germany, and Centre for the Study of Metals in Biology and Medicine, King's College, University of London, Campden Hill Road, London W8 7AH, U.K.

Received January 2, 1996; Revised Manuscript Received April 3, 1996⊗

ABSTRACT: Two different types of "Rieske" [2Fe-2S] clusters have been observed in proteins, one in the *bc* complexes of the respiratory chain and the other in bacterial dioxygenases. We have compared the circular dichroic (CD) spectra and redox properties of the water soluble fragment of the Rieske center of the bovine heart mitochondrial bc_1 complex (ISF) and of the ferredoxin from benzene dioxygenase in *Pseudomonas putida* ML2 (FD_{BED}). Spinach ferredoxin was also measured for comparison. The redox potential of both proteins could be determined in solution by cyclic voltammetry (CV) and by CD-monitored spectroelectrochemistry using a specially constructed optically transparent thin layer (OTTLE) cell. Whereas the redox potential of the ISF (+312 ± 5 mV at pH 7.0) depended both on the pH above pH 7 and on the ionic strength, the redox potential of the FD_{BED} (−155 ± 5 mV at pH 7.0) was observed to be independent of pH and ionic strength. The ISF showed a marked dependence of its redox potential on temperature, while the FD_{BED} showed no temperature dependence. The entropy of the redox reaction $\Delta S°_{rc}$ was calculated as −88 ± 11 J K^{-1} mol^{-1} for the bc_1 Rieske center and ∼0 J K^{-1} mol^{-1} for the Fd_{BED}. The CD spectra of Rieske type clusters are significantly different from those of plant type [2Fe-2S] ferredoxins. A strong negative CD band is present at 20 000 cm^{-1} (500 nm) in all reduced Rieske clusters. The possible assignment of this band is discussed as arising from the highest energy magnetically allowed d → d transition ($d_{z^2} \rightarrow d_{xz}$) of the Fe^{II} site. If so, this band is highly indicative of the distortion of the ligand field of the Fe^{II} site.

The bc_1 complexes contain, besides two hemes *b* and heme c_1, a [2Fe-2S] cluster which was first described by Rieske *et al*. (1964). The EPR[1] spectra of reduced clusters of this type have a $g_{av} = 1.91$ in comparison to typical values of $g_{av} = 1.96$ for plant type ferredoxins with a [2Fe-2S] cluster (Figure 1). They also have a high redox potential, $E_m \sim +300$ mV, compared to around −420 mV for plant type ferredoxins. From ENDOR, ESEEM, Mössbauer, and Resonance Raman spectroscopic studies, there is good evidence that "Rieske" [2Fe-2S] clusters have two histidine residues coordinated to the Fe^{II} atom (Gurbiel *et al*., 1989, 1991; Britt *et al*., 1991; Mason & Cammack, 1992) in contrast to the four-cysteine ligand pattern observed for plant type ferredoxins.

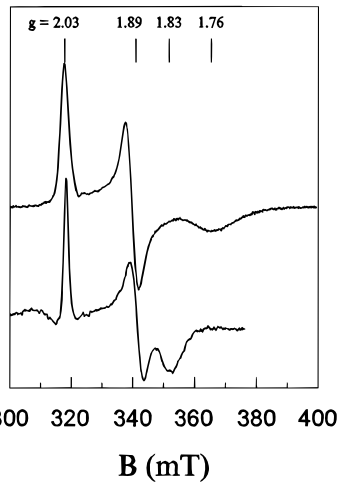

FIGURE 1: EPR spectra of the ISF (top) and the Fd_{BED} (bottom). EPR conditions were as follows (ISF/Fd_{BED}): microwave frequency, 9.021 GHz; modulation frequency, 100 kHz/25 kHz; modulation amplitude, 1 mT/0.9 mT; microwave power, 1 mW/9 mW; temperature, 17 K/30 K; and protein concentration, 100 μM/37 μM.

On the basis of the classification of dioxygenases by Batie *et al*. (1991), benzene dioxygenase is a class IIB dioxygenase which has a flavoprotein and two iron−sulfur proteins, Fd_{BED} and ISP_{BED}. ISP_{BED} is the terminal dioxygenase, and it contains [2Fe-2S] clusters of the Rieske type (Shergill *et al*., 1995) as well as a catalytic site. The other iron−sulfur protein is a small ferredoxin, Fd_{BED} ($M_r = 11.9$ kDa),

† Supported by grants from the Deutsche Forschungsgemeinschaft (DFG, Li 474/2) to T.A.L. and from the BBSRC (GR/J 00151) to J.R.M. and J.K.S. and a studentship from the BBSRC to P.U. and from MASIMO to R.C.

* Corresponding author: Dr. Thomas A. Link, Therapeutische Biochemie, Universitätsklinikum Frankfurt, ZBC, Theodor-Stern-Kai 7, Haus 25B, D-60590 Frankfurt/Main, Germany. Telephone: +49-69-6301-6451. Fax: +49-69-6301-6970. E-mail: Link@zbc.klinik.uni-frankfurt.de.

‡ Universitätsklinikum Frankfurt.

§ University of London.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: ISF, water soluble fragment of the iron−sulfur protein of the bc_1 complex; Fd_{BED}, ferredoxin from benzene dioxygenase in *Pseudomonas putida*; ISP_{BED}, iron−sulfur protein from benzene dioxygenase in *P. putida*; Fd_{SP}, [2Fe-2S] ferredoxin from spinach; SHE, standard hydrogen electrode; 2D-HYSCORE, two-dimensional hyperfine sublevel correlation spectroscopy; CD, circular dichroism; CV, cyclic voltammetry; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; ESEEM, electron spin echo envelope modulation; OTTLE, optically transparent thin layer electrode; PATS, pyridine-3-carboxaldehyde thiosemicarbazone.

S0006-2960(96)00004-9 CCC: $12.00

which is the electron donor to ISP_{BED} and has one [2Fe-2S] cluster. The EPR spectrum of the reduced form has $g_{av} = 1.91$ (Axcell & Geary, 1975).

A comparison of sequences with other Rieske type iron−sulfur proteins indicates that the [2Fe-2S] center in Fd_{BED} is homologous with those that have two histidine ligands (Mason & Cammack, 1992). Evidence for the presence of non-sulfur ligands to iron atoms of the [2Fe-2S] center has been provided by Mössbauer spectroscopy (Geary & Dickson, 1981). However, the redox potential of the dioxygenase ferredoxin ($E_m \sim -150$ mV; Geary *et al*., 1984) is some 400 mV more negative than that of the bc_1 Rieske center despite its apparently similar ligand environment.

We have undertaken a comparative study of the Rieske centers of the bc_1 complex and of bacterial dioxygenases in order to gain an insight into the factors determining the redox potential and the spectroscopic properties of the cluster.

MATERIALS AND METHODS

A water soluble fragment (ISF) of the bc_1 complex from bovine heart mitochondria containing the Rieske [2Fe-2S] cluster was isolated as described (Link *et al*., 1996). The oxidized protein was obtained by oxidation with $K_3[Fe(CN)_6]$ which was then removed by filtration over a short G-25 gel filtration column. The ferredoxin (Fd_{BED}) of the benzene dioxygenase from *Pseudomonas putida* was expressed in *Escherichia coli* JM109 pJRM506 (Tan *et al*., 1994) and isolated using a modification of the procedure of Geary *et al*. (1990). Instead of the preliminary $(NH_4)_2SO_4$ precipitation, the protein was loaded directly onto DEAE-Sepharose, followed by separation over Sephacryl S-100 (Unalkat, unpublished data). The concentration of Fd_{BED} was 8.24 mg/mL; the cluster content, 1.7 mol of Fe per mole of protein, was determined by EPR, comparing the spin concentration to a 1 mM Cu-EDTA standard. Spinach ferredoxin, prepared according to Hirasawa *et al*. (1984), was a kind gift from Dr. M. Hirasawa & Dr. D. B. Knaff (Lubbock, TX).

EPR spectra of the Fd_{BED} were recorded at 30 K using a Bruker ESP300 EPR spectrometer fitted with an Oxford Instruments ESR900 helium flow cryostat. Samples were reduced under argon by the addition of a small excess of sodium dithionite. UV/vis spectra were recorded using a Bruins Omega 10 spectrophotometer.

Cyclic voltammetry of the ISF was performed as described by Link *et al*. (1992) using an apparatus described by Hagen (1989). Cyclic voltammetry of the Fd_{BED} was performed in the same apparatus using a gold electrode modified with PATS (Baymann *et al*., 1991). The Fd_{BED} was used either isolated in 50 mM MOPS (pH 7.0) or diluted into a Good's buffer solution containing each of the following at 100 mM: 2-(*N*-morpholino)ethanesulfonic acid (MES), 3-(*N*-morpholino)propanesulfonic acid (MOPS), *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS), 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), and 3-(*N*-cyclohexylamino)propanesulfonic acid (CAPS), adjusted with KOH. Direct cyclic voltammetry of the spinach ferredoxin was performed at a pyrolytic graphite electrode in 50 mM Tris-HCl (pH 7.2) in the presence of 200 μg/mL polymyxin.

Circular dichroism (CD) spectroscopy was performed as described earlier (Link, 1994) using a Jasco J-720 spectropolarimeter. A CD-monitored redox titration was performed in an OTTLE cell with a path length of 0.1 mm built in-house according to the design of Moss *et al*. (1990) (Figure 2). The transparent electrode was a PATS-modified gold grid with a mesh size of 200 wires per inch and transmission of 78% (Buckbee-Mears, St. Paul, MN). The Pt foil counter electrode and the miniaturized Ag/AgCl/KCl (3 M) reference electrode were in contact with the solution at the edges of the quartz windows; contacts to the gold grid and the Pt foil were made with electrically conducting paint (RS Components, Mörfelden). The micro reference electrode was calibrated against a standard saturated Ag/AgCl electrode (Radiometer, Kopenhagen). The potential was controlled through a Wenking MP 87 potentiostat (Bank Elektronik, Clausthal). In order to facilitate redox equilibration, the following mediators were added at a final concentration of 80 μM each to a concentrated (0.8 mM) protein solution: benzylviologen ($E° = -358$ mV), anthraquinone-2-sulfonate ($E° = -225$ mV), 2-hydroxy-1,4-naphthoquinone ($E° = -145$ mV), menadione ($E° = -13$ mV), and phenazine methosulfate ($E° = +80$ mV). Neither the oxidized nor the reduced form of the mediators showed any CD signal even at a 10-fold concentration. All potentials have been recalculated with reference to the standard hydrogen electrode (SHE).

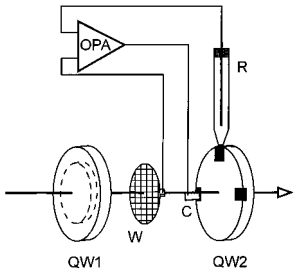

FIGURE 2: Schematic drawing of the OTTLE cell: R, reference electrode; W, working electrode; C, counter electrode; QW1 and QW2, quartz windows; and OPA, operational power amplifier. The arrow indicates the direction of the measuring beam.

RESULTS

Cyclic Voltammetry of the ISF and of the Fd_{BED}. Cyclic voltammetry of the ISF can be performed using a nitric acid-modified glassy carbon electrode (Link *et al*., 1992). The Fd_{BED} gave only a very weak reponse at this electrode. We therefore tested other electrode systems and obtained good responses using a PATS-modified gold electrode (Figure 3). The midpoint potential versus the saturated calomel electrode was determined to be −397 mV, corresponding to $E° = -155 \pm 5$ mV. The same value has been previously reported by Geary *et al*. (1984) using mediator titrations with EPR detection. At a potential scan rate of 10 mV/s, the peak separation of the anodic and cathodic peaks was 105 mV. From this value, the heterogeneous rate constant, $k°$, can be calculated (Nicholson, 1965) as 6×10^{-4} cm/s, assuming the same diffusion coefficient D as for the ISF, 10^{-6} cm^2/s. The latter value is consistent with the observed peak current which is similar to that observed for the ISF.

Spinach ferredoxin gave cyclic voltammograms only when it was added to the electrode together with a coadsorbent. We have used polymyxin at a concentration of 200 μg/mL. During the first half-cycle, a strong peak of an adsorbed species was observed, followed by quasi reversible voltammograms during the following cycles. The potential was determined from the second cycle since the response

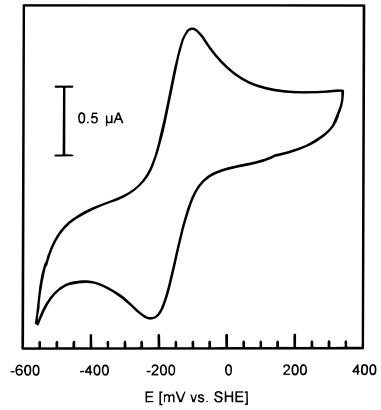

FIGURE 3: Cyclic voltammetry of the Fd_{BED} at the PATS-modified gold electrode. The protein concentration was 3.5 mg/mL in 50 mM K^+/MOPS and 125 mM NaCl (pH 7.0); the potential scan rate was 10 mV/s. The potentials have been recalculated versus the SHE. $T = 25$ °C.

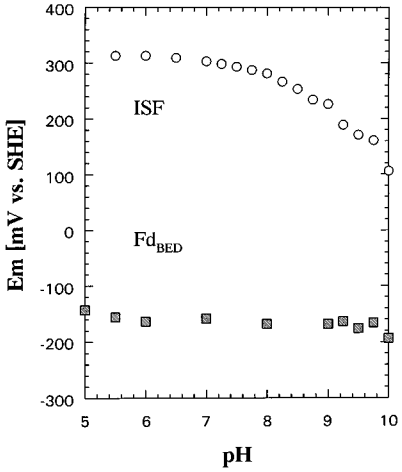

FIGURE 4: pH-dependence of the redox potential of the ISF and of the Fd_{BED} determined by cyclic voltammetry. The ISF [2.4 mg/mL in 20 mM K^+/MOPS (pH 7.2)] was diluted 1:5, and the Fd_{BED} [8.4 mg/mL in 50 mM K^+/MOPS (pH 7.0)] was diluted 1:1 in a buffer mixture of 200 mM MES, MOPS, EPPS, CHES, and CAPS of the desired pH. Cyclic voltammograms were taken at $T = 25$ °C at a potential scan rate of 10 mV/s.

deteriorated rapidly. The midpoint potential was −419 mV versus SHE in 50 mM Tris-HCl (pH 7.2); this value is in excellent agreement with published values (Tagawa *et al*., 1968; Ke *et al*., 1974).

pH Dependence of the Redox Potential. For the determination of the pH dependence, the protein stock solution was diluted into a Good's buffer mixture of the desired pH. Cyclic voltammograms were obtained over the range pH 5−10.75 for the ISF and pH 5−10 for the Fd_{BED}; outside these ranges, the cyclic voltammograms were broad so that the potentials could not be determined reliably. The midpoint potential of the ISF (Link *et al*., 1992), like that of the Rieske protein in the whole bc_1 complex, is pH dependent above pH 7 (Prince & Dutton, 1976). In contrast, the potential of the Fd_{BED} was essentially pH independent over the range pH 5−10 (Figure 4).

Thermodynamics of the Redox Reaction. Cyclic voltammograms at different temperatures were obtained in a nonisothermal setup. The lower part of the cell and the last wash bottle of the argon line were thermostatted in a water bath while the reference electrode was maintained at ambient temperature. In the nonisothermal setup, the temperature coefficient $dE°/dT$ is related directly to the reaction entropy, $\Delta S°_{rc} = S°_{red} - S°_{ox}$:

$$\Delta S°_{rc} = nF(dE°/dT)$$

(Taniguchi *et al*., 1980). From the reaction entropy, the standard entropy change for the cell reaction can be calculated using the equation

$$\Delta S° = \Delta S°_{rc} - \Delta S°(H_2)$$

with $\Delta S°(H_2) = +65$ J K^{-1} mol^{-1} (Taniguchi *et al*., 1980).

Using the nonisothermal setup, we have obtained a value for $dE°/dT = -0.91 \pm 0.12$ mV/K for the ISF at $I = 50$ mM (Figure 5) corresponding to

$$\Delta S°_{rc} = -88 \pm 11 \text{ J K}^{-1} \text{ mol}^{-1}$$

and

$$\Delta S° = \Delta S°_{rc} - \Delta S°(H_2) = -153 \pm 11 \text{ J K}^{-1} \text{ mol}^{-1}$$

These values are in excellent agreement with the values determined previously in an isothermal setup (Link *et al*., 1992), thus confirming the validity of the method.

The midpoint potential of the Fd_{BED} was found to be much less temperature dependent; between 0 and 30 °C, the midpoint potential slightly decreased with $-dE°/dT < 0.2$ mV/K. From this value, the reaction entropy can be calculated as

$$\Delta S°_{rc} = -10 \pm 10 \text{ J K}^{-1} \text{ mol}^{-1}$$

and

$$\Delta S° = \Delta S°_{rc} - \Delta S°(H_2) = -75 \pm 10 \text{ J K}^{-1} \text{ mol}^{-1}$$

From these values, the standard Gibbs free energy change, $\Delta G°$, and the enthalpy change $\Delta H°$ at $T = 298.15$ K can be calculated using the equations

$$\Delta G°_{rc} = -nF\Delta E°$$

and

$$\Delta H° = \Delta G° + T\Delta S°$$

The values obtained for the ISF and for the Fd_{BED} are compared in Table 1.

Ionic Strength Dependence of the Redox Potential. The midpoint potential at different ionic strengths was obtained after diluting the protein into buffer with different KCl concentrations. The midpoint potential of the ISF decreased linearly with $\sqrt{I}$ up to $I = 2.5$ M. The midpoint potential of the Fd_{BED} was invariant within ±3 mV over $I = 0.025-2$ M (Figure 6). Good cyclic voltammograms of Fd_{SP} were obtained up to $I = 0.2$ M; in this range, the midpoint potential increased with a slope of +37 mV $M^{-1/2}$.

Visible Absorption and CD Spectroscopy. The absorption and CD spectra of the ISF and of the Fd_{BED} are shown in Figure 7; the spectral maxima are given in Table 2. Spectra of the reduced Fd_{BED} were obtained after reduction with dithionite, while the ISF was isolated in the reduced state (Link *et al*., 1996). There is a good correspondence between the absorption spectra of the two proteins. The CD spectra of both oxidized proteins showed two positive bands between

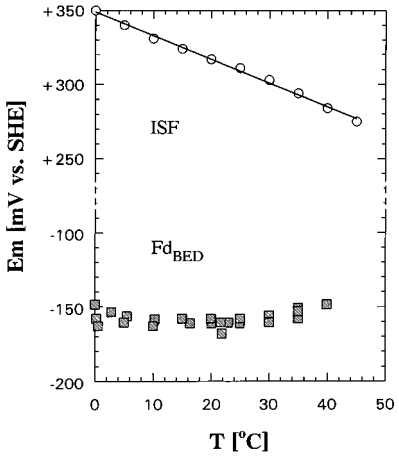

FIGURE 5: Temperature dependence of the redox potential of the ISF and of the Fd_{BED} determined by cyclic voltammetry: ISF, 2.4 mg/mL in 20 mM K^+/MOPS (pH 7.2); Fd_{BED}, 8.2 mg/mL in 50 mM K^+/MOPS (pH 7.0). Cyclic voltammograms were taken at a potential scan rate of 10 mV/s.

Table 1: Thermodynamic Parameters of the ISF and of the Fd_{BED}

	ISF	Fd_{BED}
$E°$ (mV)	$+312 \pm 5$	-155 ± 5
$\Delta G°$ (kJ mol^{-1})	-30 ± 1	$+15 \pm 1$
$\Delta S°$ (J mol^{-1} K^{-1})	-153 ± 11	-75 ± 10
$\Delta H°$ (kJ mol^{-1})	-76 ± 4	-7 ± 4
$dE°/d\sqrt{I}$ (mV M$^{-1/2}$)	-26 ± 1	0

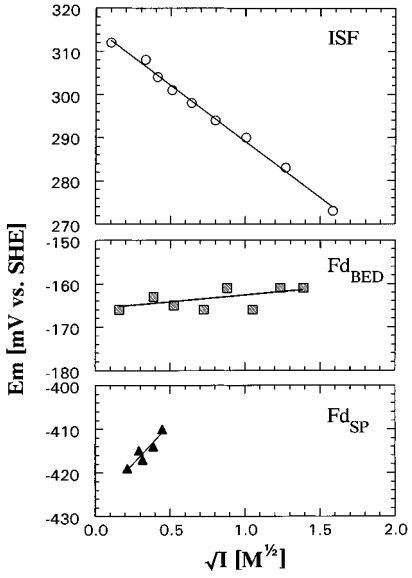

FIGURE 6: Ionic strength dependence of the redox potential of the ISF, Fd_{BED}, and Fd_{SP} determined by cyclic voltammetry. The ISF [2.4 mg/mL in 20 mM K^+/MOPS (pH 7.2)] was diluted 1:5, and the Fd_{BED} [8.2 mg/mL in 50 mM K^+/MOPS (pH 7.0)] and the Fd_{SP} [2.1 mg/mL in 50 mM Tris-HCl (pH 7.2)] were diluted 1:1 with NaCl to obtain the desired ionic strength. Cyclic voltammograms were taken at $T = 25$ °C at a potential scan rate of 10 mV/s (Fd_{SP} at 2 mV/s).

310 and 350 nm, a negative band at 377 nm, and several positive bands between 400 and 500 nm. The CD spectra of the reduced proteins showed positive bands at 314 nm, negative bands at 388−390 nm, a set of positive bands around 440 nm, and negative bands at 498−505 nm. The similarities are even more clearly visible in the (reduced − oxidized) difference spectra (Figure 8); for comparison, we have also included the catalytic subunit of the benzene

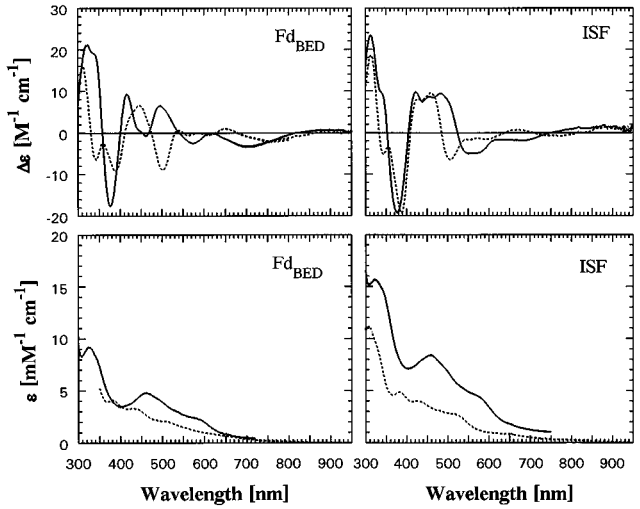

FIGURE 7: Comparison of the absorption and CD spectra of the ISF and of the Fd_{BED}: (- - -) reduced proteins and (—) oxidized proteins. ISF in 50 mM sodium phosphate (pH 7.5); Fd_{BED} in 50 mM K^+/MOPS (pH 7.0). Top, CD spectra; bottom, absorption spectra.

Table 2: Extinction Coefficients and Ellipticities of the ISF and the Fd_{BED}

	Absorption			
	ISF		Fd_{BED}	
	λ (nm)	ϵ (mM^{-1} cm^{-1})	λ (nm)	ϵ (mM^{-1} cm^{-1})
oxidized	323	15.7	325	9.0
	458	8.4	457	4.8
	579	4.3	573	2.7
reduced	305	11.1		
	383	4.8	382	4.1
	428	4.0	432	3.3
	520	2.7	505	2.1
	CD			
	ISF		Fd_{BED}	
	λ (nm)	$\Delta\epsilon$ (mM^{-1} cm^{-1})	λ (nm)	$\Delta\epsilon$ (mM^{-1} cm^{-1})
oxidized	314	+22.7	323.5	+21.2
	379	−18.8	376.5	−17.7
reduced	314.5	+18.1	312	+16.1
	389.5	−19.4	386.5	−9.3
	506	−6.6	502	−8.9

dioxygenase (ISP_{BED}), which incorporates a Rieske type [2Fe-2S] center, and spinach ferredoxin. The CD difference spectra of all three proteins containing a Rieske cluster had negative peaks at 340 nm, positive peaks at 365−370 nm, and negative peaks at 390−410 and at 500 nm. The set of positive and negative peaks at 340, 370, and 400 nm in the difference spectra can be explained by redox dependent shifts of the CD bands at 340 and 380 nm of the oxidized proteins, while the negative peak at 500 nm is solely due to a negative CD band of the reduced protein. In contrast, the difference spectrum of Fd_{SP} showed predominantly a strong negative peak centered at 430 nm which is due to a positive CD band of the oxidized protein (Palmer *et al*., 1967).

Spectroelectrochemical Titration in an OTTLE Cell Monitored by CD. Since the CD spectra of Rieske clusters show significant differences between the oxidized and reduced forms, CD spectroscopy can be effectively used to monitor the redox state of these proteins. We have determined the redox potential in an OTTLE cell with a 0.1 mm path length, using a PATS-modified transparent gold mesh as the working electrode. The three-electrode setup allows the adjustment

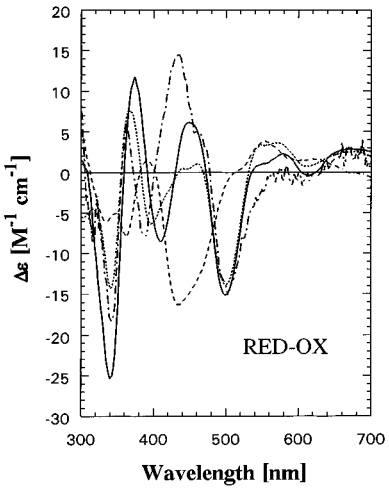

FIGURE 8: Difference CD spectra (reduced − oxidized) of the Rieske proteins: (•••) ISF in 50 mM sodium phosphate (pH 7.5) (—) Fd_{BED} in 50 mM K^+/MOPS (pH 7.0), (—•—•) ISP_{BED} in 50 mM MOPS (pH 7.0), and (— — —) Fd_{SP} in 50 mM Tris-HCl (pH 7.2).

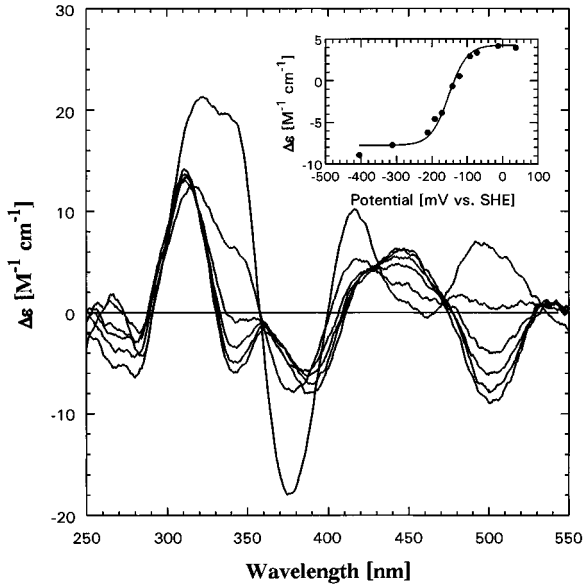

FIGURE 9: CD-monitored electrochemical redox titration of the Fd_{BED}. CD spectra were recorded in an OTTLE cell with a path length of 0.1 mm at the following potentials versus the SHE: +205, −122, −142, −172, −212, −312, and −405 mV (with increasing intensity of the negative band at 500 nm). The protein concentration was 0.58 mM. The inset shows a Nernst curve calculated from the CD intensity at 500 nm and fitted using a single Nernstian curve with the following parameters: $n = 1$ and $E° = -156$ mV.

of the potential of the working electrode through a potentiostat without external addition of oxidant or reductant. Figure 9 shows a set of spectra obtained at different potentials, and the inset shows the titration curve obtained from the intensity of the redox dependent CD band at 500 nm. The midpoint potentials obtained by CD-monitored potentiostatic redox titration were identical within ±5 mV to the potentials obtained by cyclic voltammetry.

DISCUSSION

Electrochemistry of the Rieske Cluster. The ISF has an almost neutral p*I* and gives a good electrochemical response at the negatively charged nitric acid-treated glassy carbon electrode. At this electrode, the strongly acidic dioxygenase ferredoxin showed no response. However, a good response was obtained using a modified gold electrode; this response was fully reversible and stable over many cycles. The redox potential obtained in solution was identical to that obtained by EPR-monitored redox titration (Geary *et al*., 1984) or by CD-monitored spectroelectrochemistry.

For the ISF, we have reported a linear dependence on the square root of the ionic strength with $dE°/d\sqrt{I} = -26$ mV $M^{-1/2}$ up to high ionic strengths (Link *et al*., 1992). An ionic strength dependence of the redox potential is expected from the Debye−Hückel theory since the oxidized and reduced species bear different net charges and therefore show different electrostatic interactions with the solvent dipoles; however, the Debye−Hückel limiting law is generally valid only at low ionic strengths, and deviations from linearity occur at higher ionic strengths due to ionic association. The linear ionic strength dependence up to high ionic strengths has been ascribed to the fact that the ISF is an almost electroneutral species around neutral pH; therefore, the protein will have weak electrostatic interactions with the solvent in both oxidation states (Link *et al*., 1992).

The redox potential of the Fd_{BED} was invariant when the ionic strength was varied between 0.025 and 2 M. The lack of ionic strength dependence of the Fd_{BED} can be understood if the [2Fe-2S] cluster is buried within the protein so that the solution dipoles do not "see" the charge difference of the cluster. In contrast, spinach ferredoxin (Fd_{SP}) shows a strong ionic strength dependence (+37 mV $M^{-1/2}$) at low ionic strengths; here, the cluster is exposed at the surface of the protein (Tsukihara *et al*., 1981). The different ionic strength dependence of the ferredoxins is not due to the overall charge of the protein since both Fd_{BED} and Fd_{SP} are highly acidic.

An important difference between plant type and Rieske type [2Fe-2S] clusters is the formal charge of the cluster (0 for oxidized [2Fe-2S] Cys_2His_2; −2 for oxidized [2Fe-2S] Cys_4); therefore, a greater ionic strength dependence of the redox potential of plant type [2Fe-2S] clusters is to be expected, in agreement with the experimental results.

The redox potential of the Fd_{BED} was found to be pH independent over pH 5−10, while that of the ISF was pH dependent (Link *et al*., 1992). It is likely that the groups of the ISF with redox dependent p*K* values are the histidine ligands to the cluster. Since the same ligand pattern is also present in the Fd_{BED}, these histidines are most likely hydrogen-bonded and/or secluded from the aqueous environment, though no deprotonation of the oxidized form occurs up to pH 10. The low value obtained for the reduction entropy, $\Delta S°_{rc}$, of the Fd_{BED} indicates that little reorganization (if any) takes place upon oxidation/reduction. The conclusion that the [2Fe-2S] cluster is buried more deeply in the Fd_{BED} than in the ISF is also supported by the observation that the heterogeneous rate constant for electron transfer with the electrode is almost 1 order of magnitude lower in the Fd_{BED} than in the ISF.

The differences between the two proteins can be understood in view of their different functions. The Rieske cluster of the bc_1 complex is the primary electron acceptor at the hydroquinone oxidation site of the bc_1 complex; its quinone binding site is accessible to solvent. In contrast, the dioxygenase ferredoxin has purely an electron transfer function and no substrate binding site; here, the cluster is buried and not accessible to solvent.

Table 3: Bands of the Reduced Rieske Clusters

ISF				Fd_{BED}			
λ (nm)	ϵ (M^{-1} cm^{-1})	$\Delta\epsilon$ (M^{-1} cm^{-1})	$\lvert\Delta\epsilon/\epsilon\rvert$	λ (nm)	ϵ (M^{-1} cm^{-1})	$\Delta\epsilon$ (M^{-1} cm^{-1})	$\lvert\Delta\epsilon/\epsilon\rvert$
500.3	nd	−18.5	nd	499.4	nd	−17.0	nd
762.4	348	−2.4	0.007	755.8	159	−2.2	0.014

Electronic Spectroscopy of the Rieske Cluster. The visible spectra of iron−sulfur proteins in general are characterized by a large number of $S \rightarrow Fe^{III}$ charge transfer bands and a lack of significant spectral characteristics (Noodleman & Baerends, 1984). [2Fe-2S] clusters both of the four-cysteine coordinated plant ferredoxin type and of the Rieske type have intense CD spectra, while [4Fe-4S] clusters show only weak CD spectra. This is in agreement with expectations from group theory. The [2Fe-2S] cluster belongs to the point group D_{2h}, while the [4Fe-4S] cluster belongs to the point group T_d, both of which are inherently CD inactive. The CD is induced by perturbations leading to a reduction of symmetry. In chromophores with higher symmetry, smaller optical activity is likely to be induced by static perturbations (Charney, 1979). Therefore, systems belonging to high-symmetry point groups in general show less intense CD spectra than systems belonging to lower-symmetry point groups. Since in Rieske clusters the basic symmetry is further reduced to C_{2v}, Rieske clusters display particularly intense CD spectra. These characteristic CD spectra are significantly different from those of other iron−sulfur proteins so that CD spectroscopy can be used to identify Rieske clusters even in the absence of other evidence (Degli Esposti *et al*., 1987).

$S \rightarrow Fe^{III}$ charge transfer bands are more intense in oxidized than in reduced iron−sulfur proteins. Upon reduction, the absorption intensity drops to approximately half the value of the oxidized protein. However, we have observed the appearance of CD bands at 500 and at 760 nm in the reduced protein only; the assignment of these CD bands is of particular interest.

Strong CD bands are expected for magnetically allowed transitions, *i.e.*, d−d bands. Oxidized high-spin Fe^{III} has no spin-allowed d−d transitions, while reduced high-spin Fe^{II} has spin-allowed d−d transitions derived from excitation of an electron from the lowest-lying d_{z^2} orbital into the d_{xz} and d_{yz} orbitals; in the CD spectrum, these are characterized by large dissymmetry ratios, $\Delta\epsilon/\epsilon > 0.05$. In reduced plant type ferredoxin [2Fe-2S] clusters, these transitions have been identified at energies ΔE_{xz} and ΔE_{yz} of 6000 and 3800 cm^{-1} (Eaton *et al*., 1971). A ligand field analysis of Rieske clusters indicated an increased energy difference ΔE_{xz} compared to plant type ferredoxin [2Fe-2S] clusters (Bertrand *et al*., 1985). A d−d energy splitting of 20 000 cm^{-1} has been observed in the mineral Gillespite where the iron has square planar coordination by four oxygen atoms; the iron atom was found to be completely high-spin up to room temperature (Burns *et al*., 1966). The only strong CD bands of the reduced Rieske clusters between 7000 and 22 000 cm^{-1} are those at 13000 cm^{-1} (760 nm) and 20 000 cm^{-1} (500 nm) (Table 3). The dissymmetry ratio of the band at 760 nm can be calculated for the ISF and for the Fd_{BED} as 0.007 and 0.014, respectively (Table 3); since d−d bands should have dissymmetry ratios >0.05, we conclude that this band is not a d−d band. Therefore, the highest energy d−d transition ($d_{z^2} \rightarrow d_{xz}$) must be assigned to the band at 500 nm, giving $\Delta E_{xz} = 20\,000$ cm^{-1}. The dissymmetry ratio cannot be calculated for the d−d band at 500 nm since the absorption spectrum is dominated by the charge transfer bands.

The high value of ΔE_{xz} is indicative of a strong distortion of the ligand field of the Fe^{II}, giving a local symmetry close to C_2. As the ligand field splitting of the ISF and of the Fd_{BED} is identical within 40 cm^{-1}, we expect the local symmetry and therefore also the protein fold around the cluster to be similar in both proteins despite the fact that the amino acid sequences are not conserved except for the four residues coordinating the Rieske cluster.

ACKNOWLEDGMENT

We thank Drs. M. Hirasawa and J. B. Knaff, Texas Tech University, Lubbock, TX, for a generous gift of spinach ferredoxin and Dr. T. Ohnishi, University of Pennsylvania, Philadelphia, PA, for recording the EPR spectra of the ISF.

REFERENCES

Axcell, B. C., & Geary, P. J. (1975) *Biochem. J. 146*, 173−183.
Batie, C. J., Ballou, D. P., & Correll, C. J. (1991) in *Chemistry & Biochemistry of Flavoenzymes* (Müller, F., Ed.) pp 544−554, CRC press, Boca Raton, FL.
Baymann, F., Moss, D. A., & Mäntele, W. (1991) *Anal. Biochem. 199*, 269−274.
Bertrand, P., Guigliarelli, B., Gayda, J.-P., Beardwood, P., & Gibson, J. F. (1985) *Biochim. Biophys. Acta 831*, 261−266.
Britt, R. D., Sauer, K., Klein, M. P., Knaff, D. B., Kriauciunas, A., Yu, C.-A., Yu, L., & Malkin, R. (1991) *Biochemistry 30*, 1892−1901.
Burns, R. G., Clark, M. G., & Stone, A. J. (1966) *Inorg. Chem. 5*, 1268−1272.
Charney, E. (1979) *The Molecular Basis of Optical Activity*, p 162, John Wiley & Sons, New York.
Degli Esposti, M. D., Ballester, F., Solain, G., & Lenaz, G. (1987) *Biochem. J. 241*, 285−290.
Ding, H., Robertson, D. E., Daldal, F., & Dutton, P. L. (1992) *Biochemistry 31*, 3144−3158.
Eaton, W. A., Palmer, G., Fee, J. A., Kimura, T., & Lovenberg, W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3015−3020.
Geary, P. J., & Dickson, D. P. E. (1981) *Biochem. J. 195*, 199−203.
Geary, P. J., Saboowalla, F., Patil, D., & Cammack, R. (1984) *Biochem. J. 217*, 667−673.
Geary, P. J., Mason, J. R., & Joannou, C. L. (1990) *Methods Enzymol. 188*, 52−60.
Gurbiel, R. J., Batie, C. J., Sivaraja, M., True, A. E., Fee, J. A., Hoffman, B. M., & Ballou, D. P. (1989) *Biochemistry 28*, 4861−4871.
Gurbiel, R. J., Ohnishi, T., Robertson, D. E., Daldal, F., & Hoffman, B. M. (1991) *Biochemistry 30*, 11579−11584.
Hagen, W. R. (1989) *Eur. J. Biochem. 182*, 523−530.
Hirasawa, M., Gray, K. A., Sung, I.-D., & Knaff, D. B. (1989) *Arch. Biochem. Biophys. 275*, 1−10.
Ke, B., Bulen, W. A., Shaw, E. R., & Breeze, R. H. (1974) *Arch. Biochem. Biophys. 162*, 301−309.
Link, T. A. (1994) *Biochim. Biophys. Acta 1185*, 81−84.
Link, T. A., Hagen, W. R., Pierik, A. J., Assmann, C., & von Jagow, G. (1992) *Eur. J. Biochem. 208*, 685−691.
Link, T. A., Saynovits, M., Assmann, C., Iwata, S., Ohnishi, T., & von Jagow, G. (1996) *Eur. J. Biochem. 237*, 71−75.
Mason, J. R., & Cammack, R. (1992) *Annu. Rev. Microbiol. 46*, 277−305.
Moss, D. A., Nabedryk, E., Breton, J., & Mäntele, W. (1990) *Eur. J. Biochem. 187*, 565−572.
Nicholson, R. S. (1965) *Anal. Chem. 37*, 1351−1355.

Noodleman, L., & Baerends, E. J. (1984) *J. Am. Chem. Soc. 106*, 2316−2327.
Palmer, G., Brintzinger, H., & Estabrook, R. W. (1967) *Biochemistry 6*, 1658−1664.
Prince, R. C., & Dutton, P. L. (1976) *FEBS Lett. 65*, 117−119.
Rieske, J. S., MacLennan, D. H., & Coleman, R. (1964) *Biochem. Biophys. Res. Commun. 15*, 338−344.
Shergill, J. K., Joannou, C. L., Mason, J. R., & Cammack, R. (1995) *Biochemistry 34*, 16533−16542.
Tagawa, K., & Arnon, D. I. (1966) *Biochemistry 5*, 6−13.
Tan, H.-M., Joannou, C. L., Cooper, C. E., Butler, C. S., Cammack, R., & Mason, J. R. (1994) *J. Bacteriol. 176*, 2507−2512.
Taniguchi, V. T., Sailasuta-Scott, N., Anson, F. C., & Gray, H. B. (1980) *Pure Appl. Chem. 52*, 2275−2281.
Tsukihara, T., Fukuyama, K., Nakamura, M., & Katsube, Y. (1981*) J. Biochem. (Tokyo) 90*, 1763.

BI960004+