# ISOLATION OF THE RIESKE IRON–SULFUR PROTEIN FROM THE CYTOCHROME $b_6/f$ COMPLEX OF SPINACH CHLOROPLASTS

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## 1. Introduction

The organization of cytochrome $b/c$ complexes in electron-transfer chains of mitochondria [1], chloroplasts [2,3] and photosynthesizing bacteria [4], is strikingly similar. In addition to cytochrome $b$ and $c_1$ (called cytochrome $f$ in chloroplasts), an essential component of these complexes is a Fe–S center with a midpoint potential of ~300 mV [1,3–7]. It was first isolated from beef heart mitochondria in [5], and has been called the Rieske Fe–S protein thereafter. In [8,9] this Fe–S protein has been purified in active form, reconstituting electron transport from succinate to cytochrome $c$ in depleted preparations of the succinate–cytochrome $c$ reductase complex from beef heart mitochondria. The Rieske Fe–S center has been detected in chloroplasts [6] and found in a highly purified, enzymatically active preparation of cytochrome $b_6/f$ complex [3]. Here, we report on the identification of the second smallest polypeptide of the complex as the Rieske Fe–S protein. It can be isolated from the complex retaining the characteristic EPR signals. The deficient complex is inactive in plastoquinol–plastocyanin–oxidoreductase activity and lacks the EPR signals of the Fe–S center. An antibody, prepared against the isolated Rieske Fe–S protein inhibits the oxidoreductase activity of the intact complex. The effects of the inhibitory plastoquinone analogue, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, (DBMIB) which has been found to shift the $g$-value of the EPR signals of the Rieske Fe–S center in chloroplast membrane [10,11], on the EPR-spectrum of the isolated cytochrome $b_6/f$ complex and its subunit IV have also been studied.

*Abbreviations:* Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-glycine; DBMIB, 2,5-dibromomethylisopropyl-*p*-benzoquinone; SDS, sodium dodecylsulfate

## 2. Materials and methods

The isolation of the cytochrome $b_6/f$ complex from spinach chloroplasts has been published [3]. Subunit IV of the complex was isolated as follows: 5 ml of the complex in its final purification stage, containing ~20 μM cytochrome $f$, was brought to 5 mM potassium phosphate and was loaded onto a hydroxyapatite column (1.5 × 3 cm) which was equilibrated with 20 mM potassium phosphate (pH 6.8), 0.5% Triton X-100. Washing with 25 ml of the buffer resulted in elution of subunit IV from the column, and this was collected and dialyzed against 1 liter 10 mM Tricine–NaOH (pH 8.0) at 4°C with 2 changes of the buffer. The dialyzed fraction was then loaded onto a small DEAE-cellulose column (0.5 × 2.0 cm) equilibrated with 10 mM Tricine–NaOH (pH 8.0), 0.5% Triton X-100. The adsorption of subunit IV was visible by formation of a faint brown band on top of the column. After further washing with the equilibration buffer, the protein was eluted with the equilibration buffer containing 0.05% Triton, 200 mM NaCl. The brownish fractions were collected and contained 0.3–0.5 mg protein/ml. Recovery of the subunit was ~40% with respect to the intact complex. A cytochrome $b_6/f$ complex deficient in the Rieske Fe–S center could be eluted from the hydroxyapatite column with 200 mM potassium phosphate (pH 6.8), 0.05% Triton X-100.

An antiserum against the isolated subunit IV of the complex was prepared by injecting a rabbit weekly 5 times with 0.1 mg protein in complete

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Freund's adjuvant, followed by a peritoneal booster injection of 0.3 mg protein in 1 ml saline. The purity of the serum was checked by immune replication of SDS–PAGE [12]. The γ-globulin fraction was prepared from immune and control sera by precipitating with 40% ammonium sulfate.

Acid-labile sulfur was estimated according to [13]. EPR spectroscopy at 10–15 K was done on a modified JEOL X-band spectrometer as in [14]. Settings of the EPR measurements are specified in the legends. Hydroxyapatite was prepared as in [15]. Other assays and materials were as in [3].

## 3. Results and discussion

Fig.1 shows the polypeptide patterns of the isolated, intact cytochrome complex, and of the preparations eluted from the hydroxyapatite column. It is obvious that subunit IV, the second smallest with $M_r$ 20 000, is eluted at low salt concentration, leaving the rest of the polypeptides behind (the particular preparation of the cytochrome complex shown in fig.1 contained a contamination of 38 000 $M_r$, which is sometimes seen if one takes the maximum yield of the sucrose gradient; this contamination stays on the column). Separation is critically dependent on relatively high concentrations of Triton X-100; at lower concentrations, only part of the subunit can be removed. Plastoquinol–plastocyanin oxidoreductase activity [3] is correlated with the presence of subunit IV in the complex: it is lost in the deficient complex, which is eluted at higher salt concentration, when all of the subunit is removed in the presence of high concentrations of the detergent, but is partially retained when the chromatography is carried out at 0.05% Triton X-100. This observation is very similar to that in [16], where an active cytochrome $b/c_1$ complex from beef heart mitochondria was isolated from hydroxyapatite with low concentrations of Triton X-100 only. At higher concentrations of Triton X-100, activity and a 24 500 $M_r$ subunit, the Rieske Fe–S center, are lost in parallel from the mitochondrial complex. We therefore conclude that the association of the two analog subunits with their respective complexes is similar.

Isolated subunit IV contained 3,1 non-heme irons and 1,9 acid-labile sulfur per polypeptide, which is suggestive of a $Fe_2S_2$-cluster. The excess iron is probably present as a contamination. Fig.2 shows the

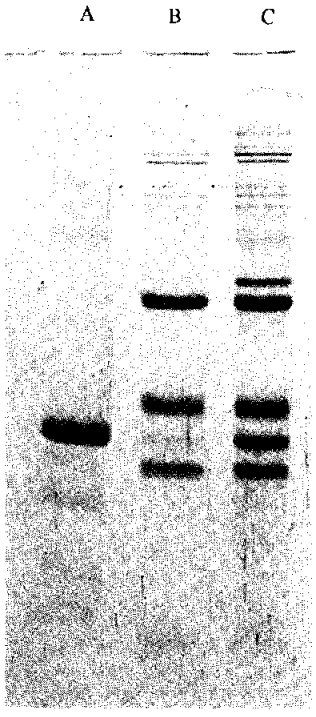


Fig.1. SDS–PAGE of intact and depleted cytochrome $b_6/f$ complex and isolated subunit IV. Electrophoresis was done as in [3]: (A) 3.7 μg protein of the isolated subunit IV was applied; (B,C) 0.1 and 0.15 nmol cytochrome $f$ of the depleted and the intact cytochrome $b_6/f$ complex was applied, respectively.

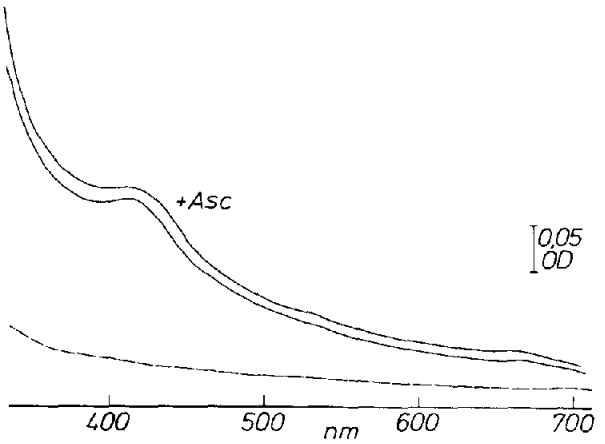


Fig.2. Absorption spectrum of the isolated Rieske Fe–S protein. The solution contained 0.37 mg protein of the isolated subunit IV in 200 mM NaCl, 10 mM Tricine–NaOH (pH 8.0) and 0.05% Triton X-100. For the upper spectrum ascorbate was added. The dashed spectrum corresponds to the baseline.

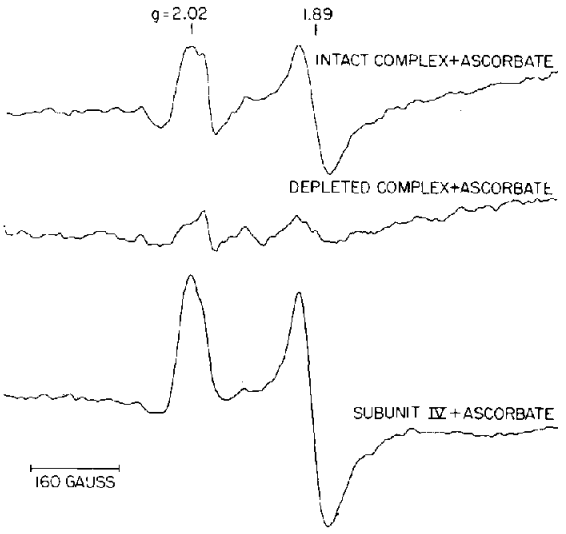


Fig.3. EPR spectra of intact and depleted cytochrome $b_6/f$ complex and isolated subunit IV. EPR spectra of the intact and depleted cytochrome complexes (cytochrome $f$ was 10 $\mu$M) were recorded after the addition of 5 mM sodium ascorbate; subunit IV was ~0.37 mg/ml. EPR conditions: field setting, 3400 ± 500 G; modulation amplitude, 12.5 G; microwave power, 5 mW; temperature, 13 K.

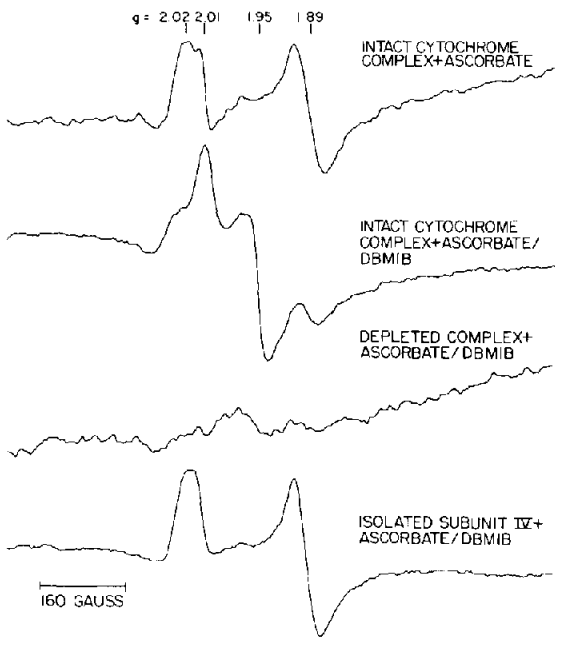


Fig.4. Effect of DBMIB on the Rieske Fe–S center of the cytochrome $b_6/f$ complex and isolated subunit IV. Sample preparations and concentrations were as in fig.3 except for the addition of 50 nmol DBMIB. EPR conditions were as in fig.3.

absorption spectrum of the preparation in the visible region and indicates only a very small contamination by residual chlorophyll (absorbance at 670 nm). Besides a shoulder around 420 nm, the spectrum lacks characteristic absorption bands, showing less structure than the one of the Rieske Fe–S protein of mitochondria [5]. Ascorbate did not alter the spectrum, suggesting that the Fe–S center is in the reduced form as isolated.

The EPR spectrum of the intact cytochrome $b_6/f$ complex in the presence of ascorbate (fig.3) is characterized by $g$-values at 2.02 and 1.89. These originate from the reduced form of the Rieske Fe–S center. After resolution of subunit IV from the complex (fig.3), these characteristic signals are almost totally absent. As shown in fig.3, the isolated subunit shows the typical EPR spectrum of the Rieske center and the $g$-values are identical with those of the center in the intact complex. It seems established, therefore, that we have isolated the chloroplast Rieske Fe–S protein in pure form. Also, from a comparison of the amplitudes of the EPR signal at $g = 1.89$ in subunit IV and the intact complex, assuming 1 subunit IV/cytochrome $f$ in the complex, we conclude there is no loss of the Fe–S cluster during isolation.

The effect of DBMIB on the Rieske center in the intact cytochrome complex is shown in fig.4. As has been shown for chloroplast membranes [10,11,17], the addition of DBMIB causes a shift in the $g$-values of the Rieske center to $g = 2.01$ and 1.95. When DBMIB is added to the Rieske-depleted cytochrome complex, no $g$-value shift is observed, and this result confirms that the site of action of DBMIB in this region of the electron-transfer chain is the Rieske Fe–S center. The isolated Rieske Fe–S protein does not show any alteration of its EPR spectrum on the addition of DBMIB (fig.4). We have also isolated the protein from the chloroplast complex by the procedure in [8], and this isolated protein also showed no interaction with DBMIB based on alterations in EPR signals. It would seem that for the interaction of DBMIB with the Rieske Fe–S center, the structural organization of the complex is required. Further studies with the intact and depleted complex may lead to a more complete understanding of the nature of this interaction.

Attempts to reconstitute plastoquinol–plastocyanin–oxidoreductase activity in the deficient complex with the isolated Rieske Fe–S protein, as has been

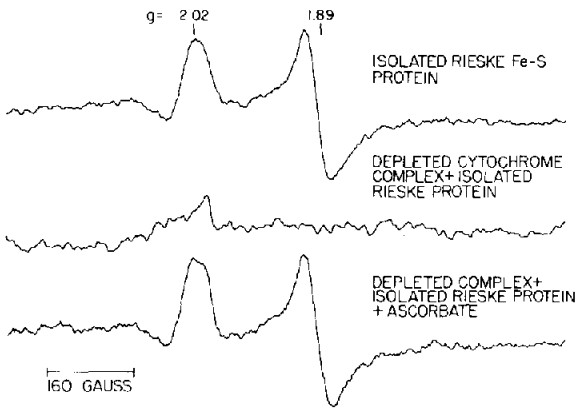


Fig.5. Interaction of the soluble Rieske Fe–S protein with the depleted cytochrome $b_6/f$ complex. Sample preparations and EPR conditions were as in fig.3.

reported for the mitochondrial activity [8], have not been successful. Also, the prearation made according to [8] was not reconstitutively active with either the deficient chloroplast or the deficient mitochondrial complex. Interaction of the isolated Rieske protein with the depleted complex can occur (fig.5). Addition of the reduced Rieske Fe–S protein to oxidized, depleted complex leads to the oxidation of the Fe–S center.

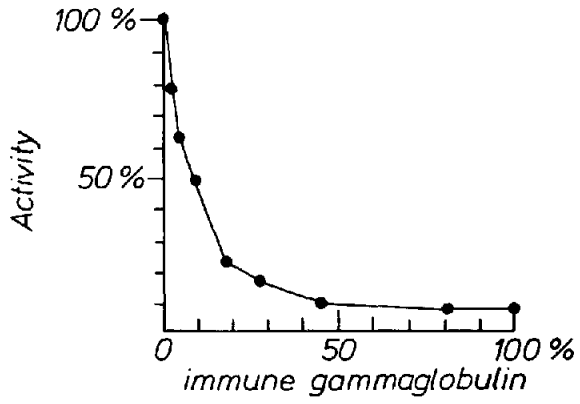


Fig.6. Inhibition of plastoquinol–plastocyanin–oxidoreductase activity by an antibody against the Rieske Fe–S protein. Activity was measured as in [3]. The reaction mixture contained in 0.5 ml: 0.15 M NaCl; 20 mM 4-morpholino ethane sulfonic acid–NaOH (pH 6.2); 5.6 $\mu$M plastocyanin; 125 nM cytochrome $f$ in form of the cytochrome $b_6/f$ complex; and 11 mg $\gamma$-globulin. The amount of the immune gammaglobulin was varied from 0–100%. The reaction was started by addition of plastoquinol-1 to 50 $\mu$M. 100% activity corresponds to 20 $\mu$mol plastocyanin reduced . nmol cytochrome $f^{-1}$ . $h^{-1}$.

Fig.6 demonstrates that plastoquinol–plastocyanin–oxidoreductase activity with the intact $b_6/f$ complex is fully sensitive to an antibody against the Rieske Fe–S protein. The antibody did not inhibit electron transport from water, or from plastoquinol to photosystem I in chloroplasts, which indicates that the Rieske Fe–S center is either buried in the membrane or located on the inner surface of the thylakoids.

As indicated before [3,18], oxidant-induced reduction of cytochrome $b$, a general characteristic of cytochrome $b/c$ complexes [19,20], can also be seen with our purified cytochrome $b_6/f$ complex. It is lost by removal of the Rieske Fe–S protein, and this behavior is analogous to that of the mitochondrial cytochrome complex [19].

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