# The Rieske Protein from Purple Sulfur Bacteria Is an Extrinsic Protein

Andrzej Szczepaniak[a], Joachim Uhrig[b,*], Beata Gubernator[a]

[a] Institute of Biochemistry, University of Wrocław, 50–137 Wrocław, Tamka 2, Poland
[b] Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität, D-44780 Bochum, Germany

Z. Naturforsch. **53c,** 15–20 (1998); received September 30/October 16, 1997

Chromatophor, Cytochrome $bc_1$ Complex, Rieske Protein, *Rhodospirillum rubrum*

The mode of membrane attachment of the Rieske iron-sulfur protein from cytochrome $bc_1$ complex of *Rhodospirillum rubrum* has been studied using biochemical approaches.

In contrast to cytochrome $c_1$ the bacterial Rieske protein was extracted from chromatophores using chaotropic agents (NaSCN, urea, guanidine), an alkaline pH and relatively low concentration of Triton X-100.

The results presented here lead to the conclusion, that the Rieske protein from chromatophores is extrinsic and that their association with the rest of the complex involves hydrophobic interactions.

## Introduction

The cytochrome $bc_1$ complex is the most common of the energy-transducing electron transfer protein. Cytochrome $bc_1$ complex have been isolated from many bacteria including oxygenic and anoxygenic photosynthetic bacteria, and from mitochondria of lower and higher eucaryotes. A very similar cytochrome $b_6f$ complex occur in algae and the chloroplast of higher plants (Cramer *et al.*, 1996; Kallas, 1994). Although they have a different peptide content (from 3 to 12 subunits) they all possess the universal composition of three redox carriers: Cyt $b_6$ or Cyt $b$ containing two $b$ type heme; Cyt $f$ or Cyt $c_1$ containing one $c$ type heme and the Rieske protein containing iron-sulfur cluster.

The location and folding patterns of Cyt $b$, Cyt $b_6$ and subunit IV, Cyt $f$ and Cyt $c_1$ are well established (Cramer *et al.*, 1991; Trumpower, 1990). Most unclear is the folding pattern of the Rieske iron-sulfur protein. Based on the sequence data the folding pattern with one or with two membrane spanning helices has been proposed for the iron-sulfur protein (Schägger *et al.,* 1987; Willey and Gray, 1988; Stepphuhn *et al.*, 1987; Harnisch *et al.*, 1985). On the other hand, it has been suggested that the Rieske protein in mitochondria may be a peripheral membrane protein that does not span the membrane bilayer (Hartl *et al.*, 1989). Recently, using a variety of biochemical approaches it has been shown that the Rieske protein of *Chlamydomonas reinhardtii* (Breyton *et al.*, 1994) and of pea thylakoids and yeast mitochondria (Szczepaniak *et al.,* 1995) is an extrinsic protein.

In the present study, we have examined the mode of membrane association of the Rieske protein from *Rhodospirillum rubrum* chromatophores using biochemical methods.

## Materials and methods

### *Chromatophore membranes preparation*

Culture of *Rhodospirillum rubrum* FR1 (DSM 1068) were grown under anaerobic conditions according to Omerod *et al.*(1961). In the end-logarithmic phase they were harvested by centrifugation at 10,000 x *g* for 20 min and were washed twice in 10 mM Tris-HCl (pH 7.6). Chromatophores were prepared by the method of Collins and Niderman (1976), modified according to Majewski (1989) and were used fresh or stored in −20 °C.

*Abbreviations:* Cyt, cytochrome; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

* Present address: Max-Planck-Institut für Zuechtungsforschung, Abteilung Schell, Carl-von-Linnee-Weg 10, 50829 Koeln, Germany.

Reprint requests to A. Szczepaniak.
Telefax: 71-40 26 08.

0939–5075/98/0100–0015 $ 06.00  

*Polypeptide extraction from chromatophores*

Chromatophore membranes were centrifuged at 150,000 x *g* for 45 min. The resultant centrifuge pellet was resuspended in 5 mM Hepes-NaOH (pH 7.5), 10 mM EDTA at a protein concentration 1.0 mg/ml and centrifuged (150,000 x *g*, 60 min). The sediments were resuspended in 10 mM Caps-NaOH, pH 10, 10.5, 11, 11.5 and 12 at a protein concentration 0.5 mg/ml. The pH was checked after addition of membranes and in supernatant after centrifugation. As a control chromatophores were incubated in 10 mM Hepes-NaOH, pH 7.5. After incubation for 30 min on ice the membranes were centrifuged (150,000 x *g*, 60 min), the sediments were washed once with distilled water, subjected to SDS-PAGE and analyzed by Western blotting. In case of extraction with chaotropic agents the pellet was resuspended in 20 mM Tris-HCl, pH 7.6 (at protein concentration 0.5 mg/ml) containing either 6 M guanidine, 8 M urea or different concentration of NaSCN (detailed in the figure legends). Detergent permeabilized membranes were prepared by incubation of chromatophores (2.5 mg of protein/ml) with 0.1% Triton X-100 for 10 min on ice. After centrifugation (150,000 x *g* for 60 min) the sediments were resuspended in the appropriate buffer with concentration of NaSCN as indicated in the figure legends. Protein concentration was determined by the Micro-BCA (bicinchoninc acid, Pierce Chemical, Rockford, IL) method.

*SDS-PAGE and Western blotting*

These procedures are as described by Szczepaniak and Cramer (1990), except that Western blotting was carried out at a current of 200 mA for 90 min using semi-dry transfer unit. The antibodies to Cyt $c_1$ and Rieske iron-sulfur protein were kindly provided by Trebst, Bochum, and were prepared as described by Majewski and Trebst (1990).

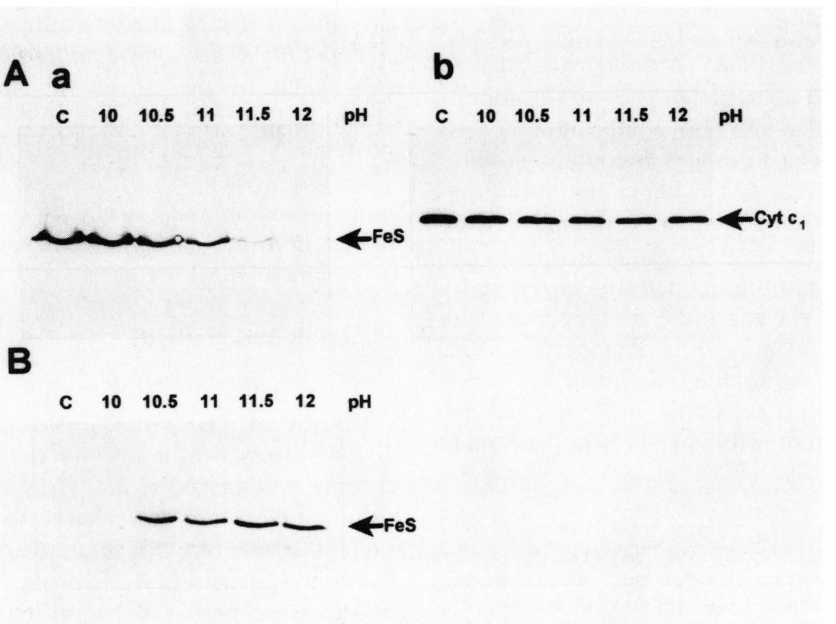


Fig. 1. Western blot analysis of the polypeptides of the cytochrome $bc_1$ complex extracted from the *Rhodospirillum rubrum* chromatophores at alkaline pH (10–12). (A) Samples (40 μg of protein) were subjected to SDS-PAGE and analyzed by Western blotting using polyclonal antibodies against (a) Rieske iron-sulfur protein (arrow), (b) cytochrome $c_1$ (arrow). (B) Western blot analysis of the supernatant resulting from the chromatophores extracted at alkaline pH. Samples (equivalent of 40 μg of protein) were subjected to SDS-PAGE and analyzed by Western blotting using polyclonal antibodies against Rieske iron-sulfur protein (arrow). Western blot analysis of the samples with polyclonal antibodies against Cyt $c_1$ resulted in not detectable signal. Other conditions as under "Materials and Methods".

## Results

The effect of extreme alkaline pH (>10) on the association of Rieske iron-sulfur protein of the $bc_1$ complex with chromatophore membrane was tested in order to determine whether the Rieske protein was an intrinsic membrane protein or peripheral. Incubation of membrane at alkaline pH is a well documented procedure for removing peripheral or extrinsic (Steck and Yu, 1973; Capaldi and Vanderkooi, 1972) proteins from membrane.

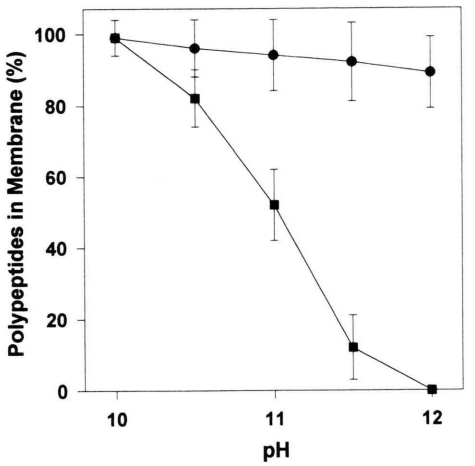


Fig. 2. Graph of the polypeptides from cytochrome $bc_1$ complex remaining in the chromatophores after alkaline pH (10–12) extraction derived from data shown in Figure 1. The relative protein concentrations were measured by densitometric analysis of Western blots. The points are expressed as percentages of the control sample and are the means of five different experiments; (□) Rieske iron-sulfur protein, (○) cytochrome $c_1$; standard deviations resulting from the average of five measurements are shown.

At pH value >11, the iron-sulfur protein was, indeed, released from the chromatophore membrane (Fig. 1A, panel a) and appeared quantitatively in the supernatant (Fig. 1B). The pH value at which 50% of the iron-sulfur protein was lost from the chromatophore membrane was 11.1 (Fig. 2). The loss of cytochrome $c_1$ polypeptide from the membrane was small and was in the range of the method error (Fig. 2).

Similar experiments were made using other dissociating media. As shown by Fig. 3 at 8 M urea and at 6 M guanidine the 75% and 90% (respectively, average from three different experiments) of iron-sulfur protein was released from the chromatophores and appeared quantitatively in the supernatant.

The extraction of iron-sulfur protein by NaSCN was examined in more detail. Release of the Rieske protein from intact chromatophores, detergent permeabilized and sonicated chromatophores at different concentrations of NaSCN was tested (Fig. 4). In intact chromatophores the Rieske protein was not completely released even at 5 M concentration. But after permeabilization of the chromatophores with Triton X-100 the iron-sulfur protein was more readily released. At concentration of 3 M NaSCN the Rieske protein was released completely.

Detergent should not only permeabilize chromatophore membranes but should weaken the hydrophobic protein-protein or protein-lipid interac-

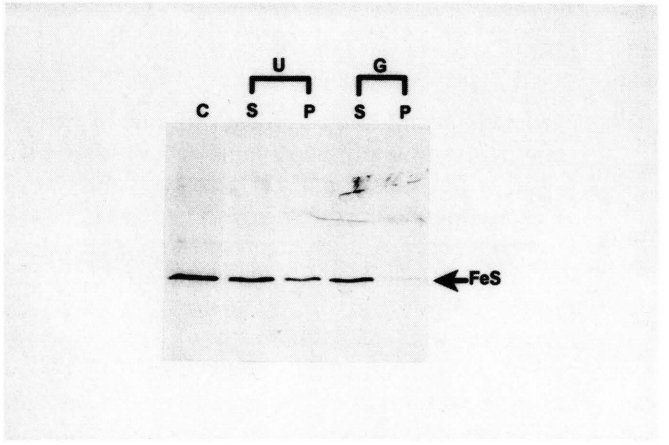


Fig. 3. Extraction of chromatophore membrane proteins by dissociating treatments. The supernatants (S) and the pellets (P) were subjected to SDS-PAGE and analyzed by Western blotting using polyclonal antibodies against Rieske iron-sulfur protein. Urea (U) and guanidine (G) were used as dissociating reagents. Lane c, control chromatophores not subjected to dissociatingon treatments. Other conditions as in Fig. 1 and under "Materials and Methods".

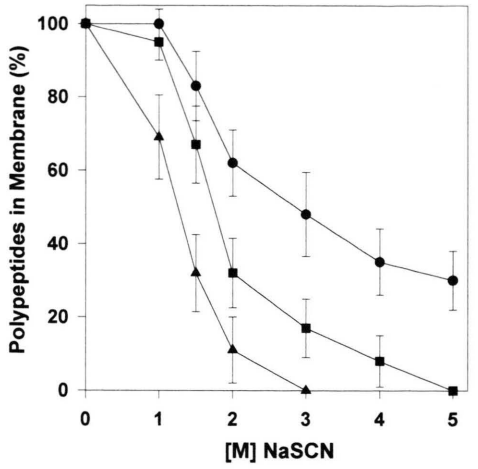


Fig. 4. Graph of relative extraction of the Rieske protein by NaSCN from (○)intact chromatophores, (Δ) Triton X-100 permeabilized chromatophores and from (□) sonicated chromatophores. Other conditions as in Figure 2. The points represent the means of five different experiments; standard deviations resulting from the average of five measurements are shown.

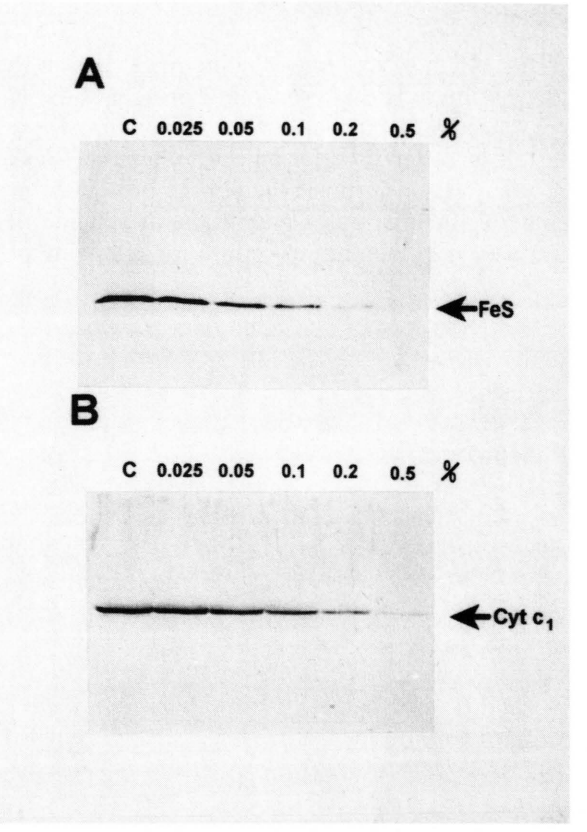


Fig. 5. Western blot analysis of the polypeptides of the cytochrome $bc_1$ complex extracted from the *Rhodospirillum rubrum* chromatophores by Triton X-100.
(A) Chromatophores (0.5 mg of protein/ml) were incubated with different concentrations of Triton X-100 (indicated on the figure) for 30 min on ice. After centrifugation (150,000 x *g*, 60 min), sediments were washed once with distilled water, subjected to SDS-PAGE and analyzed by Western blotting with polyclonal antibody against Rieske iron-sulfur protein (arrow). Lane c, control not treated with Triton. Other conditions as in Fig. 1 and under "Materials and Methods".
(B) Conditions as in (A) except that the Western blot used the antibody against cytochrome $c_1$.

tions. In order to find out why detergent pre-treatment facilitates release of Rieske protein from chromatophores, we introduced the sonication to disrupt the chromatophore membranes. The chromatophores were resuspended in indicated concentration of NaSCN and subjected to sonication. In this case the release of the iron-sulfur protein was improved, although sonication was less effective than pre-treatment with Triton X-100.

The effect of different concentrations of Triton on the association of polypeptides of cytochrome $bc_1$ complex to the chromatophore membranes was tested (Fig. 5). The Rieske protein was more readily released from the chromatophore membrane than Cyt $c_1$. Most of the iron-sulfur protein was released at a protein/Triton ratio of 1:4 (Fig. 5A). This conditions were ineffective in releasing of Cyt $c_1$ from chromatophore membranes. Releasing of Cyt $c_1$ from membrane required the higher detergent concentration at a protein/Triton ratio 1:10 (Fig. 5B).

## Discussion

The mode of association of the Rieske protein with membrane has been controversial for years with regard to both the cytochrome $b_6f$ and cytochrome $bc_1$ complexes. Models with one or two N-terminal transmembrane α-helices have been proposed (Schägger *et al.*, 1987; Willey and Gray, 1988; Stepphuhn *et al.*, 1987; Harnisch *et al.*, 1985). However, recently introducing variety of biochemical approaches it has been shown that the Rieske protein of mitochondria (Hartl *et al.*, 1989) of *Chlamydomonas* thylakoids (Breyton *et al.*, 1994) and pea thylakoids (Szczepaniak *et al.*, 1995) is an extrinsic protein. In our work we introduced similar biochemical methods to study the mode of

membrane attachment of the Rieske protein from *Rhodospirillum rubrum* chromatophores.

Alkaline wash of the Rieske subunit from chromatophores was efficient only above pH 11 and is consistent with results on higher plant thylakoids, on yeast mitochondria (Szczepaniak *et al.,* 1995) and green algae thylakoids (Breyton *et al.*, 1994). The results imply a similar mode of an association of this protein with these membranes.

Chaotropic agents are known to disrupt protein/protein interaction and not to extract intrinsic protein (Tanner, 1979). The Rieske protein was extracted by chaotropic agents (8 M urea and 6 M guanidine) from chromatophores almost completely. Extraction with NaSCN (1–5 M) was less effective and was improved by sonication, suggesting that in these experimental conditions part of chromatophore vesicles were still intact and the protein was trapped in vesicles. Even more efficient in improving the extraction of Rieske protein by NaSCN was pre-treatment with low concentration of Triton. Detergent facilitates the removal of iron-sulfur protein not only by permeabilization of vesicles but also by weakening of the hydrophobic interaction of the protein with the complex and/or membrane. Similar effect of Triton was observed in the case of mitochondria and tylakoids. Rieske iron-sulfur protein was released from chromatophore membranes at lower concentration of Triton than Cyt $c_1$ implying much weaker bounding of the Rieske protein with chromatophore membranes than Cyt $c_1$.

In conclusion, the evidence presented in this paper strongly suggests the Rieske iron sulfur protein from *Rhodospirillum rubrum* $bc_1$ complex being an extrinsic protein, although its association with the complex and/or membrane involves primarily hydrophobic interactions. This is with agreement to previously published data regarding Rieske protein from mitochondria (Hartl *et al.* 1986, Hartl *et al.* 1989, Gonzalez-Halphen *et al.* 1991), from *Chlamydomonas reinhardtii* (Breyton *et al.*1994) and from pea thylakoids (Szczepaniak *et al.,* 1995). The conclusion from the present work, therefore, must extend to Rieske proteins from other organisms as well.

*Acknowledgements*

This research was supported by grants from the State Committee for Scientific Research (project no. 6 6263 92 03) to AS and from Deutsche Forschungsgemeinschaft. We thank Prof. A. Trebst for gifts of antibodies to the Rieske iron-sulfur protein, Cyt $c_1$ and for helpful discussions.

Breyton C., de Vitry C. and Popot L-J. (1994), Membrane association of cytochrome $b_6$f subunits. The Rieske iron-sulfur protein from *Chlamydomonas reinhardtii* is an extrinsic protein. J. Biol. Chem. **269**, 7597–7602.

Capaldi R. A. and Vanderkooi G. (1972), The low polarity of many membrane proteins. Proc. Natl. Acad. Sci. USA **69,** 930–932.

Collins M. L. P., Niederman R. A. (1976), Membranes of *Rhodosoirillum rubrum*: Physicochemical properties of chromatophore fractions isolated from osmotically and mechanically disrupted cells. J. Bacteriol. **126,** 1326–1338.

Cramer W.A, Furbacher P., Szczepaniak A. and Tea G-S. (1991), Electron transport between photosystem II and photosystemI. In: Current Topics in Bioenergetics **16**, (C. P. Lee, ed.), Academic Press, Orlando, pp. 179–222.

Cramer W. A., Soriano G. M., Ponomarev M., Huang D., Zhang H., Martinez S. E. and Smith J. L. (1996), Some new structural aspects and old controversies concerning the cytochrome $b_6$f complex of oxygenic photosynthesis. Annu.Rev.Plant Physiol. Plant Mol. Biol. **47,** 477–508.

Gonzalez-Halphen D., Vazquez-Acevedo M. and García-Ponce B. (1991), On the interaction of mitochondrial complex III with the Rieske iron-sulfur protein (subunit V). J. Biol. Chem. **266**, 3870–3876.

Harnisch U., Weiss H. and Sebald W. (1985), The primary structure of the iron-sulfur subunit of ubiquinol-cytochrome *c* reductase from *Neurospora*, determined by cDNA gene sequencing. Eur.J.Biochem. **149**, 95–99.

Hartl F. U., Schmidt E., Wachtert E., Weiss H. and Neupert W. (1986), Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome *c* reductase. Cell **47**, 939–951.

Hartl F. U., Pfanner N., Nicolson D. W. and Neupert W. (1989), Mitochondrial protein import. Biochim. Biophys. Acta **988**, 1–45.

Kallas T. (1994), The cytochrome $b_6$f complex. In: The Molecular Biology Cyanobacteria (D. A. Bryant, ed.). Kluwer Academic Publishers, The Netherlands, pp. 259–317.

Majewski C. (1989), The cytochrome $bc_1$ complex of *Rhodosoirillum rubrum*. Isolation of the complex and the coding gene. Thesis, Ruhr-University, Bochum.

Majewski C. and Trebst A. (1990), The *pet* genes of *Rhodospirillum rubrum*: cloning and sequencing of the genes for the cytochrome $bc_1$ complex. Mol. Gen. Genet. **224,** 373–382.

Omerod J. G., Omerod K. S., Gest H. (1961), Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen 18 by photosynthetic bacteria: relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94,** 449–463.

Schägger H., Borchart U., Macheldt W., Link T. A. and Jagow G. (1987), Isolation and amino acid sequence of the Rieske iron-sulfur protein of beef heart ubiquinol-cytochrome *c* reductase. FEBS Lett. **219**, 161–168.

Steck T. L. and Yu J. (1973), Selective solubilization of proteins from red blood cell membranes by protein perturbants. J.Supramol. Struct. **1,** 220–232.

Steppuhn J., Rother C., Hermans J., Jansen T., Salnikow J., Hauska G. and Herrmann R. G. (1987), The complete amino acid sequence of the Rieske Fe-S precursor protein from spinach chloroplasts deduced from cDNA analysis. Mol. Gen. Genet. **210**, 171–177.

Szczepaniak A. and Cramer W. A. (1990), Thylakoid membrane protein topography. Location of the termini of the chloroplast cytochrome $b_6$ on the stromal side of the membrane. J.Biol.Chem. **265**, 17720–17726.

Szczepaniak A., Frank K. and Rybka J. (1995), Membrane association of the Rieske iron-sulfur protein. Z.Naturforsch. **50c,** 535–542.

Tanner M. J. A. (1979), Isolation of integral membrane proteins and criteria for identyfying carrier proteins. Curr. Top. Membr. Transp. **12**, 1–55.

Trumpower B. (1990), Cytochrome $bc_1$ complex of microorganisms. Microbiol. Rev. **54**, 101–129.

Willey D. L. and Gray J. C. (1988), Synthesis and assembly of the cytochrome *bf* complex. Photosynth. Res. **17**, 125–144.