Membrane Association of the Rieske Iron–Sulfur Protein

Andrzej Szczepaniak[a], Karin Frank[b] and Jacek Rybka[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, Bundesrepublik Deutschland

Z. Naturforsch. **50c,** 535–542 (1995); received January 1/February 27, 1995

Chloroplast, Mitochondria, Cytochrome b_6f Complex, Cytochrome bc_1 Complex, Rieske Protein

The mode of membrane attachment of the Rieske iron–sulfur proteins from cytochrome b_6f complex of pea thylakoids and from cytochrome bc_1 complex of yeast mitochondria has been studied using biochemical approaches.

The relative sensitivity of the Rieske protein to trypsin in the thylakoid membrane shows that all trypsin sites of the Rieske protein are on the lumen side of the thylakoid membrane.

In contrast to cytochrome *f* the chloroplast Rieske protein was extracted from thylakoids using chaotropic agents (NaSCN, urea), an alkaline pH and relatively low concentrations of Trinon X-100. The cytochrome bc_1 complex Rieske protein from mitochondrial membranes of yeast was also released by NaSCN and alkaline treatment.

The results presented here led us to the conclusion, that the mitochondrial and chloroplast Rieske proteins are extrinsic and that their association with the rest of the complex involves hydrophobic interactions.

Introduction

The cytochrome b_6f complex is an intrinsic membrane protein complex, that mediates electron transport between photosystem II and photosystem I complexes in oxygenic photosynthesis (Cramer *et al.,* 1991; Malkin, 1992). In terms of function and composition, the photosynthetic cytochrome b_6f complex is analogous to the extensively studied complex III of the mitochondrial respiratory chain (Rieske, 1976) and the cytochrome bc_1 complex of photosynthetic bacteria. 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 hemes; Cyt *f* or Cyt c_1 containing one *c* type heme and the Rieske iron–sulfur protein containing one 2Fe–2S iron–sulfur cluster.

The locations and folding patterns of Cyt *b*, Cyt b_6 and subunit IV, Cyt *f* and Cyt c_1 are well established. The folding model of Cyt b_6 (Szczepaniak and Cramer, 1990; Widger and Cramer, 1991), and subunit IV has a high degree of similarity to that of Cyt *b* (Trumpower, 1990; Widger and Cramer, 1991), considering the sidedness and the amount and location of hydrophobic transmembrane domains (Cramer *et al.,* 1987; Widger and Cramer, 1991). The same type of similarity exists between Cyt *f* (Gray, 1992) and Cyt c_1 (Cramer *et al.,* 1987; Widger and Cramer, 1991).

Most unclear is the folding pattern of the Rieske iron–sulfur protein. Based on sequence data, different folding patterns have been proposed. A folding model with two membrane spanning helices has been proposed for the Rieske protein from beef heart mitochondria (Schägger *et al.,* 1987) and from chloroplasts (Willey and Gray, 1988). A model with one hydrophobic transmembrane helix has been proposed for the Rieske protein from spinach chloroplasts (Stepphuhn *et al.,* 1987) and from mitochondria of *Neurospora crassa* (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* is an extrinsic

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

Reprint requests to A. Szczepaniak.
Telefax: 71-402608.

0939–5075/95/0700–0535 $ 06.00 N

protein (Breyton *et al.*, 1994). In the present study, we have examined the mode of membrane association of the Rieske protein from pea *(Pisum sativum)* chloroplasts using biochemical methods (proteolysis and extraction). The effect of alkaline pH and chaotropic agents on the association of the Rieske protein from pea chloroplasts with the membrane was compared to that of the mitochondrial Rieske protein from yeast. We conclude that the Rieske protein is an extrinsic protein, whose binding to the complex and/or to the membrane involves hydrophobic interactions.

Methods

Chloroplast preparation

Peas (*Pisum sativum* cv. Nefryt) were grown for 14 days in a growth chamber under 12-h-day fluorescent light. Freshly harvested pea leaves (50 g) were homogenized in 250 ml buffer (0.3 M sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5) for about 5 s, filtered through four layers of cheesecloth, then subjected to centrifugation at $1000\times g$ for 3 min. The sediment was resuspended in 50 mM sucrose, 10 mM NaCl and Hepes, pH 7.5, centrifuged again at $1000\times g$ for 30 s, the sediment discarded, and the supernatant centrifuged at $3000\times g$ for 5 min. The resultant pellet was resuspended in homogenization buffer.

Mitochondria and mitochondrial particles of commercially available yeast *(Saccharomyces cerevisiae)* used for the preparation of inner membrane vesicles according to Pedersen and Hullihen (1979) were isolated according to Clejan and Beattie (1986). Inner mitochondrial vesicles (called mitochondrial membrane) were used for washing experiments.

Proteolysis

Thylakoids (100 µl, 1 mg of Chl/ml) were incubated at room temperature (about 20 °C) with chymotrypsin-free trypsin for 15 min using the chlorophyll/trypsin ratios given in the figure legends. The trypsin reaction was terminated by addition of 5 mM phenylmethylsulfonyl fluoride. The membranes were immediately washed with 90% acetone, recovered by centrifugation (4 min, $10{,}000\times g$) and then solubilized prior to electrophoresis.

Polypeptide extraction from thylakoids

Thylakoid membranes (100 µl, 1 mg Chl/ml) were centrifuged for 10 min at $30{,}000\times g$. The pellets were resuspended in 500 µl of the following media: 50 mM Hepes-NaOH, pH 7.5 (control), the same buffer containing either 2 M NaCl, 2 M $CaCl_2$, 2 M NaSCN (or other concentrations detailed in the figure legends) or 6 M urea. One pellet was resuspended in 0.1 N NaOH. After 30 min incubation on ice samples were centrifuged for 30 min at $30{,}000\times g$. The sediments were washed once with distilled H_2O, subjected to SDS-PAGE and analyzed by Western blotting. In case of alkaline extraction the membranes were resuspended in 5 mM Hepes-NaOH (pH 7.5), 10 mM EDTA at a chlorophyll concentration of 0.4 mg/ml (thylakoids) or a protein concentration of 1.0 mg/ml (mitochondrial membranes) and centrifuged ($30{,}000\times g$ for 30 min or $100{,}000\times g$ for 60 min, respectively). The sediments were resuspended in 10 mM Caps-NaOH, pH 10, 10.5, 11, 11.5 and 12, at a chlorophyll concentration of 0.2 mg/ml (thylakoids) or a protein concentration of 0.5 mg/ml (mitochondrial membranes). The pH was checked after addition of membranes and in supernatant after centrifugation. As control membranes were incubated in 10 mM Hepes-NaOH, pH 7.5. After incubation for 30 min on ice the membranes were centrifuged, the sediments were washed once with distilled water, subjected to SDS-PAGE and analyzed by Western blotting. Detergent permeabilized membranes were prepared by incubation of membranes (1 mg of Chl/ml or 2.5 mg of mitochondrial protein/ml) with 0.1% of Triton X-100 for 10 min on ice. After centrifugation (thylakoids at $30{,}000\times g$ for 30 min, mitochondria at $160{,}000\times g$ for 45 min) the sediments were resuspended in the appropriate buffer with concentrations of NaSCN as indicated in the figure legends. Protein concentrations were determined by the Micro-BCA (bicinchoninic acid, Pierce Chemical Co., Rockford, IL) method. Chlorophyll concentrations were determined in 80% acetone.

Preparation and purification of antibodies

The cytochrome b_6f complex isolated from spinach chloroplasts (Black *et al.*, 1987) and the cytochrome bc_1 complex from yeast mitochondria (Ljungdahl *et al.*, 1987) were subjected to prepara-

tive gel electrophoreses. After a short (1 h) staining period the bands of interest were cut out of the gel and proteins were electroeluted using the Laemmli system buffer (Laemmli, 1970). The preparations routinely yielded a single protein band in the region of interest. The four proteins from spinach (Cyt *f*, Cyt b_6, Rieske protein and subunit IV) and Rieske protein from cytochrome bc_1 complex of yeast (about 1 mg/ml) were mixed with an equal volume of complete Freund's adjuvant each. 1 ml (0.5 mg of protein) of the resulting emulsion was injected into a rabbit. The first booster injection was given three weeks later. Similar booster injections were given at two additional two week intervals. Sera were collected one week after the last booster injection.

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 using a semi-dry transfer unit.

Results

Relative rate of trypsin proteolysis of the Rieske protein in thylakoids

The accessibility to trypsin of the Rieske iron–sulfur protein in thylakoids was compared to that of the 16 kDa OEC extrinsic polypeptide located on the lumen side of the membrane. It was detected by immunoblotting with polyclonal antibodies (Figs 1 and 2). The iron–sulfur protein was found to be relatively resistant to trypsin added to thylakoids compared to the 16 kDa OEC protein. At a chlorophyll/trypsin ratio of 1/5 the 16 kDa polypeptide was digested almost completely whereas less than 50% of the Rieske protein were digested. The explanation of the degradation of the 16 kDa protein by trypsin added on the stromal side is, that at high concentrations of proteinase and/or prolonged incubation time, the thylakoids are somewhat leaky to the trypsin. The slower digestion of the iron–sulfur protein compared to the OEC polypeptide implies, that sites for the action of trypsin are located on the lumen side of the thylakoid membrane. Trypsinolysis of the Rieske protein results in only one visible prod-

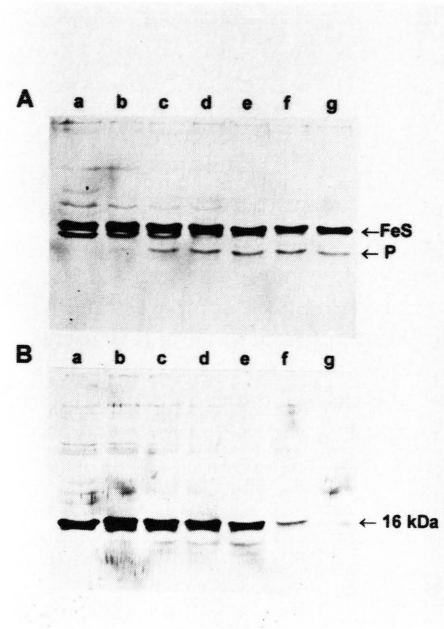

Fig. 1. Relative rate of trypsinolysis of the Rieske protein and the OEC extrinsic protein (16 kDa) in thylakoids. Thylakoids were incubated with trypsin for 15 min at room temperature (1:200, 1:100, 1:40, 1:20, 1:10, 1:5 (w/w), trypsin:chlorophyll, lanes b, c, d, e, f, g). Lane a, control not treated with trypsin. Other conditions as under "Material and Methods". (A) Immunodetection of trypsinolysis of the Rieske protein; (B) immunodetection of trypsinolysis of the 16 kDa OEC extrinsic protein.

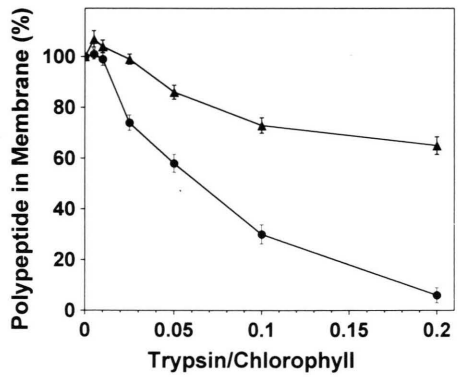

Fig. 2. Relative sensitivity of the Rieske protein (▲) and the 16 kDa OEC extrinsic protein (●) to trypsin added from the stromal side. Conditions as in Fig. 1. Relative protein concentrations were measured by densitometric analysis of the Western blots. The sensitivity is expressed as percentage of the control sample. It was obtained as the mean of three different experiments. The standard deviations are shown.

uct at $M \approx 16{,}000$ (Fig. 1 A, lanes c–g) which subsequently was degraded.

Extraction of the Rieske protein from the thylakoid membrane

The effect of salts, chaotropic agents and high pH (0.1 N NaOH) on the association of the iron–sulfur protein with the thylakoid membrane was tested in order to determine whether the Rieske protein of the cytochrome b_6f complex is an intrinsic membrane protein or peripheral. At high pH (13), at 6 M urea and at 2 M NaSCN, the iron–sulfur protein was released from the thylakoid membrane (Fig. 3 A) and appeared quantitatively in the supernatant (Fig. 3 A, lanes g–i). 2 M NaCl and 2 M $CaCl_2$ were ineffective in releasing the Rieske protein from intact thylakoid membranes (Fig. 3 A) and from Triton permeabilized thylakoid membranes (not shown).

The cytochrome *f* topography is well established. The protein is held in the thylakoid membrane by a single transmembrane span (Gray, 1992). A similar topography has been suggested for the Rieske protein (Harnisch *et al.*, 1985; Stepphuhn *et al.*, 1987). In all cases tested no extraction of cytochrome *f* from pea thylakoids could be observed (Fig. 3 B). These data imply a different mode of association to the membrane for the two proteins.

The alkaline extraction of pea iron–sulfur protein was examined in more detail. Thylakoid membranes were resuspended in 10 mM Caps buffer, pH 10.0, 10.5, 11.0, 11.5, and 12.0, incubated for 30 min on ice and centrifuged. The sediments were washed once with distilled water and analyzed by Western blotting. The Rieske protein was particularly resistant to alkaline extraction, only at pH 12.0 the protein was almost completely extracted. The pH value at which 50% of the iron–sulfur protein were lost from the thylakoid membrane was pH 11.3 (Fig. 8). These data concerning the release of the Rieske protein from higher plant thylakoids at alkaline pH are consistent with existing results (Szczepaniak *et al.*, 1991).

A similar experiment was made using NaSCN as dissociating medium. The release of the iron–sulfur protein from intact thylakoids and from detergent permeabilized thylakoids at different

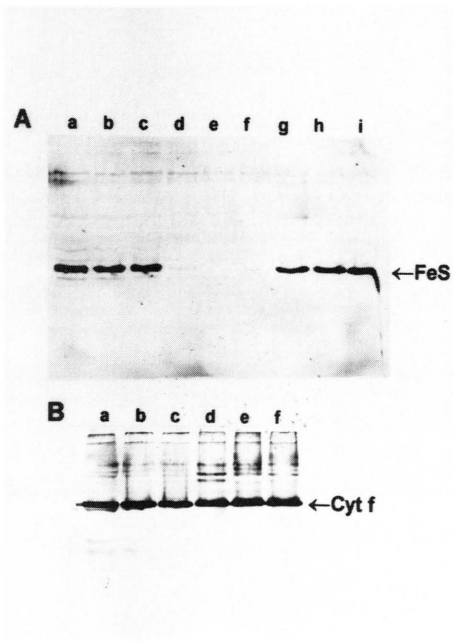

Fig. 3. Western blot analysis of the extraction of proteins from pea thylakoid membranes by dissociating treatment. Lane a, 50 mM Hepes, pH 7.5, only; lane b, extraction with 2 M NaCl; lane c, with 2 M $CaCl_2$; lane d, with 2 M NaSCN; lane e, with 6 M urea; lane f, with 0.1 N NaOH; lane g, supernatant from thylakoids extracted with 2 M NaSCN; lane h, supernatant from thylakoids extracted with 6 M urea; lane i, supernatant from thylakoids extracted with 0.1 N NaOH. Samples (equivalents of 10 µg Chl) were subjected to SDS-PAGE and analyzed by Western blotting using polyclonal antibodies against (A) the Rieske iron–sulfur protein (FeS) and (B) cytochrome *f* (Cyt *f*). Other conditions as under "Materials and Methods".

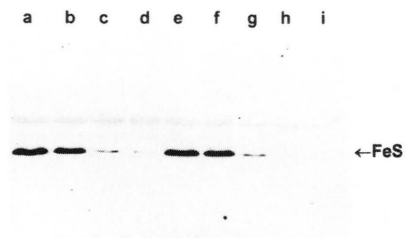

Fig. 4. Western blot analysis of the Rieske iron–sulfur protein extraction by different concentrations of NaSCN from intact pea thylakoid membranes (lanes a–d) and Triton X-100 permeabilized thylakoid membranes (lanes e–i). Intact thylakoids were extracted with 0, 1.0, 1.5 and 2 M NaSCN (lanes a, b, c, d, respectively). Triton permeabilized thylakoids were extracted with 0, 0.25, 0.75 and 1 M NaSCN (lanes e, f, g, h, i, respectively). Other conditions as in Fig. 3 and under "Materials and Methods".

concentrations of NaSCN was tested (Fig. 4). In intact thylakoids the Rieske protein was completely released at a concentration of 1.5 M. The concentration at which 50% of the protein were lost from the thylakoids was 0.8 M (Figs 4 and 6). After permeabilization of the thylakoid membranes with Triton X-100, the iron–sulfur protein was even more readily released. At a concentration of 0.75 M NaSCN the Rieske protein was released completely. 50% of the protein were released at 0.35 M.

Detergent should not only permeabilize thylakoid membranes but should weaken the hydrophobic protein–protein or protein–lipid interactions. In order to find out why detergent pretreatment facilitates release of the iron–sulfur protein from thylakoids, we introduced the sonication to disrupt the thylakoid membranes. The thylakoids were resuspended in indicated concentrations of NaSCN and subjected to sonication. In this case the release of the Rieske protein was almost not facilitated (Figs 5 and 6). The efficiency of the disruption of thylakoids by sonication was checked by the liberation of plastocyanin. After sonication and centrifugation, Western blots show only 20% of plastocyanin in thylakoid membranes

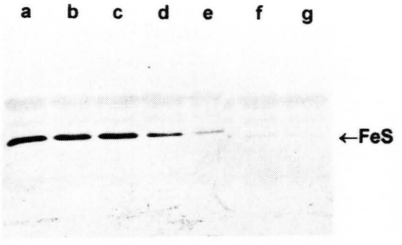

Fig. 5. Extraction of the Rieske iron–sulfur protein from sonicated thylakoid membranes by different concentrations of NaSCN. Thylakoids were incubated with NaSCN (0, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 M, lanes a, b, c, d, e, f, g, respectively) on ice, subjected to sonication 4×30 s with 60 s intervals by a small probe sonifier. After centrifugation the Rieske protein was detected by Western blotting. Other conditions as under "Materials and Methods".

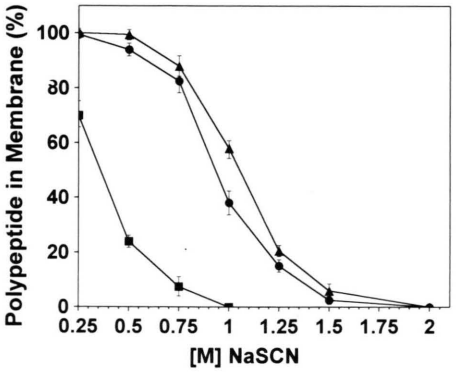

Fig. 6. Graph of relative extraction of the Rieske protein by NaSCN from (▲) intact thylakoids, from (■) Triton X-100 permeabilized thylakoids and from (●) sonicated thylakoids derived from data similar to those shown in Figs 5 and 6. Other conditions as in Fig. 2. The points represent the means of three different experiments; standard deviations resulting from the average of three measurements are shown.

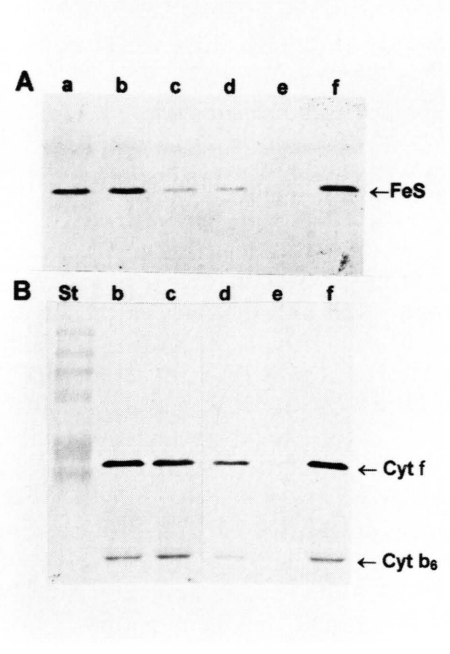

Fig. 7. Western blot analysis of the polypeptides of the cytochrome b_6f complex extracted from pea thylakoid membranes by Triton X-100. (A) Thylakoids (1 mg of Chl/ml) were incubated with different concentrations of Triton X-100 (0.025, 0.05, 0.1, 0.2, 0.5%, lanes a, b, c, d, e, respectively) for 30 min on ice. After centrifugation (30,000×*g*, 1 h), sediments were washed once with distilled water. Lane f, control not treated with Triton. Samples (equivalent of 10 μg Chl) were subjected to SDS-PAGE and analyzed by immunodetection using polyclonal antibodies against the Rieske protein (FeS). (B) Conditions as in (A) except that for the Western blot antibodies against cytochrome *f* (Cyt *f*) and cytochrome b_6 (Cyt b_6) were used. Lane St, prestained molecular weight standard (Sigma).

in comparison to not sonicated thylakoids (not shown).

Differential solubilization of the cytochrome b_6f complex polypeptides from thylakoid membranes by detergents

The effect of different concentrations of Triton (Fig. 7) and cholate (not shown) on the association of polypeptides of the cytochrome b_6f complex to the thylakoid membrane was tested. The Rieske protein was most readily released from the thylakoid membrane. Most of the protein was released at a chlorophyll/Triton ratio of 1/1 (Fig. 7A). This conditions were ineffective in releasing other subunits of the cytochrome b_6f complex from pea thylakoids. Cyt f and Cyt b_6 were released at the same detergent concentration at a chlorophyll/Triton ratio of 1:5 (Fig. 7B).

Extraction of the Rieske protein from yeast mitochondria

The effect of high alkaline pH (>10) on the association of the Rieske protein with the mitochondrial membrane was tested. At pH values >11, the iron–sulfur protein was released completely and appeared quantitatively in the supernatants (not shown). The pH value at which 50% of the Rieske protein were lost from mitochondrial membranes was 10.8 (Fig. 8). Alkaline pH extracted the iron–sulfur protein from mitochondrial membranes a little easier than from thylakoids. It should be noted that the difference between pH values at which 50% of the protein were released was only 0.5 (Fig. 8).

The extraction of the Rieske protein by NaSCN from mitochondria and detergent permeabilized mitochondria (Fig. 9) was very similar to that of pea thylakoids (Fig. 4). In both cases at 1.5 M NaSCN the iron–sulfur protein was released from the intact membrane. After permeabilization with detergent these values are very close: 0.5 M for thylakoids and 0.75 M for mitochondria.

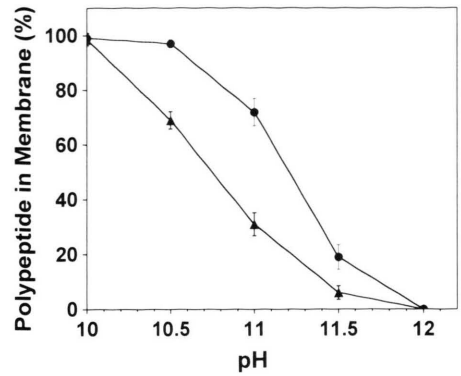

Fig. 8. Graph of relative alkaline extraction of the Rieske iron–sulfur protein from thylakoid membranes of pea and from mitochondrial membranes of yeast derived from densitometric analysis of the Western blots (not shown). Conditions as in Fig. 2 and under "Materials and Methods". The points represent the means of five different experiments. (●) Rieske protein from thylakoids, (▲) Rieske protein from mitochondria; standard deviations resulting from the average of five measurements are shown.

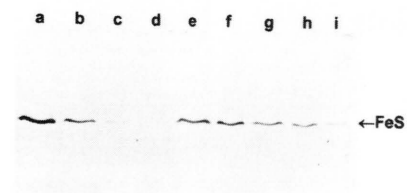

Fig. 9. Western blot analysis of the iron–sulfur protein extraction by different concentrations of NaSCN from yeast mitochondrial membranes (lanes a–d) and from Triton X-100 permeabilized yeast mitochondrial membranes (lanes e–i). Mitochondrial membranes were extracted with 0, 1.0, 1.5 and 2 M NaSCN (lanes a, b, c, d), Triton permeabilized mitochondrial membranes were extracted with 0, 0.25, 0.5, 0.75 and 1.0 M NaSCN (lanes e, f, g, h, i). Other conditions as under "Materials and Methods".

Discussion

There is a general agreement regarding the location of the iron–sulfur cluster to an extramembrane, COOH-terminal domain of the protein, located in the luminal compartment. The mode of association of the Rieske protein with the membrane has been controversial for years with regard to both the cytochrome b_6f and the cytochrome bc_1 complexes. Models with 0, 1 or 2 NH_2-terminal transmembrane α-helices have been proposed (Harnisch *et al.*, 1985; Schägger *et al.*, 1987; Steppuhn *et al.*, 1987; Willey and Gray, 1988; Hartl *et al.*, 1989; Breyton *et al.*, 1994).

The use of trypsin does not allow a conclusion about transmembrane helices. It shows only that the Rieske protein has all trypsin sites on the

lumen side of the thylakoid membrane. Treatment of biological membranes with high concentrations of salt is frequently used for the release of electrostatically bound components as many peripheral polypeptides. Washing of thylakoids or detergent permeabilized thylakoids (not shown) with salt is ineffective in releasing the Rieske protein from the membrane, implying that this protein is not associated with the thylakoid membrane by electrostatic interactions.

Alkaline extraction of the Rieske protein in various experimental systems has yielded conflicting results. Alkaline extraction (100 mM Ca_2CO_3, pH 11.5) released 80% of the Rieske protein from the mitochondrial membrane of *Neurospora crassa* (Hartl *et al.*, 1986). Slightly higher pH (100 mM Na_2CO_3, pH 12.0) removes only one-third of the Rieske protein from chromatophores of *R. sphaeroides* (Van Doren *et al.*, 1993). Washing of the pea thylakoids with Caps buffer removed the Rieske protein at pH values above 11.5. This is consistent with results on spinach (Szczepaniak *et al.*, 1991) and *Chlamydomonas reinhardtii* (Breyton *et al.*, 1994). A little more effective is Caps buffer in removing the Rieske protein from mitochondrial membranes of yeast. The pH value at which 50% of the Rieske protein were lost from mitochondrial membranes was 10.8 and is slightly lower than that for pea (pH 11.3). This value was 11.1 for the iron–sulfur protein of chromatophores from *Rhodospirillum rubrum* (Szczepaniak, in preparation). The rather small differences in removing of the Rieske protein from thylakoids, mitochondria and chromatophores imply a similar mode of association of this protein with the membrane. Protein extraction at extreme alkaline pH is a consequence of increased Coulomb repulsion. Increasing the pH causes an increase of negative net charge in the peripheral segments of the protein and in consequence extrusion of the protein from the membrane.

Chaotropic agents are known to disrupt protein/protein hydrophobic interactions and not to extract intrinsic proteins (Tanner, 1979). The Rieske protein was extracted by a chaotropic agent (1.5 M NaSCN) from thylakoids and mitochondria. After pre-treatment of membranes with Triton or cholate (0.5%, not shown) this protein was removed at much lower concentrations of thiocyanate. Because the ultrasonication does not facilitate release of the iron–sulfur protein from thylakoids by thiocyanate, we conclude that detergent facilitates the removal of the Rieske protein rather by a weakening of the hydrophobic interactions of the Rieske protein with the complex and/or the membrane than by permeabilization of the thylakoids. This result suggests that the hydrophobic interactions are important for the association of this protein with the complex/or the membrane. The effect of detergent on the association of the Rieske protein with thylakoid membranes confirms this conclusion. The Rieske protein is the first one to be released by Triton from thylakoids at relatively low concentrations (above 0.05% Triton at the experimental conditions used). The Cyt *f*, Cyt b_6 and subunit IV (not shown) were removed at higher (0.5%) concentrations of Triton, most probably as a subcomplex. The concentration which would release the Rieske protein was different depending on the growth conditions of the plants. It is higher (0.2% Triton) for field plants and lower for plants grown in a phytotron.

In conclusion, the evidence presented in this paper strongly suggests that the Rieske iron–sulfur protein from cytochrome b_6f complex of pea and from cytochrome bc_1 complex of yeast is an extrinsic protein, although its association with the complex and/or the membrane involves primarily hydrophobic interactions. This is in agreement with previously published data regarding the Rieske protein from mitochondria (Hartl *et al.*, 1986; Hartl *et al.*, 1989; Gonzales-Halphen *et al.*, 1991) and from thylakoids from *Chlamydomonas reinhardtii* (Bryton *et al.*, 1994).

Acknowledgements

This research was supported by grants from the State Committee for Scientific Research (project No. 6 6263 9 2 03) to A. S. and from the Deutsche Forschungsgemeinschaft.

Black M. T., Widger W. R. and Cramer W. A. (1987), Large-scale purification of active cytochrome b_6f complex from spinach chloroplast. Arch. Biochem. Biophys. **252,** 655–661.

Breyton C., de Vitry C. and Popot L.-J. (1994), Membrane association of cytochrome b_6f subunits. The Rieske iron–sulfur protein from *Chlamydomonas reinhardtii* is an extrinsic protein. J. Biol. Chem. **269,** 7597–7602.

Clejan L. and Beattie D. S. (1986), Preparation of complex III from yeast mitochondria and related methodology. In: Meth. Enzymol. **126,** pp. 173–180.

Cramer W. A., Black M. T., Widger W. R. and Girvin M. E. (1987), Structure and function of photosynthetic bc_1 and b_6f complexes. In: The Light Reactions (J. Barber, ed.). Elsevier, Amsterdam, pp. 447–493.

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

Gray, J. C. (1992), Cytochrome *f:* Structure, function and biosynthesis. Photosynth. Res. **34,** 359–374.

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.

Knaff D. B. (1993), The cytochrome bc_1 complexes of photosynthetic purple bacteria. Photosynth. Res. **35,** 117–133.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Ljungdahl P. O., Pennoyer J. D., Robertson D. E. and Trumpower B. L. (1987), Purification of highly active cytochrome bc_1 complex from phylogenetically diverse species by a single chromatographic procedure. Biochim. Biophys. Acta **891,** 227–241.

Malkin R. (1992), Cytochrome bc_1 and b_6f complexes of photosynthetic membranes. Photosynth. Res. **33,** 121–136.

Pedersen P. L. and Hullihen J. (1979), Resolution and reconstruction of ATP synthesis and ATP-dependent functions of liver mitochondria. In: Meth. Enzymol. **55,** pp. 736–741.

Rieske J. S. (1976), Composition, structure and function of complex III. Biochim. Biophys. Acta **456,** 195–247.

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.

Stepphuhn 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., Huang D., Keenan T. W. and Cramer W. A. (1991), Electrostatic destabilization of the cytochrome b_6f complex in the thylakoid membrane. EMBO J. **10,** 2757–2764.

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

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

Van Doren S. R., Yun C.-H., Crofts A. R. and Gennis R. B. (1993), Assembly of the Rieske iron–sulfur subunit of the cytochrome bc_1 complex in the *Escherichia coli* and *Rhodobacter spheroides* membranes independent of the cytochrome *b* and c_1 subunits. Biochemistry **32,** 628–636.

Widger W. R. and Cramer W. A. (1991), The cytochrome b_6f complex. In: Cell Culture and Somatic Cell Genetic of Plants **7b** (L. Bogorad and I. K. Vasil, eds.). Academic Press, London, pp. 149–176.

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