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Protein Expression and Purification 29 (2003) 8–14

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## Overexpression and reconstitution of a Rieske iron–sulfur protein from the higher plant

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Received 1 August 2002, and in revised form 31 December 2002

### Abstract

The iron–sulfur protein subunit, known as the Rieske protein, is one of the central components of the cytochrome *b<sub>6</sub>f* complex residing in chloroplast and cyanobacterial thylakoid membranes. We have constructed plasmids for overexpression in *Escherichia coli* of full-length and truncated Rieske (PetC) proteins from the *Spinacia oleracea* fused to MalE. Overexpressed fusion proteins were predominantly found (from 55 to 70%) in cytoplasm in a soluble form. The single affinity chromatography step (amylose resin) was used to purify about 15 mg of protein from 1 liter of *E. coli* culture. The isolated proteins were electrophoretically pure and could be used for further experiments. The NifS-like protein IscS from the cyanobacterium *Synechocystis* PCC 6803 mediates the incorporation of 2Fe–2S clusters into apoferritin and cyanobacterial Rieske apoprotein in vitro. Here, we used the recombinant IscS protein for the enzymatic reconstitution of the iron–sulfur cluster into full-length Rieske fusion and truncated Rieske fused proteins. Characterization by EPR spectroscopy of the reconstituted proteins demonstrated the presence of a 2Fe–2S cluster in both full-length and truncated Rieske fusion proteins.

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The Rieske iron–sulfur proteins are a unique group of membrane residing polypeptides, which contain 2Fe–2S clusters. They are known as essential components of cytochrome *bc<sub>1</sub>* and cytochrome *b<sub>6</sub>f* complexes in both prokaryotic and eukaryotic cells and play an important role in the electron transport chain within these complexes; in detail, they mediate the electron transfer between a quinol and a *c*-type cytochrome (cytochrome *c<sub>1</sub>* or cytochrome *f*) [1,3]. The Rieske protein consists of an N-terminal transmembrane helix and a C-terminal soluble domain on the P-side of the membrane. The three-

dimensional structure of the hydrophilic part of Rieske protein was solved at atomic resolution: 1.8 and 1.5 Å for protein from the cyt. *b<sub>6</sub>f* and the cyt. *bc<sub>1</sub>* complex, respectively [1,2]. The structure of the soluble fragments reveals two domains: a small “cluster binding” subdomain and a large scaffold subdomain. In contrast to most 2Fe–2S cluster containing proteins, the Rieske proteins have an unusual high midpoint potential of +105 to +305 mV [4,5], which can partially be explained by the presence of two histidines replacing two of the four cysteine residues normally found in the coordination sphere of Fe–S clusters [6]. Because of their vital role in energy generation, there is considerable interest in determining how Rieske proteins bind their essential iron–sulfur cofactors.

The Fe–S proteins appeared at an early stage of evolution and are present in cells of virtually all species. Biosynthesis of these proteins is a complex process involving numerous compounds [7]. Initial evidence indicating the involvement of specific gene products for post-translational formation of protein-bound Fe–S clusters was obtained by genetic analysis of the major

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<sup>1</sup> Abbreviations used: BCA, biconchonic acid; BSA, bovine serum albumin; cyt. *b<sub>6</sub>f*, cytochrome *b<sub>6</sub>f* complex; cyt. *bc<sub>1</sub>*, cytochrome *bc<sub>1</sub>* complex; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; MBP, maltose-binding protein; MBP-R420, N-terminally truncated Rieske protein fused to maltose binding protein; MBP-R540, full-length Rieske protein fused to maltose binding protein; OG, *N*-octyl β-D-glucopyranoside.

nitrogen fixing (*nif*) gene cluster of the nitrogen-fixing bacterium *Azotobacter vinelandii* [8,9]. More recently, similar gene clusters involved in the assembly of Fe–S centers have also been identified in *Escherichia coli* [10].

Among these gene products, the *nifS* gene product was the first identified enzyme to be involved in Fe–S cluster biosynthesis. Biochemical analysis showed that NifS protein exhibits a cysteine desulfurase activity converting cysteine into alanine and enzyme bound sulfur [11,12]. Subsequently, it was shown that in the presence of ferrous iron, mobilized sulfur can be used for Fe–S cluster synthesis at nitrogenase Fe protein in vitro [13]. The NifS-mediated assembly of Fe–S clusters, has in the meantime, become a routine method for the reconstitution of Fe–S clusters as proteins other than nitrogenase [14,15]. For instance, an NifS-like protein from the cyanobacterium *Synechocystis* PCC 6803 was recently reported to mediate the incorporation of the Fe–S clusters into apoferrredoxin [16] and cyanobacterial Rieske apoprotein in vitro [17].

Here, we report the overexpression of full-length and the N-terminally truncated Rieske apoprotein from *Spinacia oleracea* as fusion proteins with the maltose-binding protein. These fusion proteins were purified by affinity chromatography and the Fe–S cluster was enzymatically reconstituted.

## Materials and methods

### Construction of the expression plasmids

The coding regions for full-length spinach as well as truncated (amino acid residues 43–179) Rieske proteins were amplified by PCR from cDNA, which encodes the entire precursor. The cDNA was a generous gift from R.G. Herrmann. The direct primers 5'-CTGACAGAA TTCGCTACTAGCATTCCAGCT for full-length protein or 5'-CGGAATTCATGCCTCCTGGTGGAGGA GCTGGC for truncated protein carrying *EcoRI* sites (underlined) and the reverse primer 5'-ACAGAAGCTT ATGCAGACCACCATGGTGC carrying *HindIII* (underlined) were designed according to the known sequence of the *petC* cDNA [18]. The PCR products were cleaved by *EcoRI* and *HindIII* (New England Biolabs) and purified using a purification kit (Qiagen). The purified products were cloned into the *EcoRI* and *HindIII* sites of the expression vectors pMal-cRI (New England Biolabs) by a standard T4 DNA ligase procedure, resulting in plasmids for the expression of target gene products as fusion proteins [polypeptides fused to the C-terminus of the maltose-binding protein (MBP)]. The resulting plasmids, designed pMal-cRI-R540 (encoding the full-length Rieske protein) and pMal-cRI-R420 (encoding the truncated Rieske protein including car-

boxy-terminal domain from 43 to 179 residue), were checked by restricted analysis and verified by DNA sequencing. As the plasmids pMal-cRI-R540 and pMal-cRI-R420 are devoid of a signal sequence, the expressed fusion protein should remain in the cytoplasm.

### Protein expression and purification

*Escherichia coli* strain TB1 cells transformed with pMal-cRI-R540 or pMal-cRI-R420 plasmids were grown from overnight cultures in Luria growth medium containing 80 µg ampicillin to mid-log phase, induced by the addition of 0.5 mM IPTG, and harvested after 3.0 h. The cells were lysed through freeze–thawing, followed by sonication in 20 mM Tris–buffer, pH 7.4 (containing 200 mM NaCl, 1 mM EDTA, and 1 mM PMSF), and centrifuged for 20 min at 12,000g. The supernatants were used for purification of the soluble fusion proteins (MBP-R540 and MBP-R420) by affinity column chromatography (amylose resin, New England Biolabs). The column (2 × 3.5 cm) was equilibrated with 20 mM Tris–buffer, pH 7.4 (containing 200 mM NaCl, 1 mM EDTA) and the protein was eluted with 10 mM maltose (solubilized in the same buffer), followed by concentration with Centricon-10 ultrafiltration units (Amicon).

### Determination of the subcellular localization of the expressed proteins

Periplasm, inclusion bodies, membrane fraction, and cytoplasmic fraction were isolated from induced *E. coli* cells as in [19]. In detail 50 ml induced cell cultures containing expression plasmids pMal-cRI-R540 or pMAL-cRI-R420 were harvested by centrifugation at 4000g for 10 min at 4°C. Cells were resuspended in 12.5 ml of a Tsu buffer (30 mM Tris, pH 8.0, containing 20% saccharose) and EDTA was added to 1 mM. The suspension was gently shaken for 5–10 min at RT and centrifuged at 8000g for 10 min at 4°C. Harvested cells were resuspended in a cool (4°C) 5 mM MgSO<sub>4</sub> solution, gently shaken for 10 min at 4°C, and centrifuged as described above. The supernatant was preserved for further analysis (periplasm). The pellet was washed in a TNEP buffer (50 mM Tris, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 1 mM PMSF) and then resuspended in 3.75 ml TNEP buffer and cells were broken by seven 30 s cycles of sonication when within 1 min a break in the ice occurred. Sonicated suspension was cleared by centrifugation at 27,000g for 15 min at 4°C. The pellet was preserved for further analysis (inclusion bodies). The retained supernatant was centrifuged again at 140,000g for 2.5 h at 4°C. The obtained pellet (membrane fraction) and the supernatant (cytoplasmic fraction) were preserved for further analysis.

### Refolding of the Rieske protein and reconstitution of the iron–sulfur center

Reconstitution of the Rieske protein was done as described by Schneider et al. [17] with some modifications. In detail, the overexpressed and purified protein was dialyzed for 15 h against a 100-fold volume of 20 mM Tris–HCl buffer, pH 8.0, at 4 °C and then used for reconstitution; alternatively, the protein was denatured and refolded before reconstitution. Refolding of the protein was achieved in two ways: (a) The protein was unfolded by the addition of 6 M urea and incubated for 30 min. in RT. Refolding was achieved by an 8-fold dilution in 20 mM Tris–HCl buffer, pH 8.0, in RT. (b) The unfolded protein was dialyzed for 36 h in six steps against 50-fold volume of 20 mM Tris–HCl buffer, pH 8.0, containing decreasing concentrations of urea (from 6 to 4 M in RT, from 3 to 0 M in 4 °C).

To reconstitute the Fe–S cluster, a solution of the fused protein (0.6–1.6 mg/ml in 20 mM Tris–HCl buffer, pH 8.0) was sparged with argon for 10 min in RT while DTT was added to 2.5 mM and pyridoxal phosphate to 10  $\mu$ M. With further sparging for 5 min, 1 mM L-cysteine, 2 mM  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ , and 6  $\mu$ g IscS [16] were added and the solution was incubated for 2 h at 30 °C. The reaction was terminated by the addition of 10 mM EDTA and the reconstitution mixture was dialyzed against a 20 mM Tris–HCl buffer, pH 8.0, in 4 °C (containing 1 mM EDTA, 1 mM *o*-phenanthroline, and 1 mM OG). Samples were concentrated by Centricon-30 ultrafiltration units (Amicon) to a final volume of 0.3 ml. The Fe–S cluster incorporation was analyzed by EPR spectroscopy.

### EPR spectroscopy

EPR spectra were recorded at the Section de Bioenergetique, CEA-Saclay, France, on a Bruker EPR200 spectrometer. Sample temperature of 15 K was maintained by a liquid helium cryostat (Oxford Instruments). Other parameters for the measurement include 6.3 mW microwave power, 10 G modulation amplitude and 9.44 GHz microwave frequency. Measurements include 3–4 scans. The samples of reconstituted proteins in 20 mM Tris, pH 8.0, containing 1 mM OG were reduced by adding DTT or sodium ascorbate. The concentration of protein in samples was 1.2–2 mg/150  $\mu$ l.

### Measurements of protein concentration

MBP concentration was determined directly from the absorbance at 280 nm with an extinction coefficient of  $\epsilon = 68.750 \text{ M}^{-1} \text{ cm}^{-1}$  [20]. The concentration of the fusion proteins was determined using the BCA reagent (Sigma) according to manufacturer's instructions with BSA as a standard.

### SDS–PAGE and Western blot

Proteins were separated on a 15% acrylamide gel. Western blotting was carried out as in [21]. The polyclonal antibody against spinach Rieske protein was generated in rabbits as in [22]. In details the cytochrome *b<sub>6</sub>f* isolated from spinach chloroplast [23] was subjected to preparative gel electrophoresis. After a short (1 h) staining period, the band of interest (Rieske protein) was cut out of the gel and protein was electroeluted using the Laemmli system buffer. The preparation routinely yielded a single protein band in the region of interest. The Rieske protein was mixed with an equal volume of Freund's complete adjuvant. One milliliter (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. Rabbit anti-MBP antiserum was purchased from New England BioLabs.

## Results

### Production and purification of the Rieske fusion proteins

Plasmids for the production of full-length and truncated spinach Rieske protein were generated by PCR-amplification of the *petC* coding regions; the resulting 540 and 420 bp DNA fragments were cloned into the expression vector pMal-cRI and sequenced. Constructed plasmids pMal-cRI-R540 and pMal-cRI-420 (Fig. 1) were used for overproduction of the spinach full-length or truncated Rieske protein fused to a maltose binding protein designed as MBP-R540 and MBP-R420, respectively. Induction of the plasmid pMal-cRI-R540 in *E. coli* TB1 led to the overproduction of a protein of expected size ~63 kDa (not shown) that cross-reacted with antibodies against the Rieske proteins (Fig. 2, panel B, line 1) and the MBP (not shown). Induction of the plasmid pMal cRI-R420 led to the overproduction of a protein of expected size ~58 kDa (not shown) that cross-reacted with an antibody against the Rieske protein (Fig. 2, panel A, lane 1) and the MBP (not shown). The expressed proteins represent ~30% (MBP-R540) or 20% (MBP-R420) of the total cellular protein, as estimated from gel scans of Coomassie blue stained gels.

To determine the subcellular localization of the expressed proteins, periplasm, cytoplasm, inclusion bodies, and membranes were isolated from induced cells. The protein content of these fractions was investigated by Western blotting (Figs. 2A and B). Almost 70% of the MBP-R420 was found in a soluble form in cytoplasm, 15% in inclusion bodies, and 15% bound to the cytoplasmic membrane. In the case of MBP-R540

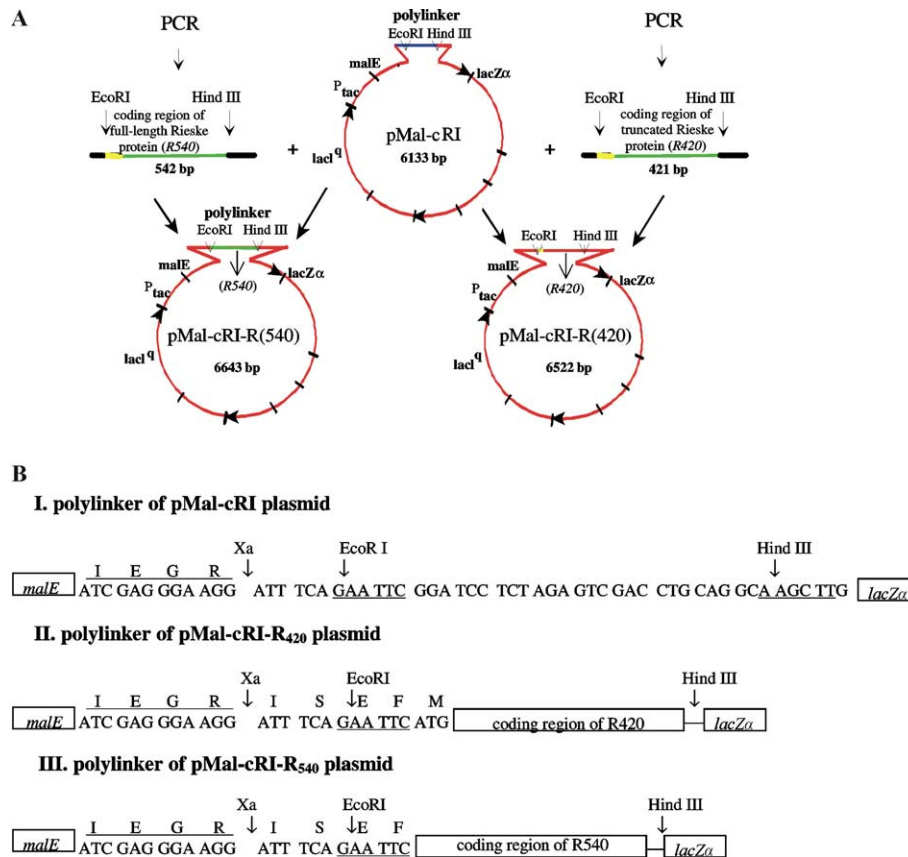


Fig. 1. (A) Construction of the expression vectors for overproduction of MBP-R540 i MBP-R420 fusion proteins. PCR-based strategy was employed to subclone the *R540* or *R420* coding regions into pMal-cRI plasmid. The sequences of primers used for PCR amplification of *R540* and *R420* are given in the text. The restriction sites *EcoRI* and *HindIII* were introduced on either side of coding regions to facilitate subsequent subcloning. The resulted expression vectors were described as pMal-cRI-R540 and pMal-cRI-R420. (B) A schematic illustration of polylinkers of the vectors used in the experiments. I—polylinker of pMal-cRI plasmid. Restriction sites *EcoRI*, *HindIII*, and *Xa* cleavage site are indicated by arrows; II—polylinker of pMal-cRI-R420 plasmid. The *R420* copy generated by PCR was cloned behind the *malE* coding region and simultaneously five additional codons were introduced between them; and III—polylinker of pMal-cRI-R540 plasmid. The *R540* copy generated by PCR was cloned as in II simultaneously four additional codons were introduced between them. For details see in the text.

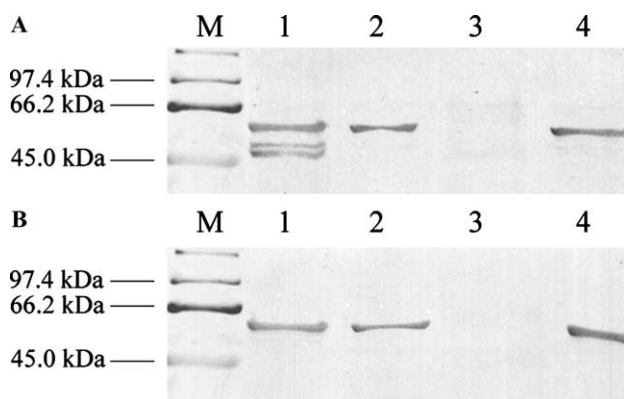


Fig. 2. Western blot analysis of isolated cell subfractions from induced cells. (A) Cells overexpressed MBP-R420. Lane M—molecular mass standard; lane 1—cytoplasmic fraction; lane 2—inclusion bodies; lane 3—periplasmic fraction; and lane 4—membrane fraction. (B) Cells overexpressed MBP-R540. Description as in (A). Lanes 1 and 2 contain the equivalent of 50  $\mu$ l culture cells; lane 3 contains the equivalent of 0.5 ml culture cells; and lane 4 contains the equivalent of 100  $\mu$ l culture cells. For immunodetection, polyclonal antibody against spinach Rieske protein was used.

protein, a substantial amount ( $\sim 30\%$ ) was bound to the cytoplasmic membrane, almost 55% was found in a soluble form in the cytoplasm, and the remainder (15%) in inclusion bodies. In both cases, no fusion protein was detected in the periplasmic protein fraction (Figs. 2A and B, lanes 3).

To purify the expressed proteins, the *E. coli* cells were broken by sonication and the released cytoplasmic proteins were purified by a single affinity column step (amylose resin). One liter of *E. coli* culture yields about 15 mg MBP-R540 or MBP-R420. The isolated proteins were electrophoretically pure (Fig. 3).

#### Reconstitution of the 2Fe–2S center

As MBR-R540 and MBP-R420 did not yield the characteristic EPR spectrum, we tried instead in vitro reconstitution of the iron–sulfur center with  $\text{Fe}^{2+}$ , cysteine, and the *Synechocystis* IscS protein (see Materials and methods). In the reconstitution experiments samples prepared in different ways were used: (i) isolated by

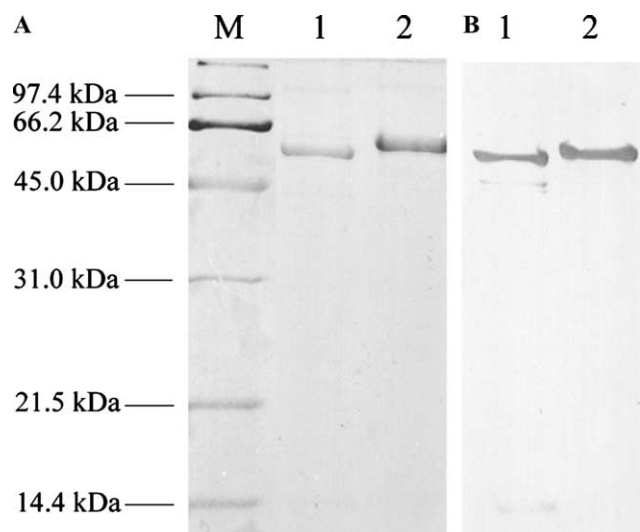


Fig. 3. Analysis of purified on amylose column fusion proteins. (A) SDS-PAGE. Lane M—molecular mass standard; lane 1—MBP-R420 ( $\approx 58$  kDa); lane 2—MBP-R540 ( $\approx 63$  kDa). (B) Western blotting. Description as in (A). For immunodetection, polyclonal antibody against spinach Rieske protein was used.

affinity chromatography, (ii) isolated by affinity chromatography, denatured, and refolded by dilution, and (iii) isolated by affinity chromatography, denatured, and refolded by dialysis. Only the last approach allowed assembly of the Rieske 2Fe–2S cluster into the spinach Rieske protein, as demonstrated by the intense EPR signals at  $g_y = 1.89$  and  $g_z = 2.03$  observed at 15 K (Figs. 4A and B). The similarity of this spectrum with EPR spectra obtained from the isolated cytochrome  $b_6f$  complex and other Rieske proteins [6] strongly suggests that the overproduced Rieske proteins were correctly refolded and that functional iron–sulfur clusters were incorporated. However, both full-length and truncated fusion proteins showed a significant difference in the amount of reconstituted iron–sulfur clusters.

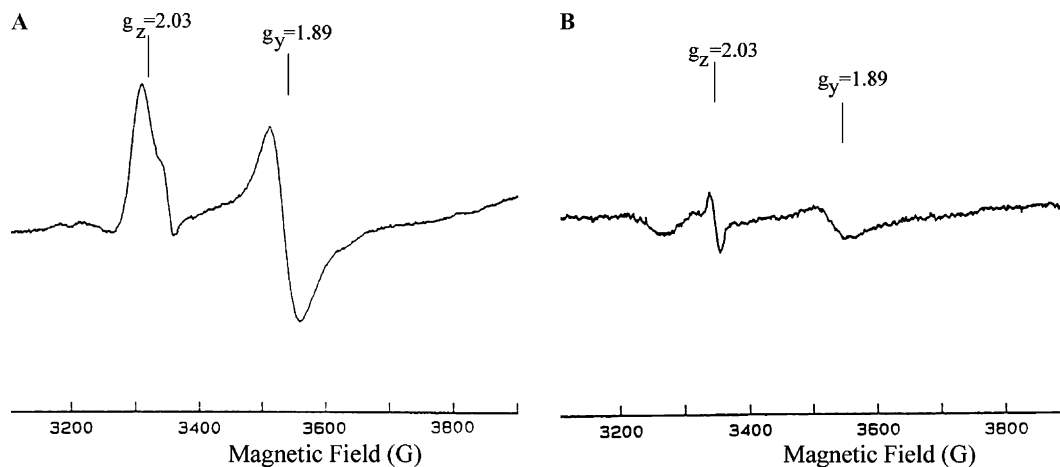


Fig. 4. EPR spectra of the reconstituted fused Rieske proteins. (A) MBP-R540; (B) MBP-R420. For experimental conditions see Materials and methods.

## Discussion

We have reported here the overproduction of a full-length and a truncated Rieske protein from the higher plant *S. oleracea*; in both cases, the Rieske protein was fused to the maltose binding protein. The fusion of Rieske proteins to MBP allowed purified fusion proteins in one step affinity of chromatography and kept them in a soluble form. Expression of Rieske proteins from the cyanobacteria *Nostoc* sp. and *Synechocystis* in *E. coli* produced copious quantities of full-length Rieske protein predominantly as inclusion bodies [17,24], which did not show any typical EPR Rieske signal. This indicates that the protein was overexpressed in a denatured form. In contrast, our full-length and truncated spinach Rieske proteins were produced in *E. coli* in soluble forms. However, according to the EPR-measurements both in the MBP-R540 and the MBP-R420 proteins the Rieske 2Fe–2S cluster was still lacking.

Recent progress in the field of molecular biology has made it possible to clone the genes for Fe–S proteins from a wide variety of organisms and highlight them in foreign hosts such as *E. coli*. Genes encoding adrenodoxin and plant-type ferredoxin have been efficiently expressed in *E. coli*, which resulted in high level production of the holoproteins with correctly assembled 2Fe–2S cluster (see for example [26–31]). The production in *E. coli* of active enzymes which require “Rieske type” Fe–S centers is shown [32–34]. A cyanobacterial Rieske protein (*Nostoc* sp.) fused to thioredoxin and expressed in *E. coli* shows the insertion of a 2Fe–2S cluster into this soluble fusion protein in vivo [24]. Recently, it was shown that the iron–sulfur cluster was correctly inserted into a full-length cyanobacterial Rieske protein bound to *E. coli* membranes [25]. Also the Rieske protein II from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* was expressed in *E. coli* [35]. The iron–sulfur cluster was correctly inserted in

vivo into a fraction of full-length protein (strictly bound to the *E. coli* membrane) and much more effectively into a soluble form created by the deletion of 45 N-terminal amino acids [35,36]. Using the same system (as described in [35]) we were unable to detect a 2Fe–2S cluster in full-length and truncated spinach chloroplast Rieske proteins fused to MBP and expressed in *E. coli* (not shown).

The NifS-like protein IscS from *Synechocystis* was recently shown to be able to mediate the incorporation of an iron–sulfur cluster in vitro into apoferritin [16] and a cyanobacterial Rieske apoprotein [17] by the activation of sulfur from a cysteine. We used this approach for the reconstitution of the refolded Rieske protein and showed the successful reconstitution by the characteristic Rieske EPR spectrum. It should be noted that we were unable to obtain reconstituted Rieske proteins from isolated MBP-R540 or MBP-R420 by affinity chromatography when proteins were not refolded or refolded by dilution. Only the refolding of the Rieske fusion proteins by slow dialysis facilitated the formation of a structure, which can incorporate the iron–sulfur cluster. It is important to note that all samples used in the reconstitution experiments yielded a properly folded MBP-moiety of the fused protein, as indicated by binding to the amylose resin (not shown). This implies that all three samples differ only in the structure of the Rieske protein moiety. The data presented in this paper support the conclusion that the fusion proteins isolated by affinity chromatography and refolded by dilution contain misfolded Rieske protein. Only denaturation and refolding by dialysis result in the conformation of the Rieske protein, which is able to incorporate a 2Fe–2S cluster. The inability of *E. coli* to assemble the Rieske center into full-length and truncated spinach Rieske proteins, thus, appears to originate from misfolding rather than from the lack of specific co-factors. The suggestion that the spinach Rieske protein must be in native or near-native conformation to incorporate an appropriate iron–sulfur cluster was supported by chemical reconstitution of Rieske clusters according to Holton et al. [22]. Using this method we were able to reconstitute an iron–sulfur cluster into our fused proteins, refolding only by slow dialysis (in preparation).

A similar observation was made for Rubisco, although large and small subunit polypeptides from higher plants have been synthesized in *E. coli*, an active plant Rubisco could never be obtained [37]. This has been attributed specifically to misfolding of large subunit polypeptides [38,39]. In contrast, the large Rubisco subunit of cyanobacteria (identified at 80% in sequence to higher-plant polypeptides) folds correctly and assembles with the small subunit into an active enzyme in *E. coli* [38]. The inability of *E. coli* to fold Rieske protein is in agreement with the observation that the imported Rieske protein associates with chloroplast specific chaperonins: Cpn60 and Hsp70 in the stroma [40].

In the scope of the present report and literature or data perspective, it seems that Rieske proteins derived from different sources (bacteria, cyanobacteria, and higher plants) require a distinct mechanism of protein path folding.

Analysis of the data presented in this paper and in the literature [24,36] reveals that the hydrophobic N-terminus of the Rieske polypeptide has no general influence on the incorporation of the 2Fe–2S center. The observed significant lower amount of reconstituted iron–sulfur cluster into MBP-R420 (Fig. 4B) is rather related to a steric hindrance between the IscS enzyme and the fused protein.

### Acknowledgments

We thank Dr. A.W. Rutherford for his support with the EPR measurements at CEA-Saclay. This research was supported by a grant from the State Committee for Scientific Research (Grant No. 6 P04 019 13) to A.Sz., by the Deutsche Forschungsgemeinschaft (SFB480, M.R.), and by the Ministry of Sciences and Research of Northrhine-Westfalia (A.S.).

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