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Biochimica et Biophysica Acta 1185 (1994) 239–242

BBA Biochimica et Biophysica Acta

Rapid Report

Evidence for two different Rieske iron-sulfur proteins in the cytochrome *bf* complex of spinach chloroplast

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(Received 31 January 1994)

Abstract

Spinach thylakoids, grana vesicles, stroma lamellae vesicles and also isolated cytochrome *bf* complex, were analysed by two-dimensional polyacrylamide gel electrophoresis employing isoelectric focusing in the first dimension and sodium dodecylsulfate polyacrylamide gel electrophoresis in the second dimension. About 100 thylakoid membrane proteins were resolved. In all cases the Rieske FeS protein separated into two polypeptide spots having the isoelectric points of 5.1 and 5.4, respectively. The Rieske FeS protein was identified by immunoblot analysis and by microsequences of the first 23 N-terminal amino acids. The intensity of the Coomassie brilliant blue stain of the two spots was stronger for the Rieske FeS protein of the grana vesicles than for that of the stroma lamellae vesicles.

Key words: Thylakoid membrane; Iron-sulfur protein; Cytochrome *bf* complex; Rieske iron-sulfur protein; Electrophoresis, two-dimensional; Microsequence

The Rieske iron-sulfur protein is one of four major components of the cytochrome *bf* complex of chloroplasts. This complex functions in linear electron transport between Photosystem II and I and also in cyclic electron transport around Photosystem I. It is a plastoquinol-plastocyanin oxidoreductase and is also involved in proton translocation. However, the role of the Rieske iron-sulfur protein in the cytochrome *bf* complex is still under debate [1–5].

The Rieske FeS protein is encoded by nuclear DNA and synthesized in the cytoplasm, while the other three main polypeptides of the cytochrome *bf* complex, cytochrome *f*, cytochrome *b* and the 17 kDa protein, are encoded by the chloroplast DNA. The Rieske FeS protein may therefore due to its nuclear origin play a key role for the assembly of the cytochrome *bf* complex [6,7].

Rieske FeS protein has been isolated and purified from several plant species, such as spinach, tobacco and pea [8–10]. The isolated Rieske protein from different plant species appears on one-dimensional SDS-PAGE as a unique band with a molecular mass of 20 kDa. Its primary structure has also been partially determined by amino acid sequencing [8] and completely deduced from the cDNA nucleotide sequence in spinach [11]. Recently, two types of cDNA clones in tobacco named TR3 and TR6, were reported [9]. When the amino acid sequences, deduced from the nucleotide sequences of these two cDNAs, were compared it was found that these two amino acid sequences differ in only four amino acid residues. On this basis it was suggested that chloroplast Rieske FeS protein is encoded by two different genes in the tobacco nuclear genome [9].

In this communication we present results, obtained by two-dimensional electrophoretic analysis, which show that two types of Rieske iron-sulfur protein are present in the spinach cytochrome *bf* complex.

Using a modified two-dimensional electrophoresis procedure recently described [12], about 100 spinach thylakoid membrane proteins have been resolved of which approximately 12, directly involved in the photosynthetic process, have been identified [12]. During this study it was observed that there are two well-separated polypeptide spots having apparent molecular mass of

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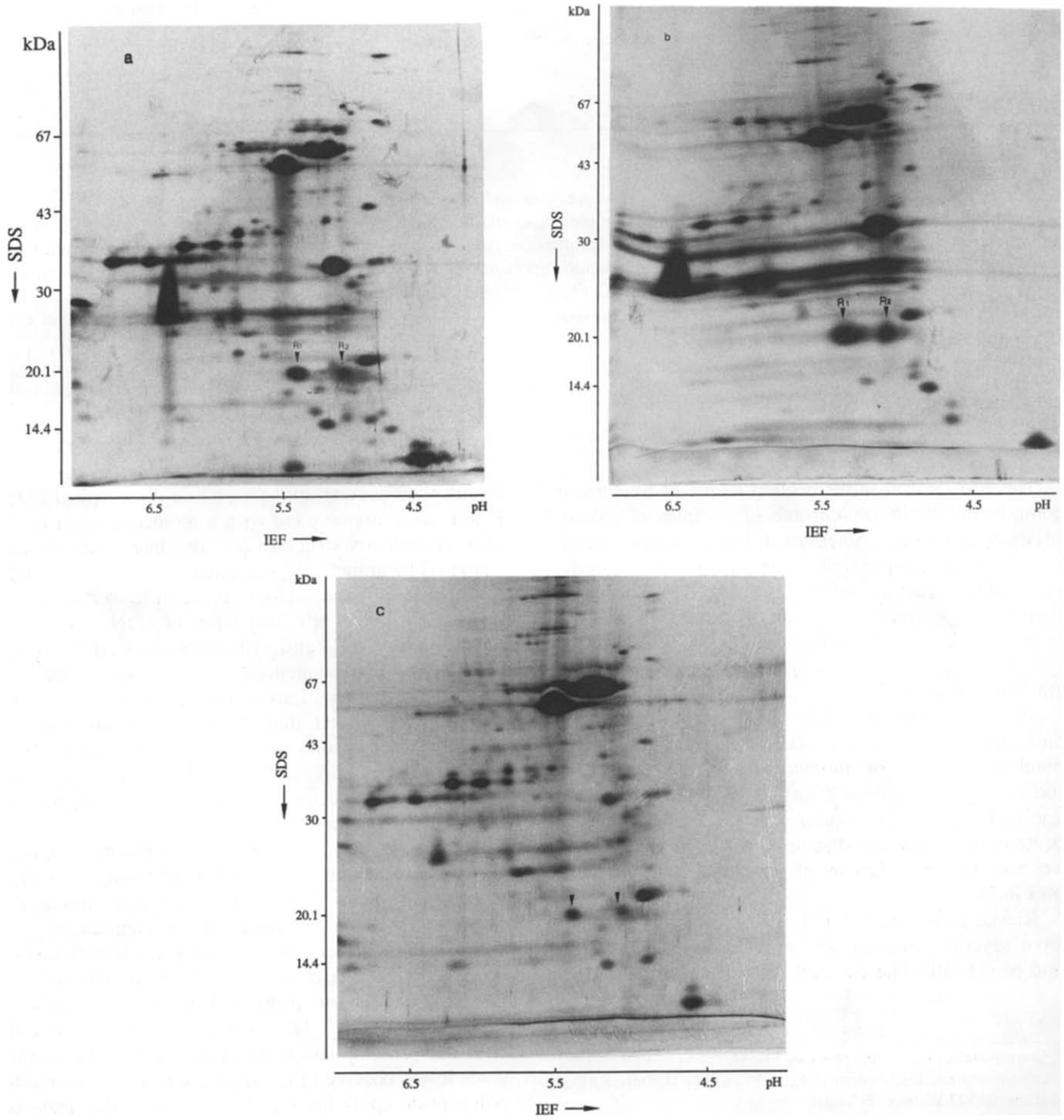
SSDI 0005-2728(94)00019-2

20 kDa and isoelectric points of 5.40 (named R_1) and 5.1 (named R_2), respectively (Fig. 1a). Both spots reacted positively using the western blot procedure with antibodies raised against isolated Rieske FeS protein (Fig. 2).

The two-dimensional electrophoresis was also applied on isolated grana vesicles (Fig. 1b), stroma lamellae vesicles (Fig. 1c) and also on a purified cytochrome *bf* complex (Fig. 3). In all cases the two Rieske FeS spots were found at the same position as with thylakoids. The intensity of the Coomassie brilliant blue stain of the two spots was stronger for the Rieske FeS protein of the grana vesicles than of the stroma lamellae vesicles, in agreement with the distribution of the cytochrome *bf* complex between these two membrane domains [13–16].

In order to further characterize them, the two Rieske proteins were isolated separately by transblotting and



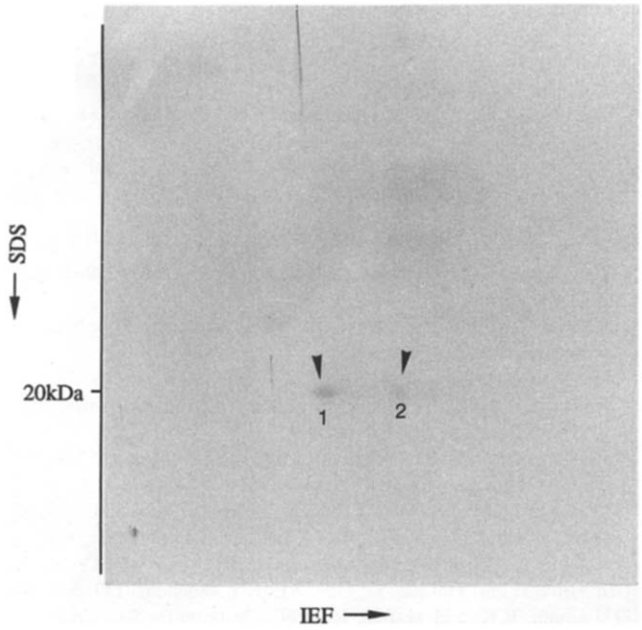


Fig. 2. Western blotting for the identification and the localization of the Rieske iron-sulfur protein in the two-dimensional electrophoretic protein pattern of Fig. 1a. The arrowheads indicate the two immunostained Rieske FeS polypeptide spots. The polypeptides were electroblotted onto a Nitrocellulose membrane and immunostained using antisera against the Rieske protein. This was purified from isolated cytochrome *bf* complex according to Hurt et al. [19].

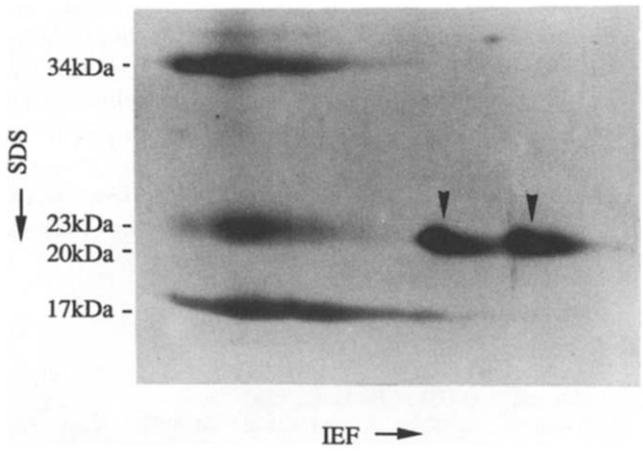


Fig. 3. Two-dimensional electrophoresis protein pattern of the purified cytochrome *bf* complex from spinach. The arrowheads indicate the Rieske FeS proteins R_1 and R_2. The cytochrome *bf* complex was prepared from spinach chloroplasts as described by Hurt and Hauska [20].

were microsequenced using Applied Biosystem model 477 Sequenator. The sequence of the first 23 N-terminal amino acids was the same for both R_1 and R_2 and was as follows:

Ala-Thr-Ser-Ile-Pro-Ala-Asp-Asn-Val-Pro-Asp-Met-
Gln-Lys-Arg-Glu-Thr-Leu-Asn-Leu-Leu-Leu-Leu-

This is identical to the N-terminal sequence of purified spinach Rieske protein [8] and the deduced sequence from cDNA nucleotide sequence [11]. This further confirms that both R_1 and R_2 are Rieske FeS proteins rather than some other copurified proteins. A difference between isoelectric points of 0.30 pH units indicates a charge difference due to a difference in amino acid composition between the two Rieske FeS polypeptides. Since no such difference appears among the N-terminal amino acid residues determined, one might expect a difference in primary structure in other regions of the polypeptide. Such a suggestion is not unreasonable, since the two mature tobacco Rieske proteins differ in only three amino acid residues, viz. at positions 40 (Gly: Ala), 44 (Val: Ala) and 176 (Asp: Ala). The charged residue 176 (Asp) is located in the C-terminal of the tobacco Rieske protein I and results in a calculated 0.24 p*I* difference between the two Rieske proteins of tobacco. Furthermore, the amino acid sequence of the spinach Rieske FeS protein deduced from the cDNA is very similar to that of tobacco, the identity being about 86% [9,11].

At the moment we can only speculate about the physiological significance of the presence of two iso-

Fig. 1. Two-dimensional electrophoretic protein pattern for (a) whole thylakoids, (b) the grana derived subthylakoid fraction and (c) the stroma lamellae subthylakoid fraction. R_1 and R_2 indicate the two Rieske Fe S protein spots. Spinach thylakoids, grana vesicles and stroma vesicles were prepared as described earlier [15]. Thylakoid membrane proteins were extracted with phenol and treated following the procedure described below. The membrane fractions suspended in 10 mM sodium phosphate buffer pH 7.4, 5 mM NaCl and 100 mM sucrose were diluted with homogenization medium containing 50 mM Tris-HCl (pH 8.0), 20 mM $CaCl_2$, 0.1 mM EDTA, 0.5 mM DTT (dithiothreitol) and 2-mercaptoethanol (2% [v/v] was final concentration) to give a chlorophyll concentration of 1 mg/ml and then were subjected to sample treatment. An equal volume of water saturated phenol was added to the sample and the phases were separated by centrifugation. The phenol phase was collected and reextracted with an equal volume of the homogenization medium. Proteins were precipitated from the phenol phase by addition of 2.5 vol. of 0.2 M ammonium acetate in methanol and incubated at −20°C overnight. The pellets were gathered by centrifugation at 14000 rpm in an Eppendorf microfuge for 20 min and washed three times with the ammonium acetate in methanol and once with acetone. Dried protein samples were either stored at −20°C or, when used immediately, solubilized in lysis buffer consisting of 9.8 M urea (Bio-Rad Cat. No. 161-0731), 2% of Nonidet P40 (LKB, Bromma, Sweden), 2% Ampholytes pH range 7–9 (Pharmacia LKB, Bromma, Sweden. Code No. 80-1125-84) and 100 mM DTT (Sigma, USA). After thorough shaking and mild sonication in a water bath for 10 min at room temperature, the samples in the lysis buffer were centrifuged at 14000 rpm in an Eppendorf microfuge for 20 min at room temperature. The supernatants were recovered and immediately loaded. The protein concentration was measured with Bearden's method [17]. 100 μg of the solubilized polypeptides (in lysis buffer) was loaded on to the isoelectric focusing gel. The two-dimensional electrophoresis was carried out essentially as described by O'Farrell [18]. Silver staining was applied in order to get high resolution.

forms of the Rieske iron sulfur protein in the cytochrome *bf* complex. It is of interest that the isolated cytochrome *bf* complex is dimeric [21]. It might be, therefore, that each dimeric complex contains one of each isoform and this might be necessary for its proper in vivo function.

We thank Mrs. Ingun Sundén for skillful technical assistance. This work was supported by the Swedish Natural Science Research Council.

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