Volume 319, number 1,2, 115–118 FEBS 12219 March 1993

A 5 kDa protein (SCS23) from the 30 S subunit of the spinach chloroplast ribosome

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Received 18 January 1993; revised version received 27 January 1993

The proteins of the 30 S ribosomal subunits from spinach chloroplasts were investigated using a radical-free and highly reducing (RFHR) method of two-dimensional polyacrylamide gel electrophoresis (PAGE). Twenty-three proteins were resolved on the gel down to the smallest protein of 5 kDa. The N-terminal amino acid sequence of the 5 kDa protein showed no homology with that of any other protein stored in databases, and the copy numbers were estimated to be 0.88±0.16 and 0.72±0.04 in the 30 S subunits and the 70 S ribosomes, respectively. The results suggest that the 5 kDa protein, which we have called SCS23, may be an essential ribosomal protein specific to spinach chloroplasts.

Chloroplast ribosome; Ribosomal protein; Spinach chloroplast

1. INTRODUCTION

In a previous study [1] we made substantial improvements to the Kaltschmidt and Wittmann (K-W) method of two-dimensional PAGE [2]. This was accomplished by removing free radicals from the gel, keeping the gel under highly reducing conditions and decreasing the pH of the second dimensional gel. This radical-free and highly reducing (RFHR) method enabled us to separate clearly and reproducibly small and basic proteins, as well as cysteine-containing proteins. With this method we found four new small ribosomal proteins (r-proteins) A, B, C, and D in *E. coli* [1,3,4]. Previously, 50–60 different chloroplast r-proteins with molecular weights (MWs) of 10 kDa or more have been identified with the K-W method or modifications of it [5]. However, the MW of some chloroplast-encoded proteins, homologous to *E. coli* r-proteins, are smaller than 10 kDa [6], and Markmann-Mulisch and co-workers [7] isolated, using HPLC, a small r-protein (4.4 kDa) from the spinach 50 S subunit, homologous with *E. coli* r-protein B (L36) [4]. These results suggest that it is likely that some small chloroplast r-proteins may not have been detected by the PAGE systems used in previous studies.

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Abbreviations: ID, integrated density; K-W, Kaltschmidt and Wittmann's; MW, molecular weight; q, net charge; PAGE, polyacrylamide gel electrophoresis; RFHR, radical-free and highly reducing; r-protein, ribosomal protein.

In this report, we analyzed the component proteins of the chloroplast ribosome by RFHR-PAGE and found a new chloroplast-specific r-protein in the 30 S subunit.

2. MATERIALS AND METHODS

2.1. *Preparation of chloroplast ribosomes*

Chloroplast ribosomes were isolated according to the method described by Bourque and co-workers [8]. Spinach leaves were purchased at a local market and homogenized in buffer A (0.35 M sorbitol, 2 mM EDTA, 40 mM 2-mercaptoethanol and 25 mM Tris-HCl, pH 7.5). The homogenate was filtered through a layer of nylon mesh, and centrifuged at 1,500×*g* for 10 min. The pellet was gently resuspended in buffer A, and the suspension was loaded on 30% Ludox-AM in buffer A and centrifuged at 5,000×*g* for 15 min. The pellet (intact chloroplasts) was resuspended in buffer B (25 mM $MgCl_2$, 25 mM KCl, 6 mM 2-mercaptoethanol and 25 mM Tris-HCl, pH 7.5) and centrifuged at 40,000×*g* for 30 min. KCl was added to the supernatant to a final concentration of 0.5 M and the mixture was loaded on a sucrose cushion (1 M) in buffer B. After centrifugation at 100,000×*g* for 16 h the 70 S ribosomes were obtained from the pellet. To dissociate to the 50 S and 30 S subunits, the pellet was suspended in buffer C (1 mM $MgCl_2$, 25 mM KCl, 6 mM 2-mercaptoethanol and 25 mM Tris-HCl, pH 7.5) and dialyzed overnight against buffer C. The suspension was centrifuged at 10,000×*g* for 10 min. The supernatant was loaded onto a 10–40% linear sucrose gradient in buffer C and centrifuged at 200,000×*g* for 3 h. The 50 S and 30 S fractions were pooled and centrifuged at 400,000×*g* for 4 h to collect the subunits as pellets.

2.2. *Preparation of chloroplast r-protein*

r-Proteins were extracted from chloroplast 70 S ribosomes, and 50 S and 30 S subunits by the acetic acid method [9] and dialyzed against 2.5% acetic acid for lyophilization. Lyophilized proteins were stored at −20°C.

2.3. *Electrophoresis*

Lyophilized proteins were analyzed by RFHR-PAGE as described previously [1], except that 6 M urea was replaced with 8 M urea and 3 mm thick gels were changed to 2 mm thick gels.

2.4. *Determination of MW*

To determine the apparent MW of spinach chloroplast r-proteins, *E. coli* r-proteins, the amino acid sequences of which have been determined, were used as MW markers. The mixture of spinach chloroplasts and *E. coli* r-proteins was subjected to RFHR-PAGE, and 2D migration distances, which are based mainly on MW, were measured on the gel. For each chloroplast r-protein, a standard line was drawn by plotting the 2D migration distances against the MW of *E. coli* r-proteins having values of net charge per MW (q/MW) close to that of the chloroplast r-protein. In this way, the contribution of the difference in net charge to the 2D mobility was eliminated. To select the *E. coli* r-protein MW markers, q/MW values were calculated conventionally from the 1D migration distances at pH 8.2, rather than using q/MW values determined in the second dimensional analysis [1].

2.5. *Estimation of the copy number of r-protein*

After electrophoresis, the gels were stained with Amido black 10B and scanned with a personal densitometer PD110 (Molecular Dynamics Co.) to measure the integrated density of the stained spots of 30 S r-proteins. The integrated density per MW (ID/MW) values were subsequently compared. Then, r-proteins which had large ID/MW values were detected and defined as unit copy proteins. The copy number of SCS23 was determined by normalizing its ID/MW value to the average value of ID/MW for two unit copy proteins, SCS21 and SCS22.

2.6. *Determination of the amino acid sequence of SCS23*

The r-proteins were electroblotted onto a Polyvinylidene difluoride membrane at 10 volts for 4 h using the 2D gel buffer of RFHR-PAGE. A sample of the proteins was cut from the membrane and applied to a 470A gas-phase protein sequencer (Applied Biosystems Inc.).

3. RESULTS AND DISCUSSION

3.1. *Characterization of chloroplast r-proteins*

In order to determine the protein composition of chloroplast ribosomes and their subunits, we first investigated whether the ribosomes were contaminated by stromal proteins or cytoplasmic ribosomes. Cytoplasmic r-proteins and stromal proteins were prepared and separated by RFHR-PAGE and the profiles of the proteins on the 2D gels were compared with those of the r-proteins. When chloroplast ribosomes were prepared

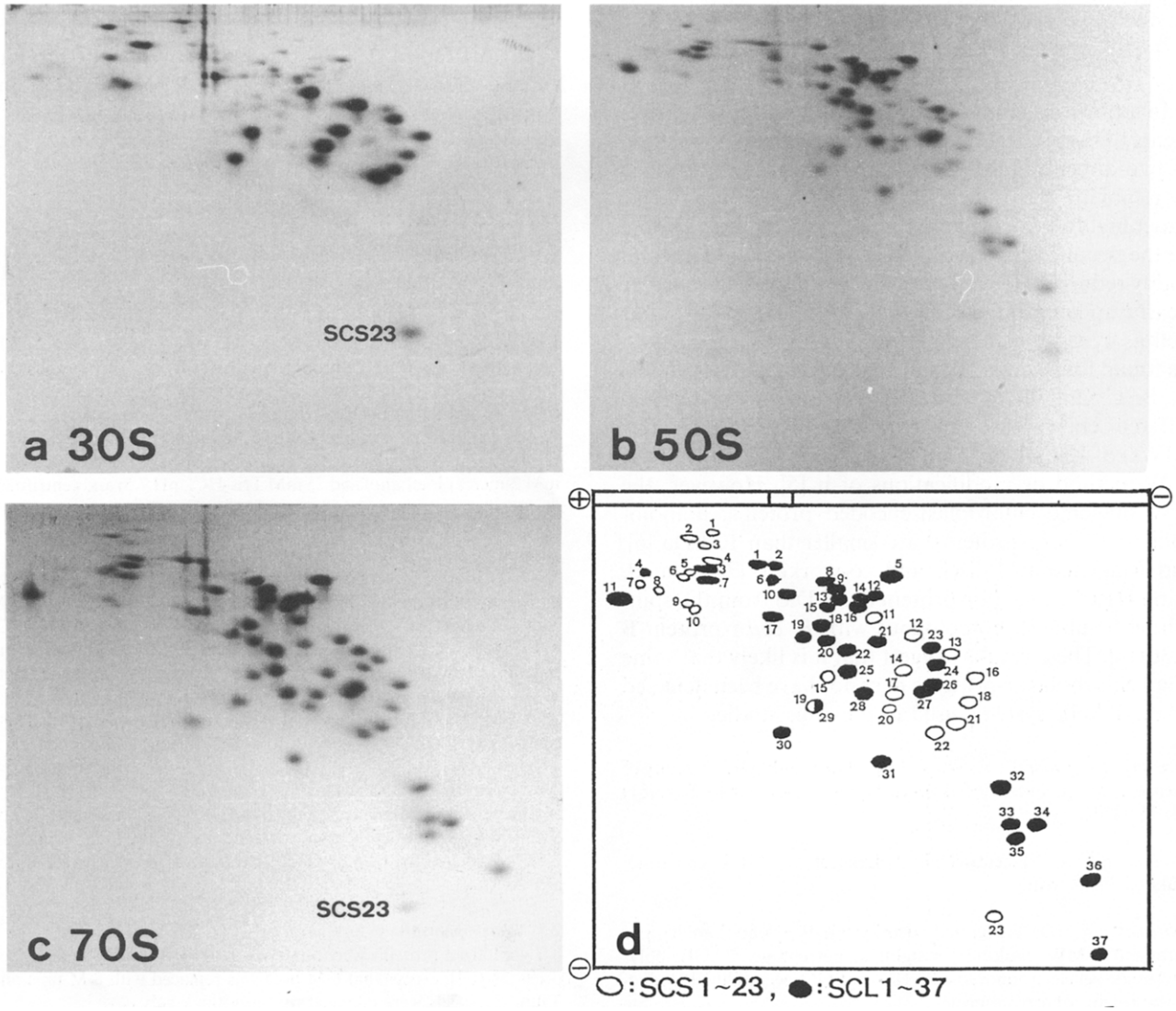

Fig. 1. Spinach chloroplast 30 S (a), 50 S (b), and 70 S (c) r-proteins on 2D gels of RFHR-PAGE, and the diagram of the 70 S r-proteins (d).

from intact chloroplasts, they were completely free of cytoplasmic ribosomes (data not shown). Stromal proteins were not found in the chloroplast ribosomal fraction either (data not shown). We then compared the protein profiles of the 30 S and 50 S subunit fractions (Fig. 1a and b) with the profile of the 70 S ribosome (Fig. 1c) and determined the protein composition of the two subunits (Fig. 1d). The numbering of the r-proteins of spinich chloroplast was carried out according to that in *E. coli* [10]. The 30 S and 50 S subunits contained 23 (SCS1–SCS23) and 37 (SCL1–SL37) proteins, respectively. The MW of the 30 S r-proteins ranged between 10.5 and 53.0 kDa except for an extremely small protein (SCS23). The MW of the 50 S subunit proteins ranged between 4.9 and 23.8 kDa.

3.2. *MW of SCS23*

The MW of SCS23 was determined electrophoretically to be 5,000 Da by using thirteen *E. coli* r-proteins with q/MW ratios similar to that of SCS23 as MW markers (Fig. 2) as described in section 2. SCS23 is one of the smallest chloroplast 30 S r-proteins known.

3.3. *Copy number of SCS23*

As described in section 2, to estimate the copy number of each 30 S r-protein in ribosomal particles, densitometry was carried out after staining the spots with Amido black 10B. The ID of each spot of r-protein was measured and the ID/MW values were then compared. Ten r-proteins (SCS 11–18, 21 and 22) which had large ID/MW values were found and defined as unit copy proteins. The copy number of SCS23 was estimated by normalizing its ID/MW value to the average value of ID/MW for two unit copy proteins, SCS21 (MW 10,700) and SCS22 (MW 10,500) (Fig. 1) which have a basicity similar to that of SCS23. This was done to eliminate the weak positive dependency of Amido black 10B staining on basicity. The difference in color yield (absorbance/mg of protein) of different proteins, however, is not large for Amido black 10B (within ±20% of the average value) [11]. In 30 S particles, the copy number of SCS23 was determined to be 0.88±0.16 as the average of four experiments, and in 70 S particles, it was found to be 0.72±0.04 using three gels as the average of three experiments. These results strongly suggest that SCS23 is an essential protein component of spinach chloroplast 30 S subunits.

3.4. *NH_2-terminal sequence of SCS23*

SCS23 was purified by RFHR-PAGE and subjected to automated NH_2-terminal amino acid sequencing. It was analyzed in 22 cycles and was found to have the following sequence:

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1         10              20
A-V-G-D-R-K-T-A-K-G-K-R-F-N-( )-S-F-( )-N- A-Y-P- ●●●
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The 15th and 18th amino acid residues could not be identified. This sequence showed no significant similarity to any known protein or gene sequence in our databases, including chloroplast genomes of tobacco, liverwort and rice. Therefore the gene seems not to be in the chloroplast but in the nucleus.

Small basic proteins, with MW similar to that of SCS23, have not been reported for chloroplast 30 S subunits. MWs of r-proteins of the 30 S subunit, which are derived from nuclotide sequences in the tobacco [6] and liverwort [12] chloroplast genome, are larger than

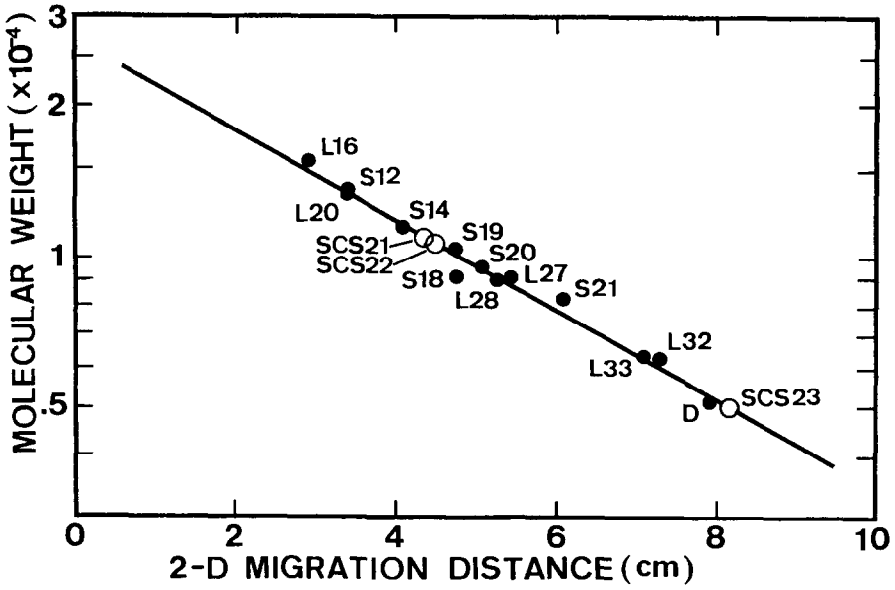

Fig. 2. Determination of the MW of SCS23.

10.4 and 8.9 kDa, respectively. The MW of chloroplast r-proteins determined by PAGE ranged from 8.8 kDa to 35.7 kDa for *N. tabacum* [13] and from 13 kDa to 40.5 kDa for rye [14]. The small basic proteins cannot be resolved by ordinary PAGE systems because they become stuck in the front line or run off the second dimension. With the improved method of PAGE (RFHR-PAGE), several small basic proteins from chloroplast ribosomes with MW less than 8.8 kDa were clearly resolved on the gel. One of these proteins, SCS23, belongs to the 30 S subunit and has not been reported. This indicates that RFHR-PAGE is a useful tool for the analysis of chloroplast r-proteins.

In many respects chloroplast ribosomes resemble those of *E. coli* [15]. Most chloroplast r-proteins therefore have homology with *E. coli* r-proteins. However, some r-proteins which have no homology with any *E. coli* r-protein have been reported for pea [16] and spinach [17] chloroplasts. These chloroplast-specific r-proteins might not originate in eubacterial ribosomes. SCS23 is also a chloroplast-specific r-protein because the amino acid sequence of SCS23 showed no homology with the sequence of any *E. coli* r-protein, including D protein which migrated very close to SCS23 on the gel. Chloroplast-specific r-proteins of pea and spinach are neutral or acidic proteins, while SCS23 is a basic protein and has a unit copy number. SCS23 seems to be an essential component of the chloroplast 30 S subunit.

Acknowledgements: This work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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