The 70-kDa major DNA-compacting protein of the chloroplast nucleoid is sulfite reductase

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Abstract The chloroplast nucleoid is a complex of chloroplast DNA and various, mostly uncharacterized proteins. An abundant 70-kDa protein of the isolated nucleoids of pea chloroplasts was identified as sulfite reductase by N-terminal sequence analysis as well as immunoblot analysis, spectrophotometry and enzyme activity analysis. Recombinant maize sulfite reductase was indeed able to compact chloroplast DNA and to form nucleoid-like particles in vitro. The role of sulfite reductase in the structural organization of the nucleoid is discussed.

Key words: Chloroplast nucleoid; DNA-binding protein; Sulfite reductase; *Pisum sativum*

1. Introduction

The chloroplast nucleoid is a complex of chloroplast DNA (cpDNA) and various, mostly uncharacterized proteins, which is thought to be the functional unit of replication and gene expression of cpDNA [1–3]. The chloroplast nucleoids are known to change their morphology, size and localization within the chloroplast during the development of the leaf (for reviews, see [1,3]). Isolated chloroplast nucleoids retain the activity of replication and transcription [2]. The replication activity of nucleoids was also demonstrated in vivo [4].

However, proteins of the nucleoid have been poorly characterized. Hansmann et al. [5] described the protein composition of nucleoids from various types of plastids. Only a few of the polypeptides have been characterized biochemically, such as CND41 [6]. Topoisomerase II was localized to the nucleoids [7]. PEND protein was identified as a candidate that plays a role in the binding of nucleoids to the envelope membrane during the replication of nucleoids [8,9]. Nemoto et al. [10,11] analyzed nucleoid proteins of plastids and chloroplasts in tobacco, and found that a 69-kDa DNA-binding protein is abundant in the plastid nucleoids. A previous study [12] showed the presence of an abundant 70-kDa protein in the nucleoids of developing chloroplasts of pea. A 68-kDa protein of chloroplast nucleoids in soy bean cultured cells has been claimed to suppress replication [13]. Whether the 69-kDa tobacco protein, the 70-kDa pea protein and the 68-kDa soy bean protein are homologous proteins still remains unknown.

In the present study, we show that the major 70-kDa protein of pea plastid nucleoids is a DNA-compacting protein, and this protein is identified as sulfite reductase (SiR). We also show that recombinant SiR is able to compact cpDNA and to form a structure similar to the chloroplast nucleoid.

2. Materials and methods

2.1. Plant material and preparation of nucleoids

Growth of plants and preparation of chloroplasts have been described previously [8]. Nucleoids were prepared as described previously [12] using TAN buffer (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 M sucrose, 7 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, 1.2 mM spermidine), and then stored in the presence of 33% glycerol at −80°C until use.

2.2. Salt treatment of nucleoids

The nucleoids were washed twice in TAN buffer, and then suspended in 0 M TEMP buffer (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride; *x* M TEMP buffer contains *x* M NaCl in 0 M TEMP buffer) containing various concentrations of NaCl. After standing on ice for 2 h, the suspension was centrifuged at $22\,000 \times g$ for 1 h at 4°C. The supernatant was removed and the pellet was resuspended in TAN buffer. The supernatant obtained by treatment with 5 M TEMP buffer was dialyzed against 0 M TEMP buffer for 2 h at 4°C, and then centrifuged as above to obtain reconstituted DNA–protein complex.

2.3. Immunoblot analysis

The 5 M supernatant, which contained essentially the 70-kDa protein, was dialyzed against 0 M TEMP buffer, and then used to immunize mice to obtain antiserum against the 70-kDa protein. Only the antisera that showed a specific reaction to the 70-kDa protein were used. SDS–PAGE and immunoblotting were performed using a 12% polyacrylamide gel as described in previous papers [9,12]. Antisera against maize SiR were described in [14].

2.4. SiR activity

SiR activity was measured by the production of cysteine by the SiR–cysteine synthase coupled system [15] as described in [14]. Either ferredoxin III or methyl viologen was used as the electron donor for SiR. Dithionite was used to reduce ferredoxin III or methyl viologen.

2.5. In vitro reconstitution of DNA–protein complex

The cDNA for the maize SiR (DDBJ/EMBL/GenBank accession number: D50679) was overexpressed with the siroheme synthase gene (*cysG*) in *Escherichia coli* cells, and then the active enzyme was purified by MonoQ column chromatography [16]. cpDNA was prepared from developing chloroplasts of pea as described previously [8]. cpDNA (2.5 μg) and SiR (5 μg) were mixed in 100 μl of TAN buffer,

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Abbreviations: cpDNA, chloroplast DNA; SiR, sulfite reductase

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and stored at 4°C for 20 h. Then the mixture was examined by fluorescence microscopy.

2.6. Microscopy

The reconstituted DNA–protein complex in TAN buffer (4 μl) was mixed with 4 μl of 1% glutaraldehyde in TAN buffer, and then stained with 4 μl of 1 μg/ml DAPI in TAN buffer. The specimens were examined with a fluorescence microscope Olympus model BX-60 with the WU filter cube set.

3. Results and discussion

3.1. Partial decomposition of plastid nucleoids by NaCl

When the isolated nucleoids were washed with various concentrations of NaCl, an abundant 70-kDa polypeptide, among other minor ones, was released (Fig. 1A). About 30% of this polypeptide was released by 0.2 M NaCl (see lanes 6 and 7), and the release was almost complete in 2 M NaCl (lanes 10 and 11). Concomitant with the release of the 70-kDa protein, cpDNA was also released from the nucleoids by NaCl treatment (Fig. 1B). A small amount of cpDNA was released by 0.2 M NaCl (lane 7), but about 50% of cpDNA was released by 0.5 or 2 M NaCl. No further release of cpDNA was found to occur at higher concentrations of NaCl or after prolonged (overnight) incubation.

When the supernatant obtained by washing nucleoids with 5 M NaCl (Fig. 1C, lane 1) was dialyzed against 0 M TEMP buffer and then centrifuged, half of the 70-kDa protein became associated with the precipitate again (lane 3). At the same time, a significant part of the cpDNA was recovered in the precipitate (Fig. 1D, lane 3). These results suggest that the 70-kDa protein is involved in compacting cpDNA within the nucleoids.

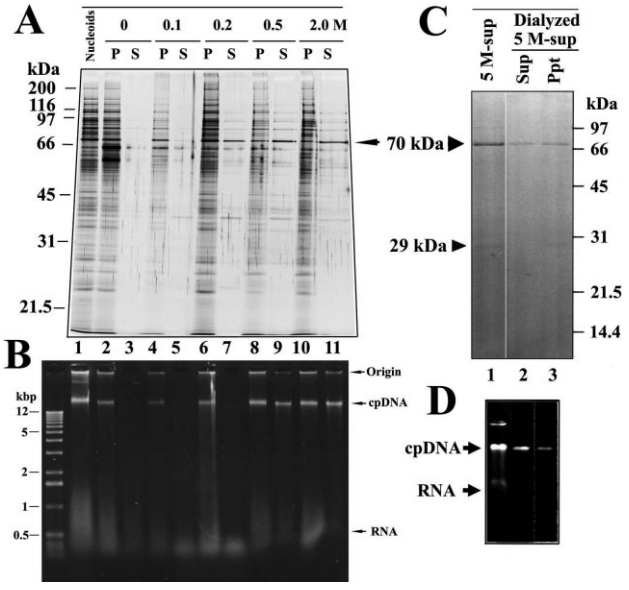

Fig. 1. Decomposition of nucleoids by NaCl treatment and reconstitution of DNA–protein complex. A, B: Decomposition of nucleoids by NaCl treatment. Plastid nucleoids were isolated from 6-day-old pea leaves in TAN buffer (lane 1), and then suspended in TEMP buffers with various concentrations of NaCl. After standing for 1 h on ice, the supernatant (S) and precipitate (P) were separated by centrifugation. Protein composition (A) was analyzed by SDS–PAGE using a 12% gel. Protein bands were visualized by silver staining. The numbers on the left indicate molecular size markers. The arrowhead indicates the 70-kDa protein. Nucleic acids were analyzed with 0.8% agarose gel and stained with ethidium bromide (B). The lanes in panel B correspond to the lanes in panel A. The leftmost lanes indicate DNA size markers. C, D: Reconstitution of nucleoid-like complex by dialysis. The supernatant obtained by washing the nucleoids with 5 M TEMP buffer was dialyzed against 0 M TEMP buffer for 2 h at 4°C, and then centrifuged to obtain supernatant and precipitate. Protein (C) and DNA (D) were analyzed as described above.

3.2. The 70-kDa protein is a pea homologue of SiR

The N-terminal sequence of the 70-kDa protein (Fig. 2C) was similar to those of the mature form of SiRs from tobacco, maize and *Arabidopsis thaliana*. Exact N-termini were somewhat variable depending on the plant species, but they were also consistent with the N-termini of cyanobacterial enzymes that have no transit sequence.

To confirm this result, immunoblot analysis was performed (Fig. 2A,B). The antiserum raised against the 70-kDa protein specifically reacted with recombinant maize SiR (Fig. 2A, lane 6). The level of the 70-kDa protein in the supernatant after the sedimentation of nucleoids was below the detection limit in this analysis (lane 7). The antiserum raised against maize recombinant SiR reacted with the 70-kDa protein of pea (Fig. 2B, lanes 8–10), as did the antiserum against the 70-kDa protein. SiR was not detected in the supernatant fraction (less than 1/10 concentration), but this fraction contained a 25-kDa polypeptide that cross-reacted with the antiserum. Whether this polypeptide is a degradation product of SiR or an unrelated protein is not clear at present. These results suggest that the 70-kDa nucleoid protein of pea chloroplasts is SiR. The small difference in the mobility during the electrophoresis can be explained by a small difference in the size of two SiR proteins. In fact, according to the cDNA sequences, the size of SiR precursor is somewhat variable in maize (635 residues), tobacco (693 residues) and *A. thaliana* (642 residues).

Spectrophotometric analysis of the 70-kDa protein in the 2 M supernatant (Fig. 2Da) showed two small but distinct absorption maxima at 392 and 580 nm, which coincided with the absorption maxima of authentic maize SiR assembled with the siroheme and [4Fe–4S] cluster (Fig. 2Db). The high background in this measurement was due to the presence of DNA in the 2 M supernatant. These spectral characteristics indicate the presence of a heme prosthetic group. The Fe–S cluster does not show a distinct absorption maximum. Comparison of SiR concentrations determined by spectrophotometry and SDS-PAGE (inset in Fig. 2Da) indicated that most (70%) of the 70-kDa protein released from the nucleoids is holoenzyme retaining the prosthetic group.

The SiR activity was measured in the nucleoid fractions (Table 1). The isolated nucleoids exhibited a high activity of ferredoxin-dependent SiR activity. Methyl viologen also acted as an electron donor at a lower efficiency. A major part of this activity was recovered in the supernatant after salt treatment. Although the amounts of SiR estimated by dividing the activity values by the specific activity of maize recombinant SiR (407 nmol Cys/min/mg protein) were roughly two times higher than the values estimated by the densitometry of the 70-kDa band in SDS–PAGE, this discrepancy could be accounted for by the difference in specific activity of pea and maize enzymes. These results clearly indicate that the major 70-kDa protein in the nucleoid is SiR. However, most of the SiR activity in the chloroplast was present in the post-nucleoid supernatant though at a lower concentration (results not shown).

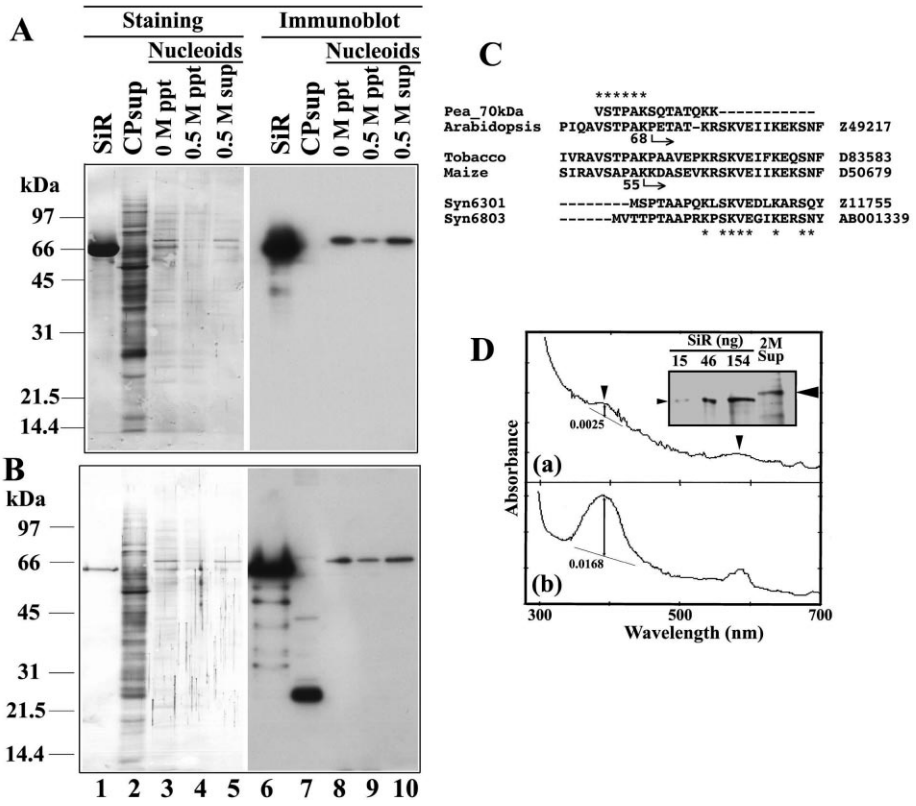

Fig. 2. Identification of the 70-kDa protein as SiR. A, B: Immunological cross-reactivity of the 70-kDa protein and maize SiR. Recombinant maize SiR and various fractions of pea nucleoids were subjected to immunoblotting with anti-70-kDa protein (A) and anti-SiR (B) antisera. Lanes 1 and 6, recombinant SiR (5 μg in A, 0.04 μg in B); lanes 2 and 7, supernatant (1.5 μg protein) obtained by centrifugation of the 6-day-old chloroplasts solubilized with Nonidet P-40 (post-nucleoid supernatant); lanes 3 and 8, nucleoids (equivalent to 0.1 μg protein before washing) washed with 0 M TEMP buffer (see lane 2 of Fig. 1A); lanes 4 and 9, precipitate obtained after washing nucleoids with 0.5 M TEMP buffer (see lane 8 of Fig. 1A); lanes 5 and 10, supernatant obtained after washing nucleoids with 0.5 M TEMP buffer (see lane 9 of Fig. 1A). Two identical sets of samples were electrophoresed and blotted, and then one part was stained with colloidal gold (Aurodye, Amersham). Another part was immunostained, and finally the signal was detected by chemiluminescence. C: Alignment of the N-terminal sequence of the 70-kDa protein with plant and cyanobacterial SiRs. The cleavage site of transit peptide of plant enzymes, if known, is shown by an arrowhead. The accession numbers are shown on the right. D: Presence of prosthetic group in the 70-kDa protein in the 2 M supernatant. a: Absorption spectrum of the supernatant obtained by washing the nucleoids with 2 M TEMP buffer; b: Absorption spectrum of authentic maize SiR (31 μg/ml). The absorption peaks at 392 and 580 nm (downward arrowheads) are attributed to the Soret and α bands of siroheme, respectively. The numbers within panels a and b indicate estimated absorbance at 392 nm due to the prosthetic group. Based on the absorbances, the concentration of SiR was estimated to be 4.6 μg/ml. Inset in a: Estimation of SiR protein by SDS–PAGE and silver staining. Small and large arrowheads indicate maize SiR and pea counterpart, respectively. Based on the band intensities, the concentration of SiR was estimated to be 6.5 μg/ml.

3.3. Reconstitution of nucleoid-like structure from recombinant SiR and chloroplast DNA

As SiR is known to be an enzyme catalyzing the reduction of sulfite to sulfide, its presence in the nucleoid was unexpected. In addition, maize SiR is a slightly acidic protein as a whole as evidenced by adsorption to MonoQ column. To confirm the DNA-packing activity of the SiR, recombinant maize SiR was mixed with pea cpDNA and incubated in TAN buffer. After incubation for 1 h, tiny particles began to appear (Fig. 3C). After incubation for 20 h, a number of small particles similar to nucleoids were formed (Fig. 3D). In the control experiments, either cpDNA alone (Fig. 3A) or

Table 1
Sulfite reductase activity of nucleoids and various fractions

	Cys (nmol/min/fraction)			Cys (nmol/min/mg protein)	SiR (μg/fraction)	
	No add.	+Fd	+MV	(+Fd)	Activity	PAGE
Nucleoids	0	22	17	18.5	54	26
1 M supernatant	0	21	12		52	24
1 M precipitate	0	16	2		39	13
Maize SiR				407		

SiR activity is expressed as the rate of synthesis of cysteine, which is produced by cysteine synthase from sulfide. Two types of electron donor systems were used: dithionite plus ferredoxin or dithionite plus methyl viologen. No add., no addition; +Fd, 10 μM ferredoxin III; +MV, 0.5 mM methyl viologen without ferredoxin. The amount of SiR was estimated from the enzyme activity using the value for the maize SiR or by densitometry of the 70-kDa protein in SDS–PAGE. The discrepancy between the two sets of data could be accounted for by the difference in specific activity of pea and maize enzymes.

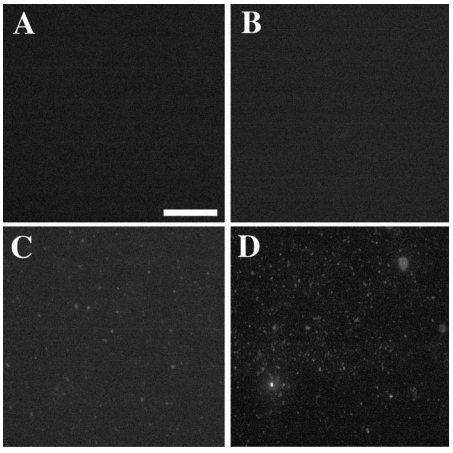

Fig. 3. Complex formation of cpDNA and SiR. Pea cpDNA was mixed with recombinant maize SiR, and incubated on ice. The mixture was stained with DAPI and examined by fluorescence microscopy. A: Control experiment with no SiR. B: Control experiment with bovine serum albumin instead of SiR. C: Mixture of cpDNA and SiR, 1 h incubation. D: Mixture of cpDNA and SiR, 20 h incubation. Scale bar represents 10 μm.

cpDNA plus bovine serum albumin (Fig. 3B) did not generate such nucleoid-like particles. Similar results were obtained with plasmid DNA such as pBluescript (results not shown). These results clearly indicate that SiR is a non-specific DNA-binding protein that has the ability to condense DNA into small particles.

3.4. Novel role of sulfite reductase in the structural organization of DNA

The results of the present study suggest that the chloroplast nucleoid consists of a salt-resistant core and a peripheral domain associated by electrostatic interaction. The peripheral domain is composed of SiR and 50% of cpDNA. The association of SiR to the nucleoid is not considered to be an artifact of nucleoid isolation, because a significant amount of cpDNA was also released with SiR by salt treatment (Fig. 1). If the binding of SiR to the nucleoid were an artifact, this pool of cpDNA would have been present in the chloroplast stroma as free molecules. However, microscopic observation clearly indicated that all cpDNA is present as nucleoids in vivo [1,3]. The bipartite domain structure might correspond to the hetero- and euchromatin of the cell nucleus. High-resolution microscopic localization of SiR in vivo will be important to determine the fine structure of nucleoid.

We should also ask a question about the role of SiR in the chloroplast nucleoid. Obviously, this is an enzyme involved in the reductive assimilation of sulfite to sulfide. However, the significance of this catalytic function in the nucleoid is not clear, because ferredoxin, which is known to be an electron donor for SiR [14], was not detected in the nucleoids (results not shown). One possibility is that SiR protects cpDNA from oxidative damage. Another possibility is that SiR protects cpDNA from chemical modification by bisulfite (HSO_3^-) ion. Cytosine residue is converted to uracil residue in the presence of a high concentration of bisulfite [17]. Further study is necessary to shed light on the role of SiR in the chloroplast nucleoid.

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