Identification of a novel prokaryotic HEAT-repeats-containing protein which interacts with a cyanobacterial IscA homolog

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Received 21 February 2002; revised 15 April 2002; accepted 15 April 2002

First published online 25 April 2002

Edited by Gunnar von Heijne

Abstract IscA homologs are known to be involved in iron–sulfur cluster formation in various organisms. Recombinant proteins of two IscA homologs from the cyanobacterium *Synechocystis* PCC 6803, designated SLR1417 and SLR1565, were purified. The absorption spectrum of purified SLR1565 was typical for [2Fe–2S] cluster-containing proteins, whereas that of SLR1417 predominantly showed the presence of the iron ion alone. In the cyanobacterial cell extracts, only SLR1565 was found to form a complex with a novel prokaryotic HEAT-repeats-containing protein, SLR1098. Thus, the two cyanobacterial IscA protein homologs exist in distinct molecular states, suggesting different cellular roles for these proteins.

Key words: IscA; Iron–sulfur cluster; HEAT-repeat; Cyanobacterium; Ferredoxin

1. Introduction

While a number of iron–sulfur cluster-containing proteins have been the focus of extensive genetic, biochemical, or biophysical characterization [1,2], detailed information on the complex process of iron–sulfur cluster biosynthesis is only recently emerging. Much of what is currently known stems from investigations on the function of two *nif* (nitrogen fixation) gene products involved in nitrogenase assembly in *Azotobacter vinelandii* [3,4]. NifS catalyzes the conversion of cysteine to alanine and sulfane to sulfur via a protein-bound cysteine persulfide intermediate [5,6]. By comparison, NifU serves as a scaffold for NifS-directed assembly of a transient iron–sulfur cluster [7,8]. The non-nitrogen-fixing cyanobacterium *Synechocystis* PCC 6803 contains only one *nifU*-like gene encoding a protein that corresponds to the C-terminal one third of *A. vinelandii* NifU [9]. We previously demonstrated that this cyanobacterial NifU-like protein can assemble a labile [2Fe–2S]-type iron–sulfur cluster and possesses the ability to deliver its [2Fe–2S] cluster to an apoferredoxin protein [10]. We proposed that the cyanobacterial NifU-like protein plays a central role in iron–sulfur cluster biosynthesis as a scaffold for iron–sulfur cluster assembly and delivery.

Like NifS, NifU and their homologs [11–13], IscA proteins are thought to be involved in iron–sulfur cluster biosynthesis [14–18]. Recently, *Escherichia coli* IscA was shown to assemble an air-sensitive [2Fe–2S] cluster, which served as a source for holo-ferredoxin synthesis in vitro [19]. The ^{Nif}IscA of *A. vinelandii* was proposed to function as an alternative scaffold protein in iron–sulfur cluster biosynthesis based on its NifS-directed assembly of a labile [4Fe–4S] cluster via a transient [2Fe–2S] cluster [20].

It is well known that several organisms, including prokaryotes and eukaryotes, possess multiple copies of *iscA*. It is not clear if these IscA isologs perform complementary or differentiated functions in the same cell. The cyanobacterium *Synechocystis* PCC 6803 was also found to contain two genes, *slr1417* and *slr1565*, the translation products of which are similar to IscA (Fig. 1A). The predicted amino acid sequences of the two cyanobacterial IscA homologs, SLR1417 and SLR1565, show 39% sequence identity to each other and 38% and 39% to the *A. vinelandii* IscA protein. To elucidate the function of the two cyanobacterial IscA homologs, we analyzed the molecular status of these proteins and characterized recombinant proteins purified from *E. coli* cells.

2. Materials and methods

2.1. Bacterial and cyanobacterial strains and their culture conditions

The *E. coli* strains TG1 and BL-21(DE3) RIL (Stratagene) were used for plasmid propagation and over-expression of the recombinant proteins, respectively. The cyanobacterium *Synechocystis* PCC 6803 was grown at 30°C under white light in BG11H (BG11 with 20 mM HEPES–NaOH, pH 7.5) liquid or solid agar medium.

2.2. Expression and purification of SLR1417, SLR1565, and SLR1098 in E. coli

The plasmid used for expression of SLR1417, pET-21/*slr1417*, was constructed by cloning the *slr1417* gene into pET-21d (Amp^r) (Novagen). The plasmid used for over-expression of SLR1565, pET-9/*slr1565*, was constructed by cloning the *slr1565* gene into pET-9d (Km^r) (Novagen). The SLR1098 over-expression plasmid, pET-21/*slr1098*, was constructed in the same manner. Expression and purification of recombinant proteins was performed essentially according to the methods described previously [10].

2.3. Subcellular fractionation of the cyanobacteria

Cyanobacterial cells were harvested by centrifugation. The cell pellet was suspended in five volumes of a buffer containing 50 mM HEPES–NaOH (pH 8.5), 25 mM NaCl and 5 mM dithiothreitol (DTT). Cells were ruptured by sonication. After removal of unbroken cells, cell extracts were centrifuged for 20 min at $9000 \times g$ to precipitate large cellular debris. The soluble and membrane fractions were obtained by ultra-centrifugation at $100\,000 \times g$ for 30 min. The soluble protein fraction was further fractionated with a buffer containing 50 mM HEPES–NaOH (pH 8.5), 150 mM NaCl and 5 mM DTT using Superdex-75 gel filtration chromatography (Amersham Pharmacia)

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0014-5793/02/$22.00
PII: S0014-5793(02)02736-9

[21]. The molecular marker proteins used were bovine serum albumin (BSA) (69 kDa), ovalbumin (43 kDa), FNR (35 kDa), myoglobin (17.6 kDa), and ribonuclease A (13.7 kDa). The peak fractions containing either SLR1565 or SLR1417 were used for chemical cross-linking with 2.5 mM disuccinimidyl suberate (DSS) or 5 mM bis-(sulfosuccinimidyl)-suberate (BS^3). The cross-linked products were detected by Western blotting.

2.4. Purification and identification of the SLR1565-interacting 30-kDa protein

A HiTrap™ NHS-activated HP affinity column (Amersham Pharmacia), covalently conjugated with affinity-purified anti-SLR1565 antibody, was used for affinity purification of any proteins interacting with SLR1565 in the cyanobacterial soluble fraction. After separation of the affinity-purified proteins by SDS–PAGE, proteins were electrotransferred onto an Immobilon™ transfer membrane (Millipore), and the desired protein bands were subjected to proteolytic digestion with lysyl-endopeptidase followed by peptide isolation and peptide sequencing according to conventional methods.

2.5. Other methods

UV/visible absorption spectra of protein solutions were recorded using a UV-2500PC UV/visible recording spectrophotometer (Shimadzu). The concentration of [2Fe–2S] clusters was estimated using the molar extinction coefficients of 9.68 mM^{-1} cm^{-1} at 422 nm [22]. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) with BSA as a standard. Western blotting was carried out using horseradish peroxidase-protein A (Zymed) as a secondary antibody and an ECL™ detection system (Amersham Pharmacia) [11].

3. Results and discussion

Two cyanobacterial IscA homologs, SLR1417 and SLR1565, were expressed in *E. coli*, and each protein was purified to homogeneity as shown in Fig. 1B. Purified SLR1565 had a UV/visible absorption spectrum characteristic of [2Fe–2S] cluster-containing proteins that have an absorption peak at 330 nm and lower broad peaks at around 420 nm and 460 nm (Fig. 1C, plain line). By comparison, the UV/visible absorption spectrum of SLR1417 had a clear peak at 330 nm and only minor absorption at around 450 nm compared to SLR1565 (Fig. 1C, dotted line). In a typical [2Fe–2S] cluster, absorption from 420 nm to 460 nm is attributed to the vibration between iron and bridging inorganic sulfur, whereas absorption at 330 nm is a result of vibration between the iron and cysteinyl sulfur of the polypeptide backbone. Therefore, SLR1565 appears to possess an [2Fe–2S] cluster, while SLR1417 appears to be able to bind an [2Fe–2S] cluster but more stably bind only iron ions, probably in a cysteinyl-ligated coordination.

E. coli IscA and *A. vinelandii* ^{Nif}IscA proteins have been purified in a metal-free form [19,20]. In these studies an [2Fe–2S] cluster could only be assembled with purified IscA proteins under anaerobic conditions in vitro, and the reconstituted [2Fe–2S] cluster in either the *E. coli* or *A. vinelandii* IscA protein was fairly labile. By comparison, the [2Fe–2S] cluster found associated with purified SLR1565 was stable, as indicated by the persistence over several days in a closed tube at 4°C of its brown-red color (data not shown).

Using the purified proteins as antigens, we prepared antibodies to specifically identify each IscA homolog (Fig. 2A). Western blotting analysis of total cyanobacterial cell lysates showed that the purified antibodies detected only one specific protein band with an electrophoretic migration consistent with recombinant SLR1417 or SLR1565 (Fig. 2B, lanes 1). As expected, both SLR1417 and SLR1565 were localized exclusively in the soluble fraction (lanes 4). Since the intensity of

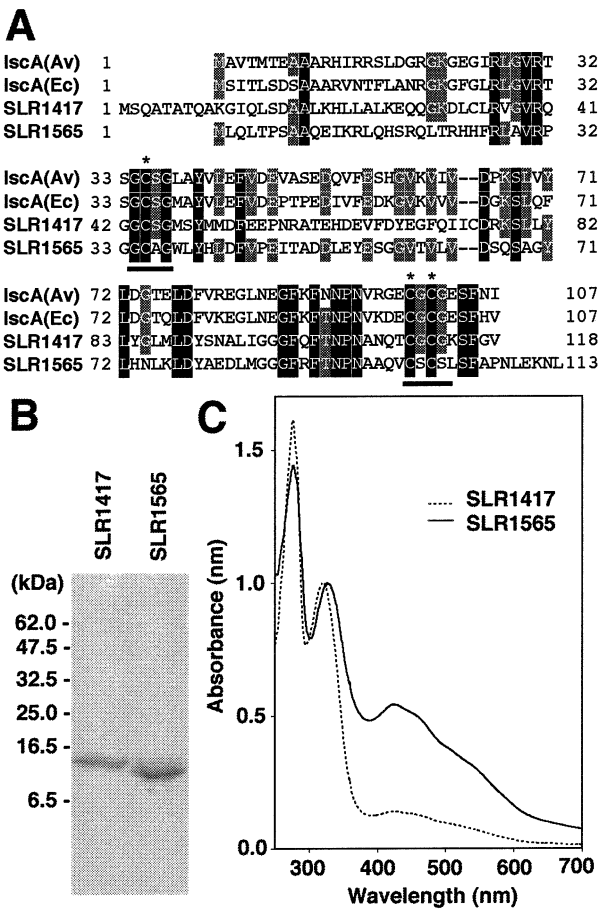

Fig. 1. Purification and spectroscopic characterization of recombinant SLR1417 and SLR1565. A: Sequence alignment of SLR1417 and SLR1565 with IscA proteins from *A. vinelandii* (Av) and *E. coli* (Ec). Highly conserved cysteine residues are denoted by asterisks and amino acids conserved in at least three of four IsaA proteins are highlighted. B: Purified SLR1417 (lane 1) and SLR1565 (lane 2) were analyzed by 17.5% SDS–PAGE. Protein molecular weight markers are indicated on the left. C: UV/visible absorption spectra of purified SLR1417 (dotted line) and SLR1565 (plain line) were adjusted by the absorption values at 330 nm.

the SLR1417 and SLR1565 protein bands detected by Western blotting were similar to those of 10 ng of the corresponding purified proteins, we concluded that nearly equivalent amounts of SLR1417 and SLR1565 existed in the soluble compartment of cyanobacterial cells (lanes 5) and that each SLR1417 and SLR1565 represents an estimated 0.01% of the total soluble proteins in the cyanobacterial cell.

Gel filtration of the soluble cyanobacterial protein fraction resulted in SLR1565 and SLR1471 eluting as single homogeneous peaks with molecular masses estimated at 17 kDa and 69 kDa, respectively (Fig. 2C). By comparison, recombinant SLR1417 and SLR1565 eluted as 17-kDa and 15-kDa proteins, respectively (data not shown). These results suggest that SLR1565 may be part of a complex in vivo.

Chemical cross-linking experiments were performed to further analyze the oligomeric status of each IscA homolog in the soluble cyanobacterial protein fraction. When the amine-reactive cross-linker DSS was incubated with gel-filtration fraction 9, which contained SLR1565, two adjacent immunoreactive bands at around 42.5 kDa were detected by Western blotting using anti-SLR1565 antibodies (Fig. 2D, right panel). Anoth-

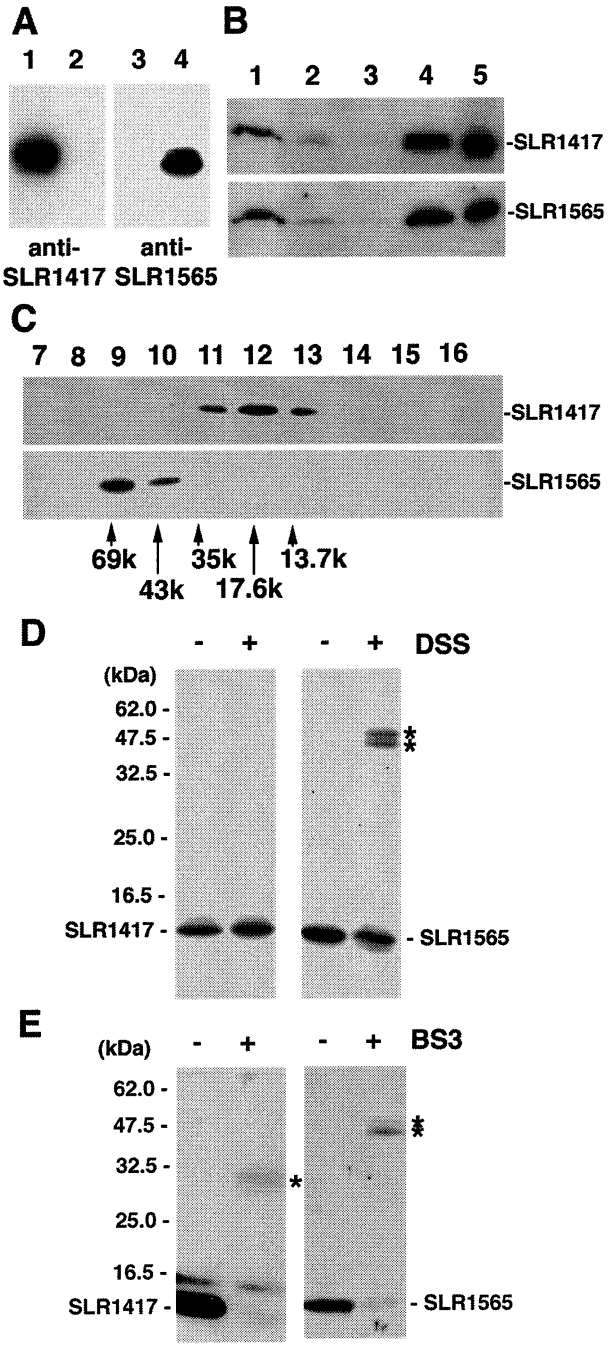

Fig. 2. Western analyses of SLR1417 and SLR1565 in the cyanobacterial cell extracts. A: 10 ng of purified SLR1417 (lanes 1 and 3) and SLR1565 proteins (lanes 2 and 4) were analyzed by 17.5% SDS–PAGE, followed by Western blotting using the affinity-purified anti-SLR1417 antibody (left panel) and anti-SLR1417 antibody (right panel). B: The cyanobacterial cell extracts (lanes 1) were fractionated into cell debris (lanes 2), membranes (lanes 3) and the soluble fraction (100 μg, lanes 4). These fractions were subjected to Western blotting with the anti-SLR1417 antibody (upper panel) and anti-SLR1565 antibody (lower panel). 10 ng of purified SLR1417 (upper panel, lanes 5) and purified SLR1565 (lower panel, lane 5) were also analyzed. C: The cyanobacterial soluble proteins were fractionated by gel filtration and fractions 7–16 were subjected to Western blotting with the affinity-purified anti-SLR1417 antibody (upper panel) and with anti-SLR1565 antibody (lower panel). Molecular weight marker proteins are BSA (69 kDa), ovalbumin (43 kDa), FNR (35 kDa), myoglobin (17.6 kDa), and ribonuclease A (13.7 kDa). D: Fractions 9 (right panel) and 12 (left panel) were used for chemical cross-linking experiments and analyzed by Western blotting with anti-SLR1565 antibody (right panel) and anti-SLR1417 antibody (left panel), respectively. Samples incubated with the cross-linker DSS were loaded to lanes denoted by +. Asterisks denote cross-linked products. E: Same as in D except for the use of BS^3 instead of DSS.
←

er cross-linker, BS^3, resulted in a similar immunoreacted band (Fig. 2E, right panel). Since the calculated molecular mass of SLR1565 is 12.5 kDa, SLR1565 appears to form a complex with a protein about 30 kDa. No other immunoreactive cross-linked products were detected at around 25 kDa, or larger than 47.5 kDa, suggesting that the SLR1565 complex does not contain an SLR1565 dimer or other additional component.

When gel-filtration fraction 12, which contained mostly SLR1417, was incubated with DSS, the migration pattern of SLR1417 on SDS–PAGE was not affected and showed good agreement with its calculated monomeric molecular mass of 13 kDa (Fig. 2D, left panel). However, when another cross-linker, BS^3, was used, a band at 30 kDa was detected by Western blotting using anti-SLR1417 antibodies (Fig. 2E, left panel). Since the recombinant SLR1417 was eluted in the same fractions as the endogenous SLR1417 by gel filtration as described above and since reported IscA protein family members are known to form a dimer, SLR1417 appeared to exist as a dimer in the cyanobacterial cells. In the case of

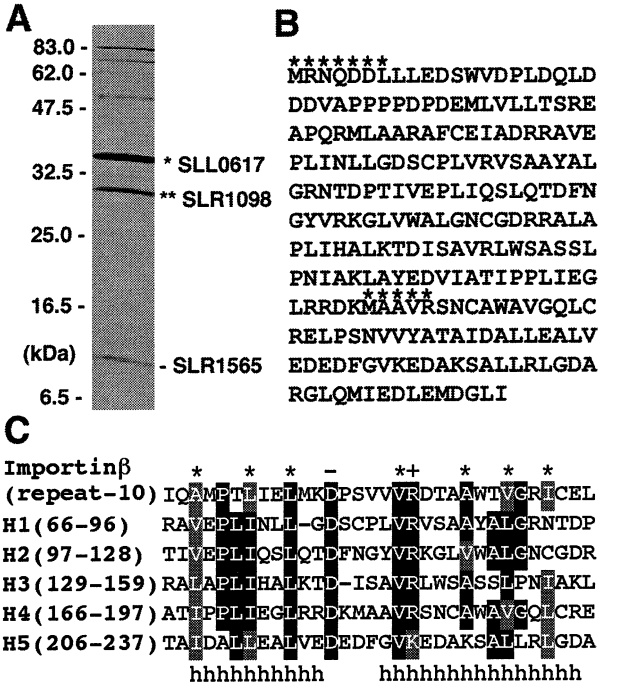

Fig. 3. Purification and identification of the SLR1565-interacting 30-kDa protein. A: The cyanobacterial soluble fraction was applied to anti-SLR1565 antibody-conjugated affinity column, and eluate was analyzed by SDS–PAGE. B: The deduced amino acid sequence of SLR1098. Asterisks correspond to regions whose amino acid sequences were identified by peptide sequencing. C: Alignment of HEAT-repeats found in SLR1098 (H1–H5) with a typical HEAT-repeat (named repeat-10) found in importin β (PDB 1QGK) [24]. h denotes the helix-forming sequences in the repeat-10.

purified recombinant SLR1565, we could observe a cross-linked band corresponding to the dimeric form of SLR1565 (data not shown), although such a cross-linked dimeric form of SLR1565 was hardly observed in the cyanobacterial lysates as shown in Fig. 2D. Thus, the solely expressed SLR1565 might form a dimer like SLR1417.

To identify the 30-kDa protein interacting with SLR1565, the cyanobacterial soluble fraction was applied to an anti-SLR1565 antibody-conjugated affinity column. In addition to SLR1565, two proteins with molecular masses of 30 kDa and 35 kDa were purified (Fig. 3A). The partial amino acid sequence of proteolytic fragments of these two candidate proteins was determined. The 35-kDa protein was found to be SLL0617, which is a cyanobacterial homolog of the chloroplast membrane-associated 30-kDa protein [23]. However, we concluded that this protein was not a true SLR1565-interacting protein for the following reasons: (1) SSL0617 could not form a protein complex with SLR1565 when the two proteins were co-expressed in *E. coli*, and (2) recombinant SSL0617 could bind to the anti-SLR1565 antibody even in the absence of SLR1565 (data not shown).

The affinity-purified 30-kDa protein contained the peptide sequences MRNQDDL and MAAVR, and was found to be SLR1098, a protein with no known function. SLR1098 is 246 amino acids long and has a calculated molecular weight of 26 968 Da (Fig. 3B). The amino acid sequence of SLR1098 is not homologous to any proteins with a known function, although HEAT-repeat motifs appear several times throughout the sequence (Fig. 3C). The HEAT motif was found initially in a rather diverse family of eukaryotic proteins including huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A, and importin β [24]. Although HEAT-repeat proteins are involved in many different cellular processes, a common function is thought to be mediation of protein–protein interactions. The canonical HEAT-repeat consists of two helices, which form a helical hairpin, and several hydrophobic and electrostatic interactions between the helices to stabilize the structural unit. Neighboring repeats stack together into a single domain with a continuous hydrophobic core. It would be interesting to determine if the HEAT-repeats in SLR1098 form a binding site for SLR1565 and/or for other protein substrate(s).

When SLR1565 and SLR1098 are co-expressed in *E. coli*, SLR1098 forms a stable complex with SLR1565 that can be purified to homogeneity (Fig. 4A). The protein band densities of SLR1565 and SLR1098 from the SDS–PAGE gel indicate that the complex forms with 1:1 stoichiometry. The UV/visible absorption spectrum of the purified SLR1565/SLR1098 complex closely resembles but is sharper than that of SLR1565 alone, suggesting the presence of a more stabilized [2Fe–2S] cluster (Fig. 4B). Upon gel filtration of the cyanobacterial soluble fraction, SLR1565 and SLR1098 co-eluted (Fig. 4C) with an estimated 1:1 stoichiometry (data not shown). There was no detectable SLR1565 or SLR1098 alone suggesting that all available protein goes to form the SLR1565/SLR1098 complex. The binding of SLR1098 to SLR1565 is specific because no such complex was formed when SLR1098 was co-expressed with SLR1417 in *E. coli* (data not shown).

In summary, the two cyanobacterial IscA homologs, SLR1417 and SLR1565, are molecularly distinct; SLR1417 binds predominantly the iron ion alone whereas SLR1565

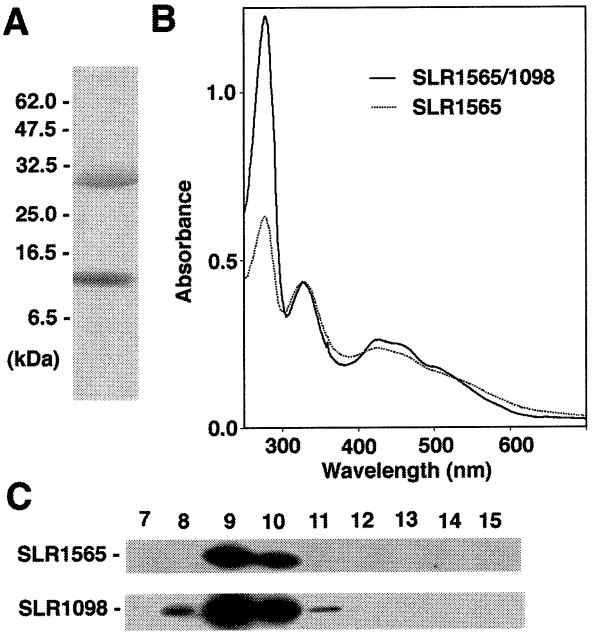

Fig. 4. Purification and characterization of the SLR1565/SLR1098 complex. A: SLR1098 and SLR1565 were co-expressed in the same *E. coli* cell and the recombinant protein complex was purified and analyzed by SDS–PAGE. B: UV/visible absorption spectrum of purified SLR1565/SLR1098 complex (plain line). That of SLR1565 is also shown (dotted line). C: Fractions 7–15 obtained from the similar gel filtration as shown in Fig. 2C were analyzed by Western blotting with the affinity-purified anti-SLR1098 antibody (lower panel) and with the anti-SLR1565 antibody (upper panel).

binds a [2Fe–2S] cluster and can also form a stable protein complex with the HEAT-repeats-containing protein SLR1098. These results suggest that the two IscA homologs have different physiological roles in the cyanobacterial cell. Although the degree of homology between SLR1417 and SLR1565 is similar to the homology between other bacterial IscA proteins encoded in the *isc* operons, only SLR1417 contains conserved short stretches of the sequence, GCSG and CGCG, in which the cysteine residues are thought to play an important functional role (Fig. 1A) [16,17,20]. This suggests that SLR1417 is more closely related to the IscA proteins encoded in the bacterial *isc* operon than SLR1565, and thus we speculate that SLR1565 may have evolved in the ancestral cyanobacteria to play a specialized role with SLR1098.

Based on the UV/visible spectra of purified SLR1565 and the SLR1565/SLR1098 complex, it was estimated that one [2Fe–2S] cluster was present per SLR1565 monomer or per complex, using the molar extinction coefficients at 422 nm derived from a typical [2Fe–2S] cluster containing ferredoxin. Since the two spectra were essentially similar, it appears that the [2Fe–2S] cluster binds to the SLR1565 moiety in the SLR1565/SLR1098 complex. While four conserved cysteinyl ligations are commonly found in [2Fe–2S] cluster-containing proteins, some variations are known, including the presence of histidine and aspartic acid which can serve as ligands for iron atoms [1,2]. In addition to three cysteine residues that are highly conserved among IscA homologs, SLR1565 possesses four histidine and several aspartate residues, one of which might serve as the fourth ligand for the [2Fe–2S] cluster (Fig. 1A).

A more detailed biochemical characterization of the two

IscA homologs and their possible involvement in iron–sulfur cluster biosynthesis in the cyanobacterial cell is now under investigation.

Acknowledgements: We thank M. Sugita (Nagoya University) for providing the *Synechocystis* PCC 6803 cells and Dr. S. Norioka and Dr. Y. Yoshimura (Osaka University) for peptide sequencing. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) and (C) and by a Grant-in-Aid for Encouragement of Young Scientists.

References

[1] Beinert, H., Holm, R.H. and Münck, E. (1997) Science 277, 653–659.
[2] Beinert, H. (2000) J. Biol. Inorg. Chem. 5, 2–15.
[3] Jacobson, M.R., Cash, V.L., Weiss, M.C., Laird, N.F., Newton, W.E. and Dean, D.R. (1989) Mol. Gen. Genet. 219, 49–57.
[4] Dean, D.R., Bolin, J.T. and Zheng, L. (1993) J. Bacteriol. 175, 6737–6744.
[5] Zheng, L., White, R.H., Cash, V.L., Jack, R.F. and Dean, D.R. (1993) Proc. Natl. Acad. Sci. USA 90, 2754–2758.
[6] Zheng, L., White, R.H., Cash, V.L., Jack, R.F. and Dean, D.R. (1994) Biochemistry 33, 4714–4720.
[7] Fu, W., Jack, R.F., Morgan, T.V., Dean, D.R. and Johnson, M.K. (1994) Biochemistry 33, 13455–13463.
[8] Yuvaniyama, P., Agar, J.N., Cash, V.L., Johnson, M.K. and Dean, D.R. (2000) Proc. Natl. Acad. Sci. USA 97, 599–604.
[9] Nakamura, Y., Kaneko, T., Hirosawa, M., Miyajima, N. and Tabata, S. (1998) Nucleic Acids Res. 26, 63–67.
[10] Nishio, K. and Nakai, M. (2000) J. Biol. Chem. 275, 22615–22618.
[11] Nakai, Y., Nakai, M., Hayashi, H. and Kagamiyama, H. (2001) J. Biol. Chem. 276, 8314–8320.
[12] Agar, J.N., Krebs, B., Frazzon, J., Huynh, B.H., Dean, D.R. and Johnson, M.K. (2000) Biochemistry 39, 7856–7862.
[13] Smith, A.D., Agar, J.N., Johnson, K.A., Frazzon, J., Amster, I.J., Dean, D.R. and Johnson, M.K. (2001) J. Am. Chem. Soc. 123, 11103–11104.
[14] Zheng, L., Cash, V.L., Flint, D.H. and Dean, D.R. (1998) J. Biol. Chem. 273, 13264–13272.
[15] Tokumoto, U. and Takahashi, Y. (2001) J. Biochem. 130, 63–71.
[16] Kaut, A., Lange, H., Diekert, K., Kispal, G. and Lill, R. (2000) J. Biol. Chem. 275, 15955–15961.
[17] Jensen, L.T. and Culotta, V.C. (2000) Mol. Cell. Biol. 20, 3918–3927.
[18] Pelzer, W., Mulenhoff, U., Diekert, K., Siegmund, K., Kispal, G. and Lill, R. (2000) FEBS Lett. 476, 134–139.
[19] Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y. and Fontecave, M. (2001) J. Biol. Chem. 276, 22604–22607.
[20] Krebs, C., Agar, J.N., Smith, A.D., Frazzon, J., Dean, D.R., Huynh, B.H. and Johnson, M.K. (2001) Biochemistry 40, 14069–14080.
[21] Nishio, K., Nakai, M. and Hase, T. (1999) in: Photosynthesis: Mechanisms and Effects (Garab, G., Ed.), pp. 3155–3158, Kluwer Academic Publishers, Dordrecht.
[22] Tagawa, K. and Arnon, D.I. (1968) Biochim. Biophys. Acta 153, 602–613.
[23] Westphal, S., Heins, L., Soll, J. and Vothknecht, U.C. (2001) Proc. Natl. Acad. Sci. USA 98, 4243–4248.
[24] Neuwald, A.F. and Hirano, T. (2000) Genome Res. 10, 1445–1452.