J. Biochem. **134**, 211–217 (2003)
DOI: 10.1093/jb/mvg131

A HEAT-Repeats Containing Protein, IaiH, Stabilizes the Iron-Sulfur Cluster Bound to the Cyanobacterial IscA Homologue, IscA2

Kozo Morimoto[1], Shusei Sato[2], Satoshi Tabata[2] and Masato Nakai[1,*]

[1]Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita 565-0871; and [2]Kazusa DNA Research Institute 1532-3 Yana, Kisarazu, Chiba 292-0812

Received March 29, 2003; accepted May 1, 2003

IscA homologues are involved in iron-sulfur cluster biosynthesis. In the non-nitrogen-fixing cyanobacterium *Synechocystis* PCC 6803, there are two IscA homologues, SLR1417 and SLR1565 (designated IscA1 and IscA2), of which only IscA2 exists as a protein complex with the HEAT-repeat-containing protein, SLR1098 (IaiH). We observed that the absorption spectrum of the recombinant IscA2/IaiH complex resembles that of IscA2 alone, although it is sharper. In the presence of dithiothreitol, the [2Fe-2S] cluster of IscA2 alone, but not of the IscA2/IaiH complex, became reductively labile upon the addition of sodium dithionite. This implies that the IscA2 moiety of the [2Fe-2S] cluster is stabilized by the presence of IaiH. The [2Fe-2S] cluster of the IscA2/IaiH complex was destabilized by sodium dithionite in the absence of dithiothreitol, suggesting that the *in vivo* stability of the iron-sulfur cluster in the IscA2/IaiH complex is influenced by the redox state of cellular thiols. When any one of three conserved cysteine residues in IscA2, potential ligands for the [2Fe-2S] cluster, was replaced with serine, the amount of assembled [2Fe-2S] cluster and protein complex was significantly reduced in *E. coli* cells. The cysteine mutated IscA2/IaiH complexes that were present all contained a [2Fe-2S]-like cluster suggesting that the assembly of a stable iron-sulfur cluster bound to IscA2 is required for efficient and stable complex formation. Truncated IaiH proteins were analyzed using the yeast two-hybrid assay to identify the essential domain of IaiH that interacts physically with IscA2. At least 2 of the 5 N-terminal HEAT repeats of IaiH were found to be required for interaction with IscA2.

Key words: biosynthesis, cyanobacteria, HEAT-repeats, iron-sulfur cluster, *Synechocystis*.

IscA, together with other Isc proteins, has been identified in a wide range of organisms, and participates in the process of iron-sulfur cluster biosynthesis (*1*). Although gene disruption studies in *Escherichia coli (E. coli)* (*2*) and *Saccharomyces cerevisiae* (*3–5*) have demonstrated an important, albeit nonessential, role for IscA proteins in iron-sulfur cluster biosynthesis, the precise role of these proteins is unclear.

IscA is about 110 amino acid residues in length and contains three evolutionally conserved cysteine residues thought to bind iron or iron-sulfur clusters. Ollagnier-de-Choudens, et al. purified IscA from recombinant *E. coli* in a colorless metal-free form (*6*). This apo-IscA protein was shown to assemble an air-labile [2Fe-2S] cluster under anaerobic conditions, and the resultant holo-IscA, but not the apo-IscA, was able to form a complex with the holo-Fdx protein, a [2Fe-2S]-containing ferredoxin encoded in the *isc* operon. Krebs et al described the purification and characterization of an IscA homologue encoded in the *nif* operon of the nitrogen-fixing bacterium *Azotobacter vinelandii* (*7*). When this homologue, termed ^{Nif}IscA, was purified from recombinant *E. coli*, it was also colorless and metal-free. Further investigation revealed that ^{Nif}IscA could bind the ferrous ion in a tetrahedral, predominantly cysteinyl-ligated, coordination environment. In addition, like the previously identified IscA, ^{Nif}IscA was able to assemble an air-labile [2Fe-2S] cluster under anaerobic conditions. They also found that the [2Fe-2S] cluster could be converted to a [4Fe-4S] cluster *in vitro*, although the resultant [4Fe-4S] cluster was extremely labile even under anaerobic conditions. The IscA homologues of *E. coli* and *A. vinelandii* are thought to act as alternative scaffold proteins to NifU or IscU for iron-sulfur cluster biosynthesis (*6*, *7*).

Genome analyses of different organisms have revealed the presence of IscA isologues, even in a same organism. For example, *E. coli* contains at least three IscA isologues and yeast mitochondria contain two IscA isologues, Isa1p and Isa2p. Disruption of either Isa1p or Isa2p results in respiratory deficiency (*3*). The disruption of both genes did not produce an additive effect suggesting that these IscA homologues are not redundant in yeast. Functional differences, similarities or cooperation between the two yeast IscA homologues, or among other IscA isologues, have not been determined.

We reported recently that the non-nitrogen-fixing cyanobacterium, *Synechocystis* PCC 6803, contains two IscA homologues, SLR1417 and SLR1565 (designated IscA1 and IscA2) (*8*, *9*). The absorption spectrum of purified recombinant IscA2 is typical of [2Fe-2S]-cluster-containing proteins, whereas the absorption spectrum of IscA1 shows predominantly the presence of the iron ion

*To whom correspondence should be addressed. Tel: +81-6-6879-8612, Fax: +81-6-6879-8613, E-mail: nakai@protein.osaka-u.ac.jp

(*9*). Although both IscA1 and IscA2 localize in the soluble fraction of the cyanobacterial cell and represent about 0.01% of soluble cellular proteins, their molecular states are remarkably different with IscA1 residing in the cell as a homo-dimer and IscA2 existing as a complex with another 30-kDa protein. This 30-kDa protein was identified as SLR1098 (termed IaiH for IscA-interacting Heat-repeats-containing protein), the function of which has not been elucidated. When IscA2 and IaiH were co-expressed in *E. coli*, they formed a stable complex with 1:1 stoichiometry and exhibited the UV-visible spectral characteristic of [2Fe-2S] cluster-containing iron-sulfur proteins. The stable nature of the [2Fe-2S] cluster bound to IscA2 and IscA2/IaiH complexes is in stark contrast to the extremely labile nature of the reconstituted iron-sulfur cluster bound to IscA from *E. coli* or *A. vinelandii* (*6*, *7*). In the present study, we characterized the purified IscA2/IaiH complex and identified a HEAT-repeats domain in IaiH required for complex formation with IscA2. We also used amino acid substitutions to investigate the role of the three conserved cysteine residues of IscA2 in [2Fe-2S]-cluster binding and IaiH complex formation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The *E. coli* strain TG1 was used as the host for plasmid propagations. *E. coli* strain BL-21(DE3)RIL (Stratagene) was used for the over-expression of recombinant proteins. Both *E. coli* strains were grown using standard procedures in LB-liquid or agar-solidified medium with appropriate antibiotics. The expression and purification of recombinant proteins have been described (*9*, *10*).

Spectroscopic Methods—UV-visible absorption spectra of proteins were recorded using a UV-2500PC UV-visible recording spectrophotometer (Shimadzu). The concentration of the [2Fe-2S] clusters bound to IscA2, the IscA2/IaiH complex, or ferredoxin (Fd) were estimated using the molar extinction coefficient 9.68 mM^{-1} cm^{-1} at 422 nm as described previously (*9*).

Spectroscopic changes in the presence of a metal chelator, ethylenediaminetetraacetic acid (EDTA), were analyzed as follows: Purified IscA2 or IscA2/IaiH complexes were prepared in 500 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM dithiothreitol (DTT) and 10 mM EDTA, to give a final protein concentration of about 10 μM. The mixture was then divided into aliquots and the UV-visible absorption spectra of each aliquot was recorded at 0, 30, and 60 min. Spectroscopic changes in the presence of the reducing agent, sodium dithionite ($Na_2S_2O_4$), were analyzed as follows: Purified ferredoxin, IscA2 or IscA2/IaiH complex was prepared in 100 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, with or without 5 mM DTT, to give a protein concentration of about 10 μM. UV-visible absorption spectra of the basic states of these proteins were recorded first. The reduced state was analyzed following the addition of 1 μl of 100 mM sodium dithionite to the protein solution. Oxidized states were then analyzed after exposure to air for 20 min.

Yeast Two-Hybrid Analysis of IscA2 and Various Truncated Fragments of IaiH—The *iscA2* gene was PCR-amplified from *Synechocystis* PCC6803 genomic DNA, and cloned as an *EcoRI*/*Pst*I fragment into pAS2-1, a bait vector for yeast two-hybrid analysis (Clontech). The resultant plasmid was called pAS2-1/*iscA2*. The prey vector for two-hybrid assay derivatives, pACT2 (Clontech), was used to construct truncated *IaiH* derivatives. Intact *IaiH* and several truncated *IaiH* fragments were amplified independently by PCR from cyanobacterial genomic DNA. Each PCR product was cloned into pACT2. Individual pACT2 derivatives, along with pAS2-1, were co-transformed into the yeast strain PJ69-4A. Co-transformed yeast cells were transferred onto histidine-lacking synthetic media supplemented with 5 mM 3-aminotriazole (3-AT), and grown at 30°C for 4 days for analysis of hybrid formation. Yeast two-hybrid analyses were carried out essentially as described in the Yeast Protocols Handbook (Clontech). The two-hybrid assay was also used to evaluate mutated *iscA2* genes cloned into pAS2-1.

Site-Directed Mutagenesis of IscA2—Site-directed mutagenesis of IscA2 was performed using the PCR-based QuikChange™ site-directed mutagenesis kit (Stratagene). Plasmids carrying the desired amino acid substitutions were confirmed by DNA sequencing with the BigDye™ terminator cycling sequencing ready reaction (ABI Prism) and used to transform *E. coli* strain BL-21(DE3)RIL cells with or without pET/*IaiH*. The resultant transformed cells were spotted onto a nitrocellulose membrane filter (Millipore) placed on a LB-agar solid medium supplemented with appropriate antibiotics, 0.3 mg/ml ammonium iron citrate, and 1 mM L-cysteine. Cells were grown at 37°C for 16 h, and then the expression of recombinant proteins was induced by transferring the membrane onto LB-agar solid medium supplemented with 0.4 mM IPTG. Cells were then grown for an additional 16 h at 20°C.

Other Methods—Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Western blot analysis was carried out using HRP-Protein A (Zymed) as a secondary antibody and the ECL™ detection system (Amersham Pharmacia) (*11*, *12*).

RESULTS

Effect of EDTA on the Spectroscopic Properties of IscA2 and the IscA2/IaiH Complex—As reported previously, IscA2 and the IscA2/IaiH complex can bind [2Fe-2S] clusters and have a characteristic UV-visible absorption peak at approximately 330 nm and lower broad peaks at around 420 nm and 460 nm. Stably assembled [2Fe-2S] clusters, such as those bound to photosynthetic ferredoxin, are difficult to disrupt with the metal chelator EDTA (data not shown). In contrast, the cyanobacterial NifU-like protein, now termed CnfU for a protein homologous to the carboxy-terminal domain of NifU and proposed as a major scaffold protein for iron-sulfur cluster biosynthesis in cyanobacteria, carries a labile [2Fe-2S] cluster that is sensitive to chelating agents such as EDTA (*10*). To determine whether the [2Fe-2S] cluster bound by IscA2 or the IscA2/IaiH complex is stable or labile in the presence of a metal chelator, we analyzed purified proteins in the presence of EDTA. When purified IscA2 was incubated with 10 mM EDTA for 60 min at 25°C, the intensity of the visible absorption spectrum characteristic of the [2Fe-2S] cluster decreased slightly

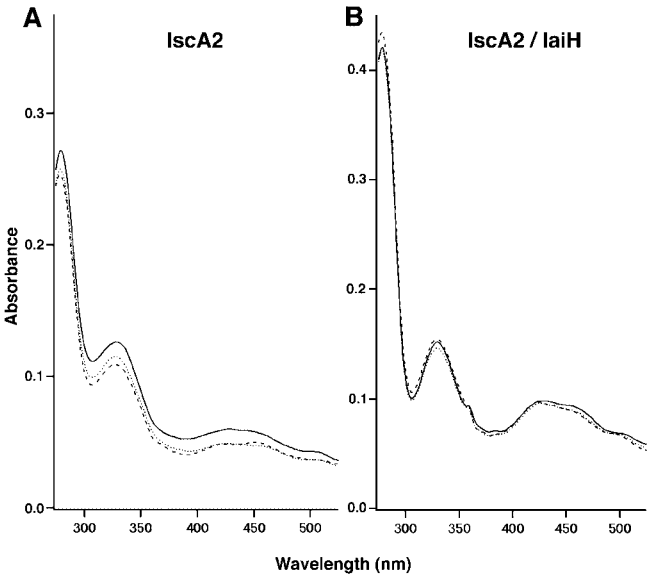

Fig. 1. **Effect of EDTA on the spectroscopic properties of IscA2 and the IscA2/IaiH complex.** Samples containing either purified IscA2 (A) or IscA2/IaiH complex (B) were prepared in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, and 10 mM EDTA, to give a final protein or protein complex concentration of about 10 μM. UV-visible absorption spectra were recorded at 0 min (plain lines), and after 30 min (dotted lines) and 60 min (dashed lines).

(Fig. 1A). By comparison, the absorption spectrum of the IscA2/IaiH complex was unaffected by the addition of EDTA (Fig. 1B). Therefore, unlike the [2Fe-2S] cluster bound to CnfU, the [2Fe-2S] cluster bound by IscA2 is stable in the presence of a metal chelator, and this stability is greater in the presence of IaiH.

Effect of Sodium Dithionite on the Spectroscopic Properties of IscA2 and the IscA2/IaiH Complex—To analyze whether [2Fe-2S] clusters bound to IscA2 or the IscA2/IaiH complex are redox-active or reductively labile, spectroscopic changes were monitored after the addition of a powerful reducing agent, sodium dithionite. As shown in Fig. 2A, the absorption spectrum of ferredoxin (Fd), a cyanobacterial photosynthetic protein typical of [2Fe-2S] cluster-containing electron carrier proteins, disappears at 420–460 nm in the presence of excess dithionite. This is due to the reduction of all bound [2Fe-2S] clusters. The original absorption spectrum, characteristic of the presence of oxidized [2Fe-2S] clusters, can be regained by air-oxidation. The absorption change observed at around 280 nm is due to the presence of the reduced form of excess dithionite. The absorption spectrum of IscA2 at 420–460 nm also dropped off drastically in a few seconds following the addition of dithionite (Fig. 2B). In this case, air-oxidation could not recover the original absorption spectrum. In the absence of dithionite, the [2Fe-2S] cluster bound to IscA2 was resistant to air-oxidation (data not shown). These data show that the [2Fe-2S] cluster bound to IscA2 is removed or disassociated from the protein upon reduction by dithionite.

The effect of IaiH in the presence of dithionite on the stability of the [2Fe-2S] cluster bound to IscA2 was then analyzed. As shown in Fig. 2C, the absorption spectrum

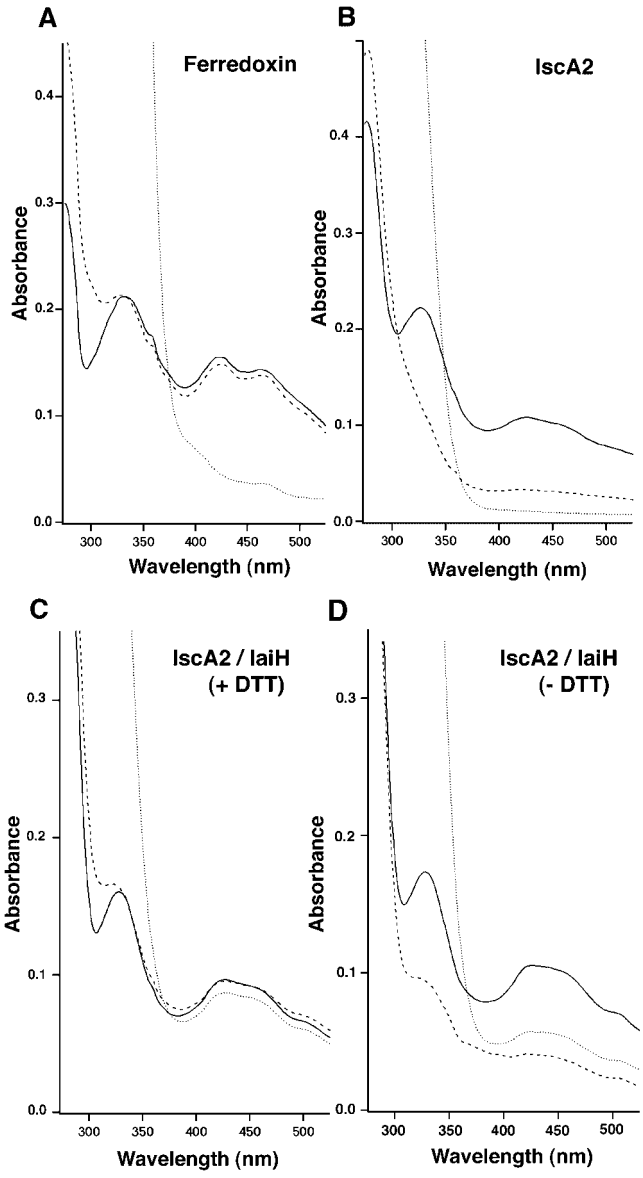

Fig. 2. **Effect of sodium dithionite on the spectroscopic properties of IscA2 and the IscA2/IaiH complex.** Samples containing either purified ferredoxin (PetF; SSL0020) (A), IscA2 (B) or IscA2/IaiH complex (C) were prepared in 100μl of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 5 mM DTT, to give a final protein or protein complex concentration of about 10 μM. UV-visible absorption spectra were recorded initially to generate a base line (solid lines). Then, 1 μl of 100 mM sodium dithionite ($Na_2S_2O_4$) was added to the mixture. Immediately following gentle, but thorough mixing, the absorption spectra of the reduced state were recorded (dashed lines). Absorption spectra of the oxidized state (dotted lines) were recorded following oxidation of the mixture, achieved by exposing the mixture to air and mixing thoroughly. Spectra of the IscA2/IaiH complex shown in (D) were recorded as in (C) with DTT omitted from the buffer.

of the [2Fe-2S] cluster-containing IscA2/IaiH complex at 420–460 nm was essentially unaffected by dithionite or by subsequent air-oxidation. Although sodium dithionite is a small inorganic compound (Mw = 174.11), it is possible that the [2Fe-2S] cluster bound to the IscA2 moiety of the IscA2/IaiH complex is largely covered by IaiH render-

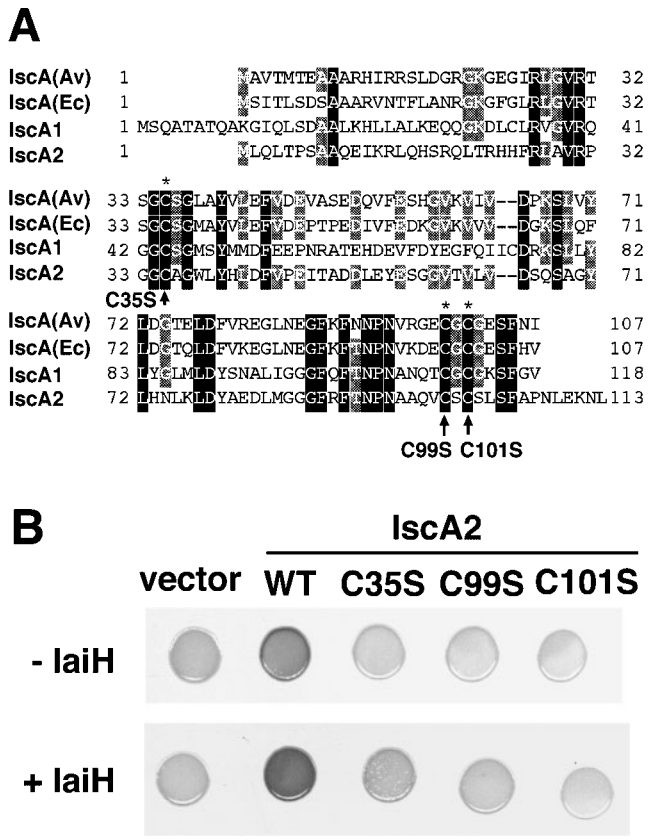

Fig. 3. **Iron-sulfur cluster assembly in wildtype and cysteine-substituted mutant IscA2 proteins in *E. coli*.** A: Alignment of IscA proteins from *Azotobacter vinelandii* (Av), *E. coli* (Ec), and *Synechocystis* PCC6803 (IscA1 and IscA2). Arrows indicate positions of amino acid substitutions in IscA2. B: Formation of holo-IscA2 in *E. coli* was analyzed as described in Experimental Procedures. Vector alone (vector) or plasmid encoding either the wildtype (WT) or cysteine-substituted IscA2 (C35S, C99S, C101S) was transformed into *E. coli* with (+IaiH) or without (–IaiH) a co-expression plasmid for IaiH. Transformed cells were spotted onto a nitrocellulose membrane layered on solid LB-medium supplemented with iron and cysteine. The expression of IscA2 and IaiH was induced by transferring the membrane onto LB-media containing 0.4 mM IPTG.

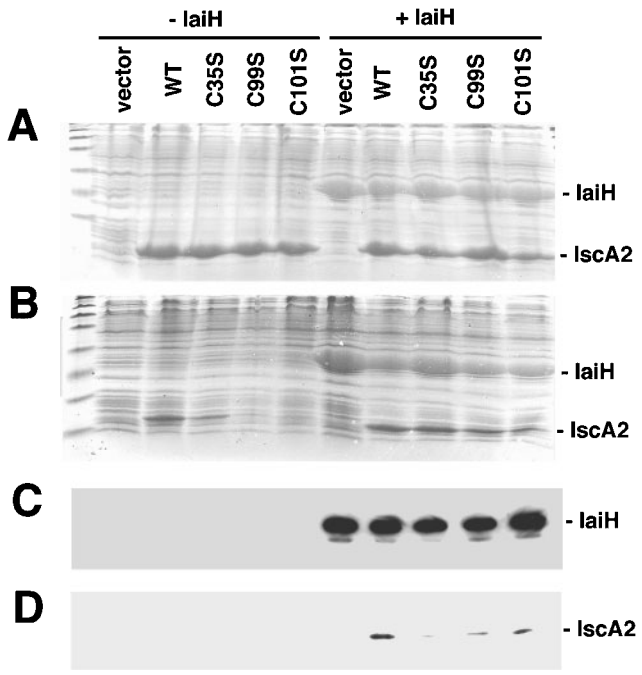

Fig. 4. **Complex formation between IaiH and various IscA2 proteins as analyzed by immunoprecipitation.** Various transformed *E. coli* cells used in the experiments shown in Fig. 3 were cultivated in liquid LB-medium supplemented with iron and cysteine, and the expression of IaiH and IscA2 proteins was induced by the addition of 0.4mM IPTG. After further cultivation for 16 h at 20°C, the cells were harvested, ruptured by sonication, and fractionated into soluble and membrane fractions. Total cell lysates (A) and soluble fractions (B) were analyzed by staining with Coomassie Brilliant Blue following SDS-PAGE. Soluble fractions were used for immunoprecipitation with purified anti-IaiH antibodies, and precipitated proteins were analyzed by Western blotting either with anti-IaiH antibodies (C) or anti-IscA2 antibodies (D).

ing the [2Fe-2S] cluster-binding pocket inaccessible. Alternatively, IaiH may mechanically stabilize the binding of the [2Fe-2S] cluster to IscA2 thereby lowering the redox potential of the iron sulfur cluster and the effectiveness of dithionite in reducing the [2Fe-2S] cluster.

All experiments described above were carried out in the presence of 5 mM DTT. Omitting DTT resulted in the [2Fe-2S] cluster bound to the IscA2/IaiH complex becoming reductively labile (Fig. 2D). Possible explanations for this phenomenon will be discussed later.

Site-Directed Mutagenesis of IscA2—IscA has three conserved cysteine residues, which are the best candidates for binding the [2Fe-2S] clusters (*3*, *5*, *7*). Cyanobacterial IscA2 also has three conserved cysteine residues, Cys35, Cys99, and Cys101, and there are no other cysteine residues in the sequence (Fig. 3A). To investigate the role of these cysteine residues, we used site-directed mutagenesis to convert each cysteine residue to a serine residue as described in Experimental Procedures. Resultant IscA2 mutants were called C35S, C99S, and C101S, with the S referring to the replacement of the serine residue at positions 35, 99, and 101, respectively. These mutants were expressed independently in *E. coli*.

Analysis of the color of transformed *E. coli* colonies was used as an initial assessment of the [2Fe-2S] cluster-binding ability of each IscA2 mutant. As shown in Fig. 3B, the expression of wildtype IscA2 in *E. coli* resulted in brown red colonies, even in the absence of IaiH, and colonies expressing the IscA2 mutant proteins were colorless, apart from C35S IscA2, which turned a very pale pink. Although the expression levels of the mutant IscA2 proteins were similar to that of wildtype IscA2 (Fig. 4A), most of the mutant proteins were not recovered in a soluble form (Fig. 4B).

Co-expression of IaiH resulted in colonies that expressed the wildtype IscA2 protein being a deeper red brown color (Fig. 3B). The presence of co-expressed IaiH had no effect on the color of mutant IscA2 colonies (Fig. 3B), but greatly enhanced the solubility of all mutant IscA2 proteins (Fig. 4, A and B).

Physical interactions between IaiH and the IscA2 mutants were confirmed by immunoprecipitation (Fig. 4, C and D). Although all IscA2 mutants could form complexes with IaiH, the efficiency of complex formation was

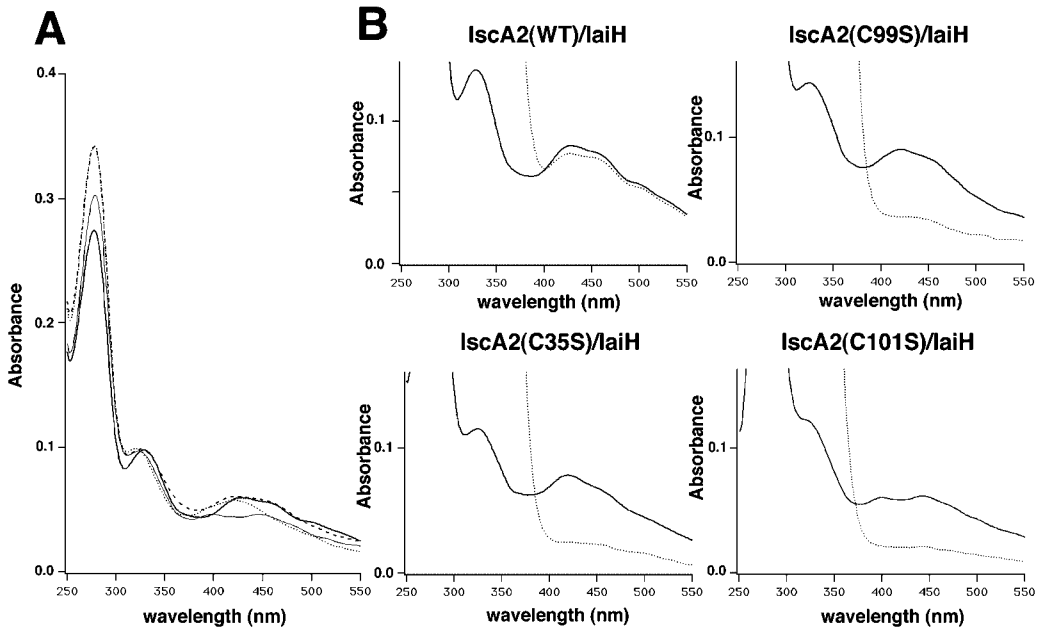

Fig. 5. **UV/visible absorption spectra of purified mutant IscA2/IaiH complexes.** A: Various IscA2/IaiH proteins were purified as described previously (*9*). UV/visible absorption spectra of purified wildtype IscA2/IaiH (solid line), IscA2(C35S)/IaiH (dotted line), IscA2(C99S)/IaiH (dashed line), and IscA2(C101S)/IaiH (thin line) complexes were adjusted using the absorption values at 330 nm and compared. B: Changes in the absorption spectra of various IscA2/IaiH complexes as indicated were monitored before (solid lines) and after (dotted lines) reduction by the addition of 1mM sodium dithionite as described in the legend to Fig. 2.

low. This was confirmed by yeast two-hybrid analyses as described below (Fig. 6B).

Protein complexes of IaiH and IscA2 mutants were purified. Although significant levels of individual proteins were present in the extracts, the recovery of mutant complexes was low, implying that these complexes are either unstable or inefficiently assembled in *E. coli*. Fig 5A shows that all purified mutant complexes displayed absorption spectra similar to [2Fe-2S] clusters. The absorption spectra at 420–460 nm of the C35S and C101S IscA2/IaiH complexes were obviously affected by the cysteine substitutions, whereas the absorption spectrum of the C99S IscA2/IaiH complex rather resembled that of the wildtype complex. However, by contrast with the [2Fe-2S] cluster of wild-type complex, all [2Fe-2S]-like clusters bound to the mutant complexes, including C99S IscA2/IaiH, were reductively labile even in the presence of DTT (Fig. 5B).

These results suggest that substituting serine for cysteine either decreases the efficiency of [2Fe-2S] cluster assembly or renders the assembled cluster unstable. For this reason we conclude that all three conserved cysteine residues are important for [2Fe-2S] cluster assembly, and most likely act as ligands for iron-sulfur cluster binding. However we could not totally exclude the possibility that these substitutions might have an indirect effect (*e.g.* improper folding of the IscA2 molecule) that causes inefficient [2Fe-2S] cluster binding. In any case, without the assembled iron-sulfur cluster, IscA2 and IaiH are unable to form a stable complex. This result was also confirmed by yeast two-hybrid analyses (Fig. 6, A and B).

Yeast Two-Hybrid Analysis between Mutant IscA2 Proteins and IaiH—The physical interaction between IscA2 and IaiH was first identified by yeast two-hybrid analysis in a genome-wide survey of interactions among thousands of cyanobacterial proteins (Sato, S. and Tabata, S., unpublished results). The DNA fragment encoding IaiH was fused in frame to the coding sequence of the GAL4 transcriptional activation domain on pACT2, and the gene for wildtype or mutated IscA2 was fused in frame to the coding sequence of the GAL4 DNA-binding domain on pAS2-1 (Fig. 6A). Derivatives of pACT2 and pAS2-1 were then co-transformed into the host yeast cells. An interaction between IaiH and IscA2 will lead to autotrophic growth of the yeast on histidine-omitted media. Figure 6D shows that yeast cells expressing either IaiH or IscA2 do not display histidine-autotrophic growth, but those expressing full-length IaiH and IscA2 do display histidine-autotrophy. This result shows that IaiH and IscA2 interact physically. Figure 6B shows that only wildtype IscA2 could form a complex allowing the transcriptional activation of *HIS3*. Mutant IscA2 proteins do not allow the [2Fe-2S] cluster to be assembled or retained, thereby inhibiting the formation of a stable complex with IaiH.

Yeast Two-Hybrid Analysis between IscA2 and Truncated IaiH—IaiH is 246 amino acid residues long (Mw = *ca.* 27 k) and shows no strong sequence similarities to proteins with known functions. As described previously, *in silico* analysis of the IaiH amino acid sequence revealed the presence of HEAT-repeats throughout the molecule (*9*). Figure 6C shows 5 and a half HEAT motifs in tandem repeat following the extra 50 residues on the N-terminus; these are designated H0–H5. The HEAT-repeats was initially found in a diverse family of eukaryotic proteins that includes huntington, elongation factor 3, the PR65/A subunit of protein phosphatase 2A, the lipid kinase TOR, and importin β (*13*, *14*). Although

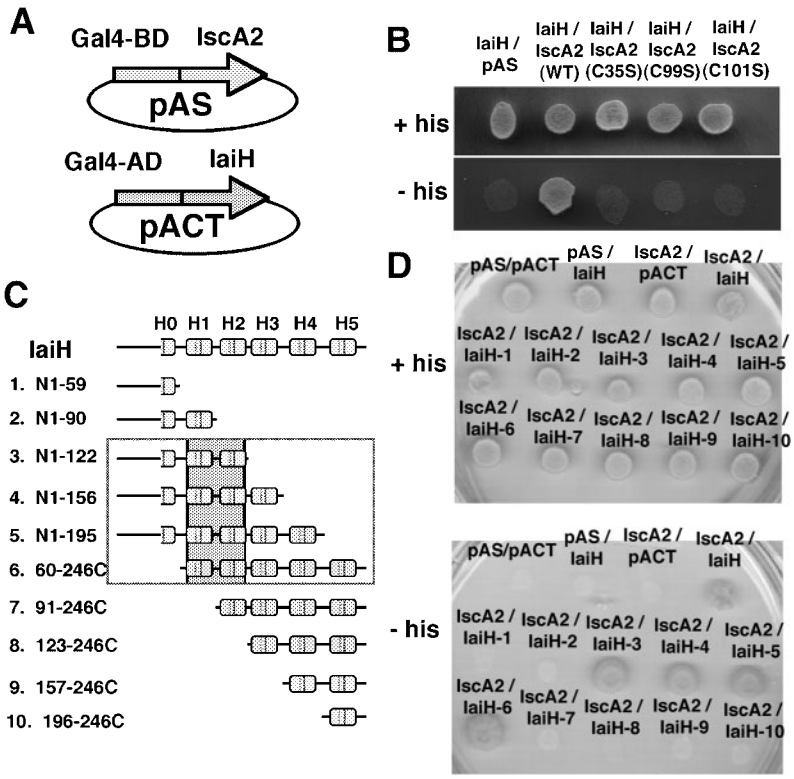

Fig. 6. **Yeast two-hybrid analysis of the interaction between IscA2 and IaiH proteins.** A: Schematic representations of pAS and pACT plasmids used for the yeast two-hybrid analysis of the interaction between IscA2 and IaiH variants. B: Various pAS derivatives, each encoding wildtype IscA2 or one of the cysteine-substituted IscA2 mutants, as indicated, were co-transformed with the wildtype IaiH-encoding pACT derivative. All co-transformed yeast cells grew on medium with added histidine (+his). Only wildtype IscA2 was able to interact with IaiH sufficiently to allow growth of the co-transformed yeast cells on medium lacking histidine (–his). C: Various truncated IaiH proteins were designed so each contained an N-terminal or C-terminal fragment; IaiH-1 (N1–59), IaiH-2 (N1–90), IaiH-3 (N1–122), IaiH-4 (N1–156), IaiH-5 (N1–195), IaiH-6 (60–246C), IaiH-7 (91–246C), IaiH-8 (123–246C), IaiH-9 (157–246C), and IaiH-10 (196–246C). D: All co-transformed yeast cells grew on medium with added histidine (+his). Only the N1–122, N1–156, N1–195, 60–246C, and wildtype (IaiH) constructs were able to interact with IscA2 sufficiently to allow growth of the co-transformed yeast cells on medium lacking histidine (–his).

HEAT-repeats containing proteins are involved in a great variety of cellular processes, the mediation of protein-protein interactions is thought to be a common function. For example, importin β binds to various protein substrates destined for nuclear import, and the PR65/A subunit of protein phosphatase 2A interacts with various regulatory subunits of PP2A. The crystal structures of several proteins containing HEAT repeats have already been determined (*13*, *14*). Structural information has revealed that the canonical HEAT-repeat consists of two helices forming a helical hairpin. Between the two antiparallel helices, one electrostatic interaction and several hydrophobic interactions play important roles in stabilizing this structural unit. Neighboring repeats stack together into a single domain with a continuous hydrophobic core, forming an elongated curlicue-like or solenoid-like super-helix. The resultant concave surface of the super-helix in the HEAT repeats-containing protein is known to serve as a ligand-binding domain.

The interaction of IscA2 with IaiH may occur via some or all of these HEAT-repeats. Yeast two-hybrid analysis was performed to investigate this possibility. Figure 6C shows the various truncated IaiH proteins constructed with different N-terminal or C-terminal fragments, including N1–59, N1–90, N1–122, N1–156, N1–195, 60–246C, 91–246C, 123–246C, 157–246C, and 196–246C, where numberings correspond to the amino acid residues of IaiH. DNA fragments were constructed as described in Fig. 6A and yeast two hybrid analysis was performed as described above.

Among the fusion constructs containing truncated IaiH domains, only N1–122, N1–156, N1–195, and 60–246C fragments were found to interact with IscA2. These fragments contain amino acid residues 60 to 122, a region containing two HEAT-repeats designated H1 and H2. We conclude that the two HEAT repeats H1 and H2 of IaiH are the minimum requirement for the interaction of this protein with IscA2.

DISCUSSION

In this study we demonstrate that IscA2 forms a stable protein complex with the HEAT repeats-containing protein, IaiH, which functions to stabilize the [2Fe-2S] cluster bound to IscA2. Three evolutionarily conserved cysteine residues in IscA2 are important for efficient [2Fe-2S] cluster binding, and the stable retention of the iron-sulfur cluster bound to IscA2 is required for the tight interaction with IaiH. Normally, four conserved cysteine residues are found in [2Fe-2S] cluster-containing proteins, although histidine or aspartic acid residues can also serve as ligands for [2Fe-2S] cluster binding. IscA2 possesses four histidine and several aspartate residues, one of which might serve as the fourth ligand for [2Fe-2S] cluster binding. It is also possible that an amino acid residue, such as cysteine, in IaiH acts as a ligand for [2Fe-2S] cluster binding in the IscA2/IaiH complex. However, this possibility is unlikely because purified IscA2 alone shows the characteristic [2Fe-2S]-type absorption spectrum that is essentially similar to that of the IscA2/IaiH complex. Further investigation is necessary to define other residues in IscA2 that act as ligands for [2Fe-2S] cluster binding.

The physiological role of the IscA2/IaiH complex is unknown. IscA proteins from other organisms (*6*, *7*), such as IscU and NifU-like proteins (*10*, *15–17*), function as scaffold proteins in iron-sulfur cluster assembly. The iron-sulfur cluster bound to these proteins is extremely

labile, suggesting that the cluster may be preformed transiently on IscA and transferred either directly or indirectly to an apoprotein. IscA1, another cyanobacterial IscA homologue, may function in such a way. By contrast, the iron-sulfur cluster bound to cyanobacterial IscA2 is stable and is further stabilized when complexed with IaiH. Data from our previous experiments showed that nearly all cellular IscA2 and IaiH exist in IscA2/IaiH complexes. In the present study there was virtually no stable complex formed between apo-IscA2 and IaiH *in vitro*, suggesting that all cellular IscA2/IaiH complexes bind an iron-sulfur cluster. The stable nature of the holo-IscA2/IaiH complex implies that cyanobacterial IscA2 may be involved in physiological functions other than iron-sulfur cluster delivery. It is also possible that the [2Fe-2S] cluster bound to the IscA2/IaiH complex becomes transferable following other protein interactions. This interaction may be via the remaining HEAT-repeats domain of IaiH, which leads to the formation of a transient tertiary complex and transfer of the iron-sulfur cluster. The possibility that the holo-IscA2/IaiH complex functions as a redox electron carrier is unlikely because we found that the iron-sulfur cluster bound to the IscA2/IaiH complex is not reduced by dithionite *in vitro*. Instead, the iron-sulfur cluster became labile when the complex was incubated with dithionite in the absence of DTT, a thiol reductant. It is possible that the iron-sulfur cluster can be destabilized and transferred when the redox state of cellular thiols is largely oxidized. Alternatively, the iron-sulfur cluster bound to IscA2 could be a molecular sensor by which the redox state of cellular thiols is monitored. These questions may be answered in further investigations that aim to identify and characterize proteins that interact with the IscA2/IaiH complex. We are also currently investigating the physiological role of the IscA2/IaiH complex by analyzing cyanobacterial mutants in which one or both genes have been disrupted.

We thank Dr. K. Nishio (Osaka University) for technical assistance and useful suggestions in the initial stage of this study. 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. Lill, R. and Kispal, G. (2000) Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem. Sci.* **25**, 352–356
2. Tokumoto, U. and Takahashi, Y. (2001) Genetic analysis of the *isc* operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. *J. Biochem.* **130**, 63–71
3. Jensen, L.T. and Culotta, V.C. (2000) Role of *Saccharomyces cerevisiae* ISA1 and ISA2 in iron homeostasis. *Mol. Cell. Biol.* **20**, 3918–3927
4. Pelzer, W., Mulenhoff, U., Diekert, K., Siegmund, K., Kispal, G., and Lill, R. (2000) Mitochondrial Isa2p plays a crucial role in the maturation of cellular iron-sulfur proteins. *FEBS Lett.* **476**, 134–139
5. Kaut, A., Lange, H., Diekert, K., Kispal, G., and Lill, R. (2000) Isa1p is a component of the mitochondrial machinery for maturation of cellular iron-sulfur proteins and requires conserved cysteine residues for function. *J. Biol. Chem.* **275**, 15955–15961
6. Ollagnier-de-Choudens, S., Mattioli, T., Takahashi, Y., and Fontecave, M. (2001) Iron-sulfur cluster assembly: characterization of IscA and evidence for a specific and functional complex with ferredoxin. *J. Biol. Chem.* **276**, 22604–22607
7. Krebs, C., Agar, J.N., Smith, A.D., Frazzon, J., Dean, D.R., Huynh, B.H., and Johnson, M.K. (2001) IscA, an alternate scaffold for Fe-S cluster biosynthesis *Biochemistry* **40**, 14069–14080
8. Nakamura, Y., Kaneko, T., Hirosawa, M., Miyajima, N., and Tabata, S. (1998) CyanoBase, a www database containing the complete nucleotide sequence of the genome of Synechocystis sp. strain PCC6803. *Nucleic Acids Res.* **26**, 63–67
9. Morimoto, K., Nishio, K., and Nakai, M. (2002) Identification of a novel prokaryotic HEAT-repeats-containing protein which interacts with a cyanobacterial IscA homolog. *FEBS Lett.* **519**, 123–127
10. Nishio, K. and Nakai, M. (2000) Transfer of iron-sulfur cluster from NifU to apoferredoxin. *J. Biol. Chem.* **275**, 22615–22618
11. Nishio, K., Nakai, M., and Hase, T. (1999) Fe-S cluster formation of ferredoxin in chloroplast stroma in *Photosynthesis: Mechanisms and Effects* (Garab, G., ed.) pp. 3155–3158, Kluwer Academic Publishers, Dordrecht
12. Nakai, Y., Nakai, M., Hayashi, H., and Kagamiyama, H. (2001) Nuclear localization of yeast Nfs1p is required for cell survival. *J. Biol. Chem.* **276**, 8314–8320
13. Neuwald, A.F. Hirano, T. (2000) HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. *Genome Res.* **10**, 1445–1452
14. Andrade, M.A., Petosa, C., O'Donoghue, S.I., Müller, C.W., and Bork, P. (2001) Comparison of ARM and HEAT protein repeats. *J Mol. Biol.* **309**, 1–18
15. Yuvaniyama, P., Agar, J.N., Cash, V.L., Johnson, M.K., and Dean, D.R. (2000) *Proc. Natl Acad. Sci. USA* **97**, 599–604
16. Agar, J.N., Krebs, B., Frazzon, J., Huynh, B.H., Dean, D.R., and Johonson, M.K. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Biochemistry* **39**, 7856–7862
17. Smith, A.D., Agar, J.N., Johnson, K.A., Frazzon, J., Amster, I.J., Dean, D.R., and Johnson, M.K. (2001) Sulfur transfer from IscS to IscU: The first step in iron-sulfur cluster biosynthesis. *J. Amer. Chem. Soc.* **123**, 11103–11104