Screening and identification of a protein interacting with IscU

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Summary

The mechanisms of biosynthesis and repair of iron-sulfur clusters are still unclear. In *E. coli*, the *iscU* gene is proposed to be involved in the formation of iron-sulfur cluster. We cloned *iscU* gene from *E. coli* and expressed IscU tagged with six histidines at its C-terminus. Proteins which specifically interact with IscU were screened by "pull-down" assay by using a nickel-chelating column, resulting in finding that IscS interacts with IscU. A disulfide bond was formed between IscS and IscU, which was induced in the presence of 0.1 mM hydrogen peroxide in a cultivation medium. The disulfide bond was formed specifically between Cys63 of IscU and Cys328 of IscS. Cys63 of IscU is thought to play an important role as a ligand for iron-sulfur cluster. Cys328 of IscS is essential for its enzymatic activity. Cys63 of IscU and Cys328 of IscS probably exist in a short distance in the IscU-IscS complex, and they may form a covalent complex under oxidative condition.

Introduction

Iron-sulfur (Fe-S) proteins are present in all organisms and are involved in various cellular processes such as electron transfer and gene regulation[1]. Their structures, functions and redox properties have been studied well. However, little is known about the mechanisms of biosynthesis and repair of their active sites, iron-sulfur clusters. Recently, Zheng et al. reported that *isc* genes are involved in the assembly of iron-sulfur clusters[2]. These genes exist in a gene cluster in the genome of *E. coli*. One of the gene products, IscS is the best studied in the *isc* genes[3]. IscS has a cysteine desulfurase activity and plays a role in supplying sulfur atoms to iron-sulfur clusters. Another gene product, IscU is thought to play an essential function for biosynthesis of iron-sulfur cluster. However, function and property of IscU are still not clear. In this study, we investigate the interaction between IscU and IscS by using "pull-down" analysis.

Materials and methods

Materials Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA) ; oligonucleotides from Espec Origo Service (Tsukuba, Japan) ; molecular mass markers for SDS-PAGE from Amersham Pharmacia Biotech (Uppsala, Sweden) ; nickel-chelating resin from Novagen (Wisconsin, USA). All other chemicals were of analytical grade from Nacalai Tesque (Kyoto, Japan).

Construction of expression plasmids For the expression of IscU-His6, IscU tagged with six histidines at its C-terminus, the *iscU* gene was amplified by PCR with the Kohara miniset clone No. 430 as a template and inserted into the *Nde*I and *Xho*I sites in pET21a to yield pUH12. For expression of both IscS and IscU-His6, a DNA fragment containing both *iscU* and *iscS* was obtained, and the expression plasmid pFH6 was constructed. The expression plasmids producing mutants of IscU and IscS were constructed by PCR with mutagenic primers. Mutations were intro-

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duced into pUH12 to yield mutant IscU, in which each cysteine residue, Cys37, Cys63 or Cys106, was replaced by serine. Mutation was introduced into pFH6 to yield C328A mutant of IscS.

Pull-down assay To identify proteins which interact with IscU, we used "pull-down" assay with a nickel-chelating column. *E. coli* BL21 (DE3) pLysS harboring pUH12 or its derivative plasmids were cultured aerobically in 500 ml of LB broth supplemented with IPTG (1 mM), ampicillin (200μg/ml) and chloramphenicol (40μg/ml) at 37℃ for 12 h. *E. coli* BL21 (DE3) pLysS harboring pFH6 or its derivative was cultured at 26℃ for 20 h. The cells were harvested by centrifugation, suspended in a standard buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 5 mM imidazole, and disrupted by sonication. The cell debris was removed by centrifugation, and the supernatant solution was applied to a nickel-chelating column (7 ml) and washed with 100 ml of binding buffer (20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 5 mM imidazole) and 250 ml of washing buffer (20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 60 mM imidazole). Proteins were eluted with 25 ml of elution buffer (20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 1 M imidazole).

Analytical methods The eluate from a nickel-chelating column was analyzed by SDS-PAGE[4]. Determination of the N-terminal sequence of a protein was performed with a Shimadzu PPSQ-10 protein sequencer.

IscU-IscS complex formation *E. coli* BL21 (DE3) pLysS was cultured in LB medium at 37℃ for 12 h, and 50μl of the culture was inoculated into 5 ml of LB broth. After 2 h, hydrogen peroxide was added to the medium at a concentration of 0 mM-1 mM, and the cells were incubated at 37℃ for 30 min with shaking. The cells were harverested and washed with 0.85% NaCl three times. The cell pellets were suspended in 1 ml of 0.85% NaCl, and iodoacetic acid was added at a concentration of 30 mM. Finally, the sample was mixed with 1/10 volume of a saturated solution of trichloroacetic acid. The denatured proteins were collected by centrifugation, washed with acetone, and dissolved in 50 mM Tris-HCl (pH7.4) containing 1% SDS, 1 mM EDTA and 10 mM iodoacetic acid. The proteins were then separated by SDS-PAGE without addition of any reducing agent. IscU and IscS were detected by Western blotting analysis.

Results and discussion

Pull-down assay The cell-free extract of *E. coli* BL21 (DE3) pLysS harboring pUH12 was applied to a nickel-chelating column. The eluate was analyzed by SDS-PAGE. A number of protein were co-eluted with IscU-His6. Most of them were non-specific proteins as they were also observed in a control experiment which employed the cell-free extract of *E. coli* BL21 (DE3) pLysS harboring pET21a. However, a 42-kDa protein specifically interacted with IscU-His6. The N-terminal sequence of the protein was determined to be MKLPIYLDYSATTPV, which is identical to that of IscS. To confirm the interaction between IscU and IscS, we used the expression plasmid pFH6 producing both IscS and IscU-His6. Efficient co-expression of IscS and IscU-His6 required cultivation at 26℃ for 20 h. The cell-free extract was applied to a nickel-chelating column. The eluate was analyzed by SDS-PAGE, and the result showed that IscS clearly interacts with IscU-His6 (Fig. 1).

Interestingly, when 2-mercaptoethanol was omitted from a sample buffer, the proteins of molecular masses of approximately 68 kDa and 36 kDa were formed. The 68 kDa-protein is a covalently-bound IscU-IscS heterodimer and the 36 kDa-protein is a covalently-bound IscU dimer. N-terminal analysis and the molecular masses of the proteins revealed that disulfide bonds exist between IscU and IscS and between two subunits of IscU. Although IscU is an iron-sulfur protein[5], IscU was purified as an apo-form as judged by its absorption spectrum. The iron-sulfur cluster in IscU was broken in the pull-down procedure and the cysteine residue(s) of IscU may form the disulfide bond.

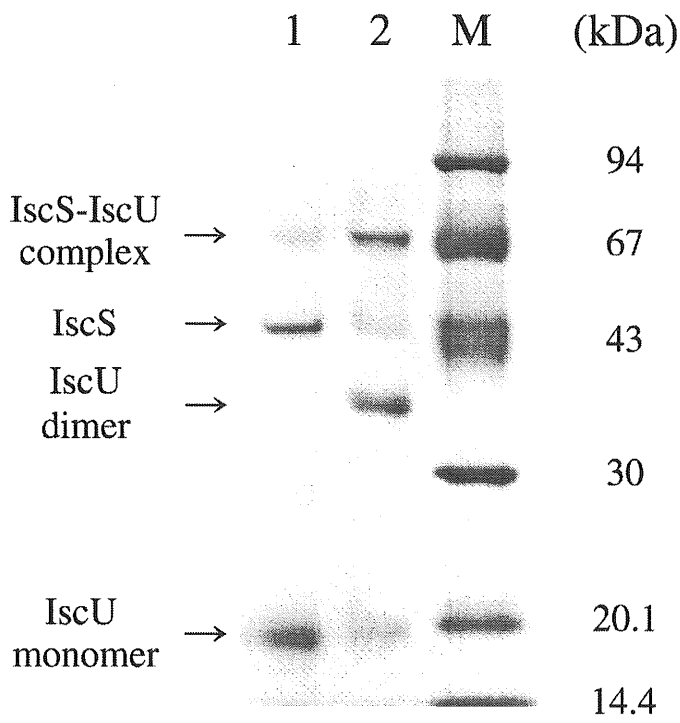

Fig. 1 Pull-down assay of the cell-free extract of *E. coli* BL21 (DE3) pLysS harboring pFH6. The eluate was analyzed by SDS-PAGE in the presence (lane 1) or absence (lane 2) of 0.1% 2-mercaptoethanol in the sample buffer.

IscU-IscS complex formation We examined whether a disulfide bond between IscU and IscS is formed under oxidative condition. *E. coli* BL21 (DE3) pLysS was cultured in LB broth containing hydrogen peroxide. The cells were treated with 30 mM iodoacetic acid to block free cysteine residues and denatured with trichloroacetic acid. The proteins were separated with non-reductive SDS-PAGE. IscU and IscS were detected by Western blotting using anti-IscS and anti-IscU serum (Fig. 2).

In the absence of hydrogen peroxide, the IscS-IscU complex was not observed. On the other hand, the complex was observed in the sample treated with 0.1 mM or 1 mM hydrogen peroxide. This indicates that IscU and IscS contain reactive cysteine residues which possibly form a disulfide bond by oxidative stress such as hydrogen peroxide. The amount of IscU is increased with an increase in the concentration of hydrogen peroxide. However, the IscU dimer was not observed in this experiment.

Site-directed mutagenesis To identify the cysteine residues participating in the formation of a disulfide bond between IscU and IscS, we constructed the expression plasmids producing the mutants of IscU and IscS, where a cysteine residue was replaced by serine or alanine. Each cysteine residue, Cys37, Cys63 or Cys106, in IscU was replaced by serine. The active-site Cys328 of IscS was replaced by alanine. The ability of each mutant of IscU and IscS to form a disulfide bond was examined by pull-down assay as described above. The eluate was applied to SDS-PAGE in the presence or absence of 2-mercaptoethanol, and IscU and IscS were determined by Western blotting analysis (Fig. 3).

The mutant of IscS (C328A) did not form a IscU-IscS heterodimer, and the mutant of IscU (C63S) did not form neither a IscU-IscS heterodimer nor a IscU dimer. The results suggest that a disulfide bond is formed between Cys63 of IscU and Cys328 of IscS. IscU has three cysteine residues which were thought to play important roles as ligands for an iron-sulfur cluster[5]. Cys328 of IscS is also essential for its enzymatic activity[6]. Therefore, the IscU-IscS complex having a disulfide bond formed between the essential cysteine residues should be inactive. Cys63 of

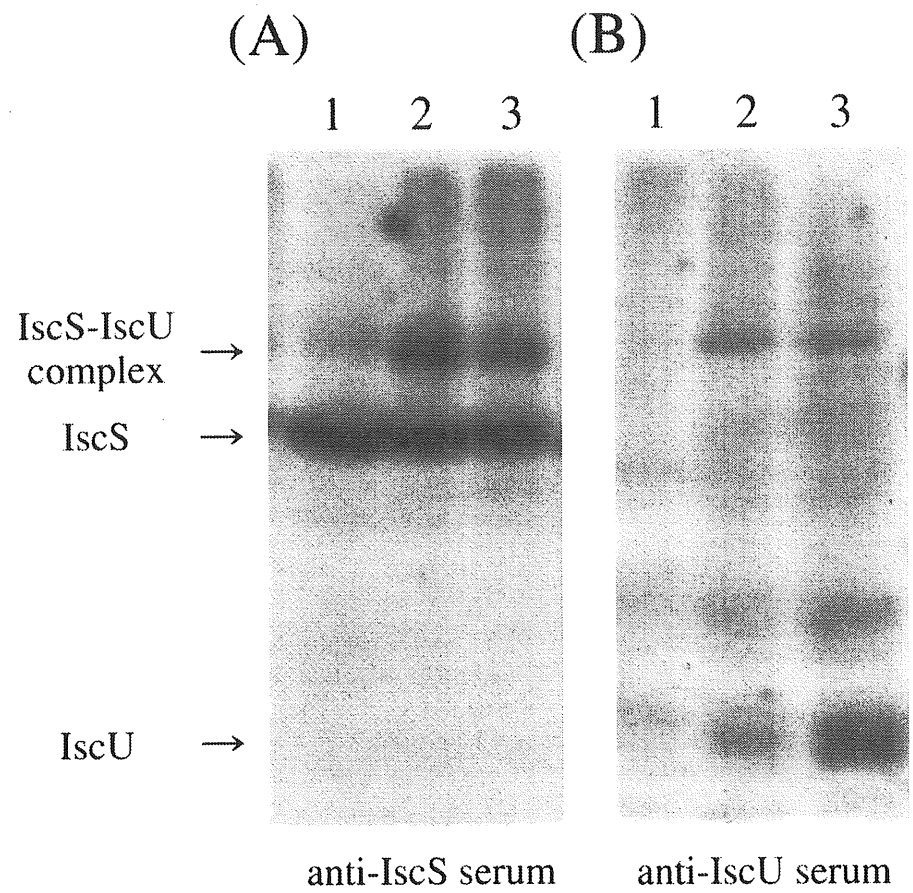

Fig. 2. Western blotting analysis using anti-IscU (A) and anti-IscS (B) antisera. The proteins were separated by non-reductive SDS-PAGE. *E. coli* BL21 (DE3) pLysS was cultured in the absence (lane 1) or presence of hydrogen peroxide at concentrations of 0.1 mM (lane 2) and 1 mM (lane 3).

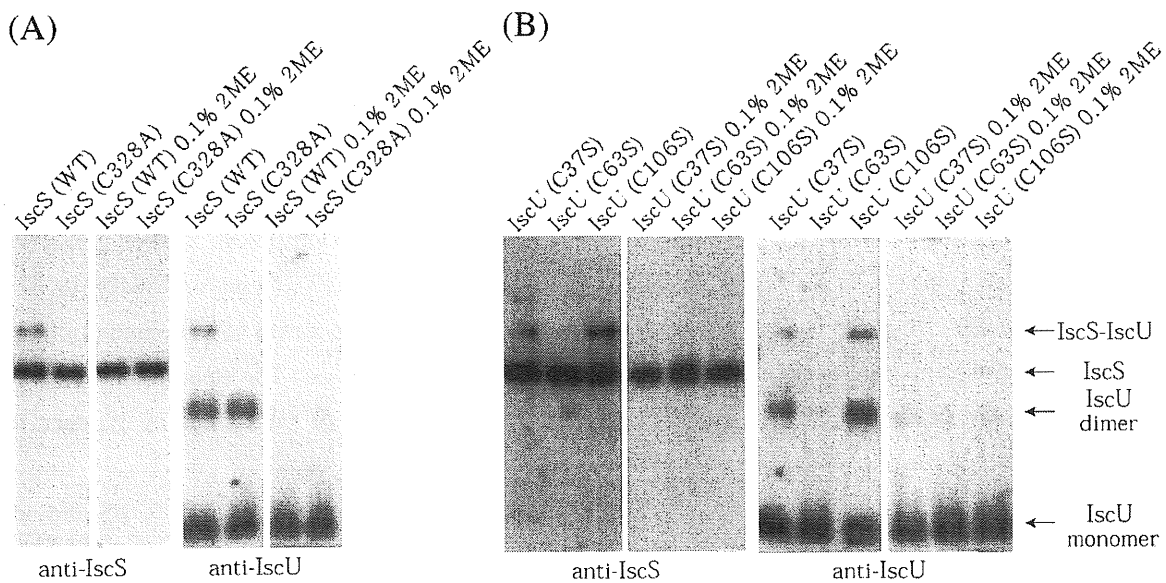

Fig. 3. Detection of the disulfide bond formation by Western blotting analysis. The eluates containing the IscS (C328A) mutant (A) and IscU (C37S), (C63S) and (C106S) mutants (B) were separated by SDS-PAGE in the presence or absence of 0.1% 2-mercaptoethanol.

IscU and Cys328 of IscS probably exist in a short distance in the IscU-IscS complex, and they may form the covalent complex under oxidative condition. We examined the effect of IscU on the cysteine desulfurase activity of IscS. We found that the addition of IscU significantly increased the activity of IscS (data not shown). The activity in the presence of IscU was about 6 times higher than that in the absence of IscU. On the other hand, the mutant of IscU (C63S) did not increase the activity of IscS. IscS forms persulfide on Cys328 during the catalytic cycle[6], and the persulfide-form of the enzyme is probably inactive. The enhancement of the IscS activity by IscU, but not by IscU (C63S), suggests that Cys63 of IscU removes the sulfur atom from cysteine persulfide formed on Cys328 of IscS.

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