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Network of Protein–Protein Interactions among Iron-Sulfur Cluster Assembly Proteins in *Escherichia coli*[1]

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The assembly of iron-sulfur (Fe-S) clusters is mediated by complex machinery which, in *Escherichia coli*, is encoded by the *iscRSUA-hscBA-fdx*-ORF3 gene cluster. Here, we demonstrate the network of protein–protein interactions among the components involved in the machinery. We have constructed $(His)_6$-tagged versions of the components and identified their interacting partners that were co-purified from *E. coli* extracts with a Ni-affinity column. Direct associations of the defined pair of proteins were further examined in yeast cells using the two-hybrid system. In accord with the previous *in vitro* binding and kinetic experiments, interactions were observed for the combinations of IscS and IscU, IscU and HscB, IscU and HscA, and HscB and HscA. In addition, we have identified previously unreported interactions between IscS and Fdx, IscS and ORF3, IscA and HscA, and HscA and Fdx. We also found, by site-directed mutational analysis combined with the two-hybrid system, that two cysteine residues in IscU are essential for binding with HscB but not with IscS. Despite the complex network of interactions in various combinations of components, heteromultimeric complexes were not observed in our experiments except for the putative oligomeric form of IscU-IscS-ORF3. Thus, the sequential association and dissociation among the IscS, IscU, IscA, HscB, HscA, Fdx, and ORF3 proteins may be a critical process in the assembly of Fe-S clusters.

Key words: cysteine desulfurase, Fe-S cluster, *isc* operon, molecular chaperone, protein–protein interaction.

Iron-sulfur (Fe-S) proteins are present in almost all living organisms and exhibit diverse functions including electron transfer, redox and non-redox catalysis, and sensing for regulatory processes (*1, 2*). They contain Fe-S clusters composed of iron and sulfur atoms, which are ligated to the polypeptides by the thiolate side chains of cysteine residues. During the last 4 years, significant advances have been made in our understanding of a complex apparatus termed ISC (iron-sulfur cluster) machinery involved in the assembly of Fe-S clusters, a key step in the post-translational maturation of the Fe-S proteins (*3–6*). The components of the ISC machinery are conserved from bacteria to higher eukaryotes and, in *Escherichia coli*, are encoded by the so-called *isc* operon (*iscRSUA-hscBA-fdx*-ORF3).

Genetic studies in *E. coli* and *Saccharomyces cerevisiae* have demonstrated crucial roles for IscS, IscU, IscA, HscB, HscA, and Fdx in Fe-S cluster biosynthesis, since mutations in the respective genes decrease the activity of many Fe-S proteins (*7–22*). Furthermore, overexpression of the *isc* operon improves the ability of *E. coli* to assemble Fe-S clusters, leading to a marked increase in the yield of recombinant Fe-S proteins (*23, 24*). In contrast to the direct participation of IscS, IscU, IscA, HscB, HscA, and Fdx in the assembly reaction, a regulatory role has recently been demonstrated for IscR, which contains a [2Fe-2S] cluster and functions as a transcriptional repressor of the *isc* operon (*25*). Until now, little has been known about the ORF3 product, although it appears likely that the ORF is cotranscribed with other genes. IscR and ORF3 are conserved in several bacterial species but not found in eukaryotic organisms.

To understand the mechanistic details of the ISC machinery in the formation of Fe-S clusters, it is important to dissect the biochemical properties of each component. IscS is a cysteine desulfurase that catalyzes the removal of sulfur from cysteine to form alanine and IscS-bound elemental sulfur (*3, 26, 27*). The sulfur is transferred directly to a dimer of IscU on which an unstable [2Fe-2S] or [4Fe-4S] cluster is constructed (*28–31*). IscA can also assemble an unstable Fe-S cluster *in vitro* (*32, 33*). It has been proposed that the Fe-S clusters subsequently are delivered from IscU or IscA to other apo-Fe-S proteins, but the pre-

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Abbreviations: GAD, activation domain of GAL4; GBD, DNA binding domain of GAL4; Fe-S, iron-sulfur; IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, *o*-nitrophenyl β-galactoside; ORF, open reading frame.

cise mechanism is not yet understood. HscA and HscB, an Hsp70-type molecular chaperon and a J-domain containing co-chaperone, respectively, interact with IscU in a synergistic manner (*34–36*). Fdx is an adrenodoxin-type ferredoxin containing a stable [2Fe-2S] cluster that probably serves as an electron donor to a hitherto unknown step in Fe-S cluster assembly (*37, 38*).

It is also of interest to understand how these components cooperate in the assembly of Fe-S clusters. We have previously demonstrated tightly coupled functions among the IscS, IscU, HscB, HscA, and Fdx proteins by mutational analyses in *E. coli*, in which the depletion of one component causes the loss of function of the remaining proteins (*8*). This renders it likely that these proteins work in concert either in a linear way or together as a complex. Up to now, *in vitro* complex formation has been demonstrated for combinations of IscS and IscU, IscU and HscB, IscU and HscA, and IscA and Fdx (*28, 30–32, 35, 36, 39*), but the interactions of other components or combinations are not defined.

In this paper, we describe a comprehensive analysis of the protein-protein interactions among the components (here called ISC proteins) encoded by the *isc* operon. By using a biochemical co-purification method and a genetic approach with the yeast two-hybrid system, we identified at least eight interactions among ISC proteins. The network of protein–protein interactions may indicate sequential reactions in the assembly of Fe-S clusters that involve specific associations and dissociations of the ISC proteins.

MATERIALS AND METHODS

Plasmid Construction—For N-terminal fusion constructs, genes were amplified by PCR from the second codon to the stop codon using primer sets containing *Bam*HI and *Sal*I restriction sites (Table I). The mutated *iscS* gene was amplified by PCR from the plasmid pEFC328A, a pET-21a(+) derivative carrying the gene for IscS(C328A) (*27*). Similarly, the mutated *iscU* genes were amplified from the plasmids pUHCS1, pUHCS2, and pUHCS3, carrying C37S, C63S, and C106S substitutions, respectively (*39*). The PCR products were initially cloned into the pCR2.1-TOPO vector (Invitrogen) by a TA cloning method, and their correct sequences were verified. For N-terminal $(His)_6$-fusion constructs, the *Bam*HI/*Sal*I fragments were excised and cloned into the corresponding sites of pQE-30 (Qiagen). For construction of the plasmids used in the two-hybrid analysis, the genes cloned in pCR2.1-TOPO were excised by digestion with *Eco*RI (located in the multiple cloning site of the vector) and *Sal*I and then ligated into *Eco*RI/*Sal*I sites of pGAD424 and pGBT9 (Clonetech). The IscU-$(His)_6$ fusion construct was created by PCR amplification of *iscU* using primers *IscU*-5′FN and *IscU*-3′RB (Table I) containing *Nco*I and *Bgl*II restriction site, respectively, for subsequent cloning into the expression vector pQE-60 (Qiagen).

TABLE I. **PCR primers used in this work.** The underlined bases indicate restriction sites.

IscR-5′F	5′-GGATCCAGACTGACATCTAAAGGGCG-3′
IscR-3′R	5′-GTCGACTATTAAGCGCGTAACTTAACGTC-3′
IscS-5′F	5′-GGATCCAAATTACCGATTTATCTCGACTAC-3′
IscS-3′R	5′-GTCGACTATTAATGATGAGCCCATTCGAT-3′
IscU-5′F	5′-GGATCCGCTTACAGCGAAAAAGTTATCG-3′
IscU-3′R	5′-GTCGACTATTATTTTGCTTCACGTTTGCTTTTATAG-3′
IscA-5′F	5′-GGATCCTCGATTACACTGAGCGACAG-3′
IscA-3′R	5′-GTCGACTATCAAACGTGGAAGCTTTCG-3′
hscB-5′F	5′-GGATCCGATTACTTCACCCTCTTTGGC-3′
hscB-3′R	5′-GTCGACTATTAAAAATCGAGCAGTTTTTCTTCG-3′
hscA-5′F	5′-GGATCCGCCTTATTACAAATTAGTGAACC-3′
hscA-3′R	5′-GTCGACTATTAAACCTCGTCCACGGAATG-3′
fdx-5′F	5′-GGATCCCCAAAGATTGTTATTTTGCCTC-3′
fdx-3′R	5′-GTCGACCATTAATGCTCACGCGCATGGT-3′
ORF3-5′F	5′-GGATCCGGACTTAAGTGGACCGATAG-3′
ORF3-3′R	5′-GTCGACTATTATTCGGCCTCGTCCAGC-3′
IscU-5′FN	5′-GCCGCCATGGCTTACAGCGAAAAAGTT-3′
IscU-3′RB	5′-GAAGATCTTTTTGCTTCACGTTTGCTTTTA-3′

Affinity Co-Purification—The $(His)_6$-tag constructs were introduced into *E. coli* M15 cells harboring the pREP4 (Qiagen) and pRKISC (*23*) plasmids, carrying the *lacI* gene and the *isc* operon, respectively. The cells were grown in Terrific broth supplemented with 0.1 mg/ml ferric ammonium citrate, and the coexpression of a $(His)_6$-tagged protein and the ISC proteins was induced by the addition of 1 mM IPTG. Expression of the IscU-$(His)_6$, $(His)_6$-IscA, $(His)_6$-HscB, $(His)_6$-Fdx, and $(His)_6$-ORF3 fusion proteins was allowed for 16 h at 37°C. For the expression of $(His)_6$-IscR, $(His)_6$-IscS, and $(His)_6$-HscA, cells were cultivated for 16 h at 28°C to prevent the formation of inclusion bodies. The cells were harvested by centrifugation, suspended in a solution containing 50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol, and disrupted by lysozyme treatment and sonication. After centrifugation (15 min, 15,000 ×*g*, 5°C), soluble proteins were subjected to purification with a Ni-NTA affinity column (Qiagen) under the conditions recommended by the manufacturer. The column was washed extensively with a buffer containing 50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol, and the bound proteins were eluted from the column with 250 mM imidazole in the same buffer. Aliquots of the eluted samples were subjected to immunoblotting analysis using antisera raised against each ISC protein. Detection was with an ECL Plus kit (Amersham Pharmacia Biotech) according to the manufacturer's specifications.

Yeast Two-Hybrid Assay—The chimeric plasmids of pGAD424 and pGBT9 derivatives were introduced together into yeast SFY526 cells carrying the β-galactosidase reporter gene. Expression of β-galactosidase was measured by a liquid culture assay as described previously (*40*). At least three independently derived transformants were subjected to assay and their β-galactosidase activity is presented in Miller units; the release of *o*-nitrophenol from the substrate ONPG was recorded at 420 nm and normalized to cell density measured photometrically at 600 nm. β-Galactosidase activity was also measured using a filter assay (not shown), and the results obtained by the two methods were fully compatible.

RESULTS

Co-Purification of ISC Proteins from E. coli Cells—The physical associations of the ISC proteins were first examined using an affinity co-purification method. We constructed a plasmid in which either IscR, IscS, IscU, IscA, HscB, HscA, Fdx, or ORF3 was fused to $(His)_6$-tag at the N-terminus. As reported previously, mutant strains with inactivated *iscS*, *iscU*, *hscB*, *hscA*, or *fdx* genes show very low

activities of many Fe-S proteins and a slow-growth phenotype (*7, 8*). Transformation of the mutant cells with the respective $(His)_6$-tag constructs resulted in complementation of the phenotypic effects except for the $(His)_6$-IscU construct (not shown). Another plasmid, in which $(His)_6$-tag was fused to the C-terminus of IscU, restored the growth of Δ*iscU* cells. Thus, the IscU-$(His)_6$ construct was preferred to $(His)_6$-IscU and used for subsequent analysis. Next, the $(His)_6$-tag construct was introduced into *E. coli* cells harboring the pREP4 and pRKISC plasmids, and co-overexpression of the *isc* operon and a $(His)_6$-tagged protein was induced by IPTG. Under the co-expression conditions, the levels of non-fused ISC proteins increased 10–20-fold and those of $(His)_6$-tagged proteins ~500-fold. Among the $(His)_6$-tagged ISC proteins, only Fdx was expressed in a [2Fe-2S] cluster-containing holo-form.

We performed affinity purification of the $(His)_6$-tagged proteins from cell extracts using Ni-NTA metal affinity resin. The $(His)_6$-tagged proteins were obtained at a purity of more than 80% as judged by SDS-PAGE, but minor contaminants co-migrating with some of the ISC proteins were visible on the gel (not shown). For identification, the fractions were subjected to immunoblotting analysis using antibody raised against each ISC protein. As shown in Fig. 1, three proteins, IscS, HscB, and HscA, were co-purified with IscU-$(His)_6$. With $(His)_6$-Fdx, co-purification of IscS and HscA was observed and with $(His)_6$-ORF3, IscS, and IscU. Significant interactions were also observed for the combinations $(His)_6$-IscS and IscU, $(His)_6$-IscA and HscA, $(His)_6$-HscB and IscU, and $(His)_6$-HscA and IscU. Thus, interactions between IscS and IscU, IscU and HscB, and IscU and HscA were detected even when the $(His)_6$-tag fusion partners were exchanged. In control experiments using an unfused $(His)_6$ sequence, hardly any ISC proteins were co-purified with Ni-NTA resin. No interactions were seen for IscR with any ISC proteins. Taken together, these data indicate a complex network of protein–protein interactions among the ISC proteins, the only exceptional case being IscR.

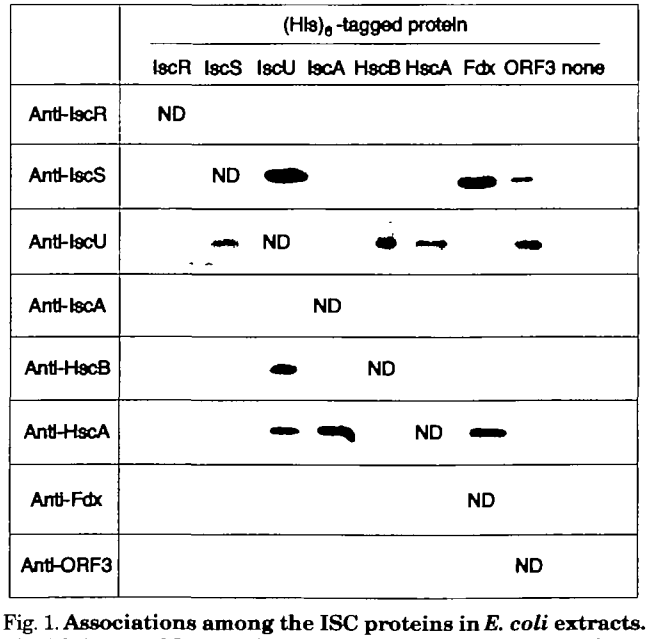

Fig. 1. **Associations among the ISC proteins in *E. coli* extracts.** The $(His)_6$-tagged IscR, IscS, IscU, IscA, HscB, HscA, Fdx, and ORF3 were purified from *E. coli* extracts with Ni-NTA resin. The ISC proteins co-purified with the $(His)_6$-tagged protein were analyzed by SDS-PAGE in 15% gels followed by immunoblotting using anti-ISC antisera. An unfused $(His)_6$ sequence expressed from the pQE-30 vector was used as a negative control. ND, not done.

Interactions Detected by Two-Hybrid Analysis—The results of the co-purification experiments do not show conclusively that two proteins bind to each other, because all of the ISC proteins are coexpressed within the same cell and interactions could be indirect. To investigate the interactions between defined pairs of proteins, we made use of the yeast two-hybrid system. We constructed plasmids in which each ISC protein was fused to the GAL4 activation domain (GAD) and the GAL4 DNA binding domain (GBD), using cloning vectors pGAD424 and pGBT9, respectively. These constructs were introduced into *S. cerevisiae* strain SFY526 carrying the *lacZ* reporter gene under the control of the GAL4-dependent promoter. If the two fusion proteins interact, the GAD and GBD are brought together to the correct localization to allow the production of β-galactosidase, which was detected by a liquid culture assay using ONPG as a substrate. Under the culture conditions, however, it is unknown whether the IscR, IscU, IscA, and Fdx proteins fused to GAD or GBD can accommodate the Fe-S clusters in yeast cells.

All pairwise combinations of the two ISC proteins were examined by the two-hybrid system. However, GBD fused to HscB or Fdx gave high background activities of β-galactosidase, which made it difficult to detect any interacting proteins (not shown). In other fusion constructs, low background activities were confirmed by control experiments in combination with unfused GAD or GBD (Fig. 2). Among the pairwise combinations examined, GBD-IscU and GAD-HscB yielded the highest expression of the reporter gene with a 64-fold increase over background, indicating a stable interaction between IscU and HscB. A weak but significant interaction was also observed for combinations of IscS and IscU, IscS and ORF3, IscA and HscA, and HscB and HscA. Of these combinations, it is noteworthy that the interactions of IscS and IscU, IscS and ORF3, and IscA and HscA could be detected even when the fusion partners of these proteins were exchanged. A homotypic interaction was observed for the GBD-IscS and GAD-IscS pairing, which might reflect a homodimeric structure of IscS. Two-hybrid analysis using GAD-IscR (Fig. 2) or GBD-IscR (not shown) gave only the background level of β-galactosidase activity in any combination with other proteins. Thus, no interactions were observed for IscR, which is consistent with the co-purification experiments described above, and the proposed role for the protein as a repressor of the *isc* operon (*25*).

Interaction of IscS(C328A) with IscU or ORF3—IscS was shown to have cysteine desulfurase activity, which catalyzes the conversion of cysteine to alanine and elemental sulfur via the formation of a persulfide intermediate on a conserved cysteine residues at position 328. The essential role of Cys-328 has been demonstrated by the specific alkylation of that residue and by site-directed mutagenesis experiments (*26, 27*). In addition, recent biochemical studies have provided evidence for the transfer of elemental sulfur from IscS to IscU and a covalent complex between the two proteins via disulfide linkage (*30, 31, 39*). We therefore as-

sessed whether mutated IscS with Cys-328 replaced by Ala could interact with IscU in the two-hybrid system (Fig. 3, left). The mutation caused a marked decrease in the interaction with IscU as detected by β-galactosidase expression. However, the residual activity in the combination of GAD-IscS(C328A) and GBD-IscU was still higher than that in control cells expressing unfused GAD and GBD-IscU. Thus, it appears likely that in IscS, Cys-328 is crucial but not essential for binding with IscU. In parallel experiments using the combination of GAD-IscS and GBD-ORF3, replacing C328A did not cause a major alteration in β-galactosidase expression, showing rather a stimulating effect (Fig. 3, right). The results suggest that C328A substitution does not give rise to a major conformational change in GAD-IscS, and that the residue is not directly involved in the binding between IscS and ORF3.

Interactions of Mutated IscU with IscS or HscB—IscU has been proposed to serve as a scaffold for the assembly of intermediate Fe-S clusters prior to delivery to other apo-Fe-S proteins (*28, 29*). IscU contains three conserved cysteine residues at positions 37, 63, and 106, all of which are essential for *in vivo* function (*14*, Tokumoto U., unpublished observations). Two of the cysteine residues appear to coordinate a transient [2Fe-2S] or [4Fe-4S] cluster that is presumed to bridge two subunits of IscU. In addition, the cysteines are likely to be involved in both binding with IscS via disulfide linkage and accepting elemental sulfur provided by IscS (*30, 31, 39*), although the roles of the individual cysteines have yet to be fully characterized. Here, we analyzed three mutant IscU proteins in which Cys-37, Cys-63, or Cys-106 was individually substituted by Ser. In the two-hybrid system using the combination of GAD-IscS and GBD-IscU, however, none of the substitutions caused a major alteration in β-galactosidase expression (Fig. 4, left). A small decrease in activity was observed for GBD-IscU-(C63S), although 60% activity was retained compared with non-mutated GBD-IscU. The results indicate that the three cysteine residues in IscU do not serve as a major determinant in binding with IscS. In experiments using the combination of GAD-HscB and GBD-IscU, we also examined the effect of mutations in IscU on binding with HscB. As shown in Fig. 4 (right), β-galactosidase expression was almost completely abolished by the C37S and C63S substitutions. In contrast, hardly any change was detected in IscU-

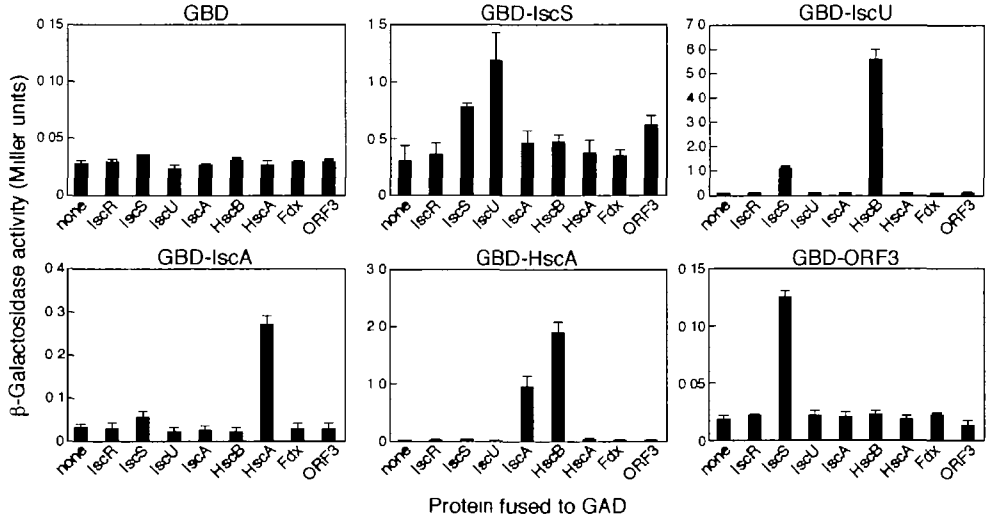

Fig. 2. **Interactions between ISC proteins in yeast cells.** Plasmids carrying the genes for the indicated fusion proteins were co-introduced into yeast SFY526 by the lithium acetate method. Unfused GAD and GBD were used as negative controls. Transformants containing two plasmids were selected on SD plates lacking Trp and Leu and then grown to mid-log phase in liquid YPD medium. β-Galactosidase activity was monitored by the liquid culture assay and is shown in Miller units. Experiments were carried out using at least three independent transformants and the values are the mean ± SD.

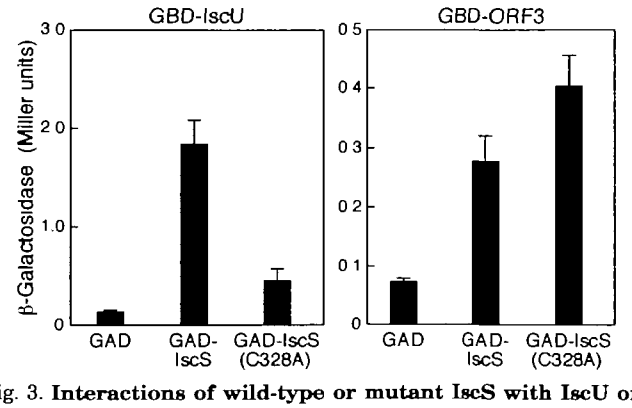

Fig. 3. **Interactions of wild-type or mutant IscS with IscU or ORF3 in the yeast two-hybrid system.** A C328A substitution was introduced into the GAD-IscS fusion construct. Interactions were examined in combination with GBD-IscU or GBD-ORF3 by the two-hybrid analysis as described in the legend of Fig. 2.

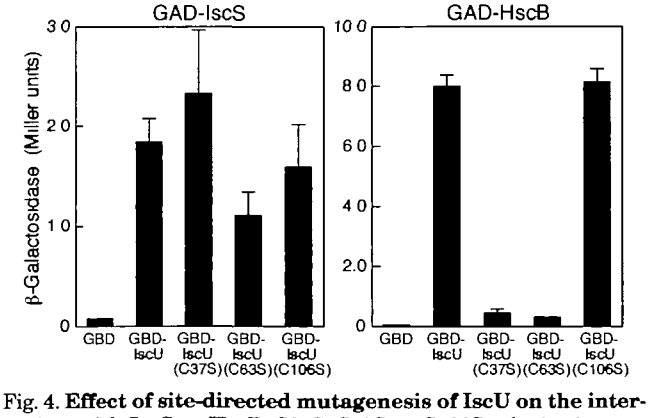

Fig. 4. **Effect of site-directed mutagenesis of IscU on the interaction with IscS or HscB.** C37S, C63S, or C106S substitution was introduced into the GBD-IscU fusion construct. Interactions were examined in combination with GAD-IscS or GAD-HscB by two-hybrid analysis as described in the legend to Fig. 2.

(C106S), suggesting that the interaction involves the specific recognition of Cys-37 and Cys-63 in IscU.

DISCUSSION

We demonstrate that most ISC proteins associate in various combinations in both *E. coli* extracts and yeast cells (Fig. 5). The binding profiles by the two methods are consistent for the following combinations: IscS and IscU, IscS and ORF3, IscU and HscB, and IscA and HscA. In addition, an interaction between HscB and HscA was observed in the two-hybrid analysis. Co-purification experiments revealed additional interactions between IscS and Fdx, IscU and HscA, IscU and ORF3, and HscA and Fdx. The two-hybrid system is advantageous for examining interactions between defined pairs of proteins without any influence of other components. For instance, an interaction between IscU and ORF3 was observed in the co-purification experiments but not in the two-hybrid system, which might be an indication of indirect binding of an IscU-IscS-ORF3 complex. However, such an oligomeric complex could not explain the other discrepancies between the two methods, the typical case being the interaction between IscS and Fdx. The $(His)_6$-Fdx purified from *E. coli* shows an absorption spectrum characteristic of a [2Fe-2S] cluster (not shown), whereas it is unknown whether the Fdx expressed in the nucleus of yeast cells accommodates the cluster. The interaction of holo-Fdx and holo-IscA observed in the previous *in vitro* binding experiment (*32*) was not detected in our studies. Therefore, in order to interpret the data, it may be necessary to take into account some limitations of the techniques. Nevertheless, the previous biochemical analyses have demonstrated direct associations of IscS-IscU, IscU-HscB, and IscU-HscA (*28, 30, 31, 35, 36, 39*). The interaction of HscB and HscA has also been inferred from *in vitro* kinetic studies (*34–36*). Our findings are compatible with these observations and, basically, reliable for a comprehensive under-standing of protein–protein interactions among ISC proteins.

How do the interactions among ISC proteins contribute to the assembly of Fe-S clusters? According to a current model (*30, 31, 39*), the assembly process is initiated by the binding of IscS to IscU to transfer elemental sulfur (S^0). A transient Fe-S cluster is then constructed on the scaffold protein IscU (*28, 29*), although the mechanistic details remain unclear. The S^0 should be reduced to S^{2-} prior to the assembly of the Fe-S cluster, and Fe should be recruited by an unknown mechanism. In this report, we have demonstrated the physical associations of IscS with IscU, Fdx, and ORF3. Of these, the IscS-Fdx complex appears noteworthy since Fdx has a redox-active [2Fe-2S] cluster with a low midpoint potential (*37*). In addition, we have recently found that IscS binds to holo-Fdx but not to apo-Fdx (not shown). Thus, the binding may facilitate the reduction of S^0 to S^{2-} by providing reducing equivalents from Fdx. Consistent with this view, the recent crystallographic analysis of Fdx demonstrates a unique cysteine residue at position 46 that is located on the molecular surface and near the [2Fe-2S] cluster (*38*).

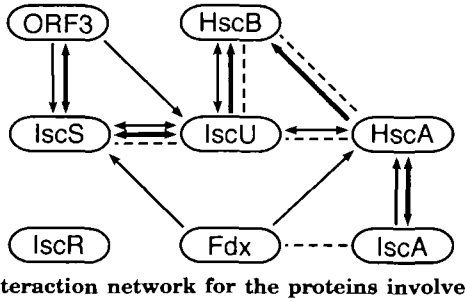

Fig. 5. **Interaction network for the proteins involved in the assembly of Fe-S clusters.** Thin and thick lines indicate interactions detected by the co-purification experiments and the two-hybrid analysis, respectively. Dashed lines indicate biochemical interactions described in previous studies. Arrows point away from the protein used as the $(His)_6$-tagged or GBD-fused construct to the interaction partner.

Similar biochemical properties have been observed for IscA and IscU, that is, an oxygen-labile [2Fe-2S] or [4Fe-4S] cluster bridging the two homologous subunits (*28, 29, 32, 33*). Based on these findings, IscA has been proposed to provide an alternative scaffold to IscU for the assembly of intermediate Fe-S clusters. In our analyses, however, no interaction was observed for the combination of IscS and IscA, in marked contrast with the IscS-IscU complex. Therefore, a direct sulfur-transfer from IscS to IscA appears less likely. Instead, both IscU and IscA associate with HscA, an Hsp-70 type molecular chaperone that is dedicated to the biogenesis of Fe-S clusters. To date, a synergistic interaction among IscU, HscB and HscA has been demonstrated by *in vitro* binding and kinetic analyses using the purified proteins (*34–36*). Our results are compatible with the previous reports and provide further information about the chaperone activity. First, the interaction between IscA and HscA, which was detected in both the co-purification and two-hybrid experiments, supports stable binding between the two proteins and suggests a role for IscA as a substrate for the chaperone as in the case of IscU. Second, the chaperone interacts with apo-IscU and apo-IscA since the purified IscU-$(His)_6$ and $(His)_6$-IscA proteins from *E. coli* extracts display no spectroscopic evidence of an Fe-S cluster (not shown). Third, HscB specifically recognizes two essential cysteines in IscU for binding (discussed below). It appears from these findings that the chaperone system may assist both IscU and IscA in forming transient Fe-S clusters by stabilizing an apo-state conformation suitable for cluster assembly. Whether the chaperone can interact with other Fe-S proteins including apo- or holo-Fdx remains to be clarified.

The two-hybrid system for ISC proteins will be useful in further genetic studies to identify residues involved in protein-protein interactions. As a first step, we analyzed mutant IscS and IscU proteins with essential cysteine residues replaced with other amino acids. The substitution of Cys-328 to Ala in IscS resulted in a marked decrease of its interaction with IscU, whereas none of the three cysteines in IscU was found to be essential for binding (Figs. 3 and 4). This finding is not surprising because previous results showed a non-covalent IscS-IscU complex as well as a covalent one with a disulfide linkage (*28, 30, 31, 39*). On the other hand, we have unexpectedly identified two cysteines in IscU at positions 37 and 63 that are crucial for the interaction with HscB (Fig. 4). A recent crystallographic analysis of HscB revealed a three-helix bundle structure of the C-terminal domain that is proposed to bind with target proteins (*41*). The surface of the domain is mainly acidic but

also contains some hydrophobic side chains. At present, it is unclear which residues are involved in binding with IscU and how the two cysteines are recognized by HscB.

In conclusion, the present findings demonstrate a network of protein–protein interactions among ISC proteins. From interactions in various combinations, one might expect a large, multimeric protein complex involving several components. However, no indications of such a complex were observed in the present study other than the putative oligomeric form of IscU-IscS-ORF3. Attempts to identify high-molecular-mass complexes by gel filtration have also been unsuccessful (not shown). We therefore speculate that ISC proteins work in concert during the sequential association and dissociation to assemble transient Fe-S clusters and transfer them to other apo-Fe-S proteins. The identification of the new interactions will provide important information for detailed investigations of the molecular mechanism of Fe-S cluster biosynthesis. Further, both the co-purification and two-hybrid techniques have the potential to be used not only to investigate interactions between known proteins, but also to look for unknown binding partners. We are currently using these methods to try to identify novel proteins that interact with each of the ISC components.

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