Iron-Sulfur Cluster Biosynthesis

CHARACTERIZATION OF ESCHERICHIA COLI CYaY AS AN IRON DONOR FOR THE ASSEMBLY OF [2Fe-2S] CLUSTERS IN THE SCAFFOLD IscU

The biogenesis of iron-sulfur [Fe-S] clusters requires the coordinated delivery of both iron and sulfide. Sulfide is provided by cysteine desulfurases that use L-cysteine as sulfur source. So far, the physiological iron donor has not been clearly identified. CyaY, the bacterial ortholog of frataxin, an iron binding protein thought to be involved in iron-sulfur cluster formation in eukaryotes, is a good candidate because it was shown to bind iron. Nevertheless, no functional in vitro studies showing an involvement of CyaY in [Fe-S] cluster biosynthesis have been reported so far. In this paper we demonstrate for the first time a specific interaction between CyaY and IscS, a cysteine desulfurase participating in iron-sulfur cluster assembly. Analysis of the iron-loaded CyaY protein demonstrated a strong binding of Fe$^{3+}$ and a weak binding of Fe$^{2+}$ by CyaY. Biochemical analysis showed that the CyaY-Fe$^{3+}$ protein corresponds to a mixture of monomer, intermediate forms (dimer-pentamers), and oligomers with the intermediate one corresponding to the only stable and soluble iron-containing form of CyaY. Using spectroscopic methods, this form was further demonstrated to be functional in vitro as an iron donor during [Fe-S] cluster assembly on the scaffold protein IscU in the presence of IscS and cysteine. All of these results point toward a link between CyaY and [Fe-S] cluster biosynthesis, and a possible mechanism for the process is discussed.

Iron-sulfur [Fe-S] clusters are ubiquitous and evolutionary ancient prosthetic groups that are required to sustain fundamental life processes. They are involved in electron transfer, substrate binding/activation, iron/sulfur storage, regulation of gene expression, and enzyme activities (1). Formation of intracellular [Fe-S] clusters does not occur spontaneously but requires a complex biosynthetic machinery. In Escherichia coli three different types of [Fe-S] cluster biosynthesis systems have been identified so far, namely the iron-sulfur cluster, sulfur mobilization, and cysteine sulfinate desulfinase systems (2, 3). These different machineries have in common the involvement of a cysteine desulfurase that allows utilization of cysteine as source of sulfur atoms (4). The ISC and SUF systems, furthermore, both contain scaffold proteins that provide an intermediate assembly site for [Fe-S] clusters or [Fe-S] cluster precursors (5–8).

Important questions related to [Fe-S] cluster biosynthesis include: (i) the molecular mechanism by which iron and sulfide are assembled on the scaffold protein; (ii) how accessory proteins (chaperones in particular) participate in the process; and (iii) how the cluster is transferred from the scaffold to an apo target protein. Another essential question is the identity of iron and sulfur donors for the formation of [Fe-S] clusters. Whereas L-cysteine has been identified as the ultimate source of sulfur, the question “Where does the iron come from?” still remains unanswered. Whereas some preliminary answers have been provided to most of the above issues, very little is known regarding the last question. It is simply assumed that, because of its toxicity, iron has to be stored and transported by proteins from which it can be mobilized for assembly of iron sites. In bacteria, IscA and YggX, which were shown to be able to bind iron and to be, to some extent, involved in [Fe-S] metabolism, are potential candidates requiring further investigations (9–12). However, this is controversial because IscA was proposed to be an [Fe-S] scaffold protein (5, 7), whereas a recent report could not establish iron binding to YggX (13).

More information concerning a putative iron donor protein is available in eukaryotic systems. In eukaryotes, [Fe-S] cluster assembly requires two biosynthetic protein machineries. One is localized in the mitochondria and functions in the assembly of all cellular [Fe-S] proteins, whereas the other one is cytosolic, specifically involved in the maturation of cytosolic and nuclear [Fe-S] cluster proteins (14). Several lines of evidence support the proposed role of the frataxin gene as an iron donor for assembly of [Fe-S] clusters in eukaryotes: (i) low expression of the frataxin gene in humans results in Friedreich’s ataxia, a neurodegenerative disease characterized by reduced levels of mitochondrial [Fe-S] enzyme activities (15); (ii) the yeast frataxin (Yfh1p) is involved in the regulation of iron homeostasis (16, 17) and is required for maturation of [Fe-S] cluster containing proteins (18), and its inactivation results in iron accumulation in mitochondria and is correlated to oxidative stress (18–20); (iii) frataxin binds iron and, with some exceptions, the holomform of the protein oligomerizes (21–24); (iv) Yfh1p was shown to directly interact, within the cell, with proteins of the [Fe-S] cluster assembly machinery, for example the scaffold ISU1p and the cysteine desulfurase Nfs1p (18, 25, 26), as well as with aconitase, a mitochondrial [Fe-S] enzyme (27); and (v) its ability to deliver iron to ISU-type proteins and aconitase was demonstrated in vitro (27–29). On the other hand, a YFH1 deletion is not lethal in Saccharomyces cerevisiae, indicating that another iron donor might exist in mitochondria (17, 30).
The bacterial ortholog of frataxin is the CyaY protein, which displays ~25% sequence identity to human and yeast frataxin. The situation in bacteria appears to be more complex. Indeed, a knock-out of the cyaY gene alone confers no phenotype: no defect in iron content, no sensitivity to oxidative stress, and no auxotrophy (31). It is possible that the specific growth conditions wherein the cyaY gene expression plays a prominent role have not been defined yet. Interestingly, during the preparation of this manuscript, it was reported that a double knock-out in cyaY and yggX in Salmonella enterica resulted in defects in [Fe-S] cluster metabolism (32). Structural analysis of E. coli CyaY (x-ray structure at 1.4 Å resolution and NMR structure) and yeast and human frataxin revealed a common protein fold for all frataxin homologs (33–36). NMR spectroscopic studies on CyaY showed that it is able to bind iron in both Fe²⁺ and Fe³⁺ oxidation states through conserved Asp and Glu residues in the same region of the protein as in the case of human frataxin (34). It was shown that CyaY can accommodate 2 Fe²⁺/protein when incubated with Fe²⁺ an aerobically. Under these conditions the protein was shown to be a tetramer in solution. Upon ferrous iron oxidation CyaY is able to bind an initial group of 6 Fe³⁺/protein and up to 26 Fe³⁺/protein using a large excess of iron (35, 37). The iron-loaded form of CyaY is present in solution as a mixture of monomer and oligomers, with only the latter containing iron (35). Furthermore, iron-containing oligomers are resistant to iron chelation by EDTA or citrate, showing strong affinity of CyaY oligomers for Fe³⁺ (35).

So far, no functional in vitro studies showing an involvement of CyaY in [Fe-S] cluster biosynthesis have been reported. In this work we demonstrate for the first time a specific interaction between E. coli CyaY and an ISC protein (IscS), which provides a link between CyaY and [Fe-S] cluster biosynthesis. Furthermore, we show a functional role for the iron-containing CyaY oligomers during [Fe-S] cluster assembly on the scaffold protein IscU in vitro.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals were of reagent grade and obtained from Sigma-Aldrich or Fluka unless otherwise stated. ⁷⁷Fe₂O₃ was converted into ferric chloride by dissolving it in hot concentrated (35%) hydrochloric acid of analytical grade (Carlo Erba) and repeatedly concentrated in water. Plasmid pET-22b-CyaY for production of recombinant E. coli CyaY was kindly provided by Prof. Se Won Suh (33). Plasmid pTrcIscU for production of non-His-tagged recombinant E. coli IscU was kindly provided by Prof. Larry E. Vickery (38), and plasmids pQE-IscS and pRKISC for production of E. coli IscU, IscS, and all proteins from the isc operon, respectively, were provided by Prof. Y. Takahashi (39–41). Plasmid pSL219 and pEB586 for production of non-His-tagged IscU and IscS (between 0.5 and 1 equivalent with regard to CyaY).

Expression and Purification of Proteins—Recombinant E. coli CyaY containing a C-terminal His₆ tag was expressed and purified as previously described with some minor modifications (36). Following metal-chelate chromatography on nickel-nitrilotriacetic acid resin (Amersham Biosciences) a gel filtration on a HiLoad 16/60 Superdex 75 prep-grade column (Amersham Biosciences) was performed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM DTT). After these two purification steps the protein was judged 99% pure by SDS-PAGE. The protein was concentrated by ultrafiltration using an YM10 membrane (Amicon) and was stored at ~80°C.

Recombinant E. coli IscU (non-His-tagged), E. coli His-tagged IscU, E. coli His-tagged IscS (both N-terminal His-tagged), E. coli IscS (non-His-tagged), and E. coli IscS_C328S (non-His-tagged) were expressed and purified as previously described (38–40, 42, 43). For non-His-tagged IscU an additional gel filtration step on a HiLoad 16/60 Superdex 75 prep-grade column (Amersham Biosciences) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM DTT was added to further purify the protein. Concentrated protein solutions were stored at ~80°C.

Preparation of Cell-free Extracts—E. coli BL21(DE3)-RIL harboring plasmid pRKISC, E. coli BL21(DE3) harboring plasmid pTrcIscU and E. coli BL21(DE3) harboring plasmid pET-22b-CyaY were grown as previously described (36, 38, 44). Overexpression of proteins of the isc operon, of non-His-tagged IscU and of His-tagged CyaY was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. After collecting the cells by centrifugation, the bacterial pellets were resuspended in buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl), and cells were broken by sonication (10 × 10 s; amplitude 25%). The soluble protein fraction was obtained by centrifugation at 4500 rpm (Ti50.2 rotor), 4°C for 90 min in a Beckman ultracentrifuge (Beckman Coulter, Inc.). The obtained supernatant (cell-free extract) was directly used for protein-protein interaction studies (see below).

Protein-Protein Interactions between CyaY and Proteins of the isc Operon—His-tagged pure CyaY was loaded onto a gravity flow 0.2-ml nickel-loaded metal chelating Sepharose column (Amersham Biosciences). Cell-free extracts from a 2- or 3-litter culture of E. coli cells overexpressing the complete isc operon or non-His-tagged IscU were passed over this CyaY column. The column was washed with 30 column volumes of buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0–20 mM imidazole), and bound proteins were eluted using buffer containing 0.5 M imidazole. These experiments were performed aerobically at 20°C. Eluted proteins were analyzed by SDS-PAGE and identified by N-terminal sequencing. The same experiment was performed either aerobically or anaerobically with pure non-His-tagged IscU and non-His-tagged wild-type or mutated (C328S) IscS (between 0.5 and 1 equivalent with regard to CyaY).

Iron Binding to CyaY—Purified CyaY (235 μM) was incubated with a 15-fold molar excess of either FeCl₃ (aerobically) or Fe(NH₄)₂(SO₄)₂ (anaerobically) in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl for 2 h at 4°C. After incubation the mixtures were centrifuged for 10 min at 13,000 rpm in an Eppendorf table centrifuge (Eppendorf AG) and subsequently desalted on a NAP10 column (Amersham Biosciences). Aliquots of iron-loaded CyaY were stored at ~80°C. The iron binding properties of CyaY-Fe³⁺ and CyaY-Fe²⁺ were investigated by dilution with subsequent desalting, incubation with iron chelators like EDTA or citrate, and incubation with chemical reducing agents like DTT or dithionite. To study the dilution effects the iron-loaded protein was passed over a NAP10 column, which resulted in a dilution of 1:1.5. After 1 h of incubation the diluted protein was again passed over a NAP 10 column, which again resulted in a dilution of 1:1.5. These steps were repeated three times and the iron content before and after each dilution step was determined. To study the effects of iron chelators, iron-loaded CyaY (80 μM in iron) was incubated with a 60-fold molar excess (over iron) of either EDTA or citrate for 1 h. After desalting the iron content was determined and compared with that before chelator treatment. The effect of chemical reducing agents on the iron-loaded CyaY was investigated by incubation of CyaY (300 μM in iron) with a 10–20-fold molar excess of either DTT or dithionite for 1 h. After incubation the mixtures were desalted, and the iron content was determined and compared with that before the treatment.

Oligomerization State of Iron-loaded CyaY—The oligomerization state of CyaY loaded with either Fe³⁺ or Fe²⁺ was determined by gel filtration on an analytical Superdex 75 HR 10/30 (Amersham Biosciences).
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—Different oligomeric forms of Fe^{3+}-loaded CyaY were separated by preparative gel filtration on a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl at a flow rate of 0.8 ml/min. Fractions of different oligomeric forms were collected, combined, and concentrated. The integrity of the separated forms after concentration was verified by reinsertion onto a Tricorn high performance Superdex 200 10/300 GL column.

—Protein-protein interactions can provide a strong enough to allow trapping of IscS in natural abundance. The third visible band in lane 4 corresponds to another histidine-rich protein not belonging to the isc operon. Finally, the same experiment was repeated with purified non-His-tagged IscU. In that case no interaction was detected between CyaY and IscU (Fig. 1, lane S), showing that CyaY

Figure 1. Interaction of CyaY with Isc proteins. His-tagged CyaY was loaded onto a nickel-loaded metal chelating Sepharose column (0.2 ml), and either cell-free extracts from E. coli overexpressing the whole isc operon (lane 2), pure non-His-tagged IscS (lane 3), cell-free extracts from E. coli overexpressing only non-His-tagged IscU (lane 4), or pure non-His-tagged IscU (lane 5) were passed over this column. Bound proteins were eluted with buffer containing 0.5 M imidazole, and eluted fractions were analyzed by SDS-PAGE. Lane 1, molecular mass markers (kDa).

RESULTS

CyaY Interacts with IscS—Protein-protein interactions can provide a useful hint for identifying unknown protein functions. Because the CyaY protein is proposed to be a potential iron donor protein for [Fe-S] cluster biosynthesis, observable interactions of the protein with proteins involved in this process (ISC proteins) would provide an important hint toward CyaY function. To study the possible interactions of CyaY with proteins of the isc operon, an affinity chromatography approach was chosen. The His₆-tagged CyaY, expressed and purified as already described (36), was loaded on a gravity flow Ni²⁺-charged metal chelating column, and in a first experiment, an E. coli cell-free extract (≈400 mg) prepared from a 3-liter culture of BL21(DE3)-RIL cells overexpressing the whole isc operon was passed over the CyaY-loaded column. Because all of the cellular proteins were not His-tagged, proteins retained on the column and eluted together with CyaY are those that interact with CyaY specifically. Bound CyaY and potential partners were then eluted in the presence of 0.5 M imidazole. Fig. 1 (lane 2) shows the analysis of eluted proteins by SDS-PAGE (15%). In addition to the band corresponding to CyaY (12200 Da), other bands are visible on the gel. N-terminal sequencing of the most intense bands revealed that the upper one, more intense, corresponds to IscS (≈45,000 Da), the lower one (≈14,000 Da), less intense, to IscU and that the intermediate ones (≈25000 Da) did not correspond to proteins of the isc operon (Fig. 1, lane 2). Binding of these proteins was due to high histidine contents favoring direct interaction with the nickel-loaded chelating Sepharose column. Apart from IscS and IscU, no other proteins from the isc operon were detected in significant amounts by SDS-PAGE. In a control experiment, performed either aerobically or anaerobically, using pure non-His-tagged IscS in place of the extracts, we observed that IscS eluted with CyaY in the 0.5 M imidazole fraction (Fig. 1, lane 3). Under identical conditions, no IscS could bind to the column in the absence of CyaY (data not shown). This demonstrated a specific interaction between CyaY and IscS.

Because IscU had been previously shown to form a tight complex with IscS (47, 48), we then addressed the question whether the presence of a small amount of IscS from the extracts on the gel (lane 2) was an indirect consequence of its binding to IscS rather than caused by direct binding to CyaY. To verify this hypothesis the same experiment as described above was repeated by passing extracts from a 2-liter culture of BL21(DE3) cells overexpressing only the IscS protein over a CyaY-loaded nickel-loaded chelating Sepharose column. Under these conditions IscU was again observed in small amounts in the imidazole fraction as shown by gel electrophoresis (Fig. 1, lane 4) and N-terminal sequencing. More surprising was the presence of a much higher amount of IscS in this fraction. This indicated that the interaction CyaY-IscS is strong enough to allow trapping of IscS in natural abundance. The third visible band in lane 4 corresponds to another histidine-rich protein not belonging to the isc operon. Finally, the same experiment was repeated with purified non-His-tagged IscU. In that case no interaction was detected between CyaY and IscU (Fig. 1, lane S), showing that CyaY
interacts only with IscS and that binding of IscU was due to the presence of IscS. When the same experiment was repeated with extracts from cells overexpressing either SufS or SufS-SufE, the cysteine desulfurase system of the suf operon, no bands corresponding to these proteins were detectable on the SDS gel, whereas the band corresponding to IscS was visible, once again demonstrating the affinity of IscS for CyaY (data not shown). These results demonstrate that CyaY interacts with the cysteine desulfurase IscS. Because a bioinformatics study had shown that the frataxin/cyaY gene has the same phylogenetic distribution as the chaperone iscAB/jac1 genes, suggesting strongly that they are involved in the same process, namely iron-sulfur cluster biosynthesis (49), we repeated the same kind of experiment as above with cells over-expressing HscA and HscB to analyze the ability of CyaY to interact with the chaperones of the isc operon. Once again no interaction could be observed (data not shown). Finally, we addressed the question of whether this CyaY-IscS interaction was the result of an intermolecular disulfide bridge. We thus carried out incubations and CyaY affinity chromatography anaerobically within a glove box and also used, in place of wild-type IscS, an IscS mutant protein, in which the redox active cysteine (cys328) had been changed into serine. In both cases the interaction was observed.

All of these results together demonstrate that CyaY interacts with IscS specifically and that this interaction does not depend on the formation of an intermolecular disulfide bridge between the two proteins. These results thus provide evidence for a role of CyaY in sulfur metabolism or iron-sulfur cluster biosynthesis. Further experiments are required to better characterize the IscS-CyaY complex.

Properties of Iron-loaded CyaY—After purification the recombinant CyaY is a monomeric protein that contains no detectable amounts of iron. To test the putative role of CyaY as an iron donor protein during iron-sulfur cluster biosynthesis, iron-loaded CyaY was required. Therefore we prepared iron-loaded CyaY, based on procedures already described with slight modifications (see “Experimental Procedures”), and studied its properties. The ability to bind iron was investigated by incubation of apo CyaY with a 15-fold molar excess of either Fe²⁺ (anaerobically) or Fe³⁺. After 2 h the incubation mixtures were desalted over a G25 column to remove unspecific iron, and the oligomerization state and iron content of the protein were determined. Fig. 2 shows the Superdex-75 chromatograms obtained with three different purified protein forms: the apoprotein (Fig. 2A), the Fe²⁺-containing protein (Fig. 2B) and the Fe³⁺-containing protein (Fig. 2C). The former, as already mentioned, is a monomer. The Fe³⁺-containing form contains up to 2.5 Fe²⁺/polypeptide chain, in agreement with previous reports, and exists in solution as a mixture of monomer, as a major component, together with dimers and trimers (34, 50). The Fe³⁺-loaded protein contains up to 8 Fe³⁺/polypeptide chain (averages of three independent experiments) and is mainly in the form of larger oligomers. In the case of the Fe³⁺ form, we cannot exclude the possibility that some oxidation had occurred during the experiment.

To analyze the binding properties of the iron bound to CyaY (Fe²⁺ or Fe³⁺), the effects of dilution, iron chelators, and chemical reducing agents were studied. The amount of protein-bound iron was determined after treatment followed by desalting and compared with that of the starting material. Upon dilution or treatment with a 60-fold excess of EDTA or citrate for 1 h, ferrous iron was removed from the protein (data not shown). In contrast, no loss of iron could be observed during treatment of the ferric form of CyaY under the same conditions, showing a strong resistance of CyaY to Fe³⁺ chelation. Removal of Fe³⁺ was observed during treatment with strong reducing agents such as dithiothreitol (data not shown). All of the above results demonstrated weak binding of Fe²⁺ and strong binding of Fe³⁺ by CyaY. Because of the presence of different oligomerization states of CyaY containing Fe³⁺, it was impossible to determine a binding constant of Fe³⁺ to CyaY. However, incubation of CyaY containing Fe³⁺ with 100 mM citrate during 1 h could not remove any iron from the protein, indicating an iron association constant higher than 1.0 × 10¹⁷ M⁻¹, which corresponds to that of ferric citrate (51). In the following, only the Fe³⁺-containing protein was used.

Biochemical and Spectroscopic Characterization of CyaY-Fe³⁺—A more detailed study of the aggregation states of CyaY containing Fe³⁺ and their respective iron contents was performed by loading the protein onto a Superdex 200 column that separates proteins over a 10,000–600,000 Da range (Fig. 3). Using this gel filtration column we observed that CyaY-Fe³⁺ was a mixture of monomers, intermediate forms (dimers to pentamers), and large oligomers (beyond 600,000 Da), which could be isolated by collecting and pooling the corresponding fractions and analyzed separately for their iron content. As already mentioned, the monomeric form was found to be iron-free. The inter-
mediate forms contained ~20 iron atoms/polypeptide chain (average of four independent experiments). Both forms were stable after isolation and concentration as verified by reinjection onto an analytical Superdex 200 column (data not shown). On the contrary, large oligomeric forms of CyaY precipitated after isolation and could not be further analyzed for their iron content. From these results only the intermediate oligomers (dimers to pentamers) corresponded to a stable and soluble iron-containing CyaY form, at least under our in vitro conditions. We analyzed again the ability of CyaY to interact with IscS this time with this Fe3+-loaded CyaY form. Again, with no significant difference with respect to apo CyaY, the iron-containing protein was found to bind IscS (data not shown). In the following the term CyaY-Fe3+ protein will refer to this preparation of intermediate forms.

CyaY-Fe3+ shows a yellow color and displays a characteristic UV-visible spectrum with a broad absorption band from 300 to 400 nm corresponding to Fe3+ oxo(hydroxo) species bound to the protein as already reported by Bou-Abdallah et al. (37) (Fig. 4). The large 280/300 nm ratio observed is explained by the high iron content of the protein (20 iron/polypeptide chain).

Studies of CyaY-Fe3+ reduction led to the discovery that cysteine was an excellent reducing agent. This is shown in Fig. 5. In the presence of 2 mM cysteine, the intensities of the 300–400 nm absorption band decreased with time, indicating Fe3+ reduction (Fig. 5A). A plot of the yield (%) of Fe2+ as a function of time was fitted to a rate equation for a first order decay process. The apparent rate constant (kobs) for this reaction was estimated to be 0.034 min⁻¹. Reduction was directly demonstrated in an experiment in which ferrozine, an effective Fe3+ chelator and a Fe2+ probe, was added to the reaction mixture. Under these conditions, formation of the pink ferrozine-Fe2+ complex was observed, as shown from the increase of its characteristic absorption band at 562 nm (Fig. 5B), indicating reduction of CyaY-Fe3+ by cysteine and mobilization of Fe2+ by ferrozine. However, formation of the ferrozine-Fe2+ complex was slower than reduction of CyaY-Fe3+ (kobs ~0.0016 min⁻¹). Indeed, whereas reduction of CyaY-Fe3+ was completed in ~1 h, 6 h were required for conversion of 89% of the initial iron content into the Fe2+-ferrozine complex. This seems to indicate that iron sites are not easily accessible. This reduction was specific to cysteine because, as shown in Fig. 5 (C and D), no reduction of Fe2+ and no production of Fe2+-ferrozine complex could be observed during incubation of CyaY-Fe3+ with reduced glutathione, another abundant cellular reducing thiol.

CyaY-mediated Reconstitution of IscU—CyaY is able to bind specifically ferric iron in a stable form. We further tested the ability of CyaY-Fe3+ to behave as an iron donor in the assembly of iron-sulfur clusters. For that purpose E. coli IscU, a scaffold protein of the ISC system, was chosen as a target. IscU (50 nM) was incubated anaerobically with a catalytic amount of E. coli IscS (1 μM) and cysteine (2 mM) for 2 h. Iron was then provided in the form of CyaY-Fe3+ (150 μM in iron). At time intervals, formation of iron-sulfur clusters on IscU was monitored by UV-visible absorption spectroscopy from the increase of characteristic absorption bands in the 300–700-nm region (Fig. 6A). The absorption bands at 320, 410, and 456-nm and the shoulder ~510 nm present in the UV-visible spectrum at the end of the reaction are characteristic for [2Fe-2S] clusters assembled in IscU as already published (8). From the absorption band at 456 nm after 2-h reaction and based on published extinction coefficients (8), we estimated that 90% IscU dimer contained a [2Fe-2S] cluster with variations of ±10% from one experiment to the other. A plot of the yield (%) of [Fe-S] formation as a function of time was fitted to a rate equation for a first order process (Fig. 6A, inset). We estimated that the apparent rate constant (kobs) of this reaction was 0.127 min⁻¹. This value is slightly larger than that corresponding to the reduction of CyaY-Fe3+ by cysteine, indicating that under cluster synthesis specific conditions, electron transfer and iron transfer might be facilitated. The same rate constant was observed for iron citrate added in the absence of CyaY (data not shown).
three doublets: A, B, and C. For A and B we have assumed equal intensities, and the obtained parameters are: $\delta = 0.30$ mm/s, $\Delta E_Q = 0.60$ mm/s for A and $\delta = 0.34$ mm/s and $\Delta E_Q = 0.88$ mm/s for B. Within experimental error these hyperfine parameters are similar with those reported by Agar et al. (8) for the [2Fe-2S]$^{2+}$ IscU protein reconstituted chemically with IscS, cysteine and ferrous iron. The determined parameters indicate that one iron site of the pair is not Cys-bound. For the spectrum of Fig. 6B these doublets account for ~70% of total iron. An asymmetry at the right part of the 1-mm/s line prompted us to incorporate an additional doublet C in the fit. As a control experiment we assume an isomer shift of 0.44 mm/s and an average quadrupole splitting of 1.10 mm/s. From the fitting procedure this species represents ~20% of total iron. As a control experiment we recorded a Mössbauer spectrum from a sample with IscU chemically reconstituted with iron by the method of Agar et al. (8). A spectrum almost identical to that of Fig. 6B was obtained (data not shown). All of these results demonstrate that CyaY-Fe$^{3+}$ can be used as an iron donor for cluster formation on IscU resulting mainly in [2Fe-2S] with minimal amount of [4Fe-4S].

Control experiments in which either cysteine or IscS was omitted from the reaction mixture (conditions under which no sulfur was available for cluster assembly) demonstrated that IscU cannot acquire Fe$^{2+}$ or Fe$^{3+}$ directly from CyaY as shown from iron determination in the final IscU protein after separation from the reaction mixture (data not shown).

DISCUSSION

Iron-sulfur cluster assembly in proteins in vitro has been investigated using ferrous or ferric salts as the iron source. Even though, under these conditions, clusters can be correctly synthesized, it is very unlikely that the in vivo biological process utilizes free iron because the cellular concentration of free iron is expected to be maintained at a very low level to limit its toxicity. This assumption has led to the generally accepted concept that the iron donor should be a soluble protein which has the potential to store and transport ferric or ferrous iron but also to deliver it under controlled conditions to [Fe-S] scaffold proteins. Such a system should display sufficiently strong affinity for iron atoms as well as a mechanism for iron release. On the other hand, the fact that [Fe-S] clusters can be assembled with free iron in vitro without accessory proteins strongly suggests that the importance of the iron donor protein in the biosynthetic process is more in relation with its ability to solubilize, transport, and sequester iron than with an essential catalytic function for the reaction. To our knowledge, there are only very few studies of [Fe-S] cluster biosynthesis in vitro using an iron donor protein, so far. As far as eukaryotes are concerned, human frataxin was shown to transfer iron to the scaffold ISU protein because in the presence of a sulfur donor it is able to mediate [Fe-S] cluster assembly in ISU (29). In the case of bacterial systems, possible candidates as iron donor proteins were IscA and YggX, so far. Indeed IscA, a member of the [Fe-S] cluster assembly machinery, has been shown to behave, under certain conditions, as an iron-binding protein that can provide iron for [Fe-S] cluster synthesis in IscU, a scaffold protein of the bacterial isc operon (9, 52). On the other hand, IscA was also shown to assemble [Fe-S] clusters and was suggested to function as a scaffold protein (5–7). As a consequence, it is still unknown which role IscA is playing: an iron donor protein or an [Fe-S] scaffold protein. Even though indirect evidence has been provided regarding the YggX ability to bind iron, comparable biochemical studies in vitro are required to support the hypothesis that YggX is an iron scaffold protein. Even though indirect evidence has been provided regarding the YggX ability to bind iron, comparable biochemical studies in vitro are required to support the hypothesis that YggX is an iron scaffold protein.

**FIGURE 6. Reconstitution of [2Fe-25] IscU with CyaY-Fe$^{3+}$, IscS, and cysteine.**

A. UV-visible absorption. ApolscU (50 μM) was incubated with 1 μM IscS, 2 μM cysteine, and CyaY-Fe$^{3+}$ (150 μM iron) in 0.1 M Tris-HCl, pH 8, 50 mM KCl. The reaction was followed by UV-visible absorption spectroscopy. Thin line, initial spectrum; bold line, spectrum after 2 h of reaction. Inset, a plot of the yield (%) of [Fe-S] formation as a function of time was reported by Agar et al. (8) for the [2Fe-2S]$^{2+}$ IscU protein reconstituted chemically with IscS, cysteine and ferrous iron. The determined parameters indicate that one iron site of the pair is not Cys-bound. For the spectrum of Fig. 6B these doublets account for ~70% of total iron. An asymmetry at the right part of the 1-mm/s line prompted us to incorporate an additional doublet C in the fit. This doublet is proposed to arise from [4Fe-4S]$^{2+}$ (S = 0) species as has been observed earlier for IscU reconstituted chemically with IscS, cysteine, and ferrous iron (8). For this doublet we assumed an isomer shift of 0.44 mm/s and an average quadrupole splitting of 1.10 mm/s. From the fitting procedure this species represents ~20% of total iron. As a control experiment we recorded a Mössbauer spectrum from a sample with IscU chemically reconstituted with iron by the method of Agar et al. (8). A spectrum almost identical to that of Fig. 6B was obtained (data not shown). All of these results demonstrate that CyaY-Fe$^{3+}$ can be used as an iron donor for cluster formation on IscU resulting mainly in [2Fe-2S] with minimal amount of [4Fe-4S].

Control experiments in which either cysteine or IscS was omitted from the reaction mixture (conditions under which no sulfur was available for cluster assembly) demonstrated that IscU cannot acquire Fe$^{2+}$ or Fe$^{3+}$ directly from CyaY as shown from iron determination in the final IscU protein after separation from the reaction mixture (data not shown).
serves as an iron donor (9, 56, 57). We also failed to observe an Fe-IscU complex using E. coli IscU and CyaY-Fe$_{3+}$ as an iron donor (this work). We thus exclude the mechanism in which iron is coming first at least for E. coli and A. vinelandii. A second mechanism, on the contrary, involves first sulfur binding followed by iron binding. This is supported by the finding that sulfur transfer from IscS to IscU through transpersulfuration reactions is effective (48, 57). However, no evidence for [Fe-S] cluster formation upon the addition of iron to sulfur-contain-
ing forms of IscS has been reported either (55). Further work is needed to show whether this is possible. In this context and based on the observation reported here that CyaY forms a complex with IscS, we would like to propose a third mechanism that would involve transfer of Fe$_{3+}$ to intermediate IscS persulfides to generate protein-bound Cys-sulfur-sulfur-iron species followed by transfer of iron-sulfur units to apoSISC (Fig. 7). This speculative mechanism is experiment-
testable and is currently under investigation in our laboratory. In conclusion, the biochemical studies reported here strongly support the hypothesis that CyaY is an iron transport/donor protein involved in [Fe-S] cluster assembly. Even though CyaY is probably not unique in this function, the present work together with a very recently reported study showing CyaY specific phenotypes (32) establishes both in vivo and in vitro a link between CyaY and [Fe-S] cluster biosynthesis.

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REFERENCES

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52. Ding, H., Clark, R. J., and Ding, B. (2004) J. Biol. Chem. 279, 37499–37504