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Distinct Iron Binding Property of Two Putative Iron Donors for the Iron-Sulfur Cluster Assembly

*IscA AND THE BACTERIAL FRATAXIN ORTHOLOG CyaY UNDER PHYSIOLOGICAL AND OXIDATIVE STRESS CONDITIONS**

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Frataxin, a small mitochondrial protein linked to the neurodegenerative disease Friedreich ataxia, has recently been proposed as an iron donor for the iron-sulfur cluster assembly. An analogous function has also been attributed to IscA, a key member of the iron-sulfur cluster assembly machinery found in bacteria, yeast, and humans. Here we have compared the iron binding property of IscA and the frataxin ortholog CyaY from *Escherichia coli* under physiological and oxidative stress conditions. In the presence of the thioredoxin reductase system, which emulates the intracellular redox potential, CyaY fails to bind any iron even at a 10-fold excess of iron in the incubation solution. Under the same physiologically relevant conditions, IscA efficiently recruits iron and transfers the iron for the iron-sulfur cluster assembly in a proposed scaffold IscU. In the presence of hydrogen peroxide, however, IscA completely loses its iron binding activity, whereas CyaY becomes a competent iron-binding protein and attenuates the iron-mediated production of hydroxyl free radicals. Hydrogen peroxide appears to oxidize the iron binding thiol groups in IscA, thus blocking the iron binding in the protein. Once the oxidized thiol groups in IscA are re-reduced with the thioredoxin reductase system, the iron binding activity of IscA is fully restored. On the other hand, hydrogen peroxide has no effect on the iron binding carboxyl groups in CyaY, allowing the protein to bind iron under oxidative stress conditions. The results suggest that IscA is capable of recruiting intracellular iron for the iron-sulfur cluster assembly under normal physiological conditions, whereas CyaY may serve as an iron chaperon to sequester redox active free iron and alleviate cellular oxidative damage under oxidative stress conditions.

Frataxin is a small mitochondrial protein that has been linked to Friedreich ataxia, an autosomal recessive neurodegenerative disease (1). Most Friedreich ataxia patients are homozygous for a large GAA repeat expansion in the first intron of the frataxin gene which impairs transcription and causes severe reduction in the level of frataxin in mitochondria (1, 2). Frataxin is highly conserved from bacteria to humans (3). Deletion of frataxin results in disruption of iron homeostasis and mitochondrial function in *Saccharomyces cerevisiae* (4), embryonic lethality in the mouse (5), and developmental arrest in the nematode *Caenorhabditis elegans* (6). Structural studies of human frataxin (7), yeast frataxin (8), and the bacterial frataxin ortholog CyaY[2] (9, 10) revealed a well conserved three-dimensional structure. However, the specific function of frataxin/CyaY is still not fully understood. Recently, it has been proposed that one of the functions of frataxin/CyaY may be involved in biogenesis of iron-sulfur clusters, a group of ubiquitous redox co-factors in cells. This notion is primarily based on the observations that (i) depletion of frataxin in Friedreich ataxia patients is associated with deficiency of iron-sulfur proteins in mitochondria (11), (ii) Frataxin/CyaY binds both ferrous and ferric iron with relatively weak iron binding affinities (12–17), (iii) Frataxin/CyaY has specific protein-protein interactions with IscU, a proposed iron-sulfur cluster assembly scaffold protein (18–21), cysteine desulfurase IscS (21, 22), and the mitochondrial electron transfer components (23) and aconitase (24), and (iv) the iron-loaded human frataxin (18) and *Escherichia coli* CyaY (22) can provide iron for the iron-sulfur cluster assembly in IscU *in vitro*. Taken together, these results suggested an attractive idea that frataxin/CyaY may act as a physiological iron donor for biogenesis of iron-sulfur clusters. However, genetic studies indicated that the frataxin orthologs are not essential for biogenesis of iron-sulfur clusters in *E. coli* (25), *Salmonella enterica* (26), and *S. cerevisiae* (27). Instead, deficiency of frataxin has been shown to cause mitochondrial and nuclear oxidative damages in yeast cells (28, 29), and expression of human mitochondrial ferritin rescues the respiratory function of mitochondria in the frataxin-deficient cells (30). Furthermore, Condo *et al.* (31) recently reported that an extra-mitochondrial pool of frataxin can efficiently prevent mitochondrial oxidative damage and apoptosis in different cellular systems and fully replace mitochondrial frataxin in promoting survival of Friedreich ataxia cells (31). Accordingly, it was suggested that the primary function of frataxin/CyaY is to detoxify the redox active free iron in cells (28–34).

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[2] The abbreviations used are: CyaY, bacterial frataxin ortholog; apoCyaY, CyaY devoid of any iron; apoIscA, IscA devoid of any iron; IscU, a proposed iron-sulfur cluster assembly scaffold protein; IscS, cysteine desulfurase.

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In searching for specific iron donor(s) for biogenesis of iron-sulfur clusters, we have discovered that IscA, a key member of the iron-sulfur cluster assembly machinery found in bacteria (35–37), yeast (38), and humans (39), is a novel iron-binding protein (40). In the presence of the thioredoxin reductase system, IscA binds iron with an iron association constant of 2.0×10^{19} M^{-1} (40, 41). The iron center in IscA can be readily mobilized by L-cysteine (42) and transferred for the iron-sulfur cluster assembly in a proposed scaffold protein IscU (40–44). Although IscA was previously characterized as an alternative iron-sulfur cluster assembly scaffold (45–48) and as a regulatory protein (49), the strong iron binding affinity (40, 41) and ease to mobilize the iron center in the protein by L-cysteine (42) led us to hypothesize that the primary function of IscA is to recruit intracellular free iron and deliver the iron for the iron-sulfur cluster assembly (44).

To further elucidate the role of frataxin/CyaY and IscA in biogenesis of iron-sulfur clusters, here we have compared the iron binding property of IscA and CyaY from *E. coli* under physiological and oxidative stress conditions. The results indicate that in the presence of the thioredoxin reductase system, which emulates the intracellular redox potential, CyaY, unlike IscA, fails to bind any iron even at a 10-fold excess of iron in the incubation solution. In the presence of hydrogen peroxide, however, CyaY becomes a competent iron-binding protein and attenuates the iron-mediated production of hydroxyl free radicals, whereas IscA completely loses its iron binding activity. The possible physiological role of IscA and CyaY in biogenesis of iron-sulfur clusters and in the intracellular iron metabolism under oxidative stresses will be discussed.

EXPERIMENTAL PROCEDURES

Protein Preparation—The DNA fragment encoding CyaY was amplified from wild-type *E. coli* genomic DNA using the PCR. Two primers, CyaY-1, 5′-GATACAACCATGGACGA-CAGTGAA-3′, and CyaY-2, 5′-CATGCAAAGCTTGCG-GAAACTGAC-3′, were used for the PCR amplification. The PCR product was digested with two restriction enzymes HindIII and NcoI and ligated into an expression vector pET28b^{+} as described previously (40). The cloned DNA fragment was confirmed by direct sequencing using the T7 primers. Recombinant CyaY was overproduced and purified as described previously for IscA (40), IscU (40), and IscS (50). The precise molecular weight of purified CyaY was confirmed using the electrospray ionization-mass spectrometry (Chemistry Department, Louisiana State University). ApoIscA and apoCyaY (proteins devoid of any iron) were prepared by incubation with L-cysteine (2 mM) at 37 °C for 30 min followed by re-purification of the protein using a HiTrap desalting column or a Mono Q column. *E. coli* thioredoxin-1 and thioredoxin reductase were produced from the expression vectors pDL59 (51) and pTrR301 (52), respectively, and purified as described in Ding *et al.* (41). The expression vectors pDL59 (51) and pTrR301 (52) were kindly provided by Dr. Scott B. Mulrooney (University of Michigan). Both thioredoxin-1 and thioredoxin reductase were purified as the native form without any tags. The purity of purified proteins was greater than 95% as judged by the SDS-polyacrylamide gel electrophoresis analyses. The concentration of apoIscA was determined using an extinction coefficient at 260 nm of 2.4 $mM^{-1}cm^{-1}$ (40). The concentrations of apoCyaY, apoIscU, IscS, thioredoxin-1, and thioredoxin reductase were determined using extinction coefficients at 280 nm of 30.0, 11.2, 39.7, 14.2, and 17.7 $mM^{-1}cm^{-1}$, respectively (40).

Iron Binding Assay—For the iron binding assay, apoIscA and apoCyaY were incubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ in the presence of the thioredoxin reductase system (thioredoxin-1 (5 μM), thioredoxin reductase (0.5 μM) and NADPH (500 μM)) in buffer containing NaCl (100 mM) and Tris (20 mM) (pH 8.0) in open-to-air microcentrifuge tubes at 37 °C for 30 min. CyaY and IscA were then re-purified from the incubation solutions using a Mono Q column as described in Yang *et al.* (44). The re-purification procedure using the Mono Q column did not significantly affect the iron binding in CyaY or IscA as >90% of the iron content in the iron-bound CyaY or IscA remained after passing through the Mono Q column. The eluted proteins from the Mono Q column were analyzed using a SDS-polyacrylamide gel electrophoresis. The total iron content in the eluted fractions was determined using the inductively coupled plasma mass spectroscopy (Department of Geology and Geophysics, Louisiana State University) or an iron indicator ferrozine as described in Yang *et al.* (44). The iron-ferrozine complex was measured at 564 nm using an extinction coefficient of 27.9 $mM^{-1}cm^{-1}$ (53). The results from both iron analysis methods were similar to each other.

Iron-Sulfur Cluster Assembly Assay—For the iron-sulfur cluster assembly assay, either IscA or CyaY was preincubated with apoIscU and IscS in the presence of the thioredoxin reductase system in buffer containing NaCl (100 mM) and Tris (20 mM) (pH 8.0) at 37 °C. The reaction solutions were purged with pure argon gas and preincubated at 37 °C for 5 min before L-cysteine was added to initiate the iron-sulfur cluster assembly reaction. The iron-sulfur cluster assembly in IscU was monitored in a Beckman DU640 UV-visible absorption spectrometer equipped with a temperature controller as described previously (41, 54).

Measurements of Hydroxyl Free Radicals—The iron-mediated production of hydroxyl free radicals was measured after the procedure described by Halliwell *et al.* (55). Briefly, hydroxyl free radicals degrade 2-deoxyribose to form a malondialdehyde-like compound that reacts with thiobarbituric acid to generate a chromogen. In the experiments, apoIscA or apoCyaY was preincubated with $Fe(NH_4)_2(SO_4)_2$ in buffer containing K_2HPO_4/KH_2PO_4 (10 mM) (pH 7.4), NaCl (60 mM), 2-deoxyribose (4 mM), and the thioredoxin reductase system at 37 °C for 10 min before hydrogen peroxide (0.5 mM) was added to initiate Fenton reaction. The reactions were continued at 37 °C for additional 25 min. A developing solution containing 1% thiobarbituric acid and 2.8% trichloroacetic acid (400 μl) was then mixed with the above incubation solutions (600 μl) and boiled for 15 min. The reaction mixtures were centrifuged at 14,000 rpm in a desktop microcentrifuge for 15 min to remove the precipitates. The relative amounts of the chromogen in the solutions were measured from the emission at a wavelength of 553 nm using an excitation wavelength of 532 nm in a PerkinElmer LS-3 fluorescence spectrophotometer (55).

Measurements of the Total Free Thiol Contents—The total free thiol contents in the protein samples were analyzed using the Ellman reagent (5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) (56). 5,5′-Dithiobis(2-nitrobenzoic acid) (at a final concentration of 100 μM) was added to the protein samples pretreated with methanol and incubated at room temperature for 20 min followed by centrifugation to remove the precipitates. The amounts of the total free thiols in the protein samples were calculated from an absorption amplitude at 412 nm using *N*-acetyl-L-cysteine as a standard.

EPR Measurements—The EPR spectra were recorded at X-band on a Bruker ESR-300 spectrometer using an Oxford Instruments ESR-9 flow cryostat (Chemistry Department, Louisiana State University). The routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 2.0 millitesla; sample temperature, 4.5 K; receive gain, 1.0×10^5.

RESULTS

Iron Binding Activity of CyaY and IscA in the Presence of the Thioredoxin Reductase System—In previous studies, the frataxin ortholog CyaY and IscA from *E. coli* have been shown to bind iron with the iron association constants of 2.6×10^5 M^{-1} (13, 15) and 2.0×10^{19} M^{-1} (40–44), respectively. However, the iron binding studies for IscA and CyaY were carried out by different groups and under different experimental conditions. Because both CyaY (22) and IscA (40–44) are proposed as the potential iron donor for biogenesis of iron-sulfur clusters, it is imperative to re-evaluate the iron binding property of CyaY and IscA under the same physiologically relevant conditions.

In cells the intracellular redox potential is estimated to be in the range of −260 mV to −280 mV (57, 58). The relatively low intracellular redox potential is largely maintained by the redundant thiol reducing systems (59). To emulate the intracellular redox potential we have reconstructed the thioredoxin reductase system using *E. coli* thioredoxin-1 (51), thioredoxin reductase (52), and NADPH as described in Ding *et al.* (41). In the system, NADPH provides electrons to reduce thioredoxin-1 via thioredoxin reductase (59).

To compare the iron binding activity of IscA and CyaY under the physiologically relevant conditions, we incubated apoCyaY (100 μM) and apoIscA (100 μM) with freshly prepared ferrous iron (50 μM) in the presence of the thioredoxin reductase system at 37 °C for 30 min in open-to-air micro-centrifuge tubes followed by re-purification using a Mono Q column. As described under "Experimental Procedures," re-purification procedure using the Mono Q column did not significantly affect the iron binding in CyaY or IscA. The SDS-polyacrylamide gel electrophoresis analysis showed that IscA was mostly eluted in fractions 8 and 9, whereas CyaY was eluted in fractions 12 and 13 (Fig. 1*B*) under the experimental conditions. The iron content analyses of the eluted fractions showed that >94% of the total iron content in the incubation solution was bound to IscA and less than 4% to CyaY (Fig. 1*A*). The UV-visible absorption measurements of the eluted IscA fractions revealed an iron-loaded IscA with a dominating absorption peak at 315 nm and a shoulder at 435 nm (Fig. 2*A*). The ratio of the absorption at 315 nm to that at 260 nm was about 1.02, indicating that IscA was almost fully saturated with iron (40). The specific iron binding in IscA was further confirmed from the EPR measurements, which revealed an EPR signal at g = 4–6 of a unique $S = 3/2$ ground spin state mononuclear iron center in IscA (Fig. 2*C*) (40). In contrast, the UV-visible absorption (Fig. 2*B*) and EPR measurements (Fig. 2*C*) indicated no iron binding in the eluted CyaY samples.

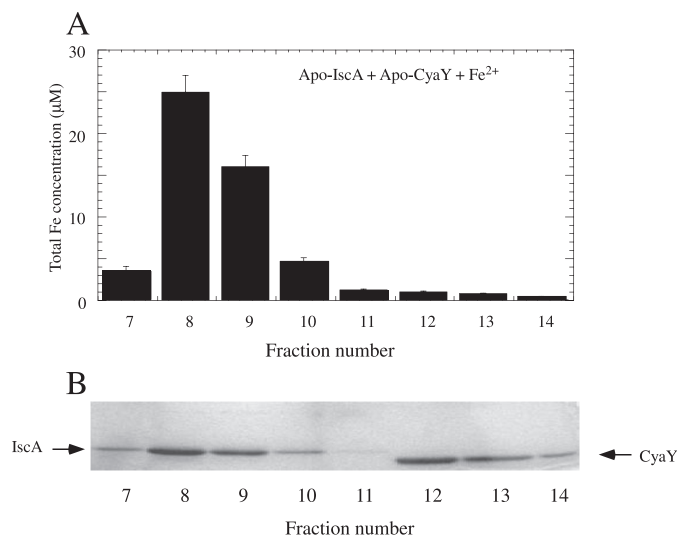

FIGURE 1. **The iron binding activity of IscA and CyaY in the presence of the thioredoxin reductase system.** ApoIscA (100 μM) and apoCyaY (100 μM) were incubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (50 μM) in the presence of the thioredoxin reductase system (thioredoxin-1 (5 μM), thioredoxin reductase (0.5 μM), and NADPH (500 μM)) at 37 °C for 30 min followed by re-purification of the proteins using a Mono Q column as described under "Experimental Procedures." *A*, total iron content analyses of the eluted fractions. *B*, SDS-polyacrylamide electrophoresis gel of the eluted fractions. The positions of IscA and CyaY on the SDS-polyacrylamide electrophoresis gel are indicated. The results are representative of three independent experiments.

To further examine the iron binding activity of CyaY under the physiologically relevant conditions, apoCyaY was incubated alone with ferrous iron in the presence of the thioredoxin reductase system at 37 °C for 30 min followed by re-purification of the protein. Again, the UV-visible absorption measurements and the total iron content analyses showed no iron binding in the re-purified CyaY (data not shown). On the other hand, the iron binding in IscA was essentially the same with or without apoCyaY in the incubation solution. These results suggest that in the presence of the thioredoxin reductase system, CyaY fails to bind any iron, whereas IscA is a strong iron-binding protein as reported previously (40–44).

We then asked whether the iron center in IscA is available for the iron-sulfur cluster assembly under the same physiologically relevant conditions. In the experiments the re-purified IscA from Fig. 1*A* was preincubated with IscU, a proposed iron-sulfur cluster assembly scaffold (54) and cysteine desulfurase IscS in the presence of the thioredoxin reductase system at 37 °C for 5 min. The iron-sulfur cluster assembly reaction was initiated by adding L-cysteine to the incubation solution. The results demonstrated that the iron center in the re-purified IscA was efficiently transferred to IscU for the iron-sulfur cluster assembly after L-cysteine was added to the incubation solution as

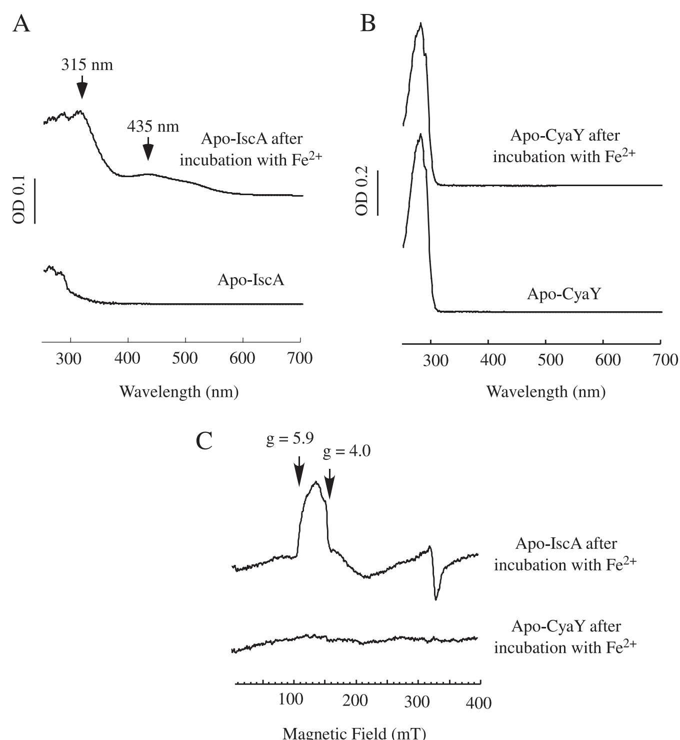

FIGURE 2. **Spectroscopic analyses of IscA and CyaY after incubation with ferrous iron in the presence of the thioredoxin reductase system.** *A*, UV-visible absorption spectra of the re-purified IscA before and after incubation with ferrous iron in the presence of the thioredoxin reductase system. *B*, UV-visible absorption spectra of the re-purified CyaY before and after incubation with ferrous iron in the presence of the thioredoxin reductase system. *C*, EPR spectra of the re-purified IscA and CyaY after incubation with ferrous iron in the presence of the thioredoxin reductase system. *mT*, millitesla.

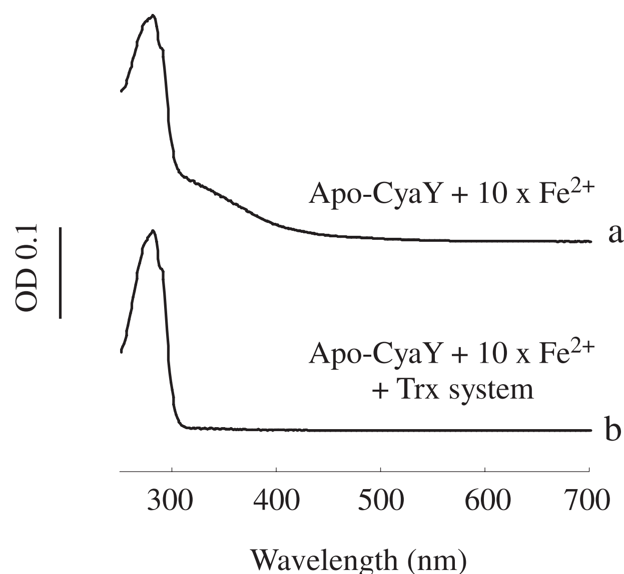

FIGURE 3. **CyaY does not form the iron-mediated aggregate complexes in the presence of the thioredoxin reductase system.** ApoCyaY (100 μM) was incubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (1 mM) in the presence or absence of the thioredoxin reductase system in buffer containing NaCl (100 mM) and Tris (20 mM) (pH 8.0) at 37 °C for 30 min. CyaY was then re-purified from the incubation solutions. *Spectrum a*, CyaY purified from the incubation solution without the thioredoxin reductase system. *Spectrum b*, CyaY purified from the incubation solution with the thioredoxin reductase system (*Trx*). Similar results were obtained from three independent experiments.

reported previously (41). In contrast, when the re-purified IscA was replaced with the re-purified CyaY in the preincubation solution, no iron-sulfur clusters were assembled in IscU (data not shown). Collectively, the results described above suggest that in the presence of the thioredoxin reductase system, CyaY fails to bind any iron, whereas IscA is able to recruit iron and transfer the iron for the iron-sulfur cluster assembly in IscU.

CyaY Does Not Form the Iron-mediated Aggregate Complexes in the Presence of the Thioredoxin Reductase System—Lack of iron binding in CyaY in the presence of the thioredoxin reductase system would preclude CyaY from being a major iron donor for the iron-sulfur cluster assembly. However, it has been reported that CyaY may form the aggregate complexes in the presence of excessive amounts of iron and low concentrations of salts under aerobic conditions *in vitro* (13, 15, 22), and that the aggregate complexes can provide iron for the iron-sulfur cluster assembly in IscU (22).

To test whether CyaY can form the aggregate complexes under the physiologically relevant conditions, apoCyaY was incubated with a 10-fold excess of iron in the absence or presence of the thioredoxin reductase system at 37 °C for 30 min followed by re-purification of the protein. Without the thioredoxin reductase system in the incubation solution, CyaY showed an increased absorption at around 300–400 nm (Fig. 3*a*), indicative of the formation of the oxo/hydroxoferric iron species in the protein. The total iron content analyses showed that the ratio of iron to the CyaY monomer was about 4.5:1. Finally, the gel filtration profile of the re-purified CyaY indicated that in the absence of the thioredoxin reductase system, CyaY formed the aggregate complexes (data not shown). These results are consistent with the previous studies showing that CyaY can form the aggregate complexes in the presence of excessive amounts of iron and low concentrations of salts *in vitro* (13, 15, 22).

However, when apoCyaY was incubated with a 10-fold excess of ferrous iron in the presence of the thioredoxin reductase system at 37 °C for 30 min, the re-purified CyaY showed no absorption increase at around 300–400 nm (Fig. 3*b*), and the iron content in the re-purified CyaY was not detectable. Furthermore, the gel filtration analysis showed no CyaY aggregate complexes after incubation. Taken together, these results suggest that in the presence of the thioredoxin reductase system, CyaY fails to bind iron or form the iron-mediated aggregate complexes.

Hydrogen Peroxide Promotes the Iron Binding in CyaY and Blocks the Iron Binding in IscA—Oxidative metabolism will inevitably generate reactive oxygen species in cells. An elevated level of intracellular free iron will further enhance the cellular oxidative damage by promoting the production of hydroxyl free radicals via a Fenton reaction (60). As iron-binding proteins, both CyaY and IscA could have the potential to sequester redox active free iron and prevent the production of hydroxyl free radicals.

To compare the iron binding activity of IscA and CyaY under oxidative stress conditions, we incubated apoIscA (100 μM) and apoCyaY (100 μM) with freshly prepared ferrous iron (50 μM) in the presence of the thioredoxin reductase system and hydrogen peroxide (2 mM) at 37 °C for 30 min in open-to-air microcentrifuge tubes followed by re-purification of IscA and CyaY using

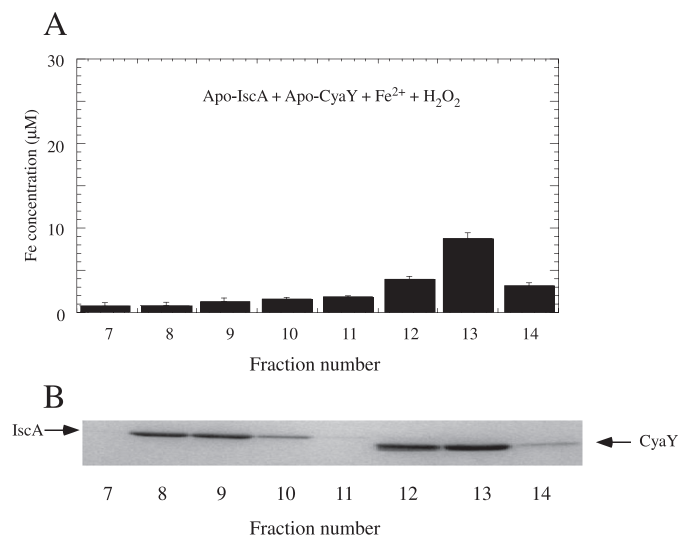

FIGURE 4. **The iron binding activity of IscA and CyaY in the presence of hydrogen peroxide.** ApoIscA (100 μM) and apoCyaY (100 μM) were incubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (50 μM) in the presence of hydrogen peroxide (2 mM) and the thioredoxin reductase system at 37 °C for 30 min followed by re-purification of the proteins using a Mono Q column as described under "Experimental Procedures." *A*, the total iron content analyses of the eluted fractions. *B*, the SDS-polyacrylamide electrophoresis gel of the eluted fractions. The positions of IscA and CyaY on the SDS-polyacrylamide electrophoresis gel are indicated. The results are the representatives of three independent experiments.

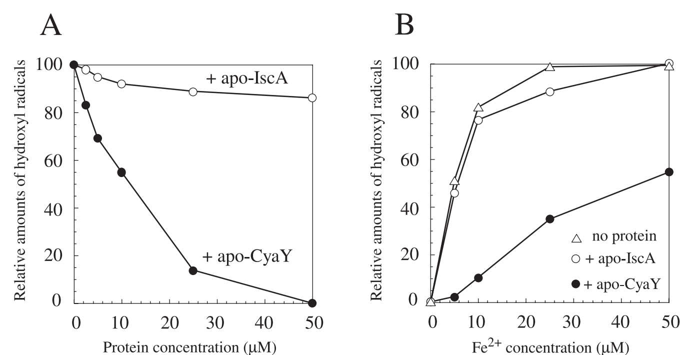

FIGURE 5. **CyaY prevents the iron-mediated production of hydroxyl free radicals in the presence of hydrogen peroxide.** *A*, apoCyaY or apoIscA (0–50 μM) was preincubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (5 μM) in the presence of the thioredoxin reductase system and 2-deoxyribose (4 mM) at 37 °C for 10 min before hydrogen peroxide (0.5 mM) was added to initiate Fenton reaction. The relative amounts of hydroxyl free radicals produced in the incubation solutions were plotted as a function of the protein concentrations of apoCyaY (*filled circles*) or apoIscA (*open circles*). *B*, apoCyaY (50 μM) or apoIscA (50 μM) was preincubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (0–50 μM) in the presence of the thioredoxin reductase system and 2-deoxyribose (4 mM) at 37 °C for 10 min before hydrogen peroxide (0.5 mM) was added to initiate Fenton reaction. The relative amounts of hydroxyl free radicals produced in the incubation solutions were plotted as a function of the concentrations of $Fe(NH_4)_2(SO_4)_2$ in the incubation solutions. *Open triangles*, no protein; *filled circles*, apoCyaY; *open circles*, apoIscA. The samples without any ferrous iron were used as negative controls. The data are the representatives of three independent experiments.

the Mono Q column (Fig. 4*B*). The iron analyses of the eluted samples showed that less than 3% of the total iron content was bound to IscA and about 15–20% of the total iron content was bound to CyaY (Fig. 4*A*), indicating that in the presence of hydrogen peroxide, IscA completely loses its iron binding activity, whereas CyaY is able to bind iron at a relatively weak iron binding affinity.

To further explore the iron binding activity of CyaY and IscA in the presence of hydrogen peroxide, we measured the iron-mediated production of hydroxyl free radicals in the presence of apoCyaY or apoIscA. In the Fenton reaction, ferrous iron reduces hydrogen peroxide to deleterious hydroxyl free radicals. Sequestration of redox active free iron by proteins could prevent the iron-mediated production of hydroxyl free radicals. In the experiments ferrous iron was preincubated with either apoCyaY or apoIscA in the presence of the thioredoxin reductase system and hydrogen peroxide. The production of hydroxyl free radicals was measured using 2-deoxyribose and thiobarbituric acid as described in Halliwell *et al.* (55).

Fig. 5*A* shows the titration of apoCyaY or apoIscA (0–50 μM) with a fixed concentration of ferrous iron (5 μM) in the presence of the thioredoxin reductase system and hydrogen peroxide (0.5 mM). As the concentration of apoCyaY was gradually increased, the iron-mediated production of hydroxyl free radicals was progressively decreased. When a 10-fold excess of apoCyaY was used, the iron-mediated production of hydroxyl free radicals was almost completely eliminated. In contrast, apoIscA (0–50 μM) had only a very little effect on the iron-mediated production of hydroxyl free radicals under the same experimental conditions.

Fig. 5*B* shows the production of hydroxyl free radicals as a function of the iron concentrations in the presence of the thioredoxin reductase system and hydrogen peroxide (0.5 mM). As the iron concentration was gradually increased from 0 to 50 μM, the iron-mediated production of hydroxyl free radicals was quickly increased. The addition of apoCyaY (50 μM) to the incubation solutions greatly attenuated the iron-mediated production of hydroxyl free radicals. On the other hand, apoIscA (50 μM) had little or no effect on the iron-mediated production of hydroxyl free radicals in the incubation solutions containing different concentrations of iron. The results demonstrate that in the presence of hydrogen peroxide, CyaY is a competent iron-binding protein that attenuates the iron-mediated production of hydroxyl free radicals, whereas IscA completely loses its iron binding activity.

Hydrogen Peroxide Oxidizes the Free Thiol Groups in IscA and Blocks the Iron Binding in the Protein—The available structure studies indicate that CyaY binds iron via the carboxyl groups of the conserved aspartate and glutamate residues (10, 13, 15), whereas IscA binds iron through the thiol groups of the conserved cysteine residues (41, 61, 62). It is conceivable that hydrogen peroxide may have no effect on the iron binding carboxyl groups in CyaY, allowing the protein to bind iron under oxidative stress conditions and attenuate the iron-mediated production of hydroxyl free radicals. On the other hand, hydrogen peroxide may oxidize the iron binding thiol groups in IscA, thus blocking the iron binding in the protein.

To test the idea that oxidization of the thiol groups in IscA blocks the iron binding in the protein, we simultaneously analyzed the iron binding activity and the total free thiol groups of IscA after incubation with ferrous iron and different amounts of hydrogen peroxide in the presence of the thioredoxin reductase system. As the concentration of hydrogen peroxide was gradually increased, the iron binding in IscA (Fig. 6*A*) and the total thiol groups in the protein (Fig. 6*B*) were progressively

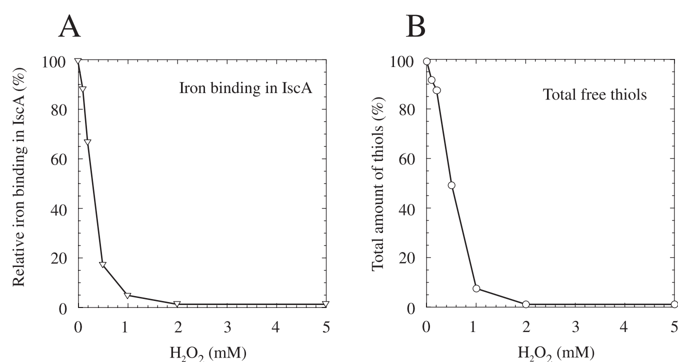

FIGURE 6. **Hydrogen peroxide inhibits the iron binding in IscA and oxidizes the free thiol groups in the protein.** ApoIscA (100 μM) was incubated with freshly prepared $Fe(NH_4)_2(SO_4)_2$ (50 μM) and hydrogen peroxide (0–5 mM) in the presence of the thioredoxin reductase system at 37 °C for 30 min. *A*, hydrogen peroxide blocks the iron binding in IscA. The iron binding in IscA was plotted as a function of the hydrogen peroxide concentrations in the incubation solution. *B*, hydrogen peroxide oxidizes the free thiol groups in IscA. The relative amounts of the total free thiol contents in IscA were plotted as a function of the hydrogen peroxide concentrations in the incubation solution.

decreased. A correlation between the iron binding in IscA and the amount of the free thiol groups in the protein strongly suggests that oxidation of the free thiol groups in IscA leads to the failure of iron binding in the protein.

If oxidization of the free thiol groups blocks the iron binding of IscA, we reasoned that re-reduction of the hydrogen peroxide-oxidized IscA should re-establish its iron binding activity. Indeed, when the hydrogen peroxide-treated IscA was re-incubated with the thioredoxin reductase system, the iron binding activity of IscA was fully restored (Fig. 7).

DISCUSSION

Throughout evolution, iron-sulfur clusters have become integral parts of diverse physiological processes ranging from energy metabolism to the regulation of gene expression (63–65). Increasing evidence suggests that sulfur in iron-sulfur clusters is derived from L-cysteine via cysteine desulfurase IscS (66–68). However, the iron donor(s) for the iron-sulfur cluster assembly still largely remains elusive. In this study we have compared the iron binding property of two putative iron donors for the iron-sulfur cluster assembly; CyaY, the bacterial ortholog of human frataxin (1, 2), and IscA, a key member of the iron-sulfur cluster assembly machinery found in bacteria (35–37), yeast (38), and humans (39). The results demonstrate that IscA and CyaY have distinct iron binding properties under normal physiological and oxidative stress conditions. In the presence of the thioredoxin reductase system, which emulates the intracellular redox potential, CyaY fails to bind any iron or form the iron-mediated aggregate complexes, whereas IscA binds iron with an iron association constant of 2.0×10^{19} M^{-1} (41) and delivers iron for the iron-sulfur cluster assembly in a proposed scaffold IscU (40–44). In the presence of hydrogen peroxide, however, IscA completely loses its iron binding activity, whereas CyaY becomes a competent iron-binding protein and attenuates the iron-mediated production of hydroxyl free radicals. Hydrogen peroxide appears to oxidize the iron binding thiol groups in IscA, thus blocking the iron binding in the protein. Re-reduction of the hydrogen peroxide-oxidized IscA with

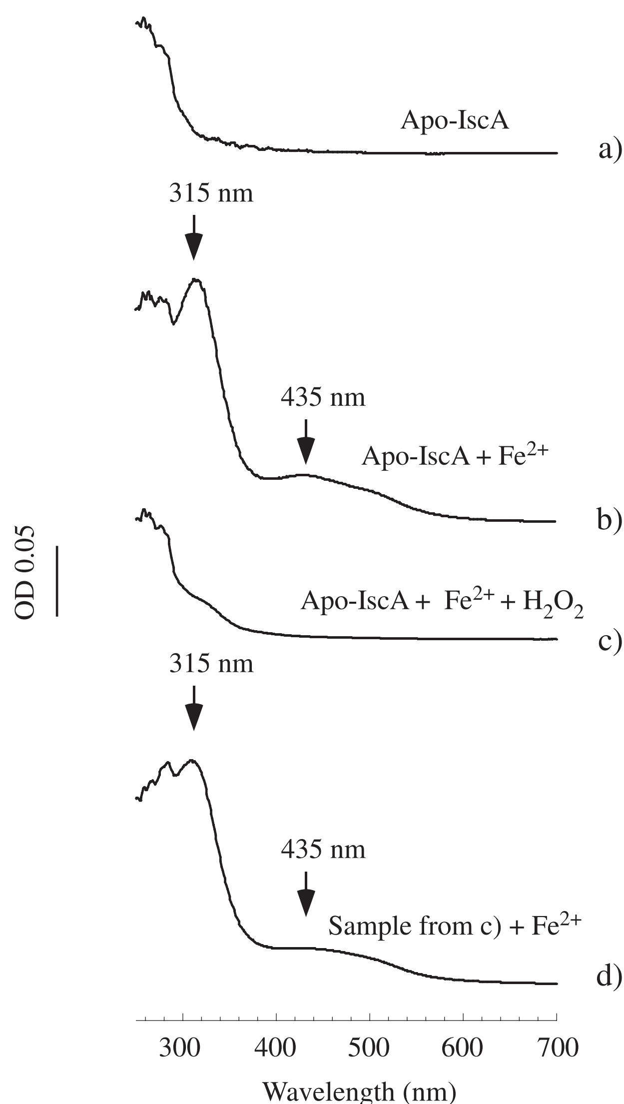

FIGURE 7. **Recovery of the iron binding activity of the hydrogen peroxide-treated IscA by the thioredoxin reductase system.** *Spectrum a*, apoIscA. *Spectrum b*, apoIscA was incubated with an equivalent amount of freshly prepared $Fe(NH_4)_2(SO_4)_2$ in the presence of the thioredoxin reductase system at 37 °C for 30 min. *Spectrum c*, as *spectrum b*, except hydrogen peroxide (2 mM) was included in the incubation solution. *Spectrum d*, the hydrogen peroxide-treated IscA (*spectrum c*) was re-incubated with an equivalent amount of freshly prepared $Fe(NH_4)_2(SO_4)_2$ in the presence of the thioredoxin reductase system at 37 °C for 30 min. All protein samples were re-purified from the incubation solutions using a Mono Q column. The protein concentration in each sample was adjusted to 40 μM. The absorption peaks at 315 and 435 nm indicate the iron binding in IscA.

the thioredoxin reductase system fully restores the iron binding activity of IscA. On the other hand, hydrogen peroxide has no effect on the iron binding carboxyl groups in CyaY, allowing the protein to bind iron under oxidative stress conditions. These results led us to propose that the primary function of IscA is to recruit intracellular iron for the iron-sulfur cluster assembly under normal physiological conditions, whereas CyaY may serve as an iron chaperon to sequester the redox active free iron and alleviate the iron-mediated production of hydroxyl free radicals under oxidative stress conditions.

IscA is highly conserved from bacteria (35–37) to yeast (38) to humans (39). In *E. coli* IscA is a member of an operon *iscRSUA*, which also encodes IscR, IscS, and IscU (35–37). IscR

is a repressor of the operon *iscRSUA* and contains an iron-sulfur cluster (37). Disassembly of the iron-sulfur clusters in IscR, an indicator of deficiency or damage of iron-sulfur clusters in cells, will inactivate IscR as a repressor and stimulate the expression of IscS, IscU, and IscA (37). IscS is a cysteine desulfurase that catalyzes desulfurization of L-cysteine and transfers sulfane sulfur to a proposed scaffold protein IscU for the iron-sulfur cluster assembly (66–68). Although previous studies indicated that IscA may act as an alternative scaffold protein (45–48) or a regulatory protein (49), our results shown here and in previous studies (40–44) suggest that the primary function of IscA could be to recruit intracellular iron for the iron-sulfur cluster assembly. The x-ray crystallographic studies revealed that IscA likely exists as a dimer or a tetramer with three invariant cysteine residues (Cys-35, Cys-99, and Cys-101, *E. coli* numbering) projected to form a "cysteine pocket" that could accommodate the iron binding in the protein (61, 62). The site-directed mutagenesis studies further showed that the invariant cysteine residues in IscA are essential for the iron binding activity *in vitro* (41) and its physiological function *in vivo* (38, 69). In the presence of the thioredoxin reductase system, the iron association constant of IscA (2.0×10^{19} M^{-1}) (41) is almost comparable with that of human transferrin (4.7×10^{20} M^{-1}) (70). Such a high iron binding affinity may ensure the iron binding in IscA for biogenesis of iron-sulfur clusters under physiological conditions. Although the iron-bound IscA is stable under both anaerobic and aerobic conditions (44), the iron center in IscA can be readily mobilized by L-cysteine and transferred for the iron-sulfur cluster assembly in IscU (42). In this context we propose that IscS (sulfur donor), IscU (a scaffold), and IscA (an iron donor) may constitute the core of the iron-sulfur cluster assembly machinery in cells (44).

Frataxin/CyaY and their homologues share a well conserved three-dimensional structure belonging to the α-β sandwich motif family (7–10). The highly conserved patch of aspartic and glutamic acid residues on the surface of frataxin/CyaY have been suggested to be directly involved in the iron binding (7–10). Using the isothermal titration calorimetry, the ferrous iron association constants for human frataxin (18) and *E. coli* CyaY (15) were estimated to be 1.8×10^4 and 2.6×10^5 M^{-1}, respectively. Considering the relatively weak iron binding affinity, the iron binding in CyaY could be insignificant under physiological conditions. Indeed, in the presence of the thioredoxin reductase system, CyaY fails to bind any iron *in vitro* (Fig. 1*A*). Interestingly, it has been reported that frataxin/CyaY may form the iron-mediated aggregate complexes in the presence of excessive amounts of iron and low concentrations of salts under aerobic conditions (12–16). However, the physiological relevance of the iron-mediated aggregation of frataxin/CyaY was questioned by a recent study showing that a yeast frataxin mutant defective in the iron-mediated aggregation has a wild-type phenotype (71). Here, we found that in the presence of the thioredoxin reductase system, CyaY does not form the iron-mediated aggregate complexes even at a 10-fold excess of iron, further supporting the notion that the iron-mediated aggregation of frataxin/CyaY may be not a critical function of the protein (17, 71). On the other hand, under oxidative stress conditions, frataxin/CyaY may have an important role in detoxifying the redox active free iron (4, 11, 12, 28–34). The iron binding carboxyl groups in CyaY are resistant to hydrogen peroxide, which allows CyaY to bind iron under oxidative stress conditions and attenuate the iron-mediated production of hydroxyl free radicals (Fig. 5). This is consistent with the observations that a deficiency of frataxin in cells is associated with the iron-mediated production of reactive free radicals and the increased cellular oxidative damage (28–31).

In cells, iron-sulfur clusters are considered the primary targets of reactive oxygen species (72). Disruption of iron-sulfur clusters will not only inactivate the proteins containing iron-sulfur clusters but also release iron and sulfur to promote cellular oxidative damage via the Fenton reaction. Under these conditions, frataxin/CyaY may sequester the free iron released from the modified iron-sulfur clusters and prevent the iron-mediated production of hydroxyl free radicals. On the other hand, organisms may also transiently reduce the iron-sulfur cluster assembly activity under oxidative stress conditions to avoid the vulnerable targets of reactive oxygen species. Blocking the iron binding activity of IscA could at least in part contribute to an overall decrease of biogenesis of iron-sulfur clusters when cells are under oxidative stress conditions. It should be pointed out that inhibition of the iron binding activity of IscA by hydrogen peroxide is reversible, as re-reduction of the hydrogen peroxide-oxidized IscA with the thioredoxin reductase system fully restores the iron binding activity of IscA (Fig. 7). Although the relative amounts of IscA and CyaY in cells under physiological and oxidative stress conditions remain to be further investigated, we envision that dynamic iron exchange between IscA and frataxin/CyaY may reflect an important interplay between biogenesis of iron-sulfur clusters and cellular iron metabolism under oxidative stresses.

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