ARTICLES

Bacterial frataxin CyaY is the gatekeeper of iron-sulfur cluster formation catalyzed by IscS

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Frataxin is an essential mitochondrial protein whose reduced expression causes Friedreich’s ataxia (FRDA), a lethal neurodegenerative disease. It is believed that frataxin is an iron chaperone that participates in iron metabolism. We have tested this hypothesis using the bacterial frataxin ortholog, CyaY, and different biochemical and biophysical techniques. We observe that CyaY participates in iron-sulfur (Fe-S) cluster assembly as an iron-dependent inhibitor of cluster formation, through binding to the desulfurase IscS. The interaction with IscS involves the iron binding surface of CyaY, which is conserved throughout the frataxin family. We propose that frataxins are iron sensors that act as regulators of Fe-S cluster formation to fine-tune the quantity of Fe-S cluster formed to the concentration of the available acceptors. Our observations provide new perspectives for understanding FRDA and a mechanistic model that rationalizes the available knowledge on frataxin.

Friedreich’s ataxia (FRDA) is a relentlessly progressive neurodegenerative disease which leads to the death of the affected individuals. Although classified as rare, this recessive pathology has ~1 carrier in every 120 individuals1. Discovery of the responsible gene in 1996 established that FRDA is caused by deficiency of frataxin, a small essential protein highly conserved from bacteria to humans2. In eukaryotes, frataxin is nuclearly encoded, translated in the cytoplasm and then imported into mitochondria, where it is finally matured3.

The cellular function of frataxin remains controversial. It is commonly accepted that frataxin is involved in iron metabolism: partial depletion of frataxin has been shown to increase mitochondrial iron levels and to decrease the activity of Fe-S cluster proteins (reviewed in ref. 1). In vitro, frataxins from different species bind both Fe2+ and Fe3+ with a defined stoichiometry, although with relatively low affinity and specificity4–7. Bioinformatics, genetic and biochemical evidence has shown that frataxin binds to ferrochelatase5,8–10, an essential function does not involve large aggregates of Fe-S clusters. This function does not involve large aggregates of Fe-S clusters. This function does not involve large aggregates of Fe-S clusters. Therefore, the role of frataxin in iron metabolism is still not entirely understood, that requires several steps: conversion of cysteine into alanine with formation of a persulfide, its transfer to an acceptor and the formation of the cluster. We followed the effect of CyaY on the kinetics of the reaction in vitro using purified proteins. In this assay, the cluster is formed under strict anaerobic conditions on the scaffold protein IscU, either chemically, using sulfide and ferric ammonium citrate as sources of sulfur and iron, respectively, or enzymatically13,16,17. In the latter case, the desulfurase IscS transfers sulfur from cysteine onto IscU, with the concomitant uptake of iron to form a Fe-S cluster. IscU further transfers the Fe-S cluster to its final acceptors.

Using this assay, we show that Escherichia coli frataxin is not merely an iron chaperone with a neutral involvement in the enzymatic process but an integral part of the cluster-assembly machinery. Bacterial frataxin works as a molecular regulator able to inhibit, depending on the extent to which it is iron saturated, formation of 2Fe-2S clusters. This function does not involve large aggregates of frataxin, which could store iron in a bioavailable form. Our work provides a new perspective on the cellular role of frataxin and suggests a molecular mechanism to explain FRDA.

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RESULTS

**CyaY is an inhibitor of enzymatic Fe-S cluster formation**

We implemented an in vitro reconstitution assay by adapting published protocols. Specific care was paid to choosing the appropriate concentrations of this complex, multicompartment system and to ascertain the state of folding of each component (see the Results section of Supplementary Methods online).

Fe-S reconstitution on IscU was first monitored by absorbance spectroscopy: 2Fe-2S clusters are characterized by maxima at 400 nm and 456 nm and a shoulder at 510 nm, whereas 4Fe-4S clusters have a broad absorption centered at ~390 nm. Formation of Fe-S clusters was detected as an increase of absorbance in the range of 400–550 nm and by the appearance of a brown-red color, as previously described. As expected, the IscS-mediated enzymatic reaction was both faster and more efficient than the non-enzymatic reaction (Fig. 1b).

When CyaY was added to the enzymatic reaction, we observed two main effects: the absorption spectrum at the end of the reaction changed shape, with a marked attenuation of the band at 456 nm and almost complete disappearance of the shoulder at 510 nm (Fig. 1a).

The absorbance ratio between the bands at 400 nm and 456 nm increased progressively as the reaction proceeded. In the presence of CyaY, the kinetics of the process were also distinctly slower (Fig. 1b and Supplementary Fig. 1 online). The brown-red color was observed also in this case, although it was less intense than in the absence of CyaY.

To check whether the presence of CyaY could facilitate iron delivery, as would be expected for an iron chaperone, we compared the kinetics when iron was added to the reaction solution to those obtained when CyaY was preloaded with iron. We incubated a CyaY solution with an excess of either citrate or calmodulin. The first is a well-known iron chelator; the second has been seen to form iron-loaded aggregates in the presence of excess iron (S.A. and A.P., unpublished data). In neither case did we observe interfering effects on the kinetics of the enzymatic reaction (data not shown).

Taken together, this evidence suggests that the role of CyaY is not simply that of an iron chaperone but that of an inhibitor.

**CyaY specifically inhibits formation of 2Fe-2S clusters**

We also used CD spectroscopy to follow the effect of CyaY on Fe-S cluster formation. This technique is complementary to absorbance spectroscopy as it avoids complications due to the overlapping absorption spectra of other iron-bound components. It is also sensitive to the cluster nucleation: 2Fe-2S and 4Fe-4S clusters can clearly be distinguished by CD. 2Fe-2S gives intense contributions in the range 300–700 nm. 4Fe-4S clusters, with the exception of 2[4Fe-4S] bacterial ferredoxins, have weak bands that can barely be distinguished by CD than by absorbance spectroscopy. This is probably because CD monitors only formation of 2Fe-2S clusters on IscU, whereas absorbance also detects other iron-containing species.

To check whether the observed effect was due to the presence of any agent able to bind iron and interfere with the enzymatic activity in a nonspecific way, we repeated the experiments replacing CyaY with an excess of either citrate or calmodulin. The first is a well-known iron chelator; the second has been seen to form iron-loaded aggregates in the presence of excess iron (S.A. and A.P., unpublished data). In neither case did we observe interfering effects on the kinetics of the enzymatic reaction (data not shown).

Enzymatic cluster formation on IscU showed clear evidence for formation of a 2Fe-2S cluster with maxima at 340 nm, 430 nm and 510 nm, and minima at 370 nm and 560 nm, in agreement with the literature. In the presence of CyaY, the rate of 2Fe-2S cluster formation was much slower and the reaction efficiency greatly reduced, as shown by the strong drop (>80%) in the plateau intensity (Fig. 1d). Minor differences in the kinetics of the different experiments were due to several factors. Different enzyme preparations had slightly different activities. CD measurements were overall slightly faster because, by this technique, we followed only one pathway and used larger IscU–IscS ratios. This was possible because, for technical reasons, the reaction could be initiated directly in the spectrophotometer, thus the early events of the reaction were not lost. This could not be done for the absorbance measurements, for which we had to transfer the cuvette from the chamber to the spectrophotometer.

The inhibitory effect of CyaY seemed to be stronger when monitored by CD than by absorbance spectroscopy. This is probably because CD monitors only formation of 2Fe-2S clusters on IscU, whereas absorbance also detects other iron-containing species.
To confirm that inhibition is due to CyaY and is independent of the order in which the components are added, we recorded kinetics in which CyaY was injected into the mixture only after starting the reaction (Fig. 1d). Again, we observed no appreciable differences in the presence of CyaY preloaded with iron or when Fe2+ was used instead of Fe3+ (data not shown).

These results indicate that CyaY has a strong and specific inhibitory effect on the formation of 2Fe-2S clusters.

**Dissecting Fe-S cluster assembly into its components**

During Fe-S cluster assembly, IscS-mediated formation of a cluster on IscU is followed by cluster transfer from IscU to the final acceptor. The second process is known to be slower than the first24. To assess which step is affected by CyaY, we dissected the pathway as follows. First, we carried out non-enzymatic (chemical) cluster formation on IscU in the presence and in the absence of CyaY and followed the process by CD (Fig. 2a). The absence of any observable effect indicates that CyaY intervenes in the enzymatic reaction.

We then followed the enzymatic reaction in the absence of IscU, using isc-encoded ferredoxin (Fdx) as the final acceptor. This protein can form Fe-S clusters in the presence of IscS or other desulfurases, also in the absence of IscU22,23. (Fig. 2b). We observed a strong decrease in the kinetics in the presence of CyaY. The effect was comparable with that observed in the presence of IscU as a scaffold (Fig. 1d), indicating that the inhibitory effect of CyaY involves IscS and not IscU.

To confirm these results and to assess further the role of IscS, we analyzed cluster transfer from chemically reconstituted holo IscU (that is, Fe-S cluster–loaded IscU) to Fdx in the presence and in the absence of CyaY (Fig. 2c). Transfer is known to occur when the two proteins are mixed because of the lower affinity of IscU for the cluster21. Because the CD signal of cluster-loaded Fdx is more intense than that of cluster-loaded IscU24 (Supplementary Fig. 2 online), the reaction led to an overall increase of the signal intensity. CyaY had little, if any, effect on the rate of cluster transfer.

To further circumscribe the mode of action of CyaY, we tested whether CyaY acts on the IscS desulfurase activity by assessing the efficiency of enzymatic cysteine-to-alanine conversion. We started the reaction in the presence of CyaY (5 μM) using low cysteine concentrations (10 μM). When the reaction reached a plateau, we injected additional cysteine to reach a total added concentration at 20 μM, causing the reaction to start again and to proceed with the same rate observed after the first addition (Fig. 2d). We collected aliquots of the mixture at several time points. The alanine concentration, as estimated by amino acid analysis, increased steadily and at the two plateaus was

![Figure 2](https://example.com/fig2.png)

**Figure 2** Dissecting the pathway of cluster reconstitution in the absence and in the presence of CyaY. (a) Kinetics of chemical reconstitution of IscU. The reaction mixtures contained, in addition to 3 mM DTT and 250 μM cysteine, which were common to all measurements in the figure, 40 μM L2S, 40 μM iron ammonium citrate and, when present, 5 μM CyaY. (b) Enzymatic reconstitution of Fdx as the final acceptor in the absence of IscU. The reaction mixtures contained 48 μM Fdx, 1 μM IscS, 80 μM ferric ammonium citrate and, when present, 5 μM CyaY. (c) Cluster transfer from chemically reconstituted holo IscU to Fdx as the final acceptor. The reaction was started by adding Fdx (48 μM) to chemically preloaded IscU (50 μM) and, when present, an excess of CyaY (50 μM) to enhance its effect, if any. (d) Dosage of alanine production during enzymatic Fe-S assembly, as followed by absorbance spectra recorded at 456 nm. The experiments were carried out in the absence and in the presence of 5 μM CyaY using 10 μM cysteine, 50 μM IscU, 25 μM Fe(NH4)2(SO4)2, 3 mM DTT and 1 μM IscS. In the experiment with CyaY, additional 10 μM cysteine solution was added once the reaction had reached a plateau. The alanine concentration was dosed by amino acid analysis.

![Figure 3](https://example.com/fig3.png)

**Figure 3** Interaction of CyaY with IscS. (a) GST pull-down assay using E. coli crude lysate. GST-saturated beads were used as a control. The band of IscU was expected around the 14-kDa region, where we instead identified lysozyme. (b) Titration of labeled IscU* with IscS in the absence and in the presence of an excess of CyaY, as followed by fluorescence. (c) Comparison of the NMR HSOQC spectra of 15N-labeled CyaY recorded at 25 °C and 800 MHz in the absence (red) and in the presence (black) of unlabeled IscS (at a protein ratio of 1:0.8). Residues affected by the titration are marked. (d) Mapping the observed effects on the CyaY structure (PDB 1EW4)6. The backbone of the protein is shown in blue (helical regions) and red (β-sheet). The side chains of the residues affected are shown in yellow. The positions affected and mutated are shown in green. (e) Effect of CyaY mutations of residues involved in IscS interaction on cluster reconstitution as followed by absorbance at 456 nm.
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IscS because the enzyme continues converting its substrate into
cysteine initially added has been converted into alanine.
results from a direct effect of CyaY on IscS and is independent of the
CyaY does not inhibit the desulfurase activity of IscS because the enzyme continues converting its substrate into alanine until it is completely consumed.
CyaY forms a specific complex with IscS
Our results point to a direct interaction between CyaY and IscS, which is in agreement with previous glutathione S-transferase (GST) pull-down studies using E. coli extracts containing over-expressed isc operon proteins. To strengthen these results, we tested whether a pull-down also 'fished out' the endogenous proteins: we bound GST-tagged CyaY to a glutathione Sepharose column and then passed an E. coli extract through the column. Under these conditions, we detected the presence of endogenous IscS but not IscU (Fig. 3a).

Because IscU also interacts with IscS, we tested whether CyaY competes with IscU for the same site on IscS using fluorescence spectroscopy. As all three proteins contain tryptophan residues, we attached a fluorophore, AlexaFluor532, to the more reactive sulfhydryl group on IscU. The monophasic curve obtained by titrating labeled IscU (IscU*) with IscS confirmed the presence of a direct interaction between IscU and IscS with a dissociation constant consistent with the value previously reported (1 µM) (Fig. 3b). The fluorescence intensities obtained by individually titrating IscU* with IscS in the presence of an excess of CyaY (apo or Fe³⁺ loaded) were super-imposable with those obtained in the absence of CyaY, suggesting an absence of competition.

We next used NMR to map the surface of interaction on CyaY. When iron-free and iron-preloaded ⁵⁷⁷ labeled CyaY was titrated aerobically or anaerobically with unlabeled IscU, no spectral perturbations were observed (data not shown). In contrast, titration of CyaY with IscS produced two effects: a progressive disappearance of the whole CyaY spectrum, as expected from formation of the large molecular complex of CyaY with the IscS dimer (90 kDa), and a chemical shift perturbation that specifically affected a limited number of peaks (Fig. 3c). The residues strongly affected already at a 1:0.5 CyaY:IscS molar ratio were Asp22, Asp23, Ser28, Asp29, Glu33, Ile34, Phe43 and Glu44. Other residues affected at higher CyaY:IscS molar ratios (1:0.75) were Leu21, Asn35, Thr42, Gly46, Lys48, Thr64 and Gln98. These residues all map onto the protein surface that contains the iron binding sites (Fig. 3d).

The effect of CyaY on IscS is therefore the consequence of a direct interaction between the two molecules that does not compete with IscU binding.

CyaY mutations affect cluster reconstitution kinetics
To explore the role of CyaY residues in or around the surface of IscS binding, we produced different mutants, chosen among those already well characterized and known to have an effect in vivo or in vitro. CyaY_D22K, CyaY_D31K, CyaY_E19K D22K and CyaY_E18K E19K D22K affect residues that are completely or partially conserved and are known to be involved in the main iron binding site.²⁵,²⁶ The equivalent mutations in yeast frataxin led to progressively severe phenotypes in vivo.²⁷ We also tested a CyaY_W61R mutant because the equivalent position in human frataxin is associated with a severe FRDA case.²⁸ Although not immediately perturbed in our titration with IscS, this residue is in a region contiguous to the iron binding interface. A mutant of residues not perturbed by IscS titration and far away from the iron binding surface (CyaY_H7K D76K)³ was used as a control. We had previously confirmed that these mutants retain their fold.²⁹

With the exception of the negative control CyaY_H7K D76K, which behaved like the wild type, the mutants showed progressively faster kinetics of Fe-S formation on IscU, with initial rates similar to that observed in the absence of CyaY (Fig. 3e). CyaY_W61K has a comparatively smaller effect. These data validate the surface for CyaY-IscS interaction and confirm a role of CyaY in Fe-S cluster reconstitution.

The effect of CyaY is iron-concentration dependent
As the surface of CyaY interacting with IscS is also involved in iron binding, we tested whether CyaY’s inhibitory effect could be sensitive to variations in iron concentration. The initial reaction rates depend on the iron concentration, both in the presence and in the absence of CyaY, which is expected because iron is a substrate in the overall cluster-reformation reaction (Fig. 4a). However, the difference in rates at the same Fe³⁺ concentration in the absence (closed symbols) and in the presence (open symbols) of CyaY becomes much more marked at higher iron concentrations.
As an independent control, we measured the rate of cluster formation as a function of CyaY concentration, while keeping the iron and enzyme concentrations constant (Fig. 4b). We observed a decrease in the rate of cluster formation with increasing CyaY concentrations. The effect saturated at 5 μM CyaY, much lower than the IscU concentration used in our assays but close to the concentration of IscS, confirming that CyaY affects IscS rather than IscU.

These results indicate that the CyaY effect depends on the amount of iron present in the environment.

**CyaY inhibition does not form formation of multimer**

Frataxin has been suggested to act as a ferritin-like iron scavenger by forming large, spherical polymers of distinct stoichiometry. We used gel filtration to test whether oligomerization of CyaY could be important for the observed effects (Fig. 5). Freshly prepared iron-free CyaY eluted as a monomer, as previously described. Under the conditions of the kinetic measurements, CyaY eluted again as a monomer. We did not observe species of large molecular weight, even when the protein was treated with an Fe³⁺ excess to create conditions favorable for oligomers formation. Detectable amounts of oligomers could be observed only when the experiment was carried out at low ionic strength, in the absence of DTT and using HEPES rather than Tris-HCl.

These results indicate that the effect of CyaY is not linked to formation of oligomeric species. It must be completely attributed to the monomeric form of CyaY and its stoichiometric interaction with IscS.

**DISCUSSION**

We have investigated the effect of frataxin in the enzymology of Fe-S cluster biogenesis using purified proteins from E. coli. In using the bacterial system, we postulated that, given the high homology and structural conservation within the frataxin family, the main features of the mechanism(s) by which frataxins functions must be the same, albeit with species-specific adaptations. This assumption is supported by complementation studies in yeast using different frataxin orthologs.

We reasoned that, if frataxins were iron chaperones—that is, molecules that simply escort iron to its final destination—the presence of CyaY should either enhance the enzymatic rates (if the rate-limiting step of the reaction depended on iron delivery) or otherwise have no effect. We observed instead that, far from facilitating iron delivery, the presence of CyaY inhibits the reaction.

By dissecting the complex pathway of Fe-S cluster formation, we have shown conclusively that IscS is indispensable for the CyaY action and that the effect of CyaY is mediated through a direct interaction with IscS, in agreement with a previous report. Although we cannot in principle exclude an interaction of CyaY also with IscU in the context of the tertiary complex (IscS–IscU–CyaY), we see that CyaY has little or no effect on the kinetics of the processes involving IscU or other scaffold proteins. CyaY does not alter the IscS desulfurase activity but is an inhibitor of cluster formation. Finally, we have shown that CyaY does not compete with the IscU binding site on IscS, and its effect does not depend on the specific acceptor.

We can rationalize why a function of frataxin as an inhibitor of Fe-S formation was not observed earlier. In vitro experiments, although crucial for the identification of the hallmarks of the disease, could not provide details on the mechanism of the process. Most of the previous in vitro work has, on the other hand, compared the effect of frataxin on the chemical reaction, thus missing the most important component, the desulfurase enzyme. The only report that compares the effect of CyaY on the enzymatic reaction was carried out by incubating IscS with the substrate (cytC) for 2 h before adding Fe⁵⁷⁻-preloaded CyaY. Under these conditions, cytC is almost completely converted into alanine, as we have observed (Fig. 6).

The mapped IscS interface includes CyaY residues that are highly conserved and that have been implicated in iron binding, indicating that our overall conclusions can be generalized, although there might be some degree of species variability. Accordingly, we observed an iron-dependent effect of CyaY on Fe-S cluster formation, in agreement with the observation that, in yeast, binding of the frataxin ortholog Yfh1 with the Isu1–Nfi1 complex (equivalent to IscU–IscS) is iron dependent. Notably, an iron-dependent effect of the activity of ferrochelatase, the enzyme involved in another frataxin pathway, has also been reported.

We propose that frataxin function as iron sensors and suggest a mechanism for their action (Fig. 6). By a negative-regulation mechanism, frataxins would act as gate keepers of Fe-S assembly by fine tuning the quantity of Fe-S clusters formed to match the concentration of the apo acceptors. Frataxins would have low affinity for the IscS–IscU system at normal iron levels. At any small iron imbalance (that is, an excess of iron as compared to final acceptors), the affinity of the protein for IscS would increase. One may wonder why such a regulation mechanism is needed, considering that the isc operon is under the control of the transcription factor IscR.

The necessity of regulation both at the transcriptional and post-translational levels can be explained by considering that IscR regulates the whole operon, and its action will require more time than the immediate response that a component that interacts directly on the central component of the machinery, the enzyme, could have by sensing the iron concentrations.

In FRDA, and even more so in knockout models, where the phenotype is exacerbated, such a regulatory mechanism would be absent. As a regulator that tunes the quantity of Fe-S clusters to the availability of the apo acceptors, any reduction or depletion of frataxin levels would upset this equilibrium and lead to an imbalance in the amount of the Fe-S clusters produced with respect to the apo acceptors. Even a small iron accumulation could result in the formation of Fe-S clusters at a rate incompatible with the concentration of final acceptors. Fe-S clusters are labile species that cannot exist without an acceptor or carrier. They would therefore fall apart, producing free iron, which in turn would give rise to Fenton chemistry. The FRDA phenotype, including the early damage of mitochondria, the presence of CyaY inhibits the reaction.

In the context of the Fe-S cluster assembly, frataxin is a central component of the machinery, the enzyme, could have by sensing the iron concentrations. The necessity of regulation both at the transcriptional and post-translational levels can be explained by considering that IscR regulates the whole operon, and its action will require more time than the immediate response that a component that interacts directly on the central component of the machinery, the enzyme, could have by sensing the iron concentrations.

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intramitochondrial iron accumulation in frataxin-deficient organisms is observed after the onset of the pathology and after inactivation of the Fe–S–dependent enzymes. Finally, a role for the CyaY monomer rather than an aggregate is in agreement with previous work showing that an oligomerization-deficient mutant of Yh1 can still participate in Fe–S cluster biogenesis or heme assembly.

We believe that, although more work is needed to establish the details of the molecular mechanism of IscS inhibition exerted by CyaY and provide a direct description of the eukaryotic system, our work opens an entirely new perspective to understanding the role of frataxins. This will hopefully promote new studies to clarify its links with the FRDA pathology.

METHODS

Protein production. We overexpressed and purified the proteins, all from *E. coli*, as previously described. Fdx was obtained courtesy of L.E. Vickery (University of California at Irvine). We prepared cluster-free Fdx by acidic precipitation of holo Fdx. We checked the purity of all proteins by SDS-PAGE and by MS of the final product.

Absorbance experiments. We performed cluster reconstitution in an anaerobic chamber (Belle) under a nitrogen atmosphere. We followed the reaction by absorbance spectroscopy using a Cary 50 Bio (Varian) spectrophotometer. Variations in the absorbance at 456 nm were measured as a function of time. Unless otherwise specified, we incubated 50 mM solutions of purified IscU in sealed cuvettes typically using 3 mM DTT and 25 μM Fe(NH₄)₂SO₄ for 30 min in 50 mM Tris-HCl buffer, pH 7.5, and 150 mM NaCl. Subsequently, we added 1 μM IscS and 250 mM cysteine to start the reaction. Chemical reconstitution was carried out under similar conditions but replacing 1 μM IscS with 250 μM L-S as a source of sulfur. We studied the effects of iron and CyaY concentrations by varying them individually in the range 5–100 μM and 4–5 μM, respectively, with the other components fixed.

The control experiments of the effects of other iron carriers were done using sodium citrate or calmodulin (50 μM).

To assess the effect of preloading CyA with iron, we mixed CyaY (200 μM) anaerobically with two equivalents of Fe(NH₄)₂SO₄ (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and incubated the mixture for 1 h before adding an aliquot to the enzymatic mixture to reach a final concentration of CyaY (10 μM) preloaded with two equivalents of Fe²⁺.

To check the effect of the CyaY mutants, we added 5 μM of these proteins to the enzymatic mixture before starting the reaction. Other controls are described in the “Results” section of Supplementary Methods.

Circular dichroism experiments. We obtained anaerobic conditions for CD studies by using septum-capped 1-cm quartz cuvettes, stainless steel cannules and anaerobic syringes for sample transfer. Cluster reconstitution was monitored by following the increase of the CD signal at 435 nm using a Jasco J-715 spectropolarimeter.

We diluted concentrated protein stocks to their final concentrations (20–50 μM IscU and 4–5 μM CyaY or CyaY mutants) into 50 mM Tris-HCl, 150 mM NaCl, pH 7.5–8.0, containing 3 mM DTT. Cysteine was added to a final concentration of 250 μM, followed by ferric ammonium citrate at a final concentration less than or equal to that of IscU (40–50 μM). The reaction was typically started by the addition of IscS at a final concentration of 1–2 μM.

The experiments in Figure 2 were carried out as follows: chemical reconstitution of IscU was performed with 3 mM DTT, 250 μM cysteine, 40 μM L-S and 40 μM ferric ammonium citrate. We proved the effect of CyaY on cluster formation using Fdx as the final acceptor in the absence of IscU under similar conditions to those used for IscU (1 μM IscS, 250 μM cysteine and 3 mM DTT), but the final reaction mixtures contained 40–50 μM of Fdx, a twofold molar excess of ferric ammonium citrate with respect to Fdx and, when present, 5 μM CyaY. Transfer of the cluster from holo IscU to Fdx was followed using 50 μM of chemically reconstituted IscU in the presence and in the absence of CyaY (40 μM). For this experiment, IscU was chemically reconstituted by adding in small aliquots 500 μM ferric ammonium citrate and 500 μM L-S to maximize the yield.

GST pull-down. We equilibrated GST-beads of Glutathione-Sepharose (500 μl) in a buffer containing 20 mM Tris-HCl, 100 mM NaCl and 2 mM β-mercaptoethanol and incubated them with an excess of purified GST-CyaY in a final volume of 2 ml for 1 h at 4 °C. As a control we used GST. After extensive washing with the same buffer, the saturated beads were mixed overnight with *E. coli* crude lysate (DH5α strain). Potential protein partners bound to the beads were eluted with 1 ml of 1 M NaCl in 50 mM Tris-HCl buffer and separated by 12% SDS-PAGE. To ensure that no protein would be retained on the beads, we used harsher conditions for the control: GST-saturated beads were eluted with 20 mM glutathione in 50 mM Tris-HCl buffer. The gels were stained by Novex colloidal Coomassie blue for 4 h. Stained bands were cut out, processed and analyzed by MS.

Fluorescence and nuclear magnetic resonance measurements. We performed fluorescence experiments at 25 °C on a Jasco fluorimeter with excitation at 465 nm and emission at 546 nm. We kept the concentration of the species being titrated constant throughout the titration. A 0.6 mM solution of IscU in 20 mM Tris-HCl buffer, pH 7.0, and 150 mM NaCl was reacted for 1 h with a fourfold excess of AlexaFluor 532 fluorescent probe (Invitrogen). We separated the labeled product from the free fluorophore on a PD10 gel-filtration column and eluted with 20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 20 mM β-mercaptoethanol. Labeled IscU (2 μM) was titrated with CyaS (up to a 5-molar excess) in the absence and in the presence of CyaY (200 μM).

We recorded NMR spectra at 25 °C on a Varian spectrometer operating at 800 MHz. ¹H frequencies equipped with a 5 mm cryoprobe. All proteins were in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 20 mM β-mercaptoethanol to which 10% D₂O was added. Iron-preloaded CyaY was obtained by adding Fe²⁺ or Fe³⁺ (at protein-to-ratio of 1:2 or 1:6, respectively). Fe³⁺ was added aerobically.

Alanine dosage. We started enzymatic IscU reconstitution in the presence of CyaY (5 μM) as in other absorbance assays (1 μM IscS, 50 μM IscU, 3 mM
DTT) but adding 20 μM cysteine in total, with a starting concentration of 10 μM. When the reaction reached a plateau, further cysteine was added to give a total concentration of 20 μM. We collected aliquots in triplicates at different reaction times (360, 720, 1,500, 2,200, 2,800 and 3,400 s). We then quenched the reaction on each aliquot by adding 20% (v/v) trichloroacetic acid (TCA) and collected samples. After keeping the samples on ice for 10 min, we left them at −20 °C overnight to allow slow protein precipitation. The solution was centrifuged at 11,000g for 5 min. We quantified the alanine content in the supernatant by amino acid analysis.

**Analysis of the oligomerization state of CyaY.** We probed the oligomerization state of CyaY during cluster reconstitution by gel filtration. CyaY (20 μM) was incubated in a solution containing 3 mM DTT, 25 μM Fe(NH4)2(SO4)2, 50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl and 250 μM cysteine, that is, the same composition used for cluster reconstitution except for the absence of IscS and IscU. We incubated the solution at room temperature (25 °C) for 1 h and injected it into the gel-filtration column. The experiment was repeated after removing DTT and cysteine to eliminate reducing agents. In a separate experiment, we incubated 20 μM CyaY with 100 μM Fe(NH4)2(SO4)2 in 20 mM HEPES, pH 7.4. These samples were loaded on an analytical Superdex 75 HR 10/30 column (Amersham Biosciences).

**Note:** Supplementary information is available on the Nature Structural & Molecular Biology website.

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