Transfer of Iron-Sulfur Cluster from NifU to Apoferredoxin*

Received for publication, April 24, 2000, and in revised form, June 2, 2000
Published, JBC Papers in Press, June 2, 2000,
DOI 10.1074/jbc.C000279200

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Iron-sulfur proteins are present in a wide variety of organisms and are known to play important physiological roles, not only in electron transfer and metabolic reactions, but also in transcriptional regulation. However, little is known about how iron-sulfur clusters themselves are synthesized and assembled within polypeptides. Here we show that a [2Fe-2S] cluster-containing NifU of cyanobacterium Synechocystis PCC6803, SyNifU, possesses the ability to deliver its [2Fe-2S] cluster to an apoferredoxin without the aid of other proteinaceous or nonproteinaceous factor(s). Upon delivery the reconstituted holoferreredoxin regained electron transfer ability. The [2Fe-2S] cluster contained within SyNifU was labile upon exposure to the iron-chelating reagent EDTA, suggesting that the iron-sulfur cluster is abnormally exposed to solvent. We propose that NifU serves as a scaffold for iron-sulfur cluster assembly and functions as a mediator for iron-sulfur cluster delivery.

Iron-sulfur proteins are distributed among various organisms and are known to play important physiological roles not only in electron transfer or metabolic reactions but also in gene regulation (1–3). Although cellular functions and enzymatic reactions of many iron-sulfur proteins have been extensively studied, molecular mechanisms involved in the assembly of the iron-sulfur cluster into the polypeptides still remain unclear. Recent evidence in prokaryotes and eukaryotes has led to the identification of complex machinery for the biosynthesis of iron-sulfur clusters, including NifS/IscS and NifU/IscU (4–16). NifS/IscS has been shown to catalyze the formation of elemental sulfur from cysteine to provide the inorganic sulfur necessary for in vivo formation of the iron-sulfur cluster (4, 5). Under certain experimental conditions NifS alone has been reported to catalyze the formation of the iron-sulfur cluster in vitro (5). However, this reaction required a significantly higher concentration of ferrous ion. Moreover, because NifS was found to catalyze cysteine cleavage both in the absence and the presence of acceptor apoprotein, the participation of additional factor(s) in in vivo iron-sulfur cluster formation has been proposed. In contrast, the precise role of NifU/IscU during iron-sulfur cluster formation remains unknown. Recently, several reports concerning the possible involvement of yeast NifU/IscU homologs in mitochondrial iron-sulfur cluster assembly and iron metabolism have been published (13–16).

Originally identified Azotobacter vinelandii NifU encoded in the nif gene cluster, which has been known to be involved in the biosynthesis of nitrogenase, is 242 amino acids long and has been proposed to consist of three distinct domains (17). The amino-terminal domain, which shows high sequence identity to the bacterial IscU proteins (6) and also to the yeast IscU proteins (10, 13, 14), contains three evolutionarily conserved cysteine residues. The middle domain of Azotobacter NifU shows significant sequence similarity to internal domains of nitrate reductases and nitrite reductases (17). The carboxyl-terminal domain contains the conserved Cys-X-X-Cys motif. Although bacterial isc (iron-sulfur cluster formation) operons encode IscU proteins that correspond only to the amino-terminal domain of Azotobacter NifU, some bacteria and some eukaryotes seem to possess other NifU-related proteins, corresponding to the carboxyl-terminal domain of A. vinelandii NifU, in addition to or instead of, such IscU proteins (6). Thus, the relationship among those NifU-related proteins of prokaryotes and eukaryotes is fairly complex. Interestingly, A. vinelandii NifU was shown to be a homodimer containing two permanent [2Fe-2S] clusters, and the [2Fe-2S] cluster-coordinating ligands were provided by four cysteine residues of the middle domain (18). Moreover, Dean and co-workers (19) reported recently that the three conserved cysteines in the amino-terminal domain comprised a transient [2Fe-2S] cluster binding.

To elucidate a role of NifU-like protein on iron-sulfur cluster assembly in detail, we chose a NifU homolog of the cyanobacterium Synechocystis PCC6803 for biochemical characterization, since the entire genome of this organism was determined and was found to contain only one nifU-like gene (sul2667) (20). The cyanobacterial NifU homolog shares high sequence homology with the carboxyl-terminal domain of A. vinelandii NifU. In the present study, the NifU homolog of Synechocystis PCC6803 has been shown to possess the ability to transfer its [2Fe-2S] cluster to an apoferredoxin without the aid of other proteinaceous or nonproteinaceous factor(s). From these observations, we propose that NifU functions as an intermediate site for the iron-sulfur cluster assembly and delivery.

EXPERIMENTAL PROCEDURES
Expression and Purification of SyNifU-h6 Proteins—Escherichia coli BL21(DE3) and a vector pET-21d (Novagen, Inc., Madison) were used for the overexpression of SyNifU and SyNifU-h6. SyNifU-h6 contained an additional Leu-Glu sequence plus hexahistidine at the carboxyl terminus of SyNifU. The expression of SyNifU and SyNifU-h6 was induced by the addition of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside to exponentially growing cells in LB medium at 23 °C. Cells were harvested after overnight induction and broken by sonication in the buffer containing 50 mM Hepes-KOH (pH 8.0) and 150 mM NaCl. After removal of the membrane pellets and any insoluble materials by centrifugation, the soluble supernatants were used for further purification. SyNifU-h6 was purified by successive column chromatography with a TALON affinity column (CLONTECH), a hydroxyapatite column, and an anion-exchange Resource Q column (Amersham Pharmacia Biotech). By contrast, SyNifU in the supernatant was first precipitated with 10–30% saturated ammonium sulfate. The precipitates were then solubilized and separated by butyl-Tylosep hydrophobic column chromatography. The elute was then desalted through a Sephadex G-50 column, and the resulting protein fraction was further purified by hydroxyapatite column chromatography followed by anion-exchange Resource Q column chromatography. Finally, the buffers of purified
protein fractions were exchanged with 50 mM Hepes-KOH (pH 8.0) and 25 mM NaCl by Fast-desalting column chromatography (Amersham Pharmacia Biotech). Apo-form of SyNifU-h6 was purified by the same procedure as described above from overexpressor cells grown at 30 °C in the presence of 1 mM isopropyl-1-thio-β-β-galactopyranoside. A cyanobacterial Fdx (slr0148 gene product of Synechocystis PCC6803) and a yeast mitochondrial adrenodoxin homolog (mtAd) were also purified to homogeneity from corresponding overexpressor E. coli cells. Both purified proteins showed typical UV/visible absorption spectra characteristic for [2Fe-2S]-containing proteins (data not shown).

Preparation of Apoferredoxin—Apoferredoxin was prepared by boiling holoferredoxin in the presence of 100 mM EDTA and 500 mM dithiothreitol to trap iron atoms liberated from the holoproteins and ensure that the side chains of the four cysteines previously participating in [2Fe-2S] cluster ligation were reduced to free sulfhydryl groups. After boiling, apoferredoxin was purified by gel filtration column chromatography using a Fast-desalting column (Amersham Pharmacia Biotech) which had been equilibrated with 50 mM Hepes-KOH (pH 8.0) and 25 mM NaCl.

In Vitro Reconstitution of Holoferredoxin—Typically, apoferredoxin (40 μg) was incubated with either the purified SyNifU (200 μg) or SyNifU-h6 (80 μg) for 1 h at 30 °C in buffer containing 50 mM Hepes-KOH (pH 8.0) and 25 mM KCl. Because of the higher content of the apo-form in the purified SyNifU than in SyNifU-h6, the former was used in 2.5-fold excess of the latter. Samples were separated by 20% nondenaturing PAGE1 as described previously (21). The appearance of the gel before staining, after iron-staining (22), and after further staining with CBB was recorded by two-dimensional color image scanning. The amount of newly formed holoferredoxin and the corresponding decrease of holo-SyNifU(-h6) were densitometrically quantified.

Electron Transfer Activity of Holoferredoxin—After incubating apoferredoxin (40 μg) with SyNifU-h6 (80 μg) as described above, electron transfer activity of the reconstituted holoferredoxin was assayed by measuring the rate of cytochrome c reduction. The reaction mixture contained 0.25 mM NADPH, 40 mM FNR (pea ferredoxin-NADP⁺ oxidoreductase), 0.1 mM horse heart cytochrome c, 50 mM Tris-HCl (pH 7.5), and 100 mM NaCl. The reaction was initiated by addition of the reconstituted ferredoxin solution, which contained ferredoxin polypeptide equivalent to 10 μM of the initially added apoferredoxin, and recombination of cytochrome c was monitored by the increase in absorbance at 550 nm. As a control, activity of purified SyNifU-h6 (20 μg) and apoferredoxin (10 μg) was also separately analyzed, and activities were compared with those of the holoferredoxin (10 or 5 μg).

RESULTS AND DISCUSSION

The cyanobacterium Synechocystis PCC6803 contains only one nifU-like gene (ssl2667) (20). The 76-amino acid translation product shows significant sequence homology to the carboxyl-terminal domain of NiF encoded in the nif one gene cluster of nitrogen-fixing bacteria (6) and possesses the conserved -Cys-X-X-Cys- motif. Recombinant proteins, either with (SyNifU-h6) or without (SyNifU) a carboxyl-terminal hexahistidine tag, were overexpressed in E. coli cells and purified (Fig. 1A). UV/visible absorption spectrum of the purified SyNifU-h6 showed a characteristic spectrum with peak absorbance at 330, 420, and 460 nm, suggesting the presence of the [2Fe-2S] cluster. Although SyNifU showed essentially similar absorption spectrum, the ratio of absorbances at 330 versus 280 nm was smaller in SyNifU than in SyNifU-h6, indicating a higher content of the apo-form in the purified SyNifU protein fraction. Interestingly, based on gel filtration, the SyNifU-h6 fraction also contained a considerable amount of the apo-form. It seemed most likely the apo-form existed as a monomer, and the cluster-containing holo-form of both proteins existed at low concentrations (data not shown). Apo- and holo-forms were also observed in the purified SyNifU-h6 under nondeaturing PAGE conditions (21); the purified SyNifU-h6 clearly forming two distinct bands detected by CBB staining as shown in Fig. 1C. The slower migrating band seemed to correspond to the [2Fe-2S] cluster-containing holo-form and the faster migrating band to the monomeric apo-form, because only the former showed the pink-red color characteristic of the [2Fe-2S] cluster-containing protein in the unstained gel and was detected by iron staining (Fig. 1C). Because SyNifU contains two evolutionarily conserved cysteine residues, we speculate that the oligomeric holo-SyNifU(-h6) carries one [2Fe-2S] cluster between two identical monomers.

The presence of a considerable amount of the apo-form in purified SyNifU(-h6) fractions was probably due to limited formation of the iron-sulfur cluster of SyNifU(-h6) in the overexpressor cells. It is also possible that instability of the iron-sulfur cluster, during the prolonged purification procedure necessary for SyNifU, caused the higher content of the apo-form. Because the holo- and apo-SyNifU(-h6) showed similar behavior through various chromatographic separations, complete removal of the apo-form proved difficult. The labile property of the [2Fe-2S] cluster contained in the holo-SyNifU(-h6) was demonstrated by a significant decay in its characteristic UV/visible absorption spectrum upon exposure to the iron-chelating reagent EDTA (Fig. 1D). Such EDTA-induced destruction of an iron-sulfur cluster was not observed in typical [2Fe-2S] cluster-containing proteins such as ferredoxin (data not shown), suggesting that the iron-sulfur cluster of the holo-SyNifU(-h6) is abnormally exposed to solvent.

The presence of the [2Fe-2S] cluster in purified SyNifU(-h6)

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue.
lead us to analyze the ability of purified SyNifU(-h6) to transfer its iron-sulfur cluster to another substrate apoprotein. To examine this, a [2Fe-2S] cluster-containing photosynthetic ferredoxin of Synechocystis PCC6803, a petF (ssl0020) gene product, was chosen as a model substrate protein (20). The ferredoxin was first purified from E. coli overexpressor cells as the holoform (21) and converted to the apo-form by boiling in the presence of EDTA and dithiothreitol. Apoferredoxin was further purified by desalting to remove low molecular weight molecules such as sulfur, iron, or their derivatives liberated from the ferredoxin polypeptide by denaturation, and also to remove any excess EDTA and dithiothreitol. As shown in Fig. 2A (left and middle panels), the prepared holo- and apoferredoxins were easily separated during nondenaturing PAGE (21); the holoferredoxin migrated much faster than the apoferredoxin, a fairly efficient formation of the holoferredoxin (solid line) and apoferredoxin (dotted line) devoid of inorganic elements. UV/visible absorption spectra were adjusted by the absorption values at 275 nm.

Purified SyNifU(-h6) was incubated with apoferredoxin to investigate iron-sulfur cluster transfer at a simple level. Surprisingly, as shown in Fig. 3A, a fairly efficient formation of the holoferredoxin could be observed, while a corresponding amount of SyNifU(-h6) lost its cluster. Essentially no difference could be observed between the result obtained with SyNifU and that obtained with SyNiU-h6. More than 50% of the added apoferredoxin seemed to be converted to its holo-form, based on the color intensity of the bands corresponding to reconstituted holoferredoxin and authentic holoferredoxin in the unstained gel after electrophoresis, whereas color loss in the apo-form was complete. The absence of the iron atom in the prepared apoferredoxin indicated that more than 97% of its iron-sulfur cluster to another substrate apoprotein. To examine this, a [2Fe-2S] cluster-containing photosynthetic ferredoxin of Synechocystis PCC6803, a petF (ssl0020) gene product, was chosen as a model substrate protein (20). The ferredoxin was first purified from E. coli overexpressor cells as the holoform (21) and converted to the apo-form by boiling in the presence of EDTA and dithiothreitol. Apoferredoxin was further purified by desalting to remove low molecular weight molecules such as sulfur, iron, or their derivatives liberated from the ferredoxin polypeptide by denaturation, and also to remove any excess EDTA and dithiothreitol. As shown in Fig. 2A (left and middle panels), the prepared holo- and apoferredoxins were easily separated during nondenaturing PAGE (21); the holoferredoxin migrated much faster than the apoferredoxin, a fairly efficient formation of the holoferredoxin (solid line) and apoferredoxin (dotted line) devoid of inorganic elements. UV/visible absorption spectra were adjusted by the absorption values at 275 nm.

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**Fig. 2. Preparation of apoferredoxin.** A, separation of holoferredoxin (holo-fd) from apoferredoxin (apo-fd) through nondenaturing PAGE (21). Purified holo- and apoferredoxin (40 and 200 μg, respectively) were applied to 20% nondenaturing PAGE. The CBB-stained (left), the unstained (middle), and the iron-stained (left) gels (22) are shown. Note that the prepared apoferredoxin lost not only the native color characteristic of the [2Fe-2S] cluster but also all the bound iron atoms. B, UV/visible absorption spectra of holoferredoxin (solid line) and apoferredoxin (dotted line) devoid of inorganic elements. UV/visible absorption spectra were adjusted by the absorption values at 275 nm.

**Fig. 3. Iron-sulfur cluster transfer from SyNifU(-h6) to apoferredoxin.** A, apoferredoxin (40 μg) was incubated with either the purified SyNifU (200 μg) or SyNifU-h6 (80 μg) for 1 h at 30 °C in the buffer containing 50 mM Hepes-KOH (pH 8.0) and 25 mM KCl. To monitor the intactness of SyNifU(-h6) during the incubation in the absence of apoferredoxin, a mock incubation was carried out. The heat-denatured apo-SyNifU(-h6) was also analyzed by this assay. Samples were separated by 20% nondenaturing PAGE; the unstained gel image is shown in the upper panel and iron-stained gel in the lower (quantified by immunoblotting data not shown). Note that the purified SyNifU migrated faster than the SyNifU-h6. Proteins that appeared after the incubation of the apoferredoxin with the purified SyNifU(-h6) and migrated faster than the dye front were confirmed as holoferredoxin by immunoblotting (data not shown). B, aliquots of 20 μg of apoferredoxin were incubated with 100 μg of SyNifU(-h6) for 0, 5, 10, 30, or 60 min at 30 °C (solid line) or 4 °C (dotted line). Proteins were then separated through nondenaturing PAGE at 4 °C, and the amount of newly formed holoferredoxin was densitometrically quantified. C, reconstitution assays were performed as in A in the presence or absence of 1 mM ferrous ammonium sulfate (iron) and/or 1 mM Na2S (sulfide). Apo- or holo-SyNifU(-h6) (100 μg) was also included as indicated. The amount of newly formed holoferredoxin was quantified as in (B). D, electron transfer activity of the reconstituted holoferredoxin was assayed by measuring the rate of cytochrome c reduction. The reconstitution reaction was carried out as described in A and under "Experimental Procedures," and one-fourth of the reaction mixture (initially containing 10 μg of apoferredoxin and 20 μg of SyNifU(-h6)) was used for the assay. As a negative control, activity was analyzed in the purified SyNifU-h6 (20 μg) or apoferredoxin (10 μg) fractions alone, and as a positive control activity was analyzed in the purified authentic holoferredoxin (10 μg or 5 μg). E, after incubating apoferredoxin with holo-SyNifU(-h6) as in A, SyNiU-h6 was removed by TALON affinity resin, and ferredoxin was further purified by Mono Q (Amersham Pharmacia Biotech) ion-exchange column chromatography. UV/visible absorption spectra of the purified ferredoxin after reconstitution reaction (b) were compared with that of the authentic holoferredoxin (a). Spectra were adjusted by the absorption values at 275 nm. F, the purified cyanobacterial Fdx (50 μg of protein) or the yeast mitochondrial adrenodoxin homolog (mtAd) (50 μg of protein) were incubated with apoferredoxin (40 μg) and analyzed as in A in place of SyNifU(-h6). The left panel shows the unstained appearance of the nondenaturing gel after electrophoresis, and the right panel shows the same gel after iron staining.
cysteines in the apoferredoxin participating in the [2Fe-2S] cluster ligation was prerequisite for SyNiFU-h6-dependent holoferrodoxin formation (data not shown). As shown in Fig. 3C, a relatively low level of reconstitution of apoferredoxin (~5% as compared with that by holo-SyNiFU-h6) by simple addition of ferrous ion and Na₂S was observed. Such chemical reconstitution was not enhanced by the presence of apo-SyNiFU-h6 in the reaction mixture, suggesting that the observed efficient holoferrodoxin formation by holo-SyNiFU-h6 was caused by the direct transfer of the [2Fe-2S] cluster from the holo-SyNiFU-h6 to the apoferredoxin.

As shown in Fig. 3D, upon incubation with SyNiFU-h6, approximately 50% of the added apoferredoxin seemed to regain the electron transfer activity comparable with that of the authentic holoferrodoxin. This percentage of the recovered electron transfer activity was in good agreement with that of the reconstituted holoferrodoxin observed under nondenaturing PAGE as shown in Fig. 3A. Moreover, the ferredoxin purified from the reconstitution assay mixture showed typical UV/visible absorption spectra characteristic for the [2Fe-2S] ferredoxin (Fig. 3E). These results indicate that the [2Fe-2S] cluster of SyNiFU-h6 was transferred and assembled accurately into the apoferredoxin polypeptide to form the biochemically active holoferrodoxin. Such cluster transfer was not observed when other [2Fe-2S] cluster-containing proteins such as a cyanobacterial Fdx or an adrenodoxin homolog of yeast mitochondria (mtAd) were used as donors in place of SyNiFU-h6 (Fig. 3F).

In conclusion, holoferrodoxin can be formed in vitro by transfer of the [2Fe-2S] cluster from the holo-SyNiFU protein to the apoferredoxin polypeptide. From these results, we propose that the SyNiFU-mediated delivery of the iron-sulfur cluster could be a key step in the biosynthesis of iron-sulfur proteins in the cell. NiFU encoded in the nif gene cluster, originally identified in Azotobacter vinelandii, has been proposed to consist of three distinct domains (17, 18). The amino-terminal domain shows a high sequence identity to bacterial IscU proteins encoded in the isc (iron-sulfur cluster formation) operon and also to the yeast mitochondrial Isu proteins (6, 13, 14). In addition to, or instead of, such IscU/Isu proteins, some bacteria and eukaryotic mitochondria seem to possess additional SyNiFU-like NiFU-related proteins with identity to the carboxyl-terminal domain of Azotobacter NiFU (6, 20). Interestingly, Azotobacter NiFU was shown to be a homodimer containing two permanent [2Fe-2S] clusters in the middle domain and one transient [2Fe-2S] cluster in the amino-terminal IscU/Isu domain (18, 19). Therefore, it seemed that the carboxyl-terminal domain of Azotobacter NiFU, which shares high sequence similarity to SyNiFU, remained as apo-form when prepared. This means that a [2Fe-2S] cluster might be present also in the carboxyl-terminal domain in vivo but it might be too unstable to be detected, or it might be transferred rapidly to other iron-sulfur cluster acceptor proteins or domains (e.g. the amino-terminal or middle domains of Azotobacter NiFU itself). However, most recently, two cysteine residues contained in the carboxyl-terminal domain of Azotobacter NiFU were found to be unnecessary for the full physiological function (24). The proposed function of the transient [2Fe-2S] cluster is to provide the Fe-S cores of the nitrogenase metalclusters (19). Thus, it is possible that the IscU/Isu domain/proteins and the SyNiFU-like proteins perform somewhat similar or otherwise overlapped functions in the iron-sulfur cluster assembly in the cell.

A more detailed molecular mechanism that is involved in the transfer of the [2Fe-2S] cluster from holo-SyNiFU to apoferredoxin remains to be elucidated on the basis of both chemical and structural points of view. Because the donor and the acceptor proteins in the in vitro [2Fe-2S] cluster transfer reaction used in this study are rather small and even soluble, this system should be a useful and powerful model to understand this key reaction of the assembly of iron-sulfur clusters into proteins. It has yet to be elucidated how the iron-sulfur cluster of SyNiFU itself is formed in vivo. NiF/S/IscS, NiF/IscA, and/or Fdx might participate in this process (6, 8, 18, 19, 25).

Acknowledgments—We thank M. Sugita for providing the Synechocystis PCC6803 cells. We also thank Y. Takahashi and T. Hase for discussions and G. Hanke for helpful advice in manuscript preparation.

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