Repair of Nitric Oxide-modified Ferredoxin [2Fe-2S] Cluster by Cysteine Desulfurase (IscS)*

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Iron-sulfur proteins are among the sensitive targets of the nitric oxide cytotoxicity. When Escherichia coli cells are exposed to nitric oxide, iron-sulfur clusters are modified forming protein-bound dinitrosyl iron complexes. Such modified protein dinitrosyl iron complexes are stable in vitro but are efficiently repaired in aerobically growing E. coli cells even without any new protein synthesis. Here we show that cysteine desulfurase encoded by the gene iscS of E. coli can directly convert the ferredoxin dinitrosyl iron complex to the ferredoxin [2Fe-2S] cluster in the presence of L-cysteine in vitro. A reassembly of the [2Fe-2S] cluster in the dinitrosyl ferredoxin iron complex does not require any addition of iron or other protein components. Furthermore, a complete removal of the dinitrosyl iron complex from ferredoxin prevents reassembly of the [2Fe-2S] cluster in the protein. The results suggest that cysteine desulfurase (IscS) together with L-cysteine can efficiently repair the nitric oxide-modified ferredoxin [2Fe-2S] cluster and that the iron center in the dinitrosyl iron complex may be recycled for the reassembly of iron-sulfur clusters in proteins.

Iron-sulfur proteins are ubiquitous in living organisms. Their pervasive occurrence and multiplicity of function are comparable to other biological prosthetic groups such as hemes and flavins (1, 2). Ironically, iron-sulfur proteins are highly sensitive to reactive free radicals such as nitric oxide (3). When purified iron-sulfur proteins are treated with nitric oxide in vitro, the iron-sulfur clusters are modified forming protein-bound dinitrosyl iron complexes that have a characteristic EPR signal at \( g_{av} = 2.04 \) (4–6). The same EPR signal at \( g_{av} = 2.04 \) has been observed in activated macrophages (7–9), indicating that the protein dinitrosyl iron complexes may be produced by nitric oxide physiologically. Direct isolation of ferredoxin protein from the Escherichia coli cells treated with nitric oxide demonstrated that up to 40% overproduced ferredoxin [2Fe-2S] clusters are converted to the ferredoxin dinitrosyl iron complex (10). Many iron-sulfur proteins become inactivated when their iron-sulfur clusters are modified (4–6, 11, 12). Using isolated aconitases from mammalian cells, the interrelation between formation of the dinitrosyl iron complex and inactivation of the enzyme activity has been quantitatively demonstrated (6). In some cases, iron-sulfur proteins are considered the nitric oxide sensor (12–14). For example, the redox transcription factor SoxR of E. coli is switched on when the SoxR [2Fe-2S] cluster is modified by nitric oxide forming the SoxR dinitrosyl iron complex (14).

Although the protein dinitrosyl iron complexes are stable in vitro, they are efficiently repaired in aerobically growing E. coli cells even in the presence of the protein synthesis inhibitor chloramphenicol (10, 14). This observation is consistent with a previous report (15) that proteins with modified iron-sulfur clusters are not degraded; rather the modified iron-sulfur clusters are repaired in E. coli cells. Surprisingly, when intact E. coli cells are disrupted, the protein dinitrosyl iron complexes become very stable (10), suggesting that the specific cellular activity for repairing the protein dinitrosyl iron complexes is inactivated in the cell extracts. We proposed that repair of the protein dinitrosyl iron complexes requires cellular reducing equivalents, which are oxidized and diluted when intact E. coli cells are disrupted. In a search for such reducing equivalents, we found that L-cysteine, but not N-acetyl-L-cysteine or reduced glutathione, can effectively decompose the protein dinitrosyl iron complexes in the cell extracts prepared from the E. coli cells treated with nitric oxide (10). Furthermore, L-cysteine is equally effective in decomposing the purified protein dinitrosyl iron complexes, implying that other cellular components are not needed for the reaction (10). Nevertheless, the mechanism for the L-cysteine-mediated decomposition of the protein dinitrosyl iron complexes is not fully understood. One hypothesis we postulated is that L-cysteine removes the nitric oxide moieties from the dinitrosyl iron complexes, leaving an unstable iron center transiently associated with the proteins. Such an intermediate may be recycled for the reassembly of new iron-sulfur clusters if sulfide is provided in a timely manner. In this report, we used the E. coli ferredoxin [2Fe-2S] cluster (16) as an example and found that the nitric oxide-modified ferredoxin dinitrosyl iron complex can be directly converted back to the ferredoxin [2Fe-2S] cluster by cysteine desulfurase (IscS)1 and L-cysteine in vitro. IscS (17, 18) is a key member of the iron-sulfur cluster assembly machinery encoded by the operon iscSUA-hscBA-fdx in E. coli (19–21). The physiological function of IscS and the entire operon iscSUA-hscBA-fdx of E. coli in repairing the nitric oxide-modified iron-sulfur proteins will be discussed.

EXPERIMENTAL PROCEDURES

Preparation of IscS—The coding region of the E. coli IscS protein was amplified from wild-type E. coli genomic DNA by PCR using PCR ready-to-go beads (Amersham Biosciences). Two primers were designed to contain a Ncol restriction site in one primer and a HindIII site in the other. The sequences of the primers are: IscS-1, 5’-GCATTGAGCCTGAGACGGAGTTTA-3’; and IscS-2, 5’-CCGATTAAAAGTACACCATTAGTTAG-3’. The amplified product was prepared and purified by PAGE. The IscS fragment was subcloned into the E. coli expression vector pET-17b (Novagen) with an N-termi-
A protein was applied to a Hitrap-desalting column (5.0 ml) (Amersham C (300 mM imidazole, 500 mM NaCl, 50 mM Tris, pH 8.0). The eluted centrifuged at 20,000 g disrupted by passing them through a French press once and then expression of the IscS protein. The

The IscS protein was then eluted with buffer added to the culture, and incubation continued for 2 days to induce the expression of the IscS protein. The purified ferredoxin [2Fe-2S] cluster from the ferredoxin dinitrosyl iron complex was described previously (10). The purified ferredoxin [2Fe-2S] cluster was used for the reduction of nitric oxide as described previously (10). The concentration of nitric oxide in the nitric oxide-saturated solution is characteristic of the protein dinitrosyl iron complex (4–6 M at room temperature (22).

The spin quantification of the EPR signals was carried out as described previously (14).

**RESULTS**

**Preparation of the Ferredoxin Dinitrosyl Iron Complex**—When the E. coli cells containing overproduced ferredoxin protein were treated with a single dose of nitric oxide gas at a final concentration of 10 μM anaerobically, up to 40% overproduced ferredoxin [2Fe-2S] clusters in cells were modified forming the ferredoxin dinitrosyl iron complex (10). In an attempt to separate the ferredoxin dinitrosyl iron complex from the ferredoxin [2Fe-2S] cluster, the isolated ferredoxin protein was loaded onto a gel filtration column. The gel filtration elution profile of the ferredoxin protein had two major peaks, fractions 29 and 31 (Fig. 1A). The SDS-polyacrylamide electrophoresis gel analysis showed that both fractions 29 and 31 have a single ferredoxin polypeptide band (data not shown). The UV-visible absorption measurements revealed that fraction 31 has a typical UV-visible spectrum of the ferredoxin [2Fe-2S] cluster with two absorption peaks at 415 and 459 nm (16), whereas fraction 29 does not have any noticeable features in the same region (Fig. 1B). The EPR measurements showed that fraction 29 but not fraction 31 has an EPR signal at g = 2.04 (Fig. 1C), a characteristic of the protein dinitrosyl iron complex (4–6, 10). Spin quantification of the EPR signal at g = 2.04 in fraction 29 could only account for ~1.5 μM ferredoxin dinitrosyl iron complex.

**Assay of the [2Fe-2S] Cluster Reassembly in the Ferredoxin Dinitrosyl Iron Complex**—The ferredoxin dinitrosyl iron complex was mixed with the purified IscS in a 1.0-mL solution containing 500 mM NaCl, 50 mM Tris, pH 8.0, 4 mM dithiothreitol under aerobic conditions. The mixture was incubated at 37 °C for 5 min before L-cysteine was added. The formation of the ferredoxin [2Fe-2S] cluster in the reaction solution was monitored by taking UV-visible absorption spectra in a Beckman DU640 UV-visible spectrometer equipped with a Peltier temperature controller. The concentration of the ferredoxin [2Fe-2S] cluster was estimated using an extinction coefficient of 11 mM−1 cm−1 at 415 nm (16).

**EPR Spectroscopy**—X-band EPR spectra were recorded using a Bruker model ESP-300 EPR spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR measurement conditions were as follows: microwave frequency, 9.50 GHz; microwave power, 1.0 milliwatt; modulation frequency, 100 kHz; modulation amplitude, 1.2 millitesla; sample temperature, 20 K; and receive gain, 105. The spin quantification of the EPR signals was carried out as described previously (14).

**Preparation of the Ferredoxin Dinitrosyl Iron Complex**—The E. coli cells containing overproduced ferredoxin protein were treated with a single dose of nitric oxide gas at a final concentration of 10 μM anaerobically, up to 40% overproduced ferredoxin [2Fe-2S] clusters in cells were modified forming the ferredoxin dinitrosyl iron complex (10). In an attempt to separate the ferredoxin dinitrosyl iron complex from the ferredoxin [2Fe-2S] cluster, the isolated ferredoxin protein was loaded onto a gel filtration column. The gel filtration elution profile of the ferredoxin protein had two major peaks, fractions 29 and 31 (Fig. 1A). The SDS-polyacrylamide electrophoresis gel analysis showed that both fractions 29 and 31 have a single ferredoxin polypeptide band (data not shown). The UV-visible absorption measurements revealed that fraction 31 has a typical UV-visible spectrum of the ferredoxin [2Fe-2S] cluster with two absorption peaks at 415 and 459 nm (16), whereas fraction 29 does not have any noticeable features in the same region (Fig. 1B). The EPR measurements showed that fraction 29 but not fraction 31 has an EPR signal at g = 2.04 (Fig. 1C), a characteristic of the protein dinitrosyl iron complex (4–6, 10). Spin quantification of the EPR signal at g = 2.04 in fraction 29 could only account for ~1.5 μM ferredoxin dinitrosyl iron complex.
plex in a total of 5.0 μM ferredoxin protein. This amount of the ferredoxin dinitrosyl iron complex in fraction 29 could be an underestimate, because the EPR signal at g_{av} = 2.04 only reflects the iron center in the d^7 state and the iron center in the complexes may exist in the d^6 or d^8 EPR silent state (4–6). As modifications of the ferredoxin [2Fe-2S] cluster by nitric oxide have not been fully characterized, it is also probable that fraction 29 contained ferredoxin protein with other nitric oxide-modified iron-sulfur clusters that could not be detected with EPR. Nevertheless, the result clearly showed that fraction 29 contains at least 1.5 μM ferredoxin dinitrosyl iron complex and no ferredoxin [2Fe-2S] cluster.

Repair of the Ferredoxin Dinitrosyl Iron Complex by Cysteine Desulfurase (IscS) and L-cysteine.—Previous studies (10) revealed that L-cysteine could efficiently decompose the purified protein dinitrosyl iron complex and release the ferrous iron from the complex. Other related biological thiols including N-acetyl-L-cysteine and glutathione were much less effective. Kinetics characterization showed that the protein dinitrosyl iron complex was completely decomposed by L-cysteine at ~20 min, whereas the ferrous iron release continued up to 45 min, suggesting that iron release from the protein dinitrosyl iron complex is delayed (10). We proposed that there is an intermediate in which the nitric oxide moieties are removed by L-cysteine, leaving an unstable iron center associated with the protein (10). Such an intermediate may be recycled for reassembly of the iron-sulfur clusters in proteins if sulfide is provided.

To test this model, we prepared recombinant IscS, a NifS homolog that produces sulfide from L-cysteine (17, 18). IscS protein was purified from E. coli as described under “Experimental Procedures.” When fraction 29, which contains the ferredoxin dinitrosyl iron complex, was incubated with L-cysteine and the purified IscS at 37 °C for 20 min, a typical rhombic EPR signal of the reduced ferredoxin [2Fe-2S] cluster (16) was observed. Integration of the EPR signal of the reduced ferredoxin [2Fe-2S] cluster estimated from the EPR signal at g_{av} = 2.04 in fraction 29, suggesting that IscS and L-cysteine convert all of the ferredoxin dinitrosyl iron complex to the ferredoxin [2Fe-2S] cluster in fraction 29.

The reassembly kinetics of the [2Fe-2S] cluster in the ferredoxin dinitrosyl iron complex was further characterized by taking the UV-visible absorption spectra of the samples every 5 min after L-cysteine, or IscS was added to the reaction solution. The relative amounts of the ferredoxin [2Fe-2S] cluster in the sample were calculated from the absorption change at 415 nm, a maximum peak of the ferredoxin [2Fe-2S] cluster (16) at 37 °C for ~10 min. At a fixed concentration of IscS (1 μM), the recovery of the ferredoxin [2Fe-2S] cluster was not very sensitive to the L-cysteine concentrations ranging from 50 μM to 1 mM. On the other hand, at a fixed concentration of L-cysteine (1 mM), the reassembly rate and yield of the ferredoxin [2Fe-2S] cluster were highly dependent on the IscS concentrations (Fig. 3B).

The repaired ferredoxin sample was also subjected to the gel filtration analysis. When the ferredoxin sample incubated with IscS and L-cysteine was loaded onto the gel filtration column, a new peak at fraction 31 appeared (Fig. 4) that was identical to that of the ferredoxin [2Fe-2S] cluster fraction (Fig. 1A). Integration analysis of the gel filtration profiles indicated that approximately 2 μM ferredoxin protein in fraction 29 was converted to fraction 31. Thus, IscS and L-cysteine not only reassembled the [2Fe-2S] cluster in the ferredoxin dinitrosyl iron complex but also changed the ferredoxin protein conformation.

Removal of the Dinitrosyl Iron Complex from Ferredoxin Prevents the Reassembly of the [2Fe-2S] Cluster.—The observation that reassembly of the [2Fe-2S] cluster in the ferredoxin dinitrosyl iron complex does not require any additional iron.
FIG. 2. Suggested that the iron center in the dinitrosyl iron complex may be recycled for formation of the [2Fe-2S] cluster in ferredoxin. To examine whether the dinitrosyl iron complex in ferredoxin is converted to the ferredoxin [2Fe-2S] cluster, we prepared a ferredoxin sample in which the dinitrosyl iron complex was removed.

Fraction 29 containing the ferredoxin dinitrosyl iron complex was first incubated with L-cysteine (1 mM) and an iron chelator EDTA (1 mM) at 37 °C for 1 h to completely decompose the dinitrosyl iron complex (10). In a control, fraction 29 was incubated with EDTA (1 mM) only. After incubation, both samples were passed through a Hitrap-desalting column to remove EDTA, L-cysteine, and other small molecules. The EPR measurements confirmed that incubation with both L-cysteine and EDTA removed the dinitrosyl iron complex from ferredoxin, whereas incubation with EDTA only did not have any effect on the EPR signal at $g = 2.04$ of the ferredoxin dinitrosyl iron complex (data not shown). Both protein samples were then incubated with IscS and L-cysteine at 37 °C for 20 min. As shown in Fig. 5, the ferredoxin [2Fe-2S] cluster appeared in the ferredoxin sample pretreated with EDTA alone (trace d). In contrast, no [2Fe-2S] cluster was detected in the ferredoxin sample in which the dinitrosyl iron complex was completely removed by the pretreatment of both L-cysteine and EDTA (trace c).

DISCUSSION

In activated macrophages, an excessive amount of nitric oxide is produced as a powerful weapon that kills pathogenic bacteria and tumor cells (23). The specific cellular targets of the nitric oxide cytotoxicity have not been fully understood. Early EPR measurements indicated that iron-sulfur proteins are among those that are particularly sensitive to nitric oxide, as the EPR signal at $g = 2.04$ representing the protein dinitrosyl iron complexes is readily observed in activated macrophages (7–9). In the past decade, iron-sulfur proteins have been found in several important physiological processes including energy metabolism (2, 11), iron homeostasis (13, 24), heme and biotin synthesis (12, 25), DNA repair (26), and gene expression regulation (3). Many if not all iron-sulfur proteins will be modified forming the protein dinitrosyl iron complexes when organisms are exposed to nitric oxide. If cells are to survive, modified iron-sulfur proteins must be efficiently repaired. We show here that IscS together with L-cysteine can directly convert the ferredoxin dinitrosyl iron complex to the ferredoxin [2Fe-2S] cluster in vitro (Fig. 2) and that reassembly of the [2Fe-2S] cluster in the ferredoxin dinitrosyl iron complex does not require any addition of iron. Furthermore, the removal of the...
Genetic studies demonstrated that IscS assists the inactivation of the operon iscSUA-hscBA-fdx in cells, the inactivation of the operon iscSUA-hscBA-fdx has also been found in the cytoplasm and in the nucleus. For example, the nuclear localization of the IscS homolog (Nfs1p) in yeast cells seems to be critical for the cell growth. If mitochondria are the center for the biogenesis of iron-sulfur proteins, the presence of the homologs of the operon iscSUA-hscBA-fdx in the cytoplasm and in the nucleus seems to be unnecessary. However, there are a number of iron-sulfur proteins in the cytoplasm (e.g., cytosolic aconitase (13, 24)) and in the nucleus (e.g., endonuclease III (26)) that are the potential targets of reactive free radicals. Perhaps, the homologs of the operon iscSUA-hscBA-fdx in the cytoplasm and in the nucleus are responsible for efficiently repairing the modified iron-sulfur proteins when cells are exposed to reactive free radicals.

Based on the results presented here and in the previous study, we propose that the repair of the protein dinitrosyl iron complexes has two steps. First, L-cysteine removes the nitric oxide moieties from the dinitrosyl iron complex, leaving an unstable iron center transiently associated with the protein. In this reaction, L-cysteine has a unique activity because other related biological thiols such as N-acetyl-L-cysteine, and glutathione as well as sulfide have little effect on the stability of the protein dinitrosyl iron complexes. The putative intermediate of the protein dinitrosyl iron complex after the nitric oxide moieties are removed by L-cysteine may be recycled for the formation of new iron-sulfur clusters if sulfide is provided in a timely manner. In the second step, L-cysteine as a substrate for IscS will provide sulfide for reassembly of iron-sulfur clusters in proteins. In aerobically growing E. coli cells, the intracellular L-cysteine concentration was estimated to be ~0.2 mM (45). This amount of L-cysteine seems to be sufficient for repairing the nitric oxide-modified iron-sulfur proteins in E. coli cells (10, 14). The disruption of intact E. coli cells may result in substantial dilution and oxidation of L-cysteine such that the protein dinitrosyl iron complexes are stable in the cell extracts. The addition of L-cysteine to the cell extracts efficiently decomposes the otherwise stable protein dinitrosyl iron complexes (10). It seems that L-cysteine biosynthesis has an important role in repairing the modified iron-sulfur proteins when cells are subjected to oxidative stresses. Although much has been learned about the L-cysteine biosynthesis pathway in E. coli (46), the genetic regulation of L-cysteine metabolism has not been fully understood. Recent gene array experiments revealed that the cysK gene encoding O-acetylseryine sulphydrylase A, a key enzyme for the L-cysteine biosynthesis in E. coli (46), is highly induced by both hydrogen peroxide and superoxide stresses (47), suggesting that the L-cysteine biosynthesis is part of a global response to oxidative stress.

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