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L-Cysteine-mediated Destabilization of Dinitrosyl Iron Complexes in Proteins*

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Nitric oxide is a signaling molecule in intercellular communication as well as a powerful weapon used by macrophages to kill tumor cells and pathogenic bacteria. Here, we show that when Escherichia coli cells are exposed to nitric oxide, its ferredoxin [2Fe-2S] cluster is nitrosylated, forming the dinitrosyl iron complex with a characteristic EPR signal at gav = 2.04. Such formed ferredoxin dinitrosyl iron complex is efficiently repaired in E. coli cells even in the absence of new protein synthesis. However, the repair activity is completely inactivated once E. coli cells even in the absence of new protein synthesis. In search of such cellular factors, we find that L-cysteine can effectively eliminate the EPR signal of the ferredoxin dinitrosyl iron complex and release the ferrous iron from the complex. In contrast, N-acetyl-L-cysteine and reduced glutathione are much less effective. L-Cysteine seems to have a general function, since it can also remove the otherwise stable dinitrosyl iron complexes from proteins in the cell extracts prepared from the E. coli cells treated with nitric oxide. We propose that L-cysteine is responsible for removing the dinitrosyl iron complexes from the nitric oxide-modified proteins into which a new iron-sulfur cluster will be reassembled.

Nitric oxide has multiple physiological functions. At low concentrations, nitric oxide acts as a signaling molecule for intercellular communications (1). At high concentrations, nitric oxide can be a powerful toxic agent that kills pathogenic bacteria and tumor cells (2). Among cellular components, proteins that contain iron-sulfur clusters are considered one of nitric oxide’s targets (3–8). In the last decade, iron-sulfur proteins have been found in many important biological processes including energy metabolism (6, 9), cellular iron homeostasis (10), heme biosynthesis (11), DNA repair (12–14), DNA synthesis (15), and transcription regulation (16, 17). When purified iron-sulfur proteins are treated with nitric oxide, the iron-sulfur clusters are converted to the stable dinitrosyl iron complex, which has a characteristic EPR signal at gav = 2.04 (6, 11, 18–21). The same EPR signal at gav = 2.04 has also been observed in microorganisms and animal tissues when treated with nitric oxide (3, 22, 23) and in activated macrophages where nitric oxide was physiologically produced by inducible nitric oxide synthase (5, 24). The implication of these observations is that cellular iron-sulfur proteins may be modified, forming the dinitrosyl iron complexes, when cells are exposed to nitric oxide (19). However, the origin of the EPR signal at gav = 2.04 in living organisms has been a controversy. Vanin et al. suggested that the EPR signal at gav = 2.04 observed in cells could reflect the dinitrosyl iron complexes formed with small molecular thiols and cellular “free” iron (25). The argument was based on the fact that the EPR signal at gav = 2.04 can be reproduced with small molecular thiols and the ferrous iron mixed with nitric oxide in vitro (26). Here, we use the Escherichia coli ferredoxin [2Fe-2S] cluster as an example and demonstrate that the ferredoxin protein purified from E. coli cells treated with nitric oxide has been modified, forming the stable dinitrosyl iron complex. Significantly, our results also show that such modified ferredoxin dinitrosyl iron complex is rapidly repaired in the E. coli cells even in the presence of the protein synthesis inhibitor chloramphenicol.

Modification of the iron-sulfur cluster in proteins by nitric oxide will inevitably change the protein activities (6, 11, 18, 21). If the cells are to survive, the modified iron-sulfur proteins will have to be efficiently repaired. At present, little is known about the cellular repair mechanism for the modified iron-sulfur clusters in proteins by nitric oxide. We propose that the modified dinitrosyl iron complex in proteins will have to be decomposed, followed by the reassembly of new iron-sulfur clusters into the apoproteins, probably by the products of a highly conserved gene cluster iscSUA-hscBA-fdx in bacteria (27–29). To remove the dinitrosyl iron complex from protein, cellular reducing equivalents will be required. Previous studies indicated that biological thiols could destabilize the oxidized [2Fe-2S] clusters of the redox transcription factor SoxR through the redox ligand exchange reactions (30, 31). The observation prompted us to speculate that biological thiols may also be involved in removing the dinitrosyl iron complexes from nitric oxide-modified iron-sulfur proteins. As an initial approach, we examined the effect of various cellular reducing equivalents on the stability of the ferredoxin dinitrosyl iron complex in vitro. The results showed that L-cysteine can effectively eliminate the EPR signal at gav = 2.04 of the ferredoxin dinitrosyl iron complex and release the ferrous iron. Other biological thiols are much less effective. The possible mechanisms for the L-cysteine-mediated removal of the dinitrosyl iron complexes from proteins will be discussed.

EXPERIMENTAL PROCEDURES

Cloning of E. coli Ferredoxin—The coding region for the E. coli ferredoxin (32) was amplified from wild-type E. coli genomic DNA by polymerase chain reaction using polymerase chain reaction ready-to-go beads (Amersham Pharmacia Co.). Two primers were designed to contain an NcoI restriction site in one primer and a HindIII site in the other. The sequences of the primers are as follows: fdx-1, 5’-AGGTT-TACCATTGGCAAGATGTGT-3’; fdx-2, 5’-CCCTGGTAAAACCTTACG CGCATG-3’. The NcoI/HindIII-digested polymerase chain reaction

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product was purified and ligated to the expression vector pET28b+ (Stratagene Co.) to yield pTFDX, containing a six-histidine tag attached to the C terminus of the ferredoxin polypeptide. The constructed plasmid was transformed into E. coli BL-21 (DE3) cells (Stratagene Co.) for the expression of the ferredoxin protein.

Cells and Nitric Oxide Treatments—An overnight E. coli culture (BL-21 (DE3) containing the pTFDX plasmid) was diluted 100-fold into 1000 ml of fresh LB (Luria-Bertani) medium. After 3.0 h of incubation in a 37 °C incubator with aeration (250 rpm), isopropyl-β-D-thiogalactopyranoside (200 μM) was added, and incubation continued for another 1.5 h to induce the expression of the ferredoxin protein. The cells were then spun down at 4000 rpm for 8 min using an Eppendorf 5810R desktop centrifuge and resuspended gently into 500 ml of prewarmed LB medium containing the protein synthesis inhibitor chloramphenicol (at a final concentration of 0.14 mg/ml). One-third of the culture was harvested immediately by centrifugation and used as a control. The rest of the culture was transferred to a sealed flask and incubated in a 37 °C incubator for 10 min without aeration to achieve anoxic conditions. Nitric oxide-saturated solution was added, and incubation continued for another 1.5 h to induce the expression of the ferredoxin protein. The cells were then harvested immediately by centrifugation and used as a control (not treated with nitric oxide). Sample 2 was then equally divided into three samples. Sample 3 was then equally divided into three samples. Sample 1 was used as a control (not treated with nitric oxide).

RESULTS

Ferredoxin [2Fe-2S] Clusters Are Transiently Modified when E. coli Cells Are Exposed to a Single Injection of Nitric Oxide—The E. coli ferredoxin [2Fe-2S] cluster has been used as an example in this study, because the gene encoding ferredoxin is highly conserved in both eukaryotic and prokaryotic organisms (27, 28, 33, 34). To increase the amount of ferredoxin in E. coli cells, the protein was partially overproduced using the expression plasmid pTFDX, in which a His tag was fused to the C terminus of the ferredoxin polypeptide. The His tag facilitated the quantitative protein purification from E. coli cells but did not affect the assembly of the [2Fe-2S] clusters into the ferredoxin polypeptide. After ferredoxin was induced in exponentially growing E. coli cells for 1.5 h, chloramphenicol was added to the culture to stop new protein synthesis. The E. coli culture was then equally divided into three samples. Sample 1 was used as a control (not treated with nitric oxide). Sample 2 was directly transferred to the EPR tubes for the measurement as described under “Experimental Procedures.” C, EPR spectra of ferredoxin samples reduced with dithionite. Ferredoxin samples were reduced with freshly prepared sodium dithionite (at a final concentration of 2 mM) before transferred to the EPR tubes. EPR spectra shown in B and C are in the same scale. Similar results were obtained from three different experiments.

UV-visible Spectroscopy—A Beckman DU640 UV-visible spectrometer equipped with a temperature controller was used for measuring the absorption spectra of the purified ferredoxin samples. The concentration coefficient of 11 mM⁻¹ cm⁻¹ at 415 nm (32). The spectrometer was also used for determining the ferrous iron released from the ferredoxin dinitrosyl iron complex by L-cysteine. After incubation of ferredoxin samples with or without L-cysteine, each reaction solution (1 ml) was transferred to a prechilled Centicon concentrator (Micron YM-10; Millipore Corp.) and centrifuged using an Eppendorf 5801R desktop centrifuge. The iron indicator a,a’-dipyridyl (50 μM) was then added to the flow-through samples to determine the ferrous iron concentration. The ferrous iron-a,a’-dipyridyl complex has an absorption maximum at 520 nm with an extinction coefficient of 5.2 mM⁻¹ cm⁻¹ (30).

EPR Spectroscopy—X-band EPR spectra were recorded using a Bruker model ESP-300 EPR spectrometer (Louisiana State University) equipped with an Oxford Instruments 910 continuous flow cryostat (courtesy of Prof. Brian Hales, Louisiana State University). 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 mT; sample temperature, 20 K; receiver gain, 10⁷. The relative amplitude of the EPR signal was quantified as described previously (21).
treated with nitric oxide. Sample 3 was incubated at 37 °C with aeration for an additional 30 min after treatment with nitric oxide. The amount of the ferredoxin protein purified from these three samples was almost the same, indicating that the ferredoxin protein was not quickly degraded in the E. coli cells.

The ferredoxin protein (Fdx) purified from the E. coli cells not treated with nitric oxide showed a typical absorption spectrum of oxidized ferredoxin [2Fe-2S] clusters with two peaks at 415 and 459 nm in the visible region (32) (Fig. 1A, trace 1). However, the absorption spectrum of the ferredoxin protein (Fdx-NO) purified from the E. coli cells treated with nitric oxide was changed, notably around the peak at 459 nm (trace 2), indicating that some ferredoxin [2Fe-2S] clusters were modified when E. coli cells were treated with nitric oxide. This somewhat distorted spectrum of the ferredoxin protein was fully restored (trace 3) after the E. coli cells were incubated at 37 °C with aeration for an additional 30 min.

Purified ferredoxin samples were further examined using EPR spectroscopy. As shown in Fig. 1B, the Fdx sample had no EPR signal, since the oxidized ferredoxin [2Fe-2S] clusters are diamagnetic and EPR-silent (trace 1). On the other hand, the Fdx-NO sample showed a dominant EPR signal at $g_{av} = 2.04$ (trace 2), a characteristic of the dinitrosyl iron complex (18–21). The EPR spectrum was identical to that of the purified Fdx sample treated with nitric oxide in vitro under anaerobic conditions (data not shown). The same EPR signal at $g_{av} = 2.04$ was also observed in the overproduced native ferredoxin (without the His tag) prepared from the E. coli cells treated with nitric oxide, indicating that the His tag did not contribute to the EPR signal.

Double integration of the EPR signal at $g_{av} = 2.04$ of the Fdx-NO sample (Fig. 1B, trace 2) showed that the concentration of the dinitrosyl iron complex in the sample (contains ~15 μM total ferredoxin protein) was only about 2.3 μM. While this number may be an underestimate, since some redox intermediates of the ferredoxin dinitrosyl iron complex are diamagnetic and EPR-silent (18, 21), it is likely that not all ferredoxin [2Fe-2S] clusters were converted to the dinitrosyl iron complex when E. coli cells were exposed to nitric oxide. Significantly, the EPR signal at $g_{av} = 2.04$ was almost completely eliminated in the ferredoxin sample purified from the E. coli cells incubated at 37 °C with aeration for an additional 30 min after nitric oxide treatment (Fig. 1B, trace 3).

Purified ferredoxin samples were also reduced with freshly prepared sodium dithionite to determine the amount of intact ferredoxin [2Fe-2S] cluster in each sample. When the Fdx sample was reduced with dithionite, a typical EPR spectrum of the reduced ferredoxin [2Fe-2S] cluster with $g_r = 1.94$ and $g_s = 2.02$ appeared (Fig. 1C, trace 1). The Fdx-NO sample reduced with dithionite had a more complicated EPR spectrum (Fig. 1B, trace 2). The amplitude of the EPR signal at $g_{av} = 2.04$ was substantially decreased with the appearance of a new EPR signal at $g_{av} = 2.01$, reflecting the partial reduction of the ferredoxin dinitrosyl iron complex by dithionite. The incomplete reduction of the ferredoxin dinitrosyl iron complex by dithionite is probably due to the very low redox midpoint potential of the iron center in the complex (18, 20, 21). The reduced Fdx-NO sample also showed an EPR signal at $g = 1.94$, a characteristic of the intact ferredoxin [2Fe-2S] cluster. Judging from the amplitudes of the signal at $g = 1.94$ in traces 1 and 2 of Fig. 1C, about 40% of the ferredoxin [2Fe-2S] clusters were modified when the E. coli cells were treated with nitric oxide. The removal of dithionite by passing the sample through a Hitrap desalting column restored the EPR spectrum of the Fdx-NO to at least 90% of the amplitude of the original EPR signal at $g_{av} = 2.04$ (data not shown). Evidently, the ferredoxin dinitrosyl iron complex was not disrupted by dithionite treatment, as reported in other cases (18, 21). Trace 3 in Fig. 1C showed that the amplitude of the EPR signal at $g = 1.94$ was completely restored in the ferredoxin sample purified from the E. coli cells incubated at 37 °C with aeration for an additional 30 min after nitric oxide treatment. These results demonstrate that the ferredoxin [2Fe-2S] clusters are modified, forming the ferredoxin dinitrosyl iron complex, when E. coli cells are exposed to nitric oxide and that the modified iron-sulfur clusters in ferredoxin are efficiently repaired in living E. coli cells.

**Fig. 2. Effect of biological thiols on the EPR signal at $g_{av} = 2.04$ of the ferredoxin dinitrosyl iron complex.** The purified Fdx-NO sample (10 μM) was incubated with no addition (trace b) or with 1 mM l-cysteine (Cys) (trace c), N-acetyl-l-cysteine (NAC) (trace d), or GSH (trace e) at 37 °C for 20 min under aerobic conditions. After incubation, samples were transferred directly to the EPR tubes for the measurement. The EPR spectra were recorded as described under “Experimental Procedures.”
Fig. 3. L-Cysteine stimulates iron release from the ferredoxin dinitrosyl iron complex. Both Fdx and Fdx-NO samples (containing about 15 μM ferrodoxin protein) were prepared as described under “Experimental Procedures.” A, low field EPR spectra of the ferredoxin samples. The spectra were recorded in the magnetic field from 10 to 300 mT to detect the “junk” iron (g = 4.3) in the samples. The Fdx and Fdx-NO samples were incubated with no addition (Fdx, Fdx-NO) or with 1 mM L-cysteine (Fdx + Cys, Fdx-NO + Cys) at 37 °C for 20 min under aerobic conditions. B, quantification of the ferrous iron in ferredoxin samples using a,a'-dipyridyl. After Fdx and Fdx-NO sample were incubated with or without L-cysteine, samples were transferred to prechilled Centricon centrifugal filter units (Micron YM-10) (Millipore Corp.) and centrifuged at 4000 rpm for 30 min at 4 °C. The ferrous iron indicator a,a'-dipyridyl (50 μM) was added to the flow-through for the quantification of the ferrous iron. The concentration of the ferrous iron in the flow-through was measured at 520 nm (a maximum absorption of the Fe^{2+}-a,a'-dipyridyl complex) using an extinction coefficient of 5.2 mM⁻¹ cm⁻¹ (30). The results were the averages from three different experiments.

When the Fdx sample containing 15 μM ferrodoxin protein was incubated with L-cysteine, about 10.5 μM ferrous iron was detected in the flow-through of the sample. Since the intact ferredoxin [2Fe-2S] clusters in the Fdx-NO sample are not significantly affected by the L-cysteine incubation, at least 9.0 μM iron may be released from the ferredoxin dinitrosyl iron complex in the Fdx-NO sample. Since up to 40% of the ferredoxin [2Fe-2S] clusters in the Fdx-NO sample were modified in the Fdx-NO sample (Fig. 1C), the maximum concentration of the ferredoxin dinitrosyl iron complex in the Fdx-NO sample would be about 6.0 μM. Thus, up to 1.5 mol of the iron could have been released per 1.0 mol of the ferredoxin dinitrosyl iron complex by L-cysteine. More experiments are required to accurately determine the

A possible explanation for the disappearance of the EPR signal at g_{av} = 2.04 of the Fdx-NO sample after incubation with L-cysteine is that the ferrodoxin dinitrosyl iron complex could be reduced by L-cysteine to become diamagnetic and EPR-silent (18, 25). However, reparation of the ferredoxin sample after incubation with L-cysteine did not restore any EPR signal at g_{av} = 2.04 (data not shown), indicating that L-cysteine may remove the dinitrosyl iron complex from ferredoxin proteins.

L-Cysteine Releases Ferrous Iron from Ferredoxin Dinitrosyl Iron Complexes—If L-cysteine destabilizes the dinitrosyl iron complex and eventually removes the complex from ferredoxin protein, we would expect iron to be released from the ferredoxin dinitrosyl iron complex after incubation with L-cysteine. To explore this possibility, we first examined the low field EPR spectra of both Fdx and Fdx-NO samples after incubation with or without L-cysteine. The EPR signal at g = 4.3 represents the “junk” ferric iron in a low symmetry environment with an S of ½ (35). As shown in Fig. 3A, no EPR signal at g = 4.3 was seen in both purified Fdx and Fdx-NO samples. Incubation of the Fdx sample with L-cysteine produced no EPR signal at g = 4.3 either. Remarkably, an EPR signal at g = 4.3 was observed after the Fdx-NO sample was incubated with L-cysteine, indicating that iron is released from the ferredoxin dinitrosyl iron complex.

To further quantitatively determine the iron release from the ferredoxin samples, the protein was separated from the reaction mixture using Centricon Centrifugal Filters after the sample was incubated with or without L-cysteine. The flow-through of each sample was collected, and the concentration of the ferrous iron in the flow-through was determined using the ferrous iron indicator a,a'-dipyridyl (30). As expected, very little ferrous iron was observed in both Fdx and Fdx-NO samples (Fig. 3B). The addition of dithionite to the flow-through samples to reduce any ferric iron did not increase the amount of the ferrous iron. When the Fdx sample containing ~15 μM ferrodoxin protein was incubated with L-cysteine, only a small amount of the ferrous iron (~1.2 μM) was detected in the flow-through. Further incubation of the Fdx sample with L-cysteine (up to 1 h) did not increase the ferrous iron release. In addition, over 95% of the [2Fe-2S] clusters in the Fdx sample remained intact after incubation with L-cysteine (data not shown). These results indicate that released iron in the Fdx sample did not come from the intact ferredoxin [2Fe-2S] clusters. One possible source would be the iron that loosely associates with small amount of apoferredoxin protein in the sample.
Acetyl-L-cysteine (H9262) stable EPR signal at gav mids) treated with nitric oxide. Prepared cell extracts had a 30984  was incubated with L-cysteine. Interestingly, half of the amplitude of the EPR signal at g av 2.04 was almost completely eliminated (Fig. 5 trace b). The cell extracts prepared from the E. coli cells treated with nitric oxide (trace b) were incubated at 37 °C for 20 min with no addition (trace c) or with 1 mM l-cysteine (Cys) (trace d), N-acetyl-l-cysteine (NAC) (trace e), or GSH (trace f). Trace a, the cell extracts prepared from the E. coli cells not treated with nitric oxide. B, effect of different concentrations of biological thiols on the dinitrosyl iron complexes in the cell extracts. The cell extracts prepared from the E. coli cells treated with nitric oxide were incubated with different concentrations of l-cysteine (Cys), N-acetyl-l-cysteine (NAC), and GSH, respectively. Samples were incubated at 37 °C for 20 min under aerobic conditions. The amplitudes of the EPR signal at g av 2.04 were plotted as a function of the thiol concentrations added in the cell extracts before incubation.

FIG. 5. Effect of L-cysteine on the EPR signal of the dinitrosyl iron complex in cell extracts. Exponentially growing E. coli cells (without any expression plasmids) were treated with or without nitric oxide (at a final concentration of 5 μM). The cells were centrifuged and resuspended in a buffer containing 500 mM NaCl and 50 mM Tris (pH 7.9). Cell extracts were prepared as described under “Experimental Procedures.” The concentration of the cell extracts was adjusted to about 1.0 mg of total cellular protein/ml. A, effect of biological thiols on the EPR signal at gav = 2.04 of the cell extracts. The cell extracts prepared from the E. coli cells treated with nitric oxide (trace b) were incubated at 37 °C for 20 min with no addition (trace c) or with 1 mM l-cysteine (Cys) (trace d), N-acetyl-l-cysteine (NAC) (trace e), or GSH (trace f). Trace a, the cell extracts prepared from the E. coli cells not treated with nitric oxide. B, effect of different concentrations of biological thiols on the dinitrosyl iron complexes in the cell extracts. The cell extracts prepared from the E. coli cells treated with nitric oxide were incubated with different concentrations of l-cysteine (Cys), N-acetyl-l-cysteine (NAC), and GSH, respectively. Samples were incubated at 37 °C for 20 min under aerobic conditions. The amplitudes of the EPR signal at g av 2.04 were plotted as a function of the thiol concentrations added in the cell extracts before incubation.

**Destabilization of Protein Dinitrosyl Iron Complexes**

Iron-sulfur proteins often become inactivated when their iron-sulfur cluster is modified by nitric oxide, forming the dinitrosyl iron complex (6, 11, 18, 19). Using isolated aconitases from mammalian cells, interrelation between formation of the dinitrosyl iron complex and inactivation of the enzyme activity was quantitatively demonstrated (18). Recently, it has also been proposed that the redox transcription factor SoxR in E. coli can be activated by nitric oxide through formation of the dinitrosyl iron complex from its redox-active [2Fe-2S] cluster (21). Perhaps iron-sulfur proteins are not only the targets of nitric oxide cytotoxicity (6, 7) but also the signaling receptors of nitric oxide (8, 21). However, there was no direct evidence showing that iron-sulfur proteins are modified, forming protein dinitrosyl iron complexes when organisms are exposed to nitric oxide. Here, we presented in vivo data showing that up to 40% of the E. coli ferredoxin [2Fe-2S] clusters are modified forming the dinitrosyl iron complexes when E. coli cells are exposed to a single dose of nitric oxide (Fig. 1). The partial modification of the ferredoxin [2Fe-2S] clusters could be the result of not enough nitric oxide being added to the E. coli culture in the experiments.

**DISCUSSION**

Iron-sulfur proteins are involved in many important cellular activities (8, 9), prompt repair of the modified iron-sulfur proteins will be vital if the cells are to survive. Indeed, the modified iron-sulfur clusters are efficiently repaired when E. coli cells are returned to normal growth conditions (Fig. 1). So far, little is known about the repair mechanism for the nitric oxide-modified iron-sulfur proteins. It seems that such repair activity preexists in cells, since the protein synthesis inhibitor chloramphenicol does not prevent cells from repairing the ferredoxin dinitrosyl iron complex (Fig. 1). Furthermore, when E. coli cells are disrupted, the repair activity for the protein dinitrosyl iron complexes is completely inactivated (Fig. 5). These observations imply that some cellular reducing equivalents may be essential for removing the dinitrosyl iron complexes from protein. During the past few years, a highly conserved gene cluster *iseSUA-hscBA-fdx* has been identified as...
important for biogenesis of iron-sulfur proteins (27, 28). The iscSUA cluster encodes three proteins: IscS, IscU, and IscA. IscS, a pyridoxal phosphate-containing homodimer, is an enzyme catalyzing l-cysteine desulfurization to provide sulfur for the assembly of iron-sulfur clusters (29, 36). IscU may provide a scaffold for IscS-mediated assembly of the clusters that are subsequently used for maturation of iron-sulfur proteins (37, 38). IscA is suggested to be involved in delivering iron to the sites of the iron-sulfur cluster assembly (39). The hscBA cluster encodes two proteins, Hsc66 and Hsc20, that interact with IscU protein and probably participate in the maturation of iron-sulfur proteins (40). The function of ferredoxin encoded by the gene fdx is not known, although the gene fdx has an essential role in overall assembly of iron-sulfur clusters (28). It has also been postulated that ferredoxin is important for biogenesis or repair of iron-sulfur clusters in Azotobacter vinelandii (33) and in Saccharomyces cerevisiae (34). Nevertheless, none of these proteins seem to have the activity that can directly remove the damaged iron-sulfur clusters from proteins. We propose that the modified iron-sulfur clusters such as the dinitrosyl iron complexes will have to be removed from proteins before a new iron-sulfur cluster can be reassembled into apoproteins. The reassembly process may be carried out by the products of the gene cluster iscSUA-hscBA-fdx (27, 28). In order to remove the modified iron-sulfur clusters from protein, cellular reducing equivalents such as biological thiols may be essential. Previous studies indicated that biological thiols have an important role in the metabolism of the protein-iron-sulfur clusters (30, 31). We speculate that biological thiols may also be involved in removing the dinitrosyl iron complexes from proteins. The results presented here showed that l-cysteine, but not N-acetyl-l-cysteine or glutathione, is effective in removing the EPR signal at gav = 2.04 of the dinitrosyl iron complex (Fig. 2) and releasing the iron from the complex (Fig. 3). The kinetics of the iron release was closely correlated with that of the disappearance of the EPR signal at gav = 2.04 when the Fdx-NO sample was incubated with l-cysteine (Fig. 4).

l-cysteine seems to have a general function in removing the dinitrosyl iron complexes from proteins, since incubation with l-cysteine can also eliminate the EPR signal at gav = 2.04 of the dinitrosyl iron complexes in the cell extracts prepared from the E. coli cells treated with nitric oxide (Fig. 5). In aerobically growing E. coli cells, there is about 0.39 μmol of l-cysteine/10^12 cells, which is equivalent to an intracellular concentration of 0.2 mM l-cysteine (41). This concentration of l-cysteine may be sufficient to remove the dinitrosyl iron complexes when the cells are exposed to a single dose of nitric oxide (at a final concentration of about 5 μM) used in this study (Fig. 5). When E. coli cells are disrupted, the intracellular l-cysteine may be oxidized or substantially diluted (up to 50-fold) in the cell extracts such that the concentration of l-cysteine is too low to remove the dinitrosyl iron complexes from proteins. The apparently stable proteinaceous dinitrosyl iron complexes in the cell extracts are effectively decomposed when exogenous l-cysteine is added (Fig. 5). It can be envisioned that if E. coli cells are exposed to a higher concentration of nitric oxide or for a prolonged period of exposure with a low concentration of nitric oxide, the intracellular l-cysteine may be consumed, and new l-cysteine may need to be synthesized. It is worth noting that there is a cysE-like gene upstream of the gene cluster iscSUA-hscBA-fdx, whose product could increase the intracellular l-cysteine pool (27). The physiological role of l-cysteine in repairing the modified iron-sulfur proteins must be investigated before we can ascertain whether l-cysteine is the cellular factor that is responsible for removing the dinitrosyl iron complexes from proteins in E. coli.

It is intriguing that substitution of an acetyl at the amino group of l-cysteine (N-acetyl-l-cysteine) or replacement of the thiol group with a hydroxyl group (l-serine) completely eliminates the ability of l-cysteine to remove the dinitrosyl iron complexes from proteins. The slightly different pK values of the thiol group in l-cysteine, N-acetyl-l-cysteine, and glutathione (31) cannot fully explain their different abilities in removing the dinitrosyl iron complexes from proteins, since l-cysteine is almost equally effective at pH 7.5, 7.9, and 8.5 (data not shown). We suggest that it is the unique redox property of l-cysteine that allows l-cysteine to directly interact with the dinitrosyl iron complex in protein and release the iron from the complex. There are at least two models that can explain the data presented in this work. First, l-cysteine may interact with nitric oxide moieties of the dinitrosyl iron complex in protein and form nitrosylated l-cysteine. Such interaction will leave the protein with a “naked” iron center that will eventually be released from proteins into the solution. The unstable iron center in protein may also be recycled for reassembly of new iron-sulfur clusters in cells. This model is apparently supported by the observation that the EPR signal at gav = 2.04 completely disappears at 20 min, while the ferrous iron release continues during the incubation of the Fdx-NO sample with l-cysteine (Fig. 4). Alternatively, l-cysteine may extrude the dinitrosyl iron complex from protein by replacing thiol ligands and form the l-cysteine dinitrosyl iron complex. Since the l-cysteine dinitrosyl iron complex is very unstable (the half-life of the l-cysteine dinitrosyl iron complex is less than 1 min in aqueous solution (26)), the ferrous iron will be released from the complex. The redox reactions involved in the l-cysteine-mediated removal of the dinitrosyl iron complex from proteins are currently under investigation.

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Destabilization of Protein Dinitrosyl Iron Complexes