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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 $g_{av}$ = 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 are disrupted, indicating that repairing the ferredoxin dinitrosyl iron complex requires cellular reducing equivalents. 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 $g_{av}$ = 2.04 (6, 11, 18–21). The same EPR signal at $g_{av}$ = 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 signals at $g_{av}$ = 2.04 in living organisms has been a controversy. Vanin *et al.* suggested that the EPR signal at $g_{av}$ = 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 $g_{av}$ = 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 ferredoxin 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 $g_{av}$ = 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 *Nco*I restriction site in one primer and a *Hin*dIII site in the other. The sequences of the primers are as follows: fdx-1, 5′-AGGTTTACCATGGCAAAGATTGTT-3′; fdx-2, 5′-CCTCTGTTAAAGCTTACGCGCATG-3′. The *Nco*I/*Hin*dIII-digested polymerase chain reaction



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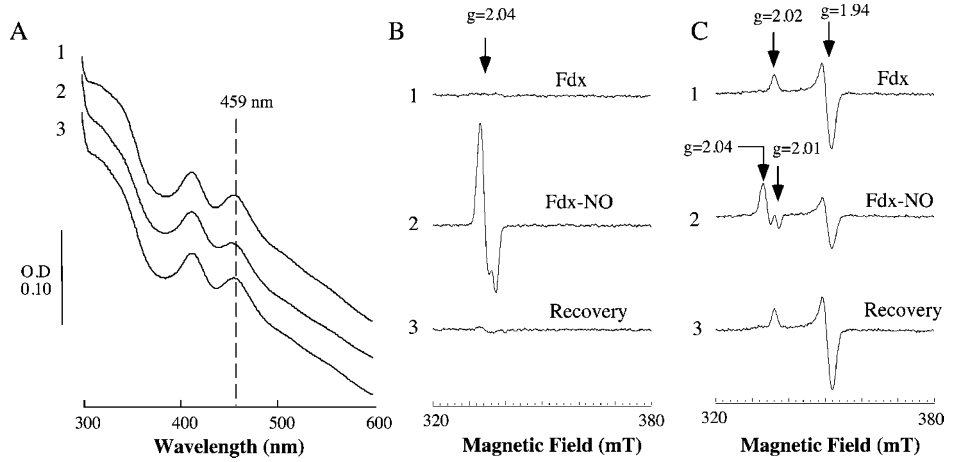


FIG. 1. **Modification and repair of the ferredoxin [2Fe-2S] clusters when *E. coli* cells are exposed to nitric oxide.** Ferredoxin proteins were purified as described under "Experimental Procedures." *A*, absorption spectra of the ferredoxin samples. *Sample 1*, Fdx. Ferredoxin protein was purified from the *E. coli* cells not treated with nitric oxide. *Sample 2*, Fdx-NO. Ferredoxin protein was purified from the *E. coli* cells treated with nitric oxide. *Sample 3*, ferredoxin protein was purified from the *E. coli* cells incubated at 37 °C with aeration for 30 min after treatment with nitric oxide. All samples contain about 15 $\mu$M ferredoxin protein. *B*, EPR spectra of the ferredoxin samples. Ferredoxin samples shown in *A* were 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.

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-$\beta$-D-thiogalactopyranoside (200 $\mu$M) 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 then injected into the *E. coli* culture using a gas-tight Hamilton syringe (the final concentration of nitric oxide was about 5 $\mu$M). After the treatment with nitric oxide, the *E. coli* culture was split into two equal volumes. One of them was harvested immediately, and the other was harvested after incubation in a 37 °C incubator with aeration for 30 min. Nitric oxide-saturated solution was prepared by bubbling pure nitric oxide gas (MG Industries, Malvern, PA), which had been first passed through a soda lime column (4–8-mesh) to remove other nitrogen oxides, through previously degassed water for 15 min (21).

*Protein Purification*—The *E. coli* cells prepared above were resuspended into 30 ml of prechilled buffer A (5 mM imidazole, 500 mM NaCl and 50 mM Tris, pH 7.9). Cells were disrupted by passing them through a French press once and then centrifuged at 11,000 rpm for 95 min to remove cell debris. The supernatants (cell extracts) were applied to a Superflow nickel-agarose column (2 ml) (Qiagen co.) attached to an ÄKTA-FPLC (Fast Protein Liquid Chromatography) system (Amersham Pharmacia Biotech). The column was then washed with three column volumes of buffer A and three column volumes of buffer B (15 mM imidazole, 500 mM NaCl, 50 mM Tris, pH 7.9). The ferredoxin protein was eluted with buffer C (300 mM imidazole, 500 mM NaCl, 50 mM Tris, pH 7.9). The eluted protein was applied to a Hitrap Desalting column (5 ml) (Amersham Pharmacia Biotech) equilibrated with buffer D (500 mM NaCl, 50 mM Tris, pH 7.9) to remove imidazole from the protein samples. The FPLC system was controlled by UNICORN software (Amersham Pharmacia Biotech) that allows a reproducible protein purification profile. The protein concentration was determined by the Bradford protein assay kit from Bio-Rad, using bovine serum albumin as a standard. The purity of the purified ferredoxin samples was greater than 90%, judging from its electrophoresis on a 15% polyacrylamide gel containing SDS followed by staining with Coomassie Brilliant Blue.

*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 of the ferredoxin [2Fe-2S] cluster was estimated using an extinction 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 Centricon concentrator (Micron YM-10; Millipore Corp.) and centrifuged using an Eppendorf 5810R desktop centrifuge. The iron indicator $\alpha,\alpha$-dipyridyl (50 $\mu$M) was then added to the flow-through samples to determine the ferrous iron concentration. The ferrous iron-$\alpha,\alpha'$-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[1]; sample temperature, 20 K; receiver gain, $10^5$. The relative amplitude of the EPR signal was quantified as described previously (21).

## 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

[1] The abbreviations used are: mT, milliteslas; Fdx, ferredoxin protein purified from the *E. coli* cells not treated with nitric oxide; Fdx-NO, ferredoxin protein purified from the *E. coli* cells treated with nitric oxide.

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. 1*A*, *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. 1*B*, 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,[2] 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. 1*B*, *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. 1*B*, *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 $g_x = g_y = 1.94$ and $g_z = 2.02$ appeared (Fig. 1*C*, *trace 1*). The Fdx-NO sample reduced with dithionite had a more complicated EPR spectrum (Fig. 1*B*, *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. 1*C*, 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. 1*C* 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.

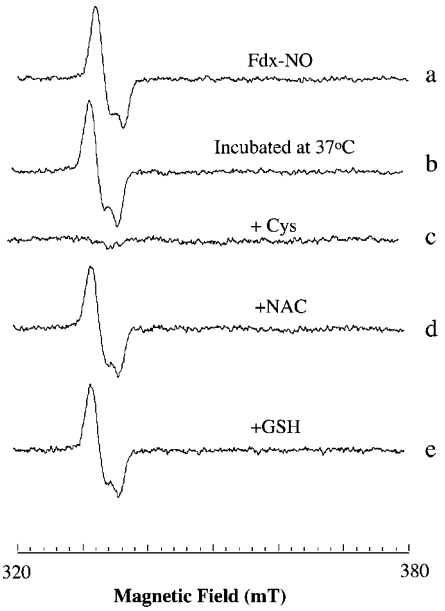


FIG. 2. **Effect of biological thiols on the EPR signal at $g_{av} = 2.04$ of the ferredoxin dinitrosyl iron complex.** The purified Fdx-NO sample (10 μM) was incubated with no addition (*trace b*) or with 1 mM L-cysteine (*Cys*) (*trace c*), *N*-acetyl-L-cysteine (*NAC*) (*trace d*), or GSH (*trace 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."

*L-Cysteine Removes the EPR Signal of the Ferredoxin Dinitrosyl Iron Complex*—Fig. 1 showed the dynamic metabolism of the ferredoxin [2Fe-2S] cluster when *E. coli* cells were exposed to a single dose of nitric oxide. During the experiments, we also found that once *E. coli* cells were disrupted, the ferredoxin dinitrosyl iron complex in the cell extracts was stable even after the cell extracts were incubated at 37 °C for 30 min aerobically (data not shown). This result indicates that cellular repairing activity for protein dinitrosyl iron complexes is inactivated when *E. coli* cells are disrupted. One candidate for such repair activity will be the cellular reducing equivalents that are potentially oxidized or substantially diluted during the disruption of the *E. coli* cells.

As an initial approach, the Fdx-NO sample prepared from the *E. coli* cells treated with nitric oxide was incubated with various biological thiols. After incubation at 37 °C for 20 min under aerobic conditions, samples were transferred to EPR tubes and frozen immediately. The EPR spectra of the samples are shown in Fig. 2. Incubation of the Fdx-NO sample alone had little effect on the EPR signal at $g_{av} = 2.04$. When the Fdx-NO sample was incubated with L-cysteine, the EPR signal at $g_{av} = 2.04$ was completely eliminated (*trace c*). In contrast, *N*-acetyl-L-cysteine (*trace d*), glutathione (*trace e*), reduced thioredoxin and L-serine (data not shown) had no observable effect on the EPR signal at $g_{av} = 2.04$ of the Fdx-NO sample.

[2] P. A. Rogers and H. Ding, unpublished data.

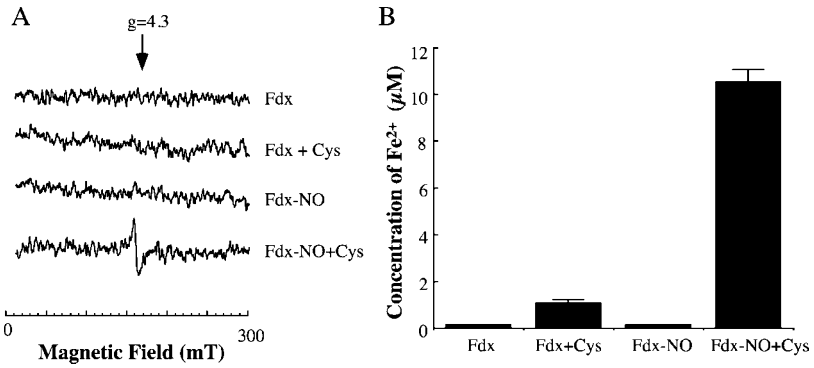


FIG. 3. **L-Cysteine stimulates iron release from the ferredoxin dinitrosyl iron complex.** Both Fdx and Fdx-NO samples (containing about 15 $\mu$M ferredoxin protein) were prepared as described under "Experimental Procedures." *A*, low field EPR spectra of the ferredoxin samples. The spectra were recorded 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 $\alpha,\alpha'$-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 $\alpha,\alpha'$-dipyridyl (50 $\mu$M) 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 $Fe^{2+}$-$\alpha,\alpha$-dipyridyl complex) using an extinction coefficient of 5.2 $mM^{-1}$ $cm^{-1}$ (30). The results were the averages from three different experiments.

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 ferredoxin dinitrosyl iron complex could be reduced by L-cysteine to become diamagnetic and EPR-silent (18, 25). However, repurification 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 ⅘ (35). As shown in Fig. 3*A*, 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 $\alpha,\alpha$-dipyridyl (30). As expected, very little ferrous iron was observed in both Fdx and Fdx-NO samples (Fig. 3*B*). 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 $\mu$M ferredoxin protein) was incubated with L-cysteine, only a small amount of the ferrous iron (~ 1.2 $\mu$M) 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.

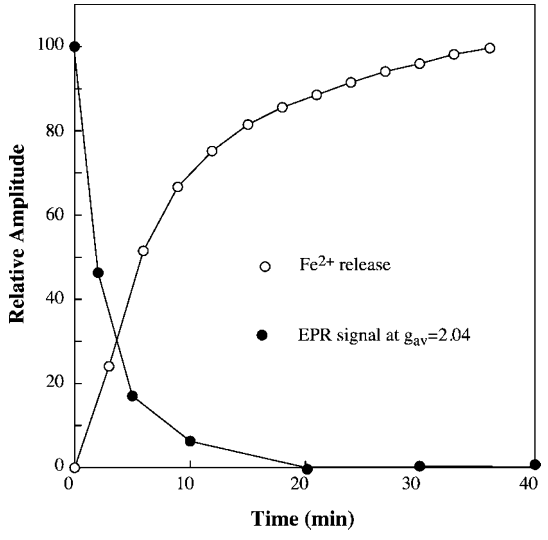


FIG. 4. **Kinetics of L-cysteine-mediated ferrous iron release and the disappearance of the EPR signal at $g_{av}$ = 2.04 of the ferredoxin dinitrosyl iron complex.** The Fdx-NO sample (containing 15 $\mu$M ferredoxin protein) purified from the *E. coli* cells treated with nitric oxide as described under "Experimental Procedures" was incubated with L-cysteine (1 mM) in a 37 °C water bath under aerobic conditions. At each time point, an aliquot (400 $\mu$l) was taken and frozen immediately for the EPR measurement. The ferrous iron release from the Fdx-NO sample by L-cysteine was monitored using $\alpha,\alpha'$-dipyridyl. The maximum amount of the ferrous iron released from the sample after incubation with L-cysteine was about 11 $\mu$M. *Open circles*, the amount of the ferrous iron released; *closed circles*, the relative amplitude of the EPR signal at $g_{av}$ = 2.04 of the ferredoxin dinitrosyl iron complex.

When the Fdx-NO sample (containing ~15 $\mu$M ferredoxin protein) was incubated with L-cysteine, about 10.5 $\mu$M 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 $\mu$M 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 were modified in the Fdx-NO sample (Fig. 1*C*), the maximum concentration of the ferredoxin dinitrosyl iron complex in the Fdx-NO sample would be about 6.0 $\mu$M. 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

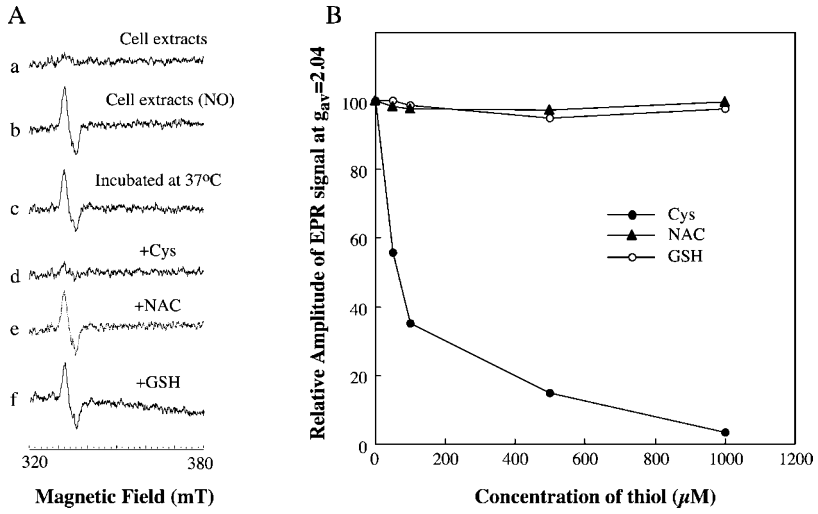


FIG. 5. **Effect of L-cysteine on the EPR signal of the dinitrosyl iron complex in cell extracts.** Exponentially growing *E. coli* cells (without any expression plasmids) were treated with or without nitric oxide (at a final concentration of 5 μM). The cells were centrifuged and resuspended in a buffer 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 $g_{av}$ = 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.

stoichiometry of the iron release from the ferredoxin dinitrosyl iron complex by L-cysteine.

The kinetics for the iron release and the disappearance of the EPR signal at $g_{av}$ = 2.04 were further characterized when the Fdx-NO sample was incubated with L-cysteine at 37 °C aerobically. As shown in Fig. 4, there was a close correlation between the amount of the iron released and the amplitude of the EPR signal at $g_{av}$ = 2.04 when the ferredoxin dinitrosyl iron complex was incubated with L-cysteine. Interestingly, half of the amplitude of the EPR signal at $g_{av}$ = 2.04 was removed at about 2 min, while half of the total iron was released from the Fdx-NO sample at about 6 min. This point will be discussed below.

*Dinitrosyl Iron Complexes in Cell Extracts Can Also Be Removed by L-Cysteine*—Since L-cysteine can efficiently remove the dinitrosyl iron complex from ferredoxin proteins, we postulate that L-cysteine may have a general role in decomposing cellular protein dinitrosyl iron complexes when *E. coli* cells are exposed to nitric oxide. Cell extracts were prepared from exponentially growing *E. coli* cells (without any expression plasmids) treated with nitric oxide. Prepared cell extracts had a stable EPR signal at $g_{av}$ = 2.04 even after being incubated at 37 °C for 20 min under aerobic conditions (Fig. 5*A*, *traces b* and *c*). The gel filtration profile of the cell extracts showed that over 95% of the stable EPR signal at $g_{av}$ = 2.04 seen in the cell extracts was associated with the fractions whose molecular mass was larger than 10 kDa (data not shown). This indicated that the majority of the dinitrosyl iron complexes in the cell extracts were associated with cellular proteins. When such prepared cell extracts were incubated with L-cysteine at 37 °C for 20 min under aerobic conditions, the EPR signal at $g_{av}$ = 2.04 was almost completely eliminated (Fig. 5*A*, *trace d*). *N*-Acetyl-L-cysteine (*trace e*) or glutathione (*trace f*) had much less effect on the EPR signal at $g_{av}$ = 2.04 of the cell extracts. The addition of either NADH or NADPH to the cell extracts had little effect on the EPR signal (data not shown). Titration experiments showed that 50 μM L-cysteine was sufficient to remove 50% of the EPR signal in the cell extracts after incubation at 37 °C for 20 min aerobically, while other thiols were not effective in the concentration range used (Fig. 5*B*).

## DISCUSSION

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.

Since 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 *iscSUA-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 in 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 $g_{av} = 2.04$ 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 $g_{av} = 2.04$ 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 $g_{av} = 2.04$ 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 $\mu$mol 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 $\mu$M) 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 $g_{av} = 2.04$ 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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