The Escherichia coli DNA damage-inducible protein DinG, a member of the superfamily 2 DNA helicases, has been implicated in the nucleotide excision repair and recombinational DNA repair pathways. Combining the UV-visible absorption and electron paramagnetic resonance (EPR) spectroscopies and the enzyme activity measurements, here we demonstrate that E. coli DinG contains a redox active [4Fe-4S] cluster with a redox midpoint potential ($E_m$) of $-390 \pm 23$ mV (pH 8.0) and that reduction of the [4Fe-4S] cluster reversibly switches off the DinG helicase activity. Unlike the [4Fe-4S] cluster in the E. coli dihydroxyacid dehydratase, the DinG [4Fe-4S] cluster is stable and the enzyme remains fully active after exposure to 100-fold excess of hydrogen peroxide, indicating that DinG could be functional under oxidative stress conditions. However, the DinG [4Fe-4S] cluster can be efficiently modified by nitric oxide (NO) forming the DinG-bound dinitrosyl iron complex with the concomitant inactivation of the helicase activity in vitro and in vivo. Reassembly of the [4Fe-4S] cluster in the NO-modified DinG restores the helicase activity, indicating that the iron-sulfur cluster in DinG is the primary target of the NO cytotoxicity. The results led us to propose that the iron-sulfur cluster in DinG may act as a sensor of intracellular redox potential to modulate its helicase activity and that modification of iron-sulfur cluster in DinG and likely in other DNA repair enzymes by NO may contribute to the NO-mediated genomic instability.
activity (15,16). Mutations that affect the iron-sulfur cluster binding or stability in XPD abolish the helicase activity (15). The X-ray crystallographic studies further revealed that the [4Fe-4S] cluster is located in the vicinity of the DNA binding site of XPD (9-11). While iron-sulfur clusters have been discovered in a large number of proteins that have specific interactions with DNA or RNA (17-29), specific functions of the iron-sulfur clusters in these proteins mostly remain elusive.

E. coli DinG has about 48% identity with human XPD in the regions of the helicase motif (5). Although DinG does not possess the corresponding conserved cysteine residues (Cys-92, Cys-113, Cys-128 and Cys-164, the T. acidophilum numbering) in XPD that host the [4Fe-4S] cluster (9-11), sequence analysis of a subset of the DinG homologs from diverse bacterial species revealed four conserved cysteine residues (Cys120, Cys194, Cys199 and Cys205, the E. coli numbering) that may provide ligands for a putative iron-sulfur cluster (6,15). In this study, we report that purified E. coli DinG contains a redox active [4Fe-4S] cluster with a redox midpoint potential (Eₐ) of -390±23 mV (pH 8.0) and that reduction of the [4Fe-4S] cluster in DinG reversibly switches off the helicase activity. Importantly, unlike the E. coli dihydroxyacid dehydratase [4Fe-4S] cluster (30), the DinG [4Fe-4S] cluster is stable and the enzyme remains fully active after exposure to 100-fold excess of hydrogen peroxide, indicating that DinG could be functional under oxidative stress conditions. In contrast, nitric oxide (NO), a physiological free radical produced in activated macrophages and other mammalian cells (31-34), can efficiently modify DinG-bound dinitrosyl iron complex (DNIC) with the concomitant inactivation of the helicase activity in vitro and in vivo. Combining with the previous studies that NO can modify the DNA repair enzyme endonuclease III [4Fe-4S] cluster and inactivate the enzyme activity (35), we propose that modification of the iron-sulfur clusters in XPD/DinG, endonuclease III and possibly other DNA repair enzymes by NO may contribute to the NO-mediated initiation of carcinogenic process and genomic instability in mammalian cells (36,37).

**EXPERIMENTAL PROCEDURES**

**Protein preparation**—The DNA fragment encoding the DNA damage-inducible protein DinG was amplified from E. coli genomic DNA using PCR. Two primers, DinG-1, 5’-GGTTTTCCCATGGCATTAACCGCC-3’ and DinG-2, 5’-CATCATTAAAGCTTCCGACGCGT-3’ were used for the PCR amplification. The PCR product was digested with HindIII and NcoI, and ligated into an expression vector pET28b' to produce pTDinG. The cloned DNA fragment was confirmed by direct sequencing using the T7 primer (Genomic Facility, LSU). Recombinant DinG was overproduced in E. coli BL21 strain in Terrific Broth (TB), and purified using a Ni-agarose column followed by a HiTrap Desalting column. The purity of purified DinG was over 95% judging from the SDS polyacrylamide gel analysis followed by the Coomassie blue staining. The precise molecular weight of recombinant DinG was confirmed using the electrospray ionization-mass spectrometry (ESI-MS) (Chemistry Department/LSU). The protein concentration of purified DinG was measured from the absorption peak at 280 nm using an extinction coefficient of 79.0 mM⁻¹cm⁻¹. The total iron content in protein samples was determined using an iron indicator ferrozine (38). The total sulfide content in protein samples was determined according to the Siegel’s method (39) as described previously (40). Site-directed mutagenesis was carried out using the Quick-change kit from Stratagene. Mutations in gene dinG were confirmed by direct sequencing (Genomic Facility, LSU). The DinG mutant proteins were expressed and purified following the same purification procedures as described for the wild-type DinG. Recombinant dihydroxyacid dehydratase (IlvD) (30) from E. coli was prepared as described in (41). The specific enzyme activity of IlvD was measured using substrate DL-2,3-dihydroxy-isovalerate, and the reaction product (keto acids) was monitored at 240 nm using an extinction coefficient of 0.19 mM⁻¹cm⁻¹ (30). DL-2,3-dihydroxy-isovalerate was synthesized according to the method of Cioffi et al. (42).
The helicase activity assay—The helicase activity of DinG was measured following the procedure described by Voloshin et al. (5) with slight modifications. Briefly, an oligonucleotide (5'CCGTAACACTGAGTTTCGTCACCAGTACAACACTAAGCTGCTCGTATCCACA-3') was labeled with $^{32}$P-$\gamma$-ATP using polynucleotide kinase (New England BioLab). The $^{32}$P-labeled oligonucleotide (5 μM) was annealed to M13mp18 ssDNA (New England BioLab) in annealing buffer containing Tris (50 mM, pH 7.5), NaCl (50 mM) and MgCl$_2$ (10 mM). The DNA solution was heated at 85°C for 5 min and cooled to room temperature over 3 hours. The annealed DNA duplex was purified using a gel filtration spin-column Chromaspin 400 (Clontech) pre-equilibrated with annealing buffer. The annealed substrate (at a final concentration of 2 nM) was incubated with DinG in 20 μl of the reaction solution containing Tris (50 mM, pH 8.0), NaCl (100 mM), MgCl$_2$ (5 mM), dithiothreitol (2 mM), glycerol (5%), and ATP (2 mM) at 30°C for 10 min. For each experiment, two controls in which the substrate was either denatured by heating at 85°C for 5 min or incubated at 30°C for 10 min without any enzymes were included. The reactions were terminated by adding 4 μl stop solution (containing 6% SDS, 60 mM EDTA and 0.3% Bromophenol Blue). The reaction products (single-stranded DNA) were separated on 1% TAE agarose gel, transferred to Nytran transfer membranes (0.45 μm) (Whatman co.), and exposed to x-ray films overnight for quantification.

Redox titration of the DinG iron-sulfur cluster—A specially designed anaerobic cuvette was used for redox titrations as described by Dutton (43). Before titration, solution containing DinG (20 μM) and safranine O (1 μM) was equilibrated with ultra-pure argon gas for 50 min at room temperature. During titration, the argon flow was maintained with gentle stirring by a small magnet on the bottom of the cuvette. The redox potential of the solution was adjusted by adding a small amount of freshly prepared sodium dithionite using a gas-tight 10-μl Hamilton microsyringe (Hamilton Co., Reno, NV). The redox potential was monitored directly with a redox microelectrode (Microelectrodes Inc., Bedford, NH). A freshly prepared ZoBell’s solution (containing potassium ferricyanide (5 mM) and potassium ferrocyanide (5 mM) dissolved in buffer Tris (20 mM, pH 8.0) and NaCl (500 mM)) was used as a standard (E$_{ox}$=238 mV) for calibration of the redox microelectrode.

NO exposure of DinG and re-assembly of the iron-sulfur cluster in the protein—Purified DinG (30 μM) was incubated with the NO releasing reagent diethylamine NONOate (0 to 0.5 mM) (Cayman Chemicals) in buffer containing Tris (20 mM, pH 7.5) and NaCl (200 mM) anaerobically at room temperature. Diethylamine NONOate releases 1.5 equivalents of NO per mole of parent compound with a half-life time of 16 min at room temperature and pH 7.5. After 20 min incubation, the protein was repurified by passing through a HiTrap Desalting column to remove residual diethylamine NONOate. For the in vivo NO exposure, the E. coli cells containing recombinant DinG were subjected to the Silastic tubing NO delivery system (44) as described previously (41). The length of the Silastic tubing (I.D. x O.D.: 0.025 x 0.047 in.) immersed in the cell culture was adjusted to such that about 100 nM NO per second was released to the cell culture in a sealed flask under anaerobic conditions. The chosen NO release rate was comparable to the reported NO production in activated polymorphonuclear leukocytes (34) or in RAW 264.7 macrophages co-cultured with arginase-deficient Helicobacter pylori (32). After the E. coli cells were subjected to the NO exposure for 0, 1, 2 4 and 10 min anaerobically, recombinant DinG was purified from the cells following the procedures described early.

For re-assembly of iron-sulfur clusters, the NO-exposed DinG (10 μM) was incubated with freshly prepared Fe(NH$_4$)$_2$(SO$_4$)$_2$ (80 μM), L-cysteine (0.5 mM), and cysteine desulfurase IscS (45) (1 μM) in the presence of dithiothreitol (2 mM) anaerobically at 37°C for 20 min as described in (45), followed by re-purification of DinG from the incubation solution. Re-purified DinG was then subjected to the EPR and helicase activity measurements.
EPR measurements—The EPR spectra were recorded at X-band on a Bruker ESR-300 spectrometer using an Oxford Instruments ESR-9 flow cryostat. The routine EPR conditions were: microwave frequency, 9.45 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 2.0 mT; sample temperature, 20 K; receive gain, 1.0 x 10^5.

RESULTS

_E. coli_ DinG contains an iron-sulfur cluster essential for the protein stability and helicase activity—_Figure 1A shows the UV-visible absorption spectrum of purified _E. coli_ DinG. The major peak at around 403 nm represents a typical absorption of iron-sulfur clusters in proteins. The overall spectrum of purified DinG was similar to that of purified XPD homolog [4Fe-4S] cluster from _Sulfolobus acidocaldarius_ (15) and the endonuclease III [4Fe-4S] cluster from _E. coli_ (35). The total iron and sulfur content analyses of purified DinG showed that each DinG monomer contained approximately 3.1±0.5 iron and 2.8±0.8 sulfide (n=3), indicating that DinG contains a [4Fe-4S] cluster per monomer. Purified DinG was further analyzed for its DNA helicase activity. Following the procedures described by Voloshin et al. (5), we demonstrated that as-purified DinG was able to unwind the double-stranded DNA in an ATP-dependent reaction (Figure 1B).

_E. coli_ DinG has eleven cysteine residues, four of them (Cys-120, Cys-194, Cys-199 and Cys-205) are conserved among a subset of DinG proteins from diverse bacteria (6,15). To test whether the conserved cysteine residues are required for iron-sulfur cluster binding, we substituted each of these four cysteine residues in DinG with serine using the site-directed mutagenesis as described in the Experimental Procedures. All four mutant proteins (C120S, C194S, C199S and C205S) purified using the same procedure as the wild-type DinG had no absorption peak at 403 nm (Figure 2A). SDS polyacrylamide gel analyses revealed that unlike the wild-type DinG, the DinG mutants expressed in _E. coli_ cells were largely degraded (Figure 2B) and had no detectable helicase activity (Figure 2C). Thus, these conserved cysteine residues appear to be essential for the iron-sulfur cluster binding in DinG and for the protein stability.

Redox state of the iron-sulfur cluster in DinG controls the helicase activity—While the iron-sulfur cluster in the wild-type DinG was stable under aerobic conditions, addition of sodium dithionite quickly bleached the absorption peak at 403 nm (Figure 3A). Nevertheless, when the reduced DinG was re-oxidized by exposure to air or by oxidant potassium ferricyanide, the absorption peak at 403 nm of DinG reappeared (Figure 3A), suggesting that the iron-sulfur cluster in DinG can be reversibly reduced by sodium dithionite.

The absorption peak at 403 nm was then used to determine the redox midpoint potential (E_m) of the DinG iron-sulfur cluster. Purified DinG was dissolved in buffer containing a redox mediator safranine O under anaerobic conditions. The redox potential of the solution was adjusted by adding freshly prepared sodium dithionite and directly monitored using a micro-electrode as described by Dutton (43). The UV-visible absorption spectra were taken at different redox potentials, and the absorption peak at 403 nm of the DinG [4Fe-4S] cluster was plotted as a function of the poised redox potentials (Figure 3B). The data from three experiments were fitted to a Nernst equation (n=1) with a redox midpoint potential of -390±23 mV, a value close to that of the intracellular redox potential in _E. coli_ (17).

The electron paramagnetic resonance (EPR) spectroscopy was further used to explore the redox state of the DinG iron-sulfur cluster. As shown in Figure 3C, purified DinG had no EPR signal at around g=2.0 region under the experimental conditions (spectrum 1). However, when freshly prepared sodium dithionite was added to purified DinG, a rhombic EPR signal with g_x=1.918, g_y=1.944 and g_z=2.005, indicative of a reduced [4Fe-4S] cluster, appeared (spectrum 2). Spin quantification revealed that there was approximately 0.8-0.9 spin per each iron-sulfur cluster in the dithionite-reduced DinG. The observed g-values were comparable to those of the reduced [4Fe-4S]^+ cluster observed in other proteins (46). The relatively small Δg could reflect the unique
property of the [4Fe-4S] cluster in DinG. The rhombic EPR signal was completely eliminated when the dithionite-reduced DinG was re-oxidized by exposure to air or by potassium ferricyanide (spectrum 3), confirming that the DinG [4Fe-4S] cluster can be reversibly reduced by sodium dithionite. It is worth mentioning that no EPR signal at g=2.018 of the [3Fe-4S] cluster (47) was observed when purified DinG was treated with potassium ferricyanide (data not shown), suggesting that the DinG [4Fe-4S] cluster is resistant to oxidation.

Because the [4Fe-4S] cluster in the archaeal XPD homologs is located in the vicinity of the DNA binding site of the enzyme (9-11), we speculated that the redox state of the iron-sulfur cluster in DinG may modulate the helicase activity. To test this idea, we compared the helicase activity of DinG when its iron-sulfur cluster was either reduced or oxidized. Figure 3D shows that the helicase activity of DinG was greatly diminished when the iron-sulfur cluster was reduced with dithionite and largely restored once the reduced iron-sulfur cluster was re-oxidized, demonstrating that reduction of the iron-sulfur cluster in DinG can reversibly switch off the helicase activity at least in vitro.

The iron-sulfur cluster in DinG is resistant to hydrogen peroxide—As a DNA-damage inducible protein, it is somewhat surprising that DinG contains a [4Fe-4S] cluster which is presumably susceptible to reactive oxygen species (30). To determine the sensitivity of the DinG [4Fe-4S] cluster to reactive oxygen species, we incubated purified DinG with 100-fold excess of H₂O₂ at 25°C for 30 min, and found that the absorption peak at 403 nm of the DinG [4Fe-4S] cluster (Figure 4A) and the helicase activity (Figure 4C) remained essentially unchanged before and after incubation. In contrast, when purified E. coli dihydroxyacid dehydratase [4Fe-4S] cluster (30) was incubated with 50-fold excess of H₂O₂ at 25°C, both the absorption peak at 415 nm of the dihydroxyacid dehydratase [4Fe-4S] cluster (Figure 4B) and its enzyme activity (Figure 4D) were abolished as reported previously (30). Thus, unlike the dihydroxyacid dehydratase [4Fe-4S] cluster, the DinG [4Fe-4S] cluster is stable and its helicase activity remains fully active after exposure to 100-fold excess of hydrogen peroxide.

The iron-sulfur cluster in DinG can be efficiently modified by nitric oxide (NO)—Nitric oxide (NO) is a physiological free radical that acts as a signal molecule (31) as well as a powerful weapon to kill pathogenic bacteria and tumor cells (32-34). Chronic exposure of NO has also been attributed to the initiation of carcinogenic process and genomic instability (36,37). Among cellular components, iron-sulfur proteins are considered the primary targets of the NO cytotoxicity (41,48). In vitro and in vivo studies have shown that NO can readily modify iron-sulfur clusters in proteins forming the protein-bound dinitrosyl iron complex (DNIC) (35,41,47,49-52). To test whether the DinG [4Fe-4S] cluster can also be modified by NO, purified DinG was exposed to NO using the NO releasing reagent diethylamine NONOate (Cayman Chemicals co.) under anaerobic conditions. Figure 5A shows that when purified DinG was incubated with an increasing amount of diethylamine NONOate (0 to 500 μM), the DinG [4Fe-4S] cluster was gradually modified by NO forming the DinG-bound DNIC with a typical EPR signal at g=2.04 as previously reported for other iron-sulfur proteins (35,47,49-52). The parallel helicase activity measurements showed that DinG was progressively inactivated by the NO exposure (Figure 5B). Thus, NO can effectively modify the DinG [4Fe-4S] cluster and inactivate the helicase activity in vitro.

To further explore the sensitivity of the DinG [4Fe-4S] cluster to NO in vivo, we exposed the E. coli cells containing recombinant DinG to pure NO gas using the Silastic tubing NO delivery system (44) as described previously (41). A releasing rate of 100 nM NO per second was chosen to emulate the NO production in activated polymorphonuclear leukocytes (34) or macrophages (32). Recombinant DinG was then purified from the E. coli cells after exposure with different amounts of NO. The EPR measurements of purified DinG showed that the DinG-bound DNIC was gradually increased with the concomitant inactivation of the helicase activity when the E. coli cells were exposed to increasing amounts of NO (data not shown).
About 4 min NO exposure at a rate of 100 nM NO per second was sufficient to completely modify the recombinant DinG [4Fe-4S] cluster and inactivate the helicase activity in E. coli cells. These results suggested that the DinG [4Fe-4S] cluster can be efficiently modified with the concomitant inactivation of its helicase activity in E. coli cells by NO.

We then attempted to re-assemble the iron-sulfur cluster in the NO-modified DinG using the iron-sulfur cluster repair system (L-cysteine, cysteine desulfurase IscS, ferrous iron and dithiothreitol) in vitro as described previously (35). Figure 6 shows that after incubation with the repair system at 37°C for 30 min, the iron-sulfur cluster was re-assembled in the NO-modified DinG (panel A), the DinG-bound DNIC was decomposed (panel B) and the helicase activity was largely restored (panel C). Thus, the iron-sulfur cluster in DinG, like other iron-sulfur proteins (41), could be the primary target of the NO cytotoxicity.

**DISCUSSION**

In this study, we report that the E. coli DNA damage-inducible protein DinG helicase contains a redox active [4Fe-4S] cluster with a redox midpoint potential (E_m) of about -390±23 mV (pH 8.0), and that reduction of the iron-sulfur cluster reversibly switches off the helicase activity of DinG. While the iron-sulfur cluster in DinG is stable in the presence of oxygen or hydrogen peroxide, it can be efficiently modified by NO forming the DinG-bound DNIC with the concomitant inactivation of the helicase activity in vitro and in vivo. The results led us to propose that the DinG helicase activity can be modulated by intracellular redox potential and by NO via its iron-sulfur cluster.

In the past decade, a large number of iron-sulfur proteins that have specific interactions with DNA or RNA have been reported. According to their functions, these iron-sulfur proteins may be divided into two groups. The first group includes the transcription or translation regulators that directly bind to DNA or RNA. The well-characterized examples are the redox transcription factor SoxR [2Fe-2S] cluster (17,18), the anaerobic growth factor FNR [4Fe-4S] cluster (19), the repressor IscR [2Fe-2S] cluster that regulates the iron-sulfur cluster biosynthesis (20), and the iron regulatory protein 1 (IRP-1) [4Fe-4S] cluster that controls the post-translational control of intracellular iron contents in mammalian cells (21). In this group of proteins, iron-sulfur clusters generally act as sensors of specific signals and modulate the subtle interactions between the protein and DNA or RNA. The second group includes the iron-sulfur enzymes that chemically modify RNA or DNA molecules. The ribosomal RNA methyltransferase (the RumA [4Fe-4S] cluster) (22) and the bifunctional radical-S-adenosylmethionine enzyme MiaB [4Fe-4S] cluster (23) are two examples of RNA modifying enzymes. More recently, the p58 subunit of human DNA primase has been shown to contain a [4Fe-4S] cluster (28,29). The iron-sulfur enzymes that chemically modify DNA are mostly the DNA repair enzymes such as the endonuclease III [4Fe-4S] cluster (24,25), the MutY [4Fe-4S] cluster (26), the family 4 uracil-DNA glycosylase [4Fe-4S] cluster (27), the DNA helicase XPD [4Fe-4S] clusters (9-11), and the E. coli DinG helicase [4Fe-4S] cluster (5,6) (this study). Evidently, the function of iron-sulfur clusters in these diverse DNA/RNA modifying enzymes could not be readily generalized. Here we show that the [4Fe-4S] cluster in DinG is stable in the presence of oxygen and hydrogen peroxide (Figure 4), a feature that could be important for the helicase activity in repairing DNA damage under oxidative stress conditions. More importantly, we demonstrate that the [4Fe-4S] cluster in DinG is redox active with a redox midpoint potential of -390±23 mV (pH 8.0) and that reduction of the [4Fe-4S] cluster in DinG reversibly switches off the helicase activity (Figure 3). We postulate that reduction of the [4Fe-4S] cluster in DinG, like that of the redox transcription factor SoxR [2Fe-2S] cluster (17), may modulate the overall structure of the catalytic center and thus inactivate the helicase activity. It should be pointed out that DinG homologs in some other bacteria do not have the conserved cysteine residues and therefore no iron-sulfur clusters (9). Whether there are other means to regulate the activity of these DinG helicases remains to be investigated.
The observed redox midpoint potential ($E_m$) of the DinG [4Fe-4S] cluster (-390±23 mV) (pH 8.0) (Figure 3) is close to that of the intracellular redox potential in *E. coli* (17). Several attempts were made to observe the redox state of the recombinant DinG [4Fe-4S] cluster in *E. coli* cells. Unfortunately, no EPR signal of the reduced DinG [4Fe-4S] cluster was observed in vivo (data not shown), likely because of insufficient amounts of the recombinant DinG expressed in *E. coli* cells. It has been reported that DNA binding shifts the redox midpoint potential ($E_m$) of the endonuclease III [4Fe-4S] cluster toward oxidation, converting the redox-inactive endonuclease III [4Fe-4S] cluster to a typical high-potential [4Fe-4S] protein (25). Here we find that unlike the endonuclease III [4Fe-4S] cluster, the DinG [4Fe-4S] cluster is redox active even without any DNA binding (Figure 3). Whether DNA binding will change the redox property of the DinG [4Fe-4S] cluster remains to be investigated. Nevertheless, based on the results presented in this study, we propose that the DinG [4Fe-4S] cluster may be partially oxidized in *E. coli* cells under normal growth conditions. When cells are subjected to oxidative stresses, the reduced [4Fe-4S] cluster is oxidized, and DinG becomes fully active to repair the inflicted DNA damage and resume the DNA replication.

NO is a physiological free radical involved in signal transduction in neuronal and cardiovascular systems (31). Excessive production of NO in activated macrophages and other mammalian cells can also act as a powerful weapon to kill pathogenic bacteria and tumor cells (32,33). In some studies, chronic NO exposure has been linked to the carcinogenic process and genomic instability (36,37). Nevertheless, the etiology of the NO cytotoxicity has not been fully understood. Here we report that the DinG [4Fe-4S] cluster can be modified by NO forming the DinG-bound DNIC with the concomitant inactivation of the helicase activity in vitro and in vivo. Because genetic defects in the human *XPD* gene (*ERCC2*) have been associated with the increase of cancer incidence and aging phenotypes (7,9,10,15), it is plausible that chronic NO exposure may inactivate the iron-sulfur cluster-containing DNA repair enzymes such as DinG, and contribute to initiation of the carcinogenic process and genomic instability (36,37).

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**Abbreviations:** EPR, electron paramagnetic resonance; DinG, DNA-damage inducible helicase; DNIC, dinitrosyl iron complex; $E_m$, redox midpoint potential.
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FIGURE LEGENDS

Figure 1. Purified *E. coli* DinG contains an iron-sulfur cluster.  A), the UV-visible absorption spectrum of purified *E. coli* DinG. The protein contraction was about 10 μM.  B), the helicase activity of purified *E. coli* DinG. Purified DinG (at a final concentration of 0 to 200 nM) was incubated with the 32P-radioactive-labeled substrate in the presence of ATP (2 mM) at 30°C for 10 min. The reaction product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described under the Experimental Procedures. Lane H, the sample was heated at 85°C for 5 min. The concentration of DinG in the reaction solution was indicated on the top of each lane.

Figure 2. The conserved cysteine residues in DinG are required for the iron-sulfur cluster binding, protein stability and the helicase activity.  The DinG mutants (C120S, C194S, C199S, and C205S) were constructed and purified as described under the Experimental Procedures.  A), UV-visible absorption spectra of purified DinG mutants. The protein concentrations were about 3 μM.  B), the SDS polyacrylamide electrophoresis gel analysis of purified wild-type DinG and DinG mutants. Same amounts of the cells containing either the wild-type DinG or the DinG mutants were used for the protein purification. Equal amounts of purified proteins were analyzed on the SDS polyacrylamide electrophoresis gel. Lanes 1-5, the wild-type DinG, the mutants C120S, C194S, C199S, and C205S, respectively.  C), the helicase activity of purified wild-type DinG and the DinG mutants. Purified proteins (200 nM) were incubated with the 32P-radioactive-labeled substrate in the presence of ATP (2 mM) at 30°C for 10 min. The reaction product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described under the Experimental Procedures. Lane H, the sample was heated at 85°C for 5 min. Lane 0, no enzyme was added. Lanes 1-5, the wild-type DinG, the mutants C120S, C194S, C199S, and C205S, respectively.

Figure 3. Redox state of the iron-sulfur cluster in DinG regulates its helicase activity.  A), UV-visible absorption spectra of the reduced and oxidized DinG. Purified DinG (25 μM) (spectrum 1) was reduced with sodium dithionite (200 μM) under anaerobic conditions (spectrum 2) and then re-oxidized by exposing to air for 30 min (spectrum 3). The absorption peak at 403 nm reflects the oxidized DinG [4Fe-4S] cluster.  B), redox titration of the DinG [4Fe-4S] cluster. Purified DinG (25 μM) in buffer containing Tris (50 mM, pH 8.0) and NaCl (500 mM) was supplemented with safranine O (1 μM) as a redox mediator. Redox titration was performed in an anaerobic redox cuvette as described under the Experimental Procedures. The x axis shows the redox potentials measured with a redox microelectrode. The y axis shows the relative absorbance at 403 nm, normalized to 0 or 100% for fully reduced or oxidized DinG [4Fe-4S] cluster, respectively. The solid line drawn through the data points was the best fit to the Nernst equation (n=1) with the redox midpoint potential (E_m) of −390 ± 23 mV. Data were from three independent experiments represented with three different symbols.  C), EPR spectra of purified DinG. Purified DinG (500 μM) (spectrum 1) was reduced with sodium dithionite (2 mM) (spectrum 2) or re-oxidized with potassium ferricyanide (2 mM) (spectrum 3).  D), the helicase activity of DinG under different redox potentials. Purified DinG (100 nM) was either reduced with sodium dithionite or re-oxidized with potassium ferricyanide, before the radioactive-labeled DNA substrate was added to the incubation solutions. After 5 min incubation at 30°C, the reaction was terminated and the product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described under the Experimental Procedures. Lane H, the sample was heated at 85°C for 5 min. Lane 0, no enzyme was added. Lane 1, purified DinG. Lane 2, purified DinG reduced with dithionite (1.0 mM). Lane 3, dithionite-reduced DinG was re-oxidized with potassium ferricyanide (2 mM).

Figure 4. The iron-sulfur cluster in DinG is resistant to hydrogen peroxide.  A), effect of H₂O₂ on the DinG [4Fe-4S] cluster. Purified DinG (10 μM) was incubated with H₂O₂ (1 mM) at 25°C. UV-visible spectra were taken every 5 min after addition of H₂O₂ for 30 min.  B), effect of H₂O₂ on the
dihydroxyacid dehydratase [4Fe-4S] cluster. Purified *E. coli* dihydroxyacid dehydratase (20 μM) was incubated with hydrogen peroxide (1 mM) at 25°C. UV-visible spectra were taken every 5 min after addition of H₂O₂ for 30 min. C), effect of H₂O₂ on the DinG helicase activity. After incubation with H₂O₂ for the indicated time, DinG (at a final concentration of 100 nM) was used for the helicase activity assay. Lane H, the sample was heated at 85°C for 5 min. Lane 0, no enzyme was added. Lanes 1 to 5, purified DinG after incubation with H₂O₂ for 0, 5, 10, 20, and 30 min, respectively. The reaction product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described under the Experimental Procedures. D), effect of H₂O₂ on the enzyme activity of dihydroxyacid dehydratase. Relative enzyme activity of dihydroxyacid dehydratase after incubation with H₂O₂ (1 mM) was measured as described under the Experimental Procedures and plotted as a function of incubation time with H₂O₂.

**Figure 5. The DinG iron-sulfur cluster is sensitive to NO.** A), modification of the DinG [4Fe-4S] cluster by NO. Purified DinG (30 μM) was incubated with different amounts of diethylamine NONOate in buffer containing Tris (20 mM, pH 7.5) and NaCl (200 mM) at room temperature under anaerobic conditions. After 20 min incubation, protein was re-purified by passing through a HiTrap Desalting column. EPR spectra 1-5, purified DinG was incubated with 0, 50, 100, 200 and 500 μM of NONOate under anaerobic conditions. The protein concentrations of re-purified DinG were about 4 μM. B), inactivation of the DinG helicase activity by NO. After incubation with different amounts of NONOate, re-purified DinG (at a final concentration of 100 nM) was used for the helicase activity assay. Lane H, the sample was heated at 85°C for 5 min. Lane 0, no enzyme was added. Lanes 1 to 5, re-purified DinG after incubation with 0, 50, 100, 200 and 500 μM of NONOate. The reaction product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described in the Experimental Procedures.

**Figure 6. Re-activation of the NO-modified DinG by re-assembly of iron-sulfur clusters.** Purified DinG (30 μM) was exposed to NO (0.5 mM NONOate) under anaerobic conditions, followed by repair using the iron-sulfur cluster repair system as described under the Experimental Procedures. A), UV-visible absorption spectra of DinG. Spectrum 1, purified DinG before the NO exposure. Spectrum 2, purified DinG after the NO exposure. Spectrum 3, the NO-exposed DinG was repaired with the iron-sulfur cluster repair system. The protein concentrations of DinG were about 5 μM. B), EPR spectra of DinG. Spectrum 1, purified DinG before the NO exposure. Spectrum 2, purified DinG after the NO exposure. Spectrum 3, the NO-exposed DinG was repaired with the iron-sulfur cluster assembly system. The protein concentrations of DinG were about 5 μM. C), reversible inactivation of DinG by NO. Two concentrations of DinG (50 nM and 100 nM) were used for the helicase activity assay. Lane H, the sample was heated at 85°C for 5 min. Lane 0, no enzyme was added. DinG, purified DinG. DinG-NO, purified DinG after the NO exposure. DinG-NO repaired, the NO-exposed DinG was repaired and incubated with the iron-sulfur cluster repair system. The reaction product (single-stranded DNA) was separated by the agarose gel (1%) electrophoresis as described under the Experimental Procedures.
Figure 1
Figure 2

A

DinG C120S
DinG C194S
DinG C199S
DinG C205S

B

C

H 0 1 2 3 4 5

Figure 2
Figure 3

A

1) As-purified DinG
2) DinG + dithionite
3) Re-oxidized DinG

Wavelength (nm)

O.D.

300 400 500 600 700

403 nm

B

Relative amount of oxidized DinG [4Fe-4S] cluster (%)

Eh (mV)

-500 -400 -300 -200

C

Magnetic field (mT)

300 400

D

Relative amount of oxidized DinG [4Fe-4S] cluster (%)

Eh (mV)

-500 -400 -300 -200

Figure 3
Figure 4
Figure 5
Figure 6
Redox control of the DNA damage-inducible protein ding helicase activity via its iron-sulfur cluster
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