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Nitric Oxide-Induced Bacteriostasis and Modification of Iron-Sulfur Proteins in *Escherichia coli*

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Summary

The nitric oxide (NO) cytotoxicity has been well documented in bacteria and mammalian cells. However, the underlying mechanism is still not fully understood. Here we report that transient NO exposure effectively inhibits cell growth of *Escherichia coli* in minimal medium under anaerobic growth conditions, and that cell growth is restored when the NO-exposed cells are either supplemented with the branched-chain amino acids (BCAA) anaerobically or returned to aerobic growth conditions. The enzyme activity measurements show that dihydroxyacid dehydratase (IlvD), an iron-sulfur enzyme essential for the BCAA biosynthesis, is completely inactivated in cells by NO with the concomitant formation of the IlvD-bound dinitrosyl iron complex (DNIC). Fractionation of the cell extracts prepared from the NO-exposed cells reveals that a large number of different protein-bound DNICs are formed by NO. While the IlvD-bound DNIC and other protein-bound DNICs are stable in cells under anaerobic growth conditions, they are efficiently repaired under aerobic growth conditions even without new protein synthesis. Additional studies indicate that L-cysteine may have an important role in repairing the NO-modified iron-sulfur proteins in aerobically growing *E. coli* cells. The results suggest that cellular deficiency to repair the NO-modified iron-sulfur proteins may directly contribute to the NO-induced bacteriostasis under anaerobic conditions.

Keywords

nitric oxide; bacteriostasis; iron-sulfur proteins; dinitrosyl iron complex

Introduction

As a neutral free radical, nitric oxide (NO) can readily penetrate cell membranes and act on a number of cellular components. At low concentrations (nM), NO is a signaling molecule for intercellular communications in neuronal and cardiovascular tissues (Ignarro, 1999). At high concentrations (μM), NO becomes a powerful weapon to kill pathogenic bacteria and tumor cells (Gobert *et al.*, 2001; Krieglstein *et al.*, 2001; MacMicking *et al.*, 1997). In response to NO stress, bacteria have developed a number of defense mechanisms (Spiro, 2007). In *Escherichia coli*, there are at least three enzymes that can directly metabolize NO: a flavorubredoxin/flavodiiron NO reductase capable of reducing NO to nitrous oxide in the absence of oxygen (D'Autreaux *et al.*, 2005; Gardner *et al.*, 2002; Gomes *et al.*, 2002), a flavohemoglobin NO dioxygenase that catalyzes oxidation of NO to nitrate in the presence of O_2 (Bodenmiller and Spiro, 2006; Gardner and Gardner, 2002; Poole and Hughes, 2000), and a pentaheme nitrite reductase that has a NO reductase activity (Poock *et al.*, 2002). In addition, a diiron protein encoded by gene *ytfE* has been shown to be critical for the cellular defense against NO (Justino *et al.*, 2007). Deletion of any of these genes significantly increases the

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sensitivity of *E. coli* cells to NO under aerobic or anaerobic conditions. However, all these genes together are still not sufficient to protect *E. coli* from the NO cytotoxicity (Hyduke *et al.*, 2007; Justino *et al.*, 2005; Mukhopadhyay *et al.*, 2004; Pullan *et al.*, 2007).

Increasing evidence has suggested that proteins containing iron-sulfur clusters or mononuclear iron centers are susceptible to NO (Spiro, 2007). So far, over 200 unique iron-sulfur proteins have been identified in diverse physiological processes such as energy conversion, sugar metabolism, heme and biotin biosynthesis, amino acids biosynthesis, RNA modification, DNA synthesis and repair, and the regulation of gene expression (Johnson *et al.*, 2005; Lill and Muhlenhoff, 2006). In vitro studies showed that purified iron-sulfur proteins can be readily modified by NO forming the protein-bound dinitrosyl iron complexes (DNICs) which have a unique electron paramagnetic resonance (EPR) signal at $g = 2.04$ (Cruz-Ramos *et al.*, 2002; Ding and Demple, 2000; Drapier, 1997; Foster and Cowan, 1999; Kennedy *et al.*, 1997; Rogers *et al.*, 2003). The in vivo studies also indicated that iron-sulfur proteins such as the aconitase [4Fe-4S] clusters (Gardner *et al.*, 1997), the ferredoxin [2Fe-2S] clusters (Rogers and Ding, 2001), the endonuclease III [4Fe-4S] clusters (Rogers *et al.*, 2003), the redox transcription factor SoxR [2Fe-2S] clusters (Ding and Demple, 2000), the anaerobic growth factor FNR [4Fe-4S] clusters (Cruz-Ramos *et al.*, 2002), the dihydroxyacid dehydratase (IlvD) [4Fe-4S] clusters (Hyduke *et al.*, 2007), and a number of other dehydratase [4Fe-4S] clusters (Woodmansee and Imlay, 2003) are highly sensitive to NO. Additional evidence came from the micro-array gene profiling experiments which revealed that NO exposure drastically elevates the expression of the genes involved in the assembly and/or repair of iron-sulfur clusters in *E. coli* cells (Hyduke *et al.*, 2007; Justino *et al.*, 2005; Mukhopadhyay *et al.*, 2004; Pullan *et al.*, 2007). Collectively, these results suggested that iron-sulfur proteins may represent one of the primary targets of the NO cytotoxicity (Spiro, 2007). Nevertheless, the physiological relevance of the NO-mediated modifications of iron-sulfur proteins and the NO cytotoxicity has still not been fully addressed.

Here, we have explored the NO-induced bacteriostasis and modification of iron-sulfur proteins in *E. coli*. The results show that transient NO exposure effectively inhibits cell growth of *E. coli* in minimal medium under anaerobic growth conditions. However, cell growth is restored when the NO-exposed *E. coli* cells are either supplemented with the branched-chain amino acids (BCAA) anaerobically or returned to aerobic growth conditions. Consistent with the studies by Hyduke et al. (Hyduke *et al.*, 2007), we find that dihydroxyacid dehydratase (IlvD), an iron-sulfur enzyme essential for the BCAA biosynthesis in *E. coli* (Flint *et al.*, 1993), is a primary target of the NO cytotoxicity. The enzyme activity measurements in the cell extracts show that IlvD is completely inactivated by NO with the concomitant formation of the IlvD-bound DNIC in *E. coli* cells. Furthermore, we discover that the NO-inactivated IlvD remains inactive in *E. coli* cells under anaerobic growth conditions but is efficiently repaired under aerobic growth conditions even without new protein synthesis. We propose that intracellular small molecules may directly contribute to the robust repair activity for the NO-modified iron-sulfur proteins in *E. coli* under aerobic growth conditions. Additional studies reveal that L-cysteine may have an important role in repairing the NO-modified iron-sulfur proteins in aerobically growing *E. coli* cells. The results led us to suggest that NO may become a potent bacteriostat under anaerobic conditions under which bacterial cells fail to repair the NO-modified iron-sulfur proteins.

Results

NO-induced bacteriostasis of E. coli under anaerobic growth conditions

To explore the NO cytotoxicity in bacteria, we adapted the Silastic tubing NO delivery system (Tamir *et al.*, 1993). The system allowed us to modulate a reproducible NO releasing by changing the length of the Silastic tubing immersed in the cell culture. Throughout the study,

a releasing rate of about 100 nM NO per second was used to emulate the pathophysiological NO productions as reported by others (Gobert *et al.*, 2001; Krieglstein *et al.*, 2001). Typically, exponentially growing *E. coli* cells were purged with pure argon gas before being subjected to the NO exposure. After NO exposure for 10 min, *E. coli* cells were re-purged with pure argon gas to remove residual NO. The NO-exposed *E. coli* cells were then divided into two flasks, one to aerobic growth conditions and the other to anaerobic growth conditions. Figure 1A shows that the NO exposure effectively inhibited cell growth of *E. coli* in minimal medium under anaerobic growth conditions. On the other hand, when the NO-exposed *E. coli* cells were returned to aerobic growth conditions (Figure 1B), cell growth was restored with only a short delay as reported previously by Hyduke et al. (Hyduke *et al.*, 2007).

The recovery of the NO-exposed *E. coli* cells under aerobic growth condition (Figure 1B) suggests that the NO exposure may have limited the production of some intermediates essential for cell growth under anaerobic growth conditions. Hyduke et al. (Hyduke *et al.*, 2007) has suggested that the NO exposure may create a transient deficiency of the branched-chain amino acids (BCAA) in *E. coli* cells under aerobic growth conditions, thus contributing to a short delay of cell growth. To test whether the NO exposure results in a prolonged BCAA auxotrophy of *E. coli* under anaerobic growth conditions, we supplemented the minimal medium with various components and found that addition of BCAA (leucine, isoleucine and valine) largely alleviated the NO-induced inhibition of cell growth under anaerobic growth conditions (Figure 1C). In control, addition of the non-branched-chain amino acids (glycine, lysine and alanine) failed to restore cell growth of the NO-exposed *E. coli* cells under anaerobic growth conditions (Figure 1C). Thus, the results indicated that the NO exposure may have created the BCAA auxotrophy of *E. coli* under anaerobic growth conditions.

Dihydroxyacid dehydratase (IlvD) in E. coli cells is inactivated by NO

The BCAA auxotrophy is a hallmark of oxidative damage to iron-sulfur clusters in *E. coli* (Jang and Imlay, 2007) and in *Saccharomyces cerevisiae* (Wallace *et al.*, 2004). The BCAA biosynthesis pathway in *E. coli* contains two iron-sulfur enzymes: dihydroxyacid dehydratase (IlvD) (Flint *et al.*, 1993) and isopropylmalate isomerase (LeuCD) (Jang and Imlay, 2007). IlvD catalyzes the conversion from 2,3-dihydroxy-isovalerate to 2-keto-isovalerate (Flint *et al.*, 1993), while LeuCD catalyzes the conversion from 2-isopropylmalate to 3-isopropylmalate (Jang and Imlay, 2007). Both IlvD and LeuCD require an intact [4Fe-4S] cluster to act as a Lewis acid for the catalytic function. Because LeuCD is a two-subunit enzyme that dissociates during purification (Jang and Imlay, 2007), we chose IlvD for further investigation.

To facilitate the rapid enzyme activity assay in the cell extracts, we expressed recombinant IlvD to about 2% of total intracellular proteins in *E. coli* cells. Figure 2A shows that when *E. coli* cells containing recombinant IlvD were subjected to the NO exposure at a rate of 100 nM NO per second anaerobically, the enzyme activity of IlvD in cells was quickly inactivated, with over 80% of the IlvD activity was eliminated after 5 min NO exposure (Figure 2B). It is worth mentioning that the enzyme activity of IlvD in *E. coli* cells was completely inactivated after 10 min NO exposure using the Silastic tubing NO delivery system (Figure 2A), whereas at least 50% of the IlvD activity still remained when *E. coli* cells were exposed to the NO-releasing reagent DeaNO (8 μM) (Hyduke *et al.*, 2007), indicating that the Silastic tubing NO delivery system may be more effective than the NO bolus exposure in treating bacterial cells.

It has been shown that iron-sulfur proteins can be readily modified forming the protein-bound dinitrosyl iron complexes (DNICs) by NO in vitro and in vivo (Cruz-Ramos *et al.*, 2002; Ding and Demple, 2000; Drapier, 1997; Foster and Cowan, 1999; Kennedy *et al.*, 1997; Rogers *et al.*, 2003). To examine whether the IlvD [4Fe-4S] cluster was also modified forming the IlvD-bound DNIC by the NO exposure, we purified recombinant IlvD from *E. coli* cells before and after the NO exposure. The unique EPR signal at $g = 2.04$ (Figure 2C) indicated that the IlvD

[4Fe-4S] cluster was indeed converted to the IlvD-bound DNIC in vivo by the NO exposure. The EPR signal of the IlvD-bound DNIC purified from the NO-exposed *E. coli* cells was essentially identical to that of the purified IlvD [4Fe-4S] cluster directly exposed to NO in vitro (Figure 2C), suggesting that formation of the IlvD-bound DNIC in *E. coli* cells by NO does not require any additional factors. As the NO exposure time of *E. coli* cells was gradually increased, we found that the inactivation of IlvD in *E. coli* cells was inversely correlated with the formation of the IlvD-bound DNIC (data not shown). Collectively, these results suggest that NO inactivates IlvD by converting the IlvD [4Fe-4S] cluster to the IlvD-bound DNIC in *E. coli* cells.

Formation of the protein-bound DNICs in E. coli cells by NO

There are at least 200 unique iron-sulfur proteins in *E. coli* (Johnson *et al.*, 2005). It is conceivable that other iron-sulfur proteins in cells may also be modified forming the protein-bound DNICs by the NO exposure. Figure 3A shows the EPR spectra of the wild-type *E. coli* cells (without recombinant IlvD) before and after the NO exposure under anaerobic conditions. The EPR signal at $g = 2.04$ indicated the formation of the protein-bound DNICs in *E. coli* cells by the NO exposure. The amplitude of the EPR signal at $g = 2.04$ reached the maximum when *E. coli* cells were exposed to NO for about 5 min under anaerobic conditions (Figure 3B). Further exposure of NO did not significantly increase the amplitude of the EPR signal at $g = 2.04$, indicating that modification of iron-sulfur proteins in *E. coli* cells by NO was nearly complete after 5 min NO exposure.

The cell extracts were then prepared from the NO-exposed *E. coli* cells and subjected to the EPR measurements. Over 80% of the EPR signal at $g = 2.04$ remained after the NO-exposed *E. coli* cells were disrupted by passing through French press (Figure 3A), supporting the idea that the protein-bound DNICs are stable in the cell extracts (Rogers and Ding, 2001). The cell extracts were further fractionated using a gel filtration column (Superdex-200) (Amersham Biosciences) as described in the Experimental Procedures. The EPR measurements of the eluted fractions showed that the protein-bound DNICs were present in almost all protein fractions (Figure 4A). Whereas the ratio of the protein-bound DNICs to the protein concentration in each fraction varied considerably (Figure 4B), the total amount of the protein-bound DNICs in the cell extracts was fully recovered in the eluted fractions. Thus, in addition to the IlvD-bound DNIC, a large number of different protein-bound DNICs were generated in *E. coli* cells by the NO exposure.

The protein-bound DNICs are efficiently repaired in E. coli cells under aerobic growth conditions but not under anaerobic growth conditions

A broad distribution of the protein-bound DNICs in the NO-exposed *E. coli* cells suggests that multiple cellular functions could have been inactivated by the NO exposure. If cells are to survive, the protein-bound DNICs must be promptly repaired. Figure 5 shows that when the NO-exposed *E. coli* cells were returned to aerobic growth conditions, the EPR signal at $g = 2.04$ of the protein-bound DNICs quickly disappeared with a half-life time of about 5 min. The efficient repair of the protein-bound DNICs in aerobically growing *E. coli* cells did not require new protein synthesis, as addition of the protein synthesis inhibitor chloramphenicol had little or no effect on decay kinetics of the protein-bound DNICs in aerobically growing *E. coli* cells (data not shown). In contrast, when the NO-exposed *E. coli* cells were returned to anaerobic growth conditions, at least 75% of the protein-bound DNICs in *E. coli* cells remained after two hours incubation, indicating that anaerobically growing *E. coli* cells fail to repair the protein-bound DNICs.

The NO-inactivated IlvD is quickly re-activated in E. coli cells under aerobic growth conditions but not under anaerobic growth conditions

To further examine whether the NO-modified iron-sulfur proteins are repaired in *E. coli* cells under aerobic growth conditions but not under anaerobic growth conditions, we measured the enzyme activity of IlvD in the cell extracts as described previously. Figure 6A shows that when the NO-exposed *E. coli* cells were returned to aerobic growth conditions, the NO-inactivated IlvD was quickly re-activated even in the presence of the protein synthesis inhibitor chloramphenicol. On the other hand, when the NO-exposed *E. coli* cells were returned to anaerobic growth conditions, the NO-inactivated IlvD remained inactive for at least two hours. Combining the results from the EPR spectra (Figure 5) and the enzyme activity measurements of IlvD in the cell extracts (Figure 6A), we concluded that *E. coli* cells can efficiently repair the NO-modified iron-sulfur proteins under aerobic growth conditions but not under anaerobic growth conditions, and that such a robust repair activity does not require new protein synthesis.

Since the NO-inactivated IlvD was efficiently repaired in aerobically growing *E. coli* cells without new protein synthesis (Figure 6A), we postulated that re-assembly of iron-sulfur clusters may be sufficient for restoring the enzyme activity of the NO-inactivated IlvD in the cell extracts. In the experiments, the cell extracts prepared from the NO-exposed *E. coli* cells were incubated with L-cysteine, cysteine desulfurase IscS and ferrous iron in the presence of dithiothreitol at 37°C anaerobically, a system that was previously used for repairing the NO-modified iron-sulfur proteins (Rogers *et al.*, 2003; Yang *et al.*, 2002). As shown in Figure 6B, the NO-inactivated IlvD in the cell extracts was quickly re-activated after incubation with the iron-sulfur cluster repair system. About 70% of the IlvD enzyme activity in the NO-exposed cell extracts (relative to the untreated *E. coli* cells) was recovered after 30 min incubation. Missing any one of the components in the iron-sulfur cluster repair system failed to restore the enzyme activity of the NO-modified IlvD in the cell extracts (data not shown). The results demonstrated that reassembly of iron-sulfur clusters in the NO-modified IlvD is necessary and sufficient for reactivation of the NO-inactivated IlvD in the NO-exposed *E. coli* cells.

Potential role of L-cysteine in repairing the protein-bound DNICs in E. coli

Because aerobically growing *E. coli* cells can efficiently repair the NO-inactivated IlvD without new protein synthesis (Figure 5 and Figure 6A), we reasoned that intracellular small molecules, but not specific proteins, may be directly responsible for the robust repair activity for the NO-modified iron-sulfur proteins observed in aerobically growing *E. coli* cells. Previous studies have shown that L-cysteine has its unique activity in decomposing the ferredoxin-bound DNIC in vitro (Rogers and Ding, 2001), and is also the substrate for cysteine desulfurase IscS (Zheng *et al.*, 1998) to provide sulfide for the re-assembly of iron-sulfur clusters in the NO-modified proteins (Figure 6B) (Rogers *et al.*, 2003;Yang *et al.*, 2002). Conceivably, L-cysteine may have an important role in repairing the NO-modified proteins in *E. coli* cells.

To test the repair activity of L-cysteine in decomposing the IlvD-bound DNIC, we prepared the cell extracts from the *E. coli* cells containing either an expression vector or recombinant IlvD before and after the NO exposure. As shown in Figure 7A, the EPR signal at $g = 2.04$ of the cell extracts prepared from the NO-exposed *E. coli* cells without recombinant IlvD was completely eliminated after incubation with L-cysteine, but not with reduced glutathione or *N*-acetyl-L-cysteine. When the *E. coli* cells containing recombinant IlvD were subjected to the same NO exposure, the amplitude of the EPR signal at $g = 2.04$ was about 3-fold higher than that of the cells without recombinant IlvD (Figure 7B), further demonstrating the formation of the IlvD-bound DNIC in *E. coli* cells by NO. Again, the IlvD-bound DNIC in the cell extracts was efficiently decomposed by L-cysteine, but not by reduced glutathione or *N*-acetyl-L-cysteine (Figure 7B). We also incubated the purified IlvD-bound DNIC with L-cysteine, and found that L-cysteine was equally effective in decomposing the IlvD-bound DNIC (Figure 7C).

While the mechanism underlying the L-cysteine-mediated decomposition of the protein-bound DNICs is still not well understood, these results suggested that L-cysteine may directly contribute to the robust repair activity for the protein-bound DNICs in aerobically growing *E. coli* cells. The physiological role of intracellular L-cysteine in repairing the NO-modified iron-sulfur proteins are currently under investigation.

Discussion

In this study, we report that transient NO exposure effectively inhibits cell growth of *E. coli* in minimal medium under anaerobic growth conditions, and that cell growth is restored when the NO-exposed *E. coli* cells are either supplemented with the branched-chain amino acids (BCAA) anaerobically or returned to aerobic growth conditions. The enzyme activity measurements reveal that dihydroxyacid dehydratase (IlvD), an iron-sulfur enzyme essential for the BCAA biosynthesis in *E. coli* (Flint *et al.*, 1993), is completely inactivated by the NO exposure with the concomitant formation of the IlvD-bound dinitrosyl iron complex (DNIC). Furthermore, we find that the NO-inactivated IlvD remains inactive in *E. coli* cells under anaerobic growth conditions, but is quickly re-activated under aerobic growth conditions even without new protein synthesis. The results not only substantiate the notion that IlvD is a sensitive target of the NO cytotoxicity (Hyduke *et al.*, 2007), but also demonstrate that NO can effectively inhibit cell growth of *E. coli* under anaerobic growth conditions under which the NO-modified IlvD cannot be efficiently repaired.

Unlike reversible binding of NO to hemes in proteins (Ramachandran *et al.*, 2002), the NO-mediated modification of iron-sulfur clusters is irreversible, associated with formation of the protein-bound DNIC (Cruz-Ramos *et al.*, 2002; Ding and Demple, 2000; Drapier, 1997; Foster and Cowan, 1999; Kennedy *et al.*, 1997; Rogers *et al.*, 2003). Re-activation of the NO-modified iron-sulfur proteins requires decomposition of the DNIC in proteins, followed by re-assembly of new iron-sulfur clusters (Rogers *et al.*, 2003). A number of proteins including cysteine desulfurase IscS (Yang *et al.*, 2002) and an diiron protein YtfE (Justino *et al.*, 2007) have been identified as critical for repairing the NO-modified iron-sulfur proteins. Both YtfE and IscS are highly induced in *E. coli* cells by NO exposure (Hyduke *et al.*, 2007; Justino *et al.*, 2005; Mukhopadhyay *et al.*, 2004; Pullan *et al.*, 2007). It is appealing to suggest that increased amounts of repair proteins such as IscS and YtfE may contribute to the robust repair activity for the NO-modified iron-sulfur proteins in aerobically growing *E. coli* cells. However, the efficient repair for the NO-modified iron-sulfur proteins in aerobically growing *E. coli* cells does not require new protein synthesis (Figure 5 and Figure 6A), suggesting that amounts of the specific repair enzymes are not the critical factors in determining the cellular repair activity for the NO-modified iron-sulfur proteins. In this context, we postulate that the amounts of intracellular small molecule(s) may be responsible for the robust repair reactivity in aerobically growing *E. coli* cells. We envision that the putative small molecules (e.g. ATP, L-cysteine, NADPH/NADH) are amply produced for repairing the NO-modified iron-sulfur proteins in *E. coli* cells under aerobic growth conditions, but not under anaerobic growth conditions. Because L-cysteine can decompose the protein-bound DNICs (Rogers and Ding, 2001) (Figure 7) and act as substrate for cysteine desulfurase IscS to provide sulfide for the iron-sulfur cluster re-assembly in proteins (Yang *et al.*, 2002) (Figure 6B), we propose that L-cysteine may directly contribute to the robust repair activity for the NO-modified iron-sulfur proteins in aerobically growing *E. coli* cells. Nevertheless, it could not be excluded that other small molecules may also have their crucial roles in the cellular repair activity for the protein-bound DNICs in cells. Experiments are in progress to explore the physiological function of L-cysteine in repairing the NO-modified iron-sulfur proteins in *E. coli* cells.

Iron-sulfur proteins are involved in diverse physiological processes with over 200 unique iron-sulfur proteins identified so far (Johnson *et al.*, 2005; Lill and Muhlenhoff, 2006). The gel

filtration fractionation of the cell extracts prepared from the NO-exposed *E. coli* cells (Figure 4) clearly demonstrates that a large number of different iron-sulfur proteins are modified forming the protein-bound DNICs in cells by NO. The results provide additional evidence that iron-sulfur proteins are the primary targets of the NO cytotoxicity (Spiro, 2007). It should be pointed out that under defined experimental conditions, some iron-sulfur proteins are not essential. For example, because the aconitase [4Fe-4S] cluster (Gardner *et al.*, 1997) of the citrate acid cycle is completely dispensable for anaerobic growth of *E. coli*, modification of the aconitase [4Fe-4S] cluster by NO would have no phenotype under anaerobic growth conditions. In contrast, because BCAA are required for cell growth of *E. coli* in minimal medium under aerobic or anaerobic growth conditions, inactivation of IlvD by NO would create the BCAA auxotrophy in *E. coli*. However, since the NO-inactivated IlvD is efficiently repaired in *E. coli* cells under aerobic growth conditions (Figure 6A), the NO exposure only induces a transitory depletion of BCAA and a brief delay of cell growth (Hyduke *et al.*, 2007) (Figure 1). In contrast, the NO-inactivated IlvD remains inactive in *E. coli* cells under anaerobic growth conditions due to the deficient repair activity for the NO-inactivated IlvD (Figure 6A), and no BCAA are synthesized to support cell growth. A proposed model for the NO-induced bacteriostasis of *E. coli* under aerobic and anaerobic conditions is summarized in the Supplemental Figure 1.

Experimental Procedures

Cell growth and NO exposure

Overnight wild-type *E. coli* cells (GC4468) were diluted 1:100 in freshly prepared Luria-Bertani (LB) medium and incubated at 37°C with aeration (250 rpm) for 2.5 hours. For some experiments, overnight *E. coli* cells were grown in freshly prepared minimal medium containing 0.2% glucose. *E. coli* cells prepared from LB medium or minimal medium had similar response to the NO exposure in general under the experimental conditions. After harvested and washed once with minimal medium, cells were re-suspended in minimal medium containing 0.2% glucose. For the NO exposure, the Silastic tubing NO delivery system was used according to (Tamir *et al.*, 1993). Briefly, pure NO gas (Air Co) was first passed through a soda-lima column to remove NO_2 and higher oxides of nitrogen before being connected to the Silastic tubing. The length of the Silastic tubing (I.D. × O.D.; 0.025 × 0.047 in.) immersed in the cell culture was adjusted in such that about 100 nM NO per second was released to the cell culture in a sealed flask. The Silastic tubing NO delivery system provided a reproducible NO exposure without significantly changing pH of the cell culture. The chosen NO release rate was comparable to the reported NO production in activated polymorphonuclear leukocytes (Krieglstein *et al.*, 2001) or in RAW 264.7 macrophages co-cultured with arginase-deficient *Helicobacter pylori* (Gobert *et al.*, 2001). Typically, exponentially growing *E. coli* cells were purged with pure argon gas before being subjected to NO exposure. After the NO exposure, residual NO gas in cell cultures was re-purged with pure argon gas. The NO-exposed *E. coli* cells were then divided into two flasks: one was returned to anaerobic growth conditions, and the other to aerobic growth conditions. Each flask (1L) contained 100 mL cell culture was incubated at 37°C with shaking of 250 rpm. When indicated, amino acids (at final concentrations of 100 μg/L) were added to the cell cultures anaerobically. *E. coli* cells not treated with NO were used as controls. The cell growth was monitored in a Klett-Summerson photoelectric colorimeter (Klett MFG. Co.).

Cloning of E. coli dihydroxyacid dehydratase (ilvD)

The DNA fragment encoding gene *ilvD* was amplified from wild-type *E. coli* genomic DNA using the Polymerase Chain Reaction (PCR). Two primers (IlvD-1, 5′-gacactgctagcaaataaagtatgcctaag-3′; IlvD-2 5′-ggttgcggctcagccattattaacccccca-3′) were synthesized to contain the *NheI* site in one primer and the *BlpI* site in the other. The PCR

product was digested with restriction enzymes *Nhe*I and *Blp*I, and the digested product was ligated to an expression vector pET28b$^+$ to produce pTIlvD. Recombinant IlvD was expressed to about 2% of total cellular protein in *E. coli* cells using isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 100 μM. In some experiments, recombinant IlvD was purified from the *E. coli* cell extracts using a Ni-agarose column followed by a HiTrap de-salting column (Amersham Biosciences) as described previously for other proteins (Rogers and Ding, 2001).

Enzyme activity assay of dihydroxyacid dehydratase (IlvD)

The cell extracts were prepared by passing the cells containing recombinant IlvD through French press once, followed by centrifugation at 34,000 g for 45 min. The specific enzyme activity was measured using substrate DL-2,3-dihydroxy-isovalerate which was synthesized according to the method of Cioffi et al. (Cioffi *et al.*, 1980). All chemical reagents used in the DL-2,3-dihydroxy-isovalerate synthesis were obtained from Sigma-Aldrich (St. Louis, MO). In the enzyme assay, 10 μL cell extracts (~5.0 mg total protein/mL) prepared from *E. coli* cells containing recombinant IlvD were added to 390 μL pre-incubated solutions containing 50 mM Tris (PH 8.0), 10 mM $MgCl_2$, and 10 mM DL-2,3-dihydroxy-isovalerate (Flint *et al.*, 1993). The reaction product (keto acids) was monitored at 240 nm using an extinction coefficient of 0.19 $cm^{-1}mM^{-1}$ (Flint *et al.*, 1993).

Re-assembly of iron-sulfur clusters in the NO-inactivated IlvD

The cell extracts prepared from the NO-exposed *E. coli* cells containing recombinant IlvD (~5.0 mg total protein/mL) were incubated with L-cysteine (0.5 mM), cysteine desulfurase IscS (2 μM), $Fe(NH_4)_2(SO_4)_2$ (100 μM), and dithiothreitol (2 mM) anaerobically at 37°C as described previously (Rogers *et al.*, 2003; Yang *et al.*, 2002). At different time points, aliquots (10 μL) of the reaction solution were taken for the IlvD enzyme activity assay as described above.

Fractionation of the cell extracts

The cell extracts were prepared from the NO-exposed *E. coli* cells by passing the cells through French press once, followed by centrifugation at 34,000 g for 45 min. The cell extracts were concentrated to about 40 mg protein/mL using the micro-concentrators (Millipore co.) and loaded onto a gel filtration column (Superdex-200) attached to a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences). The proteins were eluted from the column at a flow rate of 0.5 mL/min using a buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). The gel filtration column was calibrated using a gel filtration protein standard (Sigma co.) under the same experimental conditions. The protein concentration in each eluted fraction was determined by the Bradford assay (Bio-Rad co).

EPR measurements of the protein-bound DNICs

The X-band EPR spectra were recorded using a Bruker model ESR-300 EPR spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR measurement conditions were: microwave frequency, 9.50 GHz; microwave power, 2.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; sample temperature, 20 K; receive gain, 10^5. For quantification of the protein-bound DNICs, the *E. coli* endonuclease III-bound DNIC (Rogers *et al.*, 2003) was used as a standard. The concentration of the endonuclease III-bound DNICs was pre-determined as described previously (Rogers *et al.*, 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCAA

the branched-chain amino acids

DNIC

dinitrosyl iron complex

EPR
: electron paramagnetic resonance

IlvD
: dihydroxyacid dehydratase

LeuCD
: isopropylmalate isomerase

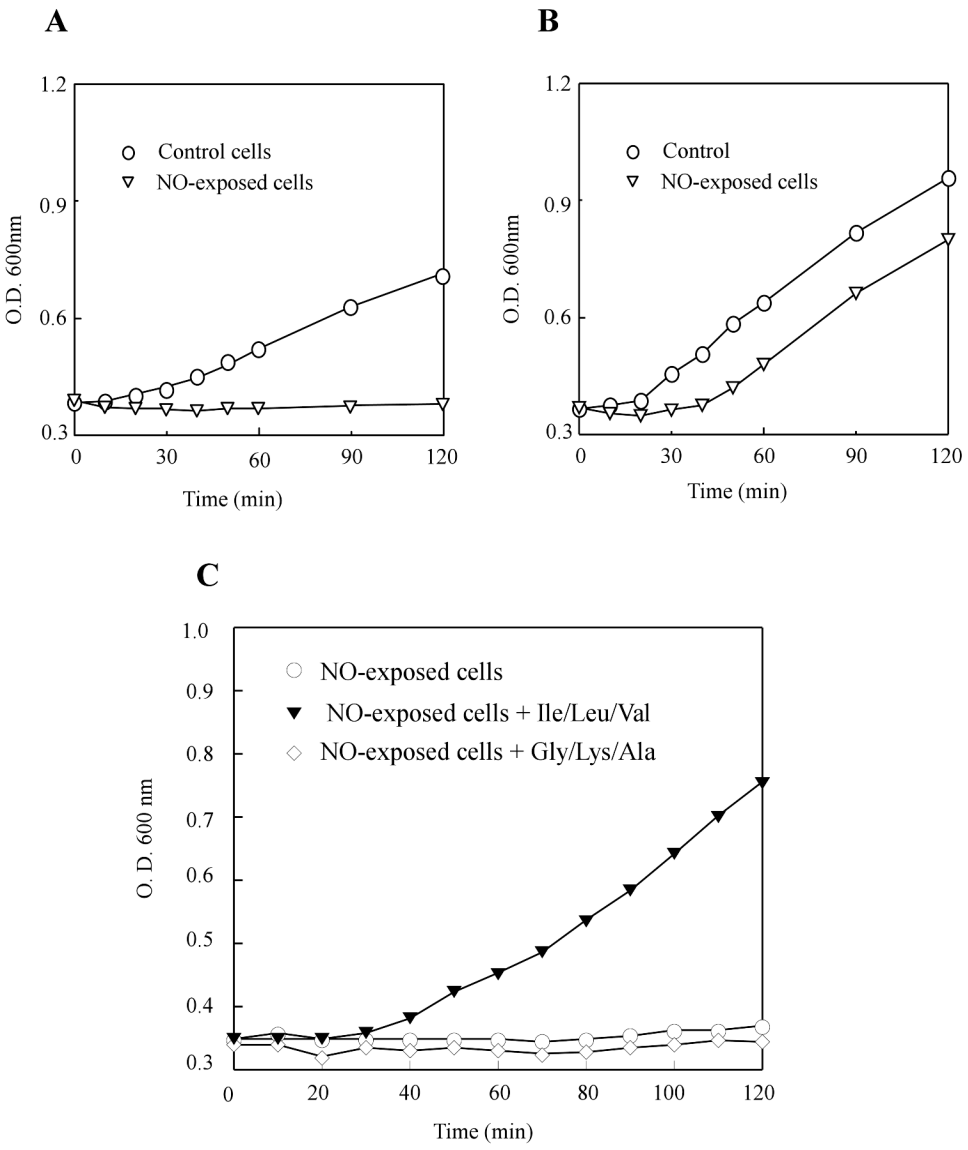

Figure 1. NO-induced bacteriostasis of *E. coli*

Exponentially growing *E. coli* cells were exposed to pure NO gas at a rate of ~ 100 nM per second for 10 min anaerobically, followed by purge with pure argon gas to remove residual NO in cell culture. The NO-exposed *E. coli* cells were returned to either **A**) anaerobic or **B**) aerobic growth conditions in minimal medium containing 0.2% glucose. Circles: untreated *E. coli* cells. Triangles: the NO-exposed *E. coli* cells. **C)**, the NO-exposed *E. coli* cells were returned to anaerobic growth conditions in minimal medium containing 0.2% glucose supplemented with three branched-chain amino acids (leucine, isoleucine, valine) (filled triangles), three non-branched-chain amino acids (glycine, alanine and lysine) (open diamonds) or an equal volume of de-gassed water (open circles) anaerobically. The final concentration for each amino acid in the minimal medium was 100 μg/ml. The cell growth was measured as described in the Experimental Procedures. The data are the representatives of three independent experiments.

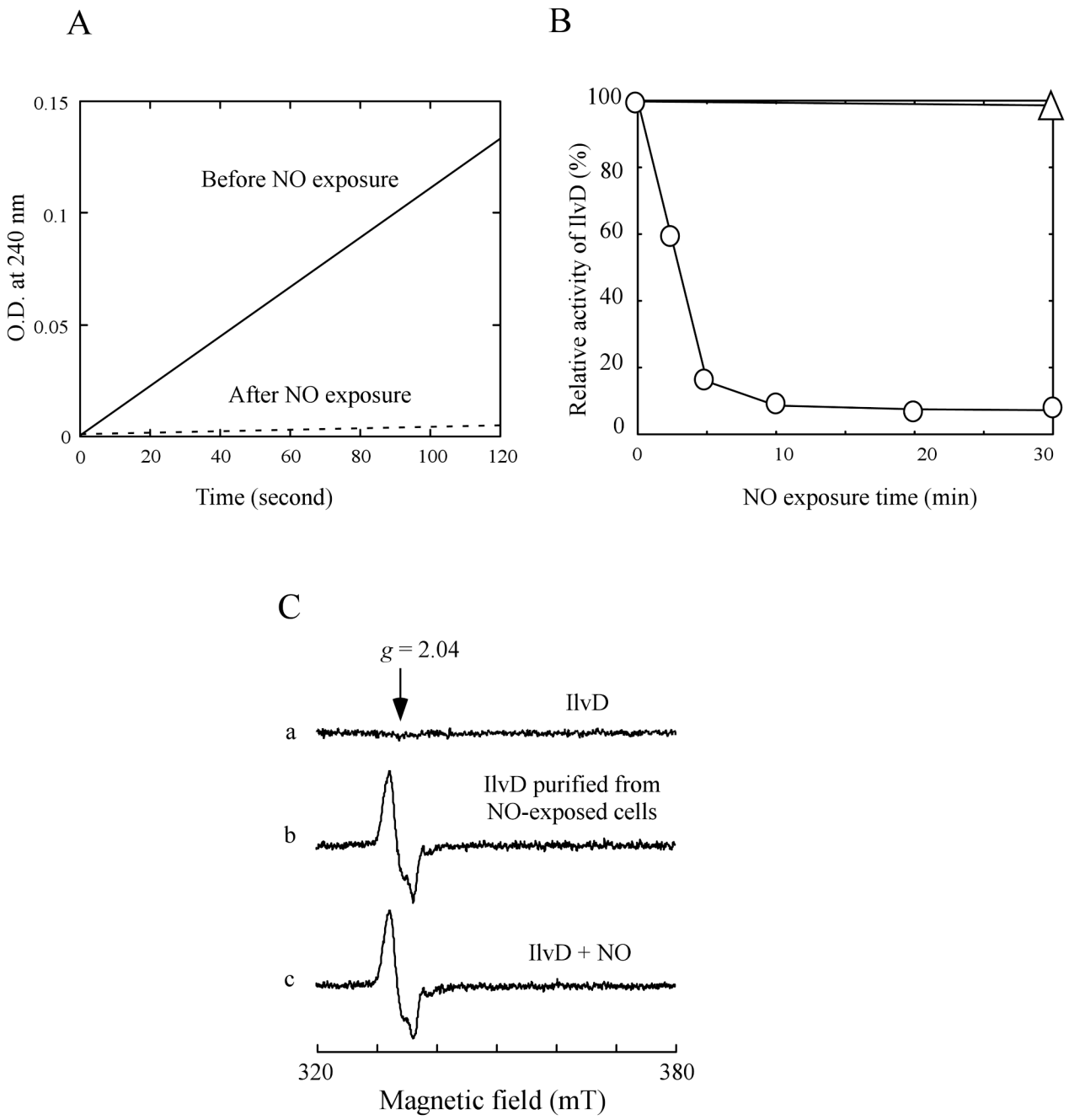

Figure 2. Inactivation of dihydroxyacid dehydratase (IlvD) in *E. coli* cells by NO

A), the enzyme activity of recombinant IlvD in *E. coli* cells before and after 10 min NO exposure. The cell extracts were prepared from the *E. coli* cells (concentrated to O.D. at 600 nm of 3.0) before and after the NO exposure. The reaction product (keto acids) catalyzed by IlvD was monitored at 240 nm as described in the Experimental Procedures. The enzyme activity of IlvD in untreated *E. coli* cells (100%) was ~11.3 μmol ketos formed per mg total cellular protein per min. **B)**, kinetics of the NO-mediated inactivation of IlvD in *E. coli* cells. Aliquots of the cell culture were taken at indicated time points during the NO exposure. The cell extracts were prepared from each aliquot. The enzyme activity of IlvD in the cell extracts

was immediately measured. The relative enzyme activity of IlvD in each cell extracts was plotted as a function of the NO exposure time (circles). IlvD remained fully active in the control *E. coli* cells after exposure to argon for 30 min (triangle). **C)**, EPR spectra of recombinant IlvD. Spectrum a), IlvD purified from the *E. coli* cells before the NO exposure. Spectrum b), IlvD purified from the *E. coli* cells after 10 min NO exposure. Spectrum c), purified IlvD directly exposed to NO under anaerobic conditions. The protein concentration in each sample was about 3 μM. The data are the representatives of at least three independent experiments.

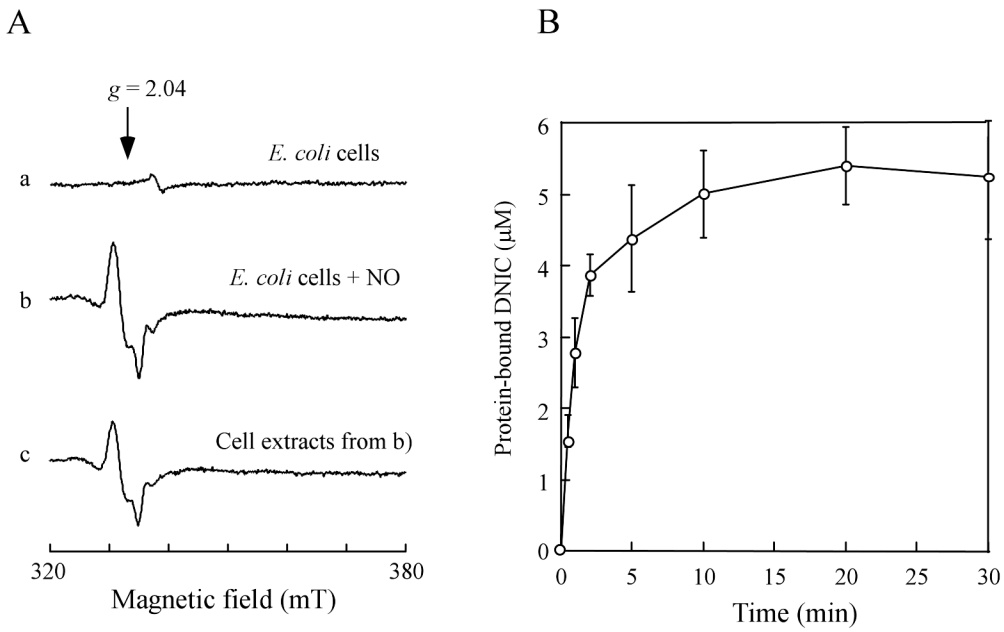

Figure 3. Formation of the protein-bound DNICs in *E. coli* cells by NO

A), formation of the protein-bound DNICs in *E. coli* cells by NO. *E. coli* cells were exposed to pure NO gas at a rate of 100 nM per second for 0 min (spectrum a) and 10 min (spectrum b) using the Silastic tubing NO delivery system under anaerobic conditions. The *E. coli* cells were concentrated to O. D. at 600 nm of 10.0 after the NO exposure. Spectrum c), the cell extracts prepared from the NO-exposed *E. coli* cells. **B)**, kinetics of the protein-bound DNICs formation in *E. coli* cells by NO. The amplitudes of the EPR signal at $g = 2.04$ in *E. coli* cells were plotted as a function of the NO exposure time. The endonuclease III-bound DNIC was used as a standard. The data in B) were the averages from three independent experiments.

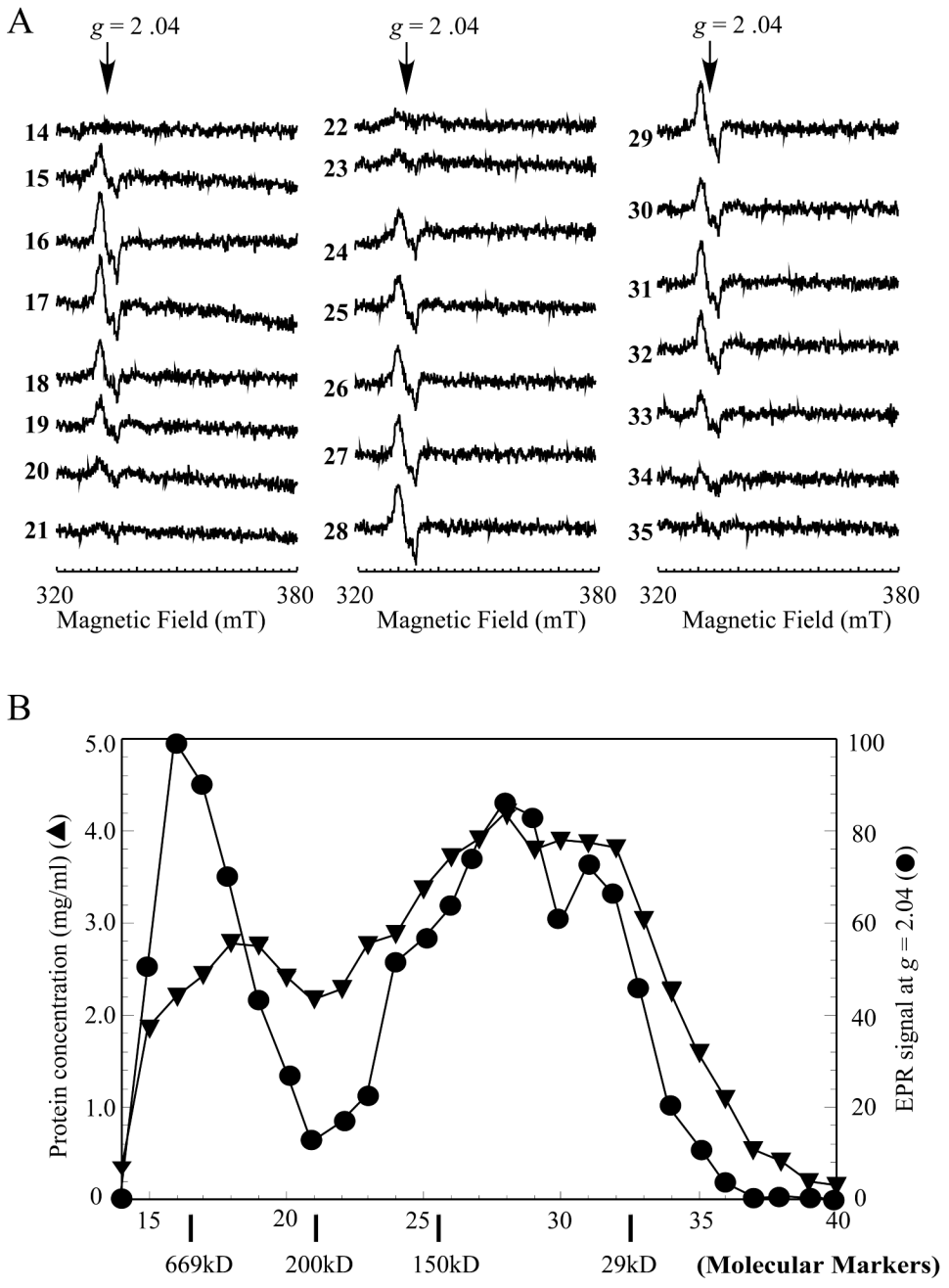

Figure 4. A broad distribution of the protein-bound DNICs in the NO-exposed *E. coli* cells

The cell extracts were prepared from the NO-exposed *E. coli* cells as described in the Experimental Procedures. The cell extracts (20 mg total protein in 0.5 ml) were loaded onto a gel filtration column (superdex-200), and eluted at a flow rate of 0.5 ml per min using buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). **A)**, EPR spectra of the fractions eluted from the gel filtration column. **B)**, the relative amplitude of the EPR signal at g = 2.04 (closed circles) and the protein concentration (mg/mL) (closed triangles) in each eluted fraction were plotted as a function of the fraction number. The molecular weights of the gel filtration protein standards are indicated on the bottom of x-axis.

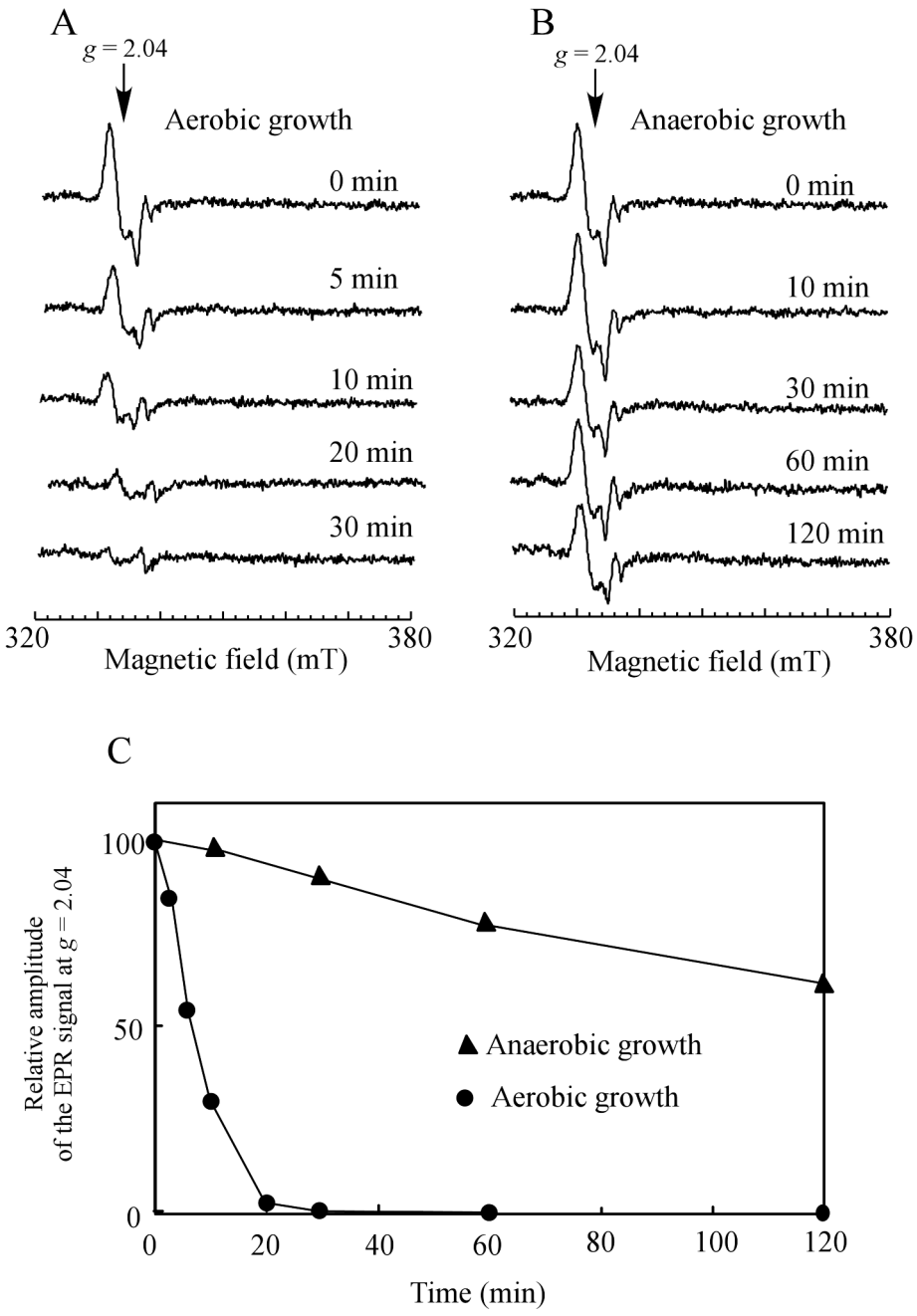

Figure 5. Aerobically growing *E. coli* cells have a robust repair activity for the protein-bound DNICs

Exponentially growing *E. coli* cells (concentrated to O.D. at 600 nm of 5.0) were exposed to pure NO gas at a rate of 100 nM per second for 10 min using the Silastic tubing NO delivery system anaerobically, followed by purge with pure argon gas. The NO-exposed cells were then returned to either aerobic or anaerobic growth conditions in minimal medium containing 0.2% glucose. **A)**, the EPR spectra of the NO-exposed *E. coli* cells at indicated time after returned to aerobic growth conditions. **B),** the EPR spectra of the NO-exposed *E. coli* cells at indicated time after returned to anaerobic growth conditions. **C),** decay kinetics of the EPR signal at *g*

= 2.04 of the NO-exposed *E. coli* cells under aerobic (closed circles) and anaerobic (closed triangles) growth conditions.

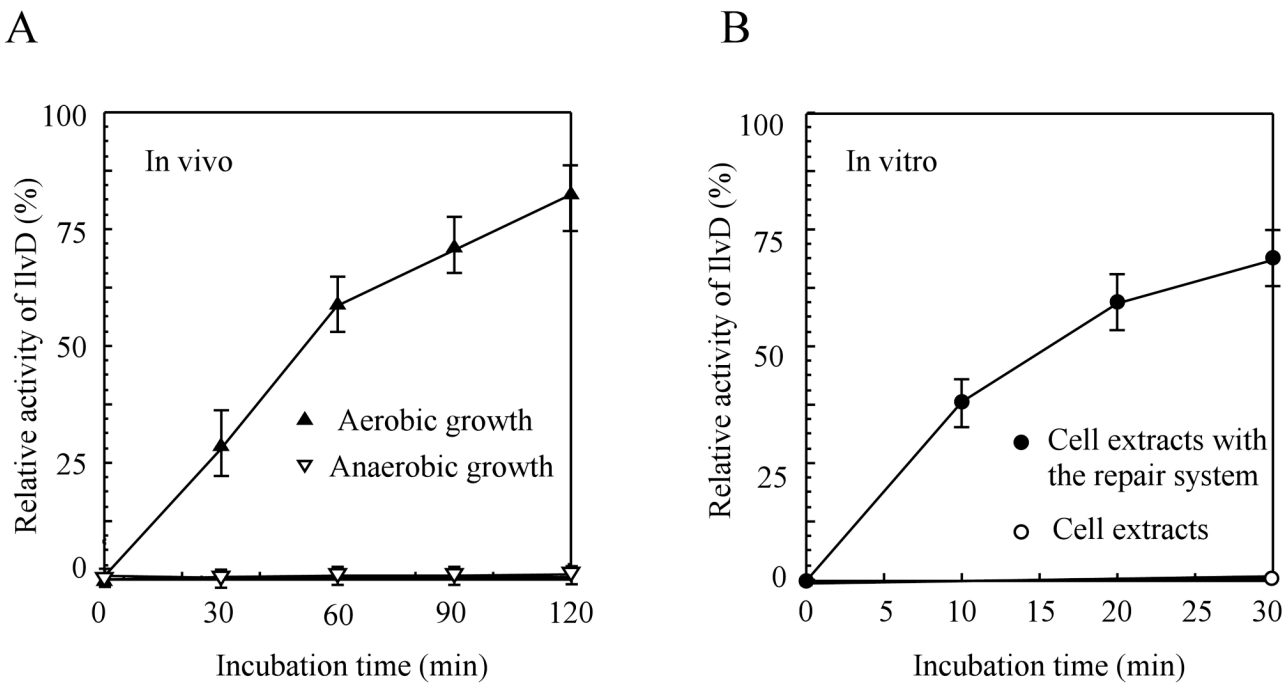

Figure 6. Re-activation of the NO-inactivated IlvD in *E. coli* cells under aerobic and anaerobic growth conditions

Recombinant IlvD was expressed to about 2% of total cellular protein in *E. coli* cells before being subjected to the NO exposure for 10 min. **A)**, re-activation of the NO-inactivated IlvD in *E. coli* cells under aerobic and anaerobic growth conditions. The enzyme activity of IlvD was measured in the cell extracts prepared from the NO-exposed *E. coli* cells after different incubation time under aerobic (filled triangles) or anaerobic (open triangles) growth conditions. Chloramphenicol (34 μg/mL) was added to the cell culture before the NO exposure to block any new protein synthesis. **B)**, re-activation of the NO-inactivated IlvD in the cell extracts by re-assembly of iron-sulfur clusters. The cell extracts prepared from the NO-exposed *E. coli* cells containing recombinant IlvD were incubated with L-cysteine (0.5 mM), cysteine desulfurase IscS (1 μM), and $Fe(NH_4)_2(SO_4)_2$ (100 μM) in the presence of dithiothreitol (2 mM) anaerobically. Samples were taken at different incubation time. Closed circles: the enzyme activity of IlvD in the cell extracts after incubation with the iron-sulfur cluster repair system. Open circles: the enzyme activity of IlvD in the cell extracts without any additions. The data are the averages from three independent experiments.

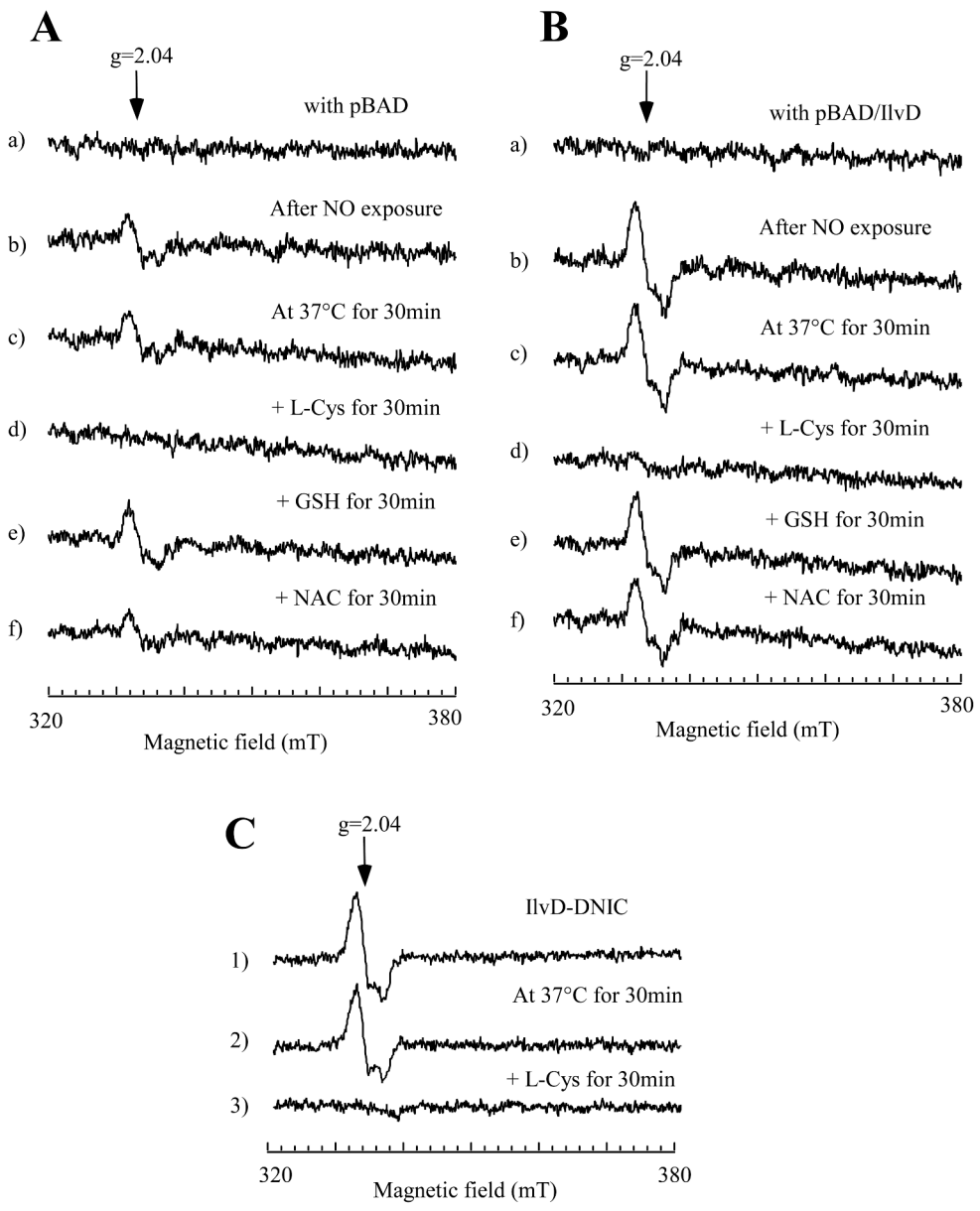

Figure 7. L-cysteine can decompose the protein-bound DNICs in the cell extracts

The cell extracts were prepared from the NO-exposed *E. coli* cells (concentrated to O. D. at 600 nm of 2.0) with the expression vector only **(A)** or with recombinant IlvD **(B)**. Recombinant IlvD was induced to about 2% of total cellular proteins in *E. coli* cells. Spectrum a), the cell extracts prepared from the *E. coli* cells before the NO exposure. Spectrum b), the cell extracts prepared from the *E. coli* cells after the NO exposure. The cell extracts prepared from the NO-exposed *E. coli* cells were incubated with no addition (c), or L-cysteine (L-Cys) (1 mM) (d), reduced glutathione (GSH) (1 mM) (e), or *N*-acetyl-L-cysteine (NAC) (1 mM) (f) at 37°C for 30 min. **C)**, purified IlvD-bound DNIC (5 μM) (1) was incubated with no addition (2) or L-cysteine (L-Cys) (1 mM) at 37°C for 30min. The samples were immediately transferred to EPR tubes after incubation. The EPR spectra were taken as described in the Experimental Procedures.