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Iron-sulfur Proteins Are the Major Source of Protein-bound Dinitrosyl Iron Complexes Formed in *Escherichia coli* Cells under Nitric Oxide Stress

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Abstract

Protein-bound dinitrosyl iron complexes (DNICs) have been observed in prokaryotic and eukaryotic cells under nitric oxide (NO) stress. The identity of proteins that bind DNICs, however, still remains elusive. Here we demonstrate that iron-sulfur proteins are the major source of protein-bound DNICs formed in *Escherichia coli* cells under NO stress. Expression of recombinant iron-sulfur proteins, but not the proteins without iron-sulfur clusters, almost doubles the amount of protein-bound DNICs formed in *E. coli* cells after NO exposure. Purification of recombinant proteins from the NO-exposed *E. coli* cells further confirms that iron-sulfur proteins, but not the proteins without iron-sulfur clusters, are modified forming protein-bound DNICs. Deletion of the iron-sulfur cluster assembly proteins IscA and SufA to block the [4Fe-4S] cluster biogenesis in *E. coli* cells largely eliminates the NO-mediated formation of protein-bound DNICs, suggesting that iron-sulfur clusters are mainly responsible for the NO-mediated formation of protein-bound DNICs in cells. Furthermore, depletion of “chelatable iron pool” in the wild-type *E. coli* cells effectively removes iron-sulfur clusters from proteins and concomitantly diminishes the NO-mediated formation of protein-bound DNICs, indicating that iron-sulfur clusters in proteins constitute at least part of “chelatable iron pool” in cells.

Keywords

nitric oxide; iron-sulfur clusters; chelatable iron pool; dinitrosyl iron complex

Introduction

NO (nitric oxide) produced from L-arginine by NO synthases [1] or from nitrite by chemical reduction [2] or nitrite reductases [3] has diverse physiological functions. At low concentrations (nM), NO acts as a signaling molecule for intercellular communication in neuronal and cardiovascular systems [4]. At high concentrations (μM), NO becomes a powerful weapon that kills pathogenic bacteria and cancerous cells [5]. Among cellular targets of NO cytotoxicity are a group of proteins that contain iron-sulfur clusters [6–14]. Iron-sulfur proteins are involved in a broad range of physiological processes from energy

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metabolism to DNA synthesis and repair [15, 16]. In vitro studies have shown that NO can directly react with iron-sulfur clusters [17, 18] forming protein-bound dinitrosyl iron complexes (DNICs) [19–27]. The NO-mediated formation of protein-bound DNICs has also been observed in bacterium *Clostridium botulinum* where NO is produced from nitrite reduction [28], in activated macrophages where NO is produced by inducible NO synthase [29, 30], in tumor cells co-cultured with activated macrophages [31], in the post-operative-day-4 allografts [32], in the gastro-oesophageal junction [11], and in cultured mammalian cells treated with various NO donors [33–36]. When *Escherichia coli* cells are exposed to pure NO gas at concentrations (μM) that emulate pathophysiological production of NO [37, 38], a large number of iron-sulfur proteins are modified forming protein-bound DNICs [12], suggesting that iron-sulfur proteins are one of primary targets of NO cytotoxicity [8, 10, 12].

NO can also react with small molecule thiols and ferrous iron to form thiol-bound DNICs which have a similar EPR (electron paramagnetic resonance) signal at $g = 2.04$ as protein-bound DNICs [39–41]. However, the existence of small molecule thiol-bound DNICs has never been demonstrated in any living cells [42, 43], likely because small molecule thiol-bound DNICs are not stable in aqueous solution (e.g. the half-life time of the cysteine-bound DNIC is less than 1 min) [27, 44] or are quickly exported by the multidrug resistance-associated protein-1 [34]. The only stable glutathione-bound DNICs in cells are found to be associated with glutathione *S*-transferases, a potential protection mechanism against excess NO [43]. More recently, it has been postulated that large molecular mass-bound DNICs, instead of small molecule thiol-bound DNICs, are formed in cultured mammalian cells under NO stress [35, 36]. However, the identities of large molecular mass-bound DNICs have not been defined [35, 36].

In this study, we combine molecular genetics and biophysics approaches and demonstrate that iron-sulfur proteins are the major source of large molecular mass-bound DNICs formed in *E. coli* cells under NO stress. Expression of recombinant iron-sulfur proteins almost doubles the amount of protein-bound DNICs formed in *E. coli* cells under NO stress. Deletion of the iron-sulfur cluster assembly proteins IscA and SufA to block the [4Fe-4S] cluster assembly [45, 46] largely eliminates the NO-mediated formation of protein-bound DNICs in *E. coli* cells, suggesting that iron-sulfur clusters, but not “free” iron, are mainly responsible for the NO-mediated formation of protein-bound DNICs. Furthermore, depletion of “chelatable iron pool” in the wild-type *E. coli* cells effectively removes iron-sulfur clusters from proteins and concomitantly diminishes the NO-mediated formation of protein-bound DNICs, suggesting that iron-sulfur clusters in proteins constitute at least part of “chelatable iron pool” in cells. The physiological relevance of the NO-mediated modification of iron-sulfur proteins will be discussed.

Materials and Methods

Gene cloning and protein purification

The genes encoding *E. coli* aconitase B [47], dihydroxyacid dehydratase (IlvD) [48], the redox transcription factor SoxR [22], the heat shock cognate protein HscA [49], and the single-stranded DNA-binding protein SSB [50] were cloned into pET28b+ (Novagen) or pBAD (Invitrogen) expression vectors as described previously [25]. Cloned genes were confirmed by direct sequencing (LSU Gene Lab). Recombinant proteins were expressed in *E. coli* strain BL21(DE3), MC4100, or the *iscA*⁻¹/*sufA*⁻¹ double mutant [45] cells, and the protein expression was confirmed by the SDS/PAGE analyses. The protein expression clones of *E. coli* ASKA library [51] were also used for the expression of IlvD, SoxR, HscA, and SSB. All protein purification solutions were purged with pure argon gas before use. Recombinant proteins were purified as described in [25], and the purity of purified proteins was over 95% judging from the SDS/PAGE analyses.

NO exposure of *E. coli* cells

Overnight *E. coli* cells containing protein expression plasmid were diluted 1:100 in freshly prepared LB (Luria-Bertani) medium and incubated at 37°C with aeration (250 rpm) for 3 hours, followed by protein induction with IPTG (100 μ M) or arabinose (0.02%) for additional 2–3 hours. For the *iscA*⁻¹/*sufA*⁻¹ double mutant, the cells were grown for 8 hours before protein induction with arabinose for additional 4 hours [45]. Both the wild-type and mutant cells were harvested and re-suspended in the M9 minimal medium to O.D. at 600 nm of 4.0–6.0. Chloramphenicol (34 μ g/mL) was added to the harvested *E. coli* cells to block new protein synthesis. Cells were then purged with pure argon gas before being treated with the NO-releasing reagent diethylamine NONOate (Cayman Chemical) at 37°C for 5 min. Diethylamine NONOate was dissolved in buffer containing Tris (20 mM, pH 10.5), and the concentration of the NONOate was determined from the absorption peak at 250 nm using an extinction coefficient of 6.5 mM⁻¹cm⁻¹. Diethylamine NONOate releases 1.5 moles of NO at a half-life time of 2 min at 37°C (pH 7.4). Released NO was monitored using an NO electrode (World Precision Instruments Inc.). The Silastic tubing NO delivery system [52] was also used for the NO exposure of *E. coli* cells as described previously [12]. Similar results were obtained when *E. coli* cells were exposed to NO using either diethylamine NONOate or the Silastic tubing NO delivery system.

Depletion of “chelatable iron pool” in *E. coli* cells

E. coli cells containing the protein expression plasmid or the vector only were diluted 1:100 in freshly prepared LB medium and incubated at 37°C with aeration (250 rpm) for 3 hours, followed by protein induction with L-arabinose (0.03%) for additional 2 hours. Cells were harvested and suspended in the M9 minimal medium to O.D. = 8.0. An iron chelator 2,2'-dipyridyl was then added to the cells, and incubated at 37°C for various time. Cells were then washed once with the M9 minimal medium before being treated with NONOate at 37°C for 5 min. For the measurements of “chelatable iron pool” content, the cells were passed through the French press once, followed by centrifugation to remove cell debris. The amount of “chelatable intracellular iron” in cells was measured from the absorption peak at 520 nm of the iron-dipyridyl complex using an extinction coefficient of 5.2 mM⁻¹cm⁻¹ [53].

Aconitase activity assay

For the aconitase activity assay, an aliquot of freshly prepared cell extracts was transferred to a pre-incubated solution containing Tris (90 mM, pH 8.0) and *D,L*-isocitrate (20 mM) at 25°C as described in [25]. The reaction was monitored at 240 nm using an extinction coefficient of 3.6 mM⁻¹cm⁻¹ for aconitate in a Beckman DU640 UV-visible spectrometer equipped with a temperature controller.

EPR measurements

The X-band EPR spectra were recorded using a Bruker model ESR-300 spectrometer equipped with an Oxford Instruments 910 continuous flow cryostat. Routine EPR conditions were: microwave frequency, 9.47 GHz; microwave power, 10.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; temperature, 25 K; receive gain, 10⁵. Freshly prepared glutathione-bound DNIC was used as a reference for quantification of protein-bound DNICs [12].

Results

Recombinant iron-sulfur proteins are modified forming protein-bound DNICs in *E. coli* cells under NO stress

When wild-type *E. coli* cells were exposed to NO using the NO releasing reagent diethylamine NONOate, a unique EPR signal at $g = 2.04$ reflecting protein-bound DNICs [19–27] appeared, as reported previously [12]. However, when recombinant iron-sulfur protein aconitase B [47] was expressed in the *E. coli* cells, the amount of protein-bound DNICs formed by the same NO exposure was significantly increased (Figure 1A). The titration of the NONOate concentration revealed that the amount of protein-bound DNICs formed in *E. coli* cells with recombinant aconitase B was about twice as much as that of the *E. coli* cells without recombinant protein when the cells were exposed to 50 μM or more of NONOate (Figure 1B).

To further explore the correlation between iron-sulfur proteins and the NO-mediated formation of protein-bound DNICs, we expressed recombinant aconitase B in *E. coli* cells to various levels, followed by NO exposure. As shown in Figure 2, when the expression level of recombinant aconitase B was gradually increased (panel C), the amount of protein-bound DNICs formed in *E. coli* cells by NO was progressively increased (panel A). In control *E. coli* cells without recombinant proteins (panel C), the total amount of protein-bound DNICs formed by NO remained essentially the same (panel B). Recombinant aconitase B was further purified from the NO-exposed *E. coli* cells. The EPR measurements confirmed that aconitase B was modified forming aconitase B-bound DNIC in *E. coli* after NO exposure (Figure 2D). Double integration analyses of the EPR signal at $g = 2.04$ indicated that purified aconitase B-bound DNIC accounted for 45–70% of the extra protein-bound DNICs formed in the NO-exposed *E. coli* cells.

To determine whether other iron-sulfur proteins also contribute to the NO-mediated formation of protein-bound DNICs *in vivo*, we expressed recombinant dihydroxyacid dehydratase which contains a [4Fe-4S] cluster [48] in *E. coli* cells, and found similar correlation between the expression level of dihydroxyacid dehydratase and the amount of protein-bound DNICs formed in *E. coli* cells by NO (data not shown). To determine whether non-iron-sulfur proteins can also contribute to the NO-mediated formation of protein-bound DNICs in *E. coli* cells, we expressed both iron-sulfur proteins and non-iron-sulfur proteins under the same experimental conditions. Figure 3 shows that expression of recombinant iron-sulfur proteins dihydroxyacid dehydratase [48] or the redox transcription factor SoxR [22] increased the amount of protein-bound DNICs in the *E. coli* cells by at least 100%. In contrast, expression of non-iron-sulfur proteins (the heat shock cognate protein HscA [49] and the single-stranded DNA binding protein SSB [50]) failed to increase the amount of protein-bound DNICs formed in *E. coli* cells, although both proteins were expressed to similar levels as dihydroxyacid dehydratase and SoxR (Figure 3B). The EPR measurements of recombinant proteins purified from the NO-exposed *E. coli* cells further confirmed that while iron-sulfur proteins were modified forming protein-bound DNICs, the non-iron-sulfur proteins did not form any DNIC (data not shown). Thus, iron-sulfur proteins, but not the proteins without iron-sulfur clusters, are modified forming protein-bound DNICs in *E. coli* cells under NO stress.

Deficiency of the iron-sulfur cluster assembly largely eliminates the NO-mediated formation of protein-bound DNICs in *E. coli* cells

In *E. coli*, iron-sulfur clusters are assembled by a group of proteins encoded by two gene clusters *iscSUA-hscBA-fdx* [54] and *sufABCDSE* [55]. Among these iron-sulfur cluster assembly proteins, IscA and SufA are paralogs with 47% sequence identity and 71%

similarity. Both IscA and SufA have been proposed as iron donors [46] or alternative scaffold proteins [56] for the iron-sulfur cluster assembly. While deletion of IscA or SufA has only a mild effect in *E. coli* cells, deletion of both IscA and SufA results in deficiency of the [4Fe-4S] cluster assembly under aerobic conditions [45, 46, 57]. Both the *iscA*⁻¹/*sufA*⁻¹ double mutant and the wild-type cells were able to express recombinant aconitase B in the rich LB medium under aerobic growth conditions (Figure 4A). However, unlike the wild-type cells, the *iscA*⁻¹/*sufA*⁻¹ double mutant failed to assemble the [4Fe-4S] cluster in proteins under aerobic growth conditions [46] (Figure 4B).

The wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells were then subject to the NO exposure. Figure 4C shows that NO exposure of the *iscA*⁻¹/*sufA*⁻¹ double mutant cells produced less than 5% of protein-bound DNICs observed in the wild-type cells. Further increase of NO concentration (up to 500 μ M NONOate) failed to increase the amount of protein-bound DNICs formed in the *iscA*⁻¹/*sufA*⁻¹ double mutant cells (data not shown). In parallel, the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant cells containing recombinant aconitase B were also exposed to NO. Again, unlike in the wild-type *E. coli* cells, expression of recombinant aconitase B in the *iscA*⁻¹/*sufA*⁻¹ double mutant cells did not significantly increase the amount of protein-bound DNICs (Figure 4C). Finally, recombinant aconitase B was purified from the NO-exposed wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells. The EPR measurements confirmed that unlike that in the wild-type cells, recombinant aconitase B in the NO-exposed *iscA*⁻¹/*sufA*⁻¹ double mutant cells was not modified by NO forming aconitase B-bound DNIC. Similar results were obtained when recombinant dihydroxyacid dehydratase was expressed in the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells (data not shown). Thus, deficiency of the iron-sulfur cluster assembly largely eliminates the NO-mediated formation of protein-bound DNICs in *E. coli* cells.

Depletion of “chelatable iron pool” effectively removes iron-sulfur clusters from aconitase B and diminishes the NO-mediated formation of protein-bound DNICs in the wild-type *E. coli* cells

“Chelatable iron pool” has been defined as the loosely bound iron that can be depleted by membrane-permeable iron chelators [34]. However, the exact chemical composition of “chelatable iron pool” has not been defined [36, 58]. It would be pertinent to explore the possible link between iron-sulfur clusters in proteins and “chelatable iron pool” in cells.

To test this idea, the wild-type *E. coli* cells containing recombinant aconitase B were treated with a membrane permeable iron chelator 2,2'-dipyridyl [59], followed by measurements of total aconitase activity in the cell extracts. A protein synthesis inhibitor chloramphenicol was added to block new protein synthesis in the cells before addition of 2,2'-dipyridyl. Because aconitase B requires an intact [4Fe-4S] cluster for its activity [20], removal of iron-sulfur cluster from aconitase B would inactivate the enzyme. Figure 5A shows that the total aconitase activity in the *E. coli* cells was gradually decreased as the concentration of 2,2'-dipyridyl in growth medium was increased and nearly abolished after the cells were incubated with 400 μ M 2,2'-dipyridyl for 40 min.

Recombinant aconitase B was also purified from the *E. coli* cells pre-treated with different concentrations of 2,2'-dipyridyl. Figure 5B shows that the amount of iron-sulfur cluster in recombinant aconitase B (as indicated from the amplitude of the absorption peak at 413 nm) was gradually decreased as the concentration of 2,2'-dipyridyl in growth medium was increased. Over 85% of iron-sulfur clusters in recombinant aconitase B expressed in *E. coli* cells was removed when *E. coli* cells were treated with 400 μ M 2,2'-dipyridyl for 40 min. Similar results were obtained when the *E. coli* cells containing recombinant dihydroxy acid dehydratase [48] were treated with 2,2'-dipyridyl (data not shown). Thus, depletion of “chelatable iron pool” effectively removes iron-sulfur clusters from proteins in *E. coli* cells.

The *E. coli* cells pre-treated with various concentrations of 2,2'-dipyridyl were then exposed to NO as described above. Figure 6A and B show that the amount of protein-bound DNICs formed in the *E. coli* cells after NO exposure was gradually decreased when concentration of 2,2'-dipyridyl in growth medium was increased, and closely correlated with the amount of iron-sulfur clusters remained in recombinant aconitase B in the cells.

We also followed the time course of the iron-sulfur cluster removal from recombinant aconitase B in *E. coli* cells in the presence of 2,2'-dipyridyl (400 μ M). As shown in Figure 6C and D, the amount of protein-bound DNICs in the *E. coli* cells was gradually decreased as the cells were incubated with 2,2'-dipyridyl (400 μ M), and closely correlated with the amount of iron-sulfur clusters remained in recombinant aconitase B in the cells. Taken together of the results shown in Figure 6, we concluded that depletion of “chelatable iron pool” effectively removes iron-sulfur clusters from proteins and concomitantly diminishes the NO-mediated formation of protein-bound DNICs in *E. coli* cells.

Deficiency of the iron-sulfur cluster assembly reduces “chelatable iron pool” in *E. coli* cells

Because depletion of “chelatable iron pool” effectively removes iron-sulfur clusters from proteins, we reasoned that iron-sulfur clusters may be closely associated with “chelatable iron pool” in *E. coli* cells. To test this hypothesis, we first compared “chelatable iron pool” in the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells. Figure 7 shows that “chelatable iron pool” in the *iscA*⁻¹/*sufA*⁻¹ double mutant cells was less than half of that found in the wild-type cells, indicating that deficiency of the iron-sulfur cluster assembly significantly decreases “chelatable iron pool” in *E. coli* cells. We then compared “chelatable iron pool” in the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells containing recombinant aconitase B, and found that expression of recombinant aconitase B increased “chelatable iron pool” in the wild-type *E. coli* cells but not in the *iscA*⁻¹/*sufA*⁻¹ double mutant cells. Similar results were obtained when dihydroxyacid dehydratase [48] was expressed in the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant cells (data not shown). On the other hand, expression of recombinant non-iron-sulfur proteins HscA [49] and SSB [50] had no effect on “chelatable iron pool” in the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells, suggesting that iron-sulfur clusters in proteins constitute at least part of “chelatable iron pool” in *E. coli* cells.

Discussion

Recent studies have shown that large molecular mass-bound DNICs are formed in cultured mammalian cells under NO stress [35, 36]. However, the exact chemical nature of large molecular mass-bound DNICs remains elusive [35, 36]. In this study, we demonstrate that iron-sulfur proteins are the major source of large molecular mass-bound DNICs formed in *E. coli* cells under NO stress. Expression of recombinant iron-sulfur proteins, but not the proteins without iron-sulfur clusters, almost doubles the amount of protein-bound DNICs formed in *E. coli* cells under NO stress (Figure 1). Purification of recombinant proteins from the NO-exposed *E. coli* cells further confirms that iron-sulfur proteins, but not the proteins without iron-sulfur clusters, are modified forming protein-bound DNICs (Figures 2 and 3). Deletion of the iron-sulfur cluster assembly proteins *IscA* and *SufA* to block the [4Fe-4S] cluster assembly in *E. coli* cells [45, 46] largely eliminates the NO-mediated formation of protein-bound DNICs. Furthermore, depletion of “chelatable iron pool” in the wild-type *E. coli* cells effectively removes iron-sulfur clusters from proteins and concomitantly diminishes the NO-mediated formation of protein-bound DNICs, indicating that iron-sulfur clusters in proteins are in dynamic equilibrium with “chelatable iron pool” in cells.

Previous studies indicated that proteins without iron-sulfur clusters such as bovine serum albumin could also form DNICs with excess ferrous iron and NO *in vitro* [60, 61]. However, we were unable to observe the DNICs formation in the proteins without iron-sulfur clusters in *E. coli* cells under NO stress (Figure 3). One possible explanation is that excess iron and NO used in the *in vitro* studies may form transient DNICs that are subsequently transferred to proteins via thiol-ligand exchange [60, 61]. It seems that exceedingly low concentration of intracellular “free” iron [62, 63] may prevent formation of DNICs in the proteins without iron-sulfur clusters in *E. coli* cells under NO stress. In contrast, iron-sulfur clusters in proteins are readily modified by NO forming protein-bound DNICs *in vitro* [19–27] and *in vivo* [8, 12], and direct modification of iron-sulfur clusters in proteins by NO has been attributed to the NO-mediated bacteriostasis of *E. coli* under aerobic and anaerobic growth conditions [8, 12] and to the NO-dependent killing of *Burkholderia mallei* [13]. Thus, at pathological NO concentrations (μM) [37, 38], iron-sulfur proteins, but not “free” iron, constitute the major source of protein-bound DNICs formed in *E. coli* cells.

The *E. coli* mutant with deficiency of the iron-sulfur cluster assembly provides a unique opportunity to further illustrate the role of iron-sulfur clusters in the NO-mediated formation of protein-bound DNICs *in vivo*. Previous studies showed that deletion of both IscA and SufA results in deficiency of the [4Fe-4S] cluster assembly in *E. coli* cells under aerobic growth conditions [45, 46]. Here we find that deficiency of the [4Fe-4S] cluster assembly due to deletion of IscA and SufA largely eliminates the NO-mediated formation of protein-bound DNICs in *E. coli* cells (Figure 4), demonstrating that iron-sulfur clusters are mainly responsible for the NO-mediated formation of protein-bound DNICs in *E. coli* cells.

Cellular “chelatable iron pool” has been described as the iron content that can be depleted with membrane-permeable iron chelators [34], and has been proposed as the major iron source for formation of large molecular mass-bound DNS under NO stress [34–36]. However, the exact chemical composition of “chelatable iron pool” has not been defined [35, 36, 58]. It is also possible that bacteria and mammalian cells may have distinct “labile iron pool”. In this study, we find that depletion of “chelatable iron pool” with a strong iron chelator 2,2'-dipyridyl effectively removes iron-sulfur clusters from recombinant aconitase B (Figure 5) or dihydroxyacid dehydratase in *E. coli* cells. We propose that iron-sulfur clusters are at least part of “chelatable iron pool” in bacterial cells. The equilibrium between “chelatable iron pool” and iron-sulfur clusters in proteins was previously implicated in regulation of the iron regulatory protein IRP-1/cytosolic aconitase in mammalian cells [62, 63]. IRP-1 is an iron-sulfur protein with cytosolic aconitase activity. When “chelatable iron pool” is depleted, the [4Fe-4S] cluster in IRP-1 is removed, resulting in apo-IRP-1 that binds the iron responsive elements (IREs) of target mRNAs to promote intracellular iron content (55,56). When intracellular iron content is replenished, the [4Fe-4S] cluster is re-assembled in apo-IRP-1 to prevent accumulation of excess intracellular iron. Similar equilibrium between “chelatable iron pool” and iron-sulfur clusters in proteins have been reported for the iron-sulfur cluster assembly regulator IscR [64, 65], the anaerobic growth factor FNR [66], and the NO sensor NsrR [14], among other iron-sulfur proteins in bacteria [10, 67]. Our results presented in this study are entirely consistent with the hypothesis that “chelatable iron pool” is in dynamic equilibrium with iron-sulfur clusters in proteins (Figure 6). Although the molecular details underlying iron exchange between “chelatable iron pool” and iron-sulfur clusters in proteins could only be speculated at present, decrease of “chelatable iron content” in the *E. coli* mutant cells with deficiency of iron-sulfur cluster assembly and increase of “chelatable iron content” in the wild-type *E. coli* cells containing recombinant iron-sulfur protein aconitase B (Figure 7) clearly suggest that iron-sulfur clusters in proteins at least contribute to part of “chelatable iron pool” in *E. coli* cells.

Throughout evolution, iron-sulfur proteins have become integral parts of diverse physiological processes [15, 16]. Over 500 unique iron-sulfur proteins have been identified in various organisms [15]. Modification of iron-sulfur proteins by NO is expected to have a broad impact on cellular functions. If cells are to survive, the NO-modified iron-sulfur proteins must be efficiently repaired. Although the biomimetic model studies suggest that DNIC may be directly converted back to iron-sulfur clusters [68], the cellular repair mechanism appears to be more complex. It has been shown that the genes encoding the iron-sulfur cluster assembly machinery are highly induced in *E. coli* cells under NO stress [6–9], suggesting that these proteins could be directly involved in repair of the NO-modified iron-sulfur clusters. Recently, we have shown that L-cysteine and oxygen are two key components for decomposition of protein-bound DNICs *in vitro* and *in vivo* [27]. However, re-assembly of new iron-sulfur clusters must occur in order to fully restore the function of NO-modified iron-sulfur proteins. Understanding the dynamic exchange between intracellular iron and iron-sulfur clusters in proteins will be crucial to elucidate cellular repair mechanism for the NO-modified iron-sulfur proteins.

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Abbreviations

AcnB	aconitase B
DNIC	dinitrosyl iron complex
EPR	electron paramagnetic resonance
NONOate	Diethylamine NONOate

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Figure 1. Recombinant aconitase B is modified forming protein-bound DNIC in *E. coli* cells under NO stress

Exponentially growing *E. coli* cells with or without recombinant aconitase B were concentrated to O.D. at 600 nm of 6.0, and treated with a freshly prepared NO-releasing reagent diethylamine NONOate under anaerobic conditions at 37°C for 5 min. **A)** EPR spectra of *E. coli* cells before and after being treated with NONOate. Spectra 1) and 2), *E. coli* cells without recombinant aconitase B before and after being treated with 200 μ M NONOate at 37°C for 5 min. Spectra 3) and 4), *E. coli* cells with recombinant iron-sulfur protein aconitase B (AcnB) before and after being treated with 200 μ M NONOate at 37°C for 5 min. **B)** Titration of NO in *E. coli* cells. *E. coli* cells with (open triangles) or without (open circles) recombinant aconitase B were treated with increasing concentration of NONOate (0, 25, 50, 100, 200, 400 μ M) at 37°C under anaerobic conditions for 5 min. The amplitude of the EPR signal at $g = 2.04$ was plotted as a function of the NONOate concentration used in the *E. coli* cells. The data are representatives from three independent experiments.

Figure 2. Correlation between the amount of recombinant aconitase B and the NO-mediated formation of protein-bound DNICs in *E. coli* cells

A) EPR spectra of the *E. coli* cells with increasing amount of recombinant aconitase B after NO exposure. *E. coli* cells containing the expression plasmid pBACNB were induced with 0.03% arabinose for 0, 0.5, 1.0, 2.0, and 3.0 hours as indicated. The cells were washed with the M9 minimal medium once, concentrated to O.D. at 600 nm of 4.0, and treated with 200 μ M NONOate at 37°C for 5 min. **B)** EPR spectra of the *E. coli* cells with no recombinant proteins after NO exposure. *E. coli* cells containing the expression vector pBAD were induced with 0.03% arabinose for 0, 0.5, 1.0, 2.0, and 3.0 hours as indicated. The cells were washed with the M9 minimal medium once, concentrated to O.D. at 600 nm of 4.0, and treated with 200 μ M NONOate at 37°C for 5 min. **C)** SDS/PAGE gel of the *E. coli* cells containing either recombinant aconitase B or an empty vector pBAD. Lane 0, purified aconitase B (AcnB). Lanes 1–5, *E. coli* cells containing pBACNB were induced with 0.03% arabinose for 0, 0.5, 1.0, 2.0, and 3.0 hours, respectively. Lanes 6–10, *E. coli* cells containing pBAD were induced with 0.02% arabinose for 0, 0.5, 1.0, 2.0, and 3.0 hours, respectively. **D)** EPR spectra of recombinant aconitase B purified from the *E. coli* cells exposed to buffer (spectrum 1) or 200 μ M NONOate (spectrum 2) at 37°C for 5 min. The protein concentration of purified recombinant aconitase B was about 5 μ M. Insert is a photograph of SDS/PAGE gel of purified aconitase B (AcnB) from the *E. coli* cells treated with buffer (1) or 200 μ M NONOate (2) 37°C for 5 min.

Figure 3. Formation of protein-bound DNICs in other recombinant iron-sulfur proteins in *E. coli* cells under NO stress

A) EPR spectra of the *E. coli* cells after exposed to NONOate. *E. coli* cells containing no recombinant protein (spectrum 1), or dihydroxyacid dehydratase [4Fe-4S] cluster (IlvD) (spectrum 2), the redox transcription factor SoxR [2Fe-2S] cluster (SoxR) (spectrum 3), the heat shock cognate protein (HscA) (spectrum 4), or the single stranded DNA binding protein (SSB) (spectrum 5), were concentrated to OD at 600 nm of 4.0, and exposed to NONOate (400 μ M) at 37°C for 5 min. The EPR spectra were taken under the same experimental conditions. **B)** The SDS PAGE gel of the *E. coli* cells with no recombinant protein (lane 1) or with IlvD (lane 2), SoxR (lane 3), HscA (lane 4), or SSB (lane 5).

Figure 4. Deficiency of the iron-sulfur cluster assembly largely eliminates the NO-mediated formation of protein-bound DNICs in *E. coli* cells

A) SDS/PAGE gel of the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells with or without recombinant aconitase B grown in the LB medium under aerobic conditions. Lane 0, purified aconitase B (AcnB). Lanes 1 and 2, the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells without any recombinant proteins. Lanes 3 and 4, the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells containing recombinant aconitase B. **B)** UV-visible absorption spectra of recombinant aconitase B purified from the wild-type (spectrum 1) and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells (spectrum 2). The absorption peak at 413 nm indicates the iron-sulfur cluster in aconitase B. The concentration of purified aconitase B was about 20 μM. Insert is a photograph of SDS/PAGE gel of purified aconitase B from the wild-type (1) and the *iscA*⁻¹/*sufA*⁻¹ double mutant (2) cells. **C)** EPR spectra of the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant *E. coli* cells after being treated with NONOate. *E. coli* cells with or without recombinant aconitase B were concentrated to OD at 600 nm of 6.0 before NO exposure. Spectra 1) and 2), the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant cells without recombinant aconitase B after the cells were treated with 200 μM NONOate at 37°C for 5 min. Spectra 3) and 4), the wild-type and the *iscA*⁻¹/*sufA*⁻¹ double mutant cells with recombinant aconitase B after the cells were treated with 200 μM NONOate at 37°C for 5 min. The data are representatives from three independent experiments.

Figure 5. Depletion of “chelatable iron pool” removes iron-sulfur clusters from recombinant aconitase B in *E. coli* cells

A) Inactivation of aconitase B in *E. coli* cells by an iron chelator 2,2'-dipyritydil. *E. coli* cells containing recombinant aconitase B were re-suspended in the M9 minimal medium to O.D. at 600 nm of 4.0. The protein inhibitor chloramphenicol was added to the *E. coli* cell culture before the cells were treated with 0, 50, 100, 400, and 1000 μM 2,2'-dipyritydil at 37°C with aeration for 40 min. The aconitase activity of the cell extracts prepared from the *E. coli* cells pre-treated with 2,2'-dipyritydil was immediately analyzed. The data were the means ± SD from three independent experiments. **B)** UV-visible absorption spectra of recombinant aconitase B purified from the *E. coli* cells pre-treated with different concentrations of 2,2'-dipyritydil for 40 min. The amplitude of the absorption peak at 413 nm represents the iron-sulfur cluster in aconitase B. The protein concentration of purified aconitase B was about 20 μM. Insert is a photograph of the SDS/PAGE gel of purified aconitase B from the *E. coli* cells pre-treated with indicated concentrations of 2,2'-dipyritydil for 40 min.

Figure 6. Removal of iron-sulfur clusters in aconitase B prevents the NO-mediated formation of protein-bound DNICs in *E. coli* cells

A) EPR spectra of the NO-exposed *E. coli* cells pre-treated with different concentrations of 2,2'-dipyridyl. The *E. coli* cells containing recombinant aconitase B were re-suspended in the M9 minimal medium to O.D. at 600 nm of 4.0, pre-treated with indicated concentration of 2,2'-dipyridyl for 40 min, and exposed to NONOate (200 μM) at 37°C for 5 min. **B)** Correlation between the iron-sulfur cluster in recombinant aconitase B and the protein-bound DNICs formed in the *E. coli* cells pretreated with different concentrations of 2,2'-dipyridyl. After *E. coli* cells were treated with indicated concentration of 2,2'-dipyridyl for 40 min, recombinant aconitase B was purified. The amount of iron-sulfur clusters in aconitase B purified from the *E. coli* cells (open squares) and the amount of protein-bound DNICs (closed circles) in the *E. coli* cells after NO exposure were plotted as a function of the 2,2'-dipyridyl concentration added in the M9 minimal medium. **C)** EPR spectra of the NO-exposed *E. coli* cells pre-treated with 2,2'-dipyridyl for different time. The *E. coli* cells containing recombinant aconitase B were re-suspended in the M9 minimal medium to O.D. at 600 nm of 4.0, pre-treated with 400 μM 2,2'-dipyridyl for indicated time, and exposed to

NONOate (200 μ M) at 37°C for 5 min. **D)** Correlation between the iron-sulfur cluster in recombinant aconitase B and the protein-bound DNICs formed in the *E. coli* cells pre-treated with 2,2'-dipyridyl for different time. After *E. coli* cells were treated with 400 μ M 2,2'-dipyridyl for indicated time, recombinant aconitase B was purified. The amount of iron-sulfur clusters in aconitase B purified from the *E. coli* cells (closed triangles) and the amount of protein-bound DNICs (closed circles) in the *E. coli* cells after NO exposure were plotted as a function of incubation time with 2,2'-dipyridyl.

Figure 7. Iron-sulfur clusters in proteins constitute at least part of “chelatable iron pool” in *E. coli* cells

The *iscA*⁻¹/*sufA*⁻¹ double mutant and the wild-type *E. coli* cells without or with recombinant aconitase B (AcnB) were treated with an iron chelator 2,2'-dipyridyl (400 μM) at 37°C with aeration for 40 min, washed with the M9 minimal medium once, and re-suspended to the O.D. at 600 nm of 6.0. The “chelatable iron” content in cells was measured after the cells were disrupted by passing through French press and centrifugation as described in the Materials and Methods. Data are the mean ± SD from three independent experiments.