Cobalt Stress in *Escherichia coli*

**THE EFFECT ON THE IRON-SULFUR PROTEINS**

Caroline Ranquet, Sandrine Ollagnier-de-Choudens, Laurent Loiseau, Frédéric Barras, and Marc Fontecave

From the Laboratoire de Chimie et Biologie des Métaux, iRTSV/LCBM, Commissariat à l’Energie Atomique/CNRS/Université Joseph Fourier, CEA-Grenoble, UMR 5249, 17 Avenue des Martyrs, 38054 Grenoble Cedex 09, France and Laboratoire de Chimie Bactérienne, UPR-CNRS 9043, IBSM, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Cobalt is toxic for cells, but mechanisms of this toxicity are largely unknown. The biochemical and genetic experiments reported here demonstrate that iron-sulfur proteins are greatly affected in cobalt-treated *Escherichia coli* cells. Exposure of a wild-type strain to intracellular cobalt results in the inactivation of three selected iron-sulfur enzymes, the tRNA methylthio-transferase, aconitase, and ferrichrome reductase. Consistently, mutant strains lacking the [Fe-S] cluster assembly SUF machinery are hypersensitive to cobalt. Last, expression of iron uptake genes is increased in cells treated with cobalt. In *vitro* studies demonstrated that cobalt does not react directly with fully assembled [Fe-S] clusters. In contrast, it reacts with labile ones present in scaffold proteins (IscU, SufA) involved in iron-sulfur cluster biosynthesis. We propose a model wherein cobalt competes out iron during synthesis of [Fe-S] clusters in metabolically essential proteins.

Cobalt is required as a trace element in procaryotes and eucaryotes to fulfill a variety of metabolic functions. Even though this metal is less frequently encountered in metalloenzymes than iron, manganese, copper or zinc, it is an important cofactor in vitamin B12-dependent enzymes and in some other enzymes in animals, yeast, bacteria, Archaea, and plants (1). At high intracellular concentration the redox active metal ion Co^{2+} is highly toxic. Cobalt toxicity is, for example, associated with various human diseases such as contact dermatitis, pneumonia, allergic asthma, and lung cancer (2). Cobalt was also shown to be immunogenic and to act as a hapten in the induction of bronchial and dermal hypersensitivity (3). Even though cobalt toxicity was attributed to cobalt-thiol group interaction of enzymes (4), surprisingly, very little has been done to investigate the mechanisms of this toxicity and to identify the principal targets of elevated intracellular concentrations of Co^{2+}.

The general strategy used by living cells to prevent damage caused by metallic toxins seems to reside in the expression of proteins that export or chelate metals. For example, the *Escherichia coli renA* gene encoding a membrane-bound polypeptide specific for nickel and cobalt resistance is induced under cobalt stress and allows cobalt efflux (5). In *Saccharomyces cerevisiae*, cobalt stress selectively induces a genetic response strikingly similar to that observed during iron starvation (6). The overexpression of iron transporters leads to a slight increase of intracellular iron concentration, which is supposed to limit cobalt toxic effects by favoring iron binding in iron enzymes (6). This strongly suggests a link between cobalt and iron metabolism and competition between cobalt and iron at iron-binding sites.

An important class of iron-containing proteins is that of iron-sulfur [Fe-S] enzymes involved in a variety of critical biological functions, including electron transfer, substrate binding/activation, regulation of gene expression, and redox and non-redox catalysis (7). The active site is made of the combination of iron and sulfur atoms mostly in the form of [4Fe-4S], [3Fe-4S], and [2Fe-2S] clusters. Formation of intracellular [Fe-S] clusters requires a complex biosynthetic machinery. In *E. coli* three different types of [Fe-S] cluster biosynthesis systems have been identified so far, namely the ISC, SUF, and CSD systems (8–10). Whereas the ISC system is thought to mature under stress conditions, the SUF machinery is thought to work under stress conditions (iron limitation and oxidative stress, which results in [Fe-S] cluster degradation) (11, 12). Whether the CSD system intervenes under specific conditions remains unknown. These different machineries have in common the involvement of a cysteine desulfurase (IscS, SufSE, and CsdAE), which allows the use of cysteine as a source of sulfur atoms. Furthermore, both the ISC and SUF systems contain scaffold proteins (IscU, IscA, SufA) that are proposed to provide an intermediate assembly site for [Fe-S] clusters or [Fe-S] cluster precursors (13–16). There are still uncertainties regarding the function of IscA: scaffold protein, iron chaperone, or [Fe-S] intermediate carrier (16–19). Finally, some ATP-hydrolyzing proteins (HscA/HscB, SupBCD) participate in the process (12, 20).

In this report we show that (i) exposure of *E. coli* cells to intracellular cobalt results in the inactivation of [Fe-S] enzymes, (ii) *E. coli* mutant strains lacking suf genes are much more sensitive to Co, and inactivation of aconitase is more severe in a sufC mutant, (iii) Co-treated cells respond by increased expression of Fur-repressed genes, and (iv) *in vitro* cobalt has a direct and specific effect on clusters chelated by scaffold proteins involved in [Fe-S] cluster biosynthesis. Together these observations indicate that cobalt toxicity is...
related to its effect on iron metabolism and, in particular, on the [Fe-S] cluster assembly process during de novo synthesis or repair.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

The majority of strains used in this paper were derivatives of E. coli K-12 MG1655. MG1655 sufC::aphA3 and MG1655 iscS::aphA3 were described in Nachin et al. (12) and Gully et al. (21), respectively. MG1655 sufA::aphA3, MG1655 sufB::aphA3, MG1655 sufD::aphA3, MG1655 sufE::aphA3, MG1655 sufS::aphA3, MG1655 iscA::aphA3, MG1655 iscSAU::aphA3, and MG1655 iscSLIA::aphA3 were constructed by using the method described in Datsenko and Wanner (22). Briefly, a fragment containing the suf or isc genes was amplified by PCR from E. coli MG1655 chromosomal DNA. Sequence of oligonucleotides used for PCR are presented in Table 1. PCR product was subcloned into pGEMT vector (Promega) or pGEM-T vector. The resulting plasmid containing the C terminus of iscS was double-digested by EcoRV/NdeI to get rid of the C terminus of iscS and a part of the aphA3 cassette. This was replaced by a DNA fragment containing the C terminus of iscA and the other part of the aphA3 cassette, obtained by double-digesting the pGEM-TiscA::aphA3 by EcoRV/NdeI. The result was plasmid pGEM-TiscA::aphA3ΔiscA. The resulting suf::aphA3 or isc::aphA3 mutant genes were excised by restriction and electroporated into E. coli BW25113/pKD46 strain, and Knr clones were selected.

All marked deletions were transduced by P1 transduction (23) into MG1655. Strains MC4100 (F−, araD139, Δ(argF, lac), U169, ptsF25, deoC1, relA1, fliB530, rpsL150, λ−) and MAM222 (MC4100 corA−) were kindly provided by M. A. Mandrand-Berthelot. Plasmid pKF191 was used for FhuF overexpression and purification (24). Plasmid pBAD-suf for production of the whole suf operon was kindly provided by W. Outten (25). A lacZ chromosomal fusion (aroB flhuF::AplacMu) (26) was used to evaluate the level of expression of the iron uptake machinery under the control of Fur. This lacZ fusion was transduced into MC4100 and MC4100 corA− strains to create strains CR100 and CR101.

Media

Bacteria were grown at 37 °C in LB-rich medium (27) or M9 minimal medium (28) supplemented with 0.4% glucose, 0.1% casamino acids, 0.0001% thiamine, 2 mM MgSO4. Minimal low phosphate MJS medium (12.5 mM HEPES, pH 7.1, 50 mM NaCl, 20 mM NH4Cl, 1 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 0.05 mM MnCl2, 0.8% casamino acids, 0.4% glucose, 0.005% thiamine) (29) supplemented with 1.5% agar was used to do the patch assays. When present in the culture medium, iron citrate was used at a final concentration of 300 μM.

Chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and ampicillin (50 μg/ml) were included when appropriate. The CoCl2 stock solution concentration was 0.1 mM (NH4)2FeSO4 was used to test aconitate reactivation. All chemicals were purchased from Sigma-Aldrich.

Patch Assays

Overnight cultures were diluted into fresh M9 supplemented medium and grown until the A600 = 0.3 at 37 °C. Cultures were then diluted 105 times, and 10-μl spots of these dilutions were spotted onto MJS supplemented plates containing different CoCl2 concentrations and incubated 3 days at 37 °C.

Enzymatic Assays

Aconitase Activity—Aconitase activity was assayed following a published protocol (30). Strains were grown in LB at 37 °C (1 liter culture), harvested in early stationary phase (A600 = 0.8), and washed with 5 ml of 50 mM Tris-HCl, pH 7.6, buffer. Because anaerobiosis is essential for maintenance of the full activity of aconitase, cell extracts were prepared with an anaerobic extract buffer (cell pellets were diluted in 6 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.6 μg/μl lysozyme in an anaerobic chamber). Three rounds of frozen/thawed were performed. Assays were performed by adding cell extracts (300 μg of protein) to 0.6 mM MnCl2, 25 mM citrate, 0.25 mM NADP+, 50 mM Tris-HCl, pH 7.6, in a 500-μl
Iron-Sulfur Proteins and Cobalt Stress

Expression and Anaerobic Purification of FhuF Proteins

E. coli competent C41(DE3) strain were transformed with a pET-derived plasmid overexpressing the His-tagged FhuF protein (pKF191 plasmid (24)). Cells were grown at 37 °C in LB medium containing 50 μg/ml ampicillin in the absence or presence of 0.5 mM CoCl₂ to an A₅₇₀ of 0.5. Expression was then induced with 0.5 mM isopropyl-β-D-galactopyranoside (E. coli) for 3 h at 37 °C. The bacterial pellets obtained from these aerobic cultures were resuspended into a glove box (Jacomex B553 (NMT)) in deaerated buffer A (100 mM Tris-HCl, pH 8, 100 mM NaCl) containing 0.6 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride, then transferred into ultracentrifuge tubes. The solution was frozen quickly (outside the glove box) and thawed (inside the glove box). This procedure was repeated 3 times and followed by an ultracentrifugation (4 °C, 45,000 rpm, 1.5 h). After anaerobic streptomycin treatment (2%), the clear supernatant solution was loaded anaerobically onto a Ni-NTA column (10 ml) equilibrated with buffer A. After an extensive washing (1 liter of buffer A) FhuF was eluted with buffer A containing 0.4 M imidazole. Pure fractions were concentrated and stored at −80 °C.

In Vitro Interaction between Cobalt and Scaffold Proteins

IscU and SufA were purified as already described (14, 15). They were incubated anaerobically in 0.1 M Tris–HCl, pH 8, buffer either under apo or holom (containing [Fe-S] cluster) with different fold excess (1–40) of CoCl₂. For the [Fe-S]-containing proteins the reaction was monitored by UV-visible spectros- copy following the decrease in the 300–700-nm absorption range. After 1 h of incubation at 18 °C, proteins were desalted and analyzed for their cobalt and iron content according to McCall and Fierke (39) and Fish (37) procedures. A UV-visible spectrum was recorded for each protein.

Transfer of Mixed Iron-Cobalt-Sulfur Complex of Scaffold Proteins to Apotargets

IscU scaffold (2 mg) was prepared as described above containing 0.8 iron, 0.6 cobalt, and 1.5 sulfur atoms/monomer and was incubated with E. coli apoMiaB (0.8 mg). After 2 h of incubation, proteins were separated onto a Ni-NTA column on which MiaB was retained since it contains a His tag at its N terminus. IscU was recovered in the run-through fraction during extensive washing with buffer A (100 mM Tris–HCl, pH 8, 50 mM KCl), whereas MiaB was collected in the 400 mM imidazole fraction. The presence of IscU and MiaB in separated fractions was checked by SDS-gel electrophoresis. Mixed iron-Co-S complex transfer was monitored by assaying each fraction for iron, cobalt, and sulfur content. The same protocol was used with ferredoxin as a target (0.8 mg), except that in this case the His-tagged protein was IscU and not the target.

Preparation of EPR Samples

200 μl of FhuF proteins (150 μM) were reduced anaerobically with 2 mM dithionite for 30 min, and EPR tubes were frozen inside the glove box. Spectra were recorded on a Bruker EMX (9.5 GHz) or ER200D EPR spectrometers equipped with an ESR 900 helium flow cryostat (Oxford Instruments).

Analysis

Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard (36). Iron and sulfide content were determined by Fish (37) and Beinert (38) methods. Cobalt content was measured colorimetrically according to McCall and Fierke (39). This method uses the feature that coordination of Co²⁺ alters the absorbance spectrum of 4-(2-pyridylazo)resorcinol at 514 nm. Briefly, the protein solution was mixed with 4 M guanidine and 7.5 mM MOPS, pH 7.5, and incubated at room temperature for a few minutes. A freshly prepared solution of 4-(2-pyridylazo)resorcinol (final concentration 100 μM) was then mixed with the sample, and the absorbance was immediately recorded at 514 nm (ε, 50 M⁻¹ cm⁻¹). Cobalt concentration was determined using a standard curve (0–100 nmol of Co). Cobalt content was also determined by atomic absorption (atomic absorption optical spectrometer).

Western Blot

Equal quantities of protein were separated on 12% SDS-PAGE acrylamide gels and transferred onto nitrocellulose filters (Amersham Biosciences). Filters were incubated with anti-aconitase (A and B) antibodies (gifts from D. Downs, University of Wisconsin, Madison, WI). Immunoblots were developed by using horseradish peroxidase-conjugated goat anti-rabbit antibody followed by enhanced chemiluminescence (Bio-Rad).
Iron-Sulfur Proteins and Cobalt Stress

TABLE 2
Cobalt and iron contents in WT and corA− strains

|                       | Wild type | corA− |
|-----------------------|-----------|-------|
| Intracellular cobalt content (µM) | +Co | +Co200 µM | -Co | +Co200 µM |
| UV (µM)                | 10       | 130    | 15    | 10       |
| Atomic absorption (µM) | 5        | 105    | -     | -        |
| Intracellular iron content (%) | 100 | 47     | 100   | 43       |

The fluF-lacZ fusion was transduced into the corA− mutant strain, and the level of cellular iron was tested as described above. Surprisingly, CoCl₂ (200 µM) induced β-galactosidase expression with similar level of stimulation as compared with the wild-type strain (Fig. 2, lanes 3 and 4). Furthermore, iron colorimetric analysis of the corA− cells showed a decrease of iron by a factor of two between the untreated and Co-treated cells, similar to that observed in the case of the wild-type strain (Table 2). This suggests that intracellular iron decrease is induced by exogenous extracellular cobalt rather than intracellular cobalt. The mechanism underlying this phenomenon needs to be clarified.

Cobalt Alters Aconitase, MiaB, and FhuF Activity in Vivo—To determine whether cobalt has an impact on [Fe-S] clusters enzymes, we selected three [Fe-S] proteins, which absolutely require a [2Fe-2S] or a [4Fe-4S] cluster for activity, and studied their integrity in E. coli cells grown in the presence or in the absence of cobalt.

Aconitase Activity—Aconitase, a [4Fe-4S] protein, catalyzes the reversible isomerization of citrate to isocitrate, a key step in the tricarboxylic acid cycle (43). Isocitrate is then transformed
Iron-Sulfur Proteins and Cobalt Stress

into α-ketoglutarate by the NADP+-dependent isocitrate dehydrogenase enzyme. Aconitase activity was assayed in anaerobic extracts as described under "Experimental Procedures." As shown in Fig. 3, aconitase activity was drastically decreased (about 70–80% loss of activity) in extracts of cells treated with a concentration of CoCl₂ of 200 μM (compare lanes 1 and 2). The amount of aconitase protein in extracts of cells treated with cobalt was shown by Western blot analysis to be comparable with that in cobalt-free extracts, supporting the conclusion that the decrease of the enzymatic activity was the consequence of a decreased specific activity (Fig. 3). We also checked that under the conditions used for the enzymatic assay, the isocitrate dehydrogenase was not inhibited (data not shown). Interestingly, only a slight drop in aconitase activity was observed in anaerobic extracts from corA− cells treated with cobalt (200 μM) (Fig. 3, compare lanes 3 and 4).

MiaB Activity—The miaB gene encodes a member of the "Radical SAM" family of enzymes and, thus, contains a catalytically essential [4Fe-4S] cluster (44). This protein participates in the modification of tRNAs with an anticodon starting with U and is required to convert 6-N-isopentenyladenosine (i6A) to 6-N-isopentenyl-2-methylthioadenosine (ms²i6A) at position A37 (34, 45). E. coli mutants lacking the miaB gene produce and accumulate i6A (45) instead of the fully modified base ms²i6A. The functionality of MiaB in cells was determined by measuring the production of ms²i6A after tRNAs isolation from bacterial cells, hydrolysis, and analysis by HPLC (45). Fig. 4 represents the HPLC chromatograms of tRNAs hydrolysates from MG1655 E. coli strain grown in LB medium with and without CoCl₂ (200 μM). The assignment of the ms²i6A peak is based on the retention time and characteristic UV-visible spectrum, displayed in the inset of Fig. 4 (46). Whereas the tRNAs from the control (LB without Co) were correctly modified (intense peak of ms²i6A and no i6A), the strain grown in the presence of 200 μM CoCl₂ (LB + Co, 200 μM) accumulated i6A and produced only a very low amount of ms²i6A. At higher CoCl₂ concentrations (0.5 and 1 mM), no ms²i6A could be detected (data not shown). This result clearly showed an in vivo inactivation of MiaB in the presence of cobalt. In contrast, the effect of 200 μM CoCl₂ on ms²i6A formation in the corA mutant was very small (supplemental Fig. S1).

FhuF [2Fe-2S] Cluster Content—FhuF contains a [2Fe-2S] cluster that is required for its in vivo activity (24). Cells overexpressing FhuF were cultivated in rich medium in the absence (control) or presence of CoCl₂ (addition of 0.5 mM CoCl₂ and isopropyl 1-thio-D-galactopyranoside during growth at A₆₀₀ = 0.5 for 2 h). Under these conditions, growth is only slightly affected by CoCl₂. The protein was then purified anaerobically from the cells, and the status of the [2Fe-2S] cluster was checked by light absorption spectroscopy and by the determination of its iron and sulfur content. Fig. 5 shows the UV-visible spectrum of pure FhuF protein from cells grown with (FhuF [+Co]) or without (FhuF [−Co]) CoCl₂. Whereas the UV-visible spectrum of the FhuF [−Co] displayed intense absorption bands at 325 and 420 nm, characteristic of the FhuF [2Fe-2S] cluster (24), that of FhuF [+Co] had much less intense bands. Furthermore, whereas FhuF [−Co] contained 1.9 iron atoms per monomer, FhuF [+Co] contained only 0.5 iron per monomer. In both cases stoichiometric amounts of iron and sulfur were determined. Both proteins were analyzed by EPR spectroscopy. In the purified as-isolated state they were shown to be EPR-silent. In contrast, after anaerobic reduction with 2 mM dithionite,
Iron-Sulfur Proteins and Cobalt Stress

Cobalt decreases [2Fe-2S] content in FhuF. A plasmid overexpressing the [2Fe-2S] protein FhuF was transformed into C41(DE3) E. coli strain. This strain was cultivated in LB with or without CoCl2 (500 μM) up to an A600 = 1, and cellular extracts were prepared under anaerobiosis. The FhuF-His-tagged protein was purified from these extracts, and the status of the [2Fe-2S] cluster was checked by UV visible spectroscopy. FhuF [−Co] and FhuF [+Co], 500 μM.

FhuF [−Co] displayed an EPR signal characteristic for a reduced [2Fe-2S]^{+1} (S = 1/2) cluster, with parameters identical to those already published (24) and which integrated for 90% of total iron. On the contrary, FhuF [+Co] displayed a low signal with similar shape but which integrated for only 14% of total iron.

Moreover, the cobalt content of the FhuF [+Co] and FhuF [−Co] pure enzyme solutions was determined by atomic absorption. The FhuF [−Co] preparation did not contain cobalt above the limit of detection, whereas the FhuF [+Co] preparation contained 0.3–0.4 Co2+ atoms per FhuF monomer. These results directly established that a [2Fe-2S] enzyme such as FhuF binds cobalt and fails to assemble a correct cluster when cells are treated with CoCl2.

Suf but not isc mutant strains are sensitive to cobalt stress. Overnight cultures of different strains were diluted into fresh M9 supplemented medium and grown until A600 = 0.3 at 37 °C. Cultures were then diluted 10^6 times, and 10 μl of these dilutions were spotted onto MJS plates containing different cobalt concentrations supplemented or not with iron citrate 300 μM (+Fe) and incubated for 3 days at 37 °C.

FIGURE 6. Suf but not isc mutant strains are sensitive to cobalt stress. Overnight cultures of different strains were diluted into fresh M9 supplemented medium and grown until A600 = 0.3 at 37 °C. Cultures were then diluted 10^6 times, and 10 μl of these dilutions were spotted onto MJS plates containing different cobalt concentrations supplemented or not with iron citrate 300 μM (+Fe) and incubated for 3 days at 37 °C.

In Vitro Effect of CoCl2 on [Fe-S] Enzymes. We further analyzed the effect of cobalt on purified or partially purified [Fe-S] proteins. Aconitase, MiaB, FhuF, and proteins involved in [Fe-S] biosynthesis (IscU and SufA) were chosen as models. Each protein was incubated with an excess of cobalt, and the integrity of the cluster was checked either by measuring enzymatic activity (aconitase and MiaB) or by measuring cobalt and iron content using colorimetric assays (FhuF, IscU, and SufA).

[Fe-S] Enzymes: Aconitase, MiaB, and FhuF. The aconitase activity of anaerobic E. coli soluble extracts (Fig. 7, bar 1) was resistant up to 0.1 mM CoCl2 and was partially inhibited (35% activity loss) in the presence of 1 mM CoCl2 (Fig. 7, compare bars 2 and 3) showing that cobalt has little direct effect on fully assembled [4Fe-4S] aconitase cluster under anaerobic conditions. Upon exposure to air for 1 h, inactivation of aconitase was observed (Fig. 7, compare bars 1 and 4), as previously reported, because the [4Fe-4S] cluster degrades upon reaction with oxygen and reduced oxygen species into iron-depleted forms unable to catalyze the enzyme reaction. The same extent of inactivation by oxygen was observed whether 0.1 or 1 mM CoCl2 was present or not in the extracts (Fig. 7, compare bars 4, 6, and 8). An active cluster in Co-free extracts could be partially regenerated (50% reactivation) simply by returning to anaerobic conditions.
Iron-Sulfur Proteins and Cobalt Stress

Reducing conditions (30, 49), as shown in Fig. 7 (compare bars 4 and 5). Anaerobic reactivation was greatly improved (activity above the initial value) when 1 mM iron was added to the extracts (Fig. 7, compare bars 5 and 10). In contrast, CoCl₂ was a powerful inhibitor of aconitase reactivation in a concentration-dependent process (Fig. 7, compare bars 5, 7, and 9 and compare bars 10 and 11). Furthermore, the results show that iron protects the enzyme from the inhibition of reactivation by CoCl₂ (Fig. 7, compare bars 9 and 11), suggesting a competition between iron and cobalt at the active site. When the purified [Fe-S] proteins MiaB and FhuF in the holoform were treated with CoCl₂ (0.5–2 mM) for 1 h under anaerobiosis, clusters remained intact, as shown by assays for enzyme activity (34) and [Fe-S] content (data not shown).

IscU and SufA Scaffold Proteins—IscU and SufA proteins are important proteins in biogenesis of [Fe-S] clusters. They are endowed with a scaffold activity that allows transient assembly of an [Fe-S] cluster and its efficient transfer to target protein (14, 15). [Fe-S]-IscU is well characterized and known to contain a [2Fe-2S] monomer characterized by light absorption bands at 325, 420, and 460 nm (15). When a [Fe-S]-IscU preparation containing 1.2 iron atoms/monomer was incubated anaerobically for 1 h with 1–40 eq of cobalt with regard to iron, we observed a decrease of the absorption in the 300–650-nm range (Fig. 8A). Iron and cobalt analysis after desalting of the protein showed that the amount of bound cobalt exactly corresponded to the amount of iron lost (Fig. 8B). Moreover, IscU containing an equivalent amount of iron and cobalt was found to be a stable species (Fig. 8B). Similar results were obtained with [Fe-S]-SufA (data not shown).

ApoIscU or apoSufA were loaded anaerobically with different excesses of CoCl₂ (4–8 eq with regard to the protein) and then desalted. The resulting protein solutions were blue and were characterized by intense absorption bands at 575 (ε = 330 M⁻¹ cm⁻¹), 620 (ε = 400 M⁻¹ cm⁻¹), and 675 nm (ε = 290 M⁻¹ cm⁻¹), which are consistent with a mononuclear tetrahedral polythiolate Co(II) species (50, 51) (Fig. 9). Colorimetric assay for cobalt revealed that proteins incorporated up to 0.9 Co/monomer. These results show that cobalt (i) has no or limited direct effect on fully assembled clusters in enzymes, (ii) can be incorporated in proteins containing degraded (Aconitase) or transient [Fe-S] clusters, and (iii) can be chelated by apoIscU and apoSufA cysteine ligands.

Transfer of Cobalt from IscU to Target Apoproteins—In this study we used the following compounds. In a first experiment, [Fe-S] IscU (2 mg) was treated with an 8-fold excess of cobalt with regard to iron and desalted under the conditions described above. This preparation contained 0.8 iron, 0.6 cobalt, and 1.5 sulfur atoms/monomer. It was further incubated with E. coli apoMiaB protein (10:1 ratio) or E. coli ferredoxin apoprotein (2.5:1 ratio). After 2 h of incubation IscU and the target proteins were separated by chromatography onto a Ni-NTA column according to the “Experimental Procedures.” Iron, Co, and sulfur transfer was monitored by assaying each isolated protein for
iron, cobalt, and sulfur content. Under these conditions MiaB was shown to contain after reaction 2.8 iron, 1.1 cobalt and 3.8 sulfur/monomer, and ferredoxin was shown to contain 0.5 iron, 0.6 cobalt, and 0.8 sulfur atoms/monomer. Altogether these results demonstrated that the mixed iron-cobalt-sulfur-loaded form of the IscU scaffold can transfer its inorganic content, including Co, to both proteins.

**DISCUSSION**

As for any other transition metal, high intracellular concentrations of cobalt are toxic to both prokaryotes and eukaryotes. The molecular basis for this toxicity is not well documented. In particular, the type of enzymes affected by a cobalt stress and the mechanisms for protecting them from the toxic agent have not been previously identified. Here we report a study identifying one such mechanism of cobalt toxicity in *E. coli*, unambiguously showing that enzyme activities depending on iron-sulfur clusters are affected by toxic concentrations of cobalt. The following results support our conclusion: (i) *in vivo* inactivation of iron-sulfur enzymes, (ii) resistance provided by the SUF system, (iii) protection by iron, and (iv) *in vitro* binding of cobalt to proteins of the [Fe-S] cluster assembly machinery and transfer to target apoproteins.

Activities and cluster content of [Fe-S] enzymes in *E. coli* cells treated with CoCl₂ are indeed greatly affected. This was clearly shown here using three different probes: MiaB, a [4Fe-4S] enzyme involved in tRNA modification; aconitase, a [4Fe-4S] enzyme of the Krebs cycle; FhuF, a [2Fe-2S] enzyme involved in ferrisiderophore reduction. In the latter, the protein isolated from Co-treated cells was shown to contain cobalt atoms and degraded clusters, suggesting that Co²⁺ competes with iron ions for the specific binding sites in [Fe-S] proteins.

In line with the observed effect of cobalt on cellular [Fe-S] enzyme activities, it is significant that the SUF protein machinery specifically and efficiently protects the cells from the toxicity of cobalt and that aconitase is more sensitive to cobalt in a *sufC* mutant. It has been clearly established that the SUF system is involved in the assembly of [Fe-S] clusters in proteins under stress conditions such as oxidative stress and iron limitation (11, 12). The present work provides an additional illustration of the importance of the SUF system under stress conditions associated with inactivation of [Fe-S] enzymes. Interestingly, the different Suf proteins, however, do not have the same impact on cobalt resistance. The great sensitivity of *E. coli* *sufS* and *sufE* mutant strains confirms the critical importance of the cysteine desulfurase activity which provides the sulfur atoms to the clusters. In contrast, ISC, the general biosynthetic [Fe-S] cluster biosynthetic machinery, does not seem to be crucial for cobalt resistance mechanisms. Again this points to a significant difference in the physiological role of the two assembly machineries.

A specific cluster repair function has been generally assigned to SUF, and the present data fit in this expectation.

How Is It Possible to Explain the Effects of Cobalt on [Fe-S] Protein Activity?—Our results show that cobalt treatment of *E. coli* cells results in changes in iron homeostasis: (i) A 2-fold decrease of cellular iron content was observed; (ii) increased intracellular iron levels, achieved by supplementation of the growth medium with iron citrate, provides partial protection; (iii) *E. coli* responded to a cobalt stress by an increased expression of the Fur-repressed gene *fluF*. Iron depletion generated by a cobalt stress was already described in *Neurospora crassa* (52). Evidently, a Co-mediated cellular iron depletion would result in incompletely assembled clusters and decreased [Fe-S] enzyme activities and would be consistent with the requirement for a complete SUF system. Yet the iron depletion *per se* cannot account for the loss of [Fe-S] enzyme activities in Co-treated cells. Indeed, *corA* mutant cells, which presented a comparable iron depletion and stimulation of the Fur-repressed genes, displayed full aconitase and MiaB activities. Because the main difference with wild-type cells is their inability to uptake Co²⁺, we conclude that the inactivation of [Fe-S] enzymes is primarily due to intracellular Co²⁺.

Oxidative stress generated by intracellular Co²⁺ would also explain at least in part Co²⁺ toxic effects on [Fe-S] enzymes. Indeed, a number of [Fe-S] enzymes in *E. coli* are sensitive to oxygen, hydrogen peroxide, and oxygen radicals (53). Furthermore, another oxidative stress would also be consistent with the requirement for a complete SUF system for resistance to Co. Here, we show that indeed an iron-sulfur enzyme with an O₂-sensitive cluster, such as aconitase, is greatly impaired by CoCl₂ when exposed to air, whereas it is resistant under anaerobic conditions (Fig. 7). However, so far we have been unable to collect experimental evidence supporting a link between Co²⁺ intracellular accumulation and oxidative stress in *E. coli*. Further studies are required to clarify this point.

An interesting observation of the present study is the sensitivity with regard to Co²⁺ of the clusters bound to the scaffold proteins IscU and SufA, critical for [Fe-S] cluster assembly, as well as their ability to bind Co²⁺. This opens the possibility that the incomplete/incorrect assembly of [Fe-S] clusters, the incorporation of Co²⁺ in [Fe-S] proteins, and their inactivation are due to cobalt binding to the scaffold proteins and to the resulting perturbation of the [Fe-S] cluster assembly process. This hypothesis is strongly supported by the observation that the mixed iron-Co-sulfur complex of the IscU scaffold is transferred to target apoproteins, either [2Fe-2S] or [4Fe-4S] proteins. The limited iron depletion might contribute to favor cobalt binding with respect to cluster assembly to these sensitive and critical proteins. Fig. 10 summarizes this hypothesis.
Iron-Sulfur Proteins and Cobalt Stress

Equation 1 shows that the scaffold proteins (Sca) can exist in four forms; the apoform (apo-Sca), the holoform (holo-Sca) containing a correctly assembled [Fe-S] cluster, the Co-Sca form containing a mononuclear Co$^{2+}$ center, and the [Fe,Co]-Sca form with a mixed [Fe,Co] cluster. The relative proportions of the metallated (Co-Sca, [Fe,Co]-Sca and holo-Sca) forms vary with the Co:Fe ratio. Equation 2 suggests that during reaction of Sca (metallated forms) with a target protein (apo-T), only holo-Sca generates an active holo-T. At large concentrations of cobalt the scaffold protein is under Co-Sca or [Fe,Co]-Sca forms which generate Co-containing and unreactive forms of the target. Direct reaction of Co$^{2+}$ with holo-T is excluded. Oxidative stress conditions can potentiate Co$^{2+}$ toxic effects. SUF contributes to resistance and/or repair mechanisms.

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