Iron is an essential element for nearly all life forms (1–3). However, free cellular iron is toxic, and the solubility of ferric iron is poor. Thus, bacteria tightly regulate cellular iron levels through multiple iron transport pathways to achieve effective homeostasis (3–5). Feo, the ferrous iron (Fe$^{2+}$) transport system, is likely a major route for transporting ferrous iron across the bacterial membrane under anaerobic or low-pH conditions, such as those in the gastrointestinal tract (6). Several systems have demonstrated the importance of Feo. Feo is critical for both survival and virulence in Helicobacter pylori (7). The Feo system is critical for virulence in Streptococcus suis (8) and for colonization in Escherichia coli and Salmonella enterica serovar Typhimurium (9, 10). Bacterial pathogens require the Feo system for enhanced colonization (1, 3, 6).

The feo operon was first identified in E. coli K-12, and its expression was shown to be under dual transcriptional control by the iron-sensing ferric uptake regulator (Fur) and oxygen-sensing fumarate nitrate reduction protein (FNR) regulators in response to different levels of iron and oxygen (6, 11). The feo operon from gamma-proteobacteria encodes 3 proteins: FeoA, FeoB, and FeoC (11–13). FeoA, present in 90% of the feo operons, is a small Src homology 3 (SH3) domain protein necessary for ferrous iron transport (6, 12, 14–16). Recent enzymatic assays have suggested that FeoA may not act as a GTPase-activating protein as originally proposed (14). FeoB, an indispensable component of the Feo system, is a large protein consisting of an intracellular amino-terminal domain (NFeoB) with high affinity for NFeoB at a site encompassing the Switch II region of the G domain and the C-terminal GDI-like domain such that the flexible W1 loop is potentially capable of interacting with residues in the nucleotide-binding site. We proposed that FeoC might coordinate the Fe-S cluster to regulate ferrous iron transport by modulating G-protein activity (G-protein modulator model) (6). However, a recent study suggested that FeoC did not regulate the promoter in Yersinia pestis (20), and no report has confirmed the DNA binding activity of FeoC.

Two studies have recently suggested that FeoC may function at the posttranslational level. We showed that apo-KpFeoC binds to the N-terminal domain of KpFeoB (KpNFeoB) with high affinity (21). In the crystal, apo-KpFeoC binds to KpNFeoB at a site encompassing the Switch II region of the G domain and the C-terminal GDI-like domain such that the flexible W1 loop is potentially capable of interacting with residues in the nucleotide-binding site. We proposed that FeoC might coordinate the Fe-S cluster to regulate ferrous iron transport by modulating G-protein activity (G-protein modulator model). However, Kim et al. found that FeoC binds to FeoB, and the presence of FeoC prevents FeoB from
proteolytic degradation by FtsH in Salmonella enterica under low-iron and low-oxygen conditions (22). This results in an elevated level of FeoB that enables Salmonella to take up Fe(II) under anaerobic and low-iron conditions (protease inhibitor model). The coordination of the Fe-S cluster on FeoC can play crucial roles in all 3 models. This study is the first to provide experimental evidence supporting the existence of an Fe-S cluster on FeoC. We present spectroscopic and mutational data proving its existence, the range of redox potentials, and the degradation of a [4Fe-4S] cluster on KpFeoC, with discussions regarding the models.

MATERIALS AND METHODS

Chemicals, bacterial strains, and vectors. [99% 15N]H4Cl, [99% U-13C]glucose, 99% D2O, and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Cambridge Isotope Laboratories (Andover, MA). Basal medium Eagle (BME) vitamins, redox reagents, and corresponding antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Novagen (Madison, WI). The expression vector pGEX-6p-1 carrying the cDNA encoding a chimera fusion protein of the glutathione S-transferase (GST) tag and FeoC from K. pneumoniae (subsp. pneumoniae) NTU-H2004 (pGEX-6p-1/GST-KpFeoC) was prepared in this lab. The fusion protein was first purified through the GST affinity column. The enzyme was digested to remove the GST tag and further purified through the size exclusion column (19). For preparing the holo-KpFeoC, the protein was purified through the GST column and concentrated aerobically, whereas the follow-up enzyme digestion was performed anaerobically (less than 5 ppm oxygen) in an anaerobic chamber from COY Lab (Grass Lake, MI) and purified through a Superdex-75 10/300 column attached to an ÄKTA purifier (GE Healthcare) inside the anaerobic chamber. Samples were kept at low temperature in a labtop cooler from Nalgene/Sigma-Aldrich (St. Louis, MO). Point mutants were cloned with the GST tag in this lab.

Protein expression and labeling. The KpFeoC protein was prepared by growing BL21(DE3)/plysS cells carrying the pGEX-6p-1/GST-KpFeoC vector in LB medium without supplementing additional iron. Uniformly 15N-labeled KpFeoC ([U-15N]-KpFeoC) protein was prepared by growing cells in the M9 medium supplemented with 1 g/liter 15NH4Cl in the presence of 50 mg/liter (wt/vol) FeCl3 and 1% (vol/vol) BME vitamins, as described previously (18, 19, 21), but with modifications: an additional 50 mg/liter FeCl3 (wt/vol) and 1% (vol/vol) BME vitamins were added to the growth medium. For preparing [U-13C, 15N]-KpFeoC protein, [U-13C]-glucose was added in 2 aliquots: 2 g/liter at the onset and 2 g/liter when the optical density at 600 nm (OD600) reached 2.5 immediately after IPTG induction. Based on optical spectrum analysis, the holo-KpFeoC expressed in the LB medium is identical to that expressed in the M9 medium with iron. The addition of iron typically increases the yield and homogeneity.

NMR and EPR sample preparation. Amicon (Millipore, Billerica, MA) tubes were used for protein concentration and buffer exchange following protein purification. Unless specified, the NMR (and EPR) buffers were 50 mM Tris and 100 mM NaCl, in 9% D2O at pH 7.8 for KpFeoC. We defined the native state of FeoC as freshly purified protein from E. coli in the anaerobic chamber. Unused samples were stored in bottles sealed with septum caps at −80°C. The DSS was added as the internal chemical shift standard. Excess molar ratios of sodium dithionite or dithiothreitol (DTT) were added to the reduced samples. Unless specified, the reduced state is the dithionite-reduced state (for NMR and EPR). NMR samples in the reduced state were prepared in an anaerobic chamber and transferred to NMR tubes with a J. Young valve (Wilmad, Vineland, NJ). EPR samples were prepared and frozen in the anaerobic chamber. Spectra were taken aerobically as solids. The pH values were measured before entering the chamber. apo-KpFeoC samples were prepared by dialyzing away iron from holo-KpFeoC, which had been exposed to oxygen for more than 5 days.

NMR spectroscopy, data processing, and analysis. NMR spectra were acquired at specified temperatures on Bruker Avance 500- or 600-MHz spectrometers equipped with triple resonance cryogenic probes, as described previously (19). To observe the fast-relaxing hyperfine shifts, the repetition times of superWETF sequences were set to 0.1 s for protons and 0.2 s for carbons and fine-tuned before measurements. Spectra were signal averaged for 160,000 scans for carbons and 40,000 scans for protons for a total acquisition time of 8 h for carbon and 2 h for protons. Spectra were obtained by subtracting 2 spectra with broad and desired line broadening (typically 60 Hz for protons and 600 Hz for carbons) and were baseline corrected using the spline protocol. Proton chemical shifts were referenced relative to internal DSS (taken as 0 ppm); carbon and nitrogen spectra were referenced indirectly by the canonical ratios (23). All spectra were processed by Topspin software (Bruker).

EPR. Unless specified, the EPR sample buffer conditions were identical to those used in NMR experiments. The EPR spectra were acquired at 9.5390 GHz (measured using a Hewlett-Packard 5246L electronic counter) and 4G modulations with 20 mW power at a 4,096-point resolution, and the average from 4 scans was reported. To avoid loss of EPR signals caused by exposure to air, the sodium dithionite-reduced samples were transferred to the EPR tube and frozen within 10 s in the anaerobic chamber. To ensure full reduction, the reductant/protein molar ratios exceeded 4 and 8 for DTT and dithionite, respectively. Cavity signals were subtracted from the EPR spectra by exact g values using Origin (OriginLab, Northampton, MA). The simulated spectra were processed with SimFonia (Bruker BioSpin, Billerica, USA) and WINEPR (Bruker BioSpin, Billerica, USA).

X-ray absorption spectra. We conducted the measurements of the Fe-K-edge X-ray absorption spectroscopy at the wigglers beamline (BL17C) with a beam size of 2 by 2 mm at the National Synchrotron Radiation Research Center in Taiwan. Beam energy was calibrated to iron foil standards. The sample cell at a volume of 120 μl was sealed with thin Fe-free Kapton tapes. We performed sample loading in the anaerobic chamber and conducted data collection with the sleeping mode that halted the exposure by approximately 1 to 8 s after 2 to 24 s of radiation. The Lytle detector collected signals ranging from 6,912 to 7,912 eV with the fluorescence mode. Samples were maintained at 283 K throughout the experiments with an air-cooling device. Each scan ran for approximately 90 min.

RESULTS

NMR evidence supports a paramagnetic center in KpFeoC. Using relaxation-optimized sequences (see Materials and Methods), we observed a proton hyperfine shift of KpFeoC isolated directly from the cell culture (native state) at −14 ppm, indicating that KpFeoC possesses a paramagnetic center (Fig. 1A) (29). Upon addition of dithionite (reduced state), the resonance shifted upfield by 5 ppm to −19 ppm, indicating that the paramagnetic...
FeoC was dark red, and the UV-Vis spectrum contained peaks at 384 nm, 490 nm, and 570 nm (Fig. 2C), suggesting that GST-FeoC likely contained a [2Fe-2S] (34, 35) or [4Fe-4S] (36–38) cluster but not a [1Fe] cluster. We further determined the fractional concentration of GST-FeoC containing a [4Fe-4S] cluster by inductively coupled plasma mass spectrometry (ICP-MS) and spectrophotometry. For spectrophotometric measurements, we used the canonical extinction coefficient of 15,000 at 410 nm per [4Fe-4S] cluster (39). The results from both methods showed that 10% of native GST-FeoC contain the [4Fe-4S] cluster.

**EPR evidence supports a [4Fe-4S] cluster on GST-FeoC.** Iron sulfur clusters exhibit the EPR spectrum characteristic of the type of clusters (40–42). Thus, we employed EPR to assign the cluster type present in GST-FeoC. At high temperature (77 K), we detected no signal within g values of 1.8 to 2.2 from native or reduced states of GST-FeoC (data not shown). Upon lowering the temperature to 14 K, we observed 2 signals at g values of 2.060 and 2.007 from the native GST-FeoC, characteristic of the [4Fe-4S]1+ state (Fig. 3A). Reducing GST-FeoC by using DTT significantly reduced the resonance intensity, and we observed only residual resonances at g values of 2.05 and 2.008 (Fig. 3B), indicating that most of the protein had been reduced to the EPR-silent diamagnetic [4Fe-4S]1+ state. After further reducing GST-FeoC with sodium dithionite, a stronger reducing agent, the EPR signals reappeared at g values of 2.038 and 1.937, reminiscent of those of the [4Fe-4S]1+ cluster (Fig. 3C) (36, 37). Thus, the redox potential of [4Fe-4S]1+ to [4Fe-4S]2+ lies between that of DTT and dithionite (43, 44). We also observed EPR signals at a g value of 4.3 for both native and DTT-reduced states (data not shown). We attribute this signal to free iron or nonspecifically bound iron on the protein (45). Table S1 in the supplemental material presents a summary of the EPR signal observed for GST-FeoC under various conditions.

**Fe K-edge X-ray absorption structures support the [4Fe-4S] cluster.** We further investigated homo-GST-FeoC using Fe K-edge x-ray absorption spectra. We conducted our initial attempt under air, and the Fe K-edge x-ray absorption near-edge structure (XANES) features of the rising edge appeared at approximately 7,125.9 eV (Fig. 4A), suggesting oxygen degradation, which was confirmed by extended x-ray absorption fine structure (EXAFS; data not shown). To protect the cluster from oxygen degradation, we repeated the experiment with samples prepared in anaerobic conditions (Fig. 4A). Consequently, the near-edge downshifted to 7,118.7 eV and the preedge (corresponding to 1s to 3d orbital transitions) peak downshifted to 7,112.7 eV, suggesting that anaerobic samples have fewer positive charges to the iron because of a reduced oxidation state (46). We then collected the EXAFS to verify the cluster and ligands (Fig. 4B). We chose the EXAFS of k ranging from 3.12 to 12.00 Å−1 for analysis (Fig. 4B, inset) and applied the Fourier transform to yield the distances of the ligating atoms (iron and sulfur; Fig. 4B). We selected three models for simulation: [4Fe-4S]-(S-Cys)3 (degradation intermediate), and [4Fe-4S]-(O-Ser) × 1-(S-Cys) × 3 (serine replacement). Among them, [4Fe-4S]-(S-Cys) × 4 was the most optimal model, which yielded the lowest R² value of 0.07%. According to the model, the average Fe-S distances were reported as 2.26 ± 0.05 Å and the Fe-Fe distance was 2.71 ± 0.09 Å, consistent with the X-ray absorption results (see below).

**Spectrophotometric evidence suggests a [2Fe-2S] or [4Fe-4S] cluster in GST-FeoC.** UV-Vis spectra were diagnostic of the presence of iron-sulfur clusters (33). Figure 2 shows the UV-Vis spectra of GST-tagged GST-FeoC (GST-FeoC) and 4 cytC-type-to-serine mutants (C56S, C61S, C64S, and C71S; Fig. 2A) and their spectra without the GST tag (Fig. 2B). For comparison, we included the spectrum of *Clostridium pasteurianum* rubredoxin (CpRd), which contains a [1Fe] cluster resembling that of another winged-helix protein, PF0610 (34). The color of the (concentrated) native state GST-FeoC was dark red, and the UV-Vis spectrum contained peaks at 417 nm, 450 nm, and 550 nm. Upon enzymatic removal of the GST tag, the wild-type GST-FeoC maintained a nearly identical UV-Vis spectrum, indicating that the absorption spectrum derived from KpFeoC (Fig. 2B). However, the peak intensity dropped 2-fold, likely because of cluster degradation during the prolonged enzyme digestion process (3 days). However, the UV-Vis spectrum of CpRd contained peaks at 384 nm, 490 nm, and 570 nm (Fig. 2C), suggesting that GST-FeoC likely contained a [2Fe-2S] (34, 35) or [4Fe-4S] (36–38) cluster but not a [1Fe] cluster.
sistent with canonical [4Fe-4S] clusters (27, 28). The EXAFS results were in good agreement with the EPR results, and a higher coordinated number of Fe-Fe bonds excluded the [2Fe-2S], supporting the temperature-dependent EPR results. Therefore, both the EPR and EXAFS results suggest that the [4Fe-4S] is the native cluster in FeoC.

The [4Fe-4S] cluster of KpFeoC is sensitive to oxygen. Similar to other iron-sulfur proteins, the holo-KpFeoC was oxygen sensitive (40). When exposed to oxygen at 4°C, native KpFeoC gradually lost its characteristic absorption peaks (Fig. 5). The change in peak height was fitted to a single exponential decay function of OD = c + A × 0.5710^(-t/t1/2), and the results yielded a half-life (t1/2) of 16.0 ± 1.0, 15.8 ± 1.0, and 18.3 ± 1.3 h for absorbance at 417 nm, 450 nm, and 550 nm, respectively, with an average half-life of 17 h. We monitored the protein using SDS-PAGE analysis, which indicated that the proteins were nondegraded (data not shown). Our data suggested that the [4Fe-4S] cluster in KpFeoC was oxygen labile. Proteins purified from a size exclusion column in air for 2 h at 4°C were depleted of the Fe-S cluster, suggesting that the degradation rate is likely less than 1 h under the chromatography conditions. The limited oxygen availability in the 1-mm by 10-mm by 10-mm cuvette without stirring might slow the degradation rate measured by the spectrophotometric experiments.

To identify the degraded products, we initially treated the freshly prepared native KpFeoC sample with 1 mM DTT anaerobically and then exposed it to air at 4°C. We used a series of EPR spectra at 14 K at various exposure times (see Fig. S1 in the supplemental material). Because DTT-reduced KpFeoC was diamagnetic, the initial spectrum showed only a signal from the residual nonreduced protein. Further exposure to oxygen resulted in the appearance of a peak at a g value of 2.010, which gradually increased in intensity and peaked at 20 h. Further exposure to oxygen resulted in a gradual loss of the EPR signal. The time course of the oxidation process is consistent with the initial buildup of the [3Fe-4S]^1⁺ cluster, the only iron-sulfur cluster with an isotropic g value at 2.01. The end product of the oxidation process is the loss of the iron-sulfur cluster and the generation of apo-FeoC and thus the loss of the EPR signal.

To assess the final state of the oxidization product, we added DTT or dithionite to reduce the cluster after 25 h of exposure to oxygen. DTT did not produce any change in the EPR spectrum (see Fig. S1D in the supplemental material), whereas dithionite greatly reduced the EPR signal (see Fig. S1E). These results suggested that 25-h O₂-exposed KpFeoC did not contain a sufficient concentration of [2Fe-2S]^2⁺ (EPR silent), because dithionite should produce [2Fe-2S]^1⁺ (EPR active). The absence of a g value of 1.96 was indicative of the depleted [4Fe-4S]^1⁺ cluster (the product of the dithionite reduction of the [4Fe-4S] cluster) in the

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**FIG 2** The UV-Vis spectra of various freshly prepared KpFeoC samples (0.5 to 3 mM) in native state. (A) The UV-Vis spectra of GST-tagged KpFeoC (GST-KpFeoC) and four cysteine-to-serine single-site KpFeoC mutants at concentrations of 2.5, 1.5, 2.6, 2.6, and 0.9 mM for C56S, C61S, C64S, C71S, and wild-type GST-FeoC, respectively. (B) The UV-Vis spectra of GST-tag-free KpFeoC and its single-site mutants at concentrations of 0.5, 0.9, 0.8, 0.5, and 0.15 mM for C56S, C61S, C64S, C71S, and wild-type FeoC, respectively. The 300-nm to 700-nm region, which exhibits the characteristic absorption of iron-sulfur clusters, is shown in the inset, shifted in the y axis direction for clarity. (C) Comparison of the UV-Vis spectrum of 3 mM KpFeoC (red) and 1.5 mM CpRd (black), which possesses [1Fe].
final oxidation product, indicating that [4Fe-4S] was completely degraded. We concluded that [3Fe-4S] is likely the intermediate oxidative degradation process of the [4Fe-4S] cluster in KpFeoC.

Cysteines in the W1 loop are the ligands for the [4Fe-4S] cluster. KpFeoC contains 4 conserved cysteine residues, which are all located in the W1 loop. To assess the roles of these cysteines in cluster formation, we generated 4 single-site Cys-to-Ser mutants (C56S, C61S, C64S, and C71S) and analyzed them with EPR (Fig. 6). Optical spectra (Fig. 2) of all GST-tagged mutants were similar to those of the wild-type KpFeoC. However, upon enzymatic removal of the GST tag, only the C61S mutant maintained the characteristic absorption spectrum of the [4Fe-4S] cluster (Fig. 2) and EPR signals similar to those of the wild-type KpFeoC (Fig. 6B, right). The intensity of [4Fe-4S] \( ^{1+} \) of C61S was approximately 30% that of the wild type at a similar concentration, indicating a less stable cluster. These results indicated that Cys56, Cys64, and Cys71 are crucial for the formation and stability of the iron-sulfur cluster, whereas Cys61 also plays a role in stabilizing the cluster but is less essential. We suggest that Cys61 is the fourth ligand with the support from EXAFS, but it is possible to substitute Cys61 with other nearby glutamic acid or serine residues in the C61S mutant. In certain cases, serine can also serve as a ligand for the iron-sulfur cluster (35).
FeoC Contains a [4Fe-4S] Cluster

**DISCUSSION**

**FeoC forms an oxygen-sensitive [4Fe-4S] cluster.** The Fe-S proteins contain inorganic iron and sulfur as cofactors (40, 47, 48). Iron and sulfur are redox active, and the cluster can undergo redox reactions under physiological conditions. Studies have documented several types of biological Fe-S clusters, including the simplest [1Fe] cluster in rubredoxin to the complex [8Fe-7S] cluster encountered several types of biological Fe-S clusters, including the [4Fe-4S] cluster. The [4Fe-4S] cluster is ligated by 4 cysteines. Protein exposure to oxygen generated the [3Fe-4S] intermediate, further supporting the [4Fe-4S] identity in native FeoC. In summary, UV-Vis, NMR, EPR, and EXAFS provide unequivocal evidence of the presence of the [4Fe-4S] cluster on FeoC.

**Redox states and oxygen-induced degradation of holo-FeoC.** Cysteine typically coordinates each tetrahedral Fe site in the form of thiolate (RS⁻). However, other residues, such as aspartate (RCO₂⁻), histidine (N=), and serine (R-O⁻), are occasionally encountered in clusters, and these ligands were shown to modify redox potential (55). The protein environment also affects the redox potential of an iron-sulfur cluster. We showed that the [4Fe-4S] cluster on FeoC is coordinated to the cysteine residues in the W1 loop. We estimated the redox potential of the [4Fe-4S] in the 3 oxidation states by examining the effect of DTT or dithionite on the native FeoC sample. We detected that most FeoC protein in the [4Fe-4S]³⁺ state reduced to the [4Fe-4S]²⁺ state by DTT and further reduced to the [4Fe-4S]¹⁺ state by dithionite. Because the redox potential of DTT is −0.33 V and that of dithionite is −0.66 V (43, 44), the results indicated that the redox potential of [4Fe-4S]³⁺/²⁺ of FeoC is higher than −0.33 V and that of [4Fe-4S]²⁺/¹⁺ is between −0.33 V and −0.66 V. However, we did not determine the precise redox potential of holo-FeoC.

The loss and gain of the iron-sulfur cluster is a common sensing mechanism for the Fe-S proteins to exhibit their biological activity. The cluster-assembling machinery assembles the iron-sulfur cluster, and degradation by oxidative agents removes the cluster. The ICP-MS results showed that 20% of the freshly prepared GST-FeoC contained the Fe-S cluster, indicating that the Fe-S cluster-assembling machinery is capable of assembling at least 20% of the overexpressed GST-FeoC. We examined the degradation of holo-FeoC by exposing the protein to oxygen and detected the presence of a [3Fe-4S]²⁺ intermediate. To assess whether the [2Fe-2S] state is the final cluster degradation product of holo-FeoC, we added dithionite to the final product to reduce the diamagnetic [2Fe-2S]²⁺ state, if present, to the paramagnetic [2Fe-2S]¹⁺ state. We did not detect the EPR signal near a g value of 2.0, suggesting that the [2Fe-2S]²⁺ state is not the final product. The pathway is similar to that of FNR, but the rates differ, suggesting various sensory/regulatory mechanisms (56–58).

**Biological implications.** Iron-sulfur proteins play key roles in catalytic reactions, in electron transfer in both oxidative phosphorylation and photosynthesis, and in gene regulation (41, 42, 48, 59, 60). The roles of bacterial iron-sulfur regulatory proteins as sensors/switches have been extensively reviewed (42, 60–62). Iron-sulfur clusters sense environmental changes by interacting with small molecules to exhibit rich chemistries and regulate cellular events. Iron-sulfur proteins acting as transcriptional regulators, such as the E. coli fumarate-nitrate reduction regulator protein (EcFNR), can alter binding affinities to specific DNA sequences by various cluster states.

**FIG 5** Kinetics of the O₂-induced degradation of the [4Fe-4S] cluster in KpFeoC (0.1 mM). The optical absorbance at 417 nm (filled squares), 450 nm (empty squares), and 550 nm (filled circles) was monitored at various time points of O₂ exposure. A total of 100 μl of sample was kept at 4°C in 50 mM Tris, 100 mM NaCl (pH 7.8), without stirring. Fittings of the curves (solid lines) yielded half-lives of 16.0 ± 1.0, 15.8 ± 1.0, and 18.3 ± 1.3 h, as monitored at 417 nm, 450 nm, and 550 nm, respectively.

Based on UV-Vis spectrophotometry, NMR, and EPR evidence, we demonstrated that KpFeoC forms an Fe-S cluster. The characteristic UV-Vis spectrum and NMR spectra suggested that the iron-sulfur cluster is not the simple [1Fe] type. The presence of the EPR signal at a g value of 1.937 in the dithionite-reduced state ruled out the possibility of the [3Fe-4S]¹⁺/₀⁺ cluster, because the reduced state of [3Fe-4S]²⁺ is diamagnetic (41, 50). As suggested in previous studies, [2Fe-2S] and [4Fe-4S] clusters can be distinguished by temperature-dependent EPR intensities (32, 50, 54). The EPR signals of [2Fe-2S] are observable above 77 K, whereas the EPR signal of the [4Fe-4S]³⁺ cluster is observable only at a temperature below 30 K. This was the case in KpFeoC (resonances disappeared above 37 K; data not shown), confirming the identity of the [4Fe-4S] cluster in KpFeoC. The EXAFS results further support the conclusion of [4Fe-4S], excluding the possibility of [2Fe-2S], and confirm that [4Fe-4S] is ligated by 4 cysteines. Protein exposure to oxygen generated the [3Fe-4S] intermediate, further supporting the [4Fe-4S] identity in native KpFeoC.
FIG 6 EPR spectra (first derivatives) of four *KpFeoC* mutant proteins at 14 K at native (left) and dithionite-reduced (right) states. (A) C56S, 1.5 mM; (B) C61S, 2.9 mM; (C) C64S, 2.4 mM; (D) C71S, 1.1 mM.
the apo and holo protein to the iron regulatory elements within the mRNA of genes related to iron metabolism (70, 71). The NreB contains a [4Fe-4S]2+ cluster, but it does not bind to nucleotides, acting as a transcriptional activator by interacting with the response regulator, NreC, to regulate the expression of the nreABC operon (72, 73). Thus, the iron-sulfur proteins regulate transcription through direct binding to the DNA or RNA. They also exhibit transcriptional activity indirectly by affecting the activity of other proteins that interact with DNA activators or repressors.

In the literature, 3 models have been proposed for FeoC function: the transcriptional regulator model (6), the G-protein modulator model (21), and the protease inhibitor model (22). Available data does not currently support the transcriptional regulator model appeared to be the only model supported by G-protein modulator model is lacking. The protease inhibitor model appeared to be the only model supported by Gel-shift or SELEX experiments (20, 22); evidence supporting the transcriptional regulator model (6), the G-protein modulator (21), and the protease inhibitor model (22). Available data does not currently support the transcriptional regulator model (6), the G-protein modulator model (21), and the protease inhibitor model (22).

In summary, by using spectrophotometric, NMR, EPR, and X-ray absorption methods, we showed that KpFeoC contains a [4Fe-4S] cluster that can be degraded by oxygen. Using single-site mutation and EXAFS techniques, we identified the crucial cysteine residues in the W1 loop as the ligands of the Fe-S cluster. Detection of the oxygen-sensitive Fe-S cluster in FeoC raises the question of how the Fe-S cluster might play a role in regulating Feo activity.

ACKNOWLEDGMENTS

We thank John L. Markley (the University of Wisconsin-Madison) for providing the plasmid encoding CpRd and Ping-Yu Chen (National Chung Hsing University) for providing the EPR service. We thank Jyh-Fu Lee, Chih-Wen Pao, and Jeng-Lung Chen for their help on the data collection of X-ray absorption at the beamline BL17C in NSRRC. We also thank Feng-Chun Lo for the discussion of the EXAFS data.

This project was supported by the National Science Council of the Republic of China, grant NSC100-2311-B-001-023. The NMR experiments were conducted on NMR spectrometers at the High-Field Nuclear Magnetic Resonance Center (HFNMRC), supported by the National Research Program for Biopharmaceuticals, the National Science Council of the Republic of China.

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