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Spectroscopic studies of cobalt-substituted ferredoxin from *Clostridium pasteurianum*

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Summary. Ferredoxin from *Clostridium pasteurianum* substituted with two Co atoms did not give any cobalt EPR signal at 8 K as isolated, but upon reduction with sodium dithionite, a broad signal appeared with g values that indicate high-spin ($S=3/2$) Co(II). These signals were distinct from Co(II)-dithiothreitol signals, and disappeared upon reoxidation with air. Under anaerobic incubation of apoferredoxin with Co(II), a green derivative showed a visible spectrum typical of tetrahedral Co(II)-thiolate coordination, which shifted dramatically upon exposure to air. The ^{1}H-NMR spectrum of the aerobically isolated protein is reported at 300 MHz; magnetic susceptibility measurements were indicative of a diamagnetic species. These spectroscopic studies indicate that Co(II)-substituted ferredoxin is oxidized to low-spin Co(III)-ferredoxin in the presence of sulfide and oxygen. The diamagnetic Co(III) state could reversibly be reduced to high-spin Co(II) by sodium dithionite.

Key words: Ferredoxin — *Clostridium pasteurianum* — Cobalt derivative — ERP — NMR

Introduction

Ferredoxin from *Clostridium pasteurianum* is a small iron-sulfur protein with two 4Fe-4S clusters ligated to eight cysteines. It functions as a two-electron carrier, to nitrogenase or hydrogenase for example, and it has a low redox potential (−410 mV) (Orme-Johnson 1973).

The protein has 55 amino acid residues, of which 12 are aspartate and glutamate. Only two aromatic and one basic residues are present; it contains no histidine or methionine (Tanaka et al. 1966).

Even though several iron-sulfur proteins have been isolated and characterized (Marsubara et al. 1987), little about their electron transfer mechanisms has been elucidated. Because of the small amount of X-ray data on the ferredoxins (Adman et al. 1973) and their oxygen sensitivity (Gersonde et al. 1971), we felt that metallosubstitution might give new insight. Our intention was to prepare a ferredoxin derivative that was stable in the presence of air, which would make it easier to handle than the native form. We have previously shown that the aerobically isolated ferredoxin with two Co atoms from *C. pasteurianum* is stable in air (Skjeldal and Ljones 1988) but there remained some questions about coordination geometry, oxidation and spin state of the two Co atoms, and the need for sulfide, which we try to answer in this paper. In the literature there is little data on cobalt coordinated to only sulfur ligands in proteins, and most of the work has been on Co(II) (May and Kuo 1978; Sugiura et al. 1975; Vasak and Kägi 1981). Cobalt can exist in many coordination geometries and oxidation states, and it can change between high-spin Co(II) ($S=3/2$), low-spin Co(II) ($S=1/2$), high-spin Co(III) ($S=2$), intermediate-spin Co(III) ($S=1$) and low-spin Co(III) ($S=0$) (Cotton and Wilkinson 1972). The rare oxidation state, Co(I), has been observed in cobalamin (Cotton and Wilkinson 1972). As in case of iron clusters (Poe et al. 1970), cobalt atoms are able to couple antiferromagnetically (Vasak and Kägi 1981).

EPR of metalloproteins provides valuable information about the oxidation and spin state or interaction of the system: whereas systems with half-integer electronic spin S (odd number of

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electrons) are EPR active, spin systems with integer or zero electronic spin (even numbers of electrons) are mostly EPR silent or difficult to detect. EPR has long been used to characterize ferredoxins (Matsubara et al. 1987); NMR has also shown to be a valuable tool, for example in determining their magnetic susceptibility (Poe et al. 1970, 1971; Phillips et al. 1974).

Materials and methods

Materials. Ferredoxin (Fd) from *C. pasteurianum W5* was purified as described previously (Skjeldal and Ljones 1988; Rabinowitz 1972); cobalt-substituted ferredoxin (Co-Fd) was isolated aerobically (Skjeldal and Ljones 1988). The green derivative refers to apoprotein with Co(II) which has not been exposed to air. Apoferredoxin was prepared by trichloroacetic acid precipitation (Rabinowitz 1972). All reagents were of the highest purity available.

Metal analyses. Cobalt and iron were measured on a Perkin-Elmer 300 flame atomic spectrophotometer in 6 M HNO_3 at 240.7 nm and 248.3 nm respectively.

Electronic paramagnetic resonance. EPR spectra were obtained on a Bruker ESP 300 EPR system equipped with a Oxford Instruments helium flow cryostat (ESR-9), operating in the X-band mode. The spectra were obtained with 1 mW microwave power at 3.6–30 K, 1 mT field modulation and the longest time constant was 0.3 s. The samples were contained and incubated in 4-mm-diameter quartz tubes, frozen and stored in liquid nitrogen.

Visible absorption spectrum. A visible absorption spectrum of the anaerobic, green derivative was recorded on an HP 8450A diode array spectrophotometer in a 1.0-ml quartz cell equipped with a serum stopper. The cell was made anaerobic by degassing on a vacuum line, and filled with highly purified nitrogen. Molar absorption coefficients, ε, were calculated from the absorbance of 2 mg/ml apoferredoxin incubated with twofold molar excess of $CoCl_2$ and Na_2S.

Proton nuclear magnetic resonance. ^{1}H-NMR spectra of Co-Fd were obtained on a Varian XL 300 spectrometer operating in the Fourier-transform mode. A typical NMR sample consisted of 1–2 mM Co-Fd in D_2O containing 50 mM borate pH* 8.0 (pH* refers to the uncorrected reading of a pH meter). A 90° pulse (29.5 μs) was employed, and the residual solvent signal was suppressed by a presaturation pulse (1 s, 0.2 W) from the decoupler, which was turned off during acquisition of the NMR signal. Chemical shifts were measured relative to the residual solvent signal, which was assumed to be 4.76 ppm downfield from sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) at 25° C. A line-broadening factor of 6 Hz was used. Magnetic susceptibility measurements were performed by the NMR technique of Evans (1959) in specially made coaxial two-chamber NMR cells consisting of an outer tube with 5-mm diameter and an inner tube with 1-mm diameter. Solutions of Co(III)-Fd (1–2 mM) in 99.77% D_2O, 50 mM borate pH* 8.0, were placed in the outer tube together with the susceptibility marker DSS. The solution in the inner tube was made to contain approximately the same total amounts of the marker compound as the outher tube but no Co-Fd. The difference in frequency, $\Delta\nu$, of the marker protons was measured in the temperature range 5–45° C. The temperature of the spectrometer sample zone was determined from the separation (measured in hertz) of the methyl and hydroxyl signals of methanol, as described by the manufacturers (Varian Associates publication 87-202-001).

Results

EPR of Co-Fd

The EPR spectra of Co-Fd, aerobically isolated, shown at 8 K (Fig. 1), does not contain any reson-

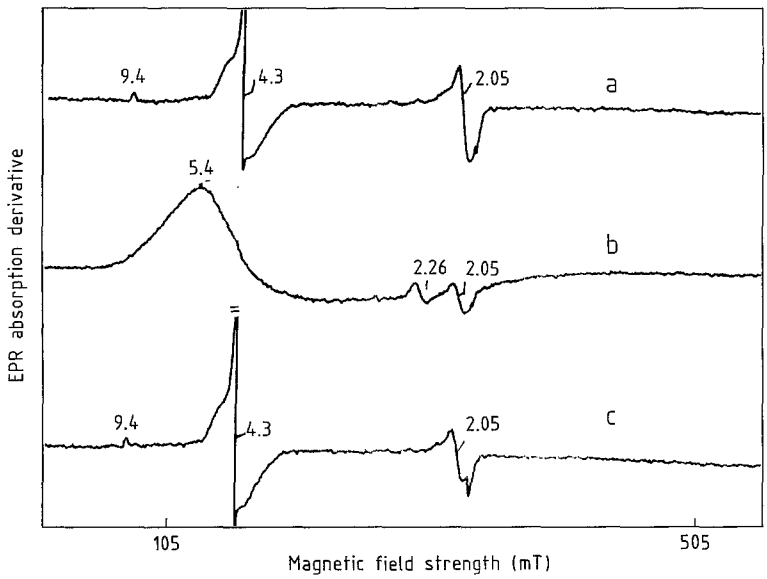

Fig. 1. The EPR spectra at 8 K, 9.24 GHz and 1 mW of (**A**) 330 μM air-isolated Co-Fd in 50 mM Tris/HCl pH 8.2 (sample contaminated with 2% iron), (**B**) after addition of sodium dithionite and (**C**) the solution, containing 3 mM sodium dithionite, after exposure to air at room temperature for 1 min. The spectra are not corrected for the 9% dilution. Each spectrum consists of four accumulations of 5 min each, *g* values are as indicated

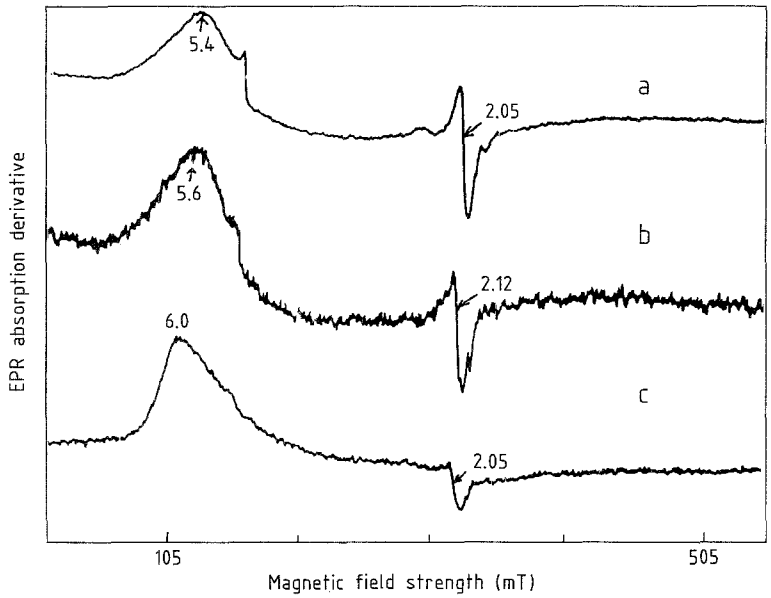

Fig. 2. The EPR spectra at 8 K, 9.24 GHz and 1 mW of (**A**) 250 μM Co-Fd after incubation with 10 mM dithionite in 50 mM Tris/HCl pH 8.2, gain 1; (**B**) 0.2 mM $CoCl_2$ light-brown sample after exposure to sodium dithionite at pH 8.2, gain 8; (**C**) 2 mM $CoCl_2$ incubated with 10 mM dithiothreitol in 75 mM Tris/HCl pH 8.2 under argon atmosphere, gain 1. The g values are indicated in the figure

ances typical of Co(II) in the temperature range 3.6–30 K, except some low-spin ($S=1/2$) impurities observed in the $g=2.0$ region, together with some Cu(II) signal. Incubation of 300 μM Co(III)-Fd at pH 1.1, or with 2 mM ferricyanide at pH 8.2, for 5 min at room temperature prior to freezing did not reveal any Co(II) signals, but increased the Cu(II) signal. The first preparation contained 660 μM Co and was contaminated with 14 μM Fe, slightly distorted rhombic high-spin ($S=5/2$) Fe(III) resonances are observed (Fig. 1A), quite similar to rubredoxin-type iron-sulfur proteins (Lovenberg 1973) with one g value at 9.4 and the two characteristic shoulders around the isotropic $g=4.28$ signal. When this sample was incubated under an argon atmosphere with 1 mM or 3 mM sodium dithionite buffered with Tris to pH 8.5 for 1 min at room temperature, the Fe(III) and most of the Cu(II) signals disappeared; a new broad signal occurred with the zero-crossing resonance around $g=4.3$ and a negative-going resonance at $g=2.0$ (possibly also in $g=2.2$–2.3) (Fig.1B). The g values and the shape of the new signal are typical of high-spin Co(II) ($S=3/2$). It is possible that this broad EPR spectrum could arise from two slightly different Co(II) species. A similar spectrum, but more intense, is observed after addition of sodium dithionite to Cd-Fd at pH 1.1.

If Co-Fd is titrated at pH 8.2 with sodium dithionite to the full development of the Co(II) signal and then exposed to air, the Co(II) resonance vanishes and the nearly rhombic high-spin Fe(III) signal reappears (Fig. 1C). This reoxidation by oxygen or hydrogen peroxide did not occur at pH 1.1. We were not able to detect any hyperfine or superhyperfine features in the reduced Co-Fd spectra as observed for cobalt-substituted ribulose-bisphosphate carboxylase (Nilsson et al. 1984). In the other preparations the buffers were filtered through Chelex-100 to avoid the contamination of iron in the preparation of Co-Fd.

The broad Co(II) resonance in dithionite-reduced Co-Fd is compared with other Co(II) signals in Fig. 2. It is clearly distinct from the green-colored argon-saturated solution of 75 mM Tris/HCl pH 8.2, 10 mM dithiotreitol 2 mM $CoCl_2$ (Fig. 2C). This EPR spectrum of the green-colored solution is more anisotropic and it has $g_{max}=6.0$ and a negative-going resonance around $g=2.0$; this spectrum is very similar that of the anaerobically isolated Co(II)-substituted metallothionein ($g=5.9$ and $g=2.0$) (Vasak and Kägi 1981); both are distinct from that of the reduced Co-Fd.

When a complex of Co(II) and dithiothreitol in the ratio 1:1 at pH 8.2 is exposed to air, the color changes from green to red-brownish as reported earlier (Skjeldal and Ljones 1988). This color turns to light brown after incubation with sodium dithionite, and the EPR spectrum from such a light-brown sample (Fig.2B) is similar to the reduced Co-Fd spectrum. Similar EPR spectra have been reported for different five- or six-coordinated complexes with Co(II)-substituted carbonic anhydrase (Bencini et al. 1981).

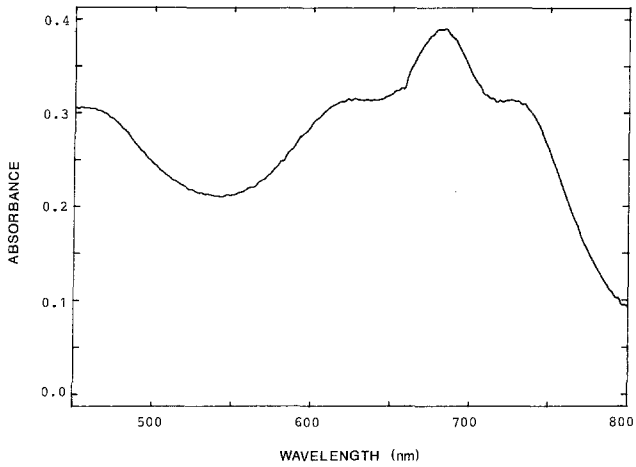

Fig. 3. Visible absorption spectrum of 2 mg/ml apoferredoxin incubated with 40 μM $CoCl_2$, 40 μM Na_2S and 0.1 M dithiothreitol in 50 mM Tris/HCl pH 8.0 under nitrogen atmosphere. The color of the solution was light green. The reference cuvette contained 40 μM $CoCl_2$, 40 μM Na_2S and 0.1 M dithiothreitol in the same buffer

Visible absorption spectrum

When apoferredoxin is incubated anaerobically with Co(II) and S^{2-} in 50 mM Tris/HCl with 0.1 M dithiothreitol at pH 8.2, a green derivative is seen. This Fd derivative is very air-sensitive, with a maximum at 680 nm ($\varepsilon = 1250\ M^{-1}\ cm^{-1}$), and shoulders at 450 nm ($\varepsilon = 1020\ M^{-1}\ cm^{-1}$), 620 nm ($\varepsilon = 1040\ M^{-1}\ cm^{-1}$) and 720 nm ($\varepsilon = 1040\ M^{-1}\ cm^{-1}$) in the visible spectrum (Fig. 3). This d-d profile, which disappears upon exposure to air, is characteristic of tetrahedral Co(II)-thiolate coordination and closely resembles that of the Co(II)-subtituted proteins rubredoxin (May and Kuo 1978) and metallothionein C (Vasak and Kägi 1981). The visible spectrum did not change when sulfide was omitted from the reaction mixture, but sulfide is needed to obtain the light-brown Co-Fd after air oxidation (Skjeldal and Ljones 1988). The light-brownish Co-Fd could not be reconverted to the green derivative by incubation in 0.1 M dithiothreitol under nitrogen atmosphere.

The ultraviolet and visible features of the aerobically isolated Co-Fd have been discussed in a previous paper (Skjeldal and Ljones 1988) and proved to be more stable in Tris/HCl and borate buffers than phosphate buffer at pH 8.2. Because Tris/HCl buffer interferes with protein resonances in the proton NMR, borate buffer was chosen as buffer in the NMR experiments.

^1H NMR at 300 MHz of the aerobically isolated Co-Fd

Diamagnetic proteins normally exhibit proton resonances from the amino acids in the range −2 ppm to +8 ppm when referenced internally to DSS; this is also the case for Co-Fd. The proton spectrum of Co-Fd, as isolated, at 300 MHz (Figs. 4 and 5) dit not show any paramagnetic downfield-shifted resonances, in contrast to native ferredoxin (Poe et al. 1970; our unpublished results). The proton spectrum differed from that of the native ferredoxin, and the signals in the aromatic region, 6.5–8 ppm, resemble more those of apoferredoxin from *Clostridium acidi-urici* (Packer et al. 1977).

None of the resonances moved away from their positions in the temperature range 4°–40° C.

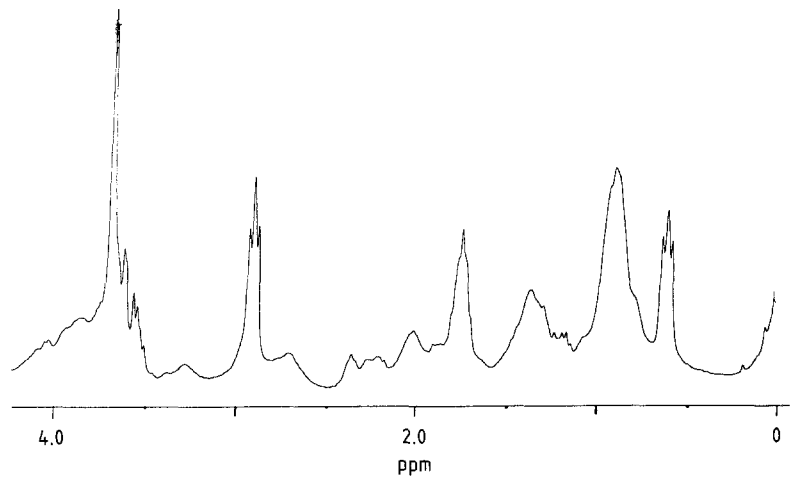

Fig. 4. Proton NMR at 300 MHz of 1 mM Co-Fd in 50 mM borate buffer pH* 8.0 showing the 0–4 ppm region. There were no signals between 0.5 ppm and −20 ppm, except the signal from DSS at 0 ppm. The spectrum is an average of 600 transients. Temperature is 297 K

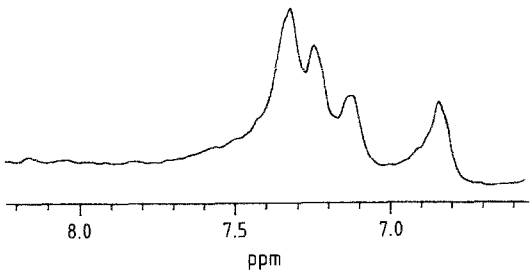

Fig. 5. Proton NMR at 300 MHz of 1 mM Co-Fd in 50 mM borate buffer pH* 8.0 showing the aromatic region, 6.0–8.0 ppm. No significant signals were found from 8 ppm down to 100 ppm. The spectrum is an average of 600 transients. Temperature is 297 K

This confirms a diamagnetic derivative, as proton resonances interacting with paramagnetic or spin-spin interacting centers show much stronger temperature dependence. No temperature-dependent magnetic susceptibility was found in the temperature range used in this work. The difference frequency (which reflects the presence of paramagnetism), $\Delta\nu$, of the marker protons was less than 1 Hz. These studies established that the air-isolated Co-Fd was diamagnetic.

The above results rule out two spin-interacting Co(II), as at these temperatures some of the excited paramagnetic states should have been thermally populated, Co(III) with $S=1$ or 2, and Co(I) with $S=1$. Thus the cobalt atoms in Co-Fd are low-spin Co(III) ($S=0$), as a Co(II) state is observed after addition of sodium dithionite. This experiment also showed that the preparation was not contaminated by detectable amounts of Fe(III) which would have influenced the magnetic susceptibility measurements.

Discussion

Co(II) can form many complexes which exhibit oxygen-carrying properties; reversible oxygen uptake in solutions of Co(II) in the presence of α-amino acids and peptides has been known for some time. The high-spin octahedral complex bis(histidinato)cobalt(II) reacts rapidly with molecular oxygen in aqueous media to give a diamagnetic, binuclear oxygenated complex. Accompanying this rapid reversible dioxygen uptake, a slow irreversible uptake of more O_2 or H_2O_2 can lead eventually to the formation of Co(III) complexes (Erskine and Field 1976).

In the case of aerobically prepared Co-Fd, we could not detect any cobalt EPR signal. Few cobalt substitutions in proteins with only sulfur ligands have been made, but Co(II)-substituted metallothionein behaves in the same way as Co(II) incubated with apoferredoxin: the green metallothionein derivative with Co(II) ligated to the sulfur atoms is highly air-sensitive and yields a light-yellow product upon exposure to air (Vasak and Kägi 1981). In that case only the green derivative was characterized, while here we primarily try to characterize the air-isolated light-brown, Co-Fd.

The EPR results indicate that Co-Fd, as isolated, has a higher oxidation state than Co(II) for three reasons.

a) After incubation of Co-Fd at pH 1.1, the color bleaches but the Co is still ERP-silent. Any magnetic interaction between two Co(II) atoms or one high-spin Co(III) and one Co(II), should have been perturbed at this low pH.

b) It is highly unlikely that dithionite could have reduced one Co(II) to the rare oxidation state Co(I), making the other magnetically coupled Co(II) EPR-active.

c) Attempts to oxidize Co-Fd with ferricyanide did not reveal any Co(II) signals, while a Co(II) signal was observed after reduction with dithionite both at pH 8.2 and pH 1.1. Furthermore, the reduction with dithionite was reversible at pH 8.2, as for Co(III)-substituted carbonic anhydrase (Shinar and Navon 1979).

The Co(II)-Fd EPR spectrum is clearly different from tetrahedral thiolate-coordinated Co(II). Ligands other than sulfur may be involved, but it is impossible to draw a conclusion on the coordination geometry based only on the shape of the EPR spectrum (Makinen et al. 1985). Furthermore, it is not possible to quantify the EPR-active high-spin Co(II) without knowledge of its zero-field splitting parameters, which are difficult to determine. Sulfur coordination is also suggested for the cobalt in Co(III)-Fd from preliminary resonance Raman experiments at 25 K, with an excitation wavelength of 472.6 nm. The resonance-enhanced Raman spectrum is similar to the spectrum obtained from the light-brown Co(III)-dithiothreitol complex without any sulfide [the corresponding Co(II) EPR spectrum is shown in Fig. 2] and they are clearly distinct from Raman spectra from Co(II)-rubredoxin (May and Kuo 1978) or tyrosinate proteins (Andersson, K. K., Lutz, M., Ljones, T. and Skjeldal, L., unpublished observations). It was not possible to reoxidize the protein at pH 1.1, probably because this low pH destroyed the protein structure.

It is interesting to note that an EPR spectrum similar to rubredoxin was observed in the prepa-

ration that was contaminated with iron. This indicates that iron is able to bind to the protein giving rise to a slightly distorted rubredoxin-like ferredoxin. The existence of a mixed cluster with iron and cobalt is hardly likely when one compares this EPR spectrum with earlier reports on mixed clusters. The cobalt derivative of *Desulfovibrio gigas* FdII was prepared by anaerobic incubation of the native [3Fe-4S] cluster for 6–10 h in the presence of dithionite, dithiothreitol and cobalt(II) (sulfide was omitted). Different spectroscopic methods showed that a [3Fe,Co-4S] cubane-like structure was formed and that this mixed metal cluster was paramagnetic. In the oxidized state the cluster exhibited eight well resolved ^{59}Co hyperfine lines in the EPR spectrum. The ^{59}Co hyperfine structure was broadened by ^{57}Fe, showing that Fe and Co were in the same complex (Moura 1987).

The absorption spectrum of the green Co(II) derivative between 600–800 nm corresponds to d-d transitions as expected for distorted tetrahedral high-spin Co(II) (May and Kuo 1978); this was not found in the air-isolated or dithionite-reduced Co-Fd. The intensities and positons of the d-d bands resemble those of isolated tetrahedral Co(II)-tetrathiolate clusters (Vasak and Kägi 1981) but disappear upon exposure to air. This d-d profile was not altered by the presence of sulfide, which indicates that sulfide is only involved in the air oxidation to form Co-Fd. The ultraviolet/visible absorption spectrum features of Co-Fd have been discussed in a previous paper (Skjeldal and Ljones 1988) but the need for the presence of sulfide in the reaction mixture to obtain a stable product is still not understood. The differences in the absorption spectra between dithionite-reduced Co-Fd and anaerobic apoferredoxin with Co(II) may reflect a change in the coordination geometry of the cobalt atoms and/or presence of non-thiolate ligands. Because sulfide is needed to obtain Co-Fd, but is not found in the product (Skjeldal and Ljones 1988), it is probably involved in a redox reaction together with oxygen.

^{1}H-NMR and magnetic susceptibility measurements of air-isolated Co-Fd showed that the protein was diamagnetic, supporting the lack of EPR signals and demonstrating once again the presence of two low-spin Co(III) ($S=0$). The NMR spectrum was much less complicated in the aromatic region than that found for the native form (Poe et al. 1970 and our unpublished results) and may be helpful in future work with the assignments of the proton signals from native ferredoxin together with the ^{1}H NMR of apoferredoxin.

In conclusion, these spectroscopic studies indicate that Co(II) can bind to apoferredoxin under anaerobic conditions, forming a green derivative with tetrahedral coordination geometry, but it is oxidized to a light-brown derivative with low-spin Co(III) in the presence of sulfide and oxygen. The Co(III)-Fd can be reduced to a Co(II) derivative with higher than tetrahedral coordination geometry (at least with only thiolate ligations), and reoxidized to the Co(III) state.

Co(III) has a d^6 configuration, and all Co(III) complexes investigated in solutions are low-spin octahedral, and therefore diamagnetic (Shinar and Navon 1979). The type of ligands and the coordination geometry of the two Co(III) atoms in Co-Fd is still, however, not well understood.

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