# High-frequency EPR on high-spin transitions-metal sites

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# Appendix C

# EPR spectroscopy on Co(II)-rubredoxin

## C.1 Introduction

In Chapter 3 of this thesis the effect is investigated of the subtle differences in geometry between the active sites of rubredoxin and desulforedoxin on the electronic structure of these sites, which contain in their oxidized state a high-spin $Fe^{3+}$ ion. EPR spectra at J band (275.7 GHz) were recorded on frozen solutions of rubredoxin, see Chapter 2, and desulforedoxin, see Chapter 3, and from these spectra an accurate set of spin-Hamiltonian parameters describing the active site of each protein could be determined. Particularly the difference in the rhombicity of the zero-field splitting tensor, $\lambda$, is remarkable: 0.26 for rubredoxin, and 0.074 for desulforedoxin.

In Chapter 3 X-band (9.5 GHz) spectra are reported on a frozen solution of Co(II)-substituted desulforedoxin. From these spectra the value of $\lambda$ of the Co(II)-substituted active site of desulforedoxin was estimated to be 0.26. In order to quantify also the effect of the geometry differences between rubredoxin and desulforedoxin on the electronic structure of the high-spin $Co^{2+}$ containing active sites, it would be desirable to have data on the electronic structure of the Co(II)-substituted active site of rubredoxin. Unfortunately we were not successful in detecting an EPR signal of a frozen solution of Co(II)-substituted rubredoxin. Here we report our attempts and discuss possible explanations for the absence of an EPR signal.

## C.2 Materials and methods

Rubredoxin from *Desulfovibrio gigas* was purified according to a previously reported method. [47] Reconstitution with $Co^{2+}$ was performed as described in reference [81]. The protein was kept in Tris buffer at pH 7.6. Samples for X-band EPR contained 20% glycerol.

Continuous-wave (cw) and pulsed X-band spectra were recorded on the Bruker Elexsys E680 spectrometer using the $TE_{102}$ rectangular cavity equipped with the ESR 900 Cryostat (Oxford Instruments) and the FlexLine probe head with the ER4118 X-MD5 resonator with a CF935W flow cryostat, respectively. Pulsed W-band (94.9 GHz) spectra were recorded on an in-house developed spectrometer. [82] The cw J-band EPR spectra were recorded on an in-house developed spectrometer [9] using a probe head, specialized for operation in cw mode [64].

## C.3 Results

Figure C.1 shows the cw X-band EPR spectra of a 400 $\mu$M frozen solution (from batch 1) of Co(II)-substituted rubredoxin at 5 and 40 K. The spectra show a broad, positive signal from 0 to about 100 mT. Figure C.2 shows the cw X-band EPR spectra of a 1.6 mM frozen solution (from batch 2) of Co(II)-substituted rubredoxin at 10 and 20 K. These spectra also show a broad, positive signal from 0 to about 100 mT, but its shape differs from the signal observed on the 400 $\mu$M frozen solution.

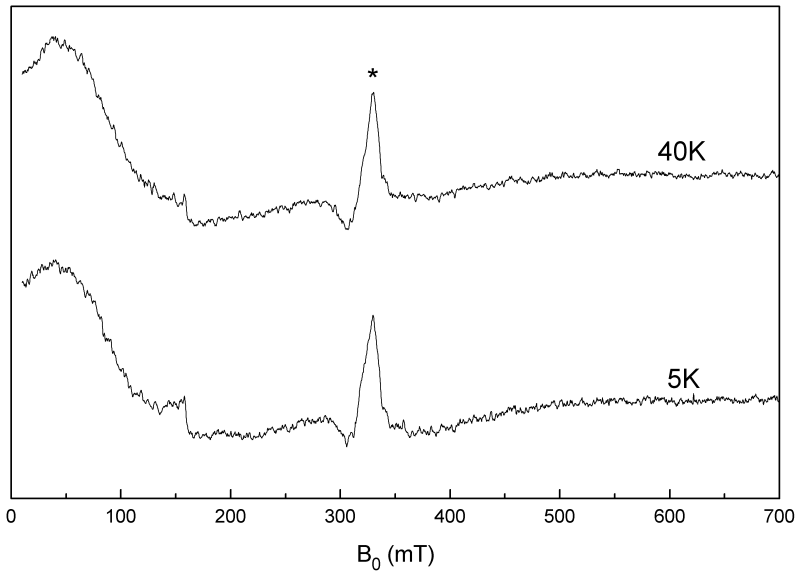


**Figure C.1:** X-band cw EPR spectra of a 400 $\mu$M frozen solution (batch 1) of Co(II)-rubredoxin from *D. gigas* at 5 and 40 K. Experimental conditions: modulation amplitude: 1.5 mT, microwave power: 200 mW, microwave frequency: 9.495 GHz. The spectra are baseline corrected. The signal marked with a $\star$ around $g = 2$ is due to an impurity in the cavity/cryostat.

All four spectra show a signal around $g = 2$ (339 mT). This signal is due to an impurity in the cavity/cryostat. The spectra for batch 1 show a signal around

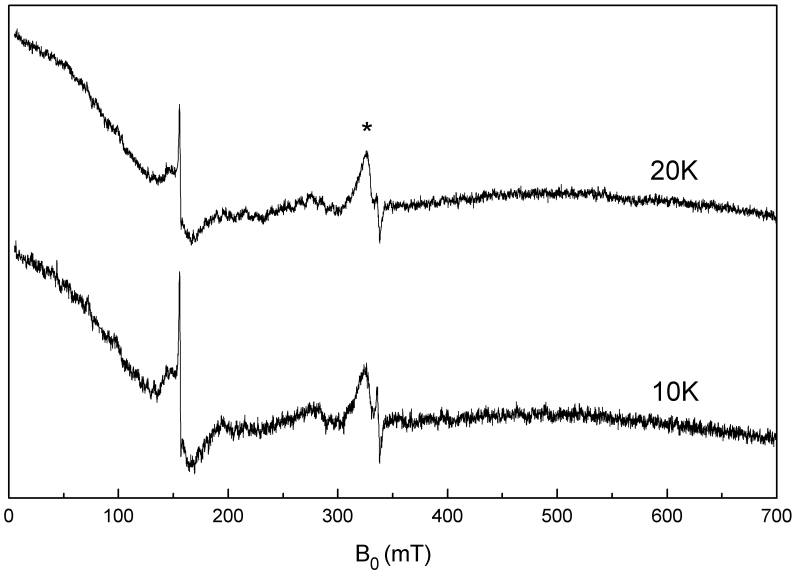


**Figure C.2:** X-band cw EPR spectra of a 1.6 mM frozen solution of Co(II)-rubredoxin from *D. gigas* at 10 and 20 K. Experimental conditions: modulation amplitude: 1 mT, microwave power: 20 mW, microwave frequency: 9.495 GHz. The spectra are baseline corrected. The signal marked with a $\star$ around $g = 2$ is due to an impurity in the cavity/cryostat.

$g = 4.3$ (158 mT), which looks like a jump down in the EPR signal. The spectra for batch 2 also show a signal around $g = 4.3$, but this signal shows a clear structure. In the spectra for batch 2 two weak positive peaks show up at 190 and 275 mT.

Figure C.3 shows the cw X-band EPR spectra of a 400 $\mu$M frozen solution (batch 1) of Co(II)-substituted rubredoxin at 6, 20, 25 and 40 K to which $NaNO_3$ was added up to a concentration of 100 mM to increase the ionic strength of the solvent and hamper aggregation of the protein. These spectra do not show the broad signal at low field, but they do show the cavity impurity at $g = 2$, the signal at $g = 4.3$ and at 20 and 25 K the two weak positive peaks at 190 and 275 mT.

Attempts to detect a cw EPR spectrum at 5 and 25 K on a concentrated 10 mM solution of Co(II)-rubredoxin (batch 1) at J band were unsuccessful, as were attempts to detect a spin echo on a 1.6 mM frozen solution (batch 2) at 1.7 K at W band and at temperatures varying from 6 to 15 K at X band. The UV/VIS optical absorption spectrum of the batch 1 solution was measured and found to be similar to spectra reported in literature. [177] [81]

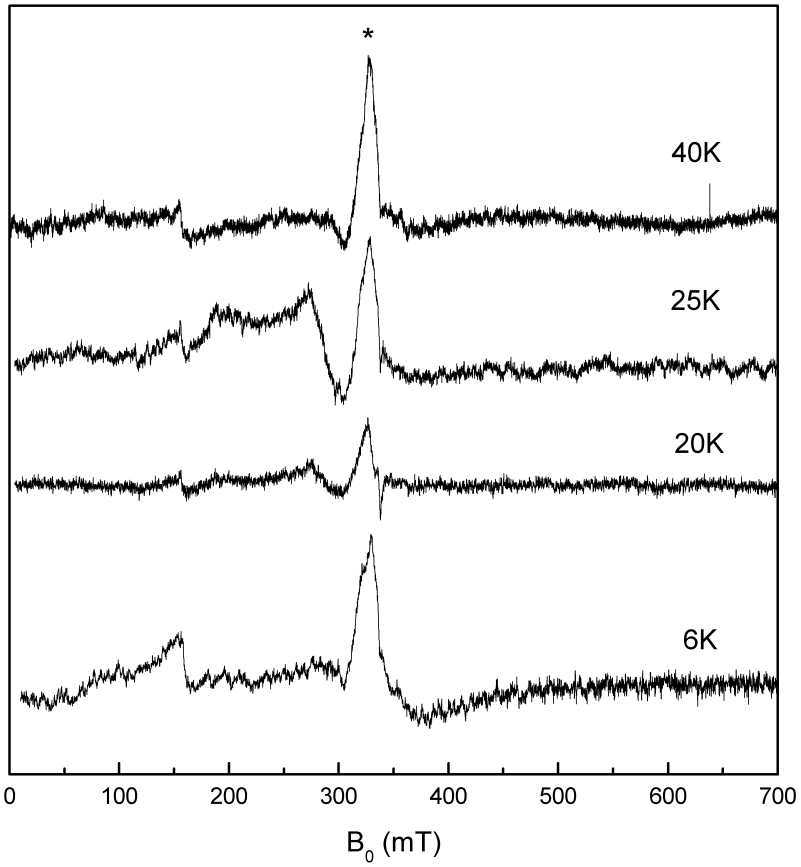


**Figure C.3:** X-band cw EPR spectra of a 400 $\mu$M frozen solution of Co(II)-rubredoxin from *D. gigas* in 100 mM $NaNO_3$ at 6, 20, 25 and 40 K. Experimental conditions spectra 6 and 40 K: modulation amplitude: 1.5 mT, microwave power: 200 mW, microwave frequency: 9.495 GHz. Experimental conditions spectra 20 and 25 K: modulation amplitude: 1 mT, microwave power: 20 mW, microwave frequency: 9.495 GHz. The spectra are baseline corrected. The signal marked with a $\star$ around $g = 2$ is due to an impurity in the cavity/cryostat.

## C.4 Discussion

The observed broad, positive signal from 0 to about 100 mT can not arise from a high-spin $Co^{2+}$, $S = 3/2$, system. A transition at $B_0 = 0$ mT can not result from a transition within a Kramers doublet. The signal can not be due to an inter-doublet transition, since the zero-field splitting between the two doublets is expected to be much larger than 9.5 GHz for $Co^{2+}$. The shape of the low-field signal is slightly different for the two batches/concentrations. The signal is no longer observed if the ionic strength of the solution is increased, which may prevent aggregation of the

protein. Possibly it is due to interacting $Co^{2+}$ sites. An $S = 3$ could give a signal starting at zero field in X band.

The EPR spectra of biological materials often show a small signal around $g = 4.3$ of the shape found in the spectra in Figure C.1 and C.3, which is thought to be due to a rhombic high-spin $Fe^{3+}$ contaminant, sometimes referred to as "garbage" iron. [178] The shape of the $g = 4.3$ signal in the spectra in Figure C.2, however, is characteristic of Fe(III)-rubredoxin. [58] In spite of the reconstitution with $Co^{2+}$, batch 2 still contains a fraction of Fe(III)-rubredoxin. This observation is remarkable, since May and Kuo state in reference [177] that the apoenzyme binds cobalt preferentially over iron. If the ZFS-tensor is close to axial and $D > 0$, a signal at $g = 4.3$ could arise at low temperatures due to high-spin $Co^{2+}$. However, the shape of this signal, taking into account broadening by conformational strain, will be considerably different from the $g = 4.3$ signal we observed.

The appearance of the two weak signals at 190 and 275 mT in the spectra is inconsistent. They likely arise from an impurity.

A possible explanation of the absence of an EPR signal from the Co(II)-substituted active site of rubredoxin is the following. If the ZFS-tensor of the site is close to axial, $\lambda \approx 0$, and $D < 0$, the transition within the lowest $\pm 3/2$ doublet is forbidden. A transition within the $\pm 1/2$ is allowed, but this doublet becomes only populated at elevated temperatures, at which the notoriously short relaxation times of high-spin $Co^{2+}$ may have broadened the transition beyond detection.

Observation of an EPR spectrum at X band from Co(II)-rubredoxin was reported by Moura *et al.* [81], but the spectrum was not shown in this paper. Instead the reader is referred to a paper by Good and Vašák [179] in which an X-band EPR spectrum of Co(II)-metallothionein is shown. According to Moura *et al.* the spectrum of Co(II)-rubredoxin is similar.