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Analyzing Xanthine Dehydrogenase Iron-Sulfur Clusters Using Electron Paramagnetic Spectroscopy

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Abstract

Analyzing Xanthine Dehydrogenase Iron-Sulfur Clusters Using Electron Paramagnetic Resonance Spectroscopy. RACHAEL HODSON (Oklahoma State University, Stillwater, OK **74078-1012)** GRAHAM GEORGE (Stanford Linear Accelerator Center, Menlo **Park, CA** 94025).

Xanthine dehydrogenase is a metalloenzyme that is present in a variety of eukaryotic and prokaryotic organisms. The oxidation of the xanthine occurs at the molybdenum site, and the catalytic cycle is completed **by** electron transfer to the iron-sulfur (Fe/S) clusters and finally the flavin, where they are accepted by nicotinamide adenine dinucleotide **(NAD).** Since the site giving **rise** to the Fe/S **I** electron paramagnetic resonance (EPR) signal is thought to be the initial recipient of the electrons from the Mo, we wish to understand which **EPR** signal is associated with which Fe/S cluster in the structure in order to develop an understanding of the electron flow within the molecule. Samples of xanthine dehydrogenase wild-type **and** mutant forms were analyzed with EPR spectroscopy techniques at low and **high** temperatures. The results showed an altered Fe/S I signal along with an unaltered Fe/S II signal. The converted Cysteine, in the mutant, did affect the Fe/S cluster immediately adjacent to it. Therefore, the Fe/S **I** signal arises from the Fe/S cluster closest to the Mo and immediately adjacent to the mutated **amino** acid, and the Fe/S II signal must arise from the more distant Fe/S cluster.

Table of Contents

Introduction

Xanthine dehydrogenase is a metalloenzyme that is present in a variety of eukaryotic and prokaryotic organisms. It catalyses the oxidation of the purine xanthine to uric acid (Figure 1) that is subsequently excreted in the urine.

Xanthine dehydrogenase is a protein of approximate molecular weight 275,000 that contains molybdenum, two different iron sulfur (Fe/S) clusters, and flavin (Figure 3). The molybdenum is coordinated by the dthiolene side chain of a novel pterin cofactor that is called molybdopterin. Molybdopterin consists of a pterin joined with a pyranoditholene moiety as shown in Figure 2 (Johnson et al., 1982). The exact function of the pterin cofactor is unknown, but it may mediate electron transfer to redox-active sites, and modulate the molybdenum reduction potential (Hille, 1996). The aromatic structure of molybdopterin allows for delocalization of electrons throughout the cofactor (Figure 2), which would make it ideally suited as a mediator of electron transfer.

The oxidation of the xanthine occurs at the molybdenum site, and the catalytic cycle is completed by electron transfer to the Fe/S clusters and finally the flavin, where they are accepted by nicotinamide adenine dinucleotide (NAD). The electron paramagnetic resonance (EPR) spectroscopic properties of the active sites of *Rhodobacter capsulatus* xanthine dehydrogenase have not yet been described, but the analogous mammalian enzymes have been extensively studied. In this enzyme each Fe/S cluster has a unique EPR signal, and we expect the *R. capsulatus* enzyme to be similar. We wish to understand which EPR signal is associated with

which Fe/S cluster in the structure in order to develop an understanding of the electron flow within the molecule.

The Fe/S I signal of the mammalian enzyme can be observed at temperatures up to 40 K. The Fe/S II signal is only observed at temperatures below 22 K, and has broader lines than Fe/S I signal. Also, the redox potentials of the two Fe/S clusters differ. Fe/S I signal has a redox potential of –310 mV at 25 degrees Celsius, while the Fe/S II signal's redox potential is –235 mV (Enroth et al., 2000). The site giving rise to the Fe/S I EPR signal is thought to be the initial recipient of the electrons from the Mo (Hille, 1996).

Here we present an EPR spectroscopic analysis of both wild-type and a mutant *R. capsulatus* xanthine dehydrogenase. In the R135C mutant arginine 135 of the native enzyme is converted to Cysteine. This amino acid is located immediately adjacent to one of the two Fe/S clusters, and is expected to alter the EPR signal of the nearby Fe/S cluster. By comparing the EPR signals of the wild-type and mutant proteins it should be possible to distinguish which Fe/S cluster gives the altered EPR signal, and thus determine the physical origin of the signal.

Materials and Methods

The samples analyzed include: xanthine dehydrogenase wild-type and mutant. These two samples were run at low and high temperatures. The high temperature spectra effectively show isolated Fe/S I signals. To prepare the samples, *R. capsulatus* xanthine dehydrogenase was expressed in *Eshcerichia coli* and purified by collaborators in Braunschweig, Germany (Truligo et al., 2002). Samples for EPR spectroscopy (0.1 – 0.5mM enzyme) were prepared in 3mm

internal diameter quartz tubes by reduction of the enzyme by an excess (10mM) of sodium dithionite solution in 0.1M bis-tris-propane buffer at pH 7.0. In this reduced state both the iron-sulfur clusters in the protein are in the fully reduced mixed valent $[Fe_2S_2]^+$ paramagnetic oxidation state (Figure 4).[1]

Electron Paramagnetic Resonance (EPR) spectra were measured on a JEOL RE1X spectrometer equipped with an Oxford Instruments ESR 9 liquid helium flow cryostat. The spectrometer was operated at X-band microwave frequency (9.1GHz). Accurate spin Hamiltonian parameters (*g*-values and linewidths) for each spectrum were obtained by conducting EPR simulations using a second order perturbation theory approximation.

Microwave frequency was measured using a Hewlett Packard 5350B microwave frequency counter. Magnetic field was calibrated with reference to a diphenylpicrylhydrazyl standard, which has a g-value of 2.0037. Magnetic field modulation amplitude was set at 10 Gauss with a modulation frequency of 100KHz.

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[1] In aqueous solution at room temperature, the dithionite anion $[S_2O_4]^{2-}$ dissociates to form the $[SO_2]^{\bullet-}$ radical anion. This is a very good one-electron reducing agent (the product being SO_2). In frozen solution the equilibrium is shifted towards the EPR-silent $[S_2O_4]^{2-}$ anion, resulting in negligible levels of the $[SO_2]^{\bullet-}$ radical anion (Cotton et al., 1999). This is convenient for EPR spectroscopy as the paramagnetic $[SO_2]^{\bullet-}$ radical anion would otherwise totally dominate the EPR signal, making protein signals difficult to observe.

Results

Figure 5 shows the EPR spectra obtained from wild-type *R. capsulatus* xanthine dehydrogenase. The EPR signals are clearly related to those previously observed for xanthine oxidases, but with some important differences. The Fe/S I spectra is seen as an axial signal. The Fe/S II signal appears very similar, and both signals have similar temperature dependence. Figure 6 shows the EPR spectra obtained from the R135C mutant enzyme. Two distinct Fe/S EPR signals are observed. The Fe/S I signal was not observed at high temperature, because the R135C mutation possibly causes a changed temperature dependence. As the temperature increased, both the Fe/S I and Fe/S II signals were both lessened, and the Fe/S I signal was not readily isolated.

The g-values calculated for the wildtype Fe/S I signal are: $g_{x,y} = 1.9217$ and $g_z = 2.0222$. The g-values calculated for the wildtype Fe/S II signal are: $g_x = 1.8964$, $g_y = 1.9711$ and $g_z = 2.073$. The g-values calculated for the mutant Fe/S I signal are: $g_x = 1.9205$, $g_y = 1.9205$, and $g_z = 2.012$. Adequate computer simulations were obtained using g-values for the Fe/S II signal of the mutant enzyme that were identical to the wild-type ($g_x = 1.8964$, $g_y = 1.9711$, and $g_z = 2.073$).

Discussion and Conclusions

The results show that the Fe/S I signal is altered in the mutant, while the Fe/S II signal remains unaffected. The conversion of arginine to cysteine is expected to most profoundly affect the Fe/S cluster immediately adjacent to it. Therefore, the Fe/S I signal must arise from the Fe/S cluster closest to the Mo and immediately adjacent the mutated amino acid. As well, the Fe/S II signal must arise from the Fe/S cluster farther away from the Mo and the mutated amino acid. For definitive evidence, a high temperature spectra of the mutant is needed to show that the Fe/S I

signal is affected and the Fe/S II signal is not. This information should allow insights into the mechanism of electron transfer within the protein. In particular, the aromatic pterin cofactor may act as a conductor to the nearby Fe/S cluster (Fe/S I), and may therefore have a direct role in electron flow from the Mo site.

Acknowledgements

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Figures

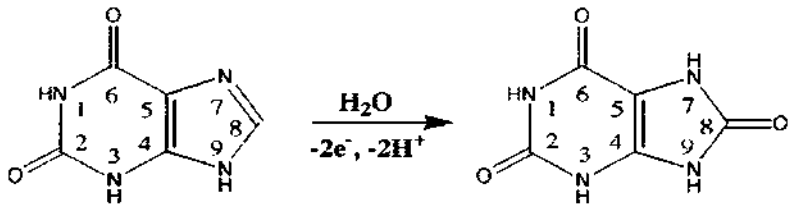

Figure 1. Oxidation of xanthine to uric acid.

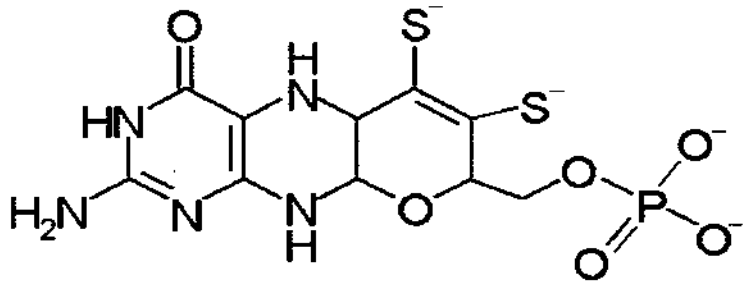

Figure 2. Structure of molybdopterin.

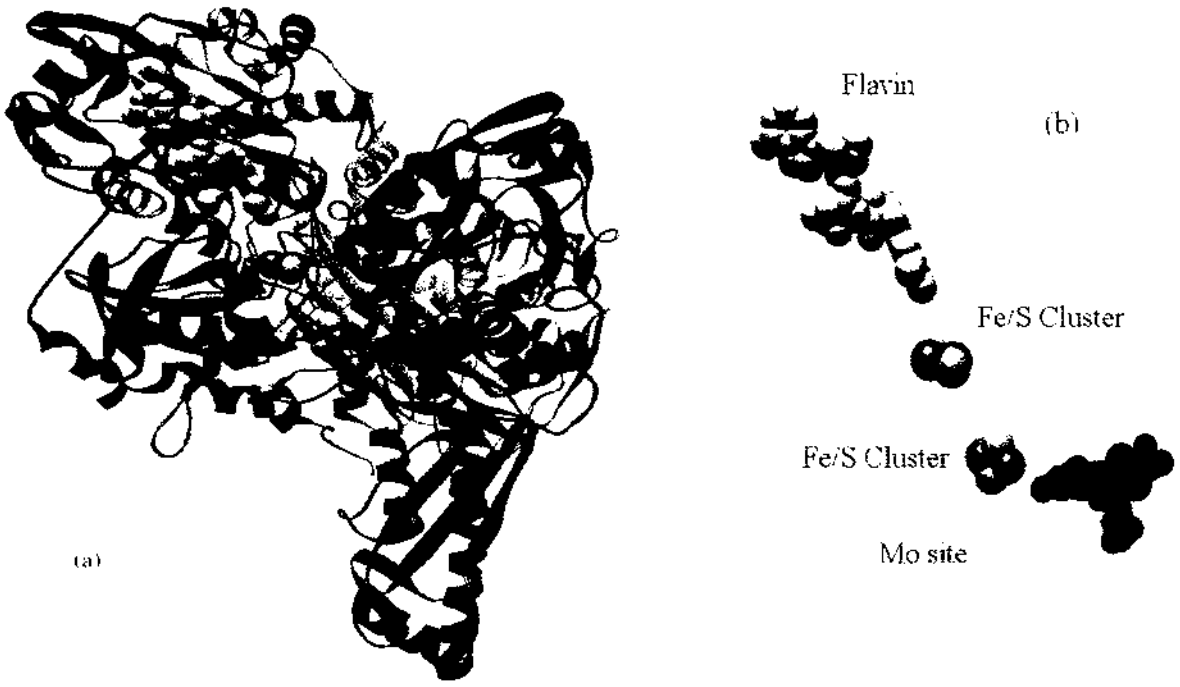

Figure 3. Crystal structure of xanthine dehydrogenase.
(a) Structure of one molecule showing the polypeptide chain as a ribbon.
(b) Detail of (a) showing the Mo site, the pterin cofactor, the Fe/S clusters, and the flavin

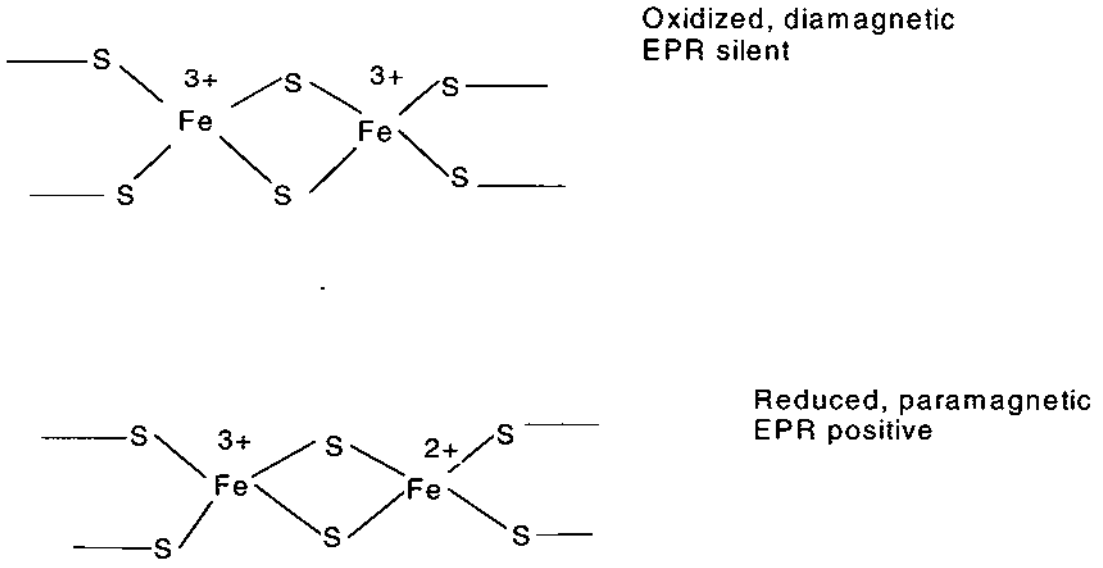

Figure 4. Fe/S clusters shown in oxidized and reduced states.

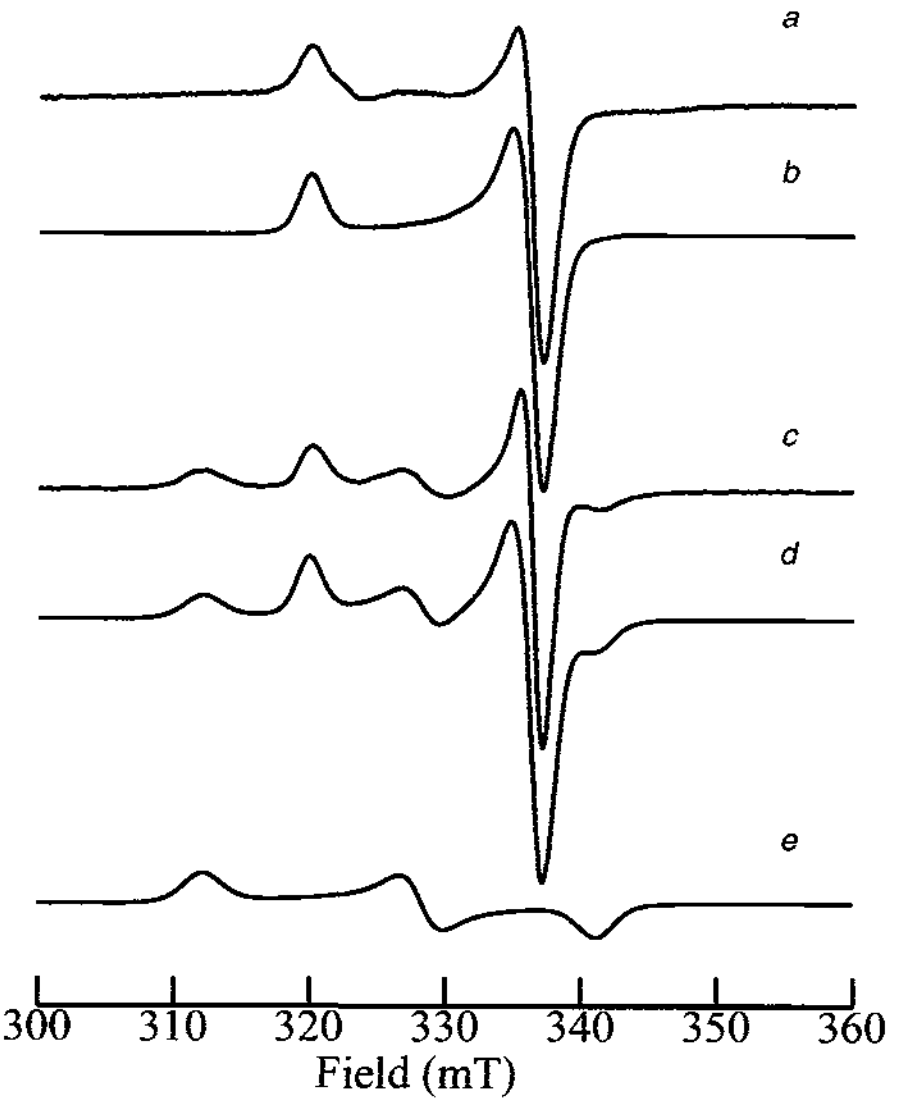

Figure 5. Wild type EPR – *a* Fe/S I signal at 42K, *b* simulation of *a*, *c* signal at 18K, *d* simulation of *c* made up of *b* + *e* added together with equal spin integrations from both. *e* simulation of Fe/S II signal, derived from summed simulation *d*.

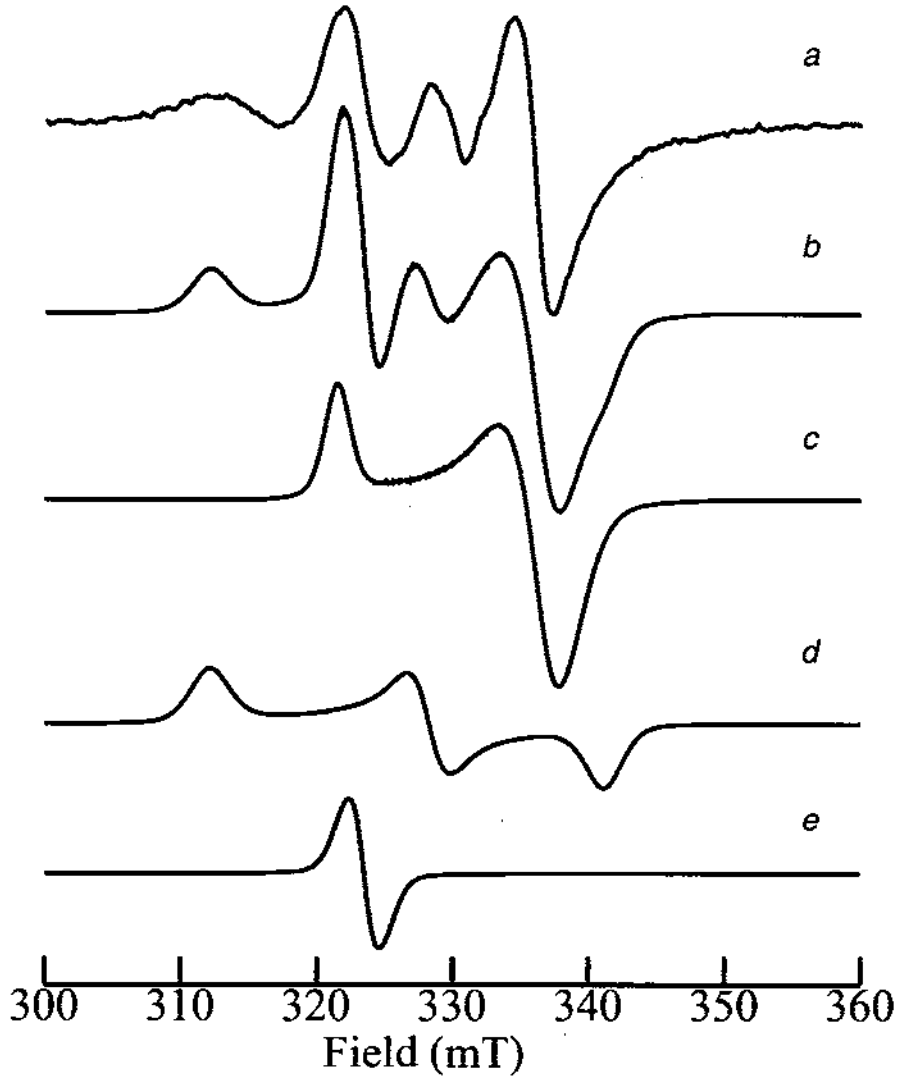

Figure 6. R135C mutant EPR – *a* Fe/S signals at 18K, *b* simulation of *a* made up of *c* + *d* added together with equal spin integrations from both, plus *e*. *c* simulation of Fe/S I signal, *d* simulation of Fe/S II signal using parameters from wild-type enzyme, *e* simulation of flavin.