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Characterization of the Iron-Sulfur Clusters in Xanthine Dehydrogenase Using Electron Paramagnetic Resonance Spectroscopy and Magnetic Coupling Interactions

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Abstract

Characterization of the Iron-Sulfur Clusters in Xanthine Dehydrogenase Using Electron Paramagnetic Resonance Spectroscopy and Magnetic Coupling Interactions. J. ROBERT SCOTT (Antioch College, Yellow **Springs,** OH 45387) **GRAHAM** GEORGE (Stanford Linear Accelerator Center, Palo Alto, **CA** 943905).

Xanthine dehydrogenase is a metalloenzyme that is present in numerous eukaryotic and prokaryotic organisms. It contains molybdenum, two different iron-sulfur clusters, and flavin. While the structures of both iron-sulfur clusters were known, it was unclear **as** to which structure was in which location. Electron paramagnetic resonance spectroscopy probes the paramagnetic qualities of molecules or ions. With this technology we wished to understand which EPR spectrum was associated with which iron-sulfur cluster by looking at magnetic coupling between the paramagnetic Mo(V) oxidation state and **the** reduced iron-sulfur clusters. We then assigned the clusters to their corresponding locations. The spin-spin interactions observed between Mo(V) and Fe-S I in xanthine dehydrogenase at low temperature show that Fe-S I is the closer site in contrast to Fe-S II.

Introduction

The metalloenzyme xanthine dehydrogenase is present in numerous eukaryotic and prokaryotic organisms, from bacteria to man. A metalloenzyme is simply an enzyme that contains a metallic element. Xanthine dehydrogenase (XDH) catalyzes the oxidation of the purine xanthine to uric acid:

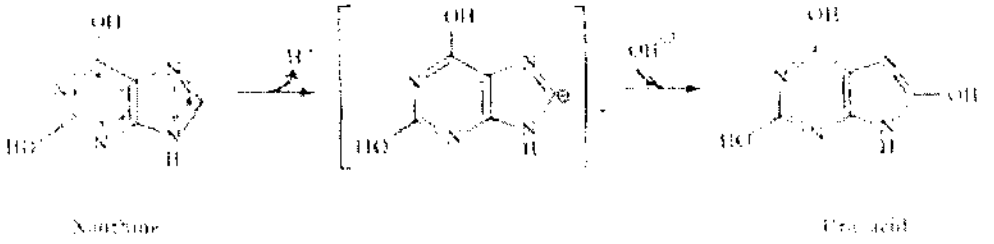

Figure 1. Oxidation of xanthine to uric acid.

Xanthine dehydrogenase contains molybdenum, two different iron-sulfur clusters, and flavin. The oxidation of the xanthine occurs at the molybdenum site, and the catalytic cycle is completed by electron transfer first to the iron-sulfur clusters and finally to the flavin where they are accepted by NAD (Olson et al, 1974). The molybdenum is part of a larger structure called the molybdenum cofactor (Hille, 1996), which is often called molybdopterin (Johnson et al, 1984) but this nomenclature can be confusing because "molybdopterin" refers to the pterin cofactor in its molybdenum-free form.

Figure 2. Structure of molybdopterin.

The function of the molybdopterin is unknown, but it probably has roles in electron transfer, and modulating the electronic properties of the molybdenum.

The structures of both iron-sulfur clusters are known, but it is unclear as to which structure is in which location. Electron paramagnetic resonance spectroscopy (EPR) is very sensitive to subtle changes in the environment of paramagnetic species so the two different clusters will give distinct EPR spectra. EPR , sometimes called electron spin resonance (ESR), probes the paramagnetic qualities of molecules or ions. To be paramagnetic a molecule or ion must contain one or more unpaired electrons. We wish to understand which EPR spectrum is associated with which cluster in the structure by looking at magnetic coupling between the paramagnetic Mo(V) oxidation state and the reduced iron-sulfur clusters. We can then assign the clusters to their corresponding locations.

Figure 3. X-ray crystal structure of xanthine dehydrogenase.

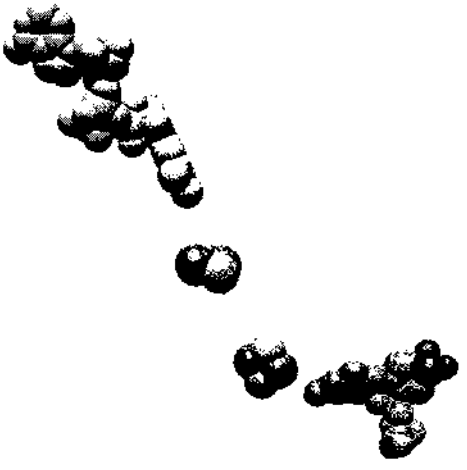

Figure 4. Flavin (yellow), iron-sulfur compounds (yellow and blue), and molybdopterin (red and green) cofactor shown without the protein (from figure 3).

Materials and Methods

Rhodobacter capsulatus xanthine dehydrogenase was expressed in *Eshcerichia coli* and purified by collaborators in Braunschweig as described (Truligo et al). In 3mm internal diameter quartz tubes, EPR spectroscopy samples (0.1 – 0.5 mM enzyme) were prepared by reduction of the enzyme with an excess (10mM) of sodium dithionite solution in 0.1M bis-tris-propane buffer at pH 7.0. Thereby putting both enzymatic iron-sulfur clusters in the fully reduced mixed valent [Fe2S2]+ paramagnetic oxidation state. Samples containing the paramagnetic Mo(V) oxidation state were prepared by mixing with 50% ethylene glycol and reduction with dithionite for 3 hours. Under these conditions a stable Mo(V) form of the enzyme was generated.

Electron Paramagnetic Resonance (EPR) spectra were measured on a JEOL RE1X spectrometer equipped with an Oxford Instruments ESR 9 liquid helium flow cryostat. Readings were taken at 42K and 100K. The spectrometer was operated at X-band microwave frequency (9.1GHz).

Microwave frequency was measured using a Hewlett Packard 5350B microwave frequency counter. Magnetic field calibrated with reference to a diphenylpicrylhydrazyl standard, having a g-value of 2.0037.

Magnetic field modulation amplitude was set to 0.2 mT using a modulation frequency of 100KHz.

Results

The high and low temperature spectra of xanthine dehydrogenase and a low temperature demolybdo sample spectra showing just Fe-S I are illustrated in Figure 5. Clear differences in both the Fe-S I and the Mo(V) EPR signals are observed when both are present (i.e. in the low temperature spectrum of Figure 5). The high temperature signal is predominantly a typical Mo(V) EPR spectrum with a small contribution from the flavin semiquinone free radical (the symmetric peak near 322.5mT, g=2.0, Figure 5) . At the high temperature the Fe-S I EPR signal is sufficiently broadened (by rapid relaxation) so as to be non-visible. Similarly, spin-spin interactions involving this site will not be visible under these conditions. At the lower temperature of 35 K the major relaxation mechanisms of Fe-S I are frozen out, and the signal is sharp enough to be observed. The Mo(V) EPR signal shows clear differences between high and low temperature forms, indicating that spin-spin interaction is occurring. The spectra of the demolybdo sample, also shown in figure 5, allows us to examine the effects of Mo(V) on Fe-S I (as opposed to the other way around). Clear broadening of the Fe-S I spectrum is observed in the presence of Mo(V) and this is attributed to the same spin-spin interaction that modifies the Mo(V) EPR spectrum. This is the first time that an effect on Fe/S I has been reported, probably because the Fe-S I EPR signal from *R. capsulatus* XDH is much sharper than the corresponding signal observed in related enzymes.

The Mo(V) spectra are more complex than those observed with xanthine oxidase. Figure 6 shows bovine milk xanthine oxidase Mo(V) EPR signals at high temperature (123K) and at low temperature (35K) (the Fe-S I features are outside the range of these spectra).

The xathine oxidase shows a simple rhombic Mo(V) spectrum which is split at low temperature by interaction with Fe-S I (figure 6). In contrast, the XDH spectrum is clearly a mixture of species (showing more than three features) and quantitative analysis by computer simulation was therefore not attempted. Nevertheless, the presence of the broadening in the Fe-S I signal and the changes in structure of the Mo(V) signal conclusively indicate the presence of electron spin coupling between the sites.

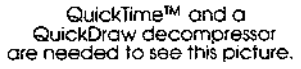

Figure 5. *R. capsulatus* xanthine dehydrogenase Mo(V) EPR signal at high and low temperatures: 100K (top), 42K (middle), and demolybdo at 35K (bottom).

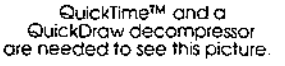

Figure 6. Xanthine oxidase Mo(V) EPR signal at high temperature and low temperatures: 123K (top) and 35K (bottom). The low-temperature EPR signal shows clear spin-spin coupling with the iron-sulfur sites.

Discussions and Conclusions

The spin-spin interactions observed between Mo(V) and Fe-S I in xanthine dehydrogenase at low temperature show that Fe-S I is the closer site in contrast to Fe-S II. In the crystal structure of the enzyme, the measurements of the distances between Mo and the two Fe-S clusters are 14.9A and 24.5A. From the reference EPR spectra, the computation of the related enzymes xanthine oxidase and aldehyde oxidase suggests a spin-spin distance of 14A. This corresponds to the measured distance between Mo and the closest Fe-S cluster. This experiment shows that the closest Fe-S cluster is likely Fe-S I. This experiment also has implications on the mechanism of electron transfer. These

results suggest that the molybdopterin acts as a wire from which the electron travels on, transferring then to Fe-S I. This is illustrated by looking at Figure 4.

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