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Pulsed EPR Studies of a Bacterial Sulfite-Oxidizing Enzyme with pH-Invariant Hyperfine Interactions from Exchangeable Protons

Arnold M. Raitsimring,[†] Ulrike Kappler,[‡] Changjian Feng,[†] Andrei V. Astashkin,[†] and John H. Enemark*,[†]

Department of Chemistry, University of Arizona, Tucson, Arizona 85721-0041, and Centre for Metals in Biology, The University of Queensland, Brisbane, Queensland 4072, Australia

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Variable-frequency pulsed electron paramagnetic resonance studies of the molybdenum(V) center of sulfite dehydrogenase (SDH) clearly show couplings from nearby exchangeable protons that are assigned to a $Mo^{V}OH_n$ group. The hyperfine parameters for these exchangeable protons of SDH are the same at both low and high pH and similar to those for the high-pH forms of sulfite oxidases (SOs) from eukaryotes. The SDH proton parameters are distinctly different from the low-pH forms of chicken and human SO.

The sulfite-oxidizing molybdenum-containing enzymes can be classified into two types, sulfite oxidases (SOs; found in animals and plants, EC 1.8.3.1) and sulfite dehydrogenases (SDHs; found in bacteria, EC 1.8.2.1), depending on their ability to transfer electrons to molecular oxygen.[1,2] X-ray crystal structures of enzymes from all three sources (chicken SO,[3] *Arabidopsis thaliana* SO,[4] and *Starkeya novella* SDH[5]) show nearly identical square-pyramidal coordination around the Mo atom, even though the overall structures of the proteins and the presence of additional cofactors vary.

Electron paramagnetic resonance (EPR) spectroscopy has been extensively used to investigate the structures of the Mo(V) centers of SOs from vertebrates (human and chicken SOs)[6−12] and from plants (*A. thaliana* SO, At-SO).[13] A characteristic feature of the EPR spectra of these eukaryotic SOs is the dependence of both their *g* values and shapes upon pH.[6,12] Distinctly different *g* values are observed at pH ≤ 7 (low pH, lpH) and at pH >9 (high pH, hpH). In addition, the EPR spectra of the lpH form of wild-type vertebrate SOs exhibit splittings from the OH ligand proton with a predominantly isotropic hyperfine interaction (hfi).[9,11] For At-SO, the observation of proton splittings at low pH depends on the method of reduction.[13] The EPR spectra of hpH SOs from eukaryotes show no detectable splittings from exchangeable protons;[6] however, pulsed EPR studies on human, chicken, and At-SO all show highly anisotropic couplings that are assigned to exchangeable protons of a $Mo^{V}OH_{(2)}$ group.[8−10,12,13]

Recently, a bacterial sulfite-oxidizing molybdenum enzyme, an SDH from *S. novella*, was reported to have EPR spectra that are *independent* of the pH and the concentration of anions and that closely resemble the spectra of hpH eukaryotic SOs.[14] No splittings from exchangeable protons are detectable at any pH. To our knowledge, SDH is the first example of a sulfite-oxidizing molybdenum enzyme whose EPR spectrum is not sensitive to the pH or anions in the medium. This EPR behavior of SDH is surprising, considering that the X-ray structures of chicken SO,[3] At-SO,[4] and SDH[5] show very similar coordination around the molybdenum atom. Here we report variable-frequency pulsed EPR measurements for SDH that clearly demonstrate the

* To whom correspondence should be addressed. E-mail: jenemark@u.arizona.edu.
† University of Arizona.
‡ The University of Queensland.

(1) Aguey-Zinsou, K.-F.; Bernhardt, P. V.; Kappler, U.; McEwan, A. G. *J. Am. Chem. Soc.* **2003**, *125*, 530.
(2) Enemark, J. H.; Cosper, M. M. Molybdenum and Tungsten: Their Roles in Biological Processes. In *Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; Marcel Dekker: New York, 2002; Vol. 39, pp 621−654.
(3) Kisker, C.; Schindelin, H.; Pacheco, A.; Wehbi, W.; Garrett, R. M.; Rajagopalan, K. V.; Enemark, J. H.; Rees, D. C. *Cell* **1997**, *91*, 973.
(4) Schrader, N.; Fischer, K.; Theis, K.; Mendel, R. R.; Schwarz, G.; Kisker, C. *Structure* **2003**, *11*, 1251.
(5) Kappler, U.; Bailey, S. *J. Biol. Chem.* **2005**, *280*, 24999.
(6) Lamy, M. T.; Gutteridge, S.; Bray, R. C. *Biochem. J.* **1980**, *185*, 397.
(7) George, G. N. *J. Magn. Reson.* **1985**, *64*, 384.
(8) Astashkin, A. V.; Mader, M. L.; Enemark, J. H.; Pacheco, A.; Raitsimring, A. M. *J. Am. Chem. Soc.* **2000**, *122*, 5294.
(9) Raitsimring, A. M.; Pacheco, P.; Enemark, J. H. *J. Am. Chem. Soc.* **1998**, *120*, 11263.
(10) Astashkin, A. V.; Raitsimring, A. M.; Feng, C.; Johnson, J. L.; Rajagopalan, K. V.; Enemark, J. H. *J. Am. Chem. Soc.* **2002**, *124*, 6109.
(11) Astashkin, A. V.; Raitsimring, A. M.; Feng, C.; Johnson, J. L.; Rajagopalan, K. V.; Enemark, J. H. *Appl. Magn. Reson.* **2002**, *22*, 421.
(12) Enemark, J. H.; Astashkin, A. V.; Raitsimring, A. M. In *Paramagnetic Resonance of Metallobiomolecules*; Telser, J., Ed.; ACS Symposium Series 858; American Chemical Society: Washington, DC, 2003; pp 179−192.
(13) Astashkin, A. V.; Hood, B. L.; Feng, C.; Hille, R.; Mendel, R. R.; Raitsimring, A. M.; Enemark, J. H. *Biochemistry* **2005**, in press.
(14) Kappler, U.; Bennett, B.; Rethmeier, J.; Schwarz, G.; Deutzmann, R.; McEwan, A. G.; Dahl, C. *J. Biol. Chem.* **2000**, *275*, 13202.

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presence of exchangeable protons with couplings that are independent of the pH and that are assigned to a $Mo^{V}OH_n$ group.

SDH was expressed and purified as described previously.[15] EPR samples were prepared using SDH in buffers containing 20 mM Hepes, 200 mM NaCl (pH 7.0, lpH), and 100 mM Bis-Tris propane (pH 9.5, hpH), respectively. The protein was reduced with a 20-fold excess of sodium sulfite and immediately frozen in liquid nitrogen. Pulsed EPR measurements were performed on a home-built X/K_u-band pulsed EPR spectrometer described elsewhere.[16] The details of individual experiments are given in the figure captions. The continuous-wave EPR spectra were recorded on a Bruker ESR-300E spectrometer at 77 K.

Because no proton hfi is observed in the EPR spectrum of SDH under any conditions, it is reasonable to assume that any exchangeable protons from a coordinated $-OH_n$ ligand should have a predominantly anisotropic hfi, similar to the hpH forms of eukaryotic SOs.[8,9,13] The method of choice for detecting such protons is electron spin−echo envelope modulation (ESEEM) spectroscopy. For example, in the spectra of two-pulse (primary) ESEEM, the protons with strong anisotropic hfi often give rise to an easily observable sum combination line, ν_σ, which is well resolved from the sum combination line due to the distant protons.[17] While the latter line is located practically exactly at the double Zeeman frequency, $2\nu_H$, the ν_σ line of the nearby protons in an orientationally disordered system is shifted to the higher frequencies by

$$\delta\nu_\sigma \approx 9T_\perp^{\,2}/16\nu_H \qquad (1)$$

where $T_\perp$ is the anisotropic hfi constant (assuming an axial hfi tensor).

Solid trace 1 in Figure 1 shows the primary ESEEM spectrum of lpH SDH in a H_2O buffer obtained at g_y, while dashed trace 1 is the similar spectrum for lpH SDH in D_2O. The solid spectrum clearly reveals two well-separated ν_σ lines. One of these lines is located at 29.27 MHz, very close to the double Zeeman frequency of 29.15 MHz, and is obviously related to distant protons. The second ν_σ line is shifted from $2\nu_H$ by $\delta\nu_\sigma \approx 2.5$ MHz and is attributed to protons having strong anisotropic hfi. In the spectrum of the sample in D_2O, this line is missing (see dashed trace 1 in Figure 1), which shows that these nearby protons are exchangeable.

For the ESEEM experiment performed at g_y, the situation is close to that of complete orientational disorder, and the characteristic anisotropic hfi constant can therefore be estimated using eq 1 as $T_\perp \approx 1.33(\nu_H\delta\nu_\sigma)^{1/2} \approx -8$ MHz. This value is of the same order of magnitude as that found

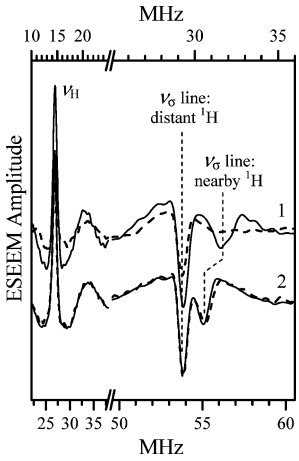

Figure 1. Solid and dashed traces 1: primary ESEEM spectra (cosine Fourier transforms) of lpH SDH in H_2O and D_2O buffers, respectively. Experimental conditions: microwave frequency, 9.4196 GHz; magnetic field, $B_o = 3423$ G (at g_y); microwave pulses, 2×10 ns; temperature, 20 K. Solid and dashed traces 2: primary ESEEM spectra (cosine Fourier transforms) of lpH and hpH SDH in H_2O, respectively. Experimental conditions: microwave frequency, 17.334 GHz; magnetic field, $B_o = 6297$ G (at g_y); microwave pulses, 2×13 ns; temperature, 20 K.

previously for the protons of the $-OH_{(2)}$ group in the hpH form of chicken[8,9] and human[10] SOs.

The X-band ESEEM measurements performed at other field positions show similar spectra, with $\delta\nu_\sigma$ always being about 2.5 MHz. When the experiment was performed in the K_u band, $\delta\nu_\sigma$ decreased in proportion with $1/\nu_H$ down to the value of ~1.4 MHz, as expected from eq 1 and shown by the solid trace 2 in Figure 1. Finally, the ESEEM spectra of hpH SDH were identical with those of the lpH sample, as exemplified by dashed trace 2 in Figure 1.

These ESEEM experiments clearly demonstrate that SDH contains a directly coordinated $-OH_{(n)}$ group at both low and high pH, which appears similar to the $-OH_{(2)}$ group of hpH chicken and human SOs. The identical ESEEM spectra observed for SDH at high and low pH are unprecedented for sulfite-oxidizing molybdenum enzymes. The nearby proton ν_σ line that is often observed in the ESEEM spectra[17] and is easily detected for SDH in this work is also unprecedented for these enzymes. For human and chicken SOs, the broad static distribution of anisotropic hfi parameters has precluded observation of the proton ν_σ line in primary ESEEM spectra obtained under the same conditions as those in this work,[9,12] and it could only be detected in refocused primary ESEEM experiments.[8] The ESEEM results for SDH suggest that it may have a somewhat more rigid protein structure or array of hydrogen-bonding interactions around the active site that restrict the possible orientations of the coordinated $-OH_{(n)}$ group and thus limit the distribution of the hfi parameters.

The fact that no hyperfine splittings are observed in the EPR spectra of SDH implies that the isotropic hfi constant, a_{iso}, of the nearby exchangeable protons is small. To get a quantitative estimate, however, one needs to perform experiments with higher spectral resolution that are capable of detecting the fundamental lines of the nearby protons. A four-pulse ESEEM experiment with integration over the time

(15) Kappler, U.; McEwan, A. G. *FEBS Lett.* **2002**, *529*, 208.
(16) Borbat, P.; Raitsimring, A. New Pulse EPR Spectrometer at the University of Arizona. *Abstracts of 36th Rocky Mountain Conference on Analytical Chemistry*, Denver, CO, July 31−Aug 5, 1994; p 94.
(17) Dikanov, S. A.; Tsvetkov, Yu. D. *Electron Spin−Echo Envelope Modulation (ESEEM) Spectroscopy*; CRC Press: Boca Raton, FL, 1992.

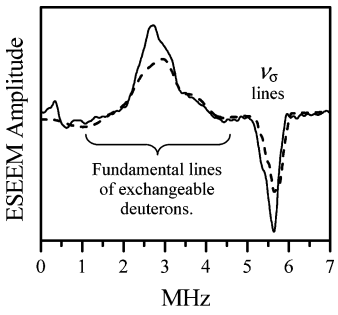

Figure 2. Solid trace: sum of integrated (over the time inerval τ between the first and second microwave pulses) four-pulse ESEEM spectra of lpH SDH in D_2O obtained at $B_o = 4072$ G (near g_z), 4120 G (near g_y) and 4140 G (near g_x). Experimental conditions: microwave frequency, 11.320 GHz; pulse sequence, 20 ns (90°)−τ−20 ns (90°)−T−15 ns (180°)−T−20 ns (90°)−τ−echo; temperature, 20 K. Dashed trace: a simulation for a nonselective excitation of the entire EPR spectrum. Simulation parameters: nucleus, ^{2}H; $a_{iso} = 0.2$ MHz; $T_\perp$ is uniformly distributed from −1.1 to −1.5 MHz; nuclear quadrupole coupling constant, $e^2Qq/h = 0.23$ MHz; the angle between the axes of the hfi and nqi tensors is 90°.

interval τ between the first two microwave pulses[18,19] is one of the possible approaches to this problem.

The solid trace in Figure 2 shows the sum of integrated four-pulse ESEEM spectra obtained at several EPR positions of lpH SDH in D_2O. The nucleus of interest in this experiment is ^{2}H, and the change of the ^{2}H Zeeman frequency, ν_D, over the range of magnetic fields used in this experiment [from $B_o = 4072$ G (near g_z) to $B_o = 4140$ G (near g_x)] is only about 0.044 MHz. The direct summation of the ESEEM spectra is, therefore, legitimate, and the solid trace in Figure 2 represents a good approximation to a spectrum in a situation of complete orientational disorder. A numerical simulation of this spectrum allows one to obtain agreement between the widths of the experimental and simulated ^{2}H fundamental lines for $a_{iso} = 0.2 \pm 0.03$ MHz (see the dashed trace in Figure 2). To reproduce the smooth shapes of the ^{2}H fundamental lines, the anisotropic hfi constant $T_\perp$ was uniformly distributed from −1.1 to −1.5 MHz. The mean value of −1.3 MHz for ^{2}H (−8.4 MHz recalculated for ^{1}H) is close to the corresponding estimate of −8 MHz for ^{1}H obtained above from eq 1. The nuclear quadrupole interaction (nqi) constant $e^2Qq/h = 0.23$ MHz, typical for a hydroxyl ^{2}H, was estimated from a hyperfine sublevel correlation (HYSCORE; see the Supporting Information) experiment. The quality of the fit for the fundamental lines, however, was not sensitive to an nqi of such magnitude or to the relative orientation of the hfi and hqi tensor axes.

The value of $a_{iso} \approx 0.2$ MHz estimated for a nearby exchangeable ^{2}H translates to a ^{1}H isotropic hfi constant of about 1.3 MHz. This hfi constant is substantially smaller than that found for the lpH form of chicken and human SO (>25 MHz).[9,11,20]

In summary, the pulsed EPR experiments performed on SDH at low and high pH confirmed that under these conditions the enzyme contains an exchangeable $-OH_{(n)}$ group with parameters that are distinctly different from those of lpH chicken and human SOs but simlar to the parameters found for the exchangeable protons of the hpH forms of eukaryotic SOs. It is tempting to use the ^{1}H hfi parameters obtained in this work to extract structural information about the Mo(V) site of SDH. However, accurate predictions of this kind from the ^{1}H hfi data may only be possible if the spin-density delocalization from Mo(V) to oxygen of the $OH_{(n)}$ ligand is taken into account.[21] The pulsed EPR experiments with SDH in a buffered solution enriched in $H_2{}^{17}O$ aimed at evaluating this spin-density delocalization are in progress.

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Supporting Information Available: ESE field sweep, primary ESEEM spectra for SDH at low and high pH, and a HYSCORE spectrum of SDH at low pH in D_2O at the g_y position. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) Van Doorslaer, S.; Schweiger, A. *Chem. Phys. Lett.* **1997**, *281*, 297.
(19) Astashkin, A. V.; Raitsimring, A. *J. Magn. Reson.* **2000**, *143*, 280.
(20) Bray, R. C.; Lamy, M. T.; Gutteridge, S.; Wilkinson, T. *Biochem. J.* **1982**, *201*, 241.
(21) Raitsimring, A. M.; Astashkin, A. V.; Feng, C.; Enemark, J. H.; Nelson, K. J.; Rajagopalan, K. V. *J. Biol. Inorg. Chem.* **2003**, *8*, 95.