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Spectroscopic Characterization of the Molybdenum Cofactor of the Sulfane Dehydrogenase SoxCD from *Paracoccus pantotrophus*

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The bacterial sulfane dehydrogenase SoxCD is a distantly related member of the sulfite oxidase (SO) enzyme family that is proposed to oxidize protein-bound sulfide (sulfane) of SoxY as part of a multienzyme mechanism of thiosulfate metabolism. This study characterized the molybdenum cofactor of $SoxCD_1$, comprising the catalytic molybdopterin subunit SoxC and the truncated *c*-type cytochrome subunit $SoxD_1$. Electron paramagnetic resonance spectroscopy of the Mo^V intermediate generated by dithionite reduction revealed low- and high-pH species with **g** and **A**(^{95,97}Mo) matrices nearly identical to those of SO, indicating a similar pentacoordinate active site in $SoxCD_1$. However, no sulfite-induced reduction to Mo^V was detected, nor could a strongly coupled ^{1}H signal or a phosphate-inhibited species be generated. This indicates that the outer coordination sphere controls substrate binding in SoxCD, permitting access only to protein-bound sulfur via the C-terminal tail of SoxY.

The aerobic chemotrophic bacterium *Paracoccus pantotrophus* is able to oxidize a variety of reduced sulfur compounds. Seven genes (*soxXYZABCD*) encode four periplasmic proteins (SoxXA, SoxYZ, SoxB, and SoxCD) that are essential for sulfur oxidation in vitro.[1] The reconstituted Sox system mediates thiosulfate-, sulfur-, and hydrogen sulfide-dependent cytochrome *c* reduction, while each protein alone is catalytically inactive.[2] SoxCD is a $\alpha_2\beta_2$-heterotetramer comprised of a SoxC subunit (containing a Mo cofactor) and the hybrid *c*-type cytochrome SoxD containing the heme domains D_1 and D_2.[3] SoxCD is part of the sulfite oxidase (SO) family of enzymes; however, in contrast with eukaryotic SO[4] and bacterial sulfite dehydrogenase (SDH),[5,6] it does not oxidize sulfite. Instead, SoxCD has been proposed to oxidize the outer sulfane−sulfur atom (oxidation state 1−) covalently bound to the exposed cysteine residue located at the C terminus of SoxY to sulfone (oxidation state 5+), with sulfate being subsequently hydrolyzed by SoxB (Figure S1 in the Supporting Information).[1] The structural features responsible for the different substrate specificities of SoxCD in sulfur

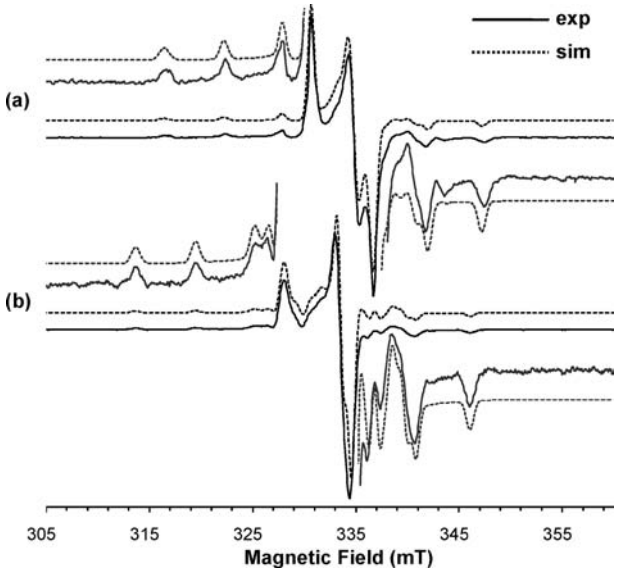

Figure 1. X-band (9.24 GHz) CW-EPR spectra of $SoxCD_1$ in (a) 25 mM Tris HCl, pH 9.0, and (b) 50 mM Tris HCl, pH 7.0, together with empirical simulations. For clarity, the ^{95,97}Mo hyperfine splittings are shown on an expanded scale. The *g* and $A(^{95,97}\text{Mo})$ parameters of each species are very similar to those of the low-pH species of SO and the high-pH species of SO and SDH (Table 1). In contrast with SO, no strongly coupled proton is observed at low pH. Experimental conditions: microwave power, 10 mW; modulation amplitude, (a) 5.5 G, (b) 2 G; modulation frequency, 100 kHz; temperature, 77 K; sweep time, 168 s; time constant, 164 ms; averages, (a) 8, (b) 6.

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(1) Friedrich, C. G.; Rother, D.; Bardischewsky, F.; Quentmeier, A.; Fischer, J. *Appl. Environ. Microbiol.* **2001**, *67*, 2873–2882.
(2) Friedrich, C. G.; Quentmeier, A.; Bardischewsky, F.; Rother, D.; Kraft, R.; Kostka, S.; Prinz, H. *J. Bacteriol.* **2000**, *182*, 4677–4687.
(3) Bardischewsky, F.; Quentmeier, A.; Rother, D.; Hellwig, P.; Kostka, S.; Friedrich, C. *Biochemistry* **2005**, *44*, 7024–7034.
(4) Enemark, J. H.; Astashkin, A. V.; Raitsimring, A. M. *Dalton Trans.* **2006**, 3501–3514.
(5) Kappler, U.; Bennett, B.; Rethmeieri, J.; Schwarz, G.; Deutzmann, R.; McEwan, A. G.; Dahl, C. *J. Biol. Chem.* **2000**, *275*, 13202–13212.
(6) Doonan, C. J.; Kappler, U.; George, G. N. *Inorg. Chem.* **2006**, *45*, 7488–7492.

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Table 1. Spin Hamiltonian Parameters of $SoxCD_1$ Determined from Empirical Simulations of CW-EPR Spectra and Comparison with Other Members of the SO Family[a]

enzyme	g_x	g_y	g_z	$\langle g\rangle$[b]	$A_{x'}$	$A_{y'}$	$A_{z'}$	$\langle A\rangle$[c]	α[d]	β[d]	γ[d]	ref
$SoxCD_1$ low pH	1.967	1.974	2.007	1.982	22.0	23.0	55.8	33.6	0	27	0	this work
$SoxCD_1$ high pH	1.950	1.961	1.985	1.965	10.0	21.4	54.2	28.5	41	21	0	this work
CSO low pH[e]	1.968	1.974	2.007	1.983	16.7	25.0	56.7	32.8	0	18	0	10
CSO high pH[e]	1.954	1.966	1.990	1.970	11.3	21.0	54.4	28.9	59	26	−56	10
CSO phosphate-inhibited	1.965	1.973	1.995	1.975	nd[f]	nd	nd	nd	nd	nd	nd	18
SDH[g]	1.951	1.963	1.988	1.967	nd	nd	nd	nd	nd	nd	nd	5
plant SO low pH[h]	1.968	1.975	2.006	1.981	nd	nd	nd	nd	nd	nd	nd	11
plant SO high pH[h]	1.956	1.964	1.989	1.970	nd	nd	nd	nd	nd	nd	nd	11

[a] Hyperfine couplings are given for ^{95}Mo in units of 10^{-4} cm^{-1}. Estimated uncertainties in the principal g and A values of $SoxCD_1$ are ±0.003 and $\pm 0.5 \times 10^{-4}$ cm^{-1}, respectively. [b] $\langle g\rangle = (g_x + g_y + g_z)/3$. [c] $\langle A\rangle = (A_{x'} + A_{y'} + A_{z'})/3$. [d] Euler rotations specifying the rotation from (x, y, z) to (x', y', z') are defined as $R = R_z(\gamma)\, R_y(\beta)\, R_z(\alpha)$. [e] From chicken liver. SH parameters have been redefined with respect to the original reference such that g_z and $A_{z'}$ are the largest principal values.[12] [f] nd = not determined. [g] From *Starkeya novella*. [h] From *Arabidopsis thaliana*.

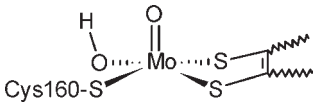

Figure 2. Postulated coordination sphere of the active site of $SoxCD_1$ following reduction by dithionite to the Mo^V oxidation state. The OH ligand is approximately perpendicular to the plane of the $d_{x^2-y^2}$-based magnetic orbital.

metabolism remain unclear. Our previous electron paramagnetic resonance (EPR) investigations have characterized the electronic structure of the dimanganese cluster of SoxB[7] and the multiheme SoxXA.[8] In this study, we present the first EPR spectroscopic characterization of the molybdenum cofactor (Moco) of SoxCD from *P. pantotrophus* by studying $SoxCD_1$, an enzyme that is missing the heme-2 domain $SoxD_2$ but in vitro is found to have a catalytic activity identical with that of SoxCD.[3]

Although no EPR-detectable $Mo^{VI} \rightarrow Mo^V$ reduction of $SoxCD_1$ occurred following the addition of sulfite (not shown), reduction of $SoxCD_1$ by dithionite to ca. +150 mV at pH 7 and ca. +50 mV at pH 9 (vs SHE) generated continuous-wave EPR (CW-EPR) signals (Figure 1) with low-symmetry **g** and **A**(^{95,97} Mo) EPR parameters that are highly comparable with those of the low- and high-pH Mo^V species observed in SO (Table 1). This indicated that SoxCD contains an active site very similar to that of SO,[4] which possesses a pentacoordinate Mo center with an axial oxo ligand, a bidentate sulfur ligand from a pyranopterin dithiolene cofactor, a sulfur ligand from a conserved cysteine residue, and an oxo (hydroxo) ligand in the Mo^{VI} (Mo^V) oxidation state (Figure 2). Q-band Davies electron nuclear double-resonance (ENDOR) spectroscopy further detected ^{1}H NMR resonances in a deuterated buffer (Figures S2 and S3 in the Supporting Information) similar in appearance to those observed for chicken liver SO (CSO).[9] These resonances were ascribed, in part, to a nearby R138 residue and the side chain protons of the equatorial C185 ligand (CSO numbering), and their observation also in $SoxCD_1$ provides evidence for coordination of the conserved Cys residue (C160 of SoxC) and the presence of nearby R114 (Figure S4 in the Supporting Information). On the basis of the structurally similar Mo^V first coordination spheres of $SoxCD_1$ and SO, we may propose a putative reaction mechanism for SoxCD similar to that of SO (Figure S1 in the Supporting Information).

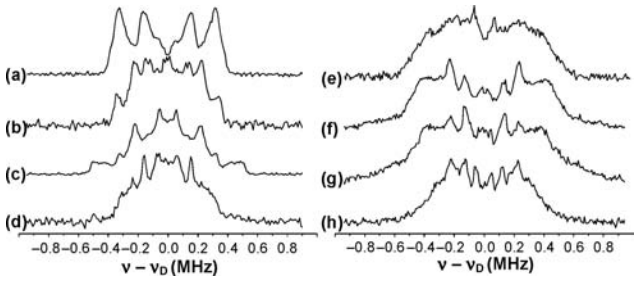

Figure 3. Q-band (34.1 GHz) Mims ^{2}H ENDOR (τ = 360 ns) of $SoxCD_1$ (left) in 50 mM Tris DCl, pD 7.0, measured at (a) 12 155 G (near g_z), (b) 12 274 G, (c) 12 357 G (near g_y), and (d) 12 393 G (near g_x) and (right) in 25 mM Tris DCl, pD 9.6, measured at (e) 12 270 G (near g_z), (f) 12 350 G, (g) 12 424 G (near g_y), (h) 12 493 G (near g_x).

A major difference between $SoxCD_1$ and SO, however, was the absence of hyperfine splitting in the CW-EPR spectra at pH 7 because of a strongly coupled exchangeable proton (Figure S5 in the Supporting Information). Davies ENDOR identified only a weakly coupled exchangeable proton detected at low pH (Figures S6 in the Supporting Information), which was subsequently isolated by Mims deuterium ENDOR (Figure 3). This proton was not affected by the presence of chloride anions (Figure S8 in the Supporting Information) or phosphate anions (Figure S9 in the Supporting Information).

Spectroscopic identification of a strongly coupled proton ($a_{iso} \approx 26-28$ MHz) is a key feature of the low-pH species of SO, which arises from a Mo^VOH intermediate that is formed both during the catalytic cycle and by exogenous reductants.[4,11] The presence of 30−40 mol equiv of Cl^- has been shown to be crucial to the observation of the strongly coupled ^{1}H in SO;[13] in particular, chloride ions occupy the positively charged substrate binding pocket of SO (displacing the product during the catalytic cycle), participating in hydrogen bonds that constrain the equatorial OH ligand in the plane.[14] However, even in the presence of 100 mM NaCl, Davies ENDOR failed to detect any strongly coupled ^{1}H features in $SoxCD_1$ at low pH (Figure S7 in the Supporting Information).

(7) Epel, B.; Schäfer, K. O.; Quentmeier, A.; Friedrich, C. G.; Lubitz, W. *J. Biol. Inorg. Chem.* **2005**, *10*, 636–642.
(8) Reijerse, E. J.; Sommerhalter, M.; Hellwig, P.; Quentmeier, A.; Rother, D.; Laurich, C.; Bothe, E.; Lubitz, W.; Friedrich, C. *Biochemistry* **2007**, *46*, 7804–7810.
(9) Astashkin, A. V.; Raitsimring, A. M.; Feng, C.; Johnson, J. L.; Rajagopalan, K. V.; Enemark, J. H. *J. Am. Chem. Soc.* **2002**, *124*, 6109–6118.
(10) Dhawan, I. K.; Enemark, J. H. *Inorg. Chem.* **1996**, *35*, 4873–4882.
(11) Astashkin, A. V.; Hood, B. L.; Feng, C.; Hille, R.; Mendel, R. R.; Raitsimring, A. M.; Enemark, J. H. *Biochemistry* **2005**, *44*, 13274–13281.
(12) Drew, S. C.; Hanson, G. R. *Inorg. Chem.* **2009**, *48*, 2224–2232.
(13) Rajapakshe, A.; Johnson-Winters, K.; Nordstrom, A. R.; Meyers, K. T.; Emesh, S.; Astashkin, A. V.; Enemark, J. H. *Biochemistry* **2010**, *49*, 5154–5159.

The presence of only a weakly coupled exchangeable proton supports the assignment of an OH ligand occupying the fourth equatorial position with the OH bond directed out of the basal plane (Figure 2) such that Fermi contact with the $d_{x^2-y^2}$ orbital is low ($|a_{iso}(^1H)| < 5$ MHz).[15,16] A weakly coupled exchangeable proton was also detected for the high-pH (pD) species; however, in contrast with low pH, the spectra were much broader across the field range (Figure 3). This suggests that the fourth ligand in the high pH species of $SoxCD_1$ is also an OH ligand directed out of the basal plane, but with a distribution of orientations, similar to that of the high-pH form of SO.[4,15]

The apparent insensitivity of the OH ligand orientation at low pH on the chloride concentration is diagnostic of the differences in the outer coordination sphere of the active sites of $SoxCD_1$ and SO that may be responsible for controlling substrate access and binding. Additional evidence for this can be gleaned from the low-symmetry EPR parameters; utilizing the results of the systematic theoretical study of the relationship between the principal axes of **g** and **A**(^{95,97}Mo) and the molybdenum(V)−dithiolene fold angle η,[12] the β Euler angles at low pH (Table 1) can be translated into fold angles of $\eta \approx 19°$ ($SoxCD_1$) and $\eta \approx 2°$ (CSO). A number of examples of related species lacking a strongly coupled proton at low pH are known, including (a) the "blocked" Mo^VOSO_2 form in SO, which occurs when sulfite displaces sulfate in the absence of excess Cl^-,[13] (b) a "trapped" Mo^VOSO_3 form in plant SO from *Arabidopsis thaliana*, which occurs following sulfite reduction and ferricyanide oxidation,[17] and (c) a "phosphate-inhibited" (Pi) form in SO, which occurs when HPO_4^{2-} displaces SO_4^{2-} or OH^-.[18] Because the low-pH form of $SoxCD_1$ was generated in the absence of a substrate, both trapped and blocked forms of Mo^V species can be ruled out. Moreover, inhibition by trace phosphate from buffers used during enzyme purification was unlikely because the Pi form has distinctly different $g_z < 2.0$ (Table 1) and ^{95,97}Mo hyperfine structure (cf. Figure 1 of ref 18) and no ^{31}P NMR signal from a coordinating HPO_4^{2-} was detected by electron spin-echo envelope modulation or ENDOR (not shown). Indeed, 25 mM phosphate also did not alter the line positions in the CW-EPR spectra at low pH (Figure S5 in the Supporting Information). The absence of a Pi form and an inability to oxidize sulfite again reaffirm the likely importance of outer coordination sphere control for substrate binding.

A detailed molecular basis for the difference in the substrate specificity of SoxCD (protein-bound sulfide) and other distantly related members of the SO family (free sulfite) will require specific consideration of the tertiary interactions of SoxCD with SoxYZ, which will be greatly aided by a crystal structure. However, sequence alignment indicates that a number of key residues located near the active site of SO are "conserved" in SoxC, despite only ≈30% sequence identity with the Moco domains of SDH and SO (Figure S4 in the Supporting Information). Specifically, R138, H140, C185, Y322, and R450 (CSO numbering) are conserved as residues R114, H116, C160, Y286, and R402, respectively, in SoxC. A potentially significant difference in the sequences of SoxCD and SO/SDH is the absence of R190 (CSO numbering) in SoxCD. This residue is conserved across all SOs and nitrate reductases and constitutes one of three Arg residues located ∼5 Å from the active site of CSO (R138, R190, and R450) that form a positively charged substrate binding pocket to hold anions such as sulfite (see Figure 4 of Kisker et al.[19]). In SoxC enzymes, R190 (CSO) is replaced with a conserved Gly residue (G165 in *P. pantotrophus*; Figure S4 in the Supporting Information), which may perturb or destroy such a binding pocket. Indeed, the inability of sulfite and phosphate anions to access the active site, together with the altered OH ligand orientation at low pH, suggests that while the active site of SoxCD shares a very similar first coordination sphere with other members of the SO family, the above differences in the outer coordination sphere are likely to control access and binding of the substrate. This may necessitate the sulfur substrate to be presented to SoxCD as a sulfane tethered to the C-terminal tail of SoxY.

Supporting Information Available: Listings of experimental methods, proposed enzyme mechanism, Davies and Mims Q-band ENDOR at low and high pH, sequence alignments, and additional CW-EPR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

(14) Klein, E. L.; Astashkin, A. V.; Ganyushin, D.; Riplinger, C.; Johnson-Winters, K.; Neese, F.; Enemark, J. H. *Inorg. Chem.* **2009**, *48*, 4743–4752.

(15) Raitsimring, A. M.; Pacheco, A.; Enemark, J. H. *J. Am. Chem. Soc.* **1998**, *120*, 11263–11278.

(16) Astashkin, A. V.; Klein, E. L; Ganyushin, D.; Johnson-Winters, K.; Neese, F.; Kappler, U.; Enemark, J. H. *Phys. Chem. Chem. Phys.* **2009**, *11*, 6733–6742.

(17) Astashkin, A. V.; Johnson-Winters, K.; Klein, E. L.; Byrne, R. S.; Hille, R.; Raitsimring, A. M.; Enemark, J. H. *J. Am. Chem. Soc.* **2007**, *129*, 14800–14810.

(18) Pacheco, A.; Basu, P.; Borbat, P.; Raitsimring, A. M.; Enemark, J. H. *Inorg. Chem.* **1996**, *35*, 7001–7008.

(19) Kisker, C.; Schindelin, H.; Pacheco, A.; Wehbi, W. A.; Garrett, R. M.; Rajagopalan, K. V.; Enemark, J. H.; Rees, D. C. *Cell* **1997**, *91*, 973–983.