Published in final edited form as:

J Biol Inorg Chem. 2010 May ; 15(4): 505–514. doi:10.1007/s00775-009-0619-0.

Pulsed EPR investigations of the Mo(V) centers of the R55Q and R55M variants of sulfite dehydrogenase from *Starkeya novella*

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Abstract

Continuous-wave and pulsed electron paramagnetic resonance (EPR) spectroscopy have been used to characterize two variants of bacterial sulfite dehydrogenase (SDH) from *Starkeya novella* in which the conserved active-site arginine residue (R55) is replaced by a neutral amino acid residue. Substitution by the hydrophobic methionine residue (SDH^{R55M}) has essentially no effect on the pH dependence of the EPR properties of the Mo(V) center, even though the X-ray structure of this variant shows that the methionine residue is rotated away from the Mo center and a sulfate anion is present in the active-site pocket (Bailey et al. in *J Biol Chem* 284:2053–2063, 2009). For SDH^{R55M} only the high-pH form is observed, and samples prepared in H₂¹⁷O-enriched buffer show essentially the same ¹⁷O hyperfine interaction and nuclear quadrupole interaction parameters as SDH^{WT} enzyme. However, the pH dependence of the EPR spectra of SDH^{R55Q}, in which the positively charged arginine is replaced by the neutral hydrophilic glutamine, differs significantly from that of SDH^{WT}. For SDH^{R55Q} the blocked form with bound sulfate is generated at low pH, as verified by ³³S couplings observed upon reduction with ³³S-labeled sulfite. This observation of bound sulfate for SDH^{R55Q} supports our previous hypothesis that sulfite-oxidizing enzymes can exhibit multiple pathways for electron transfer and product release (Emesh et al. in *Biochemistry* 48:2156–2163, 2009). At pH ≥ 8 the high-pH form dominates for SDH^{R55Q}.

Keywords

Sulfite-oxidizing enzyme; Electron spin echo envelope modulation; Sulfur metabolism; ¹⁷O labeling

Introduction

Sulfite-oxidizing enzymes (SOEs) occur in vertebrates, plants, and certain bacteria [1]. In vertebrate sulfur metabolism, sulfite oxidase (SO) converts toxic sulfite to sulfate in the final degradation step of the sulfur-containing amino acids cysteine and methionine [2,3]. Sulfite dehydrogenase (SDH), isolated from the soil bacterium *Starkeya novella*, oxidizes sulfite during chemolithotrophic growth with thiosulfate as an energy source [4,5]. Despite their different metabolic roles, both SO and SDH have nearly identical active-site geometries [6–8]. Their square-pyramidal oxomolybdenum active sites have an axial oxo ligand, one

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Electronic supplementary material The online version of this article (doi:10.1007/s00775-009-0619-0) contains supplementary material, which is available to authorized users.

equatorial sulfur from a cysteine amino acid residue, two equatorial sulfurs from the molybdopterin cofactor, and one exchangeable equatorial ligand that undergoes changes that depend on the stage of the catalytic cycle. In the fully oxidized Mo(VI) state this equatorial ligand is an oxo group [9]. In the generally accepted catalytic mechanism [10,11], this oxo ligand is attacked by sulfite to form a sulfate-bound Mo(IV) intermediate [10]. Subsequent hydrolysis of this intermediate and one-electron oxidation lead to Mo(V)–OH species that can be studied by electron paramagnetic resonance (EPR) spectroscopy [12,13].

Numerous studies of the paramagnetic Mo(V) centers of SOEs from various sources by continuous-wave (CW) and pulsed EPR techniques have revealed three distinct types of EPR spectra corresponding to different structural forms of the active center: high-pH; low-pH; and blocked [13]. For the high-pH and low-pH forms the exchangeable equatorial ligand is OH [14–19].

The spectral features of the high-pH, low-pH, and blocked forms are remarkably independent of the organism; however, the particular pH dependence of interconversions among these forms can depend upon the organism, specific mutations near the active site, and the mode of reduction. For example, for plant SO from *Arabidopsis thaliana* (At-SO) reduction by sulfite at low pH produces the blocked form in which sulfate (instead of OH) is coordinated to Mo (V), whereas reduction by titanium(III) citrate generates the low-pH form [19]. The EPR spectrum of the Mo(V) center in SDH^{WT} is independent of pH within the studied range from 4.9 to 10, and only a high-pH-type spectrum is observed [20,21]. However, substitution of the active-site tyrosine by phenylalanine (SDH^{Y236F}) results in generation of the low-pH form at pH ≤ 7 [22]. Thus, this substitution results in an effective lowering of the local pH. A qualitatively similar lowering of the local pH was obtained for the analogous mutant in human SO (HSO^{Y343F}), where the formation of the low-pH species has shifted to pH ~ 8, a typical value for observation of the high-pH form [23]. For HSO^{Y343F} only insignificant amounts of the high-pH form were generated, even at pH 10. At pH < 7 the blocked form was observed for HSO^{Y343F} [23]. For vertebrate SOs, the presence of certain anions (e.g., PO₄³⁻, AsO₄³⁻) produces additional EPR spectral forms [24–26].

Recently, we have investigated the pathogenic mutant, HSO^{R160Q}, in which an active-site arginine that is conserved in all SOEs is replaced by glutamine [27]. The high-pH form was observed at high pH (above 8). However, at pH < 7 a blocked form was generated that had a spectrum different from the spectra observed earlier in plant SO (At-SO) and HSO^{Y343F}. The electron spin echo envelope modulation (ESEEM) investigation of the blocked form of HSO^{R160Q} yielded nuclear quadrupole interaction (NQI) parameters of the ¹⁷O oxo ligand that were interpreted as indicating a six-coordinate Mo center with weak axial coordination *trans* to the oxo ligand by the substituted glutamine residue [27,28]. Additionally, pulsed electron–nuclear double resonance (ENDOR) experiments revealed the presence of a nearby (*R* ~ 3.1 Å) weakly coupled exchangeable proton that was suggested to be hydrogen-bonded to the oxygen of the equatorial sulfate ligand. At intermediate pH values (6.5–8) a completely new Mo(V) signal was observed (denoted as species 2 in [27]). The structure of species 2 is not yet known, but it is also the major EPR-active product generated upon reduction of HSO^{R160Q} by titanium(III) citrate [27]. This result for HSO^{R160Q} contrasts with the reduction of At-SO by titanium(III) citrate, which gives a typical low-pH signal [19].

It was not clear whether the unusual spectroscopic properties of HSO^{R160Q} were organism-specific or mutation-specific. Therefore, in this work we have investigated mutations of the analogous active-site arginine (R55) for an enzyme from a completely different organism, namely, the bacterial SDH from *Starkeya novella*. Two mutations are presented, SDH^{R55Q} (analogous to HSO^{R160Q}), where the hydrophilic positively charged arginine was changed to a neutral hydrophilic glutamine, and SDH^{R55M}, where the change was to the neutral, but

hydrophobic, methionine. Comprehensive studies of the overall reaction kinetics and intramolecular electron transfer kinetics for SDH^{R55M} and SDH^{R55Q} have also recently been reported [11,12].

Materials and methods

SDH^{R55M} and SDH^{R55Q} were generated as previously described [11,12], expressed in *Rhodobacter capsulatus*, and purified using previously developed procedures for SDH proteins [29].

The low pH (pH 5.8–6.0) samples of SDH^{R55M} and SDH^{R55Q} were prepared using 50 mM bis (2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis–Tris) buffer. The enzyme samples (474 μ M of SDH^{R55M} and 481 μ M of SDH^{R55Q}) were reduced with a 20-fold excess of sodium sulfite under argon and immediately frozen in liquid nitrogen. The same buffer system and procedure were used for reduction with ³³S-labeled sulfite, prepared as previously described [30].

The low pH samples in H₂¹⁷O-enriched water were prepared by first concentrating a 100 μ L solution of the enzyme in 100 mM Bis–Tris at pH 5.8 to reduce the amount of H₂¹⁶O. Next, a solution of 100 mM Bis–Tris buffer (pH 5.8) was vacuum-centrifuged to evaporate the H₂¹⁶O, and the pelleted buffer was redissolved in the same volume of H₂¹⁷O. The concentrated enzyme sample was then incubated in 30 μ L of the buffer prepared with H₂¹⁷O for approximately 3 h at 277 K. Finally, the enzyme was reduced with a 20-fold excess of sodium sulfite under argon and then immediately frozen in liquid nitrogen.

For titanium(III) citrate reduced low-pH samples of SDH in H₂O or D₂O, a stock solution of titanium(III) citrate was diluted to 3.76 mM in 100 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis–Tris propane) (pH 6.0). Approximately 850 μ M SDH^{R55Q} (22 μ L) in 50 mM Bis–Tris propane was injected into an EPR tube, which was then placed inside a Schlenk tube. The Schlenk tube was pumped to obtain a mild vacuum and then purged with argon. This procedure was repeated several times over a period of 40 min to degas the protein. Next, 5 μ L of the diluted titanium(III) citrate solution (700 μ M final concentration) in well-degassed 100 mM Bis–Tris propane buffer (pH 6.0) was injected into the protein solution under argon, and the reduced SDH^{R55Q} enzyme was frozen in liquid nitrogen immediately. The final concentration ratio of SDH^{R55Q} to titanium(III) citrate was approximately 2:1.

The X-band CW EPR experiments were performed using a Bruker ESP-300 EPR spectrometer at 77 K. The resonator used in these experiments was a standard rectangular cavity operated in TE₁₀₂ mode. The principal *g* values of the Mo(V) center were determined using a 1,1-diphenyl-2-picrylhydrazyl free radical standard (*g* = 2.0036).

The pulsed EPR experiments were performed using home-built broadband pulsed EPR spectrometers operating at the microwave frequencies from 8 to 18 GHz (X and K_u microwave bands) (<http://quiz2.chem.arizona.edu/epr>) and from 26 to 40 GHz (K_a band) [31]. The measurement temperature was about 21 K. The pulse sequences used for various ESEEM and pulsed ENDOR experiments are described in the electronic supplementary material.

Results

Continuous-wave electron paramagnetic resonance

Previous studies have shown that the CW EPR spectrum of SDH^{WT} is independent of pH [21], unlike the spectra of plant and animal SOEs, and the SDH^{WT} spectrum is characteristic of the high-pH form of eukaryotic SOEs [13]. SDH^{R55M} gives EPR spectra similar to those of

SDH^{WT}, and traces 1 and 2 in Fig. 1 show the spectra recorded at pH 9.0 and 5.8, respectively. The principal g values determined from these spectra are typical for the high-pH form of SOEs (Table 1).

In contrast, the EPR spectra of SDH^{R55Q} are distinctly different at low and high pH. The spectrum observed at high pH (trace 3 in Fig. 1, pH 9.0) is similar to the usual high-pH spectrum of all SOEs. However, the spectrum observed at low pH (trace 4 in Fig. 1, pH 5.8) represents a mixture of two species. The major species (approximately 75%, as estimated by subtracting the appropriately normalized high-pH spectrum of trace 3 and double-integrating the EPR spectra), species 1, is similar to the blocked form (large g_z and no observable hyperfine splittings), whereas the minor species (approximately 25%) is similar to the high-pH form. Decreasing the pH from 5.8 to 4.9 did not change the relative amounts of these two forms. However, in the intermediate-pH range (6–9) the amount of species 1 decreased, and the amount of the high-pH-like species increased with increasing pH.

Apart from the major EPR lines (marked by asterisks and diamonds in Fig. 1) corresponding to the Mo(V) centers containing nonmagnetic molybdenum isotopes, there are several minor features seen in the EPR spectra of Fig. 1 (e.g., at 336.5 and 342.5 mT in traces 1–3, and at 349 mT in all traces) corresponding to Mo(V) centers containing magnetic molybdenum isotopes (⁹⁵Mo and ⁹⁷Mo, both $I = 5/2$, total natural abundance approximately 25.5%). Out of these, the features at 336.5 and 342.5 mT in the spectra of SDH^{R55M} (traces 1 and 2) are at similar positions to the g_z and g_y turning points of species 1 of SDH^{R55Q} seen in trace 4. Assuming that a similar species also contributes to the spectra of SDH^{R55M}, we estimated its yield by double integration of spectra 1 and 2, which resulted in a negligible amount of approximately 5%. However, even such a minor contribution is unlikely for SDH^{R55M} because the mentioned spectral features are observed both at low pH and at high pH, whereas all of the known blocked species (to which species 1 actually belongs, as shown by the pulsed EPR experiments described later) only form at low pH. We conclude, therefore, that the spectra of SDH^{R55M} correspond to a single high-pH-like species. Additional evidence in support of this conclusion comes from the EPR spectra of SDH^{R55M} in D₂O buffer (see the electronic supplementary information).

Reduction of SDH^{R55Q} with titanium(III) citrate instead of sulfite at pH 5.8 produced only a very weak EPR signal of the high-pH-like form. This result contrasts with Ti(III) reductions of other SOEs. For At-SO the low-pH form is produced [19], and for HSO^{R160Q} a new form (species 2) is observed under these conditions [27].

To strengthen the assignments of the observed EPR-active species in SDH^{R55M} and SDH^{R55Q} to the high-pH and blocked forms, the ESEEM spectroscopic experiments described later were performed to directly establish the presence of OH or SO₄²⁻ ligands.

Exchangeable equatorial ligands in SDH^{R55M} and high-pH SDH^{R55Q}

On the basis of the principal g values, the Mo(V) species of SDH^{R55M} is likely to represent the usual high-pH form, with an exchangeable equatorial OH ligand. The OH bond makes a large angle with the equatorial plane where the Mo(V) d_{xy} orbital carrying the unpaired electron is located. This results in the isotropic hyperfine interaction constant, a_{iso} , of the OH proton being close to zero and in the absence of hyperfine splittings in the CW EPR spectrum. In contrast, the anisotropic hyperfine interaction of this proton is fairly strong, $T_{\perp} \sim 7$ –9 MHz [14,18], and the detection of such a proton verifies the presence of the equatorial OH ligand.

ESEEM methods have proved to be of great utility for investigating the protons of the equatorial OH ligand of the high-pH form [14,18]. The spectra obtained by some of the ESEEM techniques contain not only the fundamental nuclear transition lines at frequencies ν_{α} and ν_{β} ,

but also the sum and difference combination lines, $\nu_\sigma = \nu_\alpha + \nu_\beta$ and $\nu_\delta = |\nu_\alpha - \nu_\beta|$, respectively. For a nucleus of spin $I = 1/2$ in the case of weak hyperfine interaction ($A < 2\nu_I$, where A is the total hyperfine interaction constant contributed by both isotropic hyperfine interaction and anisotropic hyperfine interaction, and ν_I is the nuclear Zeeman frequency) the sum combination line is located at the frequency [32]

$$\nu_\sigma \approx 2\nu_I + 9T_\perp^2 / 16\nu_I. \quad (1)$$

Equation 1 allows one to estimate the anisotropic hyperfine interaction constant, $T_\perp$, from the position of the sum combination line detected in an ESEEM spectrum. Although this equation was derived for the situation of complete orientational disorder, it is also approximately applicable to the ESEEM spectra obtained at the orientationally disordered EPR position g_y .

The two-pulse ESEEM is the simplest technique to obtain spectra that contain the ν_σ line. However, this technique has two major drawbacks: a relatively low frequency resolution limited by the electron spin transverse relaxation; and a relatively large dead time (tens or hundreds of nanoseconds, depending on the microwave frequency band). The latter circumstance limits the observation possibilities for broad lines and results in characteristic oscillatory distortions in the cosine Fourier transform spectra phased to account for the dead time. An alternative technique that allows one to observe the sum combination feature and which is free from the above-mentioned drawbacks is the τ -integrated four-pulse technique [33,34] (where τ is the time interval between the first and second microwave pulses), and this technique was used in this work to detect the ^1H ν_σ line for the samples of SDH.

Figure 2 shows the ^1H sum combination line region of the experimental K_u band τ -integrated four-pulse ESEEM spectra of SDH^{R55M} prepared in H_2O and D_2O buffers (solid and dashed traces, respectively). The spectra were obtained at the intermediate EPR turning point corresponding to g_y . The narrow negative peak located at exactly $2\nu_{\text{H}}$ (ν_{H} is the ^1H Zeeman frequency) represents the sum combination line due to distant protons, most of which belong to the protein and are not exchangeable. The broader negative peak that is located at a somewhat higher frequency and which is not present in the spectrum of the sample prepared in the D_2O buffer arises from a neighboring exchangeable proton. The value of the shift $\Delta\nu_\sigma = \nu_\sigma - 2\nu_{\text{H}} \approx 1.58$ MHz gives an estimate of the anisotropic hyperfine interaction constant $T_\perp \approx -8.7$ MHz, quite typical for the hydroxyl ligand proton in high-pH SOEs [14,18].

Identical results were obtained for the high-pH SDH^{R55Q} enzyme, where the anisotropic hyperfine interaction constant was also estimated to be about -8.7 MHz. Thus, along with the observation of a characteristic set of principal g values by CW EPR, the ESEEM experiments establish that the Mo(V) signal of SDH^{R55M} and the high-pH signal of SDH^{R55Q} do indeed belong to the well-known high-pH form of SOEs.

Exchangeable ligand for SDH^{R55Q} at low pH

The CW EPR results suggest that species 1 of SDH^{R55Q} observed at low pH is a blocked form with coordinated sulfate. If this assumption is correct, then, similar to the previously studied blocked forms of SO [27,30], the sulfur atom of the sulfate ligand should be observable by ESEEM for a sample prepared with ^{33}S -enriched sulfite. Therefore, low-pH samples of SDH^{R55Q} were prepared with sulfite having a natural abundance of sulfur isotopes (94.93% ^{32}S , $I = 0$) and with ^{33}S -enriched sulfite (99% ^{33}S , $I = 3/2$). These samples will be referred to as ^{32}S - SDH^{R55Q} and ^{33}S - SDH^{R55Q} , respectively.

Figure 3 shows the K_a band two-pulse ESEEM spectra of ^{33}S -SDH^{R55Q} (solid traces) and ^{32}S -SDH^{R55Q} (dashed traces), obtained at the EPR turning points of species 1. The difference between the spectra of the two samples is caused by the interaction of the unpaired electron of Mo(V) with ^{33}S . The results in Fig. 3 prove that species 1 of SDH^{R55Q} is a blocked form of the enzyme, with bound sulfate, analogous to species 1 of HSO^{R160Q} that was studied previously [27]. However, in contrast to the blocked forms of At-SO and HSO studied previously [27,30] the blocked form of SDH^{R55Q} shows the largest amplitude of the ^{33}S ESEEM at the low-field and high-field EPR turning points (g_z and g_x , respectively). Toward the central parts of the spectrum the ^{33}S ESEEM rapidly decreases, and near g_y it is virtually unobservable. In the hyperfine sublevel correlation (HYSCORE) spectra of ^{33}S -SDH^{R55Q} (Fig. 4) the off-diagonal correlation lines were only seen at g_z and g_x , whereas the HYSCORE spectra obtained at the inner parts of the EPR spectrum only showed weak diagonal lines that might have originated from the contribution of a stimulated electron spin echo (ESE) signal to the HYSCORE ESE signal [35].

In our previous investigations of the blocked forms of SOEs we found that the NQI of the ^{33}S nucleus is much stronger than the Zeeman (under the K_a band experimental conditions) and hyperfine interactions (e^2Qq/h is approximately tens of megahertz, whereas $\nu_I \sim 3.5$ MHz at $B_0 \sim 1$ T, and A is approximately a few megahertz) [27,30]. The ESEEM theory for such a spectroscopic situation was developed in our previous work [30]. According to that theory, the strong NQI splits the nuclear spin states of ^{33}S into two doublets of degenerate $|\pm 3/2\rangle$ and $|\pm 1/2\rangle$ states (similar to Kramers doublets in EPR), whereas the Zeeman and hyperfine interactions lift the degeneracy. The transition frequency between the states that belong to different doublets (the interdoublet transition frequency) is

$$\nu_{\text{id}} \approx e^2Qq/2h. \quad (2)$$

The most easily observable HYSCORE cross-peaks belong to $|1/2\rangle \leftrightarrow |-1/2\rangle$ transitions of ^{33}S within the $|\alpha\rangle$ and $|\beta\rangle$ electron spin manifolds. The frequencies of these transitions, ν_α and ν_β , are

$$\nu_{\alpha,\beta} \approx c_{\text{hq}}(\nu_I \pm A/2), \quad (3)$$

where $c_{\text{hq}} \in [1, 2 + \eta]$ is a numerical factor that depends on the orientation of the B_0 vector relative to the NQI principal axes frame (η is the asymmetry parameter of the NQI tensor).

The HYSCORE spectrum obtained at g_x is qualitatively similar to that obtained earlier for HSO^{R160Q}, and it is the only spectrum that reveals an interdoublet transition(s) line at a frequency of $\nu_{\text{id}} \sim 13.7$ MHz (Fig. 4, spectrum c). Using Eq. 2, one can estimate from this frequency the quadrupole coupling constant, $e^2Qq/h \sim 2\nu_{\text{id}} \sim 27$ MHz. From the positions of the correlations peaks at g_z (4.6, 2.8) MHz and g_x (8.5, 7.2), using Eq. 3, one can estimate $A_z \approx 1.7$ MHz and $A_x \approx 0.6$ MHz. Thus, the ^{33}S NQI and hyperfine interaction parameters for species 1 in SDH^{R55Q} (at least those which could be determined) are in qualitative agreement with those reported for HSO^{R160Q} [27]: $e^2Qq/h \approx 36$ MHz, $A_z \approx 3.4$ MHz, and $A_x \sim 0$ MHz.

The most likely reason for the observed rapid decrease of the ^{33}S ESEEM amplitude in the central parts of the EPR spectrum is a static statistical distribution of the sulfate ligand orientations within certain (possibly rather narrow) limits. Such a distribution of orientations has been previously documented for the bound phosphate group in the phosphate-inhibited form of chicken SO [25]. An example of a structural model that could explain the relatively

sharp ^{33}S ESEEM spectral features at g_x and g_z and their virtual absence at g_y is as follows. Suppose the long axis of the NQI tensor is approximately in the xz -plane of the g -frame, and the static structural distribution consists of the departures of the orientation of this axis from the xz -plane by a small angle θ . The projection of this axis on the g -frame axis y will change in proportion with θ . At the same time, the projections on x and z will change in proportion with $\theta^2 \ll \theta$. The changes of the y -projection will result in a distribution of c_{hq} factors in Eq. 3, and in a dramatic broadening and decrease in amplitude of the ESEEM spectral lines at the g_y EPR position. A variation in the hyperfine interaction parameters caused by the structural distribution will also be a factor contributing to the line broadening and decrease of the ESEEM amplitude at g_y .

^{17}O electron spin echo envelope modulation

As in earlier works with SOEs [36,37], the experiments with samples prepared in a buffer containing H_2^{17}O show an exchange of the axial oxo ligand with ^{17}O . The ^{17}O NQI constant e^2Qq/h can be readily estimated from the ESEEM spectra containing the sum combination feature, ν_σ , if the NQI is weak compared with the nuclear Zeeman and hyperfine interactions: $e^2Qq/4I(2I-1)h < \nu_\sigma \pm A/2$, where ν_σ is the Zeeman frequency of ^{17}O . The ^{17}O nucleus has spin $I = 5/2$, and the sum combination feature represents a quintet with the splittings between the individual lines being determined by the NQI [38]. On the basis of the general expression derived elsewhere [38], the largest observable splitting $\delta\nu_\sigma$ translates to the quadrupole coupling constant as

$$e^2Qq/h = (40/12)\delta\nu_\sigma. \quad (4)$$

The ESEEM experiments have shown that for SDH^{R55M} the NQI splittings in the $^{17}\text{O} \nu_\sigma$ feature could only be partly resolved, even in the spectra obtained by the integrated four-pulse technique (which has high resolution limited by the electronic longitudinal relaxation time, T_1), whereas for species 1 of SDH^{R55Q} the splittings at g_z could already be resolved by the two-pulse ESEEM (resolution limited by the transverse relaxation process). The corresponding spectra are shown in Fig. 5. The maximum estimated splitting for SDH^{R55M} is about 0.22 MHz ($e^2Qq/h \sim 0.7$ MHz), whereas in SDH^{R55Q} the splittings are about 1.2 MHz ($e^2Qq/h \sim 4$ MHz).

Neighboring exchangeable proton in SDH^{R55Q} at low pH

When studying the blocked form of the Mo(V) center of $\text{HSO}^{\text{R160Q}}$, we observed ENDOR lines from a neighboring exchangeable proton. The anisotropic hyperfine interaction of this proton was significantly smaller than that of an equatorial OH ligand, but the hyperfine interaction tensor was rhombic, which is characteristic of a composite tensor with contributions from at least two centers of spin density localization. This tensor was therefore interpreted as indicative of a proton from a water molecule or an OH group that was hydrogen-bonded to the coordinated oxygen of the equatorial sulfate ligand [27]. The zero isotropic hyperfine interaction constant ($a_{\text{iso}} = 0$ MHz) of this proton has excluded an alternative explanation that the spin density was delocalized on an orbital responsible for a covalent bond of the respective hydrogen atom with another atom.

Since it was established above that the low-pH Mo(V) center of SDH^{R55Q} is in the blocked form, the possibility of a similar neighboring proton in SDH^{R55Q} was also investigated. Figure 6 shows the ^1H refocused Mims ENDOR spectra [39,40] of the exchangeable protons obtained as a difference between the spectra of the samples prepared in H_2O and D_2O . The original spectra were recorded at the EPR turning points of the blocked form of SDH^{R55Q} (species 1) and were normalized by the refocused stimulated ESE amplitude. For the g_y turning point the

minor (approximately 20%) contribution of the high-pH form was subtracted from the total ENDOR signal.

The spectra in Fig. 6 are virtually identical to the refocused Mims ENDOR spectra of exchangeable protons obtained previously for the blocked form of HSO^{R160Q} [27], and very similar numerical simulation results for the hyperfine interaction parameters are obtained for SDH^{R55Q}, where the average isotropic hyperfine interaction constant $\langle a_{\text{iso}} \rangle = 0$ MHz, and the anisotropic hyperfine interaction tensor $T = (-3.2, -1.8, 5.0)$ MHz. The spectra simulated with these parameters are shown by dashed lines in Fig. 6.

Discussion

The experiments with SDH^{R55M} have revealed that only the high-pH form of the Mo(V) center is generated at various pH values, as occurs with SDH^{WT} [21,22]. On the other hand, for SDH^{R55Q} the pH-dependent formation of two species was detected. One of the species is the usual high-pH form, which is almost exclusively present at pH 9. The second form, species 1, is the major species at pH < 7 and was shown to be the blocked form by reduction with ³³S-enriched sulfite.

SDH^{R55Q} is the second R → Q SOE active-site mutant studied by EPR. The CW EPR spectra and all of the high-resolution spectroscopic observations for the blocked form of SDH^{R55Q} are similar to those for HSO^{R160Q} reported previously [27]. Thus, the general structure of the blocked form of SDH^{R55Q} should be similar to that proposed for HSO^{R160Q} [27].

Another interesting observation for the R → Q mutants is that a typical low-pH EPR signal with large isotropic splittings from an exchangeable proton is never observed with sulfite reduction. SDH^{R55Q} shows only the blocked and high-pH forms as a function of pH. HSO^{R160Q} shows these signals and an additional low-pH EPR signal (species 2) of as yet unknown origin that can also be generated by reduction with Ti(III) [27]. For At-SO, reduction with sulfite at low pH produces the blocked form, but reduction with titanium(III) citrate at low pH generates a typical low-pH spectrum [19]. In contrast, reaction of SDH^{R55Q}, with Ti(III) at pH 5.8 resulted only in the formation of small amounts of the high-pH form.

It is also interesting to compare the g values observed for the blocked forms of the SDH^{R55Q} and HSO^{R160Q} mutants with those observed for At-SO^{WT} and HSO^{Y343F}. The values for the latter two are similar to those of low-pH SO (2.005, 1.974, and 1.963) [19,23,30], whereas the R → Q mutants exhibit a significant decrease in g_x , from 1.963 down to 1.95 (Table 1). On the basis of simple crystal field considerations, such a change in g_x can qualitatively be rationalized as resulting from a change in the energy of the d_{yz} orbital, which again may represent the effect of the hypothesized axial coordination of the glutamine residue in the HSO^{R160Q} mutant [27, 28].

One of the points of special interest for SOEs is the quadrupole coupling constant of the axial ¹⁷O-oxo ligand in the blocked forms. For both HSO and SDH, replacing the active-site arginine by a glutamine resulted in dramatic (fourfold to fivefold) increase of the quadrupole coupling constant of this ¹⁷O ligand compared with the values of $e^2Qq/h \leq 1.5$ MHz typically observed for other SOEs [38,41], for model oxo-Mo(V) compounds, [13,38,41], and for SDH^{R55M} in this work. In our previous work on HSO^{R160Q} [27] we hypothesized that this effect could be caused by weak coordination of the carbonyl oxygen of glutamine in the axial position of the Mo(V) center, *trans* to the oxo ligand. This hypothesis was supported by the oxo-¹⁷O NQI parameters obtained for a six-coordinate model complex (dtd)Mo¹⁷O(¹⁷Otms), where H₂dtd is 2,3:8,9-dibenzo-1,4,7,10-tetrathiadecane and tms is trimethylsilyl, $e^2Qq/h \approx 3$ MHz vs. 1.5 MHz in five-coordinate [Mo(V)¹⁷O(SPh)₄][−] [27]. A similar suggestion was

made earlier by Doonan et al. [28] on the basis of the extended X-ray absorption fine structure data for HSO^{R160Q}.

The high-pH form is remarkably similar for SOEs from all organisms [13], and no significant shifts in principal g values are observed among either wild-type or mutant sources (Table 1). These EPR data imply that at high pH the active-site R $\rightarrow$ Q mutations (HSO^{R160Q} and SDH^{R55Q}) do not significantly perturb the five-coordinate active-site structures observed by X-ray crystallography for the wild-type enzymes [6,7] and confirmed by pulsed EPR spectroscopy for native chicken SO [18].

Finally, the spectroscopic results presented here also reinforce our previous structural [7,11, 42] and kinetic [11,12,22,43] studies of SDH^{WT} and various R55 mutants. Earlier literature [10] has suggested that the mechanism of SOEs involves nucleophilic attack of sulfite on the oxo-Mo(VI) center to form the enzyme–substrate complex (k_1 of Scheme 1), which then dissociates to give sulfate and Mo(IV) (k_2). Two sequential one-electron oxidations of the molybdenum center (k_4 and k_7) regenerate Mo(VI). However, recently it has been proposed that one-electron oxidation to Mo(V) (k_3) can precede product dissociation in SDH [12]. This hypothesis is supported by the observation of the blocked form of SDH^{R55Q} by pulsed EPR studies in this work and by the previous observation that the one-electron intramolecular electron transfer rates between the molybdenum and heme centers for SDH^{WT}, SDH^{R55M}, and SDH^{R55Q} are all much faster than k_{cat} [12].

Conclusion

CW and pulsed EPR spectroscopy have been used to characterize two mutations of the conserved active-site arginine residue in bacterial SDH, R55M and R55Q. Both mutations replace the positively charged arginine by a neutral amino acid residue, but their effects are very different. Substitution by the neutral hydrophobic methionine residue (SDH^{R55M}) had essentially no effect on the EPR properties of the Mo(V) center, even though the crystal structure of oxidized SDH^{R55M} shows significant differences from the structure of SDH^{WT} (Fig. 7) [11]. For example, the structure of SDH^{R55M} has a sulfate bound at the active site, and the methionine side chain is bent away from the active site into a small cavity between the side chains of L121 and Q33. The space that was occupied by R55 in SDH^{WT} appears to be largely empty in SDH^{R55M}. No crystal structure is available for SDH^{R55Q}, but the substitution of the positively charged arginine by the neutral hydrophilic glutamine residue (SDH^{R55Q}) resulted in a dramatic change of the pH dependence of the EPR spectra compared with SDH^{WT}. For SDH^{R55Q} the blocked form is generated at low pH. Similar to the blocked form of HSO^{R160Q}, the quadrupole coupling constant (e^2Qq/h) of the presumed axial ¹⁷O-oxo ligand is fourfold to fivefold greater than the values typically observed for other SOEs [38,41]. This effect has been hypothesized to result from weak coordination of the carbonyl oxygen of the substituted glutamine in the axial position of the Mo(V) center, *trans* to the oxo ligand [27, 28]. However, unlike HSO^{R160Q}, the SDH^{R55Q} mutant does not show an additional EPR form at intermediate pH (approximately 7), and the high-pH form dominates at pH $\geq$ 8. The reasons for this difference between HSO^{R160Q} and SDH^{R55Q}, as well as the origin of species 2 observed for HSO^{R160Q} at pH $\sim$ 7, are not currently clear. EPR experiments on the structure of species 2 of HSO^{R160Q} are in progress and may help resolve these differences between the SDH and HSO systems.

Supplementary Material

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Acknowledgments

We gratefully acknowledge support of this research by the NIH (GM-37773 to J.H.E.) and by grants from the NSF (DBI-0139459, DBI-9604939, BIR-9224431) and the NIH (S10RR020959) for development of the pulsed EPR facility. K. J.-W. thanks the NIH for a Ruth L. Kirschstein National Service Award. We thank Eric L. Klein for preparing the sample of ^{33}S -labeled sulfite. T.D.R. is the recipient of an International Postgraduate Research Scholarship (IPRS) and a UQ Graduate School Research Travel Grant from the University of Queensland. Support of the Australian Research Council (ARC Grant DP0878525 to U.K.) is gratefully acknowledged.

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Fig. 1. Continuous-wave electron paramagnetic resonance (EPR) spectra of sulfite-reduced SDH^{R55M} at pH 9.0 (trace 1) and pH 5.8 (trace 2), and sulfite-reduced SDH^{R55Q} at pH 9.0 (trace 3) and at pH 5.8 (trace 4). The experimental conditions were as follows: $\nu_{\text{mw}} = 9.450$ GHz; microwave power, 2 mW; modulation amplitude, 0.2 mT; temperature, 77 K. The EPR turning points for the high-pH-like form are shown by *asterisks*. The turning points for species 1 of SDH^{R55Q} are shown by *diamonds*

Fig. 2.

^1H sum combination line region of cosine Fourier transform (FT) spectra of τ -integrated four-pulse electron spin echo envelope modulation (ESEEM) of the Mo(V) center of SDH^{R55M} (pH 5.8) prepared in H_2O (*solid trace*) and D_2O (*dashed trace*). The experimental conditions were as follows: $\nu_{\text{mw}} = 17.362$ GHz; $B_0 = 635.5$ mT (g_y); microwave pulses, 6, 6, 13, and 6 ns; temperature, 21 K

Fig. 3.

Amplitude FT spectra of normalized two-pulse ESEEM obtained for low-pH (pH 5.8) samples of ^{33}S -SDH^{R55Q} (solid lines) and ^{32}S -SDH^{R55Q} (dashed lines), respectively. The spectra were obtained at the EPR turning points of species 1 and have the same vertical scale. *a*, *b*, and *c* correspond to g_z , g_y , and g_x , respectively. The ^{33}S lines are clearly seen at g_z and g_x positions, but virtually disappear at g_y . The experimental conditions were as follows: $\nu_{\text{mw}} = 29.568$ GHz; $B_0 = 1,057.9$ mT (g_z), 1,078.4 mT (g_y), and 1,088.4 mT (g_x); microwave pulses, two of 16 ns; temperature, 21 K

Fig. 4.

^{33}S hyperfine sublevel correlation spectra of low-pH (pH 5.8) samples of ^{33}S -SDH^{R55Q} obtained at the EPR turning points of species 1. *a*, *b*, and *c* correspond to g_z , g_y , and g_x , respectively. The spectra were normalized by the amplitudes of the stimulated electron spin echo signal at the measurement positions. The relative scales are $\times 1$ for *a* and *c*, and $\times 2$ for *b*. The experimental conditions were as follows: $\nu_{\text{mw}} = 29.568$ GHz; $B_0 = 1,057.9$ mT (g_z), 1,078.4 mT (g_y), and 1,088.4 mT (g_x); microwave pulses, 14, 14, 25 and 14 ns; temperature, 21 K. Each spectrum represents a sum of the spectra obtained at the time intervals τ between the first and second microwave pulses of 170 and 200 ns

Fig. 5.

Cosine FT spectra of τ -integrated four-pulse ESEEM of ^{17}O -SDH^{R55M} (*trace 1*) and two-pulse ESEEM of ^{17}O -SDH^{R55Q}, species 1 (*trace 2*) obtained at the respective low-field EPR turning points (g_z). The *stick diagram* over each spectrum indicates the sum combination (ν_σ) feature split into a quintet by the ^{17}O nuclear quadrupole interaction. The experimental conditions for *trace 1* were as follows: $\nu_{\text{mw}} = 29.640$ GHz; $B_0 = 1,070$ mT (g_z); microwave pulses, 11, 11, 21 and 11 ns; temperature, 21 K. The experimental conditions for *trace 2* were as follows: $\nu_{\text{mw}} = 29.592$ GHz; $B_0 = 1,058.6$ mT (g_z); microwave pulses, two of 15 ns; temperature, 21 K

Fig. 6.

Solid traces are the ^1H electron–nuclear double resonance (ENDOR) spectra at the EPR turning points (traces 1–3) obtained as a difference between the refocused Mims ^1H ENDOR spectra recorded for species 1 of SDH^{R55Q} in H_2O and D_2O and normalized by the refocused stimulated electron spin echo amplitude. The experimental conditions were as follows: $B_0 = 1,057.5$ mT (g_z), $1,077.4$ mT (g_y), and $1,087$ mT (g_x) for traces 1, 2, and 3, respectively; $\nu_{\text{mw}} = 29.555$ GHz; microwave pulses, three of 15 ns and one of 22 ns; time interval between the first and second microwave pulses, $\tau = 80$ ns; time interval between the second and third microwave pulses, $T = 30$ μs ; time interval between the third and fourth (refocusing) microwave pulses, $\tau' = 380$ ns; RF pulse length, 20 μs ; temperature, 21 K. Dashed lines are numerical simulations for $\langle a_{\text{iso}} \rangle = 0$; $\Delta a_{\text{iso}} = 0.5$ MHz; $T = (-3.2, -1.8, 5.0)$ MHz; $(\phi_h, \theta_h, \psi_h) = (70^\circ, 80^\circ, 0^\circ)$

Fig. 7. Superimposed structures of SDH^{WT} (*blue* arginine residue) and SDH^{R55M} (*red* methionine residue), adapted from Protein Data Bank entries 2bpb and 2ca3

Scheme 1.

The sequence of steps which transform the enzyme–substrate complex, $\text{Mo}^{\text{IV}}(\text{OSO}_3^{2-})$, into product (sulfate) and the reoxidized $\text{Mo}(\text{VI})$ state of the enzyme. In the pathway colored *red*, product release precedes reoxidation of the enzyme. For the pathway colored *blue*, the enzyme–substrate complex is oxidized by one electron prior to product release, and the paramagnetic blocked form, $\text{Mo}^{\text{V}}(\text{OSO}_3^{2-})$, can be detected by electron paramagnetic resonance spectroscopy [12]

Table 1Principal *g* values of the Mo(V) center in SDH^{R55M} and SDH^{R55Q}

Mo(V) species	<i>g_z</i>	<i>g_y</i>	<i>g_x</i>	Reference
SDH ^{R55M} (high pH)	1.989	1.960	1.951	This work ^a
SDH ^{R55Q} (high pH)	1.989	1.960	1.951	This work ^a
SDH ^{WT} (high pH)	1.989	1.964	1.952	This work ^a
	1.991	1.966	1.954	[21]
HSO ^{WT} (high pH)	1.987	1.964	1.954	This work ^a
SDH ^{R55Q} species 1 (blocked)	2.006	1.968	1.949	This work ^a
At-SO (blocked)	2.002	1.972	1.962	This work ^a
	2.005	1.974	1.963	[19]
HSO ^{R160Q} (blocked)	2.006	1.971	1.951	[27]
HSO ^{Y343F} (blocked)	1.999	1.973	1.965	[23]

The *g* values for other sulfite-oxidizing enzymes are given for comparison

SDH sulfite dehydrogenase, *At-SO* sulfite oxidase from *Arabidopsis thaliana*, *HSO* human sulfite oxidase

^aThe accuracy is ± 0.0005