Spectroscopic and kinetic properties of the molybdenum-containing, NAD$^+$-dependent formate dehydrogenase from Ralstonia eutropha.

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ABSTRACT
We have examined the rapid reaction kinetics and spectroscopic properties of the molybdenum-containing, NAD$^+$-dependent FdsABG formate dehydrogenase from Ralstonia eutropha. We confirm previous steady-state studies of the enzyme and extend its characterization to a rapid kinetic study of the reductive half-reaction (the reaction of formate with oxidized enzyme). We have also characterized the EPR signal of the molybdenum center in its Mo$^{V}$ state and demonstrated the direct transfer of the substrate C$_0$ hydrogen to the molybdenum center in the course of the reaction. Varying temperature, microwave power and level of enzyme reduction, we are able to clearly identify the EPR signals for four of the iron-sulfur clusters of the enzyme, and find suggestive evidence for two others; we observe a magnetic interaction between the molybdenum center and one of the iron-sulfur centers, permitting assignment of this signal to a specific iron-sulfur cluster in the enzyme. In light of recent advances in our understanding of the structure of the molybdenum center, we propose a reaction mechanism involving direct hydride transfer from formate to a Mo=S group of the molybdenum center.

The molybdenum-containing, NAD$^+$-dependent formate dehydrogenases from bacteria such as Ralstonia eutropha catalyze the oxidation of formate to CO$_2$, reducing NAD$^+$ to NADH and are members of the NADH dehydrogenase superfamily of enzymes. These cytosolic formate dehydrogenases are expressed under aerobic conditions and are distinct from the predominant bacterial formate dehydrogenases expressed under anaerobic conditions, which are typically membrane-associated and extremely O$_2$-sensitive; they are also distinct from the cofactorless formate dehydrogenases from many eukaryotes, including humans, that catalyze the direct hydride transfer from formate to NAD$^+$ [1]. In Ralstonia eutropha, the trimeric FdsABG enzyme is encoded by the fdsGBACD operon, and is predicted to contain seven iron-sulfur clusters, FMN and a molybdenum center; each subunit bears significant sequence similarity (ca. 21% identity) and expected strong structural homology to a corresponding subunit of the matrix-or cytosol-exposed portion of NADH dehydrogenase, and the spatial layout of the several redox-active centers is particularly highly conserved. The 105 kDa FdsA is cognate to subunit Nqo3 in the crystallographically characterized T. thermophilus NADH dehydrogenase [2,3], and is predicted to have four [4Fe-4S] clusters and one [2Fe-2S] cluster [4]. The close structural homology predicted between FdsA and Nqo3 extends to the presence of a histidine ligand to one of the [4Fe-4S] clusters near the N-terminus of the FdsA/Nqo3 subunit. The C-terminus of FdsA contains a molybdenum center and has ~60% sequence similarity to the structurally characterized Mo-containing formate dehydrogenase FdhF of E. coli (part of the formate:hydrogen lyase complex), with Cys 378 occupying the homologous position to the molybdenum-coordinating Sec 140 in the latter enzyme [5]. This domain is present in Nqo3 as well, although the molybdenum center itself and one (sometimes two) of the more C-terminal iron-sulfur clusters have been lost by the NADH dehydrogenases over the course of evolution [1,3,6]. The 55 kDa FdsB subunit of the R. eutropha formate dehydrogenase has some 45% sequence identity to the FdhF of T. thermophilus, and has 34% sequence identity to the Nqo2 subunit of the T. thermophilus NADH dehydrogenase, and has a [2Fe-2S] cluster that lies off the main electron transport chain. The FdsC subunit is predicted to be the functional homolog of the Nqo3 subunit, with the Cys 378 homolog occupying the Mo-coordinating position. The 19 kDa FdsG subunit of the R. eutropha enzyme subunit has 34% sequence identity to the Nqo2 subunit of the T. thermophilus NADH dehydrogenase, and has a [2Fe-2S] cluster that lies off the main electron transport chain. The FdsABG complex is encoded by the fdsGBACD operon, and is predicted to contain seven iron-sulfur clusters, FMN and a molybdenum center; each subunit bears significant sequence similarity (ca. 21% identity) and expected strong structural homology to a corresponding subunit of the matrix-or cytosol-exposed portion of NADH dehydrogenase, and the spatial layout of the several redox-active centers is particularly highly conserved. The 105 kDa FdsA is cognate to subunit Nqo3 in the crystallographically characterized T. thermophilus NADH dehydrogenase [2,3], and is predicted to have four [4Fe-4S] clusters and one [2Fe-2S] cluster [4]. The close structural homology predicted between FdsA and Nqo3 extends to the presence of a histidine ligand to one of the [4Fe-4S] clusters near the N-terminus of the FdsA/Nqo3 subunit. The C-terminus of FdsA contains a molybdenum center and has ~60% sequence similarity to the structurally characterized Mo-containing formate dehydrogenase FdhF of E. coli (part of the formate:hydrogen lyase complex), with Cys 378 occupying the homologous position to the molybdenum-coordinating Sec 140 in the latter enzyme [5]. This domain is present in Nqo3 as well, although the molybdenum center itself and one (sometimes two) of the more C-terminal iron-sulfur clusters have been lost by the NADH dehydrogenases over the course of evolution [1,3,6]. The 55 kDa FdsB subunit of the R. eutropha formate dehydrogenase has some 45% sequence identity to the FdhF of T. thermophilus, and has 34% sequence identity to the Nqo2 subunit of the T. thermophilus NADH dehydrogenase, and has a [2Fe-2S] cluster that lies off the main electron transport chain.
transfer pathway connecting the FMN and molybdenum center.

The molybdenum center of FdsABG is the site of formate oxidation and is of the same general type seen in DMSO reductase [1,7], with two equivalents of a pyranopterin cofactor coordinated (present as the guanosine dinucleotide in the case of FdsABG) to the metal via enedithiolate side chains. Cys 378 occupies a fifth ligand position in an expected trigonal prismatic coordination geometry of the oxidized enzyme, and on the basis of the requirement of the FdsC sulfide insertase for proper maturation of active FdsABG [8] the sixth ligand coordination position is most likely a terminal Mo=S group. The makeup of the molybdenum coordination sphere of oxidized enzyme has recently been confirmed in an EXAFS study of the Rhodobacter capsulatus enzyme, specifically demonstrating a short Mo=S bond at a distance of ~2.17 Å in the oxidized enzyme [9].

Previous work with the R. eutropha FdsABG enzyme has demonstrated that the oxidized, as-isolated enzyme exhibits broad absorption throughout the visible region attributable to its FMN and iron-sulfur centers, which bleaches upon reduction by formate, NADH or dithionite [10]. The reduced enzyme exhibits multiple EPR signals to the iron-sulfur clusters of the enzyme, although the g-values for only three have been reported [11]. In the present work, we examine the rapid-reaction kinetics of the reaction of FdsABG with its reducing substrate formate, and characterize additional iron-sulfur EPR signals of the enzyme. We have also characterized the EPR signal of the molybdenum center in its MoV state, and demonstrate the direct transfer of the substrate C-H hydrogen to the molybdenum center, providing specific evidence for the enzyme operating via a hydride transfer mechanism. Finally, we observe a magnetic interaction between the molybdenum center and one of the iron-sulfur centers, permitting assignment of this signal to the [4Fe-4S] cluster nearest the molybdenum center (at a distance of 12.4 Å center-to-center, by analogy to the distance seen in the FdhF formate dehydrogenase).

**EXPERIMENTAL PROCEDURES**

Organisms and growth conditions. R. eutropha strains H16 (ATCC 17699) and HF210 were grown as previously described [1], except that the final molybdenum concentration was increased to 0.15 mM. For pre-cultures, up to 4 plates were washed with 0.5 L of minimal media (supplemented with 0.1% (wt/vol) fructose and 0.2% (wt/vol) formate) and grown at 30°C with shaking at 250 rpm in a 3 L Schott flask for ~6 hrs. Large-scale fermentation (12L) was performed at 30°C in a 15-liter fermentor (New Brunswick BioFlo 415) with a stirring rate of 300-350 rpm and aeration at 6 Lpm (using media supplemented with formate 0.2% (wt/vol) only). pH was maintained at 6.9 by the automated addition of 44-50% (v/v) formic acid. Cells were harvested after the culture had reached an OD136~6 for HF210 or ~6.5 for H16. Cells grown to higher densities (>4 g/L media) exhibited a significant decrease in FdsABG expression. Highest level of expression (60-80 Units/g cells) was obtained from HF210 cells subjected to continuous growth, where cells from one growth cycle were used as pre-cultures for subsequent cycles. Harvested cells were washed once at 4°C with 75 mM potassium phosphate, 10 mM KNO3, pH 7.2 and stored at -80°C until used.

**Purification of FdsABG.** All steps were performed at 0-4°C with an Akta FPLC system (GE Healthcare) in a procedure modified from Friedebold et al., 1993 [1]. Frozen cells, typically 48-52g wet weight, were thawed, suspended in 2-2.5 volumes of enzyme buffer, and disrupted by 1-2 passages through a French pressure cell at 20,000 psi. Cell debris was removed by centrifugation for 45 min at 200,000 x g. The supernatant was made 29% saturated in ammonium sulfate and centrifuged for an additional 45 min at 200,000 x g. The 29% fraction was then brought to 46% ammonium sulfate saturation. After centrifugation, the pellet containing FdsABG was dissolved in 80 mM K-PO4, 20 mM KNO3, pH 8.0 (saturated with 15% ammonium sulfate) at 0.4 ml/g cells to maintain a relatively small loading volume to minimize peak spreading. Supernatant treated in this fashion was loaded onto a 2.6 x 16 cm Butyl 4 Sepharose column (GE Healthcare), the column was washed with 0.5 column volume 40 mM K-PO4, 10 mM KNO3, 0.7 M ammonium sulfate, pH 7.3 (HIC-A) and the enzyme eluted with a linear gradient of 0.7 – 0 M ammonium sulfate over 4 column volumes. Active fractions (see assay conditions below) were pooled and the protein precipitated with 50% ammonium sulfate. After centrifugation the pellet was redissolved in 80 mM K-PO4, 20 mM KNO3, pH 8.0 and loaded onto a 2.6 x 20 cm G-50 desalting column equilibrated with 20 mM Tris-HCl, 10 mM KNO3, pH 7.4. Fractions from the desalting column were loaded onto a 2.6 x 14 cm Cibacron Blue Agarose 3GA, Type 3000-CL column (Sigma-Aldrich). Under these conditions, FdsABG did not bind to the gel matrix and eluted in the flow-through. The resulting fractions were applied to a

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2.6 x 14 cm Fractogel TMAE 650 (S) column (EMD-Millipore), washed with 2 column volume of 40 mM K-phosphate, 10 mM KNO₃, pH 7.0 and eluted with a linear gradient of 0 - 0.6 M NaCl over 5 column volumes. Active fractions were pooled, divided into two halves and the protein precipitated with 50% ammonium sulfate. After centrifugation one pellet was redisolved in 0.5 ml of HIC-A buffer, loaded onto a 5 ml Butyl Sepharose HP column (GE Healthcare), washed with 0.5 column volume HIC-A buffer and eluted with a linear gradient of 0.5 - 0 M ammonium sulfate over 4 column volumes. The process was repeated with the second pellet. Both HIC steps were complicated by significant peak spreading, thus total column volumes were minimized to obtain optimal resolution. Active fractions were precipitated with 50% ammonium sulfate, redisolved and loaded unto 1.6 x 60 cm Superdex 200 PG column (GE Healthcare) in buffer containing 300 mM KCl. Fractions containing only the dimer of the heterotrimer (with a molecular weight of ~358 kDa) were precipitated with 50% ammonium sulfate, redisolved and buffer exchanged into storage buffer: 75 mM K-phosphate, 10 mM KNO₃, pH 7.5 via Amicon Ultra 4 (Millipore). Concentrated protein was flash frozen and stored in liquid N₂.

Activity assays were performed at 30°C in 75 mM K-phosphate, pH 7.7 with 2 mM NAD⁺ and 40 mM sodium formate; formation of NADH was monitored at 340 nm (ε = 6,220 M⁻¹ cm⁻¹). One unit of activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD⁺ per min. Typically, 10 sec were used to calculate the initial slope. Enzyme concentrations were determined using an extinction coefficient of ε₄₁₀ cm⁻¹ = 51,500 M⁻¹ cm⁻¹ estimated from the oxidized enzyme spectrum published by Friedebold et al., 1993 [10]; activities were calculated with respect to one trimer with a molecular weight of 178,800 Da. Purified protein exhibited 70 units of activity per mg of protein, in good agreement with 80 units per mg obtained by Friedebold et al., 1993 [10].

UV/Vis absorbance measurements. Activity measurements and absorbance spectra were performed using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a thermostated cell holder. Steady-state measurements collected at a range of pH values yielded a bell-shaped curve that was fitted to the equation for a double-ionization mechanism:

\[ \text{L}_{\text{obs}} = \text{L}_{\text{max}} [1+((\text{H}^+)/10^\text{pK_a1}) + (10^\text{pK_a2})/\text{[H}^+])] \] (1)

with the maximum theoretical value for L (L_{max}), and the two pKa values (pK_a1 and pK_a2) obtained using PeakFit ver. 4 (Jandel Scientific). Reductive titrations were performed with 20 mM buffered sodium dithionite and 20 μM enzyme in 75 mM K-phosphate, pH 7.7 under anaerobic conditions at RT. Prior to titrations the enzyme was exchanged into anaerobic 75 mM K-phosphate, pH 7.7 to remove nitrate via Amicon Ultra 4 (Millipore).

Rapid Reaction Kinetics. The reaction of oxidized FdsABG with formate was followed using a SX-20 stopped-flow spectrophotometer (Applied Photophysics, Inc.) equipped with photodiode array and photomultiplier tube detection, and running ProData SX 2.2.5.6 acquisition software. The enzyme was exchanged into anaerobic 75 mM K-phosphate, pH 7.7 to remove nitrate via Amicon Ultra 4 (Millipore) prior to use. As long as the enzyme was kept under anaerobic conditions, it could be maintained for 3-4 hours without significant loss of activity. Friedebold et al., 1993 [10] showed that nitrate binding followed a mixed competitive-noncompetitive type inhibition. It is possible that nitrate binding at the Mo-site blocks O₂ from affecting the removal of the catalytically essential apical sulfur ligand [9] by some as of yet unknown mechanism. Time courses for the reaction were monitored at 450 nm at 10°C and fitted to a sum of three exponentials by nonlinear least squares regression analysis to the following equation:

\[ A_t = A_{\infty} \pm \sum A_n \exp(-t/k_n) \] (2)

where n refers to the number of kinetic phases observed. Analysis of time courses was performed using the software ProData Viewer 4.2.0 (Applied Photophysics, Inc). Observed rate constants, k_{obs}, were plotted against substrate concentrations to obtain the limiting rate constant for reduction, k_{ed}, and the dissociation constant, K_d, using the equation:

\[ k_{\text{obs}} = k_{\text{ed}} [S] / (K_d + [S]) \] (3)

EPR spectroscopy. MoV-containing EPR samples were prepared in 75 mM potassium phosphate, pH 7.5 in the presence of 10 mM KNO₃ unless otherwise stated. MoV-containing spectra could be obtained upon reduction of the enzyme with either sodium dithionite or sodium formate, although the relative signal intensities of the MoV and several iron-sulfur signals depended to some degree on both the extent of reduction and the reductant used. Maximum accumulation of MoV EPR signal was obtained when the enzyme was approximately 50% reduced (by addition of ~5e equivalents), although additional reduction did not significantly affect MoV yield as long as the sample was frozen promptly. Nitrate had no effect on the shape of the EPR signals, although the yield of MoV EPR signal was
typically reduced in the absence of nitrate, even when nitrate was removed under anaerobic conditions (likely due to loss of activity during nitrate removal). No Mo$^V$ EPR signal was observed when samples were prepared from protein that had been incubated aerobically in the absence of nitrate for at least 6 hrs at 4°C (at which point virtually all of the enzymatic activity was lost). When necessary, buffer exchange was accomplished by concentrating protein samples, diluting with appropriate buffer and re-concentrating via Amicon Ultra 4 (Millipore). Samples were made anaerobic at 4°C (5-7°C for deuterated samples) and transferred to argon-flushed, septum-sealed EPR tubes. Samples prepared in this way were subsequently frozen in an ethanol/dry ice bath and transferred to liquid nitrogen. Further details of sample preparation are included in figure captions. EPR spectra were recorded using a Bruker EMX spectrometer equipped with a Bruker ER 4119HS High Sensitivity X-band cavity and gaussmeter, operating WinEPR version 4.33 acquisition software. Temperature was controlled using a Bruker variable temperature unit and liquid nitrogen or liquid helium cryostat. For purposes of comparison, all spectra were adjusted to the same microwave frequency by converting the magnetic field values for each individual spectrum to g-values using the equation:

$$g = \frac{71.4484 \times \nu \text{ (in GHz)} / B \text{ (in mT)}}{(4)}$$

and then back to mT using a microwave frequency of 9.475 GHz. Detailed instrument settings are included in figure captions. Simulations were performed using the EasySpin 4.5.5 software package [12]. For Mo$^V$-containing spectra, Euler angles relating the g- and A-tensor frames were empirically obtained and fixed during final simulations. During simulations of Mo$^V$-containing spectra, g values for the iron-sulfur clusters were allowed to vary by no more than ± 0.001 while anisotropic line broadening (simulated as HStrain in the EasySpin software package) by no more than ± 10 MHz. Fe/S linewidths used in the Mo-containing spectra were initially estimated from experimental spectra which contained the various Fe/S components at the temperatures where the Mo-containing spectra were obtained. These estimates were then used as starting points for simulation of the Mo-containing spectra. Neither the g- nor the A-tensors were generally affected by the way in which the linewidths were treated; only the relative contribution of each component to the composite spectra was sometimes affected. Due to the complexity of the composite spectra no attempts were made to quantify the effects of temperature on the linewidths of the various signals. Every multi-component simulation included a “weight” term, which could be used to estimate the relative contribution of each component to the composite spectrum.

Electron-electron interactions between Mo$^V$ and Fe/S3 were simulated using an S = 1/2 spin system. The electron-electron spin-spin interaction module in EasySpin does not allow variable linewidths for the interacting sites and at 20K the linewidth of the $g_2$ tensor of Fe/S3 is 1.5x greater than of the corresponding Mo$^V$. Initially, only the anisotropic splitting at $g_2$ of the Mo$^V$ (no interactions were apparent at $g_1$ or $g_3$) was simulated. After the initial simulation, the resulting value for the Mo$^V$ could be fixed for the interacting Fe/S3 as well. Due to the broad nature of the iron-sulfur centers, the exclusion of electron-electron coupling for the Fe/S3 did not have a discernible effect on either the accuracy of the simulation or the resultant parameters.

RESULTS

Enzyme spectroscopy and kinetics. FdsABG, isolated as described in Experimental Procedures, has the absorption spectrum shown in Figure 1A, in good agreement with previous reports [4,10,11]. The oxidized, as-isolated enzyme absorbs throughout the visible region, as expected given the large number of chromophores present, and has a broad shoulder at ~450 nm that is attributable to the FMN cofactor. A reductive titration with sodium dithionite results in a systematic bleaching of this absorbance throughout the UV-visible region (Figure 1B). A plot of the fractional absorbance change at 550 nm, where reduction of the FMN predominates, provides information regarding the relative order of reduction (due to the relative reduction potentials) of the FMN relative to the iron-sulfur clusters. As has been shown previously in related systems [13], a deflection from the diagonal (reflecting strict proportionality in the absorbance changes at the two wavelengths) up and to the left reflects earlier reduction of Fe/S clusters relative to FMN, and a deflection down and to the right the reverse. As indicated in the inset to Figure 1B, the plot shows a deflection up and to the left from the diagonal, indicating that the FMN becomes fully reduced rather than earlier in the course of the titration relative to the iron-sulfur clusters. This suggests that the FMN has a relatively low reduction potential relative to the iron-sulfur clusters. Also consistent with previous reports [4,10,11], our enzyme preparation exhibits a broad pH optimum at ~7.5,
fits of this bell-shaped curve to equation 1 yielded pKa’s for the acidic and alkaline limbs of the plot of 5.6 and 9.3 (Figure 2A).

A full steady-state analysis, not previously reported, is shown in Figure 2B-E. Lineweaver-Burk plots for the steady-state reaction of FdsABG at 30°C (as used in previous studies) yield approximately parallel lines, as is frequently seen with enzymes in which the sites of reduction and oxidation are physically separated [14,15]. We obtain an apparent K_m of 130 µM, consistent with both the determination of Friedebold et al., 1993 [10] of 90 µM for the same enzyme, as well as the recent report of 173 µM for the enzyme from R. capsulatus [16]. Our apparent K_m of 310 µM is in good agreement with the 281 µM determined for the R. capsulatus enzyme [16] but nearly tenfold smaller than the value of 3.3 mM determined by Friedebold et al., 1993 [10]. The plots in Figure 2B-E yield an average k_cat of 201 s^{-1}, which corresponds to approximately 67 U/mg of protein calculated using a molecular weight of 178,800 daltons for the heterotrimer, consistent with earlier reports of 80 U/mg [4,10,11].

We next examined the rapid-reaction kinetics of enzyme reduction by formate at pH 7.7, 10°C, the lower temperature being dictated by the high velocity of the reaction. As shown in Figure 3, the overall reaction is triphasic, with a fast phase that accounts for approximately 32% of the total observed absorbance change at 450 nm. Consistent with the reductive titration indicating a relatively low reduction potential for the FMN, the majority of the absorbance change at 450 nm, due to reduction of FMN, occurs in the intermediate phase of the reaction (Figure 3A). A plot of the observed rate constant for the fast phase of the reaction as a function of formate concentration is hyperbolic (Figure 3C), yielding a limiting k_cat at high [formate] of 140 s^{-1}, and a K_d of 82 µM. Assuming a doubling of rate constant for every 10°C increase in temperature, this corresponds to a limiting k_cat of ~600 s^{-1} at 30°C, indicating that the reductive half of the catalytic sequence is only partially rate-limiting. The intermediate phase of the reaction is also [formate]-dependent, with a limiting k_intermediate of 19 s^{-1} and K_d of 230 µM. We attribute this phase to the reaction of formate with partially reduced enzyme that accumulates in the course of the reaction, with the reaction rate attenuated due to the increasing likelihood that the molybdenum will be reduced (and therefore unreactive) on binding formate. The slowest phase of the reaction is [formate]-independent and accounts for only ~15% of the total absorbance change. It is most likely due to the slow intermolecular transfer of reducing equivalents from the formate-reduced functional enzyme to a small population of non-functional enzyme.

When deuterated formate is used in the rapid-reaction kinetics, the same multiphasic kinetics is observed, k_cat decreases to 66 s^{-1} and yields an isotope effect on k_cat of 2.1. This is consistent with a primary isotope effect, but one in which the isotope-sensitive step of the reaction is only partially rate-limiting in the reductive-half-reaction.

Mo^V EPR and demonstration of direct hydrogen transfer from substrate to the molybdenum center. We next examined the EPR properties of the enzyme, beginning with the enzyme’s molybdenum center. Figure 4A (black) shows the EPR spectrum seen at 150K of 100 µM FdsABG that has been partially reduced with sodium dithionite. The spectrum is composed of a narrow Mo^V signal centered at ~338 mT and clearly showing hyperfine coupling due to the 25% naturally occurring 95,97Mo (I = 5/2), as well as a much broader signal attributable to an iron-sulfur center. Simulations of the Mo^V component yielded g_{1,2,3} = 2.009, 2.001, 1.992 and 95,97Mo A_{1,2,3} = 138, 82, 45 MHz, with one strongly coupled proton having approximately isotropic 1H A_{1,2,3} = 18, 21, 18 MHz (Table 1); a simulation using these parameters is shown in Figure 4C. A fivefold expanded spectrum of the Mo^V EPR signal (Figure 4C) shows in detail the Mo hyperfine splitting; the 1H hyperfine splitting is unresolved and apparent only in the broadening of the peaks. By comparison to other molybdenum-containing enzymes (e.g. DMSO reductase [17] and xanthine oxidase [18,19]), the strength of the 1H coupling clearly indicates that the proton is part of the molybdenum center. Simulation of the iron-sulfur component yields g_{1,2,3} = 2.001, 1.946, 1.918 (Figure 4B). A sum of the simulations for the individual Mo^V and iron-sulfur components is shown with the experimental spectrum shown in Figure 4A, (red spectrum). As can be seen, the fit to the experimental spectrum is excellent, and we conclude that just two signal-giving species are present: a single Mo^V species exhibiting strong coupling to a single proton and one iron-sulfur signal. The EPR signals attributable to other iron-sulfur centers of the enzyme are considered further below.

Upon preparation of the sample in D_2O rather than H_2O, the proton coupling to the Mo^V signal disappears (owing to the very much weaker nuclear magnetic moment of ^2H relative to ^1H), clearly demonstrating the solvent-exchangeability of the coupled proton and illustrating the intrinsic
anisotropy of the $\text{Mo}^\text{V}$ g-tensor (Figure 4D, Figure 5). As in the proteate sample, the experimental spectrum again includes the EPR signal of a single Fe/S cluster. A threefold expanded spectrum in Figure 4F shows in detail the Mo hyperfine splitting, narrowed by the absence of proton hyperfine splitting. Upon partial reduction of enzyme with deuterated formate in H$_2$O (pH 8.5), the Mo$^\text{V}$ signal seen at short times lacks the strong proton coupling (Figure 6D) evident in the dithionite-reduced sample, although the g-values for the signal are unchanged; over the course of 1 min, however, the proton coupling grows in (Figure 6D), yielding an EPR signal indistinguishable from that seen in the dithionite-reduced enzyme. These results clearly demonstrate that the $C\alpha$ hydrogen of formate is transferred to the molybdenum center in the course of the reaction, and that the site is solvent-exchangeable. As discussed further below, we take this to reflect direct hydride transfer in the course of the reaction. There is strong mechanistic precedence for Mo=S groups being good hydride acceptors in molybdenum-containing enzymes [20,21,22], and for Mo-SH protons being both strongly coupled and solvent-exchangeable [23,24,25,26]. The mechanistic implications of these results are considered further in the Discussion section.

**Additional Fe/S EPR signals.** We have next examined additional EPR signals attributable to the iron-sulfur centers of FdsABG, which have been only incompletely analyzed previously [2]. The spectra shown in the following figures have been generated from samples prepared under various conditions described in detail in the figure legends and experimental section. In addition to the Fe/S signal seen at 150K shown in Figures 4 and 6, additional signals are observed as the temperature is progressively lowered to 20K. Figure 7 illustrates the various experimental spectra observed in the temperature range, 20-100K. Figure 7A shows the spectrum seen at 100K, which again can be described by a single rhombic species with $g_{1,2,3} = 2.001, 1.946, 1.918$ and which we designate as Fe/S1 (this is the same Fe/S cluster which contributes to the Mo-containing spectra in Figures 4 and 6). This species is generated by brief air-reoxidation of the sample exhibiting the Mo$^\text{V}$ signal in Figure 6D. Fe/S1 can also be generated by reducing non-functional enzyme with less than 1e$^-$ equivalent and is certainly the Fe/S cluster with the highest reduction potential. On the basis of its high reduction potential, we tentatively assign the cluster giving rise to the Fe/S1 signal as that coordinated by His 112 near the N terminus of the FdsA subunit, as substitution of His for Cys in iron-sulfur clusters is known to increase their reduction potentials. Non-functional enzyme that is unable to oxidize formate is prepared by incubation of the enzyme in nitrate-free buffer for at least 6 hrs at 4°C. Upon exhaustive reduction of the non-functional enzyme with sodium dithionite, a second, nearly-axial Fe/S signal is observed below 120K with $g_{1,2,3} = 2.017, 1.947, 1.933$ which we designate Fe/S2 (Figure 7B). At 60K the integrated intensities of the two signals are identical within experimental error. The Fe/S1 and Fe/S2 signals are also observed in a sample prepared by reducing non-functional enzyme with 10 mM NADH and freezing promptly (data not shown). As the temperature is lowered to 20K, two additional signals become apparent. The first, which we designate Fe/S3, can be simulated with $g_{1,2,3} = 2.044, 1.937, 1.898$ (Figure 7C), although precise determination of $g_2$ is difficult as it overlaps with features of other signals. The second additional signal apparent in the 20K spectrum is very broad and can be observed up to 60K. This signal can be better resolved at microwave powers greater than 20 mW where Fe/S1 can be almost completely power-saturated and Fe/S2 and Fe/S3 partially so. This broad signal, designated Fe/S4, is most clearly evident with 10 Gauss modulation amplitude and 100 mW microwave power (Figure 7D). Simulation of the experimental spectrum yields $g_{1,2,3} = 2.095, 1.888, 1.862$ for Fe/S4. Upon closer inspection of the shape of the Fe/S4 signal (Figure 7E), the asymmetry and the broadness of the $g_1$ and $g_2$ tensors suggest an additional very broad signal and it is noteworthy that at non-saturating power (Figure 7C) the simulation yields a slightly different set of $g$-values with $g_{1,2,3} = 2.095, 1.885, 1.882$ which we designate Fe/S4’ in Table 1. This lends additional evidence for the presence of a second very broad signal in the envelope in Figure 7C-E. Although the total integrated intensity of the Fe/S4-Fe/S4’ contribution to the experimental spectrum is over 23% in Figure 7C and 35% in Figure 7D, the very broad nature of this signal makes precise determination of the $g$-tensor difficult, particularly with regard to $g_3$, which overlaps with features of other signals.

Comparing the region centered at $\approx$342 mT in Figure 7C with 7D, the simulations are unable to reproduce a minor feature of the experimental spectrum (see arrow in Figure 7C), which appears to be power saturated in Figure 7D. Attempts to manipulate both the position and linewidth parameters of the $g_2$-tensor of either Fe/S3 or Fe/S4 to account for this small feature were unsuccessful, and it is possible that this feature may represent $g_2$ of a sixth cluster with its $g_1$ overlapping with one or
another of the better described signals. Simulation of the composite spectrum in Figure 7C yields integrated intensities for Fe/S1 through Fe/S4 in a ratio of approximately 1:1.6:1.3:1.1, respectively. Simulations of the four well-resolved iron-sulfur clusters are shown in Figure 8.

**Mo-Fe/S interaction.** Finally, we analyzed the dithionite-reduced FdsABG in deuterated buffer at 20K and 60K. At 60K, the experimental spectrum contains contributions from both the narrow MoV signal and also three iron-sulfur signals - Fe/S1, Fe/S2 and Fe/S4 in a ratio of approximately 1:0.8:0.2, respectively (Figure 9). The simulation in Figure 9C shows the MoV, while a comparison of the sum of the MoV and iron-sulfur components with the experimental spectrum is shown in Figure 9A, (red versus black, respectively). At 20K, the experimental spectrum differs from the data collected at 60K by the appearance of an additional signal (Figure 9D, black). Simulation of the iron-sulfur component yields four signals in a ratio of approximately 1:0.9:0.9:0.7, assigned to Fe/S1, Fe/S2, Fe/S3, and Fe/S4, respectively (Figure 9E). Simulation of the MoV signal in the spectrum obtained at 20K indicates that the signal is split with a hyperfine coupling constant of approximately 19 MHz (compare Figure 9C with Figure 9F), which we attribute to anisotropic coupling to an iron-sulfur cluster. Since the experimental spectra at 60K and 20K differ only in the presence of Fe/S3 in the latter, we conclude that Fe/S3 is the magnetically-coupled partner to the MoV center. The coupling on Fe/S3 due to the MoV center is not observed owing to the broad linewidths associated with Fe/S3. We do note, however, that the extreme anisotropy of the coupling is extremely unusual, although it appears not to represent a rapid-passage artifact as the coupling persisted unchanged as the EPR modulation frequency was varied (data not shown).

In addition to the simulation of the isolated MoV signals, Figure 9D shows a comparison of the sum of the MoV and iron-sulfur components with the experimental spectrum (red versus black, respectively). As can be seen, the fit to the experimental spectrum is excellent, and we conclude that five signal-giving species are present in the experimental spectrum: a single MoV species, magnetically coupled to an adjacent iron sulfur, Fe/S3, and the EPR spectra of Fe/S3 and three other iron-sulfur signals.

**DISCUSSION**

**Kinetics of the R. eutropha FdsABG formate dehydrogenase.** The steady-state kinetics reported here demonstrate that our enzyme preparation has the characteristics previously reported, and in particular has approximately the same high specific activity. Our rapid kinetic study of the reaction of enzyme with formate indicates that the reductive half-reaction is multiphasic. The fast and intermediate phases both exhibit hyperbolic dependence on [formate], with a limiting k_fast of 140 s⁻¹ and K_d_formate of 82 µM for the fast phase, and limiting k_intermediate of 19 s⁻¹ and K_d_formate of 230 µM for the intermediate phase. Given the large number of redox-active centers and the fact that at least five equivalents of formate must react with enzyme to fully reduce it, we interpret the fast phase of the reaction as reflecting the intrinsic rate constant for reaction of formate with enzyme possessing an oxidized molybdenum center, with the intermediate phase reflecting the reaction of formate with enzyme that has already reacted with several equivalents of substrate and has a significant amount of molybdenum that is at least partially reduced and therefore unreactive. It is likely that electron transfer among the several redox-active centers of FdsABG is rapid relative to the rate of formate oxidation at the molybdenum center, so that in these partially reduced enzyme forms the distribution of electrons will be dictated by the relative reduction potentials of the centers. On the basis of the absorbance change associated with the fast and intermediate phases of the reaction with formate, FMN reduction is associated with the latter, suggesting that the FMN has a relatively low reduction potential and accumulates reducing equivalents only later in the reaction. While it is surprising that this might be the case given that the FMN is the site of reaction with NAD⁺, it is consistent with the results of the equilibrium reductive titration reported here, which indicates that the FMN is reduced only later in the course of the titration.

With regard to the fast phase of the reaction, which again is taken to reflect the intrinsic rate of reaction of formate with the fully oxidized molybdenum center, we note that the limiting rate constant of 140 s⁻¹ at 10°C corresponds to ~600 s⁻¹ at 30°C, the temperature at which, as in past work, the steady-state work was done (assuming a doubling of rate constant for every 10°C increase in temperature). This being the case, a comparison with the observed k_cat of 201 s⁻¹ indicates that the reductive half-reaction of the catalytic sequence is only partially rate-limiting. Similarly, the modest kinetic isotope of 2.1 seen when using ¹H-formate as substrate indicates that the isotope-sensitive step is
only partially rate-limiting in the reductive half-reaction.

Mo\textsuperscript{V} EPR and mechanistic implications. With regard to the EPR of the molybdenum center, we note that the very high g\textsubscript{ave} of the Mo\textsuperscript{V} EPR signal seen here is consistent with an all-sulfur molybdenum coordination sphere, as concluded in a recent XAS analysis of the homologous FdsABG from \textit{R. capsulatus} [9]. The signal is also characterized by strong coupling to a solvent-exchangeable proton, with \( \Delta_{1,2,3} = 18, 21 \) and 18 MHz. This coupling is in fact comparable to that exhibited by the Mo-SH proton of the so-called “rapid Type 1” EPR signal seen with xanthine oxidase, another molybdenum-containing enzyme possessing a Mo=S in the oxidized state. Like the strongly coupled proton in the “rapid Type 1” signal seen with xanthine oxidase, that which is coupled in the Mo\textsuperscript{V} signal of FdsABG is substrate-derived and, ultimately, solvent-exchangeable. We conclude that the substrate-derived, solvent-exchangeable and strongly coupled proton seen here with the FdsABG formate dehydrogenase is similarly due specifically to the proton of a Mo-SH group, formed by protonation of the Mo=S group of oxidized enzyme upon reduction of the molybdenum. While it is conceivable that this hydride transfer might be mediated by some intervening group in the active site, this site would have to be both redox-active and not solvent-exchangeable. An examination of the active site of the \textit{E. coli} FdhF formate dehydrogenase (Figure 11), indicates that no such group is readily identifiable. We specifically note that of the amino acid residues surrounding the molybdenum center, only two are not conserved between FdhF and FdsA: Sec 140 that coordinates the metal (and is a Cys in FdsA) and Val 338, which is a Leu in FdsA. His 141 is in the substrate binding pocket, but is not redox-active; similarly, the pyranopterin cofactors of the molybdenum center are known not to be formally redox-active [1]. The demonstration here that the strongly coupled proton seen in the cysteine-containing \textit{R. eutropha} FdsABG is substrate-derived is also consistent with previous work with the selenocysteine-containing FdhF formate dehydrogenase from \textit{E. coli}, which also exhibits a strongly coupled and substrate-derived proton [27] (we also confirm the strong pH dependence of the rate of solvent exchange seen with the FdsF protein, which shows significantly at higher pH, data not shown). These earlier workers, however, assigned the coupled proton to a conserved active site histidine of the FdhF enzyme (His 141, lying some 6.1 Å from the molybdenum) rather than to a ligand of the molybdenum coordination sphere (which at the time was believed to possess a Mo=O rather than a Mo=S in the oxidized state, and to possess only the selenocysteine and pyranopterin ligands in a five-coordinate reduced state [5]). Protonation of the selenocysteine ligand to the Mo of the \textit{E. coli} FdhF was thus the only option considered in this earlier work). We note that our present assignment of the strongly coupled proton to a Mo-SH group is in fact fully consistent with all results reported previously for the \textit{E. coli} FdhF enzyme, and provides the more plausible explanation as to why the observed proton coupling should be so strong.

On the basis of the interpretation that the substrate proton is abstracted by an active site histidine, mechanisms for the \textit{E. coli} FdhF (and by implication other molybdenum-containing formate dehydrogenases) have been proposed in which formate coordinates to the molybdenum via its oxyanion group and displaces the selenocysteine, followed by abstraction of the C\textsubscript{a} proton by His 141 (possibly involving the now-dissociated SeCys) and transfer of an electron pair into the redox-active Mo d\textsubscript{xy} orbital [5]. Given that the pK\textsubscript{a} of a molybdenum-coordinated formate is expected to be at least as high as that of formamide (pK\textsubscript{a} = 23.5, [28]), proton abstraction by a histidine residue is extremely thermodynamically unfavorable. We propose instead that the reaction for both the cysteine- and selenocysteine-containing molybdenum formate dehydrogenases (and probably also the related tungsten-containing enzymes) proceeds by a simple hydride transfer from formate, with the Mo-S serving as the hydride acceptor, as shown in Figure 10. In this mechanism, it must be remembered that in a formal valence count all four electrons in the Mo-S bond of oxidized enzyme belong to the sulfur, and the last arrow in the sequence shown represents the formal reduction of the six-coordinate molybdenum from Mo\textsuperscript{VI} to Mo\textsuperscript{V}. Retention of the catalytically essential sulfur as a ligand to the molybdenum in the reduced species is necessary since the subsequent transfer of one electron out of the molybdenum center yields the Mo\textsuperscript{V}-SH species observed by EPR (its loss in any case would lead to inactivation of the enzyme). Similarly, it is likely (although not required by the mechanism) that the cysteine ligand (or selenocysteine in the case of FdhF) is retained. We note that XAS analysis of FdhF from both \textit{E. coli} [29] and \textit{D. desulfuricans} [30] indicates that the molybdenum center is six-coordinate in the reduced state with the selenocysteine of these enzymes specifically retained; the reduced FdsABG from \textit{R. capsulatus} is also six-coordinate [9], with the cysteine retained.
The catalytic sequence is completed from the EPR-active Mo$^{V}$ state by a second electron transfer and loss of the substrate-derived proton to yield the initial Mo$^{VI}$ species.

Supporting this mechanism, we note that: (1) formate is intrinsically a good hydride donor (as reflected in the fact that the eukaryotic formate dehydrogenases are cofactorless and catalyze direct hydride transfer from formate to NAD$^{+}$ through a ternary E•formate•NAD$^{+}$ complex); and (2) Mo=S groups are facile hydride acceptors (virtually all members of the xanthine oxidase family of molybdenum enzymes possess a Mo=S group that serves as a hydride acceptor in the course of the reaction\[14,15,16\]). Other elements of this mechanism are also chemically reasonable, namely that formate need not coordinate to the molybdenum in the course of the reaction, and that the molybdenum itself is coordination-stable (apart from the conversion of a Mo$^{VI}$=S group to a Mo$^{VI}$-SH group in the course of the reaction). As in the case of xanthine oxidase and related enzymes \[31,32\], the Mo$^{V}$ state forms only at the completion of the reductive half-reaction, with transfer of a single electron to the proximal [4Fe-4S] cluster to yield the EPR-active Mo$^{VI}$-SH species. It is to be emphasized that the proton egress pathway from the molybdenum center identified by Stadtman and coworkers \[5,27\], beginning with His 141 (analogous to His 379 in the FdsABG enzyme), indeed likely plays an important catalytic role in facilitating deprotonation of the molybdenum center as it is reoxidized to the Mo$^{VI}$ state upon subsequent transfer of the second electron out of the molybdenum center.

**EPR of the iron-sulfur centers of FdsABG.**

As summarized in Figure 8 and Table 1, we have identified and characterized four EPR signals, designated Fe/S1-Fe/S4, that are attributable to the four iron-sulfur clusters of FdsABG; a fifth signal is tentatively identified but less well-characterized (Fe/S4'). Of the four well-characterized signals, two are almost axial and two are rhombic; of these, two exhibit a particularly high g-anisotropy (Fe/S3 and Fe/S4). Fe/S1 and Fe/S2, the more modestly anisotropic signals, are observed at liquid nitrogen temperatures and exhibit rather narrow linewidths that suggest slow spin-lattice relaxation rates. While Fe/S1 is observed at temperatures from 200K down to below 5K, Fe/S3 is only seen below 120K. The two more highly rhombic Fe/S3 and Fe/S4 signals are seen only at liquid helium temperatures. All of the Fe/S signals persist down to the limit of helium temperatures, below 5K.

Based on the sequence \[33\] and structural \[2\] similarities of the (cofactorless) C-terminal region of Nqo3 of the *T. thermophilus* NADH dehydrogenase \[2,3\] to the FdhF formate dehydrogenase \[5\], a model can be constructed for FdsABG that gives some indication of the disposition of its nine redox-active centers, as shown in Figure 12 \[1\]. Interestingly, in the structure of the *T. thermophilus* Nqo3 subunit the C-terminal [4Fe-4S] cluster (that nearest the position of the erstwhile molybdenum-containing domain) is some 20 Å from the nearest iron-sulfur cluster in the subunit. The alignment of Nqo3 with FdsA, however, provides clear evidence for an intervening [4Fe-4S] cluster (to the N-terminal side of the isolated cluster in Nqo3) in FdsA \[1\]. The position of this additional cluster in FdsABG in the context of the structure of Nqo3 of the *T. thermophilus* NADH dehydrogenase is readily defined by noting that the last [4Fe-4S] cluster before the gap in Nqo3 is found in a classic 2 x [4Fe-4S] bacterial ferredoxin-like structural motif that lacks a second [Fe-4S] cluster (owing to loss of cysteine residues necessary to coordinate it). This circumstance allows the approximate position of the additional [4Fe-4S] cluster of FdsA to be identified in the context of the structure of the *T. thermophilus* NADH dehydrogenase (Figure 12, circled) \[1\].

The above model provides a frame of reference for assigning the EPR signals seen with FdsABG to specific clusters, as correlated with its amino acid sequence. First, the iron-sulfur cluster that is magnetically coupled to the molybdenum center is undoubtedly that lying closest to it. We therefore assign the Fe/S3 signal with $g_{1,2,3} = 2.044, 1.937, and 1.898$ EPR to the most C-terminal [4Fe-4S] cluster of FdsA, coordinated by Cys 249, Cys 252, Cys 256 and Cys 285. This cluster is homologous to the sole iron-sulfur cluster seen in the *E. coli* FdhF enzyme, which lies to the N-terminal side of the molybdenum-binding portion of the enzyme (and whose EPR signal has $g_{1,2,3} = 2.045, 1.957$ and 1.840 \[27\]). Treating the electron-electron interaction as strictly dipolar in nature, the experimentally observed coupling of 19 MHz would correspond to a 14 Å center-to-center distance \[12\], that is generally consistent with the 12.4 Å distance seen in the *E. coli* FdhF formate dehydrogenase (PDB 1AA6). The next most straightforward assignment is Fe/S1, which with $g_{1,2,3} = 2.001, 1.946$, and 1.918 has both a relatively low $g_{ave}$ and a high reduction potential. We thus tentatively assign this signal to the [4Fe-4S] cluster of FdsA that is coordinated by His 112, Cys 116, Cys 119 and Cys 125, noting that the histidine-for-sulfur substitution is expected to have the dual effect of decreasing $g_{ave}$
and increasing the reduction potential of the cluster. Assignment of the remaining EPR signals is much more problematic, and must await future site-directed mutagenesis studies knocking out one or another center to allow definitive assignments.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
Niks performed all EPR and rapid reaction kinetic work, and assisted in preparing the manuscript. Duvvuru and Escalona assisted with the steady-state kinetics work. Hille conceived the project, directed the research and was principally responsible for preparation of the manuscript.
REFERENCES


FOOTNOTES
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ABBREVIATIONS USED
EPR: electron paramagnetic resonance; FdsABG: soluble, NAD+-dependent, molybdenum-containing formate dehydrogenase, product of the fdsABG gene cluster
FIGURE LEGENDS

FIGURE 1. Reductive titration of FdsABG. A. Oxidized (blue) and sodium dithionite-reduced (black) spectra. B. Change in absorbance as a function of reduction. Inset plots the relative absorbance at 550 nm vs. relative absorbance at 450 nm, with the diagonal (reflecting strict proportionality in the absorbance change at the two wavelengths) indicated. Details of titration are described in the Experimental Procedures.

FIGURE 2. A. pH dependence of \( k_{\text{cat}} \) for FdsABG. Reactions were performed at 30°C in a buffer containing 75 mM of malate, K\(_2\)HPO\(_4\), Tris, and glycine, brought to pH 5-10. The fit to the data (solid line) yielded two \( pK_a \) values of 5.6 and 9.3, respectively. B. Lineweaver-Burk plots for the reaction of FdsABG with formate at the following [NAD\(^+\): 1.62 mM (closed circles), 0.54 mM (open circles), 0.18 mM (closed triangles), 0.06 (open triangles). C. Lineweaver-Burk plots for the reaction of FdsABG with \( \text{NAD}^+ \) at the following [formate]: 3.24 mM (closed circles), 1.08 mM (open circles), 0.36 mM (closed triangles), 0.12 (open triangles). D. Simulation of the Mo \( \mu = \) 201 s\(^{-1}\). E. Secondary plots of 1/y-intercept taken from B. vs. 1/[NAD\(^+\)]. E. Secondary plots of 1/y-intercept taken from C. vs. 1/[formate]. Reactions in B. and C. were performed in 75 mM K-PO\(_4\), pH 7.7 at 30°C. Linear regression analysis of plots D. and E. yielded a \( K_{m\text{NAD}^+} = 130 \mu\text{M} \) and \( K_{m\text{formate}} = 310 \mu\text{M} \), respectively, and an average \( k_{\text{cat}} \) = 201 s\(^{-1}\).

FIGURE 3. Pre-steady-state kinetics for the reduction of FdsABG with formate or \(^2\text{H}\)-formate. A. Rapid scanning stopped-flow traces for the reaction of 6 \( \mu\text{M} \) of FdsABG with 5 mM formate performed at 10°C in 75 mM K-PO\(_4\), pH 7.7. B. A representative kinetic trace of the reaction of FdsABG with 0.8 mM sodium formate monitored at 450 nm. The trace is best represented by three phases with \( k_{\text{obs}} = 124 \text{ s}^{-1}, 14 \text{ s}^{-1}, \) and 2.5 \text{s}^{-1}, with \( \Delta A = 0.011, 0.017, \) and 0.006, respectively. C. Plots of \( k_{\text{fast}} \) (black circles) and \( k_{\text{intermediate}} \) (red squares) vs. formate concentrations or \( k_{\text{fast}} \) (black triangles) vs. \(^2\text{H}\)-formate concentrations. All reactions were performed at 10°C in 75 mM K-PO\(_4\), pH 7.7 with 2.5 \( \mu\text{M} \) FdsABG. Hyperbolic fits (solid lines) yielded \( k_{\text{fast}} = 140 \text{ s}^{-1}, K_{d\text{formate}} = 82 \mu\text{M}, k_{\text{intermediate}} = 19 \text{ s}^{-1} \) and \( K_{d\text{formate}} = 230 \mu\text{M} \) for reaction with formate and \( k_{\text{fast}} = 66 \text{ s}^{-1}, K_{d\text{Hformate}} = 193 \mu\text{M} \) for reaction with \(^2\text{H}\)-formate.

FIGURE 4. EPR of Mo-center of FdsABG collected at 150K. A. Mo\(^{V}\)-Fe/S EPR spectrum (black) and simulation (red) of FdsABG collected with modulation amplitude = 2 Gauss and microwave power = 4 mW. The sample was prepared under anaerobic conditions by reduction of 100 \( \mu\text{M} \) of FdsABG in 75 mM K-PO\(_4\), pH 7.5 in the presence of 10 mM KNO\(_3\), with 5 mM buffered sodium dithionite. The Mo\(^{V}\) component represents approximately 33% of total spin density. B. Simulation of the Fe/S1 contribution to the spectrum in A. C. Simulation of the Mo\(^{V}\) contribution to the spectrum in A. The hyperfine splitting due to the 25% naturally occurring \(^{95,97}\)Mo (1 = 5/2) is shown in the fivefold expanded spectrum above. D. Mo\(^{V}\)-Fe/S EPR spectrum (black) and simulation (red) of deuterated FdsABG collected with modulation amplitude = 2 Gauss and microwave power = 4 mW. The sample was prepared under anaerobic conditions by reduction of 100 \( \mu\text{M} \) of FdsABG in 75 mM K-PO\(_4\), pH 7.1, in the presence of 10 mM KNO\(_3\), with 0.4 mM sodium formate. The Mo\(^{V}\) component represents approximately 38% of total spin density. E. Simulation of the Fe/S1 contribution to the spectrum in D. F. Simulation of the Mo\(^{V}\) contribution to the spectrum in D. The hyperfine splitting due to the 25% naturally occurring \(^{95,97}\)Mo (1 = 5/2) is shown in the threefold expanded spectrum above. Simulation parameters are summarized in Table 1.

FIGURE 5. Expanded view of the EPR of Mo-center of FdsABG from Figure 4. Superposition of the composite Mo\(^{V}\)-Fe/S EPR spectra from Figure 4A (black) with Figure 4D (red). The line diagram above indicates the location of the principal g-tensors (red vertical lines) and the approximate location of the \(^1\text{H}\) hyperfine splitting (I = 1/2) in the spectrum from Figure 4A (black vertical lines).

FIGURE 6. A. Mo\(^{V}\)-Fe/S EPR spectrum (black) and simulation (red) of FdsABG collected at 150K with modulation amplitude = 3 Gauss and microwave power = 4 mW. The sample was prepared under anaerobic conditions by reduction of 50 \( \mu\text{M} \) of FdsABG in 200 mM Tris-HCl, pH 8.5, in the presence of 10 mM KNO\(_3\), with 0.25 mM sodium formate on ice and mixing for 5s before freezing. The Mo\(^{V}\) component represents approximately 10% of total spin density. B. Simulation of the Fe/S1 contribution to the spectrum in A. C. Simulation of the Mo\(^{V}\) contribution to the spectrum in A. D. Mo\(^{V}\)-Fe/S EPR spectrum (black) and simulation (red) of FdsABG collected at 150K at with modulation amplitude = 2 Gauss and microwave power = 4 mW. The sample in panel A. above was thawed in a RT water bath and incubated for an additional 55s before...
refreezing. The Mo\textsuperscript{V} component represents approximately 19% of total spin density. E. Simulation of the Fe/S1 contribution to the spectrum in D. F. Simulation of the Mo\textsuperscript{V} contribution to the spectrum in D.

**FIGURE 7.** EPR of Fe/S centers of FdsABG. A. Fe/S1 EPR spectrum (black) and simulation (red) of FdsABG collected with modulation amplitude = 4 Gauss and microwave power = 4 mW at 100K. The sample was prepared by thawing out the sample from Figure 6D under aerobic conditions in a RT water bath and incubating for an additional 1 min before refreezing. Location of \( g_1 \), \( g_2 \), and \( g_3 \) are indicated. B. Fe/S EPR spectrum (black) and simulation (red) of FdsABG collected at 60K with modulation amplitude = 4 Gauss and microwave power = 4 mW. The sample was prepared by incubation of 100 \( \mu \)M of FdsABG in 75 mM K-PO\(_4\), pH 7.7, in the absence of 10 mM KNO\(_3\) for >6hrs. (aerobically at 4°C), followed by reduction under anaerobic conditions with 5 mM buffered sodium dithionite and further incubation for 1 hr before freezing. Location of \( g_1 \), \( g_2 \), and \( g_3 \) corresponding to the Fe/S2 component of the spectrum are indicated. C. Fe/S EPR spectrum (black) and simulation (red) of FdsABG sample from panel B. collected at 20K at with modulation amplitude = 3 Gauss and microwave power = 0.2 mW. Location of \( g_1 \), \( g_2 \), and \( g_3 \) corresponding to the Fe/S3 component of the spectrum are indicated. D. Fe/S EPR spectrum (black) and simulation (red) of FdsABG sample from panel B. collected at 20K at with modulation amplitude = 10 Gauss and microwave power = 100 mW. Location of \( g_1 \), \( g_2 \), and \( g_3 \) corresponding to the Fe/S4 component of the spectrum are indicated. E. Spectrum from panel D. enlarged to show the broad peaks corresponding to Fe/S4. Simulation parameters are summarized in Table 1.

**FIGURE 8.** Simulations of EPR spectra for Fe/S1-Fe/S4 from parameters summarized in Table 1. The iron-sulfur clusters are classified by the increasing value of their \( g_1 \)-tensor.

**FIGURE 9.** EPR of deuterated Mo-center of FdsABG collected at 20-60K. A. Mo\textsuperscript{V}-Fe/S EPR spectrum (black) and simulation (red) of FdsABG sample as described in Figure 4D. collected with modulation amplitude = 2 Gauss and microwave power = 0.2 mW at 60K. The Mo\textsuperscript{V} component represents approximately 27% of total spin density. B. Simulation of the Fe/S1 and Fe/S2 contributions to the spectrum in A. C. Simulation of the Mo\textsuperscript{V} contribution to the spectrum in A. D. Mo\textsuperscript{V}-Fe/S EPR spectrum (black) and simulation (red) of FdsABG sample from panel A. above collected with modulation amplitude = 2 Gauss and microwave power = 0.02 mW at 20K. The Mo\textsuperscript{V} component represents approximately 16% of total spin density. E. Simulation of the Fe/S1-Fe/S4 contributions to the spectrum in D. F. Simulation of the Mo\textsuperscript{V} contribution to the spectrum in D. Simulation parameters are summarized in Table 1.

**FIGURE 10.** A proposed hydride transfer mechanism for the formate dehydrogenases. Beginning with the ionized substrate, the second C=O double bond forms displacing hydride, which attacks the Mo=S group, bringing about the formal two-electron reduction of the metal with concomitant transfer of the C\(_\alpha\)-H to the sulfur.

**FIGURE 11.** The active site of the FdhF formate dehydrogenase from *E. coli* (PDB 1FDO). The molybdenum is at the center of the figure, coordinated by the two equivalents of pyranopterin cytidine dinucleotide (PCD), a terminal sulfido (represented as a coordinated water in the original PDB file) and Sec 140. The substrate binding site is delineated by two loops consisting of A\(_\text{137}\)RVU\(_\text{140}\)HGP\(_\text{143}\) and G\(_\text{328}\)VNPLR\(_\text{333}\)GQNNV\(_\text{338}\)QG\(_\text{340}\). Arg 333 has been proposed to interact with the negatively charged substrate. In these two stretches, only two residues differ in the FdsA subunit of the *R. eutropha* formate dehydrogenase: Sec 140, which is a Cys that coordinates the molybdenum in FdsA, and Val 338, which is a Leu in FdsA.

**FIGURE 12.** A structural model for the *R. eutropha* formate dehydrogenase. The molybdenum center is at left and the FMN at right, with the several Fe/S clusters intervening. The model is based on homologies to the *T. thermophilus* NADH dehydrogenase (PDB 3IAM) and the *E. coli* formate dehydrogenase (PDB 1AA6), with the position of the additional [4Fe-4S] cluster present in FdsA but absent in Nqo3 indicated by the red circle.
TABLE 1. EPR simulation parameters for MoVI and reduced [2Fe-2S] and [4Fe-4S] centers of FdsABG

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a In MHz; in the absence of multi-frequency data, coupling constants are approximate
b Euler angles \( [\alpha = 10.8^\circ, \beta = 0.2^\circ, \gamma = 152.5^\circ] \) were fixed during simulations; in the absence of multi-frequency data, angles are approximate
c g-tensor uncertainty, ± 0.001
d g-tensor uncertainty not determined (see text)
e fixed during simulations
FIGURE 1.
FIGURE 2.
FIGURE 3.
FIGURE 4.
Figure 5.
Formate dehydrogenase from Ralstonia eutropha

FIGURE 7.
FIGURE 8.
FIGURE 9.
FIGURE 10.
FIGURE 11.
FIGURE 12.