Direct Oxygen Atom Transfer in the Mechanism of Action of *Rhodobacter sphaeroides* Dimethyl Sulfoxide Reductase

Brian E. Schultz,† Russ Hille,‡ and R. H. Holm*,†

Department of Chemistry, Harvard University
Cambridge, Massachusetts 02138
Department of Medical Biochemistry
The Ohio State University, Columbus, Ohio 43210

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The forward and reverse oxygen atom (oxo) transfer reactions $Mo^{VI}O_2L_n + X \rightleftarrows Mo^{IV}OL_n + XO$ resulting in oxidation/reduction of substrates X/XO have been amply demonstrated in this[1,2] and other laboratories.[1b,c,3,4] These observations are the basis of the oxo transfer hypothesis[1] for the mechanism of action of oxomolybdenum enzymes,[3,5,6] which catalyze the overall reaction $X + H_2O \rightleftarrows XO + 2H^+ + 2e^-$. Under this hypothesis, depicted as scheme 1 (Figure 1) for enzymes lacking terminal Mo=S groups, substrate XO binds cis to the $Mo^{IV}O$ group. Atom transfer ensues to afford the $Mo^{VI}O_2$ group and X; the enzyme site is returned to the Mo(IV) state by sequential electron and proton transfer. The reverse sequence applies to substrate oxidation. Existence of the $Mo^{VI}O_2$ and $Mo^{IV}O$ groups in fully oxidized and dithionite–reduced enzyme states, respectively, has been established in other enzymes by molybdenum EXAFS analysis.[3,5,6] Despite its apparent simplicity, there has been only one *direct* proof of the atom transfer pathway. By means of ^{18}O labeling, xanthine oxidase (containing the Mo^{VI}-OS group when fully oxidized) was shown to incorporate at the C-8 position of substrate the oxygen atom present at the molybdenum center, rather than that of solvent water.[7]

Two recently characterized molybdoenzymes are unusual in that they do not contain any prosthetic groups other than their molybdenum centers and their associated pterin cofactors. These are the DMSO reductases from *Rhodobacter capsulatus*[8] and *Rhodobacter sphaeroides*.[9] Both are periplasmic single–subunit enzymes (M_r 82 000) that serve as terminal reductases in the bacterial respiratory chain when Me_2SO is used as an electron acceptor ($Me_2SO + 2H^+ + 2e^- \rightarrow Me_2S + H_2O$). The similarity between the two extends to spectroscopic properties,[10–12] which provide convincing evidence for direct coordination to the pterin via its enedithiolate functionality. In a further evaluation of the oxo transfer hypothesis, we have utilized *R. sphaeroides* DMSO reductase and ^{18}O labeling methods.

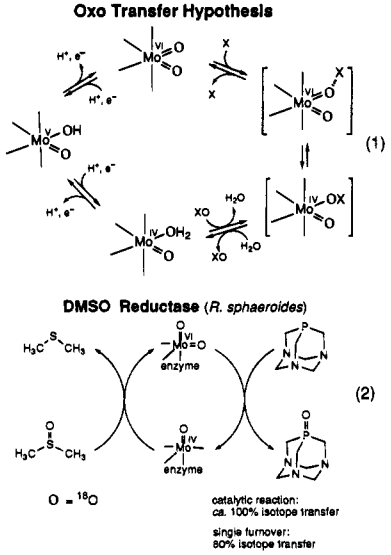

Figure 1. Top (scheme 1): The oxo transfer hypothesis for enzymes lacking terminal sulfido ligands; X/XO are reduced/oxidized substrates. Proposed intermediates are in brackets; these are anticipated on the basis of the reactions of $Mo^{VI}O_2$ complexes with tertiary phosphines.[1,2,19bc] The presence of water implies a possible role of H^+ or OH^- in certain steps.[6] Bottom (scheme 2): Coupled oxo transfer reactions of *R. sphaeroides* DMSO reductase demonstrated in this work.

Set out in scheme 2 (Figure 1) are two potentially coupled, enzyme–mediated oxo transfer reactions involving Me_2SO and a nonphysiological oxo acceptor, water–soluble 1,3,5-triaza-7-phosphatricyclo[3.3.1.1^{3,7}]decane[13] (PTA). The suitability of this tertiary phosphine as an oxo acceptor was first demonstrated by its reaction with the enzyme active site analogue complex MoO_2(*t*-BuL-NS)$_2$.[2] When a 0.44 mM solution of the Mo(VI) complex (λ_{max} 371 nm) was treated with excess (27 equiv) PTA in a heterogeneous DMF reaction mixture[13] at 25 °C, the absorption spectrum changed over 9 h to that of MoO(*t*-BuL-NS)$_2$ (λ_{max} 328, 430, 518, 700 nm) with tight isosbestic points at 341 and 404 nm. These observations are identical to those for the reaction system with Et_3P, where atom transfer to form Et_3PO was proven by ^{18}O labeling,[2] and correspond to the reaction MoO_2(*t*-BuL-NS)$_2$ + PTA → MoO(*t*-BuL-NS)$_2$ + PTAO. Reoxidation of MoO(*t*-BuL-NS)$_2$ to MoO_2(*t*-BuL-NS)$_2$ by Me_2SO has already been demonstrated.[2] On the assumption of the absence of an unduly restricted binding site in the enzyme, a similar set of reactions is expected with DMSO reductase, if it indeed operates by an oxo transfer pathway.[14]

Anaerobic treatment of a 42 μM solution of DMSO reductase[15] with a large excess of PTA resulted in replacement of the initial broad feature at 720 nm with a less intense broad band at 640 nm over *ca.* 4 h. We conclude that this absorption band, which is also generated by dithionite reduction[8–11] and corresponds to an EPR-silent state of the enzyme,[9b] derives from the reduced enzyme state formed in the reaction Enz-$Mo^{VI}O_2$ + PTA → Enz-$Mo^{IV}O$ + PTAO. Addition of excess Me_2SO completely regenerated the spectrum of the oxidized enzyme.[15] Similar spectral changes have been found in systems containing $P(CH_2OH)_3$.[16] The oxo transfer reactions implied by the present

† Harvard University.
‡ The Ohio State University.

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(14) There is as yet no *direct* proof that oxidized DMSO reductase contains an $Mo^{VI}O_2$ group. However, the EPR spectrum of the Mo(V) state and the pH dependence of the signal,[9b] as well as the EPR and other spectroscopic properties of the glycerol-inhibited Mo(V) state,[12] are consistent with other enzymes whose fully oxidized molybdenum center contains this group.

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Table 1. Isotope Transfer from [^{18}O]Me_2SO to PTA Mediated by DMSO Reductase under Single-Turnover and Catalytic Conditions[a]

	% incorporation in PTAO		% ^{18}O label transfer[b]	
run	catalytic[c]	single turnover[d]	catalytic[c]	single turnover[d]
1	86	80	100	93
2	87	76	101	88
3	86		100	

[a] All reactions were performed under a pure dinitrogen atmosphere; PTAO workup procedures were done aerobically. [b] Based on 86% enriched [^{18}O]Me_2SO. [c] [Enzyme] = 1.2 μM, [^{18}O]Me_2SO 86(3)% enriched, $[[^{18}O]Me_2SO]_0 = [PTA]_0 = 1.65$ mM, reaction time 1 day. PTAO was isolated by centrifugal filtration, lyophilization, and TLC (silica, methanol). [d] [Enzyme] = 61 μM (run 1), 53 μM (run 2); $[Me_2SO]_0 = 57$ μM (run 1), 52 μM (run 2); $[PTA]_0 = 56$ μM (run 1), 49 μM (run 2). Reactions were carried out in sequential steps of reduction of the enzyme by titration with $Na_2S_2O_4$, reoxidation of the enzyme with [^{18}O]Me_2SO, and reduction with PTA. PTAO was isolated by centifugal filtration, filtration through C_{18} silica, and lyophilization. Isotope analysis of PTAO was performed by chemical ionization mass spectrometry, using a JEOL AX-505 mass spectrometer with 3 keV ion energy and ammonia as the reagent gas.

observations with DMSO reductase were examined in reaction systems containing both [^{18}O]Me_2SO and PTA under catalytic and single-turnover conditions. Experimental conditions and results are summarized in Table 1.

Under catalytic conditions, 1.2 μM enzyme solutions were incubated with a *ca.* 1400-fold excess of [^{18}O]Me_2SO[17] (86 ± 3% enriched) and PTA for 1 day. The product phosphine oxide was detected by CI-MS at *m/z* 193 corresponding to $[PTA^{18}O{\cdot}NH_4]^+$. The catalytic experiment was performed in triplicate; the results (Table 1) demonstrate quantitative ^{18}O isotope transfer from [^{18}O]Me_2SO to PTA. In a control experiment involving much higher concentrations of reactants than in the catalytic systems, a solution with $[Me_2SO] = [PTA] = 36$ mM in 50 mM Tris buffer, pH 8.0, when monitored by ^{31}P NMR spectroscopy showed no detectable reaction after 5 days. Similarly, no reaction was observed in a solution with $[Me_2SO] = [PTA] = 30$ mM in the presence of 10 mM Na_2MoO_4. The generalized reaction $R_2SO + R'_3P \rightarrow R_2S + R'_3PO$ has an enthalpic driving force of *ca.* 45 kcal/mol.[18] Yet reaction rates under these and other conditions[19] are essentially nil in the absence of a catalyst. We conclude that the intact enzyme catalyzes oxo transfer from Me_2SO to PTA, yielding PTAO whose oxygen atom does not derive from solvent.

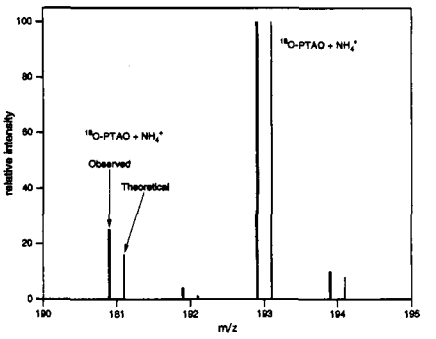

Figure 2. CI-MS spectrum of PTAO from the single-turnover reaction of [^{18}O]Me_2SO and PTA mediated by DMSO reductase (run 1) and the theoretical mass spectral signals on the assumption of quantitative ^{18}O isotope transfer from 86% enriched Me_2SO.

To provide additional confirmation of metal-centered atom transfer rather than direct reaction of Me_2SO and PTA, reactions were conducted with DMSO reductase under single-turnover conditions. Because the molybdenum center with complexed pterin is the only chromophore in the enzyme, the reaction can be monitored by changes in the UV/visible spectra. In one experiment, titration of a 39 μM solution of oxidized enzyme with dithionite resulted in the appearance of the reduced form. Reaction of the latter with 0.95 equiv of [^{18}O]Me_2SO resulted in an absorbance increase in the 700 nm region. Addition of 0.87 equiv of PTA afforded a spectrum which at 2 h closely approached that of the dithionite-reduced form. The spectral changes are nearly the same as those observed in the foregoing separate experiments with excess Me_2SO and excess PTA. The single-turnover experiment was performed in duplicate; a mass spectrum in the PTAO parent ion region is illustrated in Figure 2. The results (Table 1) show a high extent (ca. 90%) of isotope transfer from [^{18}O]Me_2SO to PTA.

The foregoing results are interpreted in terms of scheme 2 (Figure 1). In its $Mo^{IV}O$ state, DMSO reductase converts substrate to Me_2S and incorporates an oxygen atom at the catalytic center to afford the $Mo^{VI}(^{16}O^{18}O)$ state. This state evidences a substantial degree of asymmetry, at least with respect to PTA, for the substrate discriminates between the two oxo atoms; otherwise, the nearly quantitative isotope transfer observed in single turnover would not be possible. Oxidation of PTA returns the enzyme to the $Mo^{IV}O$ state and completes one cycle of ^{18}O isotope transfer. The high extent of label transfer demonstrates that exchange of the oxomolybdenum groups with bulk water is slow compared to the time scale of the experimental protocol.

The term "oxotransferase" was originally applied to oxomolybdenum enzymes because their overall reactions involve the formal transfer of an oxygen atom between substrate and water.[1a] The extent to which this term is apposite in a mechanistic reactivity sense can only be demonstrated by label transfer experiments such as those presented here. One implication of the *double* oxo transfer system of scheme 2 (Figure 1) is that enzymic $Mo^{VI}O_2$ and $Mo^{IV}O$ groups in general (e.g., those in sulfite oxidase and nitrate reductase) may be competent oxo transfer functionalities. DMSO reductase and xanthine oxidase[7] are proven examples of authentic oxotransferases; each derives from one of the two known families of oxomolybdenum enzymes that have either $Mo^{VI}O_2$ or $Mo^{VI}OS$ groups at the active site in the oxidized form.

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(15) DMSO reductase was purified from an overexpressing strain of *R. sphaeroides* by the method of McEwan *et al.*[8] In some cases it proved necessary to run a final DEAE-Sepharose column to remove a small amount of contaminating *c*-type cytochrome; the enzyme purified in this manner ran as a single band on SDS−PAGE. DMSO reductase was used as a solution in 50 mM Tris buffer, pH 8.0 (catalytic conditions), or in 10 mM $(NH_4)(HCO_3)$ buffer, pH 8.0 (single-turnover conditions). The absorption spectrum (nm) of the purified oxidized enzyme was virtually identical with that of the *R. capsulatus* enzyme ($A_{720}/A_{640} = 1.05$, $A_{720}/A_{600} = 1.00$, $A_{280}/A_{720} \approx 50$),[8] but different from that of the *R. sphaeroides* enzyme ($A_{720}/A_{640} = 1.67$, $A_{720}/A_{600} = 1.47$, $A_{280}/A_{720} \approx 57$).[9b] This discrepancy may arise from a fraction of protein lacking molybdenum but retaining pterin. Our spectrum was quantitatively reproducible in enzyme preparations from four different bacterial growths, even when the growth medium and buffer solutions were augmented with Na_2MoO_4. The difference spectrum between the oxidized and reduced DMSO reductase used here is nonetheless indistinguishable from that obtained by subtraction of previously reported spectra.[9b] Concentrations of functional enzyme reported here are based on the absorbance *change* at 720 nm observed upon reduction of the oxidized enzyme, using an extinction change of 2000 M^{-1} cm^{-1} calculated from reported spectra.[9] This approach circumvents any difficulties associated with contamination by a molybdenum-deficient form of the enzyme. The rapid and quantitative recovery of the absorption spectrum of the oxidized enzyme upon treatment of the reduced enzyme with excess Me_2SO demonstrates that the enzyme preparations used here are functional and validates the use of the spectral change at 720 nm to determine the concentration of functional enzyme.

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