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Proposed Molecular Mechanism for the Action of Molybdenum in Enzymes: Coupled Proton and Electron Transfer

(metalloenzymes/nitrogenase/nitrate reductase/xanthine oxidase/ligand acidity)

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ABSTRACT **The reactions catalyzed by Mo enzymes each find the product differing from the substrate by two electrons and two protons (or some multiple thereof). The coordination chemistry of Mo suggests that there is a distinct relationship between acid–base and redox properties of Mo complexes, and that a coupled electron–proton transfer (to or from substrate) may be mediated by Mo in enzymes. Each of the Mo enzymes (nitrogenase, nitrate reductase, xanthine oxidase, aldehyde oxidase, and sulfite oxidase) is discussed; it is shown that a simple molecular mechanism embodying coupled proton–electron transfer can explain many key experimental observations. In view of this mechanism, the reasons for the use of Mo (from an evolutionary and chemical point of view) are discussed and other metals that may replace Mo are considered.**

Molybdenum is a component of at least five distinct enzymes that catalyze seemingly diverse and unrelated reactions (1–7). Additionally, Mo has been identified as an active component of physiological systems for which isolation of a specific enzyme or identification of function has not yet occurred (8–10). The well-defined enzyme systems are nitrogenase, nitrate reductase, xanthine oxidase, aldehyde oxidase, and sulfite oxidase. These enzymes are complex, and each has additional nonprotein groups besides Mo that are redox active (11, 12). The nitrogenase system contains nonheme iron (henceforth, called Fe–S groups) (1), while nitrate reductase is a molybdoflavoprotein (2, 3). Xanthine and aldehyde oxidase each contain both flavin and Fe–S components (4, 5) and sulfite oxidase has a heme component (6, 7). The reason for this multicomponent nature involves the oxidoreductase function of the particular enzyme. Thus, each has two substrates, the oxidant and the reductant, and it is the task of the enzyme to provide a low energy pathway for electron flow from reductant to oxidant. (The enzymes derive their common names from one of the substrates; that entity will be called the substrate and the other substrate will be called the physiological reductant or oxidant.) These enzymes each have one component suited to interact with reductant and a different component adapted to react with oxidant. Additional components are (sometimes) necessary to complete the chain from the reductant to oxidant. In each of the Mo enzymes, the molybdenum is believed to be the component that interacts directly with substrate. Furthermore, the same molybdenum cofactor may be a component of each of the enzymes (13).

In this paper, I present the various reactions catalyzed by Mo enzymes and discern those features that these reactions have in common. I will discuss the coordination chemistry of Mo, with emphasis on the redox and *acid–base* behavior and, most importantly, on the relationship between the two. I then will turn to each of the Mo enzymes and show how a simple molecular mechanism can explain many key experimental observations.

The Substrate Half-Reactions. Representative half-reactions for each of the Mo enzymes are tabulated in Fig. 1. In each case (when written as an acidic half-reaction) the product differs from the substrate by transfer of some multiple of two electrons and two protons in the same direction. To function most efficiently, the active site should formally be suited to mediate a confluence of electron and proton transfer with the substrate. In previous work electron transfer has been emphasized, while the site and sequence of proton transfer have been (except for xanthine oxidase) relatively neglected. Here I suggest that electron and proton transfer are each of great importance, and that the unique relationship between them is the key to understanding the action of Mo enzymes.

Coordination Chemistry. Groups coordinated to transition metal ions often have altered chemical properties (14, 15). The metalloenzymes use this effect in altering the reactivity of substrate. Additionally, the metal is coordinated by S, N, or O donor ligands to other groups within the enzyme, and the properties of these ligands are also grossly altered. The particular alteration on which I focus is in acid–base behavior.

Many coordinated ligands can be deprotonated under various conditions. Thus, for metals in low oxidation states sodamide in liquid ammonia is often required to singly deprotonate the amine nitrogen. For example (16),

$$Ni(NH_2CH_2CO_2)_2 + NH_2^- \xrightarrow{\text{liquid } NH_3} NH_3 + Ni(NHCH_2CO_2)(NH_2CH_2CO_2)^-.$$

On the other hand, amine complexes of Os(IV) and Pt(IV) manifest their acidity in aqueous solution with $Os(en)_3^{+4}$ being a strong acid (17) ($pK_2 = 5.8$) and $Pt(en)_3^{+4}$ having $pK_1 = 5.5$. A key effect on the pKa of a complex is the oxidation number; it is generally found that (other things—such as coordination number, chemical period, and other ligands in the coordination sphere—being equal) the higher the oxidation number, the greater is the acidity of a given coordinated ligand (18).

For molybdenum, the acidity of coordinated ligands is most pronounced in formally hexavalent complexes. Here the

common molybdate ion, MoO_4^{-2}, has all four oxygens fully deprotonated at pH = 7. Even in acid solution, the Mo(VI) species remain in a partly or fully deprotonated form (19). On the other hand, $Mo(H_2O)_6^{+3}$ has fully protonated coordinated oxygens in acid solution (waters!) (20). Somewhere between the VI and III states there is a drastic change in the pKa of coordinated oxygen ligands. As another example (unpublished results of E.I.S. and J. K. Gardner), consider the complex of 2-mercaptoaniline prepared in acid solution,

$$3C_6H_4SHNH_2 + MoO_4^{-2} + 2H^+ \rightarrow Mo(NHSC_6H_4)_3 + 4H_2O.$$

In this case, when the oxygen atoms are removed, the formally hexavalent Mo complex has strongly acidic anilino protons. On the other hand, lower oxidation states of Mo favor complexes with ligands in the amino form. For example (21), the complexes $MoCl_2(NH_2)(NH_3)$, $MoCl(NH)(NH_2)_2$, and $MoCl_2(NHR)_3$ (where R = Me, Et), again show that higher oxidation states possess more highly deprotonated ligands. Thus, O or N atoms coordinated to Mo undergo dramatic changes in acidity within the range of oxidation states thought to be active in enzyme processes (22). In fact, if a complex reacts by losing an electron (as opposed to an atom or group-transfer redox process), then it is more prone to lose protons on coordinated atoms. By this mechanism *simple electron and proton transfer reactions are coupled.* As electrons flow between Mo and the substrate, protons may transfer between ligands coordinated to Mo and the substrate. This coupling lends a bifunctionality to the Mo site that may be responsible for its catalytic effectiveness. A formally similar process involving net transfer of H atom has been considered for certain redox processes (23). Here I show how this simple mechanism leads to a unified picture for the action of Mo in enzymes.

Nitrogenase is an enzyme system of two components that combine to fix and reduce molecular nitrogen to ammonia (1). The fixing protein contains an iron–sulfur component as well as Mo; in some highly purified samples, there is but one Mo per Fe–Mo protein (24). The physiological reductant is reduced ferredoxin or flavodoxin, but if the enzyme system is intact inorganic reagents such as $S_2O_4^{-2}$ and BH_4^- can act as reducing agents. Additionally, Mg^{+2} and ATP are required, with ATP being hydrolyzed during the fixation or reduction process. [The function of ATP is unknown, but suggestions (1) include its use to produce a protein conformational change

A] $N_2 + 6H^+ + 6e^- \longrightarrow 2NH_3$
$C_2H_2 + 2H^+ + 2e^- \longrightarrow C_2H_4$
$[2H^+ + 2e^- \longrightarrow H_2]$

B] $NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$
$[NO_3^- + H^+ + 2e^- \longrightarrow NO_2^- + OH^-]$

C] xanthine + H_2O → uric acid + $2H^+ + 2e^-$
[xanthine + OH^- → uric acid + $H^+ + 2e^-$]

D] RCHO + H_2O → RCOOH + $2H^+ + 2e^-$

E] $SO_3^{-2} + H_2O \longrightarrow SO_4^{-2} + 2H^+ + 2e^-$

FIG. 1. *Substrate Half-Reactions.* (*A*) Nitrogenase reactions (11, 12) [Note that in the absence of substrate H_2 is produced.] (*B*) Nitrate reductase reactions, (*C*) xanthine oxidase reactions, (*D*) aldehyde oxidase reactions, (*E*) sulfite oxidase reactions. Although reactions are written as acidic half-reactions, at physiological pH these could also be written with OH^- participating (13); this possibility is illustrated in *brackets* in parts (*B*) and (*C*).

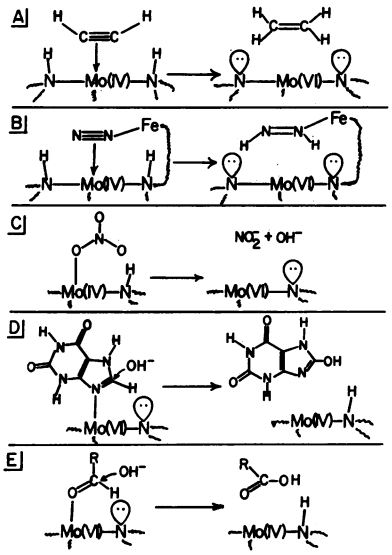

FIG. 2. *Coupled Proton–Electron Transfers.* (*A*) C_2H_2 reduction by nitrogenase, (*B*) N_2 reduction by nitrogenase, (*C*) nitrate reductase activity, (*D*) xanthine oxidation by xanthine oxidase, (*E*) aldehyde oxidation by aldehyde oxidase.

or to insure the presence of an active site on Mo.] In D_2O, ND_3 is formed from N_2, while exclusively *cis*- CHD=CHD is formed from acetylene (11, 12). In the absence of substrate, H_2 is produced.

The following scheme for the action of this enzyme is proposed. The resting (unactivated) Mo–Fe protein has Mo in the VI oxidation state coordinated by at least two nitrogens, at least one sulfur, and at most one oxo-type oxygen atom. In this situation the nitrogens will be acidic and will thus be deprotonated. (Note that while I discuss coordinated N as a key atom in this scheme, I recognize that this role could be filled by coordinated oxygens. This and the requirement for at most one oxo-type oxygen are discussed below.) The next step is activation of the Mo enzyme by transfer of two electrons to Mo from the iron protein. The role of sulfur coordinated to Mo may be to facilitate electron transport from the iron–sulfur group. The Mo is now in the oxidation state IV; thus, the coordinated nitrogens become substantially more basic and reprotonate by picking up H^+ from water (or D^+ from D_2O). The enzyme is now in its fully activated state. I first consider the substrate acetylene, which now coordinates to the Mo in a π-type (side-on) fashion. From the spacial juxtaposition of the coordinated substrate, the molybdenum, and the *trans*-amines, the enzyme–substrate complex is now poised for a coupled electron–proton transfer. Therein Mo, by giving two electrons to the substrate, goes from Mo(IV) to Mo(VI) and, in the process, its coordinated nitrogens become strongly acidic and donate their protons to the substrate. In the case of acetylene we have produced the final product, ethylene. The key step in the process is illustrated in Fig. 2*A*.

In the case of N_2, the mechanism embodies the two-site hypothesis of Hardy *et al.* (1). Thus, N_2 could coordinate to Fe in an end-on manner and the Fe–N_2 complex could be brought to the activated Mo site by a small protein conformation change. [All of the known isolated complexes of dinitrogen contain end-on σ-bonded dinitrogen (24, 25), but it is also true that these same complexes have defied all attempts at

reduction (26). Thus, as has been discussed elsewhere (27), the type of bonding needed to reduce the dinitrogen may in fact be of the π type. It is difficult to see how other substrates (e.g., acetylene) could be bound in any other way, so I call on this mode of bonding to Mo in this mechanism.] As the N_2 π bonds to the multifunctional Mo site, it is initially reduced to a coordinated diimide (28) by the coupled electron–proton transfer (see Fig. 2*B*). As the Fe-coordinated diimide leaves the Mo site, that site can now be rapidly reactivated (reduced and reprotonated) in the same manner as previously. The coordinated diimide can now be reduced to (the still Fe-coordinated) hydrazine, which could then be reduced to ammonia by the same sequence.

This model explains many key aspects of nitrogenase systems. The source of the hydrogens in H_2O is immediately apparent. The production of H_2 in the absence of substrate would clearly be a reaction of the Mo site, which contains both a strong reducing agent and a potentially strong acid. The production of exclusively *cis*-CHD=CHD in D_2O is explained by the geometric requirements of the multifunctional Mo site. (Other things such as HD formation, CO inhibition, etc., can also be explained within this model.) We can also speculate that the requirement for ATP lies in the need to remove oxygen (of the oxo type) from the Mo. Thus, ATP, after (or along with) the reduction process, may phosphorylate the (now more nucleophilic) oxo groups to phosphates, which would then more readily leave the Mo coordination sphere to form an open coordination site and a complex with appropriate acid–base properties. In Mo complexes in the V and VI oxidation state containing oxo groups, the strong σ and π donation of the oxo oxygen neutralizes much of the effective charge on Mo and destroys its acidity-enhancing effects (29, 30). Viewed in another way, the oxo Mo(VI) complex has already expressed its acidity by having oxo instead of aquo groups in the first place. Several model systems somewhat mimic the action of nitrogenase (31–35). In those cases where the resemblance is closest (31–33), the mechanism proposed here may also be applicable.

If both protons and electrons are delivered to the substrate in a concerted process, then it becomes very difficult (and perhaps not meaningful) to distinguish between transfer of two protons and two electrons, transfer of two H atoms, and transfer of a hydride ion and a proton. In the above discussion, I have given a spatial representation of the proton transfer and have suggested a compelling reason for the direction and timing of that transfer. On the other hand, while transfer of electrons through overlapping Mo and substrate orbitals seems a likely pathway, it is not the only possibility; thus, I do not expound a specific pathway for electron transfer. The details of the electron transfer would, of course, specify which of the three paths or what combination of them is the best description of the overall process. Similar considerations apply to the other reactions considered here and to other bioorganic reactions as well (36).

Nitrate reductase is known to require Mo and flavin for activity [with Fe–S groups and/or cytochromes sometimes present as additional components (3, 37)]. The path of electron transport is believed to be

$$\text{NADH} \rightarrow \text{Flavin} \rightarrow \text{Mo} \rightarrow NO_3^-$$

with Mo directly interacting with the substrate. This pathway seems quite reasonable in view of the fact that lower oxidation states of Mo are known to catalyze nitrate reduction in nonenzymatic processes (38). The enzyme is required to be in a reduced state to interact with nitrate, and I postulate the initial state as Mo(IV). As in nitrogenase, the IV state would have a fully protonated nitrogen and would be poised for nitrate reduction by coupled electron and proton transfer. As shown in Fig. 2*C*, as the Mo(IV) reduces NO_3^- by two electrons it becomes Mo(VI); the donor atom (shown here as N) becomes acidic and transfers its proton to the substrate, which then splits to nitrate and hydroxide, the products. (Alternatively, two *cis*-nitrogens might transfer one proton each to oxygen to form H_2O directly.) The enzyme in the VI state is now reduced by the flavin, which gets its reducing power from the physiological reductant, NADH (or in some cases NADPH). The appearance of a Mo(V) electron paramagnetic resonance (EPR) signal may be due to the presence of Mo(V), due to sequential one-electron transfers in the reactivation step.

Xanthine Oxidase, which contains Mo, flavin, and nonheme iron (4), catalyzes the oxidation of xanthine to uric acid, and several other similar reactions. The physiological oxidant is O_2, and there is evidence for one-electron transfer steps, with EPR spectra implicating the superoxide ion, O_2^-, as an initial product (39, 40).

Extensive EPR studies (41, 42) indicate the electron-transfer sequence to be

$$\text{Xanthine} \rightarrow \text{Mo} \rightarrow \text{flavin} \rightarrow \text{Fe–S} \rightarrow O_2$$

and present strong evidence when coupled to model studies (43–45) that Mo is coordinated to at least one sulfur. It is known further that H_2O is the source of the oxygen that enters the substrate in each case. In the proposed mechanism, I consider xanthine as the substrate. The resting active enzyme contains Mo in the (VI) state, coordinated by one deprotonated nitrogen (or non-oxo oxygen) atom. As the xanthine coordinates to Mo through its 9 nitrogen, the hydrogen atom (in the 8 position) is then positioned such that electron transfer to the Mo and proton transfer to the atom coordinated to Mo can occur in the now-familiar coupled fashion. The sequence of molecular events is as follows. As hydroxide ion [or, in general, some other nucleophile, such as enzyme persulfide (46)] attacks the 8 carbon, two electrons are transferred to Mo, producing the (IV) state, and the 8-proton is transferred to the coordinated protein nitrogen, which is now strongly basic (see Fig. 2*D*). The resultant product separates (perhaps in conjunction with Mo reoxidation) as uric acid (or perhaps in some other form attached to the protein nucleophile that then hydrolyzes to uric acid). The Mo is now in the reduced IV state, and it is probably the function of (successively) the flavin and iron components of the enzyme to bring these electrons to O_2 and, in the process, to reactivate the Mo coordination site. It seems likely that this reactivation proceeds by a series of one-electron steps. Thus, flavin first oxidizes the Mo to Mo(V), which produces the observed Mo EPR signal. [Alternatively, the reactivation mechanism may involve the presence of two Mo atoms closely situated in the active site. In the resting enzyme both are Mo(VI). After substrate reduction as described above, one becomes Mo(IV) while the other remains Mo(VI). A single electron is now transferred from Mo(IV) to Mo(VI), making two Mo(V) atoms, which are then each reactivated to Mo(VI) by the flavin $\rightarrow$ Fe–S $\rightarrow$ O_2 route.] It is clear why splitting is seen

(45) in the Mo EPR from the proton (formerly) in the 8 position of xanthine. This proton now finds itself attached to an atom coordinated to Mo, and splittings from protons on coordinated ligands have been observed in several transition metal complexes (47, 48). Furthermore, the presence of several EPR signals from Mo(V) during enzyme turnover can be explained as due to Mo(V) complexed or uncomplexed by product and by the presence of protonated or unprotonated amine ligands. Finally, as Mo is oxidized to the (VI) state it loses its amine proton stoichiometrically (in agreement with its apparent pKa) and is ready for another molecule of substrate. All of the substrates of xanthine oxidase can react by the above pathway, as in each substrate there is a potential ligand atom (O or N) two atoms away from the substrate hydrogen that is to be removed from C by the coupled electron–proton transfer.

Aldehyde Oxidase. Although this enzyme displays some unique behavior, its electron transport chain resembles (in both composition and mechanism) that of xanthine oxidase (5). A mechanism akin to that in xanthine oxidase is proposed, with the carbonyl oxygen initially coordinating to Mo. The Mo(VI) polarizes the C=O bond and makes the carbon susceptible to nucleophilic attack by OH^- or a protein nucleophile. The coupled electron–proton transfer then removes the aldehyde proton and two electrons, as is illustrated in Fig. 2*E*. The reactivation process probably resembles that in xanthine oxidase.

Sulfite Oxidase has recently been shown to contain Mo and heme iron, in the ratio 1:1. Heme is implicated as the point of electron transfer from the reduced enzyme to oxygen, while Mo is considered to be the site of sulfite oxidation (6, 7).

At pH = 7 sulfite consists 95% of SO_3H^- in equilibrium with HSO_3^- (H bound to S). Either of these forms is a candidate for oxidation by the multifunctional site in a process similar to that found in xanthine or aldehyde oxidase. As Mo(VI) goes to Mo(IV), sulfite is attacked by hydroxide and goes to sulfate, while it transfers its excess protons to the basic Mo(IV). Alternatively, reaction may first produce SO_3 (6), which then hydrates immediately to (mostly dissociated) sulfuric acid. Reactivation can now occur as in xanthine oxidase, except that heme takes the place of the flavin and Fe–S components. The proton transferred to the coordinated ligand could be the one responsible for the superhyperfine structure in the Mo signal. Its exchangeability attests to its lability, and its apparent pKa points to its participation in the catalytic process described here (7, 45).

Further Considerations. In my proposed mechanism, nitrogen was used as the key donor atom whose acidity changes as a function of electron transfer. Other atoms that are conceivable donors to Mo in enzymes are C, O, and S. Carbon is not a likely donor to high oxidation states, and even if it were it is unlikely that attached protons would show significant acidity. On the other hand, sulfur ligands are typically deprotanted in all oxidation states at pH = 7. This elimination leaves oxygen as a possible alternative to nitrogen. Here too it seems possible that the pK values of the coordinated ligands would be quite low, and at physiological pH these ligands may be deprotonated in all oxidation states involved. At least two papers (7, 45), however, have claimed that N can be eliminated as a donor atom since it should have mainifested itself by a superhyperfine splitting in the Mo(V) EPR spectra. Here I note that in paramagnetic coordination compounds with coordinated N, splitting from this atom is sometimes quite small and often not observed (49–51). The hyperfine splitting by both ^{14}N and ^1H depends on the molecular orbital in which the unpaired electron is found. Thus, while the presence of ^{14}N splitting establishes ^{14}N as a donor atom, the absence of such splitting does not rule out this possibility. It seems probable then that Mo is coordinated in the enzymes by S, N, and perhaps O, with the coupled proton transfer involving N, or possibly O.

The coupled proton–electron transfer may be involved in several chemical processes, e.g., the chromate or permanganate oxidation of alcohols or aldehydes (55). However, the chemical mechanism may be inferior to the enzymatic process due to the role of the protein. Thus, the protein will have the capacity to precisely orient the substrate and metal so that the coupled transfer can facilely occur. In chemical systems much of the proton transfer may be with solvent and, thus, only indirectly with substrate.

Finally, one might ask why Mo is used by these enzymes. In contrast to other transition metals active in biological systems, Mo is a member of the second transition series whose other members (Zr, Nb, Tc, Ru, Rh, Pd, and Ag) have no known biological function. This lack is not surprising as their natural abundances (except for zirconium) are at least 10 times less than any first-row transition element. It seems strange that nature should choose Mo out of these elements, unless it had a distinct evolutionary advantage. Vanadium has been substituted for Mo in nitrogenase (53), while W has been substituted for Mo in nitrate reductase (54), but only the vandium-substituted system displays (reduced) catalytic activity.

Thus, Mo seems particularly suited to its task, and here I suggest a reason for this suitability. What is required is a metal complex whose redox potentials will be in the appropriate range and whose polarizing effect on coordinated ligands would be such as to drastically alter their acidities through that same range of oxidation states. Mo fulfills this requirement.Tungsten complexes, while displaying the correct acid–base behavior, are too stable in the higher (VI) oxidation state. Cr complexes are too stable and inert in the (III) state, with the (VI) state being too strong and nonselective an oxidant. Vanadium probably more closely mimics these attributes of Mo and may thus display some activity. Since this effect may be an example of a diagonal relationship in the periodic table, a likely candidate to replace Mo with retention of activity is Re. It is possible that Re preparations may approach Mo activity but that Re was not selected (in the evolution of these functions) due to its extremely low abundance.

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