Molybdenum Chemistry of Biological Significance

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PREFACE

In retrospect, it was obvious that we were both, quite independently, contemplating a conference on the role of molybdenum in biology and related chemistry. At the time though, the meeting of minds on this matter was quite surprising. Although this subject has been treated in previous meetings within the overall context of, say, magnetic resonance or nitrogen fixation, it was apparent to us both that research in molybdenum-containing enzymes and molybdenum chemistry had progressed rapidly in the last several years. Jointly, we decided to organize the first meeting on Molybdenum Chemistry of Biological Significance which was held at the Hotel Lake Biwa, Shiga, Japan, on April 10-13, 1979. This volume constitutes the Proceedings of that international conference and covers the broad spectrum of interests from enzymes to coordination chemistry. It should serve not only as a source of new information on the latest research results in this area and as a useful reference tool, but should also allow a newcomer or other peripherally interested researcher to become conversant very rapidly with the "state-of-the-art" in this specialized and important area of research.

The conference was sponsored by the Japan Society for the Promotion of Science, the Japan World Exposition Commemerative Fund the Yamada Science Foundation, the Nissan Science Foundation, the Chemical Society of Japan (Kinki Regional Office) and the Agricultural Chemical Society of Japan (Kansai Branch). We thank these organizations sincerely for their interest and generosity. We also acknowledge a donation from the Climax Molybdenum Company of Michigan. We also wish to thank our Symposium President, Shiro Akabori, Emeritus Professor of Osaka University, and our colleagues on the Organizing Committee, S. Hino (Hiroshima), Y. Maruyama (Tokyo), A. Nakamura (Osaka), M. Nakamura (Tokyo), K. Saito (Sendai), Y. Sasaki (Tokyo), T. Tanaka (Osaka) and F. Egami (Tokyo) for their very worthy assistance.

The current interest in molybdenum chemistry stems from its importance in biology as an essential element in metabolism, particularly with respect to nitrogen. Our hope is that this volume will engender increased research endeavor on this vitally important subject.

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ELECTROCHEMICAL AND KINETIC STUDIES OF NITROGENASE: BRIEF REVIEW AND RECENT DEVELOPMENTS*

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INTRODUCTION

Nitrogenase is a two-component redox enzyme that catalyzes the reduction of nitrogen to ammonia in aqueous solution according to reaction (1). The two redox component proteins that comprise the

$$N_2 + 6e- + 6H^+ = 2NH_3 \qquad (1)$$

nitrogen-reducing enzyme are the MoFe protein containing 32 iron and 2 molybdenum atoms (MW = 230,000), and the $MgATP^{2-}$-binding iron protein, containing 4 iron atoms (MW = 65,000). Both proteins can be independently purified and when recombined in the presence of a source of electrons and the energy-yielding substrate $MgATP^{2-}$ catalyze reaction (1). The electron transfer sequence from the low potential electron source through the component proteins to the reducible substrate N_2 constitutes a major area of research in attempting to understand the mechanism of nitrogenase catalysis. Such questions as which protein component first accepts the low potential electrons, how MgATP and electrons are coupled during catalysis, what types of electron transfer centers are present in the proteins and what are their redox properties are all fundamental questions that are being actively investigated.

The kinetic aspects of nitrogenase catalysis constitute yet another area of nitrogenase research actively undergoing rapid development. It has been recognized by several workers[1-4] that the numerous reducible substrates of nitrogenase are all reduced at

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approximately the same rate even though their diversity of chemical properties suggest this would be unlikely. Thus, a number of questions arise. What are the kinetically discernible events during nitrogenase catalysis, which of these are rate limiting, how many substrate-reducing sites are there and what is their nature? Attempts to answer these and related queries have produced a rather extensive base of kinetic information about nitrogenase catalysis.[1]

Historically, studies of both the redox properties and kinetic properties of nitrogenase catalysis have developed more or less separately, but it is clear that both aspects must, at some point, be considered together. However, at the present level of understanding of nitrogenase catalysis, the simplification that results from separate consideration of redox reactions and kinetic properties seems to justify the approximation of separability. This article considers each area separately, first in brief review followed by recent results from our laboratory impinging on these areas.

KINETIC STUDIES OF NITROGENASE

Both steady state[1,3] and presteady state[5,6] (stopped-flow) kinetic studies of nitrogenase have provided useful insights into its mechanism of catalysis. The stopped-flow measurements of Thorneley and his colleagues[5,6] have provided interesting results for the rapid reduction of the Fe protein by reductant in the presence of the substrate MgATP and the inhibitor MgADP. They find that reduction of the Fe protein by SO_2^- is unaffected by MgATP but is significantly retarded by $MgADP^{2-}$, a known inhibitor of nitrogenase. Although such studies have been extended[7] to include the entire nitrogen-fixing system (i.e., $S_2O_4^{2-}$, MgATP, Fe Protein, MoFe protein), sufficient complexity has arisen as to make interpretation less than direct. However, these authors do conclude that a tightly bound complex forms between the Fe protein and the MoFe protein during catalysis and that internal electron transfer occurs from the Fe protein to the MoFe protein within this complex with a rate constant of 2×10^2 sec^{-1}.

Numerous kinetic studies of substrate reduction by nitrogenase have been reported using crude nitrogenase preparations[8], nitrogenase complex[3,4,9] and purified protein components[10-13] from various bacterial sources. These studies monitored the rate of product formation as either the various substrates and inhibitors or the conditions (temp, pH etc.) of catalysis were varied. Also, studies of the competition among reducible substrates (or reducible substrate against inhibitor) for available electrons in the enzyme have been made in an attempt to define the nature of the reducible substrate binding site(s).[9,12,13] In spite of the large number of these studies, the nature and number of binding sites still remains obscure

and the explanation for substrate reduction selectivity rests with two quite dissimilar models.

Kinetic Models

One model[13] postulates a number of sites, perhaps partially related, where selective substrate reduction occurs. An extensive body of information produced by Hardy et al.[10] and by Hwang et al[13] was interpreted in terms of this model. Considering the wide range of chemical reactivity, electronic properties and structures of the various nitrogenase substrates and recognizing the large size and chemical composition of the MoFe and Fe proteins and thus, the potential for numerous and varied binding sites, it is not unreasonable to propose such a model. However, this model is not only intuitively unsatistying but also newer experimental results are not easily accommodated within its scope. Consequently, a second model was proposed that suggests that only one site is required to explain reduction of the various substrates of nitrogenase. According to this view, the enzyme becomes a reservoir for electrons sufficiently energetic to be transferred to the reducible substrates in an enzyme-controlled reduction step. The electron level in the enzyme during turnover is a balance between the outflow of activated electrons to the reducible substrates and the influx of activated electrons presumably resulting from MgATP-driven electron transfer from the low potential reductant. The specificity of substrate reduction results from the number of activated electrons present in the enzyme at any given moment. When the electron level is low, substrates requiring only two electrons for reduction are preferred (H-C≡C-H, $2H^+$, N_2O etc.) to substrates requiring four ($HC{\equiv}N \rightarrow CH_3NH_2$) or six electrons ($N_2$, CH_2CN etc.) for reduction This explanation implies that an "all or nothing" binding-reduction interaction of the reducible substrates occurs with the enzyme, especially for those substrates requiring four or six electrons for reduction.

An interesting possibility arises which this latter model has not dealt with as yet. Suppose that a substrate requiring six electrons for complete reduction binds to an enzyme form possessing fewer electrons. It is not likely that the substrate would accept whatever electrons are present and "wait" until further activated electrons are made available to complete the reduction sequence? This aspect, which allows for intermediate reduction states of the six electron substrates, needs further development, particularly in view of the trapping of an enzyme-bound intermediate during nitrogen reduction[14] and the interpretation of HD production[15,16] by a nitrogen-fixing system reported by the Kettering group. Both types of experiments are clearly indicative of enzyme-bound intermediates (at the diimide or hydrazine levels) occurring during the six-electron nitrogen reduction process.

Activated Electrons

As mentioned above, MgATP is though to be involved in the production of activated electrons required for substrate reduction. Silverstein and Bulen,[9] using a nitrogenase complex from A. vinelandii with a fixed Fe protein to MoFe protein ratio of 2:1, demonstrated that, at low levels of MgATP, the two-electron reduction of protons was favored over the six-electron reduction of nitrogen. This result clearly establishes a role for MgATP in the production of activated electrons. In an extension of these studies, Davis et al[12] determined the product distribution of various reducible substrates as a function of component ratio. These experiments confirmed the conclusions of Silverstein and Bulen and further demonstrated the involvement of the Fe protein in the formation of activated electrons. The results show that, at high ratios of Fe protein/MoFe protein, six-electron substrates compete favorably with two-electron substrates while at low ratios, two-electron substrates are selectively reduced and little if any reduction of six-electron substrates occurs.

The concentration of MgATP and the ratio of Fe protein/MoFe protein are both involved in determining the electron partitioning to reducible substrates. Perhaps other unrecognized or uninvestigated variables are also involved. For example, temperature is known to vary the ATP/2e ratio[17] and may also influence substrate reduction selectivity. Similarly, effects of varying pH and ionic strength might also be interesting. Another possible variable is the potential of the reductant used as an electron source. Watt and Bulen[18] and Albrecht and Evans[19] have reported the voltage threshold value to be -380mV and -440mV for operation of A. vinelandii and chromatium nitrogenases respectively. The rate of nitrogenase activity at potentials more negative than -380mV for the Azotobacter system[18] follows a Nernstian-type curve with an operational $E_{1/2}$ of -450mV and n = 2. Conceivably, the nitrogenase system (composed of a high Fe protein/MoFe protein ratio and excess MgATP) could be poised at suboptimum potentials, where turnover is limited by the availability and energy of the electrons, to evaluate how these constraints affect the distribution of electrons to products. Results similar for limiting MgATP and low Fe protein/MoFe protein ratios might be expected.

Kinetics of $S_2O_4^{2-}$ utilization

The above experimental approach determined the amount of product formed from the reducible substrates, the rate at which product forms, and for the competition experiments, the distribution of electrons among the various reducible substrates. These results give only one view of nitrogenase catalysis, from the product formation side only. Another perspective is from the substrate or input

side which might also provide useful insights into the catalytic mechanism. Using electrochemical techniques for monitoring $S_2O_4^{2-}$ concentration and the nitrogenase complex as catalyst, Watt and Burns[3] determined the rate law (equation 2) for $S_2O_4^{2-}$ utilization under a variety of conditions. This rate law indicates that SO_2^- is

$$\frac{-d(S_2O_4^{2-})}{dt} = \frac{kE_T\,(S_2O_4^{2-})^{1/2}\,(ATP)^2}{(ATP)^2 + K_1(ATP) + K_2} \qquad (2)$$

the active electron transfer agent to the nitrogenase proteins (a result also obtained from stopped-flow studies[5]) and that two MgATP's are involved in the catalytic mechanism. The result[3] that the enzyme never becomes saturated with respect to SO_2^- is interpreted to indicate that MgATP is first bound to the Fe protein during ntirogenase catalysis followed by SO_2^- reduction.

The nonsaturation[3] of nitrogenase by SO_2^- has been questioned by Hageman and Burris[20] (a problem to be discussed more fully in a later section) who studied the kinetics of $S_2O_4^{2-}$ disappearance by optical means. Using various ratios of purified nitrogenase components, they find that the rate of $S_2O_4^{2-}$ consumption by the enzyme-catalyzed reaction is dependent on the ratio of component proteins and becomes saturated at high levels of $S_2O_4^{2-}$. From a rather involved analysis of their data, they conclude that the ATP-binding Fe protein and the MoFe protein undergo rapid association and dissociation with fleeting formation of a transient complex. During complex formation, the Fe protein transfers one activated electron to the MoFe protein which, when sufficient of these have accumulated, becomes the substrate-reducing site. Consistent with this view, Hageman and Burris[21] find, under conditions of low Fe protein to MoFe proten ratios, that hydrogen is not immediately evolved when the reaction is initiated, which suggests that electrons are stored in the MoFe protein until sufficient numbers are made available to evolve H_2. Independent studies,[22,23] using heterologous mixtures of Fe protein and MoFe proteins from different organisms which form strongly associating complexes that have low activities, are also consistent with the view that the Fe protein and MgATP act together to transfer activated electrons to the MoFe protein where substrate reduction occurs.

This brief sketch rationalizes a broad and extensive body of experimental evidence into a unified and reasonable model of nitrogenase catalysis. However, lest we become too optimistic, it should be kept in mind that many of these results have not been independently confirmed and some experiments need to be extended to include other sets of conditions. However, these views do form a useful working hypothesis from which more detailed studies of nitrogenase can be made.

The observation of Watt and Burns[3] that the nitrogenase complex from A. vinelandii becomes saturated with MgATP but not with $S_2O_4^{2-}$ is interpreted to indicate that MgATP is first bound to the Fe protein followed by SO_2^- reduction of the Fe protein. Hageman and Burris,[20] using purified component proteins from A. vinelandii and an optical method for following $S_2O_4^{2-}$ concentration, have observed saturation with $S_2O_4^{2-}$. The disagreement may be due to differences in properties of the nitrogenase complex[3] versus the purified components[20] or to the different techniques and methods of data analysis used for following $S_2O_4^{2-}$ utilization. Because the early sequence of kinetic events involving the reductant, MgATP and the Fe protein is of such fundamental importance in understanding how activated electrons are produced and ultimately appear in the reduced product, we have remeasured the kinetics for $S_2O_4^{2-}$ consumption. These newer experiments reported below were measured at various ratios of purified recombined component proteins using three independent methods for following $S_2O_4^{2-}$ utilization. These are: 1) the original polarographic method[3]; 2) the optical method used by Hageman and Burris,[20] and 3) a calorimetric technique which follows the rate of heat production during enzymatic catalysis.

Recent Kinetic Experiments

Figure 1 is a high resolution spectrophotometric curve at 375 nm resulting from $S_2O_4^{2-}$ utilization during nitrogenase-catalyzed hydrogen evolution. The curve has no linear portion which would be required if $S_2O_4^{2-}$ were saturating (i.e., if the reaction were zero order in $S_2O_4^{2-}$). Analysis of this curve by usual kinetic procedures indicates that the nitrogenase-catalyzed reaction has a well defined one-half order dependence on $S_2O_4^{2-}$ even at high $S_2O_4^{2-}$ concentrations. Increasing the Fe protein/MoFe protein ratio while maintaining the MgATP level constant and the $S_2O_4^{2-}$ at the levels shown in Figure 1 causes the rate of $S_2O_4^{2-}$ utilization to increase but it still obeys a one-half order reaction. The Fe protein/MoFe protein ratio in these experiments was varied from 0.5 to 12. The one-half order rate constants for $S_2O_4^{2-}$ utilization resulting from varying the Fe/MoFe ratio provides information about the Fe protein dependence in enzymatic catalysis. Figure 2 is a plot of enzymatic velocity against Fe protein concentration and the inset is a double reciprocal plot of these quantities. This plot shows simple hyperbolic saturation of the MoFe protein by the Fe protein. These plots give the K_m value for the Fe protein-MoFe protein interaction as 3.12×10^{-5}M and Vmax as 1.92×10^{-2} $M^{1/2}mg^{-1}min^{-1}$. These data can be combined to give equation (3) which describes the rate of $S_2O_4^{2-}$ utilization at a constant, saturating MgATP concentration. Extensive

$$\text{rate} = \frac{\text{Vmax}[\text{Fe}]}{K_m+[\text{Fe}]} \quad [\text{MoFe}][S_2O_4^{2-}]^{1/2} \qquad (3)$$