RSC Metallobiology

Molybdenum and Tungsten Enzymes

Bioinorganic Chemistry

Edited by Russ Hille, Carola Schulzke, and Martin L. Kirk

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Bioinorganic Chemistry

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Preface

In the late 1950s and early 1960s, evidence was accumulating that molybdenum was not simply present in the enzyme xanthine oxidase from cow's milk but that it was required for its activity and changed its oxidation state in the course of the reaction with substrate. In a *tour-de-force* isotopic substitution study reported in *Nature* in 1966, R.C. Bray and L.S. Meriwether demonstrated unequivocally that the EPR signals elicited by the enzyme upon treatment with xanthine arose from a molybdenum-containing active site. It is a happy coincidence but altogether fitting that this volume marks the 50th anniversary of this seminal work.

For many years, only five enzymes were recognized as possessing molybdenum in their active sites: nitrogenase from bacteria such as *Klebsiella pneumoniae* and *Azotobacter vinelandii*; xanthine oxidase from bovine milk (and other vertebrate sources); aldehyde oxidase from vertebrate as well as bacterial sources; the vertebrate sulfite oxidase; and the assimilatory nitrate reductase from plants (and algae and fungi). That began to change in the 1980s with the demonstration by K. V. Rajagopalan that an organic cofactor accompanied the molybdenum in the active sites of these enzymes (with the exception of nitrogenase), and with the contemporaneous discovery that tungsten was also found in the active sites of enzymes in certain bacteria.

There are now several dozen molybdenum- and tungsten-containing enzymes that have been crystallographically characterized, along with most of the enzymes responsible for the biosynthesis of the organic cofactor variously known as molybdopterin, tungstopterin and pyranopterin. The active site metal centres of these enzymes have proven to be fascinating and challenging targets for synthetic inorganic chemists, and both enzymes and synthetic models have proven fertile ground for the application of a range of physicochemical and spectroscopic methods probing their physical and electronic structures as well as their intrinsic reactivity. At present, well over

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50 molybdenum- and tungsten-containing enzymes have been isolated and characterized, and these have been found to catalyze a broad range of oxidation-reduction reactions, and even reactions that (at least formally) do not involve oxidation–reduction of substrate. These enzymes are found in a wide range of metabolic pathways and play particularly prominent roles in the global cycling of nitrogen, sulfur and carbon. Many have vital roles in bacterial bioenergetics, catalyzing crucial energy-conserving reactions under a variety of growth conditions. Indeed, they seem to have been among the earliest enzyme systems to have arisen, as reflected in their near-universal distribution in the biosphere. Finally, genomics analyses have led to the identification of hundreds of genes encoding putative new proteins that are likely to possess one or another metal. These systems represent an enormous frontier of new enzymes that remains to be explored.

This title provides an up-to-date account of the state of our understanding of molybdenum and tungsten enzymes and is divided into three volumes, dealing with: (1) the enzymes themselves, along with pyranopterin cofactor biosynthesis and incorporation of the mature cofactor into apoprotein (*Molybdenum and Tungsten Enzymes: Biochemistry*), (2) inorganic complexes that model the structures and/or reactivity of the active sites of each major group of molybdenum and tungsten enzymes (*Molybdenum and Tungsten Enzymes: Inorganic Chemistry*) and (3) spectroscopic and related methods of physical chemistry (including computational work) that have been applied to both enzymes and model compounds (*Molybdenum and Tungsten Enzymes: Physical Methods*). Each volume is introduced by an overview chapter written by a leading expert in the field, followed by the individual chapters that detail specific topics associated with each volume. The intent of these overview chapters is to provide an overarching and unifying theme that places each of the three major subject areas in proper context.

We are deeply indebted to each of the contributors for their efforts, which lay out the current state of our understanding in each of the many subject areas considered. The coverage of these volumes is inevitably incomplete due to space constraints, however, and for this we apologize. However, the topics that are covered are presented to the reader in considerable detail; written in a style and spirit that will be fully accessible by current researchers in the field as well as those who wish to learn more about these fascinating metalloproteins. We sincerely hope that these volumes will underscore how rapid the progress has been over the past decade or so, and also how rapidly the field is expanding. The ultimate goal is to stimulate further research on molybdenum and tungsten enzymes, and especially to encourage new investigators to take up one or another aspect of these systems. It seems inevitable that many exciting new discoveries lie in wait.

Russ Hille
Carola Schulzke
Martin L. Kirk

Dedication

It is all too fitting that these volumes dealing with the bioinorganic chemistry of molybdenum and tungsten be dedicated to three outstanding chemists whose contributions to the field over many years continues to inform, illuminate and inspire: Richard H. Holm, C. David Garner and John H. Enemark.

Prof. Holm has over 500 research publications (cited over 35 000 times) covering a wide range of nickel, iron and molybdenum chemistry (among other transition metals). He is perhaps most widely recognized for studies, beginning in the 1970s, that describe the synthesis and characterization of iron-sulfur clusters. This work came to include modelling the M and P clusters of nitrogenase, which perhaps provided the motivation to investigate models of mononuclear molybdenum-containing enzymes. His molybdenum work achieved great success with the synthesis of MoO_2 models for enzymes of the sulfite oxidase, and later the DMSO reductase family, and the characterization of their properties as oxygen atom transfer catalysts. A key contribution was his use of bulky ligands to the metal that prevented μ-oxo

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dimerization, which had long stymied work in the field. He is Higgins Professor of Chemistry at Harvard University, a member of the National Academy of Sciences and the recipient of many other awards.

Prof. Garner already had a strong track record in the synthesis of copper and molybdenum complexes when, beginning in the late 1970s, he became one of the first researchers to apply the then-new analytical method of X-ray absorption spectroscopy not only to models of molybdenum enzymes but also to the enzymes themselves. The discovery of thiolate-like sulfur, Mo=O and Mo=S ligands to the metal in the active sites of enzymes such as sulfite oxidase, xanthine oxidase and DMSO reductase was critical in establishing the molybdenum coordination environment in these enzymes and greatly focused efforts to synthesize accurate structural and functional mimics of the enzymes. With over 300 publications (having over 8000 citations), he is presently Professor Emeritus at the University of Nottingham and a Fellow of the Royal Society. He is also past President of the Royal Society of Chemistry.

Prof. Enemark was already well recognized for his work on metal nitrosyls and related systems when he began to exploit the tris-pyrazolylborate ligand as a scaffold on which to construct and study MoO_2 and MoO complexes. This work led to the synthesis and characterization of the first model that fully mimicked the catalytic cycle of oxotransferase enzymes such as sulfite oxidase. Enemark also played an instrumental role in the work that led to the first crystal structure of sulfite oxidase. Since that time, Enemark has pioneered the application of pulsed EPR methods to molybdenum enzymes and synthetic models of their active sites; work that has led to a deep understanding of not simply the physical but also the electronic structures of these systems. With over 250 publications and 10 000 citations, he is Regents Professor of Chemistry at the University of Arizona, a former Fulbright Scholar and recipient of the Humboldt Research Prize, among other national and international recognitions.

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CHAPTER 1

An Overview of the Synthetic Strategies, Reaction Mechanisms and Kinetics of Model Compounds Relevant to Molybdenum- and Tungsten-Containing Enzymes

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Introduction and Overview

Bioinorganic model chemistry in general comprises synthesizing complexes which resemble the natural active sites of metalloproteins with respect to either structure or function or (preferably) both and investigating their structural and spectroscopic characteristics and/or reactivity, most often in comparison with results from biological samples. This is important for a detailed understanding of the roles of metal, ligands and specific functional groups in the processes taking place at the active sites.

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The following chapters in *Molybdenum and Tungsten Enzymes: Inorganic Chemistry* review advances in the field of molybdenum- and tungsten-dependent oxidoreductases and nitrogenase by bioinorganic model chemistry, or more precisely the synthetic and catalytic evaluation of model systems. All this falls into the core expertise of two of the three outstanding scientists to whom this book is dedicated. Both Richard H. Holm and C. David Garner have contributed formidably to the field of molybdenum and tungsten model chemistry for the respective molybdenum cofactor (Moco) and tungsten cofactor (Wco) bearing enzymes. Although with generally the same goal, *i.e.* furthering the understanding of the electronic and steric specifics of the respective active sites and their reactivity, the approaches of the two groups were quite distinct. Holm and coworkers created model systems for the immediate coordination spheres even of those enzymes that were most difficult to mimic, their chemical composition being rather uncommon in inorganic chemistry. Garner, often in cooperation with organic chemist John A. Joule, designed ligand model systems mimicking more of the rather complex natural ligand molybdopterin by synthesizing asymmetrically substituted dithiolene ligands bearing N-heterocyclic groups, even investigating different tautomeric forms thereof; all this in relation to the pterin part now presumed (based on protein structural findings) to play a substantial role for the reactivity of the oxidoreductases. Current work by the community attempts to unify these two approaches in order to accomplish the organic–inorganic synthesis of increasingly accurate Moco and Wco models with a perfect match still being elusive.

The authors of the following chapters have in their own work necessarily been inspired by the synthetic foundations of this field laid by Holm and Garner and some were even fortunate enough to have directly participated as their coworkers.

Holm has in addition for many years been involved in nitrogenase model chemistry. Pivotal work on the enzyme includes establishing the presence of the Fe_4S_4-ferredoxins in the $\alpha_2\beta_2$ complex by their extrusion and characterization, utilizing ^{19}F NMR spectroscopy for determining the magnetic moment of molybdenum in the M-Cluster at ambient temperature and investigating the binding of small molecules to the isolated M-Cluster. His synthetic contributions comprise the earliest syntheses of Fe–Mo–S clusters, the extension of this initial work to clusters with high nuclearity, double cubanes that served as topological models of the nitrogenase P-Cluster. This work included investigations of the reactivity of these model systems, demonstrating for example that it is possible to transform the alternate nitrogenase substrate acetylene to ethylene with model clusters. All this was indispensable in developing a molecular description of the metal sites in the nitrogenase. In his work, Holm always considered the "big picture", meaning: instead of getting lost in utmost detail, he continued to question and investigate with perseverance the discrepancies observed between what was found in the proteins and what could be achieved in the chemical lab, still a key question for many areas of bioinorganic chemistry.

Today nitrogenase model chemistry has come quite a long way and some amazing science has been carried out. Still, we remain quite far away from being able to mimic the M-Cluster with all its features. This is partly due to the fact that for decades chemists tried to model a molybdenum–iron cluster, lacking an interstitial atom, now recognized to be carbon.

The structural model chemistry for both the nitrogenase and molybdopterin-containing cofactors is now well developed. At the same time, there are still important features of the active sites that await accurate modelling by bioinorganic chemists. This is what keeps this field challenging and interesting for the respective communities made up of scientists with vision and perseverance.

As will be laid out in the following chapters with respect to molybdopterin (MPT), a complex has been published by Sharon Burgmayer (co-author of Chapter 2 in this volume), which is the closest structural MPT-focused model for the active sites known today. There are only minimal differences (oxidation state of the nitrogen atoms in the pyrazine part, for instance) to the natural molecule, although these may have an important impact on reactivity. Incorporating this MPT model or another one developed by Partha Basu to a molybdenum or tungsten centre which more closely resembles the actual active site would be a fantastic achievement, although admittedly the respective chemistry is very difficult. In fact, a combination of model chemistry focusing on mimicking molybdopterin and of the immediate coordination sphere would actually involve merging Garner's and Holm's achievements and constitutes the most challenging aim of the respective bioinorganic community nowadays. Most likely it will be the active site of arsenite oxidase that will be modelled in all detail first, since in its reduced form it resembles molybdenum dithiolene complexes which are most common, *i.e.* mono-oxo bis-dithiolene molybdates (IV). For any other enzyme, model chemistry is more difficult as the active sites typically do not bear an oxo ligand together with two molybdopterins in their reduced form. Therefore, in addition to simply coordinating a more or less close molybdopterin model to molybdenum (and tungsten) it will also be necessary to fine-tune procedures allowing modification of such models, namely replacing the oxo ligand by ligands mimicking coordination to the peptide (alcoholates, thiolates *etc.*), replacing one dithiolene by a sulfido, oxo, hydroxide or hydrosulfido ligand, oxidizing the model to oxidation states V (intermediate in the catalytic cycle) and VI (the opposite end of the catalytic process) and to make the compounds water insensitive and water soluble. All this has been done before with complexes with simpler dithiolene ligands or with other ligand systems. Once the functional groups of MPT are present, however, the respective chemistry becomes unknown ground and very difficult. And this is not all. Even though a dithiolene ligand with all the functional groups of MPT is requisite for allowing detailed structure–function relationships to be studied, it will probably not be enough. In order to really understand the distinct function of a specific structural motif of MPT it will be necessary to compare the electronic states, the catalytic potential and the stability of models with and

without this specific motif. Eventually, the bioinorganic chemists' efforts will have to be united with those of the biologists. The models have to be tested with regard to being incorporated into the apoenzymes or recognized by the respective chaperones that can insert them into the enzymes.

Semisynthetic enzymes will have to be prepared and compared for their activity and need to be spectroscopically characterized. The requirements for models in a biological environment are high with respect to stability and solubility; another challenge for the bioinorganic chemist.

None of the above is trivial work and it is certainly impossible for one group only to address all these issues. Even though the community is not huge, the determination of its members is and the work of many contributors in this field is heading in one or another of the directions described above.

Only when looking at all the results of all the individual contributors to this field and combining these insights can we understand and describe the inorganic chemistry of the respective active sites really accurately.

As many cooperative efforts are required, the specific fields laid out in the following chapters, intended to inform in all needed detail, cannot be viewed completely independently from each other. Consequently there will be some overlap between the individual chapters, which should still provide a suitable reading experience for all types of readers.

In Chapter 6, Hideki Sugimoto reviews model systems for molybdenum and tungsten oxidoreductases which bear one (mimicking sulfite oxidase family, xanthine oxidoreductase family) or two (mimicking DMSO reductase family and tungsten enzymes) dithiolenes, and focuses to some extent on the first and second coordination shell of the enzymes. Sugimoto published the first structurally characterized model with dithiolene ligands bearing the pyrane ring of MPT, extending the respective model chemistry a bit farther out and contributing some interesting and detailed studies about the electronic properties of a variety of dithiolene model systems. Dithiolene ligands are special as they are non-innocent ligands, and can directly participate in oxidation–reduction reactions. This also means that it is not trivial working with these fascinating molecules. Both Garner and Holm developed procedures for the synthetic chemistry which are still widely applied today and without which there would hardly be any structural model chemistry of molybdenum and tungsten enzymes possible.

In Chapter 2, Partha Basu and Sharon Burgmayer detail the progress model chemistry has made with a focus on all the features of molybdopterin. Basu was a postdoc with John Enemark, the third outstanding scientist to whom this book is dedicated, whose work has focused on spectroscopy as much as synthesis. Burgmayer has received training in the group of Ed Stiefel, another founding father of the respective bioinorganic chemistry, and she was introduced to pterins early on in her career. Both Basu and Burgmayer have for many years, both independently and in collaboration, furthered the model chemistry of N-heterocyclic dithiolene chemistry. Burgmayer has developed the molybdenum bound model system which is most closely related to the natural molybdopterin today and is deeply involved in bringing pterin

chemistry together with pyrane and dithiolene chemistry. Basu follows different, complementing approaches towards the same goal and along the way managed to exploit the interesting chemistry these electronically flexible systems provide, for instance in the field of heavy metal ion sensing. Pterin chemistry *per se* is well understood, although elaborating pterins with pyrane and dithiolene moieties becomes incredibly complicated.

In Chapter 7, Charles G. Young outlines the bioinorganic efforts to understand enzymes of the xanthine oxidase family. These enzymes distinguish themselves from the other families in that (1) they do not catalyze the typical oxygen atom transfer reaction but rather the insertion of an oxygen atom into a C–H bond, *i.e.* they are hydroxylases; and (2), they bear a sulfido ligand in the active site which is crucial for catalytic activity. Young has worked for decades in this field and designed many model systems which have furthered our understanding of the sulfido ligand's role for the electronic states of the central metal and the complexes' (and hence enzymes') reactivity. The respective chemistry is very difficult, particularly when trying to address all ligands of the natural cofactors. Most often, for example, an auxiliary ligand system in place of the dithiolenes is used for stabilizing the respective complexes allowing the detailed investigations on the sulfide ligand's relevance to be carried out.

Young has extensively cooperated with many in the area of bioinorganic spectroscopy, including John Enemark, with whom he was a postdoc.

With respect to the catalytic evaluation of model compounds of the molybdenum and tungsten cofactors, it was Holm who first characterized a model system catalyzing oxygen atom transfer, *i.e.* the oxidation of triphenylphosphine by dimethyl sulfoxide. This model reaction is now an indispensable tool for the bioinorganic Moco/Wco community. The reaction takes place at ambient conditions only when catalyzed and it can be nicely followed by phosphorous NMR. Although DMSO actually is a natural substrate (as the DMSO reductase family evidences), triphenylphosphine is not and recent and current efforts have focused on more physiologically relevant reactions with the most difficult task of employing water as solvent and/or substrate. The authors of the two chapters dealing with catalysis and kinetics are involved in the respective developments. In particular, Sabyasachi Sarkar has been responsible for some of the most exciting findings in this field by employing a comparatively simple model system which turned out to be a fabulous work horse.

In Chapter 3, Sabyasachi Sarkar describes the achievements that were made in applying model systems in catalytic processes mimicking natural enzymatic substrate transformations and in understanding the respective mechanisms in detail. Among other achievements in this area he discovered the catalytic potential of comparatively simple dithiolene-bearing model compounds employing maleonitrile ligands $[MO_x(mnt)_2]^{2-}$ (M = Mo, W; x = 1, 2). He coaxed some surprising substrate transformations out of these small complexes by fine-tuning the respective reaction conditions. Reactions described by Sarkar and coworkers include transformation of hydrogensulfite

to hydrogensulfate relevant for sulfite oxidase, the reduction of hydrogencarbonate to formate at pH 7.5, *i.e.* basically transformation of CO_2 and relevant for tungsten-formate dehydrogenase, the oxidation of aldehydes to carboxylic acids relevant for aldehyde oxidase and the transformation of acetylene to acetaldehyde relevant for the extraordinary tungsten-dependent acetylene hydratase plus various non-natural substrates like phosphanes.

Sarkar too has cooperated with John Enemark in the past, emphasizing that many specialists with different expertise are needed in order to understand the molybdenum and tungsten oxidoreductases in detail, in particular when it comes to reactivity.

In Chapter 4, Christian Fischer reviews catalytic properties of models and enzymes more specifically. This chapter includes a detailed kinetic evaluation of the catalytic systems. As a young academic Fischer has only recently started working in the field of molybdenum- and tungsten-dependent enzymes. He has graduated from the Leibnitz Institute of Catalysis led by Matthias Beller and is an expert in the field of catalysis. His involvement will hopefully extend what we know about the kinetics of models and enzymes continuing Holm's excellent work in this field. Notably, Fischer has already developed and investigated the first catalytic system being able to operate in pure water.

In the case of the bioinorganic chemistry relevant for nitrogenase, the determination of the presence of an interstitial carbon at the centre of the M-Cluster of nitrogenase has fuelled much new model chemistry. At present, there are only three variants known of the M-Cluster with vanadium or iron in place of the molybdenum but no variation of the general cluster structure or coordination environment. Therefore structural models are rather scarce, particularly when compared with the Moco or Wco chemistry. When it comes to functional models, the achievements in particular of the last years are impressive. Many molybdenum systems and now even a few iron complexes are known which can catalytically reduce the rather inert elemental nitrogen to ammonia. These model systems are intended to mimic the natural process because it would be industrially immensely important being able to split the nitrogen triple bond catalytically at mild conditions. More importantly, from a bioinorganic point of view it would be very important to reliably identify the place on the M-Cluster where nitrogen is bound and transformed. Although recent work of Dean, Hoffman and Seefeldt strongly supports binding of N_2 on the Fe2,3,6,7 face of the M-Cluster, nitrogenase has still not been "caught in the act" of nitrogen binding crystallographically. Advocates of molybdenum- and iron-based chemistry have been fighting over this for decades and we still do not know with absolute certainty what happens at the active site of this intriguing, long-known and yet mysterious enzyme. In Chapter 5, Sonny Lee, a former coworker of Holm in this particular field, has taken on the huge task to guide the reader through the various fundamental and recent developments in the bioinorganic model chemistry of nitrogenase.

The work of each of the authors contributing to this volume has been seminal with respect to developing the most accurate models of molybdopterin and for the structures and functions of the molybdenum- and

tungsten-containing centres of nitrogenase and oxidoreductase, the best of the latter being able to catalyze biologically relevant reactions. Most of the authors have been directly involved in the outstanding achievements of those this book is dedicated to.

I am thankful to all of them for having expertly detailed the fundamental and the recent advancements in the field of molybdenum and tungsten bioinorganic chemistry. Further, I am immensely grateful to C. David Garner and Richard H. Holm in particular, and also to John Enemark for having inspired me at the beginning of my independent academic work to an extent that it was actually their work that made me choose my scientific home in this field of research.

CHAPTER 2

Pterin-Inspired Model Compounds of Molybdenum Enzymes

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2.1 Introduction

The molybdenum cofactor (Moco) is an extraordinary molecule in biology. As a small metal-containing compound, it has the unprecedented combination of a dithiolene chelate for metal binding and a pterin appended to a pyran ring. The resultant cofactor is electronically nimble due to the presence of three redox active moieties, *i.e.* the molybdenum atom, the dithiolene and the pterin, which in concert can support a range of redox events.

The long history of modeling Moco dates back nearly 50 years.[1] The early models were developed before the pterin in Moco was identified and before

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a structure of Moco had been determined by X-ray crystallography. Yet these early models provided a base of knowledge from which an understanding of possible mechanisms and of the electronic environment within the Mo inner coordination sphere was built. The chapters in this second volume discuss the contributions made by others that enhanced our knowledge of the function of molybdenum and tungsten enzymes.

The discovery of the pterin-substituted dithiolene ligand in Moco established a specific target for modeling chemists, albeit a target of considerable synthetic difficulty. There have been concerted efforts to reproduce aspects of the pterin-dithiolene ligand in Moco and their synthetic model systems have evolved over the past 30 years.[1a,2] The complexity of pterin and pyranopterin chemistry starts with the different numbering systems used, and these are shown in Figure 2.1 for the pterin and pteridine ring systems, the fully reduced, open form initially proposed for Moco and two numbering systems used for the reduced pyranopterin forms of Moco.[3]

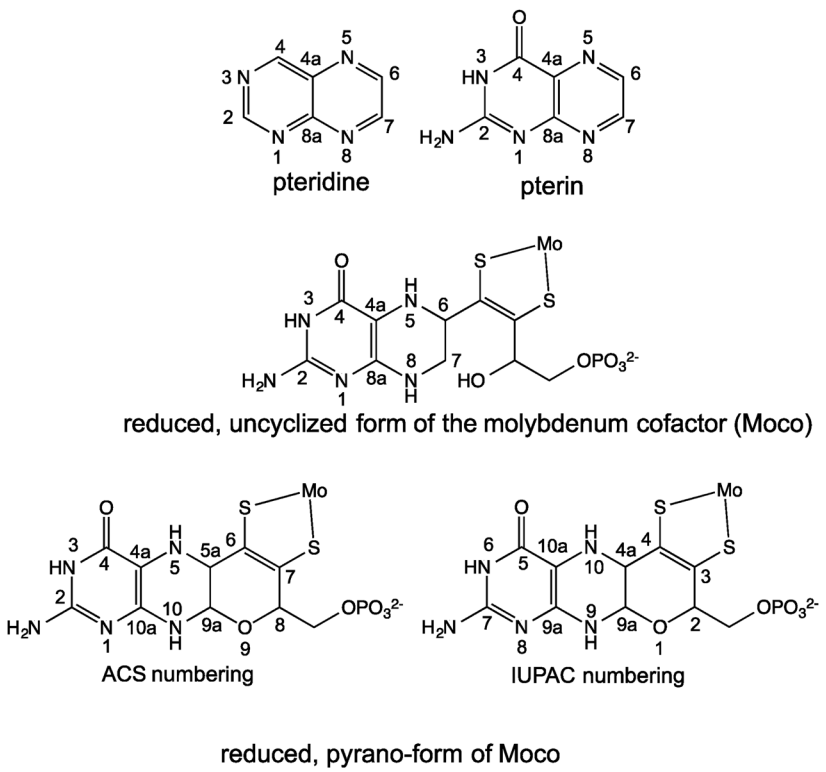


Figure 2.1 Numbering systems for pterin, a derivative of the pteridine ring system (top), in an uncyclized and fully reduced form initially proposed for Moco (middle) and as the cyclized and reduced pyranopterin form observed by crystallography in the majority of molybdenum enzymes (bottom).

The scope of this chapter is to review the model work directed towards the pterin aspects of the molybdenum cofactor. Our objective is to provide a detailed description of model work involving pterin, pteridine and related N-heterocycle systems that have been specifically developed to mimic features of Moco, as well as the study of these molecules in the absence of metal. Because the evolution of pterin models parallels the progress of revealing the pterin component of Moco, the chapter begins with a review of the key developments that unveiled the pterin ligand of Moco.

2.2 Unveiling the Pterin Component of Moco

In this section, an overview is of studies presented that revealed the presence of a pterin in molybdenum enzymes and eventually the structure of the pterin–dithiolene ligand of Moco. This background provides the context for pterin-inspired model work over the past three decades and highlights how the making of synthetic models for metal sites in enzymes follows acquisition of experimental data obtained from the metal site in the proteins.

Prior to the discovery of the molybdopterin, it had already been established that the molybdenum catalytic center of the molybdenum enzymes was part of a dissociable cofactor. Reconstitution of a Moco-deficient mutant of *Neurospora crassa nit-1* by the dissociated molybdenum cofactor from a variety of molybdoenzymes regenerated nitrate reductase activity in nit-1 further confirming that molybdenum and a particular ligand set comprised Moco.[4] Spectroscopic studies, primarily electron paramagnetic resonance (EPR) spectroscopy and extended X-ray absorption fine structure (EXAFS) spectroscopy, had suggested both oxo and sulfur coordination in the dissociated Moco samples,[2b,5] but the pterin component remained invisible in these studies. It was not until 1980, when fluorescence was detected in degraded Moco-containing protein samples, that an indication of the presence of a pterin emerged.[6]

2.2.1 The Pterin is Discovered

The investigation of the pterin component of Moco was a focus of Rajagopalan and his coworkers.[7] Initially, various oxidative treatments of Moco were shown to yield different pterin species, each having distinctive fluorescence and electronic spectral signatures, and each pterin species substituted at the C6 position on the pterin system. One of these degradation products, Form B (**1**), was proposed to have a thiophene structure similar to urothione (**2**). Urothione was found to be a metabolite of Moco in humans.[8] The sulfur atoms in **2** became the starting point for hypothesizing a dithiolene chelate attached at C6, and the dithiolene hypothesis was eventually confirmed through alkylation and trapping the dithiolene-substituted pterin.[9] The trapped pterin-dithiolene molecule also provided further support for the reduced state of the pterin. A tetrahydropterin had been suspected from the lack of fluorescent behavior of Moco, whereas oxidative degradation of Moco containing enzyme produced highly fluorescent oxidized pterins. A pictorial summary of the pterin studies is shown in Figure 2.2.

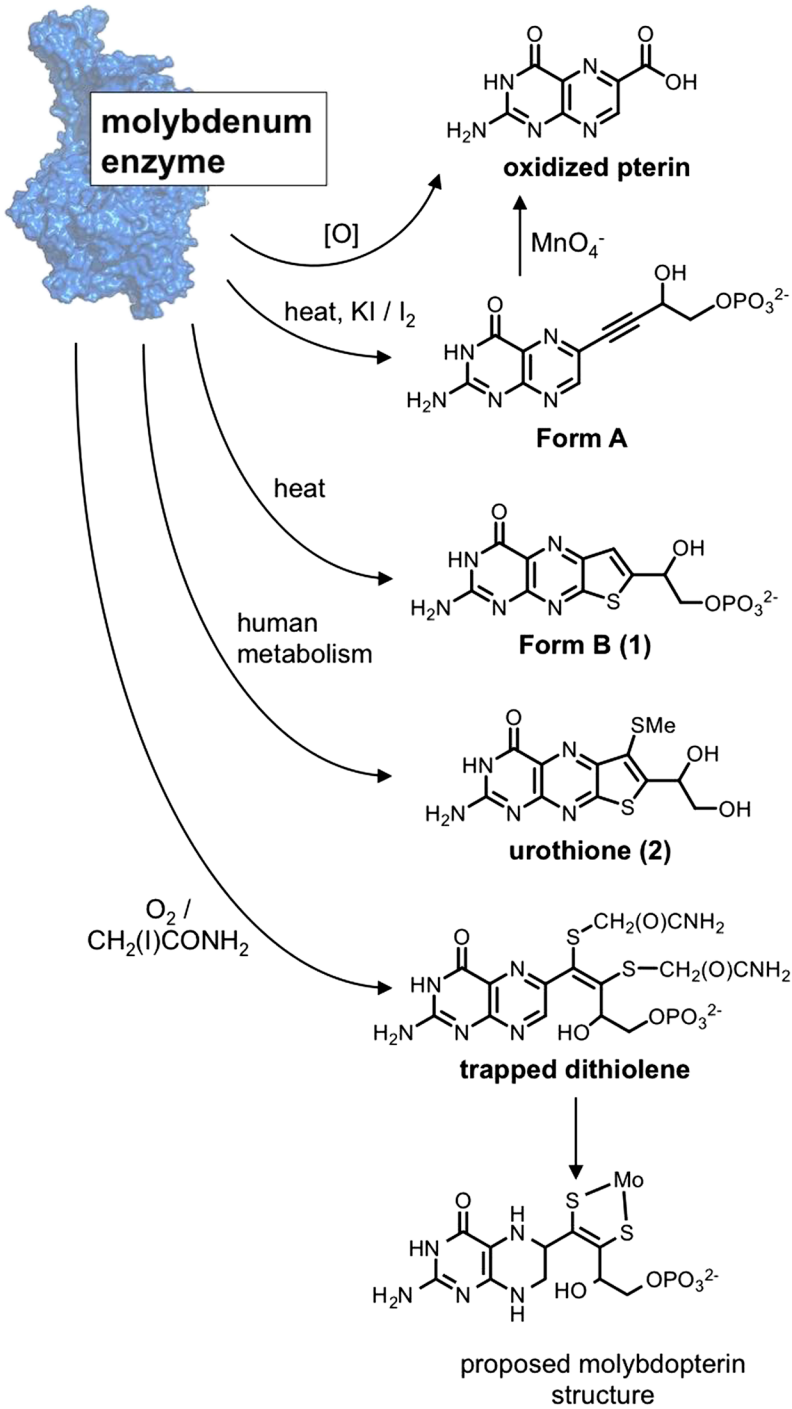


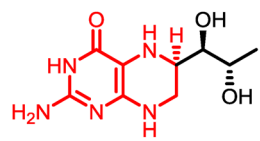
Figure 2.2 A summary of key experiments that revealed the pterin and dithiolene groups, and led Rajagopalan to propose the bottom structure for Moco.

In 1982, the culmination of these studies led to the proposal that the hidden ligand on Moco was a reduced tetrahydropterin substituted at C6 by a four-carbon chain having a dithiolene chelate at the α- and β-carbons, a hydroxyl group at the γ-carbon and terminated with a phosphate (Figure 2.1, center).[8,10] This ligand was initially named molybdopterin for "the special ligand on molybdenum", abbreviated as MPT. Since then, other names have been sought and used for this ligand, especially since the pterin-dithiolene ligand also is the special ligand in tungsten enzymes.[3,11]

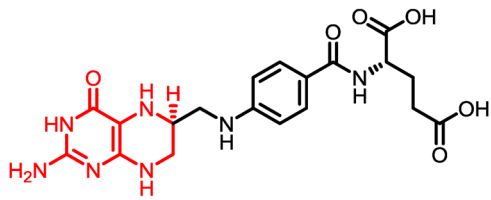
The tetrahydropterin–dithiolene ligand generated considerable excitement and speculation. It was unique in biochemistry, and is unusual in chemistry. While dithiolenes were well-known ligands for molybdenum and other metals, this was the first time a dithiolene was proposed to play a role in biochemistry. On the other hand, tetrahydropterins were already known molecules in biochemical processes, such as the tetrahydrobiopterin cofactor used by aromatic amino acid hydroxylases and tetrahydrofolate in C1 transfer in methionine synthesis (Figure 2.3). Certainly, this was the first time a pterin was found to be in combination with a dithiolene anywhere in chemistry.

The known redox roles of tetrahydropterins in biochemistry led Rajagopalan to investigate the redox behavior of the pterin unit of Moco. They titrated Moco within molybdoproteins (XO and SO) with two different oxidants, ferrocyanide and the redox dye dichlorophenol indophenol (DCIP),[12] and obtained unexpected results: two electron equivalents of either oxidant produced the spectral signature of a fully oxidized pterin (Scheme 2.1), a result only consistent with the pterin in Moco starting at the dihydro oxidation state rather than the tetrahydro state as initially proposed. The interpretation at the time was that the pterin in Moco, instead of the initially proposed tetrahydropterin structure, was a dihydropterin in an unusual tautomeric form.

Prior to the discovery of the pterin portion of Moco, it was assumed that all molybdenum enzymes (except nitrogenase) used the same cofactor, since

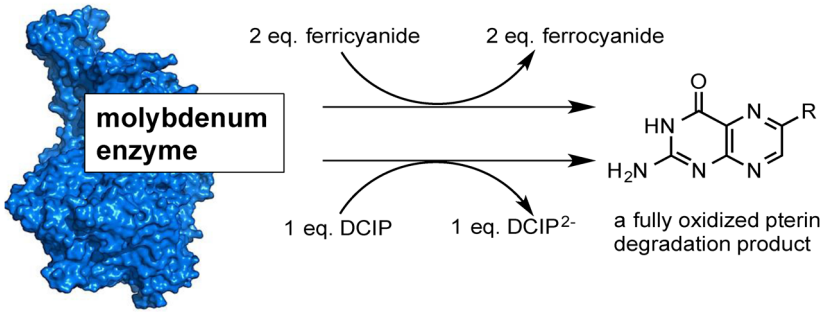


tetrahydrobiopterin



tetrahydrofolate

Figure 2.3 Structures of other pterins used in biochemical catalysis.



Scheme 2.1 The results of redox titration experiments on molybdoenzymes.

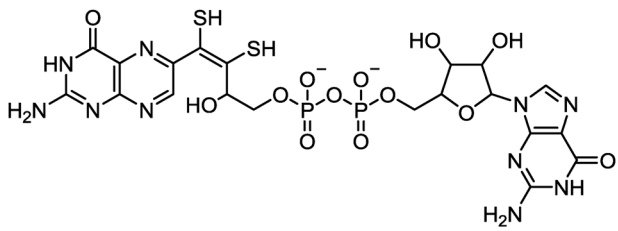


Figure 2.4 Proposed structure of MGT with appended guanosine nucleotide.

isolated Moco solutions could regenerate nitrate reductase activity in the *Neurospora crassa nit-1* mutant.[4b,4c] Krüger *et al.*[13] challenged the notion that the proposed Moco in Figure 2.2 was the universal structure in all molybdoenzymes in 1986 when they proposed a larger structure with a second phosphorylated aromatic unit. Rajagopalan's group's definitive analytical work on DMSOR from *Rhodobacter sphaeroides* proved that it possessed a modified MPT, described as a phosphoric anhydride of MPT and 5′-GMP (Figure 2.4), which they called MGD for molybdopterin guanosine dinucleotide.[14] They noted that the relationship of MPT to MGD was analogous to that between FMN and FAD.[7] By 1992 additional variants of MPT with adenine cytosine, inosine dinucleotides were identified. Dinucleotide substitution on the side chain is restricted to Moco from prokaryotic sources.

After years of speculation, in 1995, the unique structure of molybdopterin was revealed by X-ray crystallography, first in the tungsten enzyme, aldehyde ferredoxin oxidoreductase isolated from the hyperthermophile *Pyrococcus furiosus*,[15] and later as part of Moco in aldehyde oxidoreductase isolated from *Desulfovibrio gigas*.[16] These structures confirmed most of Rajagopalan's proposal with one exception: both structures showed that the dithiolene formed a third ring, a six-membered pyran ring fused to the pterin structure, presumably through a cyclization reaction involving the side chain hydroxyl group (Figure 2.5). Since these first two structures of Mo and W proteins were reported, dozens of X-ray structures have been determined and all the structures have shown the same pyranopterin–dithiolene structure for molybdopterin, with two exceptions. The first exception was observed in 2003 for a membrane bound dissimilatory nitrate