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Molybdenum-Containing Enzymes

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Abstract

An overview of modern methods used in the preparation and characterization of molybdenum-containing enzymes is presented, with an emphasis on those methods that have been developed over the past decade to address specific difficulties frequently encountered in studies of these enzymes.

Keywords

Molybdenum enzymes; Xanthine oxidase; Sulfite oxidase; DMSO reductase; Anaerobiosis

1 Introduction

Although molybdenum represents a vanishingly small portion of the earth's crust, it is the most prevalent transition metal in seawater [1, 2] owing to the high water solubility of molybdate. Given the high bioavailability of molybdenum, it is hardly surprising that living organisms have taken advantage of the chemical versatility of molybdenum and incorporated it into the active sites of enzymes. Indeed, there is accumulating evidence that the Last Universal Common Ancestor (LUCA) to all extant life forms made extensive use of molybdenum and the closely related tungsten [3]. At present, the number of molybdenum enzymes catalyzing distinct biochemical reactions is approaching 100, with representatives found in the overwhelming majority of extant organisms [4]. These enzymes can be grouped into three families based on the detailed chemical nature of their molybdenum centers, epitomized by the enzymes xanthine oxidase, sulfite oxidase and DMSO reductase [5].

The study of molybdenum-containing enzymes goes back nearly 100 years, with investigations of purified xanthine oxidase from cow's milk undertaken as early as 1924 by Dixon and Thurlow [6]. For many years, only a few additional enzymes were recognized as possessing molybdenum: aldehyde oxidase (closely related to xanthine oxidase), sulfite oxidase, the assimilatory nitrate reductase from algae and higher plants, and of course nitrogenase. Several additional enzymes from microbial sources were subsequently identified as possessing molybdenum, but it was only in the postgenomics era that the diversity and distribution of molybdenum-containing enzymes became fully appreciated.

The intent of this introductory chapter is to provide an overview of methods used in studying molybdenum-containing enzymes, with particular attention paid to a variety of issues that require special attention in their study. The intent is not to provide specific technical details, however, as these are covered in subsequent chapters. We begin with considerations relating to the isolation of native enzyme from various sources (vertebrate milk or organ tissues,

plant sources, and a variety of microorganisms), then move on to consider issues related to the expression and isolation of wild-type and variant enzyme forms in recombinant systems.

2 Native Systems

The first step in isolating enzymes of any type from vertebrate or plant sources is identifying the organisms and tissues that possess the highest levels of the target enzyme, much of this information being found in the historical literature. Enzyme preparations from these sources continue to be improved upon with, for example, modern ceramic hydroxyapatite FPLC chromatography resins replacing the notoriously poorly performing gravity columns used in earlier protocols for the purification of enzymes such xanthine oxidase from cow's milk and sulfite oxidase from chicken liver. Even with these newer methodologies, identifying the optimal sequence of chromatographic steps is nontrivial, particularly for the many molybdenum-containing enzymes that are membrane-associated or even membrane-integral. A major challenge is always ensuring that the enzyme is stable and active throughout the purification process, and activity must be carefully monitored in the course of developing new purification protocols. Some enzymes rapidly lose activity on certain column materials (for example, in the authors' laboratory the FdsABG formate dehydrogenase from *Cupriavidus necator* has been found to lose a substantial amount of its activity on GE Mono Q columns). Even with the best modern methodologies, however, preparation of useful quantities of enzyme often involves large-scale procedures, at least early on in a multistep purification. In the authors' laboratory, for example, a typical preparation of xanthine oxidase begins with 60 L of unpasteurized milk, from which several hundred milligrams of enzyme can be isolated.

A common issue in the purification of molybdenum-containing enzymes has to do with the degree of functionality of the isolated material. Most preparations of native enzymes (as distinct from what is typically seen with recombinant systems, see below) are replete with molybdenum and such other redox-active centers as may be found in the enzyme yet may still have lower than maximal specific activity. A classic problem has to do with the fact that all members of the xanthine oxidase family of enzymes and many members of the DMSO reductase family require an inorganic Mo=S ligand to the metal. The Mo=S ligand is labile, however, and can be slowly displaced by hydroxide from solvent, releasing sulfide and yielding a specific nonfunctional form of the enzyme referred to as "desulfo". This process can be accelerated by reaction of enzyme with cyanide, which releases the sulfur as thiocyanate [7]. For many members of the xanthine oxidase family, it is possible to reconstitute the activity of naturally occurring or cyanide-treated desulfo enzyme by incubation of the reduced enzyme under anaerobic conditions with sulfide [8]. This procedure is often less effective, however, with those members of the DMSO reductase family that require the Mo=S ligand. In some cases, reagents have been empirically identified (salicylate in the case of xanthine oxidase [9], nitrate in the case of the bacterial formate dehydrogenases [10]) that stabilize the Mo=S ligand and improve retention of activity. Assessing the extent of sulfurated/functional enzyme in a native preparation is important, and several different methods described in subsequent chapters discuss these in detail.

The CO dehydrogenase from organisms such as *Oligotropha carboxidovorans* represents a special case regarding sulfur incorporation, as this molybdenum enzyme possesses a unique binuclear active site in which the sulfur bridges between the molybdenum and a copper ion. As-isolated CODH is only 20–30% copperreplete and active. A reconstitution procedure involving incorporation first of the sulfur then of the copper is required to increase the enzyme activity (typically to 50–55%) [11].

For isolation of enzymes from microorganisms, a major consideration is the identification of growth conditions that lead to maximal accumulation of the target enzyme in the cell. All the issues related to isolation of enzymes from vertebrate or plant sources are relevant, however, as it is unusual that the target enzyme is ever expressed to more than 10% of total cell proteins. Among the factors to consider, particularly when using minimal media, is ensuring that the concentration of molybdate is sufficiently high to support expression levels of the desired molybdenum enzyme. Most microorganisms tolerate relative high concentrations of molybdate well, which is not always the case with vertebrates and plants: famously, cattle and other ruminants are extremely sensitive to molybdenum toxicity—even doses as low as 10 mg molybdate per day can cause serious gastrointestinal bleeding [12].

Most molybdenum enzymes from bacterial and archaeal sources are members of the DMSO reductase family and, unlike members of the xanthine oxidase and sulfite oxidase families, possess two equivalents of a pyranopterin cofactor (frequently referred to as molybdopterin, although the identical cofactor is found in tungsten-containing enzymes) coordinated to the molybdenum via an enedithiolate side chain. In the eponymous DMSO reductase from *Rhodobacter sphaeroides* or *R. capsulatus*, one of the two cofactors tends to be displaced from the molybdenum in the course of handling, being replaced in the molybdenum coordination sphere by a Mo=O group [13]; the resulting enzyme is not catalytically active. While the displaced pyranopterin can be readily reinserted into the molybdenum coordination sphere by the so-called redox cycling—reduction with sodium dithionite followed by reoxidation with substrate DMSO [14]. The two forms of the enzyme behave very differently on Q Sepharose and related materials, and the overall yield of the enzyme can be seriously compromised unless the crude cell extract is first redox-cycled [15]. To date, this has proven to be a difficulty only with the *Rhodobacter* enzymes, although there are occasional reports in the literature of X-ray crystal structures of molybdenum-containing enzymes in which one or another of the pyranopterin sulfurs has dissociated from the metal.

3 Recombinant Systems

Genomics techniques have proven extremely successful at identifying genes encoding molybdenum-containing enzymes in a variety of organisms, and many different recombinant systems have been developed for the efficient expression of both wild-type and variant enzyme forms. Heterologous expression of molybdenum-containing proteins, however, presents a number of challenges in addition to those described above for the expression of native proteins. The complex nature of the biosynthetic pathway for the pyranopterin cofactor and its subsequent insertion [16, 17] into apoprotein [18] often results in recombinant proteins that are only partially replete. More often than not, less than half of the expressed protein possesses molybdenum, even while containing its full complement

of other redox-active cofactors that may be found in the enzyme. A number of strategies have been developed for optimizing incorporation of the molybdenum center. Slowing down the growth of the *E. coli* cells so as to permit sufficient time for cofactor synthesis, primarily through a combination of low speed shaking (160–180 rpm) and low temperatures (16–25 °C), has been found to be beneficial [19]. Addition of molybdate to the growth medium prior to induction of protein expression ensures the availability of the metal during the insertion process. The amount of inducer (e.g., IPTG) can also be varied to balance the level of protein expression with endogenous cofactor biosynthesis so as to optimize cofactor saturation (lower levels of induction often promote higher levels of cofactor saturation [20]). Further, to that end, a weak promoter can also be employed [20]. A particularly important consideration in the development of bacterial heterologous expression systems for eukaryotic enzymes has to do with the fact that most bacterial molybdenum enzymes are elaborated as the dinucleotide of guanine (or less commonly, cytosine or adenine), whereas all known eukaryotic enzymes possess the mononucleotide form of the cofactor. It is thus essential to use specialized cell lines (e.g., the TP1000 series of *E. coli* strains [21]) that lack the dinucleotideforming enzymes and accumulate the mononucleotide form of the cofactor.

A major advantage of recombinant methods is the ability to apply various affinity purification protocols enabling one-step purification, and these have become widely used. His-tag methodologies relying on immobilized metal affinity chromatography (IMAC), particularly Ni-NTA and related materials, have been used most frequently, although these can be problematic for metal-containing proteins. The effectiveness of locating the His-tag at the *C*- or *N*-terminus (and in the case of multisubunit proteins, which subunit to label) is an important consideration and must be empirically determined. In the case of the FdsABG formate dehydrogenase, an *N*-terminal tag is preferable as it places the His-tag on the small FdsG subunit, well-removed spatially from the Mo-containing FdsA subunit [22]. Other factors may dictate the choice of subunit or position in the polypeptide in for example those proteins that are translocated to the periplasm (in which case an *N*-terminal His-tag would likely interfere with recognition of the *N*-terminal twin-arginine signal sequence, and in any case would be cleaved after translocation). The choice of metal used in the IMAC column material can also influence the degree to which purification results in loss of enzymatic activity. The two most widely used metals, Ni^{2+} and Co^{2+}, are sufficiently different such that where one may contribute to loss of activity, the other may be perfectly benign. The extent to which a given resin results in loss of activity can be assessed by standard enzymatic activity assays. It has occasionally been found that high concentrations of imidazole itself, used to elute bound enzyme from the IMAC column material, has contributed to protein instability and/or loss of molybdenum. This can also occur with other metal centers that might be present in the recombinant enzyme. The authors have, for example, observed loss of up to 95% of the heme cofactor in a *b*-type heme protein (part of the mARC-reductive pathway) when employing a standard Ni-NTA purification protocol. The His-tag itself may also be responsible for the loss of activity. For example, a *C*-terminal His-tag-labeled YedY protein is eight times less active than its unlabeled analogue [23]. Purification of complex His-tagged metalloproteins can be complicated by interference of the metal chelate (as well as the His-tag itself or the imidazole used to elute) with the active sites of metalloenzymes, and again quality control of the purified recombinant protein thus becomes crucial. In

cases where this is an issue, use of other forms of affinity chromatography that do not involve metal chelation (Flag tag, SUMO tag, GST, etc.) should be considered. Arnau et al. have published an extensive review of various affinity-tag and tag removal methods for the purification of recombinant proteins [24]. Finally, it should be borne in mind that in those cases where metal chelating resins themselves lead to considerable loss of activity, native purification methods may be considered as an alternative to other affinity methods, particularly in the cases where conditions have been worked out for the native form of the protein.

A number of methods can be used to assess cofactor saturation, including inductively coupled plasma mass spectrometry (ICP-MS) that can quantify the amount of molybdenum (and other metals) in a protein sample. When performed carefully, ICP-MS can be a valuable tool in the determination of the metal content in recombinant as well as native proteins. Assaying the amount of pyranopterin cofactor present in an enzyme sample is more difficult, owing to the extreme lability of the cofactor once extruded from a molybdenum enzyme. Still, an HPLC protocol has been developed for quantification of a specific degradation product of the cofactor, termed Form A, and is widely used in the field [25]; quantitative extrusion of the cofactor from the protein sample is critical to the success of the procedure. This method, in conjunction with ICP-MS, can provide an accurate determination of the level of cofactor/metal saturation.

Unfortunately, the Mo=S ligands present in the molybdenum centers of many enzymes, as referred to above, are not simply prone to spontaneous desulfuration but are frequently inserted incompletely in recombinant proteins. Like the biosynthetic pathway for the pyranopterin cofactor, the endogenous machinery for sulfur incorporation can be overwhelmed by high expression levels of the apoprotein. Coexpression of the gene encoding the sulfurase responsible for sulfur incorporation with that for the structural gene(s) for the molybdenum enzyme has been successfully employed in some cases to minimize this problem [26]. Beyond determining the extent to which a recombinant protein is replete with cofactor is the assessment of whether the cofactor is structurally and functionally intact. Electron paramagnetic resonance (EPR) spectroscopy is particularly useful, being sensitive to the protein environment of the Mo-cofactor as well as its structural integrity. In Mo-containing enzymes, only the Mo(V) state is paramagnetic and thus EPR-active. The oxidized Mo(VI) enzyme as-isolated can usually be converted to the Mo(V) state by partial reduction with sodium dithioite (or substrate), with the amount of the Mo(V) state accumulating ultimately governed by the relative reduction potentials of the Mo(VI)/Mo(V) and Mo(V)/Mo (IV) couples. In most cases, 20–50% of the Mo(V) species can be generated. The EPR spectra of functional and nonfunctional forms of many molybdenum enzymes have been characterized [27]. Diagnosing potentially compromised Mo-sites can be important not only in the characterization of full-length recombinant proteins but also for truncated forms, such as truncated versions of both the human sulfite oxidase (hSO) and plant nitrate reductase (NR) enzymes [28]. The authors have encountered preparations of the truncated form of hSO (where only the Mo-cofactor-containing domain remains) that give very unusual Mo(V) EPR signals diagnostic of compromised cofactor. Finally, it should be emphasized that the functional molybdenum centers, particularly in enzymes of the DMSO reductase family but also the isolated molybdenum-containing domains of

members of the sulfite oxidase family, exhibit distinct absorption spectra, and accurately determined extinction coefficients can provide a convenient and very sensitive tool to assess protein integrity. In particular, the molybdenum centers of DMSO reductase family members have absorption maxima above 700 nm, far from the absorption envelopes of iron–sulfur, flavin, and even heme prosthetic groups, that provide sensitive and accurate measures of the concentration of *functional* molybdenum centers.

4 O_2 Sensitivity

A final consideration regarding the handling of molybdenumcontaining enzymes is sensitivity to O_2. The vast majority of molybdenum-containing enzymes, once reduced, will reoxidize relatively rapidly to very rapidly in the presence of O_2, and it is necessary in many cases to exclude O_2 from the reaction conditions. Enzymes such as xanthine oxidase, aldehyde oxidase, and sulfite oxidase from plants use O_2 as the physiological oxidizing substrate. On the other hand, other enzymes, including nitrogenase and many members of the DMSO reductase family of enzymes (several of the formate dehydrogenases, for example) are inactivated by O_2 and must be isolated under strictly anaerobic conditions and/or in the presence of stabilizing agents such as azide or nitrate. The reader is referred to the several subsequent chapters dealing with purification and handling enzymes of the second type; here we restrict ourselves to the former group of enzymes, which are O_2-stable but can be reoxidized by O_2 once reduced.

Although anaerobic glove boxes are essential for handling O_2-sensitive enzymes, for many types of experiments the use of an anaerobic train (Schlenk line) is far more convenient, especially with the easy availability and affordability of Ar gas with built-inpurifier, containing less than 10 ppb of O_2 (Airgas ARBIP300). Ar and N_2 are both used for anaerobiosis, although Ar makes the process more efficient. Buffer solutions are conveniently made anaerobic by bubbling for 10–15 min in a serum-stoppered vessel fitted with an exit needle. Enzyme solutions, on the other hand, must be placed in an appropriate gas-tight vessel, which is then alternately evacuated and flushed with N_2 or Ar, with 6–8 cycles over the course of 60–90 min. This can be done with the vessel on ice, and is far superior to, for example, simply placing a protein solution in a glove box overnight, even with stirring. Anaerobic work involves various specialized glassware such as anaerobic cuvettes with which spectrophotometric experiments can be performed, or tonometers (sometimes equipped with sidearm cuvettes) that can be mounted on instrumentation such as a stopped-flow or freeze-quench apparatus. Other vessels that might be appropriate include vials that can be sealed with septa, frequently used in conjunction with gastight syringes for anaerobic transfer of solutions previously made anaerobic. In addition, long 20–24-gauge steel cannulae have been used in the authors' lab to directly transfer enzyme solutions from septum-sealed vials (in which the enzyme was made anaerobic) to septum-sealed EPR tubes. The reader is referred to the literature for various apparatus of this type [29, 30], which describe a wide variety of anaerobic glassware for specific applications.

5 Conclusion

There has been substantial progress in the development of protocols for the expression and purification of molybdenum-containing enzymes in both native and recombinant systems over the past decade or so as improved protocols for the purification of these enzymes continue to be developed and refined. The above is intended to highlight a number of specific points that should be borne in mind in studying these enzymes and specific methods that can be used to address them. The ensuing chapters deal in detail with many of these and other points relevant to molybdenum enzymes.

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