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Review

The bioinorganic chemistry of tungsten

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Contents

Abstract

Tungsten is widely distributed in biology, however, the majority of the tungsten-containing enzymes purified to date, originates from anaerobic archaea and bacteria. Tungsten coordination complexes incorporated in these enzymes can be studied with similar analytical and spectroscopic

Abbreviations: AH, acetylene hydratase; AOR, aldehyde oxidoreductase; ATP, adenosine triphosphate; cPMP, cyclic pyranopterin monophosphate; DMSOr, dimethyl sulfoxide reductase; FDH, formate dehydrogenase; Fdt, flavanyldithiolene; FMDH, formylmethanofuran dehydrogenase; FOR, formaldehyde oxidoreductase; GAPOR, glyceraldehyde-3-phosphate oxidoreductase; GTP, guanosine triphosphate; ITC, isothermal titration calorimetry; MCD, magnetic circular dichroism; MCP, molybdenum cofactor storage protein; MGD, molybdopterin guanine dinucleotide; Mo/WSto, molybdenum/tungsten storage protein; Moco, molybdenum cofactor; Mo-MPT, molybdopterin; MPT, metallopterin; MPT-AMP, adenylylated metallopterin; TMAO, trimethylamine-*N*-oxide; UMK, uridine monophosphate kinase; Wco, tungsten cofactor; W-MPT, tungstopterin.

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techniques as tungsten model compounds. The metal is taken up by cells in the form of tungstate, and subsequently it is processed into a sulfur-rich coordination as part of a metal-organic cofactor referred to as tungstopterin, which is equivalent to the molybdopterin forms found as active centers in several molybdenum-containing enzymes. For biology tungsten is significantly different from molybdenum and this review focuses on the (bio)molecular basis of this differential cellular use of W compared to Mo in terms of their active transport, cofactor synthesis, and functioning as catalytically active sites.

Keywords: Tungsten; Tungstoenzymes; Molybdenum; Molybdoenzymes; Pterin; Metalloprotein

1. Introduction

Tungsten is the bioelement with the highest atomic number, 74, and the only bioelement in the third transition row of the periodic table. Tungsten is widely distributed in biology, however, it is not a universal bioelement. For some species tungsten is essential: their life depends on the presence of the element; for other species tungsten is a facultative bioelement: they choose to make biological use of the element when they experience specific environmental constraints; for the remaining species tungsten is biochemically indifferent or possibly xenobiotic: they have not developed a functional use of the element, although, upon its inadvertent intake, their physiology may well be affected. Present knowledge places all eukaryotes, including man, in the last category. Two fundamental questions form the Leitmotif of this review; the first one is the 'why'-question: why do some cells go for tungsten chemistry and others not? The second is the 'how'-question: how do cells discriminate between tungsten and molybdenum?

Molybdenum is in many ways the twin element of tungsten. Also in biology the coordination chemistries of W and Mo are similar in structural and functional aspects. Molybdenum is the only bioelement in the second transition row. Like tungsten it is widely, though possibly not universally, distributed in biology. Its usage appears to be to a considerable extent the mirror image of that of tungsten. Some forms of life, e.g. humans, are strictly dependent on the availability of Mo while they are independent of W; other species, e.g. the archaeon *Pyrococcus furiosus*, have no apparent use for Mo, while they are strictly dependent on W; yet other species, e.g. the archaeon *Methanobacterium thermoautotrophicum*, appear to be able to choose between W and Mo as a function of a variable environment. And yet other species, e.g. the archaeon *Pyrobaculum aerophilum*, may have learned to employ the chemistries of Mo and W simultaneously for distinct functions. Mo-biochemistry and W-biochemistry are presently both very active areas of research, the latter in particular in relation to the fundamental why and how questions formulated above. Mo has been known to be a biological trace element for a long time, and the development of its biochemistry has commonly been taken to be an endeavor in its own right. The identification of tungsten as a bioelement is from a more recent date, and thus, it has come naturally to develop its biochemistry in comparison to that of molybdenum. Several aspects of Mo-biochemistry have been covered in recent reviews [1–4]. This review takes a tungsten vantage point and uses molybdenum for contrast. Early reviews on aspects of W-biochemistry can be found in Refs. [5–7].

2. Aqueous chemistry of tungsten

Since water is the only life-compatible solvent, the aqueous chemistry of tungsten is of relevance. The basic geochemical conditions for an element to qualify for general use in biology are a diffuse distribution over the surface of the earth and a reasonable solubility in water. Both conditions hold for tungsten (and for molybdenum as well). Scheelite and wolframite are not unusual ores, and global leaching has resulted in an average tungsten concentration in the oceans of circa 10^{-4} ppm or $\approx$1 nM. It has been argued that a circa two orders of magnitude higher concentration of molybdenum in oceanic waters could be at the basis of a presumed preference for Mo over W in biology [6,8], however, the oceanic concentration of cobalt, a universal bioelement, is of the same order, or less, of that of tungsten.

The dilute aqueous speciation of tungsten in water is presumably completely dominated by the tungsten(VI) oxoanion WO_4^{2-} over a wide range of pH and redox-potential values [9] that would cover conditions found in most of the terrestrial inhabitable environments, and so tungstate is likely to be the only molecule that cells in search of tungsten have to deal with. At increased concentrations a kinetically, and thermodynamically complex polyoxoanion chemistry evolves [10]. Thus the entry point of W-biochemistry is a stable, redox indifferent, highly soluble oxoanion; and it has a Mo congener of very similar properties including an anion radius of circa 1.74 Å [11].

3. Basics of tungsten biology

The top most systematic level of division of life on earth is into the three domains of eukaryotes, bacteria, and archaea (Fig. 1). The systematics of tungsten biochemistry is already significant at this level: there appears to be, respectively, no,

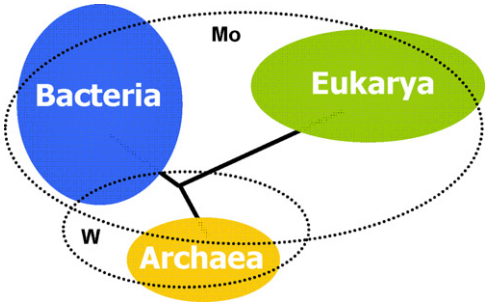
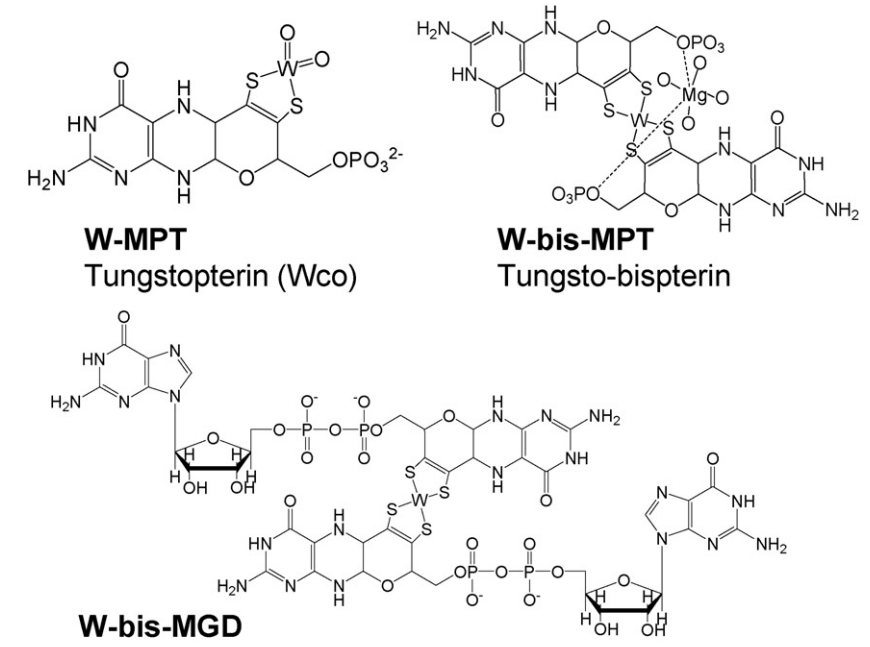


Fig. 1. The tree of the three domains of life and the distribution of tungsten- and molybdenum-containing enzymes: molybdenum enzymes are found in all forms of life whereas the occurrence of tungsten enzymes appears to be restricted to archaea and some bacteria.

occasional, and frequent usage of W in the three domains. It has been suggested that in an evolutionary sense tungsten is an 'old' element on its way to be replaced by 'modern' molybdenum (similarly nickel has been suggested to be in the course of being replaced by cobalt) [8]. This idea appears to be supported by the significant correlation between archaeal life and W-biochemistry. The name archaeon (previously: archaebacterium) is of course intended to transmit the notion that these forms of life are thought to be the most similar to 'primitive' life as it must have existed in early geological times not long after the appearance of the first living cell. It should, however, be realized that archaea, similar to bacterial or eukaryal monocellular organisms, typically have doubling times of the order of 10^{-1} to 10^{1} h and, therefore, have a life span of the order of 10^{-2} year, i.e. a very brief moment on geological time scales. Archaea are modern organisms that live today and that do not show any sign of decreased fitness for survival at all. On the other hand, there appears to be a clear over-representation of archaea in 'extreme' habitats, e.g. the vast majority of hyperthermophiles (species with optimal growth temperature above 80 °C) consists of archaea. However, a link between W usage and extremophilicity is not immediately obvious, and the rela-



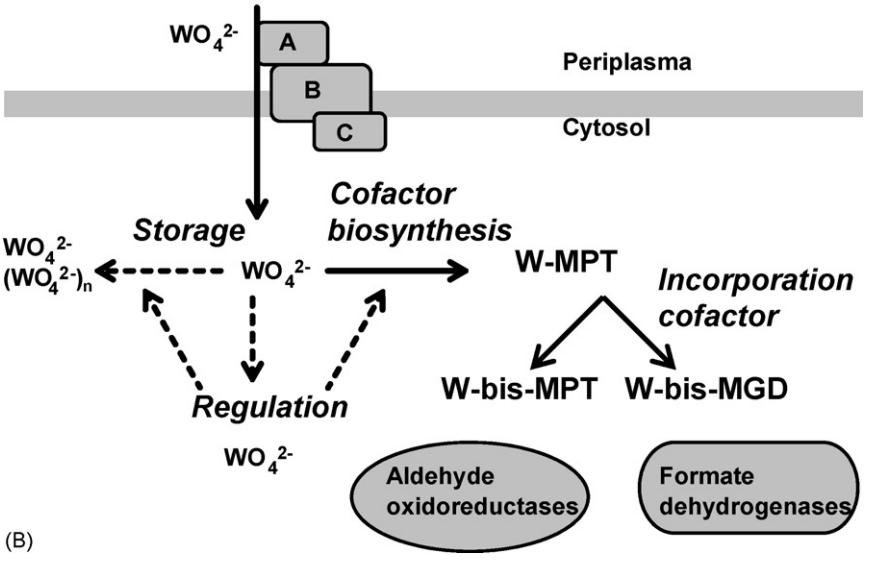


Fig. 2. The structure of tungsten-containing pterin cofactors and intermediates: tungsten-containing metal binding pterin (W-MPT) (I), tungsto-bispterin (W-bis-MPT) (II), tungsto-bispterin guanine dinucleotide (W-bis-MGD) (III) (A). A schematic overview of the stages in cellular metabolism of tungsten (in various chemical forms): uptake, storage, regulation, cofactor biosynthesis, and incorporation in enzymes. Dashed arrows correspond to hypothetical processes based on cellular processes known for molybdate (B).

tion, if any, may well be a rather indirect one: most archaea are anaerobes (or perhaps microaerophiles) and the link may simply be one of mutual exclusion of molecular oxygen and tungsten biochemistry.

In general, the biochemistry of a metal in a monocellular organism encompasses several processes (Fig. 2): sequestering and transport over the cytoplasmic membrane, storage and release, metal-cofactor biosynthesis, metalloenzyme catalysis, and metal-controlled regulation of transcription and/or translation. Our present knowledge of these processes in relation to tungsten is not particularly balanced: tungsten-based biocatalysis is by far the best studied; the processes of tungsten transport and tungsten cofactor biosynthesis are emerging research areas; we are essentially ignorant on the remaining processes of storage and regulation. For the latter there is limited information from molybdenum biochemistry, and this may help in designing experiments to determine if comparable tungsten-related processes exist.

Tungsten is sequestered and transported into the cytoplasm as tungstate. It may then enter the biosynthetic machinery to be bound to a dithiolene-functionality of a tricyclic pterin moiety to form tungstopterin (W-MPT) (Fig. 2A) and eventually end up in tungstoenzymes in the form of a complex metal-organic cofactor: tungsto-bispterin (W-bis-MPT) or tungsto-bispterin guanine dinucleotide (W-bis-MGD) (Fig. 2B).

4. Spectroscopy of tungsten

The molecular spectroscopy of tungsten associated with biomolecules poses a number of considerable challenges, which find their origin not only in the physics of the methodology but also in practical biochemical problems. A key issue is the redox chemistry of the element, i.e. the availability of the three oxidation states +IV, +V, and +VI, over a relatively narrow potential window in combination with the tendency of W(V) to disproportionation. Tungstoenzyme preparations (and molybdoenzymes similarly) are frequently found to exhibit inhomogeneity in terms of oxidation state. Furthermore, they tend to be mixtures of holo- and apo-enzyme (i.e. protein that has lost, or never received, the metal cofactor), and of active, inhibited (e.g. by substrate or product) and inactivated (e.g. by molecular oxygen) enzyme forms. Common as these problems may be at this time, they are presumably eventually solvable with careful protein production, purification, and characterization procedures. Unfortunately, this may not hold true for the problems of sensitivity and resolution intrinsic in tungsten spectroscopy.

4.1. NMR and Mössbauer spectroscopy

A decade ago we attempted an evaluation of the potential applicability of NMR and Mössbauer spectroscopy on W-proteins. It was then estimated that ^{183}W ($I = 1/2$) NMR, in which a very high resolution is counterbalanced by an extremely low sensitivity, would be applicable to proteins only in a dedicated, very labour-intensive program [5]. Since then no papers have appeared on W protein NMR, and, in fact, the literature on W NMR in general is limited, and gives indications of a detection limit (e.g. >0.1 M [12]) that would suggest it to be wise to exclude W-protein NMR for consideration in the foreseeable future.

In a similar vein, we considered ^{182}W ($I_{ground} = 0$; $I_{exited} = 2$; parent $t_{0.5} = 115$ days) as a target for biological Mössbauer spectroscopy, and we suggested that W would be somewhat more difficult than Ni, a nucleus that was, at the time, actively explored for its potential in Mössbauer studies on nickel proteins [5]. Since then no papers have appeared on the Mössbauer effect in Ni- or in W-proteins, and the literature on tungsten Mössbauer in general over the last 10 years is almost non-existent [13]. Again, one can fairly conclude that Mössbauer spectroscopy on tungstoproteins is not an advisable research subject at this time.

4.2. Optical spectroscopy

Fortunately, a somewhat brighter picture can be drawn now for optical spectroscopy on tungstoenzymes, which is another subject on which no primary literature existed 10 years ago. Dimethyl sulfoxide reductase (DMSOr) is a member of a small subgroup of molybdoenzymes that do not carry any prosthetic group in addition to the molybdopterin active center. In the DMSOr from *Rhodobacter capsulatus* Mo can be replaced with W with retention of activity and without detectable change in the 3D structure of the rest of the molecule [14]. Both the Mo(VI) and the W(VI) version of this enzyme exhibit an optical spectrum with a number of absorption bands extending all the way into the IR and with extinction coefficients of the order of $\varepsilon \approx 2\,mM^{-1}\,cm^{-1}$ (Fig. 3 [14]).

The spectra have been qualitatively interpreted in terms of metal to sulfur charge transfer with reference to model compounds [14]. Also, considerable changes occur upon metal reduction during catalytic turnover, and this has been used as a monitor in a pre-steady-state kinetics study of the molybdoenzyme [15], however, a quantitative characterization of the complete spectra of any other form than the fully oxidized enzyme has not been reported yet.

The typical molar extinction coefficient of $2 \times 10^{-3}\,M^{-1}\,cm^{-1}$ implies a problem of sensitivity in optical studies of complex enzymes. The vast majority of tungstoenzymes and molybdoenzymes carry, in addition to their metallopterin active center, one or more cofactors for electron transfer. The collection of electron transfer cofactors can be quite complex in some molybdoenzymes where one can find combinations of Fe/S clusters with flavins and/or hemes resulting in a swamping by these strong absorbers of the relatively modest absorption spectrum of the metallopterin. Tungstoenzyme members of the aldehyde oxidoreductase (AOR) family (defined below) are a special case in that they carry, in addition to the tungsto-bispterin, only a single $[4Fe–4S]^{(2+;1+)}$ cluster. Although the extinction coefficient of the latter in its oxidized form is circa an order of magnitude greater than that of the tungstopterin, the shape of the spectrum is relatively simple with only a single, broad band peaking at circa 390–430 nm and gradually falling off to zero absorption towards circa 700 nm. The use of the relatively weak tungstopterin optical spectrum from these enzymes as a

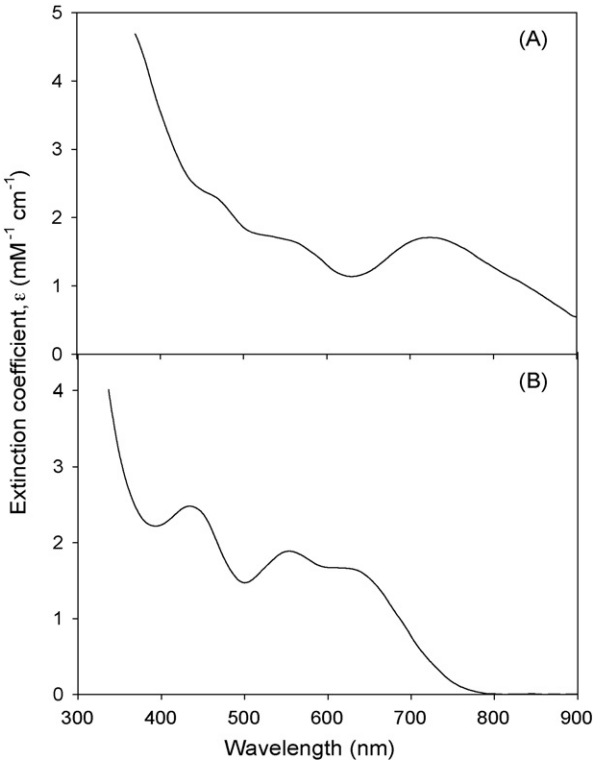


Fig. 3. UV–vis absorption spectra, recorded at room temperature for molybdenum-containing DMSOr (A) and tungsten-containing DMSOr (B) (data reproduced from [14] with permission; the spectra have been re-plotted on an absolute absorption scale).

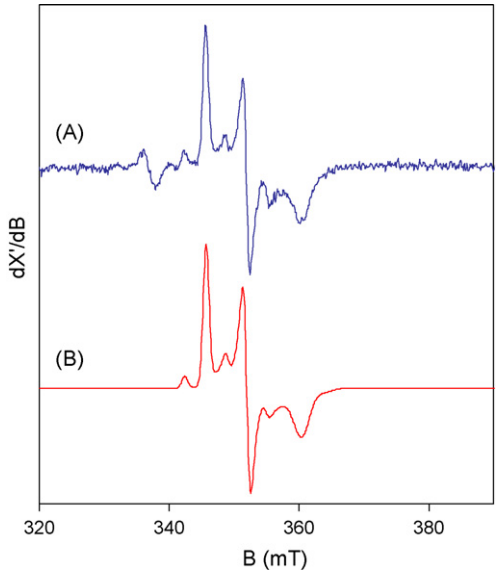


Fig. 4. W(V) $S = 1/2$ EPR from tungstoproteins is typically weak due to substoichiometry of the intermediate redox state, but is easily identified from the satellite hyperfine lines of the ^{183}W isotope (14.4%, $I = 1/2$). This example, recorded at 9.43 GHz and 22 K, is from *Pyrobaculum aerophilum* AOR (A). The simulation has $g_{zyx} = 1.952, 1.918, 1.872$ and $A_{zyx} = 6.5, 6.0, 7.0$ mT (B) (reproduced from [19]).

monitor in kinetic measurements has recently been employed in a study to delineate the redox intermediates in the reaction cycle of *P. furiosus* formaldehyde oxidoreductase (FOR) [16].

No resonance Raman data on tungsten enzymes have been reported in the primary literature. A peak at 874 cm^{-1} in the resonance Raman spectrum of *P. furiosus* FOR has been cited to be assignable to a W=O stretch frequency [17].

4.3. EPR spectroscopy

EPR spectroscopy of tungstoenzymes is relatively uncomplicated from the spectroscopist's point of view: oxidized W(VI) is diamagnetic [Xe]$5d^0$; reduced W(IV) is d^2 and could in principle be high-spin, however, the spin state has not been determined in tungstoproteins or tungstopterin model compounds, and no paramagnetism has been reported for this oxidation state. The intermediate W(V) is $5d^1$ and $S = 1/2$. From the positive sign of the spin orbit coupling constant for systems with less than half filled shells basic theory predicts the g-values to be less than that of the free electron value, $g_e = 2.0023$, however, occasionally one can find one or two of the g-values to be greater than g_e possibly related to relativistic effects, and/or extensive charge transfer from S (or Se) ligands. Experimental g-values reported for tungstoenzymes are typically in the range 1.99–1.83 [7], and the deviation from g_e as well as the overall g-anisotropy are somewhat more pronounced than in molybdoenzymes. Natural tungsten consists of five isotopes, only one of which has a nuclear spin: ^{183}W occurs in a natural abundance of 14.4% and has a nuclear spin $I = 1/2$. The isotope is frequently detectable in the $S = 1/2$ spectra of W(V) in the form of small satellite lines with circa 7% relative intensity [18] (Fig. 4 [19]) and this is a unique fingerprint for the element. The hyperfine splitting along the principal g-tensor axes is some 30–80 G, or approximately a few times the inhomogeneous line width [7], i.e. the pattern is frequently well resolved. Characteristically, for $S = 1/2$ systems that lack extreme g-anisotropy the spin-lattice relaxation rate is relatively slow, and the spectra can be observed at least up to ~77 K without significant broadening. When a cryogenic He-flow system is in place (to detect heme or iron–sulfur prosthetic groups at low temperatures) tungsten $S = 1/2$ signals are conveniently detected at circa 40–50 K. The relatively slow relaxation rate would seem to make W(V)-proteins quite suitable for high-resolution hyperfine spectroscopy through pulsed double resonance experiments (ESEEM, pulsed ENDOR). No data are available yet, but fruitful experiments on molybdoenzymes have been reported in particular by Enemark et al. [20]. Finally, the ease of the EPR spectroscopy should perhaps be put into perspective by contrasting it to the complexities of the (bio)chemistry of W(V): multiple stable W(V) forms may well be enzyme dead-end products rather than catalytically competent intermediates [16,21,22].

If EPR is easy and optical spectroscopy (of enzymes with additional cofactors) is difficult, then what about their combination in the form of magnetic circular dichroism, MCD, of tungstoenzymes? Good quality, variable cryogenic temperature W(V) MCD data have been reported for *P. furiosus* AOR [21] and FOR [22] at reduction potentials where the iron–sulfur cluster is diamagnetic (at low temperatures) with multiple bands in the 300–800 nm range plus a near-IR band at 880 nm all assigned to S-to-W(V) charge transfer transitions. Unfortunately, cryo-

genic MCD detection requires high-quality transparent glasses, which means that glassing agents, typically 50% or more glycerol, have to be added to the sample, and this causes essentially complete inhibition of enzyme activity [21,22]. Sucrose may be used instead of glycerol to overcome this problem.

4.4. EXAFS spectroscopy

EXAFS spectroscopy at the L_{III}-edge of W (circa 10.2 eV or 1.22 Å) was initially explored by Cramer et al. on formate dehydrogenase (FDH) of *Clostridium thermoaceticum* [23] (now: *Moorella thermoacetica*) but this early work was hampered by low signal intensity and an ill defined redox state of the tungsten [24]. George et al. studied *P. furiosus* AOR with cryogenic W-EXAFS before high-activity preparations of this enzyme had become available. The W-coordination in the low-activity AOR, known as "red tungsten protein", was proposed to be by three S, two oxo, and possibly one more N/O ligand [24]. The S/O ratio is lower than in the X-ray structure that was later determined for the active *P. furiosus* AOR [25] suggesting that red tungsten protein is an oxidative degradation product of AOR. A revisit of AOR EXAFS has not been reported yet. Metalloprotein EXAFS spectroscopy is most fruitfully applied when specific questions on coordination can be asked based on available X-ray structures, and the first example of such an approach is in the combined crystallographic and spectroscopic study of Stewart et al. on *R. capsulatus* W-substituted DMSO reductase [14]. The W(VI) data from oxidized enzyme were interpreted in terms of four equivalent dithiolene S ligands at 2.4–2.5 Å, the O^{γ} of Ser147 at 1.9 Å, a second O ligand at 1.9 Å, and possibly a third O close to one of the S. This coordination is consistent with X-ray structural data and is essentially identical to that previously determined for the native Mo(VI)-version of this enzyme, thus defining the protein as an excellent model to study intrinsic differences in redox properties between the two metals [26]. Tungsten oxidation state-dependent EXAFS has been initially explored by Hagedoorn on *P. furiosus* glyceraldehyde-3-phosphate oxidoreductase (GAPOR) poised at −645 mV versus −454 mV (using different substrate over product ratios) [27]. Data analysis afforded a six-coordination of four S and two O for both samples, with slightly longer distances in the more reduced sample, however these results should be considered preliminary with significant noise in the experimental data and in view of an observed mere 0.4 eV shift in edge position, which indicates that the two samples may not have been purely W(IV) and W(VI), respectively. All in all, EXAFS spectroscopy of tungstoproteins appears to have considerable potential for structure–function studies, but its exploration remains limited at this time.

5. Cellular transport of tungstate

The cellular transport system for oxoanions like tungstate, molybdate, sulphate and phosphate has been described for many organisms, in particular the molybdate uptake mechanism for *Escherichia coli* [28]. All systems are members of the adenosine triphosphate (ATP) binding cassette (ABC) transporter family. The majority of these oxoanion transporters consist of three proteins; the 'A' protein is responsible for the recognition and binding of the substrate. This protein is located in the periplasm, which is the space between the cytoplasmic membrane and: (i) the cell wall in Gram-positive bacteria, (ii) the outer membrane in Gram-negative bacteria, or (iii) the S-layer in archaea. For some ABC transporters the first component is linked to the outer surface of the cellular membrane with a so-called 'lipotail', which is a lipid-modified cysteine residue. The B component forms the transmembrane pore through which the substrate is transported into the cell, and this transport is facilitated by the ATP hydrolyzing activity of component C on the inner surface of the membrane. In Fig. 5 an overview is given of the general rules on gene and protein annotation, which are also used in this review [29].

The periplasmic molybdate-binding protein in *E. coli*, referred to as ModA, specifically binds molybdate with an equilibrium constant for dissociation (K_D) of 20 nM. ModA is also able to bind tungstate with a similar affinity [30]. After binding to the periplasmic component the molybdate or tungstate is actively transported against a concentration gradient into the cell through the transmembrane unit ModB energized by the ATP hydrolyzing activity of ModC.

A tungsten-specific transporter: Tungsten uptake protein ABC (TupABC), was identified for the first time in the mesophilic bacterium *Eubacterium acidaminophilum* and was shown to bind tungsten with a dissociation constant (K_D) of 0.5 μM [31]. The K_D for molybdate was determined to be greater by several orders of magnitude. Recently, a second, structurally different tungsten-specific ABC transporter system was discov-

Genetic properties of organisms are described in terms of phenotypes and genotypes. **The genotype** refers to the genes present in the genome of an organism and the **phenotype** describes its observable properties, for example the expression of proteins.

An **operon** contains one or more structural genes which are transcribed as a single mRNA molecule that codes for more than one protein. The operon is designated by a three-letter, lower case, italicized symbol (e.g. *mod*). The structural genes are distinguished by italicized capital letters following the name of the operon (e.g. *modA modB modC*).

Proteins (i.e. gene products) are generally referred to with the three-letter symbol of the non italicized structural gene designation with the first letter of the symbol capitalized (ModA, ModB, ModC).

Fig. 5. An overview of the annotation agreements for genes and proteins [29].

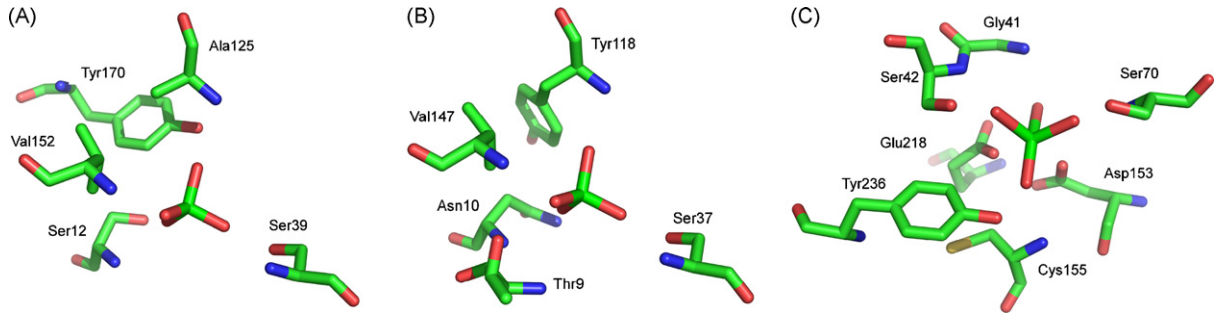


Fig. 6. Crystal structure of the binding pocket of *E. coli* ModA binding molybdate (A) [34], *A. vinelandii* ModA binding tungstate (B) [35], and the periplasmic binging protein of *A. fulgidus* binding tungstate (C) [36].

ered in the hyperthermophilic archaeon *P. furiosus* [32]. The periplasmic component: W-transport protein A (WtpA), was shown to bind tungstate specifically with a K_D in the picomolar range. Isothermal (displacement) titration calorimetry (ITC) of molybdate-saturated protein with tungstate showed clearly that tungstate replaces the molybdate in the binding pocket of the protein. These data indicate that the K_D for molybdate is several orders of magnitude greater than that for tungstate [32].

Based on a high sequence similarity with TupA (58%), the first high affinity vanadate transporter, Vanadate uptake protein ABC (VupABC), could be identified recently in *Anabaena variabilis* [33]. This organism requires vanadate for the expression of a vanadium-containing nitrogenase for the fixation of molecular nitrogen.

With the identification of these transporter systems that all have specific affinities, we can conclude that significant overall amino acid sequence similarities (ranging from 30% up to 60%) cannot be used to predict the type of oxoanion that is transported with the highest affinity. So which factors are determining the selectivity? Crystal structures of the periplasmic component in complex with the specific oxoanion could help to answer this question. The three-dimensional structure can be used to identify essential amino acid ligands that play a role in determining the specificity of the protein. Subsequently mutational studies can confirm this role in *in vitro* binding experiments.

Crystal structures are available for ModA from *E. coli* [34] and ModA from *Azotobacter vinelandii* (ModA2) [35]. Both ModA proteins were crystallized in complex with tungstate or molybdate (Fig. 6A and B). However, *in vitro* binding experiments showed that ModA was not able to discriminate between the two oxoanions, and therefore, these structures cannot be used to explain the specificity that was found for the tungstate transporters. Indeed, inspection of the structures confirms that the protein binds tungstate and molybdate in an identical way: in both ModA proteins, seven hydrogen bonds are formed between the tetrahedral oxoanion species and the amino acids of the protein. There are no (positively) charged residues and also no water molecules in both proteins within 8 Å of the oxoanion, and this determines the surface potential of the binding pocket to be neutral with an apolar character. Hu and co-authors have stated that it is energetically more favorable for an apolar pocket to bind a larger oxoanion, which could explain the selectivity for molybdate/tungstate compared to the smaller oxoanion sulfate [34]. This argument refers to simple electrostatic considerations based on Born charging energies. The authors add, however, that a more detailed analysis of the electrostatics of the anion binding, taking into account the locations and orientations of dipoles in the structure, is required to support this qualitative statement [34].

A very recent paper describes a third crystal structure of a ModA, namely, from *Archaeoglobus fulgidus* (Fig. 6C) [36]. The structure of this protein has been solved as part of the structure of the complete ABC transporter ($ModAB_2C_2$). However, in our opinion, the *A. fulgidus* protein should be referred to as WtpA homologue rather than ModA homologue. First of all, the sequence of the *A. fulgidus* periplasmic binding protein is significantly more similar to *P. furiosus* WtpA (44% identity, 64% similarity) than to *E. coli* ModA (27% identity, 45% similarity). Secondly, the crystal structure of the *A. fulgidus* protein shows a completely different coordination of the metal ion, namely an octahedral coordination, whereas the ModA of *E. coli* and *A. vinelandii* both show a tetrahedral coordination. The metal in the *A. fulgidus* protein is bound to the four oxygens of its oxoanionic structure and to one oxygen each of the carboxylate side groups of two amino acids Glu218 and Asp153, which are fully conserved only in WtpA homologues. The four oxygen atoms of the oxoanion are coordinated by eight hydrogen bonds, in a similar manner as in the ModA proteins (Fig. 6C). Finally, it is interesting to note that the genome of *A. fulgidus* contains several genes encoding putative tungsten-containing aldehyde oxidoreductases, which also suggests that tungstate is the physiological ligand of this periplasmic binding protein. However, no affinity studies have been reported yet. In addition, the resolution of the crystal structures (1.5–1.6 Å) may conceal any differences between the tungstate and molybdate binding sites [36].

Currently, no crystal structure is available of a periplasmic binding protein in complex with its ligand, for which a difference in affinity between molybdate and tungstate has been measured *in vitro*. In the coming years we expect to see crystal structures of the tungstate (and also vanadate) selective periplasmic binding proteins in complex with their ligands, and these might provide a basis to explain the molecular mechanism of the molybdenum and tungsten selectivity.

The presence of genes in the genome encoding one of these transporter systems: ModABC, TupABC or WtpABC, can explain the tungstate and molybdate uptake pathway in most bac-

teria and archaea. A recent paper reports the identification of the first eukaryal, high-affinity molybdate transporter in *Chlamydomonas reinhardtii* [37].

6. Cellular storage of tungstate

A way to insure a constant intracellular level of small essential compounds, such as metal ions, in cells is the operation of a (regulated) storage system. Proteins can act as storage units that are able to release the metal at specific locations in the cell when intracellular or local concentrations decrease. Proteins can also facilitate metal solubility or protect the cells against toxic levels of certain compounds by forming mineral cores, like in the case of ferritin. Free ferrous iron is potentially toxic to cells (notably in the presence of molecular oxygen) and therefore it is immobilized inside the ferritin protein as a mineral core of ferric iron combined together with phosphate and/or hydroxide ions. The resulting core is similar to the mineral ferrihydrite, and theoretically one protein molecule, consisting of 24 monomers, can store up to circa 3000 ferric ions [38].

So far, two classes of proteins have been described that play a role in the storage of molybdate and potentially also tungstate. The first class is formed by the so-called 'molbindin' family. These proteins consist of one or two molybdate binding domains (Mop domains) that are able to bind molybdate or tungstate [39]. They are found in bacteria and archaea, but not all organisms that use tungsten and/or molybdenum have copies of these 'Mop domain' encoding genes in their genome. Several molbindins have been crystallized: Mop from *Clostridium pasteurianum* [40], Mop from *Sporomusa ovata* [41] and di-mop (i.e. two mop domains in a single protein) ModG from *A. vinelandii* [42]. The crystal structures indicate that the mono-mop proteins form a trimer of dimers, and the di-mop proteins are trimeric, which in each case results in a native hexameric domain conformation. These hexameric molbindins can bind eight oxoanions per molecule at two different sites. Six oxoanions can bind to six high affinity sites, which are located between the faces of the dimers, and two can bind to lower affinity sites located along the trimeric symmetry axis in the middle of the three domains (Fig. 7). All three described Mop proteins were also crystallized with tungstate bound but no differences were observed in the coordination of the ligands compared to the protein complexed with molybdate [40–42]. In all cases the oxoanion was found to be coordinated by hydrogen bonds formed with the main chain and side chains of the protein. As in the ModA structures, there are no positively charged amino acids present within 8 Å of the binding sites.

Unfortunately, no quantitative *in vitro* binding experiments have been described for these three proteins that might have indicated a difference in affinity for tungstate or molybdate. The proteins were crystallized with both oxoanions and it was concluded that the higher binding affinity for molybdate and tungstate compared to an observed lower affinity for sulfate, was likely determined by the size of the binding pocket. Based on the apparently identical way in which the oxoanion is bound one would expect the Mop-domain proteins to bind tungstate and molybdate equally well.

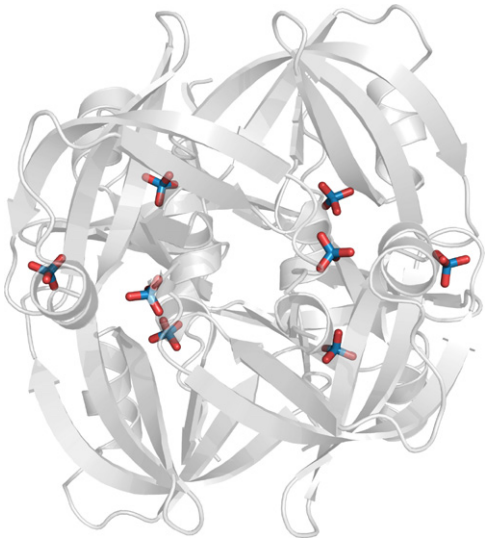


Fig. 7. Crystal structure of the *S. ovata* molbindin protein, binding eight molecules of tungstate [41].

However, so far only one molbindin protein has been described that was isolated from an organism for which we know that it actually uses tungsten: the Mop protein from *E. acidaminophilum* [43]. This organism expresses two tungsten-containing enzymes [44,45] and a specific tungsten uptake system [31] and therefore one can expect that this Mop protein serves as a tungstate storage protein *in vivo* with possibly a higher affinity for tungstate over molybdate. Unfortunately binding characteristics were determined only with a gel shift assay [43], which is not well suited for quantitative determination of the dissociation constant [32].

In addition to the molbindin proteins, a second class of oxoanion storage proteins has been characterized that store tungstate and molybdate as a metal–oxygen cluster. The first protein identified in this category was the molybdenum storage (Mo/WSto) protein from *A. vinelandii* [46]. This protein has been isolated more than 20 years ago as a molybdate and tungstate binding protein [47]. Very recently, the structure of the protein loaded with tungstate could be determined [48]. The molybdate-binding counterpart of the protein could not be crystallized so far, because all the molybdate was released during the long-term incubation required for the crystal formation. The protein appeared to be a trimer of (αβ)-dimers resulting in a hexameric native structure. The α- and β-subunits have a molecular weight of 29 and 28 kDa, respectively. The protein can store up to circa 100 molybdenum or tungsten atoms per hexamer [46] as polynuclear tungsten- or molybdenum oxide aggregates. These polyoxotungstates and polyoxomolybdates are separated from each other within the protein complex in different cavity pockets [48]. Interestingly, the formation of the cluster differs for each type of pocket [48]. The α-subunit also harbors an ATP binding site, however the binding mode of ATP and its role in the formation or breakdown of the cluster is still a subject of study [48]. It should be noticed that so far no tungsten enzyme has been purified from *A. vinelandii* so it is possible that the binding of tungstate has no physiological function.

A sequence comparison by BLAST [49] studies against the non-redundant database reveals that the α- and β-subunit of the Mo/WSto protein are related to a family of uridine monophosphate kinases (UMKs) [46]. For most Mo/WSto homologues it is not clear whether the gene product encodes a Mo/WSto or a UMK. However, there are some other nitrogen-fixing bacteria for which the gene sequence homology with Mo/WSto is so high that the gene most likely encodes a Mo/WSto protein. Perhaps this is an indication that the Mo/WSto protein is somehow linked to the biosynthesis of the FeMo-cofactor of nitrogenases.

In addition to these two types of well characterized tungstate and molybdate storage proteins, a recent paper reports on a tungstate-binding protein isolated from *Acidithiobacillus ferrooxidans* strain AP19-3 [50]. This protein has yet to be characterized in detail: the amino acid sequence is not known, the form in which the tungsten is bound is unclear, and the protein has only been shown to bind tungstate after incubation in 1.0 mM sodium tungstate at pH 3.

A very different type of storage protein related to tungsten and molybdenum metabolism is the molybdenum cofactor carrier protein (MCP). This 16 kDa protein, which forms stable tetramers in solution, has been purified from the green alga *C. reinhardtii* [51,52]. In this protein the metal is stored after it has been incorporated in the pterin cofactor, i.e. as a molybdenum cofactor (Moco) or a tungsten cofactor (Wco) (Fig. 2A). Synthesis of this cofactor is discussed in Section 8 of this review, below. Recently, the crystal structure of the apo-MCP was solved [53]. Based on the conserved surface residues, charge distribution, shape, *in silico* docking studies, structural comparisons, and identification of an anion binding site, a prominent surface depression was proposed as a Moco-binding site [53]. Recombinant MCP containing tungsten or molybdenum, dependent on the medium composition, has been isolated from an *E. coli* host. In *C. reinhardtii*, only molybdenum-dependent enzymes have been described so far, suggesting that the binding of Wco to MCP has no physiological function.

However, BLAST studies against genome databases reveal that there are homologues of MCP in genomes that also contain homologues of tungsten-containing enzymes, indicating that MCP could also have a physiological role in storing Wco *in vivo*. The genome of *A. fulgidus* for example contains a gene that shares 56% similarity and 40% identity with the *C. reinhardtii* MCP. The *A. fulgidus* genome also contains genes encoding several putative tungsten-containing aldehyde oxidoreductases and the gene of the recently crystallized putative tungstate and molybdate selective transporter (see Section 5).

In summary, two distinct molybdate and tungstate storage systems have thus far been identified: the molbindins and the Mo/WSto proteins. However they are only expressed in a small fraction of the organisms known to use one or both of the two metals. For well established molybdenum, respectively, tungsten users, like *E. coli* and *P. furiosus*, it is still not clear whether, and if so how, the metals are stored in the cell. Possibly, these cells have other proteins that function as a storage system. One can hypothesize that other proteins like ferritin might also play a role in tungstate or molybdate storage. There is experimental evidence that ferritin can incorporate vanadate, molybdate [54] and tungstate (M.N. Hasan, personal communication) as phosphate analogues in the iron mineral core *in vitro*. However, any physiological relevance of this property has not been supported by *in vivo* data.

7. Cellular regulation of tungstate metabolism

In the framework of this review, cellular regulation concerns all the functions that cells carry out to maintain metal homeostasis. In the case of tungstate metabolism this includes regulation of the tungstate uptake, the storage, and the expression of both the cofactor synthesis proteins and the enzymes that contain the tungsten cofactor. Again, more data are available on the regulatory role of molybdate, especially in *E. coli*, and therefore we will start to review those data and extrapolate these for tungstate. We will only consider metal-induced regulations. Many molybdenum- and tungsten-containing enzymes are under metabolic control (product feedback); however these regulatory networks are not considered here.

It has been known for a long time that the internal cellular concentration of molybdenum in *E. coli* is maintained within a narrow range (0–10 μM) even when its external concentration varies widely [55,56]. Grunden et al. [57] showed that this was mainly the result of a regulation of the transcription of the *modABC* operon which encodes the ABC transporter. A protein named ModE was found to be responsible for the molybdate-dependent repression of the *modABC* operon, because in *modE* deficient mutant strains this regulation was absent [57]. The dimeric protein ModE binds tungstate or molybdate with the same affinity ($K_D = 0.8$ μM) [58], and the crystal structure of both complexes has been solved as well as the structure of the apo-protein [59]. The oxoanion ligands bind between the subunits at the dimerization interface, and an oxoanion-size selectivity is determined primarily by the size of the ligand-binding pocket as was previously concluded for the ModA and molbindin proteins [34,35,40–42]. Comparisons with the structure of the apo-protein have revealed a molybdate/tungstate-dependent conformational rearrangement [59] which most likely creates a surface that has a high affinity for the DNA in the promoter region of the *modABC* operon [58]. These data indicate that the regulatory protein ModE does not seem to discriminate between tungstate and molybdate.

Only some organisms carry a *modE* homologous gene in their genome, which makes the ModE-dependent regulation not universal among molybdenum and tungsten using organisms. BLAST studies against genome databases reveal that, for example, archaeal genomes do not contain *modE* homologues. Perhaps, these organisms have other regulatory systems for the uptake of molybdate and tungstate which still have to be identified.

Besides the regulation of the transcription of the genes encoding the ABC transporter, *E. coli* ModE also regulates the transcription of several other genes, namely: *dmsA*, which encodes the molybdenum-containing subunit of the enzyme DMSO reductase [60], *hyc*, which encodes a hydrogenase [61],

narG, the molybdenum-containing subunit of nitrate reductase [61], and the *moa* operon [62], which encodes proteins involved in the first step of the molybdenum and tungsten cofactor synthesis (Section 8).

The positive influence of ModE in the presence of molybdate on the transcription of the *moa* operon was detected in molybdenum cofactor deficient strains only (these cells have a defect in the cofactor synthesis and therefore no active cofactor is synthesized). Based on this observation it has been concluded that cofactor-dependent repression of the transcription of the *moa* genes (in the presence of molybdate) is dominant over the ModE-molybdate activation [63]. *In vivo* data showed a similar positive effect of tungstate on the transcription of the *moa* operon. Surprisingly, this effect was independent of the presence of ModE or of the ability of cells to make the cofactor. Therefore, it has been suggested that the presence of tungsten results in the formation of a non-functional tungsten-containing form of the cofactor, which is not able to cause the cofactor-dependent repression observed in the presence of molybdate [63].

Of specific interest is a group of organisms that express isoenzymes with molybdenum versus tungsten in the cofactor. An example is *M. thermoautotrophicum* which expresses a tungsten-containing formylmethanofuran dehydrogenase (FMDH) (operon *fwdHFGDACB*) and a molybdenum-containing FMDH (operon *fmdECB*) [64]. The subunits FwdB and FmdB were identified as harboring the redox active tungsten, respectively, molybdenum site. The largest subunit (FwdA) probably catalyzes the formation of *N*-carboxymethanofuran from CO_2 and methanofuran [65]. Interestingly, the operon encoding the molybdenum-containing enzyme is lacking the gene encoding subunit A. Analysis revealed that subunit A in the Mo-containing FMDH has the same molecular mass and the same N-terminal amino acid sequence as subunit A of the W-containing enzyme. Therefore it was concluded that they are identical and encoded by the gene *fwdA* in the *fwd* operon. It was shown that the operon for the W-containing enzyme is constitutively transcribed, whereas the transcription of the Mo-operon appears to be induced by the presence of molybdate, independent of the presence or absence of tungstate [66]. However, later experiments showed a different effect of the tungsten concentration on the expression of the Mo-containing FMDH. These experiments concerned also a small DNA binding protein (Tfx) that was identified to specifically bind to a DNA sequence downstream of the *fmd* operon. This protein was proposed to be a transcriptional regulator of the gene-encoding part of the Mo-containing FMDH [67]. Northern blot analysis (detection of mRNA) revealed that the transcription of this regulator was repressed during growth in the presence of tungstate rather than induced by molybdate. This result is not consistent with the previous findings which indicated that the Mo-operon is transcribed in the presence of molybdate, independent of the tungstate concentration [67].

Altogether the suggestion perspires that several factors may play a role in the molybdate- and tungstate-dependent regulation and homeostasis, and many questions remain to be addressed regarding their mechanisms.

8. Biosynthesis of the cofactor

Tungsten and molybdenum associated with enzymes, occur in a similar cofactor, which consists of one or two tricyclic pterin moieties usually referred to as 'molybdopterin' (MPT) (Figs. 2 and 8) [68]. The nomenclature is confusing because MPT seems to refer only to molybdenum, and, therefore the alternative name metal-binding pterin (MPT) was introduced [5]. For both types of cofactors (Moco and Wco) the metal is coordinated by the two dithiolene sulfurs of the pterin. In the case of tungsten there are always two pterin moieties resulting in a tungsten center coordinated by four dithiolene sulfurs in the tungsto-bispterin cofactor (Figs. 2 and 8).

The pathway of Moco biosynthesis has been extensively studied in prokaryotes (*E. coli*) as well as eukaryotes (*Arabidopsis thaliana*, *Homo sapiens*) and appears to be highly conserved (Fig. 8) [69]. The pathway of Wco biosynthesis is thought to be similar to the pathway of Moco biosynthesis, at least up to the step of the metal insertion. The main ground for this assumption is that homologues of almost all genes that have an assigned function in the Moco biosynthetic pathway are also present in the genomes of organisms that use tungsten.

The first model of Moco synthesis was based on *E. coli* data [68]. Four operons have been identified to be involved in the Moco biosynthesis of this organism: *moaABCDE*, *mobAB*, *moeAB* and *mogA*. These operons encode ten proteins of which eight have an assigned function in the biosynthesis of Moco (Fig. 8). The names of the proteins follow the rules of the standard nomenclature (Fig. 5) and have no particular meaning except that Mo refers to molybdenum.

In *E. coli* the biosynthesis of Moco begins with the conversion of guanosine triphosphate (GTP) to a pterin intermediate called precursor Z or cyclic pyranopterin monophosphate (cPMP), catalyzed by two proteins: MoaA and MoaC [70]. The expression of these two proteins is regulated by ModE, described in Section 7 [62]. Subsequently, metallopterin (MPT) is synthesized from cPMP by MPT synthase, which consists of the MoaD and MoaE proteins [4]. The next step involves the ligation of the metal atom to the dithiolene sulfurs of one or two MPT moieties. The proteins MoeA and MogA play a role in this step, and very recently also MoaB was found to be involved in this stage of the cofactor synthesis [71]. The proteins MogA and MoaB catalyze the activation of MPT by adenylylation with Mg-ATP [72,73]. The trimeric MogA proteins are commonly found in bacteria and eukaryotes whereas the hexameric MoaB proteins are mostly found in archaea and in some bacteria [71]. Subsequently, MoeA is thought to bind the adenylylated MPT (MPT-AMP), and in the presence of molybdate and/or tungstate the MPT-AMP complex is hydrolyzed, molybdenum or tungsten is incorporated through binding to the dithioleno sulfurs, and AMP is released. This proposed role for MoeA in *E. coli* is based on the activity of its plant homologue: Cnx1E (Cnx: Cofactor for nitrate reductase and xanthine dehydrogenase) from *A. thaliana* [74], which catalyzes the hydrolysis of MPT-AMP in the presence of molybdate. When molybdate was replaced with tungstate, the hydrolysis catalyzed by Cnx1E was much less efficient [74], and this result suggests that Cnx1E, and homologues,

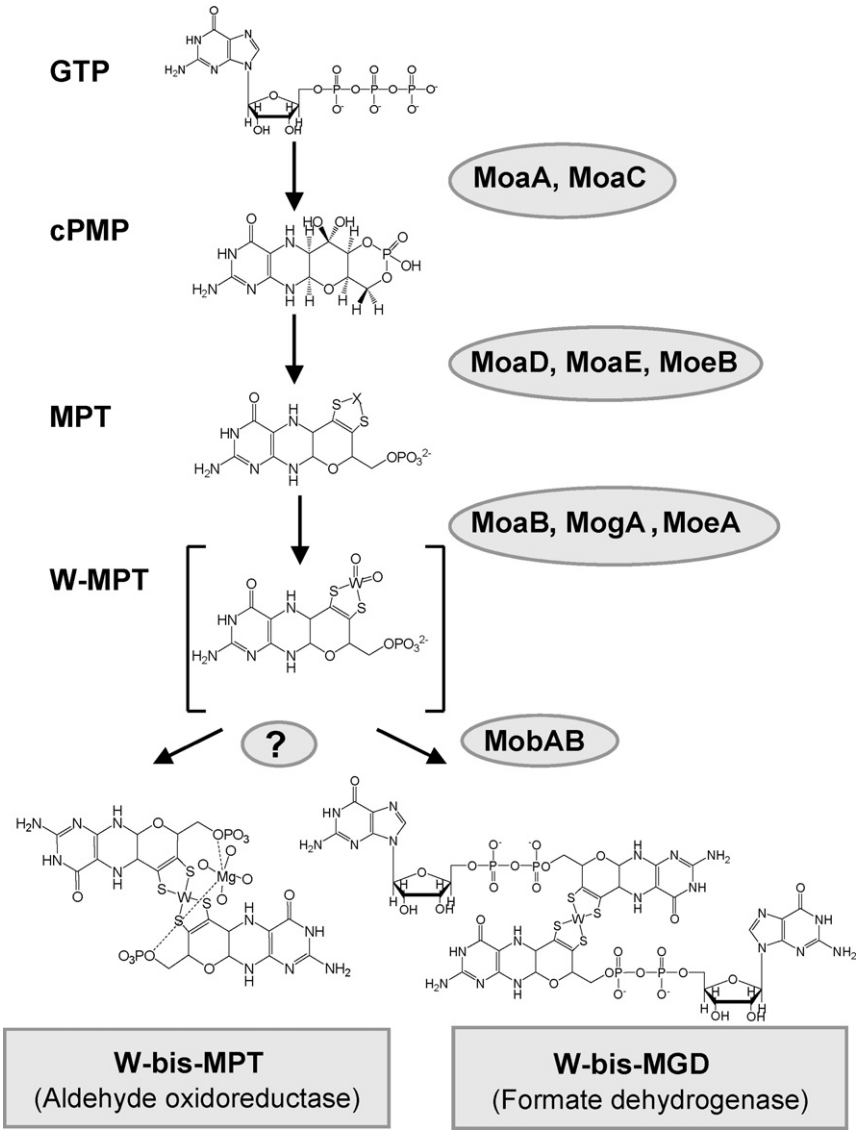


Fig. 8. Schematic overview of the tungsten cofactor biosynthesis; modified figure from Ref. [4]. Abbreviations of the intermediates are written in bold on the left side of their structures, and the enzymes that catalyze the steps are depicted on the right side of the arrows. Note that the bracketed MPT structure is a hypothetical intermediate. The metal coordinating the dithiolene ligands in MPT is indicated by an X as this atom is not known. In the crystal structure of plant Cnx1G this metal was found to be a copper ion [132].

may play a role in selectively incorporating either tungsten or molybdenum in MPT.

Interestingly, many bacterial genomes and all archaeal genomes sequenced so far, contain two different *moeA* genes which share approximately 40% sequence identity. It is tempting to speculate that perhaps one of the MoeA proteins is selective for molybdenum incorporation and the other for tungsten incorporation. The presence of these two MoeA's can then explain how organisms are able to regulate and express two enzymes, one with Moco and the other with Wco in the active center. For example, *P. aerophilum* expresses a W-containing AOR and a Mo-containing nitrate reductase [19,75]. However, *in vivo* and *in vitro* experiments are required to corroborate this hypothesis of selectivity by two different insertion catalysts.

As a final maturation step (only in bacteria and archaea), guanosine monophosphate (GMP) or cytosine monophosphate (CMP) can be attached (phosphoester condensation from GTP and CTP) to the MPT, forming a so-called molybdopterin guanine/cytosine dinucleotide (MGD/MCD) cofactor. This reaction is catalyzed by MobA and MobB [76]. For some enzymes another maturation step is required: the coupling of two Wco's or two molybdenum- or tungsten-containing MGDs leading to the formation of the bis-pterin cofactor. The formation mechanism of this so-called bis-pterin cofactor still needs to be established.

9. Tungsten enzymes

The first indication of a biological relevance of tungsten was obtained more than 30 years ago: Andreessen and Ljungdahl showed that the growth of different *Clostridia* was positively influenced by the addition of tungstate to the growth media [77]. It took another decade before formate dehydrogenase

	family-1	family-2	family-3	family-4
Mo-enzymes	Sulfite oxidase	Xanthine oxidase	DMSO reductase	
W-enzymes			Formate dehydrogenase	Aldehyde oxidoreductase

Fig. 9. Schematic overview of the four families of molybdenum- and tungsten-containing enzymes with the names of example enzymes.

could be purified from one of these acetogenic *Clostridia* as a naturally occurring tungsten-containing enzyme [78]. In the years to follow, many tungstoenzymes have been purified and characterized [19,79]. The majority was isolated from (hyper)thermophilic anaerobic archaea, and for some time it was commonly believed that tungsten enzymes mainly occur in these extremophilic organisms. A relatively high concentration of tungstate compared to molybdate in certain extreme environments like hydrothermal vents and hot-spring waters would support this hypothesis. Furthermore, most isolated tungsten-containing enzymes were very oxygen sensitive, and therefore it was also believed that they could only occur in anaerobes.

However, in the following years tungsten-containing enzymes were also purified from (mesophilic) bacteria [44,80–83] and homologous genes are even found in the genomes of aerophilic organisms, suggesting that tungstoenzymes are present in a much wider range of microorganisms. There have been no reports yet on any tungsten enzyme in eukaryotes.

Tungsten and molybdenum enzymes have been classified in different families according to: (1) sequence homology, (2) composition of the cofactor, i.e. with or without nucleotide attachment, (3) the coordination of the metal by one or two pterins, and (4) axial ligands like oxygen, sulfide or cysteine (Fig. 9).

The tungsten-containing enzymes can be divided into two families: the aldehyde oxidoreductases (AORs) that contain a non-modified tungsto-bispterin cofactor, and the formate dehydrogenases which have a guanine monophosphate attached to each pterin moiety (Fig. 8).

9.1. Aldehyde oxidoreductases

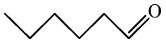
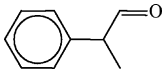
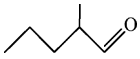
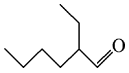
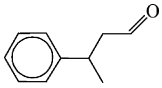
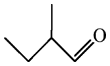
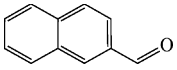
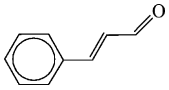
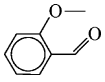
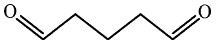
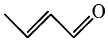
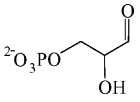
The enzymes in the AOR family catalyze the oxidation of aldehydes to carboxylic acids, and they use ferredoxin as redox partner protein. They are generally oxygen sensitive and typically have broad substrate specificities with partial overlap between enzymes from the same species. They all consist of mono-, di-, or tetramers of the catalytic subunit that contains the bis-pterin cofactor and an electron transferring [4Fe–4S] cluster. BLAST studies reveal that the genome of many organisms encodes multiple, different AOR enzymes [32]. The tungstoenzymes of *P. furiosus* have been intensively studied over the past two decades, and its complete AOR family has been purified and characterized (in some detail): aldehyde ferredoxin oxidoreductase (AOR) [79], glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) [84], formaldehyde ferredoxin oxidoreductase (FOR) [85], tungsten-containing oxidoreductase number four (WOR4) [86], and tungsten-containing oxidoreductase number five (WOR5) [87]. AOR and WOR5 have a broad substrate specificity; AOR appears to be most active on aldehydes derived from amino acids [79] whereas WOR5 has a high affinity for several substituted and non-substituted aliphatic and aromatic aldehydes with variable chain lengths (Table 1) [87]. FOR has the highest activity on small C1–C3 aldehydes and semi- and di-aldehydes [85]. In contrast to these broad substrate specificities, GAPOR is only known to convert the substrate glyceraldehyde-3-phosphate (GAP). It functions in glycolysis where it converts GAP to 3-phosphoglycerate, and it replaces the couple glyceraldehyde-3 phosphate dehydrogenase plus phosphoglycerate kinase in an unusual Embden–Meyerhof pathway of glycolysis [84]. The fourth tungsten-containing enzyme, WOR4, could only be purified from *P. furiosus* grown in the presence of S^0 [86]. No activity has been identified yet for this enzyme.

The physiological function of this AOR family of enzymes is still not clear with the exception of GAPOR [84]. However, micro-array analysis in which levels of mRNA are determined under different growth conditions indicated possible physiological functions (Table 2). In a first reported experiment *P. furiosus* was grown on peptides or on maltose [88]. Growth on peptides increased the mRNA levels of FOR and WOR4 significantly, whereas GAPOR mRNA levels increased, as expected, during growth on maltose, which is converted by glycolysis [88]. Furthermore, the cultivation temperature of the cells was dropped from their near-optimal growth temperature of 95 to 72 °C in order to elicit three different responses: an early shock response (1–2 h at 72 °C), a late shock response (5 h at 72 °C), and an adapted response (occurring after many generations at 72 °C) [89]. WOR5 mRNA levels were significantly upregulated in the case of the early and late shock, whereas AOR mRNA levels decreased. For the adapted cells, in particular, WOR4 appeared to be upregulated [89]. The results of these micro-array experiments are perhaps not conclusive, but they do indicate that FOR and WOR4 might play a role in peptide fermentation, and that WOR4 and WOR5 are possibly involved in some kind of stress response, e.g. following a cold shock.

9.2. Formate dehydrogenases

The enzymes in the FDH family catalyze the oxidation of formate to carbon dioxide. They all have the same tungsto-bispterin-MGD cofactor in common and they often contain additional [4Fe–4S] or [2Fe–2S] clusters. For most of these enzymes the physiological redox partner is still unknown. The crystal structures of four FDH proteins have been reported: one tungsten-containing enzyme from *Desulfovibrio gigas* [90],

Table 1
An example of broad substrate specificity of W-containing AORs

Substrate	Structure	V_{max} (U/mg)	K_M (mM)	K_{cat}/K_M (s^{-1} M^{-1})
Hexanal		15.6 ± 1.8	0.18 ± 0.02	80,000
Hydratropaldehyde		9.3 ± 0.7	0.12 ± 0.04	71,500
2-Methylvaleraldehyde		12.7 ± 1.3	0.27 ± 0.03	43,400
2-Ethylhexanal		8.3 ± 1.5	0.17 ± 0.02	45,100
3-Phenylbutyraldehyde		8.0 ± 0.6	0.42 ± 0.12	17,600
2-Methylbutyraldehyde		7.7 ± 0.4	0.43 ± 0.09	16,500
Isobutyraldehyde		11.8 ± 0.9	0.79 ± 0.03	13,800
2-Naphthaldehyde(β)		7.7 ± 0.8	1.3 ± 0.1	5,500
Cinnamaldehyde		7.4 ± 1.6	1.6 ± 0.1	4,600
2-Methoxybenzaldehyde		15.1 ± 0.6	4.8 ± 0.6	2,900
Acetaldehyde		0.34 ± 0.05	1.5 ± 0.2	210
Formaldehyde		8.5 ± 1.0	45 ± 12	170
Glutaraldehyde		1.4 ± 0.1	9.4 ± 0.2	140
Crotonaldehyde		1.1 ± 0.1	46 ± 6	22
Glyceraldehyde-3-phosphate		0.0	–	0

Oxidation of various aldehydes at 60 °C by *P. furiosus* WOR5 with methylviologen as electron acceptor [87].

two molybdenum-containing FDHs from *E. coli* [91,92], and recently, also a 2.6 Å resolution structure for the NADH-dependent FDH from *Pseudomonas* sp. 101 was deposited (2GO1). The *D. gigas* W-containing FDH is a heterodimeric enzyme and contains a [4Fe–4S] cluster and a tungsto-bispterin-MGD cofactor in the large subunit [90]. Functional implications of the W-FDH structure will be discussed in Section 11.2.

Table 2
The effect of different growth conditions on the mRNA levels of tungsten-containing aldehyde oxidoreductases in *P. furiosus* [88,89]

	C-source		Cold shock 72 °C	
	Peptides	Maltose	1–5 h	Adapted
AOR (PF0346)	–[a]	–	↓[b]	–
FOR (PF1203)	↑[c]	–	–	–
GAPOR (PF0464)	–	↑	–	–
WOR4 (PF1961)	↑	–	–	↑
WOR5 (PF1480)	–	–	↑	↑

[a] No significant difference.
[b] ↓Significant downregulation.
[c] ↑Significant upregulation.

A special case is acetylene hydratase from *Pelobacter acetylenicus*. Mechanistically, this enzyme does not fit into either class of tungsten enzymes because it apparently catalyzes a non-redox reaction: the hydration of acetylene to acetaldehyde [93]. The structure of this enzyme has recently been solved and encompasses a tungsto-bispterin-MGD cofactor and a [4Fe–4S] cluster [94]. The sequence and the overall structure are similar to the enzymes belonging to the FDH family.

9.3. Relationships with molybdenum-containing enzymes

The Moco-containing enzymes can be classified into three separate families based on the coordination of the molybdenum [69,95]. In the sulfite oxidase family (SOs), the molybdenum is covalently bound to a highly conserved cysteine residue. This cysteine is replaced by a non-protein sulfur atom in the family of xanthine oxidoreductases (XOs). The DMSO oxidoreductases (DMSOrs) contain a bis-pterin cofactor modified with a nucleotide attachment, forming molybdo-bispterin-MGD.

Interestingly, no naturally occurring tungsten enzymes have been isolated that belong to the SO or XO family; conversely, also no molybdenum-containing members of the AOR family are known to date. A very recent report describes the purification of active, molybdenum-containing *Methanococcus maripaludis* GAPOR recombinantly expressed in *E. coli* [96]. However, the physiological metal of the wild type enzyme has not been determined. The genome of *M. maripaludis* putatively encodes a molybdenum- and a tungsten-containing formylmethanofuran dehydrogenase and therefore the organism is likely able to process both metals into pterin cofactors. So far, only the families of the DMSOrs (molybdenum) and FDHs (tungsten) share significant sequence similarity and in addition, enzymes of both families contain the bis-MGD cofactor.

9.4. Exchange experiments

Many attempts have been made to substitute molybdenum with tungsten and vice versa in the cofactor of the various enzymes. These experiments show that not all enzymes can be synthesized with either metal. An overview of the attempts is given below.

The first substitution experiments were performed in rats that were fed with tungstate or molybdate and for which subsequently the metal content of the sulfite oxidase and xanthine oxidase was determined. It was found that both metals could be incorporated into the pterin cofactor of the enzymes, but the tungsten-containing enzymes were completely inactive [97]. In some enzymes of the DMSO reductase family, for example in the case of *R. capsulatus* DMSO reductase, expressed in *E. coli* [14], and in *E. coli* trimethylamine *N*-oxide (TMAO) reductase [98], replacing the molybdenum with tungsten afforded active enzyme. However, the activity profiles changed compared to the profile of the molybdenum-containing enzyme, presumably due to the lower reduction potential of the tungsten center [98]. Tungsten-containing DMSO reductase was found to be more active in reducing the DMSO but could not catalyze the reverse reaction, the oxidation of DMS [14]. The tungsten-containing TMAO reductase appeared to have a broader substrate specificity compared to the molybdenum enzyme because it was also able to reduce sulfoxides [98].

Not all enzymes of the DMSO reductase family could be synthesized with either tungsten or molybdenum to form a functional enzyme complex: no active tungsten substituted (prokaryotic) nitrate reductase has been obtained. Attempts have been made with nitrate reductase from *R. capsulatus* and *E. coli*, but both lead only to the formation of inactive apoenzyme [99,100].

These are all examples of naturally occurring molybdenum enzymes that were substituted with tungsten. The other way around appears to be less easy, as there is only one example of a naturally occurring tungsten enzyme, *P. acetylenicus* acetylene hydratase, in which the metal could be successfully substituted with molybdenum, leading to the formation of active enzyme [101]. The molybdenum-containing enzyme exhibits 60% of the activity compared to the wild type tungsten enzyme [101].

Metal exchange experiments were also performed with the tungsten-containing AOR enzymes from *P. furiosus*. Adams and coworkers reported that *P. furiosus* cells selectively used the trace amounts of tungsten present in the media, and that they did not incorporate any molybdenum into the cofactor of these AORs [102]. On the contrary, similar experiments performed in our laboratory, with a 1000-fold excess of molybdate over tungstate in the growth media, resulted in a significant molybdenum incorporation in two AOR enzymes: FOR (5% Mo, 2% W, 93% apo) and WOR5 (23% Mo, 2% W, 75% apo) (our unpublished results). EPR spectra confirmed the incorporation of molybdenum into the pterin cofactor. The aldehyde oxidation activities of these proteins correlated with the tungsten content, indicating that the molybdenum-containing AOR subunits are not catalytically active. Apparently, the AOR enzymes of *P. furiosus* are preferably synthesized with tungsten, despite the ability of the tungsten transporter (WtpABC) to take up molybdate from the medium [32,102].

An interesting category is formed by the isoenzymes (different genes encoding different enzymes, one with tungsten the other with molybdenum in the cofactor, which perform a similar reaction), for example, the above-mentioned formylmethanofuran dehydrogenases in *M. thermoautotrophicum* [64]. Depending on the oxoanion that is available in the growth medium the organism expresses one or both isoenzymes. The metal-dependent regulation of gene expression should be a subject of future research particularly in these organisms.

In summary, the activity (rate and specificity) of the enzyme is very much dependent on the metal present in the cofactor. Tungsten enzymes are generally faster in reducing substrates, however, in some cases they are unable to oxidize compounds, where the molybdenum counterpart can do the oxidation much faster, and, in its turn, might be unable (or less able) to catalyze the reduction. This is a result of the lower reduction potential of the W^{IV}/W^{VI} redox couple compared to the reduction potential of the Mo^{IV}/Mo^{VI} couple.

9.5. Selectivity

It is clear that the metal incorporation of W versus Mo in the cofactor of an enzyme does not only depend on the ability of the cells to take up the metal from the medium, as most organisms express either a ModA, TupA or WtpA homologue with high affinity for both oxoanions. Furthermore, selective incorporation is also not likely to be dependent on the ability of cells to insert only one of the two metals into the MPT cofactor, as we know that *E. coli* can make both Wco and Moco (e.g. TMAOr and recombinant *R. capsulatus* DMSOr [99,100]) [14] but cannot incorporate a Wco in all enzymes (e.g. nitrate reductase [103]). There is also no clear relationship between the ability to incorporate the metals in the cofactor of an enzyme and the specific kind of cofactor present in the wild type enzyme. For example, the bis-MGD in TMAO reductase can contain molybdenum or tungsten [99,100,103], whereas the bis-MGD cofactor in prokaryotic nitrate reductase exclusively contains molybdenum [99,100,103]. Perhaps the incorporation of the metal in the cofactor is mainly dependent on the structure of the apo-enzyme, possibly in combination with the occurrence of chaperone proteins that are specific for each organism and for each enzyme. So far, two chaperones have been characterized in *E. coli* that play a role in Moco incorporation in apo-molybdoenzymes: NarJ for the maturation of a nitrate reductase [104,105] and TorD for the maturation of TMAO reductase [106,107]. Recently, protein interaction assays revealed that NarJ and NarG do no longer interact with enzymes from the Moco biosynthesis pathway, MogA, MoeA and MobA, when *E. coli* strains are grown on tungstate instead of molybdate [105]. This is consistent with the above-mentioned results that attempts to synthesize tungsten-containing nitrate reductase in *E. coli* afforded only the isolation of apoenzyme. The enigmatic functioning of chaperones in the metal-insertion process calls for increased research efforts in the coming years.

10. Tungsten model chemistry

Synthetic model chemistry has helped biochemists to understand spectroscopic, structural, and mechanistic data of the molybdenum and tungsten enzymes. In general, the resolution of protein crystal structures is not high enough to determine the metal coordination by the cofactor and additional ligands in great detail. Therefore, synthetic models can aid to define accurate bond lengths and bond angles of the metals in different coordination geometries.

Due to the chemical similarity of Mo and W, nearly all W compounds have Mo counterparts. Recently the synthetic cofactor analogues of Mo and W enzymes have been thoroughly reviewed by Enemark et al. [108]. Research efforts into the total synthesis of the molybdopterin cofactor have been reviewed in Refs. [109,110].

A Moco degradation product, urothione, and a Moco derivative, FormA, have been synthesized *de-novo* (Fig. 10) [111,112]. To date no one has been able to synthesize Moco. Recently, engineered *E. coli* strains have been developed that accumulate cyclic pyranopterin monophosphate (cPMP), a precursor of Moco. The compound cPMP can be applied as therapeutic for patients with genetic Moco biosynthesis deficiency [69,113]. Moco deficiency is a rare genetic disease causing severe physical and mental retardation and death in early childhood [114], giving an added significance to research efforts into synthetic or enzymatic approaches to produce Moco and cPMP.

One of the problems with synthesizing mononuclear oxomolybdenum and tungsten complexes is the usually irreversible μ-oxo "dimerization" reaction by which $Mo^{IV}O$ and $Mo^{VI}O_2$ react to form $Mo_2{}^{V}O_3$. Therefore, ligands have been used that sterically prevent this reaction. Since all tungsten enzymes contain either bis-MPT or bis-MGD cofactors, their synthetic models are much less prone towards dimerization than synthetic models of mono-MPT cofactors. W-dithiolene compounds with different functional groups have been synthesized (Fig. 11)

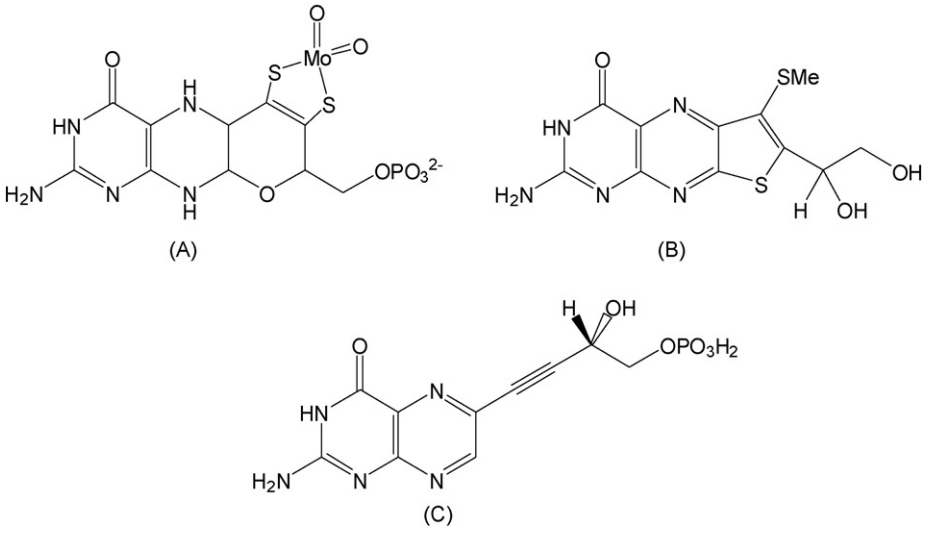


Fig. 10. The molybdenum cofactor and derivatives: molybdenum cofactor (Moco) (A), urothione (B), FormA (C).

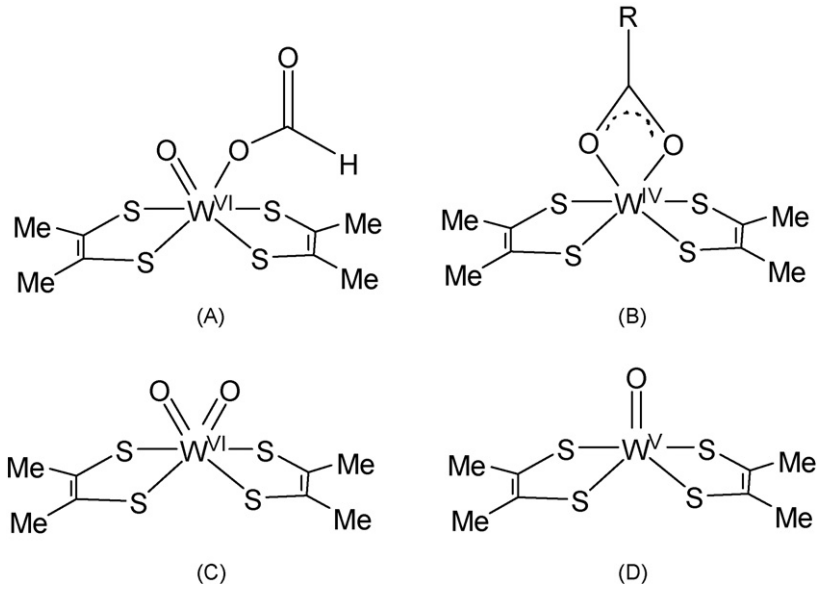


Fig. 11. Examples of biologically relevant synthetic models: $[W^{VI}O(O_2CH)(S_2C_2Me_2)_2]^{1-}$ (A), $[W^{IV}O(O_2CR)(S_2C_2Me_2)_2]^{1-}$ (B), $[W^{VI}O_2(S_2C_2Me_2)_2]^{2-}$ (C), $[W^{V}O(S_2C_2Me_2)_2]^{1-}$ (D) [108].

[115]. For all W-complexes structurally similar molybdenum counterparts have also been synthesized.

10.1. Redox chemistry

The redox chemistry of tungsten model compounds has been extensively studied. Several factors that affect the tungsten reduction potentials have been identified and characterized. In general, the reduction potentials of tungsten complexes are lower than those of analogous molybdenum coordination complexes. This effect is due to larger relativistic effects in the heavy element W which cause increased shielding of the nucleus by the innermost orbitals and thus expansion and destabilization of the 5d orbitals making them less stable than the 4d-orbitals of Mo. As a consequence, bond ionicity is enhanced and higher oxidation states are stabilized in the case of W [116]. The difference in reduction potential decreases as the bond covalency increases.

The effects of ligands on reduction potential and on electron transfer kinetics of Mo coordination complexes have been measured for compounds with different numbers of sulfur and oxygen ligands [117]. In general, replacing oxygen with sulfur increases the reduction potential of the $Mo^{V/IV}$ couple and increases the electron transfer rate. Furthermore, an aromatic framework of the ligands further increases the reduction potential, as compared to aliphatic frameworks. These observations are in line with the postulated function of the molybdopterin cofactor in modulating the reduction potentials of the metal center. Furthermore, the observations appear to be consistent with the fact that tungsten-containing enzymes always have a bis-MPT cofactor, with four dithiolene sulfurs. A mono-MPT coordination of tungsten would simply give the metal center too low reduction potentials for biologically relevant functions.

The coordination geometry also influences the reduction potentials, as has been measured for oxy Mo centers. In case of a mixed sulfur/oxo-coordination, the *cis*-oxo complex exhibits a 200 mV lower reduction potential that its *trans* counterpart [118].

Schulke has reported on the effects of temperature on the reduction potential of tungsten and molybdenum compounds [119]. Tungsten compounds structurally similar to molybdenum counterparts were found to exhibit a different temperature dependence of the reduction potentials, leading to an inverse of the Mo/W reduction potential difference at high temperature (i.e. Mo lower that W) [119]. The author, however, only observed this behavior for $MO(fdt)_2$ (fdt, flavanyldithiolene), and not for other W/Mo bis-dithiolene compounds. The reduction potential difference between $MoO(fdt)_2$ and $WO(fdt)_2$ was only 30 mV at ambient temperature, while the error in the measurements appears to be at least 50 mV. We would venture that the difference in temperature dependence that was found for Mo versus W, is probably not significant. The fact that $MoO(fdt)_2$ and $WO(fdt)_2$ have very close reduction potentials for the M^{IV}/M^{V} couple is interesting in its own right. It suggests that a conformational difference in the ligands coordinating the metals cancels out the normally found reduction potential difference of at least 200 mV. Unfortunately, the crystal structures of the $MoO(fdt)_2$ and $WO(fdt)_2$ were not obtained.

Solvent effects on the reduction potentials of oxomolybdenum complexes with dendritic thiolate ligands are determined by the dielectric constant of the solvent and the donor number [120]. This qualitatively explains why a different local protein environment can lead to dramatically different reduction potentials of biological molybdenum and tungsten centers.

Related to the redox potential is the electron transfer kinetics. Biological Mo and W centers have to transfer electrons to or from other redox centers, such as iron–sulfur clusters. Apparently, the electron transfer kinetics is faster for coordination compounds with sulfur ligands, than for the ones with more oxo-ligands [117]. Furthermore, the metal-dithiolene fold-angle has been found to be important for electron transfer. The fold-angle of

the dithiolene metallacycle along the S–S vector varies with the number of d-electrons of the metal. In general, the fold-angles are large for d^0 and small for d^2 configurations. Large fold-angles are a consequence of the stabilizing interaction between the metal in-plane and the sulfur-π orbitals. This effect is important for electron transfer reactions for the regeneration of the Mo/W active site during catalysis. Additionally, encapsulation of the metal by bulky ligands, mimicking the buried metal center inside a protein, has been found to decrease the electron transfer kinetics of the metal center [120].

10.2. Mechanism of oxo transfer

Dithiolenes are well known to be non-innocent ligands, i.e. able to do undergo redox reactions themselves. An important question that has not been answered is to what extent the pterin dithiolenes participate in the redox reactions catalyzed by W (and Mo) enzymes. To date little evidence has been obtained for such involvement of the pterin dithiolenes, except for the observation of pterin-localized radicals in Mo-containing aldehyde dehydrogenases [121] and in W-containing AORs (our unpublished observation). Recently reported crystal structures of the Mo-containing nitrate reductase A (NarGHI) from *E. coli* [122] and ethylbenzene dehydrogenase from *Aromatoleum aromaticum* [123] show that the bis-MGD cofactor can have an open pyran-ring structure for one of the two pterin moieties. The functional significance of these open-ring structures is not known. Only the closed pterin in both enzymes forms a feasible electron transfer conduit from the Mo center to the iron–sulfur clusters. Although the precise functions of the pterin cofactor have not been completely resolved the consensus at present is that the (closed) pterin cofactor serves as a conduit for electron transfer and a modulator of the reduction potentials of the metal, and does not directly participate in the catalytic mechanism [123].

Since synthetic W model compounds have Mo analogues, it has been possible to compare their oxo-transfer capabilities and to look for metal-specific differences. Only a small number of analogue functional model systems has been synthesized compared to the number of structural analogues. Using these functional analogues a kinetic metal effect has been observed: Oxo transfer from substrate to metal ($M^{IV} \rightarrow M^{VI}O$) is faster with tungsten, while from metal to substrate ($M^{VI}O \rightarrow M^{IV}$) it is faster with molybdenum [124]. Thus, enzymes that physiologically should catalyze the reduction of a substrate (DMSO reductase, TMAO reductase) are faster with W than with Mo. Enzymes that preferably oxidize a substrate (like FMDH) are expected to be faster with Mo than W.

A recent DFT study of molybdenum- and tungsten-containing model complexes of Mo-dependent nitrate reductases reported differences in the reaction energies of the different steps during the catalytic mechanism [125]. W-complexes were found to have a lower activation energy for oxygen atom transfer (OAT) and a more negative reaction energy for the nitrate reduction half reaction. These calculations would suggest that nature has chosen an energetically unfavorable metal, since no tungsten has been found in any nitrate reductase. A possible explanation of this paradox can be the ease of reducing Mo^{VI} over W^{VI} during the second half reaction which regenerates the metal center. However no calculations on this second half reaction were presented.

In summary, the metal-based differences between W and Mo that affect kinetics are: the metal–oxygen bond dissociation energies and the reduction potentials. Comparison of the catalytic properties of the W enzymes and their synthetic model compounds helps to define which factors give the enzymes their high turnover rates. In general, enzymes react orders of magnitudes faster than their synthetic model compounds.

11. Reaction mechanisms

Despite the considerable body of knowledge from synthetic model chemistry and structural and sequence information from biochemistry, it is still difficult to resolve the reaction mechanisms of tungstoenzymes. In order to prove a kinetic mechanism, it is essential to determine the reaction intermediates and kinetic parameters during a single turnover. Very recently we have reported the first pre-steady-state kinetic data of a tungstoenzyme, FOR [16]. Most mechanisms presented below still await experimental substantiation, and are based on structural, spectroscopic, and inorganic-chemical knowledge. The structures of four naturally occurring W enzymes have been deposited in the Protein Data Bank: *P. furiosus* AOR (1aor) [25], *P. furiosus* FOR (1b25) [126], *D. gigas* FDH (1h0h) [90], and *P. acetylenicus* AH (2e7z) [94]. In all four cases the exact coordination by oxo- and/or sulfide ligands, other than the dithiolene sulfurs and the selenocysteine (in the case of FDH) is under debate. Detailed structural characterization of the first coordination sphere of the W center in defined oxidation states is still lacking.

11.1. Aldehyde oxidoreductases

The crystal structures of *P. furiosus* AOR [25] and FOR [126] indicate several important conserved amino acid residues in the active site region. Of these Glu308 (FOR numbering) has been proposed to be involved in the activation of a water molecule to attack the carbonyl group of the aldehyde bound to the tungsten center. A hydride of the carbonyl carbon is then thought to be transferred to the oxo-ligand of the tungsten center (Fig. 12). A hydrogen bond from Tyr416 to the carbonyl oxygen atom of the substrate may serve to activate the substrate for nucleophilic attack. Finally, Thr240 may facilitate proton transfer as part of the coupled electron proton transfer at the active site. Future mutagenesis studies may prove or disprove the proposed roles of these amino acid residues. The W(V) intermediate of this reaction should be detectable by EPR. It would be very interesting to measure this intermediate during a single turnover, e.g. by freeze-quench methods.

Recent stopped-flow experiments on the AOR-family member FOR from *P. furiosus* showed that an activation process from the fully oxidized form of the enzyme (W^{VI}; $[4Fe4S]^{2+}$) to the 2-electron reduced form of the enzyme (W^{IV}; $[4Fe–4S]^{2+}$) has to take place before the enzyme reaches a fully active state. This

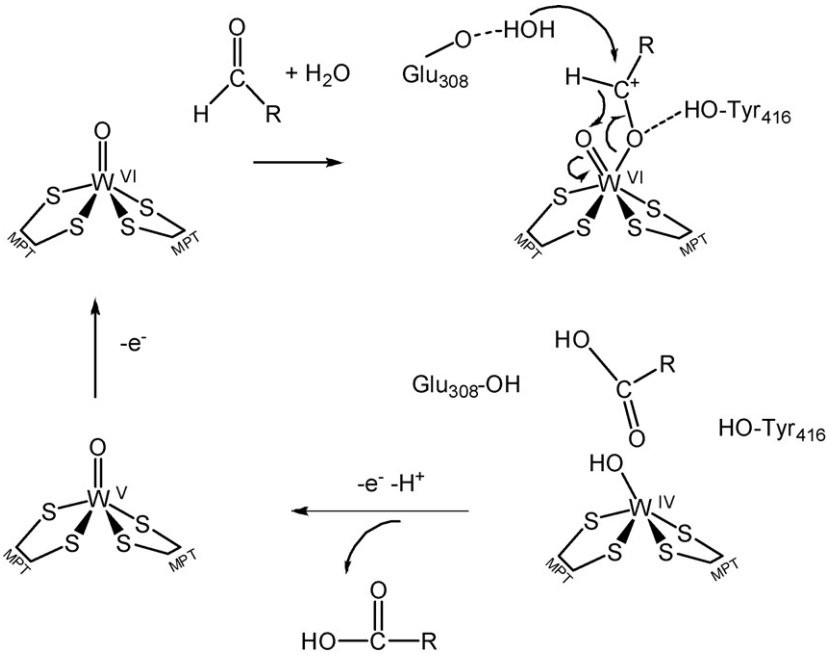


Fig. 12. Proposed mechanism of W-AOR [126]. Glu308 (FOR numbering) activates a water molecule to attack the carbonyl group of the aldehyde bound to the tungsten center. A hydride of the carbonyl carbon is then transferred to the oxo-ligand of the tungsten center. A hydrogen bond from Tyr416 to the carbonyl oxygen atom of the substrate activates the substrate for nucleophilic attack. Finally, Thr240 facilitates proton transfer as part of the coupled electron proton transfer at the active site.

activation can be achieved by reducing the enzyme with the substrate formaldehyde in the absence of an electron acceptor. During subsequent turnovers in the presence of redox partner ferredoxin, the enzyme shuttles between a 1-electron (W^{VI}; $[4Fe–4S]^{1+}$ or W^{V}; $[4Fe–4S]^{2+}$) and a 3-electron reduced state (W^{IV}; $[4Fe–4S]^{1+}$) compared to fully oxidized, resting enzyme [16].

11.2. Formate dehydrogenases

The crystal structure of the tungsten-containing formate dehydrogenase from *D. gigas* has been determined to 1.8 Å resolution [90]. A positively charged substrate channel, a putative proton channel, and a hydrophobic CO_2 channel have been proposed in this structure. Electron transfer can take place from the

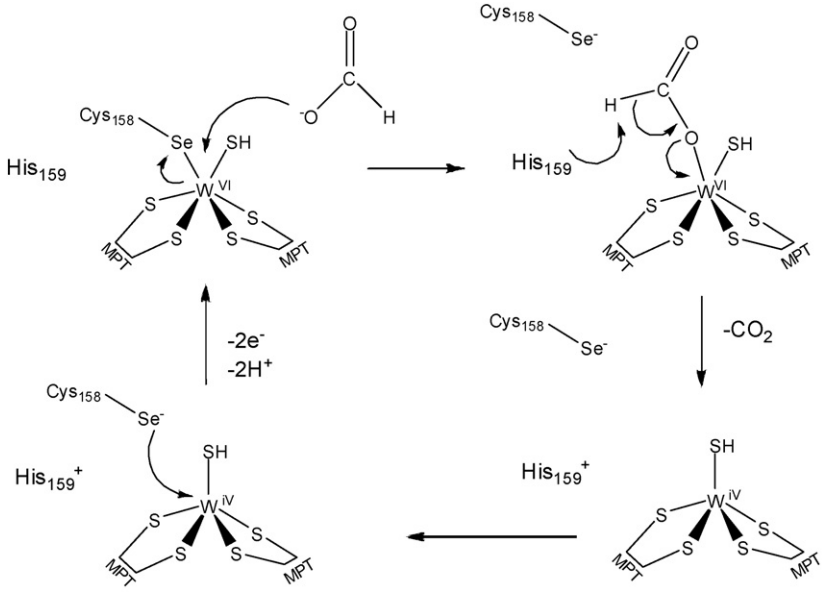


Fig. 13. Proposed mechanism of W-FDH [127]. Formate binds to the tungsten center, displacing the selenocysteine ligand (SeCys158). The selenocysteinate is stabilized by a nearby conserved arginine residue (Arg407). Subsequently, the alpha proton of the bound formate is transferred to a nearby conserved histidine residue (His159). During this step CO_2 is released and the tungsten center is reduced from VI to IV. Alternatively, the free selenocysteinate may assist by forming a selenium-carboxylated intermediate. Finally, two electrons are sequentially transferred from the tungsten center via the [4Fe–4S] cluster to an external redox partner protein.

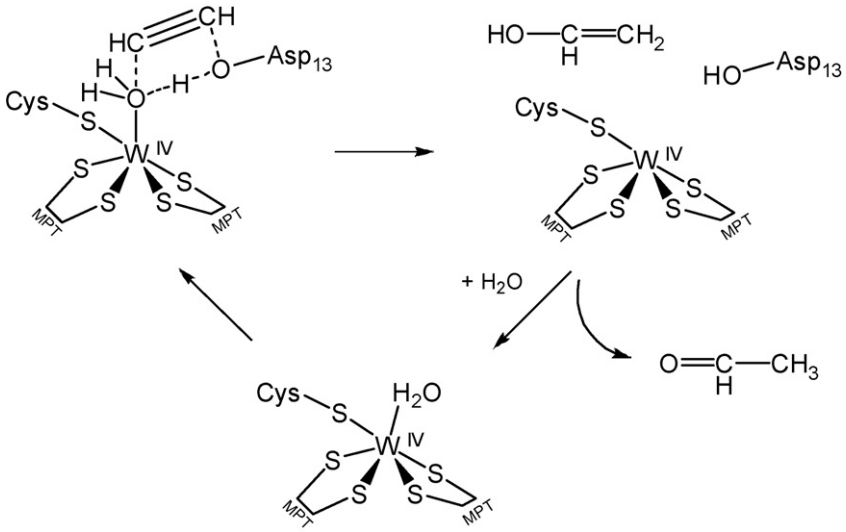


Fig. 14. Proposed mechanism of W-acetylene hydratase [94]. The reduced [4Fe–4S] cluster activates a coordinated water molecule, which gains positive charge under the influence of the proximal Asp13. Subsequently, the electrophilic water can react with the acetylene triple bond in a Markovnikov-type electrophilic addition reaction. The tungsten center is regenerated by the binding of water to the tungsten and deprotonation by Asp13. Alternatively, a hydroxo ligand would act as a nucleophile instead of a water molecule producing a vinyl-anion intermediate that is able to deprotonate Asp13.

pterin moiety via four [4Fe–4S] clusters, each circa 10 Å apart, offering an electron transfer conduit to the physiological redox partner, a monoheme cytochrome.

A recent re-evaluation of the crystal structure of the molybdenum-containing FDH-H from *E. coli*, together with the structure of the tungsten enzyme, suggests the following mechanism (Fig. 13) [127]. Formate binds to the tungsten center, displacing the selenocysteine ligand (SeCys158). The free selenocysteinate is stabilized by a nearby conserved arginine residue (Arg407). Subsequently, the alpha proton of the bound formate is transferred to a nearby conserved histidine residue (His159). During this step CO_2 is released and the tungsten center is reduced from VI to IV. Alternatively, the free selenocysteinate may assist by forming a selenium-carboxylated intermediate as has been postulated previously [128]. As the final step two electrons are sequentially transferred from the tungsten center via the [4Fe–4S] cluster to an external redox partner protein, completing the catalytic cycle. This final step involves an EPR detectable W(V) intermediate. The mechanism of FDH, however, is still a subject of debate [129].

In the absence of a crystal structure of a formylmethanofuran dehydrogenase (FMDH), any proposed reaction mechanism remains highly speculative. FMDH catalyzes the reversible reductive carboxylation of methanofuran with CO_2 to *N*-formylmethanofuran. A three-dimensional structure of the enzyme would help to explain how both substrates can get into close proximity and may indicate the involvement of additional amino acid residues in the active site pocket.

Biochemical analysis and amino acid sequence comparison show that tungsten-containing FDMH contains a bis-MGD cofactor with an additional cysteine sulfur ligand to the tungsten center, and puts this enzyme into the DMSO reductase family [130,131].

11.3. Acetylene hydratase

Acetylene hydratase is a unique tungsten-containing enzyme since it does not appear to catalyze a redox reaction. The structure has been determined at 1.26 Å resolution [94], and provides important clues on the catalytic mechanism and the roles of the W center and the [4Fe–4S] cluster (Fig. 14). The reactive species is either a hydroxo or a coordinated water molecule. The hydroxo-ligand would act as a nucleophile with a vinyl-anion product that is able to deprotonate Asp13. Interestingly, the enzyme needs to be activated by reduction of the W center from W(VI) to W(IV). The [4Fe–4S] cluster is thought to facilitate this activation step. The coordinated water molecule could gain positive charge under the influence of the proximal Asp13. Subsequently, the electrophilic water can react with the acetylene triple bond in a Markovnikov-type electrophilic addition reaction. The tungsten center is regenerated by the binding of water to the W center and deprotonation by Asp13. Theoretical calculations suggests that the W(IV) active site favors coordination of water over hydroxo, supporting the electrophilic addition mechanism [94].

12. Conclusions

In this review various aspects of the bioinorganic chemistry of the element tungsten have been considered. An overview was given of spectroscopic tools to study the metal either incorporated in proteins or as model coordination compound, followed by a description of relevant physiological processes, notably, the uptake and storage of the metal by cells and the incorporation into an organic cofactor in order to tune its redox properties required for biological activity. The two families of tungsten-containing enzymes were discussed: the aldehyde oxidoreductases containing a non-modified tungsto-bispterin

cofactor, and the formate dehydrogenases which have a guanine monophosphate attached to each pterin. Understanding their mechanisms of action on a molecular level is a field of ongoing research, for which the use of spectroscopic techniques and the availability of synthetic mononuclear oxomolybdenum and tungsten complexes, are of high importance. Repeatedly, reference was made to the literature on molybdenum, which is generally more developed both for biological and for model systems.

In the introductory part two questions were raised to serve as Leitmotif for this review. The first question is: why do organisms use tungsten? At this time a full answer cannot yet be given, although it has been shown that on an enzymatic level the presence of tungsten offers an advantage for the reduction of substrates with relatively low reduction potentials. Also, organisms that live in an environment with a relatively high tungsten over molybdenum concentration ratio and that have learned to exploit the tungsten, may have an advantage over the ones that are not able to do so, or for whom tungsten might even be xenobiotic [5–7].

The second question: 'how do organisms discriminate between tungsten and molybdenum?' also remains to be answered in full detail. However, it has become clear that the selection is not only made at the level of the transport proteins because these are all able to bind both oxoanions with affinities in the micromolar range or lower. A main act of selection must take place in the pathway of the cofactor synthesis and/or through specific enzymes that might only enable the incorporation of a specific metal-containing type of cofactor. In the coming years more experiments need to be designed and performed in order to solve the details of the selectivity process(es).

Finally, many issues are still to be addressed in the tungsten-biology fields of storage, regulation, and enzyme kinetics. Protein crystal structures of intermediates or intermediate analogues can help to solve reaction mechanisms of the tungstoenzymes. In addition, application of advanced spectroscopic techniques might contribute to a deeper insight into structure and mechanism. For these and other purposes, the synthesis of biomimetic tungsten complexes has an added importance.

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