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Review

Cellular uptake of molybdenum and tungsten

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Contents

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ABSTRACT

Cells acquire molybdenum and tungsten as their highly soluble oxoanions, $Mo^{VI}O_4^{2-}$ or $W^{VI}O_4^{2-}$, which they internalize by means of an active (i.e. energy requiring) transmembrane importer, for subsequent conversion into the metalloenzyme cofactors Moco or Wco (and FeMoco in nitrogen fixers). This import system has been studied as one of the models for the functioning of the protein complex superfamily of ABC (ATP binding cassette) transporters, but its mechanistic details are presently not clear. The complex exhibits interesting variants, known as the microbial Mod, Tup, and Wtp system, and the – less well defined – eukaryotic MOT1 system, which mutually differ in oxoanion coordination chemistry and in the control of intracellular Mo/W levels. This evolutionary diversity of Mo/W transporters has resulted in confusing nomenclature whose rectification is here proposed.

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1. Introduction

According to a basic rule of bioinorganic chemistry life will turn an element into a general bio-building block whenever the element obeys the minimal requirement of bioavailability, namely, a certain aqueous solubility combined with a not too restricted abundance and distribution over the earth's lithosphere and hydrosphere [1]. Molybdenum and, to a lesser extent, tungsten appear to come close to compliance since, although individually they are not universal bioelements, their combination (Mo and/or W) may well be one [2]. Numerous forms of life (including man) do not use W, and at least some species (*Pyrococcales*) do not use Mo [3], but a cell that does not use either one is yet to be found.

The neutral aqueous chemistry of concentrated Mo and W can be complicated by the thermodynamic and kinetic intricacies of polyoxoanion formation (e.g., [4–6]), however, in most natural environments ($<10^{-8}$ M), and in laboratory fermentors ($<10^{-5}$ M), dissolved concentrations are sufficiently low to warrant speciation to be essentially limited to the mono-oxoanions, MoO_4^{2-} and WO_4^{2-}. Thus the questions to be addressed in this review become (i) how does a cell pick up one or both of these two anions from a medium with other oxoanions? (ii) how does it transport them over an ion-impermeable membrane? and (iii) how, and in what form, are the metals finally presented to the biosynthetic machinery? A very brief answer would be: the ABC transporter binds the oxoanion with high specificity, then uses an ATP hydrolysis driven conformational change to move it through the membrane, and finally releases it on the other site. The present state of our knowledge and understanding (and the lack of it) regarding the details of these three basic steps are reviewed in what follows.

A major point emphasized in this review is the existence of three different prokaryotic transporter systems whose oxoanion binding proteins ModA, WtpA, and TupA do not only differ in their binding affinity for molybdate versus tungstate, but are also essentially unrelated at the primary sequence level, and furthermore appear to have very different oxoanion coordination chemistry. It is therefore imperative to reverse common practice of mixing up the labels Mod, Wtp, Tup.

2. The paradigmatic molybdate transporter

2.1. The molybdate system Mod

According to the principle of unity in biochemistry one should, rather than to go for taxonomic exhaustiveness, scrutinize the biochemical system from one species as a paradigm for the rest of nature. Subsequent comparison with a limited number of functional homologs from preferably distant species may lead to added understanding. Not uncommon, the model system initially chosen in molybdate transport studies was *Escherichia coli*, and the three adjacent structural genes encoding the three proteins that together form the transporter were traditionally labeled with a function-indicating three-letter name (the '*mod*' genes for the 'Mod' molybdenum transport proteins) and individually with alphabetic additions according to their position in the operon. The *modABC* genes encode the proteins ModA, ModB, and ModC, which together assemble into the ModABC transporter. Note that the ABC labeling used here has nothing to do with the ABC in 'ABC(=ATP binding cassette) transporter'; the motif (cassette) of amino acids forming the ATP binding site is on the protein ModC, which is located on the cytoplasmic site of the native ModABC complex; ModB is the transmembrane protein, and ModA is the molybdate-binding protein on the periplasmic site (Fig. 1).

Thus one could legitimately say that "this ABC transporter is an ABC transporter", meaning that the system, which binds ATP to use its Gibbs free energy of hydrolysis for active transport, is a

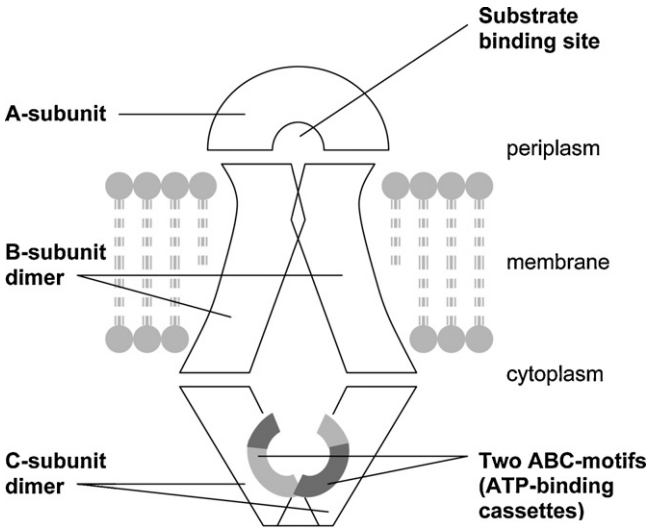

Fig. 1. Schematic drawing of a molybdate ABC transporter. This import system has subunit stoichiometry AB_2C_2. The soluble, periplasmic A-protein scavenges the oxoanion and then binds to the membrane complex which consists of a pair of translocating, transmembrane B-subunits bound to a cytoplasmic pair of C-subunits with ATPase activity.

complex made of three different subunits. In fact, the quarternary structure of the complex is presumably pentameric, AB_2C_2 [7], and furthermore, the operon encoding the transport system in *E. coli* actually contains four consecutive open reading frames, *modABCD*, that together are transcribed into a single polycistronic mRNA [8]. The function of the protein ModD is presently unknown. Upstream of the *modABCD* operon, and in opposite reading direction, are the genes *modE* and *modF* monocistronically encoding respectively for a regulator of molybdate transport and for another protein with unknown function [9]. Note that an ABC transporter does not have to be made up of three different polypeptides. Export systems are usually ABC transporters frequently made up of a homodimer of a single polypeptide with two domains homologous to *modBC* of the *E. coli* molybdate transporter.

2.2. The tungstate systems Wtp and Tup

Typically, mechanistic studies of complex proteins are based on three complementary experimental approaches: (i) genetic (modification) studies combined with phenotype analysis; (ii) biochemical and biophysical studies of the isolated complex; and (iii) X-ray crystallographic structural studies. The *E. coli* Mod system (as well as that from other gram-negative bacteria) has been the subject of several genetic and biochemical studies. Unfortunately, crystallization of membrane proteins is not trivial; at present only a crystal structure of the soluble ModA protein of *E. coli* is available [10]. No structures have been reported for individual ModB and/or ModC proteins from any species, however, the complete molybdate ABC transporter complex $ModAB_2C_2$ from the sulfate-reducing archaeon *Archaeoglobus fulgidus* has been crystallized, and its structure has been solved to 3.1 Å resolution [7]. The *A. fulgidus* molybdate transport system has hardly been studied genetically and biochemically, and so genetic, biochemical, and structural data from rather different species contribute to our present picture of the paradigmatic molybdate transporter. This is a complicating factor as is evident from the recently developed confusion in nomenclature and possibly in functional understanding. It was already known for some time that *Eubacterium acidaminophilum* produces an ABC transporter TupABC (for tungsten uptake) that is highly specific for tungstate although it has some homology with the *E. coli* ModABC system [11]. Shortly before the *A. fulgidus*

Table 1
Crystallographic structures of molybdate/tungstate transport proteins.

Species	pdb	Resolution	Ligand	Remark	Refs
ModA					
Escherichia coli	1AMF	1.75A	Molybdate		[10]
	1WOD	1.75A	Tungstate		[10]
Azotobacter vinelandii	1ATG	1.20A	Tungstate		[22]
Xanthomonas axonopodis citri	2H5Y	1.7A	Molybdate	N-His-tag	[23]
	3GZG	1.55A	Molybdate	N-His-tag; K127S	[23]
WtpA, WtpBC					
Archaeoglobus fulgidus	2ONR	1.60A	Molybdate		[7]
	2ONS	1.55A	Tungstate		[7]
	2ONK	3.10A	Tungstate	ABC complex	[7]
	3CIJ	1.07A	Tungstate		[15]
Pyrococcus horikoshii	3CG3	1.80A	Tungstate		[15]
Pyrococcus furiosus	3CG1	1.60A	Tungstate		[15]
Methanococcus janaschii	3CFZ	1.70A	Tungstate		[15]
Methanosarcina acetivorans	3CFX	1.60A	Tungstate		[15]
	3D31	3.00A	Tungstate	BC complex	[16]
	3K6U	1.95A	apo		[19]
	3K6V	1.69A	Citrate		[19]
	3K6W	2.45A	Molybdate		[19]
	3K6X	2.25A	Molybdate	Different crystal	[19]
TupA					
Geobacter sulfurreducens	3LR1	1.80A	Tungstate	Se–Met	[24]
ModE					
Escherichia coli	1B9M	1.75A	apo	Se–Met form	[25]
	1B9N	2.09A	apo	Native	[25]
	1H9R	1.90A	Tungstate	Di-mop fragment	[26]
	1H9S	1.82A	Molybdate	Di-mop fragment	[26]
	1NP6	1.90A	Molybdate		[27]

$ModAB_2C_2$ structure became available a third class of molybdate/tungstate binding proteins was characterized: WtpA (for W transport) encoded by a *wtpA* gene as part of a *wtpABC* operon for a putative ABC transporter in the hyperthermophilic archaeon *Pyrococcus furiosus* [12]. ModA, TupA, and WtpA clearly define distinct phylogenetic entities (ibidem). The dissociation constant of WtpA for tungstate, $K_D \approx 10^{-11}$ M, is some three orders-of-magnitude less than that for molybdate, which is consistent with *P. furiosus*'s strict requirement of W for growth [13], its apparent incapacity to use Mo [14], and the concentrations of tungstate and molybdate in its marine volcanic habitat [3].

In hindsight, the phylogenetic analysis of ModA, TupA, and WtpA provided a particularly surprising result. *A. fulgidus* ModA (the putative molybdate-binding protein of the molybdate ABC transporter) is clearly *not* a ModA, but a WtpA, since it has virtually no homology to the prototypical ModA of *E. coli* while it is highly homologous to the prototypical WtpA of *P. furiosus* [12]. The reason why the *A. fulgidus* system was labeled Mod in the original work describing its crystal structure [7] is not clear, since sequence data on all three systems was available at the time. In addition to specific implications for our understanding of the physiology of *A. fulgidus*, the general implication is that the protein data base contains a 3D structure of a WtpABC system, but not yet one of a ModABC system. The distinction is clearly important for the initial binding of the oxoanion, which is very different for WtpA (covalent) compared to ModA (H-bridges only); see below. Whether there are significant functional differences between the other components, is presently unknown. Sequence similarity between WtpA and ModA proteins is typically only circa 30%, while similarities between WtpBC and ModBC are in the 50–55% range [12].

2.3. Nomenclature problems and their solution

To the re-assignment of *A. fulgidus* ModA as WtpA the crystallographers have reacted by re-naming the protein as 'ModA/WtpA', for which they define the abbreviation <species>ModA, e.g., AfModA ≡ *Archaeoglobus fulgidus* ModA/WtpA [15]. On the other hand, they have chosen to continue to use the 'Mod' nomenclature for the associated transmembrane B-protein and the ATP-hydrolyzing C-protein [15–18]. This reviewer takes the liberty of expressing a firm dissent with the use of these ambivalent namings, which are now leading to a rapid build-up of misunderstandings.

The genome of the methanogenic archaeon *Methanosarcina acetivorans* contains structural genes for both a ModABC system and a WtpABC system [12]. The WtpBC complex has been crystallized in a tungstate-inhibited complex, but the crystal structure is referred to as *M. acetivorans* ModBC [16]. The structure of the isolated WtpA, which was re-named as ModA/WtpA, has recently been reported in its apo-form by another protein crystallography group using the label MaModA and with no reference whatsoever to its being a member of the Wtp family [19]. Also, in a recent review it was claimed that *A. fulgidus* has, in addition to the Wtp system, "the ABC transporter ModABC for molybdate, whose general structure could be resolved very recently for the first time" [20]. This is incorrect because *A. fulgidus* does not contain a ModABC system, and the resolved structure [7] is that of the WtpABC system. Similarly, in a very recent genomic analysis of metal-related genes one can find that one out of four *Thermococcales* with sequenced genomes (presumably *P. furiosus*) has both a WtpABC system and a ModABC system [21], which is, once more, not true. If we are to continue in this vein, what then should we call the expression product of the (yet to be explored) real *modABC* system in *M. acetivorans*?

ModA, WtpA, and TupA are distinct systems that differ in their oxoanion binding properties, whose primary sequences are essentially unrelated, and whose oxoanion binding modes are very different (see below). This reviewer proposes to consistently use the distinctive labeling Wtp, Tup, and Mod, which will facilitate concentrating on the important already detected differences in binding properties and in feed-back regulation, and on still to be explored possible differences once structural data will become available for the TupBC system and the ModBC system.

Table 2
Dissociation constants of molybdate/tungstate-binding proteins.

System	Species	K_D molybdate	K_D tungstate	Ratio	Refs
ModA	*Escherichia coli*	20 nM	Similar	~1	[34]
	Xanthomonas axonopodis citri	0.29 μM	0.58 μM	0.5	[40]
TupA	*Campylobacter jejuni*	50 nM	1 pM	0.5×10^4	[41]
	Eubacterium acidaminophilum	1.4 μM	1 nM	1.4×10^3	[20]
		nd	<0.5 μM	$\sim 10^3$	[11]
WtpA	*Pyrococcus furiosus*	11 nM	17 pM	0.6×10^3	[12]
MoT1	*Chlamydomonas reinhardtii*	7 nM[a]	nd	–	[38]
	Arabidopsis thaliana	21 nM[a]	nd	–	[37]
ModE	*Escherichia coli*	0.8 μM	Similar	~1	[42]

[a] K_M-values for overall transport.

Table 1 lists the proteins of molybdate/tungstate transporters for which crystallographic structures have been determined. Note that all the Wtp proteins of Table 1 in the protein crystallographic literature are referred to as Mod or as ModA/WtpA.

3. Bioanalytics of Mo/W transport

To monitor the transport systems in operation raises specific analytical chemical issues, which are briefly addressed in this section.

3.1. Oxoanion concentration determination

Molybdenum can be determined by a variety of colorimetric methods (e.g., [28,29]), but the biochemists appear to have settled over the years for dithiolene complexes such as toluene-3,4-dithiol [30] or methyldimercaptobenzene [31]. The procedures typically require multiple steps including a solvent extraction, the sensitivity is not fantastic, and simultaneous determination of Mo and W is problematic.

ICP–MS is frequently used as a high-end solution of significantly increased sensitivity. A less costly and even more sensitive alternative, with only minor interference issues, is the simultaneous polarographic determination of Mo and W (as oxoanions or as metalloproteins) on the hanging mercury drop electrode by adsorptive stripping voltammetry [32].

3.2. Determination of binding constants

Native polyacrylamide gel electrophoresis has been used to monitor shifts in retention time (apparent mass) of proteins upon binding of ligand. Experience with the determination of binding properties of molybdate and tungstate binding proteins ModA, TupA, and WtpA is disappointing as the method appears to afford only qualitative results at best [11,12,33].

An alternative approach is the monitoring of spectral changes upon ligand binding. Unfortunately, the relevant oxoanions are closed-shell systems with no absorption in the visible and near UV, and so one has to resort to the detection of induced minor changes in the UV absorption and/or fluorescence of the protein. This approach has been reported to be practical for molybdate and tungstate binding to ModA [12,33,34], but not for WtpA [12].

The (micro-)calorimetric technique of isothermal titration calorimetry (ITC) is in principle generally applicable to the determination of the thermodynamical constants (ΔG and K_D and, when measured as a function of temperature, ΔH and ΔS) of any binding equilibrium, and the detected heats of reaction for the systems of interest here are sufficiently pronounced to allow for accurate analyses. ITC is typically optimal for dissociation constants in the micromolar range with progressive loss of sensitivity towards higher or lower concentrations. In practice, the very strong binding of, e.g., tungstate to WtpA and TupA proteins can usually still be determined with high accuracy, but only indirectly by means of displacement experiments. First molybdate is bound, which is then subsequently displaced by the stronger binding tungstate with measurement of the difference between the two binding constants [12]. K_D-values are given in Table 2.

Dissociation constants can also be determined from Scatchard plots using radionuclide detection as bound ligand after chromatographic separation of free ligand. Suitable isotopes are ^{99}Mo ($t_{1/2}$ = 66.4 h) and ^{187}W ($t_{1/2}$ = 23.9 h). The method has been used to study binding of $^{99}MoO_4^{2-}$ to *E. coli* ModA and competition with cold WO_4^{2-} [34]. ^{187}W has not been used yet. The radionuclide experiment assumes no stripping of ligand over the column, i.e. slow binding kinetics compared to the time scale of the overall experiment, and this presents a possible problem, see Section 3.3.

The complete transport system, e.g., ModABC, in a cell (or reconstituted in a membrane) can be viewed upon as a black-box enzyme with free molybdate in the external medium as the substrate, and molybdenum, in whatever forms, in the cytoplasm as the product. The activity of the transporter can then be determined in terms of a k_{cat} and K_M value, assuming Michaelis–Menten kinetics, and we can take the K_M value as an approximation of the dissociation constant (Table 2). The definition of k_{cat} in whole-cell experiments can be ambiguous with respect to enzyme concentration. Reported low numbers, e.g., fmol molybdate (mg protein)$^{-1}$ min^{-1}, refer to total cell protein rather than transporter concentration [35].

3.3. Metalloproteomics

Radioisotopes such as ^{99}Mo or ^{187}W can also be used as markers of native Mo- or W-proteins. When cells are grown on a medium containing these tracers, and the proteins are subsequently extracted and separated by two-dimensional gel electrophoresis under native conditions (i.e. retaining the metal–protein bond) the radioactive metalloproteins can be spotted by autoradiography on a phosphorescence screen. After ten half lifes of decay of the radioactivity the spots can be excised and their protein content be prepared by tryptic hydrolysis for identification in tandem mass spectrometry. The technique has been used to determine the tungsten proteome of *P. furiosus* [36] and to monitor the substitution of W by Mo in tungsten–protein biosynthesis under pressure of high [Mo]/[W] ratio [3]. This approach appears to be problematic for the study of periplasmic molybdate- or tungstate-binding proteins possibly because the oxoanion is lost by dilution during the protein separation [36]; however, in the membrane fraction of cells grown on ^{99}Mo, labeled WtpA was found presumably as part of the transporter complex [3]. A further exploration and development of the method is called for.

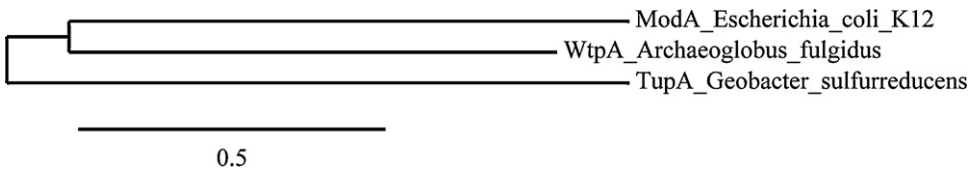

Fig. 2. Basic phylogram of ModA, WtpA and TupA. The distance scale represents the average number of changes per amino acid residue (i.e. 0.5 means 50% differences between species. The lengths of the branches indicate that evolutionary relationships between the three example A-proteins are extremely distant.

4. Natural variation: Mod, Tup, Wtp, Mot1

4.1. Phylogenetics

Molybdate and tungstate are sequestered by the gram-negative facultative anaerobic *E. coli* with ModA, by the gram-positive anaerobic bacterium *E. acidaminophilum* with TupA, and by the hyperthermophilic anaerobic marine archaeon *P. furiosus* with WtpA. Furthermore, it has recently become clear that plants (*Arabidopsis thaliana*) and green algae (*Chlamydomonas reinhardtii*) use yet another protein labeled MOT1 or MoT1 [37,38], while vertebrates, including man, do not appear to synthesize any of these four systems, and are therefore likely to have an as yet to be discovered fifth system. How different are these oxoanion-binding proteins from each other, and what is the relevance of the taxonomy for an understanding of their coordination chemistry and their role in physiology? The discovery of the Wtp system and initial sequence analysis on a limited number of species afforded a simple phylogenetic tree with three well separated main branches, i.e. an indication that based on global sequence comparison the ModA, TupA, and WtpA form three evolutionary distinct entities [12].

The number of deposited sequences from ABC transporter systems tends to grow rather rapidly, and the molybdate transporter is no exception to this rule. At the present time the total number of sequences with significant homology to ModA, TupA, WtpA, or MOT1 has become so large that even the more robust extant software hicks up when asked for a comprehensive comparison. What this means can be illustrated with an in silico experiment carried out with the robust MUSCLE + Gblocks + PhyML software on the server of phylogeny.fr [39]. First a restricted Blast (expectation value $E < 10^{-40}$) was run on each of the paradigmatic proteins, *E. coli* ModA, *E. acidaminophilum* TupA, *P. furiosus* WtpA, and *A. thaliana* MOT1. Then the resulting sets were phylogenetically compared in different combinations. This procedure affords phylogenetic trees under the assumption that all used sequences are evolutionary related through a common ancestor gene, and are so in a retraceable manner. The software failed to produce trustworthy alignments even if limited to pairwise comparisons of groups. Leaving out the MOT1 group (because the average sequence lengths is longer than that of the other groups), and reducing the number of sequences in each remaining group to a small number, say five, still led to a failure of Gblocks to identify eliminable non-overlapping sequences and to phylograms with numerous low-valued bootstrap numbers.

The conclusion should be that the four groups all are mutually so different, that it is impossible to retrace any putative evolutionary relationships between them, and thus that, for all practical purposes, they are not related. A very simple illustration of this is seen in Fig. 2 which gives a phylogram for a single representative only from the three groups ModA, WtpA, and TupA. The bar length indicates that in this example each pair is separated by a distance corresponding to circa one average replacement per amino acid site; indeed pairwise sequence identity is well below 20% for all three combinations. The implication is that phylogenetic analysis may be useful to study variation within a group; however, it is not applicable to any combination of the groups. For example, a recent conclusion, based on inconveniently low bootstrap values, that the ModA2 protein from *A. vinelandii* and *A. fulgidus* 'ModA' (that we now know to be a WtpA) are members of a sub-branch of the ModA family [23], is obviously untenable. A second implication is that, from a coordination chemical point of view, we should consider each group as independent with its own specific solution to the problem of affinity and specificity for oxoanion association. This notion of convergent functional evolution is indeed what transpires from available structural data, as discussed in Section 5.

Thus, by employing more than one of these systems, an organism could possibly fine-tune its molybdate and tungstate uptake capabilities. The bacterial and archaeal kingdoms scatter over the three families of ModA, TupA, and WtpA. The occurrence of more than one gene for molybdate/tungstate binding is quite common. *Methanosarcina mazei* has genes for ModA and TupA, *Haloarcula marismortui* encodes TupA and WtpA, and *Methanosarcina activorans* can make ModA and WtpA. *Desulfotalea psychrophila* even has the genetic information for ModA, TupA, and WtpA [12]. Some species also have paralogous genes for two variants from a single branch. Genes for two ModA's are found in *Desulfotomaculum acetoxidans*, two TupA's in *Bradyrhizobium japonicum*, two WtpA's in *Deferribacter desulfuricans*, and two MOT1's in *Physcomitrella patens*. All this suggests again that it is likely to be meaningful to explore differences with respect to coordination chemistry, physiological function, and perhaps regulation, and it underpins the necessity for an unambiguous nomenclature.

4.2. Differential binding affinities

Perhaps the most obvious and easily observable difference between the four systems is selectivity in their affinity for molybdate versus tungstate. A few illustrative selected numbers are given in Table 2.

Although as usual many more genes have been sequenced than gene products have been biochemically characterized, the limited data suggest that the TupA and WtpA systems are strongly selective in favor of tungstate, while ModA does not discriminate between the two oxoanions, nor does ModE. For the eukaryotic MOT1 system no K_D-values have yet been reported; we take the K_M values as approximation of K_D.

4.3. Three-dimensional homology

The apparently considerable evolutionary distances in primary sequence between the different molybdate/tungstate binding proteins does not appear to translate in large structural differences. It has been noted that the overall structure of *E. coli* ModA and *A. fulgidus* WtpA are quite similar in terms of alpha-helical and beta-sheet elements and in the position of the oxoanion binding site approximately in the protein's center of gravity [15]. This is perhaps not surprising since in all cases the soluble binding protein would be expected to possess an approximate two-fold symmetry designed to interact with a dimeric transmembrane translocator. All crystal structures presently available have been listed in Table 1.

The recently deposited first structure of a TupA protein at 1.80 Å resolution (with the somewhat misleading title 'The crystal

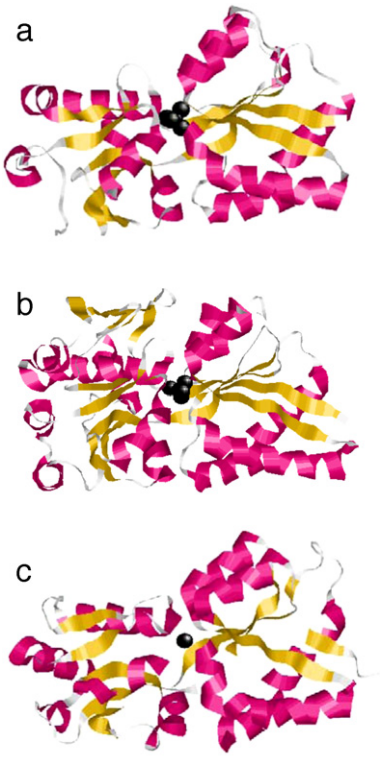

Fig. 3. Overall 3D fold of three types of A-proteins. The comparison of (a) *E. coli* ModA, (b) *P. furiosus* WtpA, and (c) *G. sulfurreducens* TupA indicates a strong similarity in three-dimensional structure, although the primary structures are unrelated. Color coding: magenta, α-helix; yellow, β-sheet; black ball(s), oxoanion atoms (in the TupA structure the tungstate is only defined as a single point). PDB files: 1AMF, 3CG1, 3LR1.

structure of the tungstate ABC transporter from *Geobacter sulfurreducens*') has not been discussed yet in the literature [24]. Bound tungstate is represented in the pdb file as a single 'atom' and the coordination chemistry of the tungsten is not clear (see also below). However, a first impression of the overall structure in comparison with a ModA and a WtpA protein, in Fig. 3, clearly indicated a considerable homology in secondary and tertiary structure. For the eukaryotic MOT1 proteins no crystal structures have been reported yet.

4.4. The 'eukaryotic' solution

The vast majority of our knowledge of molybdate transporters is based on studies of prokaryotic systems, but recently a eukaryotic system has been described from the plant *Arabidopsis thaliana* [37] and from the green alga *C. reinhardtii* [38]. The transporter consists of a single subunit of 457 amino acid residues (*A. thaliana* MOT1) or 519 residues (*C. reinhardtii* MoT1); the latter has nine putative membrane-spanning domains and there are two conserved sequence motifs – of unknown function – in between domains I–II and VI–VII [38]. The protein has not been isolated and purified yet, and the tertiary and quarternary structure are not known. Transport activity has been measured after expression in the yeast *Saccharomyces cerevisiae* [37,38]. The plant MOT1 was originally reported to be localized in the plasma membrane (when expressed in tobacco cells) based on fluorescence imaging of the protein N-terminally fused to GFP (green fluorescent protein) [37], but a subsequent study, using a C-terminally fused GFP construct in *Arabidopsis* leaf protoplasts, concluded that the localization is mitochondrial consistent with a predicted mitochondrial targeting sequence at the N-terminal [43]. In a recent study on a mutant of *C. reinhardtii*, named 'DB6', molybdate-transport deficiency was associated with mutation of another gene than the MoT1 gene, which made the authors conclude that "Without a full understanding of all the genes involved in molybdate transport, we are unable to understand the regulatory mechanism of Moco biosynthesis" [44], an opinion with which this reviewer would concur.

As a further complication, it appears from an initial phylogenetic analysis that MOT1 homologs not only occur in plants, algae, and fungi, but also in bacteria [38]. The genome of one of the species cited in the analysis, *Shewanella woodyi*, also happens to carry genes for a Tup system and two Mod systems, which leads one to wonder whether there is such a thing as a eukaryotic molybdate transporter.

5. Bio-coordination chemistry of molybdate/tungstate

5.1. Binding the substrate

How do the binding proteins discriminate between molybdate and tungstate? What is there to discriminate on? Due to the lanthanide contraction the isoelectronic MoO_4^{2-} and WO_4^{2-} tetrahedral anions are structurally very similar as evidenced, for example, by the metal–oxygen bond length in $SrMoO_4$ (176.6 ± 0.5 pm) versus $SrWO_4$ (177.9 ± 0.3 pm) [45]. A protein that would bind oxoanions through hydrogen bonds to the oxygen atoms should have no problem in excluding the isoelectronic, but much smaller sulfate ion, however an effective discrimination between molybdate and tungstate would seem to be near to impossible.

Specific ionic bonding could lead to deformation of the tetrahedron, and thus to changes in the metal-to-oxygen bonds [46], but the changes would be rather similar for the Mo and W systems

It has recently been suggested that a difference in basicity, where protonation may be part of a stable complex formation, may contribute to discriminate the two metals [20]. The cited p*K*a-values (no reference) for the first protonation of 3.8 (molybdate) and 4.7 (tungstate) are at variance with other literature values, e.g., 5.0 (molybdate) and 3.5 (tungstate) [4], or 3.7 (molybdate) and 3.8 (tungstate) [47].

A significant discrimination would require some form of direct bonding to the metal so that the chelating protein can tune into the differences in metal ionic radius, in Lewis acidity, and/or in extent of relativistic effects [48]. Whatever the relative relevance of these properties has on the stability of the protein–oxoanion complex, the overall effect is likely to be significant as inferred from the 0.3–0.8 V positive differences in reduction potential (i.e. relative stability of reduced over oxidized form) of isoelectronic Mo versus W complexes with a variety of ligands [49], including the W-substitutable Mo-enzyme DMSO-reductase from *Rhodobacter capsulatus* [50].

5.2. The ModA binding mode: hydrogen bonds only

Crystal structures have been determined for ModA complexed with its substrate from *E. coli* [10], *A. vinelandii* [22], and *X. citri* [23] (note that all other so-called "ModA's" deposited in the pdb at the time of writing are actually WtpA's; see Table 1). A schematic view of the coordination in the *E. coli* protein is depicted in Fig. 4a.

Seven atoms of the protein are in H-bond donating distance from the molybdate oxygens: three alcoholic oxygen groups (two Ser and one Tyr) and four nitrogen backbones including two from the Ser's). There is no indication for significant distortion of the molybdate (or tungstate) tetrahedron.

The binding site appears to be rather conserved. In the 67 ModA sequences considered above (Section 4.1) the two alcoholic side

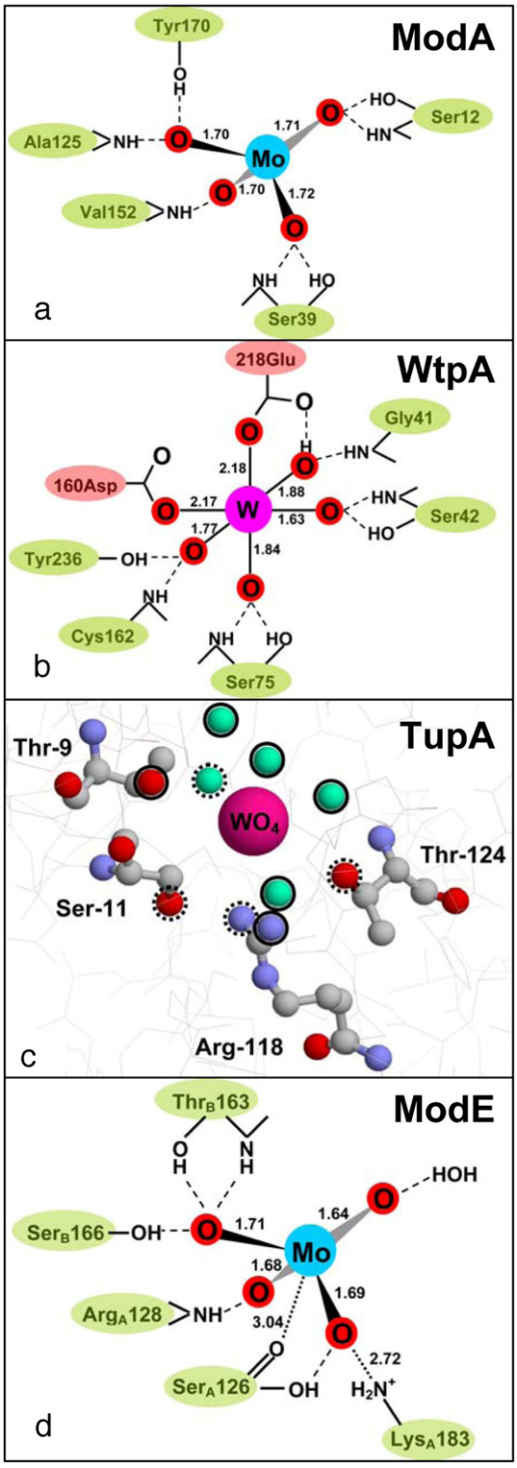

Fig. 4. Coordination details of the oxoanion in different molybdate/tungstate binding proteins. (a) *E. coli* ModA, (b) *P. furiosus* WtpA, (c) *G. sulfurreducens* TupA, (d) *E. coli* ModE. PDB files: 1AMF, 3CG1, 3LR1, 1H9S. The numbers are bond lengths in Ångström. In panel (c) putative ligands to the tungstate are indicated by solid circles (<4.0 Å from the tungstate barycenter) or broken circles (<4.5 Å) with color coding red (oxygen) blue (nitrogen) or green (water).

group residues Ser-12 (*E. coli* numbering) and Tyr-170 are fully conserved; in some proteins the equivalent of Ser-39 is replaced by Thr. Ala-125 is fully conserved in the pattern PAGxY, and Val-152 is conserved in the motif V(R/K). Apparently, not only the size of the binding pocket is relevant for the discrimination of molybdate/tungstate versus smaller oxoanions such as sulfate, but the specific coordination geometry is also of importance to bind an undeformed molybdate/tungstate tetrahedron with nanomolar dissociation constant.

Two ModA proteins of *Azotobacter vinelandii* belong to a slightly diverged group of proteins, and the binding site in the crystal structure of ModA2 [22] has some subtle modifications compared to *E. coli* ModA and *X. citri* ModA; the backbone-N and alcoholic-O hydrogen bonds of Ser-39 (*E. coli*) have been retained in Ser-37 (*A. vinelandii*), and so has the N-backbone H-bond of Val-152 (Val-147 in *A. vinelandii*). Also, the N-backbone H-bond of Ala-125 in a PAG motif has an equivalent in the backbone-N from Tyr-118 in a PYG motif. However, the alcoholic-O H-bond of *E. coli* Tyr-170 is absent in *A. vinelandii*; and the alcoholic-O and N-backbone H-bonds of *E. coli* Ser-12 become amine-N and backbone-N from Asn-10, extended with alcoholic-O and backbone-N from Thr-9. Overall, the number of hydrogen bonds is seven in both cases.

5.3. The WtpA binding mode: double carboxylate coordination

Crystal structures have been determined for the oxoanion complex of WtpA proteins from five different archaeal species: *A. fulgidus*, *M. janaschii*, *M. acetivorans*, and the hyperthermophiles *P. furiosus* and *P. horikoshii* [15]. The structure of the apoprotein has been determined for *M. acetivorans* WtpA [19]. The tungstate/molybdate binding site is very different from that in the ModA protein. In addition to multiple hydrogen bonds there are two direct coordination bonds from monodentate carboxylate oxygens to the metal (Mo or W) resulting in a major deformation of the oxoanion to what appears to be an approximately octahedral MO_6 configuration, however, with large differences in the metal–oxygen bonds. In the 23 WtpA sequences considered here the coordinating aspartate, Asp-160 (*P. furiosus* numbering) is fully conserved in a DPxGYR motif (in one case out of 23, the translated gene sequence of *Methanocorpusculum labreanum*, the Pro is substituted by Ala). The second coordinating residue is a glutamate, Glu-218, which is invariant in the species for which the WtpA 3D structure has been determined, however, it is not conserved in the set of 23 sequences (namely 12 Glu, 9 Ser, 2 Ala). The second carboxylate oxygen has been proposed to form a hydrogen bond to a protonated oxygen from the oxoanion [7,15]. Just like in the ModA structure two conserved serine residues, Ser-42 in the motif AGSLxxP and Ser-75 in the motif GS, form H-bonds through their alcoholic side groups and also with their backbone nitrogens; a third H-bond from an alcoholic side group is from conserved tyrosine-236. The maize is completed with hydrogen bonds of backbone nitrogens from conserved glycine-41 in the AGSLxxP motif and from a non-conserved cysteine-162 in the DPxGYR motif.

A recent redox chemical study on *P. furiosus* WtpA has illuminated how drastically the metal site in this protein differs from that in an oxoanion (either free or H-bonded to ModA). Bulk titration with sodium dithionite in the presence of organic-dye mediators, monitored with EPR spectroscopy, reveals a Mo(V) $S = 1/2$ species with reduction potentials $E'^0(Mo^{6+}/Mo^{5+}) \approx -0.50$ V and $E'^0(Mo^{5+}/Mo^{4+}) \approx -0.36$ V, i.e. crossed-over potentials affording a maximum of circa 3% Mo(V) at intermediate potentials [51]. The potentials for the corresponding tungstate complex have not been measured as they are presumably below the potential of dithionite (−0.55 V at pH 8 for 10 mM dithionite). The observation of this redox chemistry is remarkable since reduction of the aqueous oxoanions molybdate or tungstate only affords the insoluble $M(IV)O_2$ oxide at low potential, and, at low pH, the Mo^{3+} ion. M^{5+} species are not represented in the aqueous stability diagrams of molybdenum or tungsten, and a Mo(V) EPR species has never been detected before for free or bound oxoanion.

The quasi-octahedral MO_6 structure is not an artifact of crystallization; it has been confirmed and determined with increased

precision by tungsten L-edge X-ray absorption spectroscopy on frozen solutions of WtpA [15]. The Fourier transform of the EXAFS spectrum exhibits an unusual feature at 3.7–4.0 Å that is interpreted as evidence of multiple scattering events (i.e. involving back-scattering over more than one W–O bond), which would be possible only in a close-to-octahedral arrangement.

From a comparison of the oxoanion binding in WtpA versus ModA it would appear that the carboxylate coordination bonds provide WtpA's selectivity of tungstate over molybdate. Asp-160 could be the key residue since the Glu-218 is not conserved. However, binding characteristics and structural data have not yet been reported for any WtpA in which the Glu-218 has been substituted. Future comparative studies on Glu-218 substituted WtpA variants and on Asp-160 and/or Glu-218 site-directed mutants would presumably be helpful to further pin down the molecular basis of the selectivity.

5.4. The TupA binding mode: an arginine salt bridge?

Is the WtpA coordinating aspartate in a network of hydrogen bonds nature's unique solution for fishing out tungstate from the typical excess of molybdate? We argued, above, that TupA is apparently unrelated, or at least not strongly related, to WtpA in its primary sequence. Just like WtpA, the paradigmatic *E. acidaminophilum* TupA exhibits a tungstate over molybdate selectivity of circa three orders-of-magnitude, however with a circa 100-fold lower absolute affinity (Table 2 [11,20]). Recent work on the TupA from *Campylobacter jejuni* reports on an even more impressive selectivity of four to five orders-of-magnitude with a tungstate dissociation constant of circa 1 pM [41,52]. At this time the structural basis for this remarkable property is far from established, but it appears to be clearly different from the WtpA solution.

Crystallographic data of *E. acidaminophilum* TupA has not yet been deposited, although a 1.9 Å structure of the apo-protein with citrate in the putative oxoanion binding site was announced and briefly discussed some time ago [20] and details can also be found in a publicly available thesis manuscript [53]. Three specific conclusions were drawn from a preliminary X-ray analysis: (i) a conserved TTTS motif is a typical signature indicating a selective binding of tungstate; (ii) a conserved histidine residue might be quite important for a selective protonation of tungstate; (iii) mutagenesis of an extending (i.e. into the putative binding pocket) positively charged residue of arginine to lysine strongly diminishes the specific binding of tungstate [20,53].

Recently, the 1.8 Å structure of the *Geobacter sulfurreducens* TupA–tungstate complex has been released (3LR1.pdb), although a publication describing this structure has yet to appear [24]. Unfortunately, the tungstate oxoanion has been modeled as a single point in space, and since no EXAFS data on any TupA are available, it is presently not clear whether the coordination geometry around the tungsten is tetrahedral, or octahedral, or, for that matter, anything else. We can, however, draw some preliminary conclusions from inspection of the pdb file assuming the tungstate to have metal–oxygen bonds comparable to those in ModA and WtpA (1.7–2.2 Å cf Fig. 4a and b). In Fig. 4c the structural elements around the oxoanion "W" are highlighted; in particular, all atoms are marked that are within a radius of 4 Å (solid circles) or 4.5 Å (broken circles) of the barycenter of the tungstate. The binding pocket appears to be open on one side with several water molecules in hydrogen-bonding distance from the oxoanion. Although the 63 TupA sequences considered here have several conserved aspartates, none is even remotely in a position to form a coordination bond with the metal, and it is clear that the Asp-160 effect in WtpA has no equivalent in the TupA structure. In fact, no direct coordination to W appears to be possible for any amino acid. The predicted oxoanion-protonating histidine is not found in the *G. sulfurreducens* structure. The His nitrogen closest to the tungstate barycenter is at a distance of circa 7 Å. The TTTS motif is present and threonine-9 and serine-11 are in a position to form hydrogen bonds to the oxoanion. Another conserved threonine, Thr-124, is also in potentially hydrogen-bond forming distance. The 'extending' arginine is also in place as Arg-118 with its terminal nitrogens <4.5 Å, respectively, <4 Å away from the W-point, and this positive charge clearly represents a major difference compared to the WtpA or the ModA structure. Why this particular structural element would afford the (very) high selectivity of the TupA proteins remains to be resolved.

5.5. The MOT1 binding mode is not known

The eukaryotic MOT1 system has been studied only from two species: the monocellular green alga *C. reinhardtii* [38] and the plant *A. thaliana* [37]. A BLAST of *A. thaliana* MOT1 versus non-redundant protein sequences returns homology with the *C. reinhardtii* MoT1 at an expectation value of circa 10^{-27}, and with 35 eukaryotic sequences of even lower *E*-value typically annotated as 'putative sulfate transporter'. At least 11 residues are fully conserved amongst the 37 sequences, suggesting that also this system carries a rather well conserved binding pocket for its substrate molybdate. No crystal structure has been reported; MOT1 has no significant sequence homology to ModA, WtpA, TupA, or ModE.

5.6. ModE binding: extended H-bonding, a lysine salt bridge, and a directing carbonyl?

As the cytoplasmic *E. coli* ModE is a negative regulator of expression of the ModABC transporter, it should undergo a significant conformational change upon binding of molybdate, in order to make it ready for interaction with double-stranded DNA. This is indeed what transpires from a comparison of the apo and oxoanion-bound crystal structures [25–27]. ModE is a homodimer, with an N-terminal DNA binding domain and a C-terminal oxoanion-binding domain in each subunit, affording two oxoanion binding sites each with ligands from two subunits. Molybdate locks ModE in its regulatory conformation with a binding pattern that not only involves the significantly higher number of nine hydrogen bonds than found in the periplasmic binding proteins ModA (seven) or WtpA (seven or eight), but also a salt-bridge interaction with a lysine-183, and finally, with what appears to be a direct coordination bond with the backbone carbonyl oxygen of serine-126 (Fig. 4d). This Ser═O–Mo bond has been proposed to have a directional effect on the oxoanion tetrahedron based on the observation that the carbonyl oxygen is approximately equidistant from three of the molybdate oxygens [26]. This unusual feature has a pendant in non-biological coordination chemistry of high-valent molybdenum in the Mo(VI) enolate complex $[Mo(NAr)_2Cl\{\eta^2\text{-}C(Me_2)CO_2Bu^t\}]$ [54].

A BLAST ran for this review of *E. coli* ModE against the non-redundant protein sequences returns 33 homologs with $E < 10^{-20}$ with almost full conservation of the molybdate ligands. In particular, the putatively coordinating carbonyl Ser-126 is conserved in virtually all cases with one Ser to Thr substitution, the salt-bridging Lys-183 is fully conserved, and so are Arg-128 and Thr-163. Alcoholic-O H-bonding Ser-166 is replaced with Ala in two out of 34 cases, and backbone-N H-bonding Ala-184 is sometimes Ser (three times) or Gly (once).

An equivalent of ModE for the Wtp or Tup systems has not been identified (yet), but the WtpC protein of *M. acetivorans* (in literature known as MaModC) has a C-terminal extension, not found (yet) in any other WtpC system, that looks remarkably like the oxoanion-binding part of ModE both in its secondary and its tertiary structure [16]. The role of this WtpC extension is also a regulatory one: binding of tungstate locks the WtpBC complex in a conformation

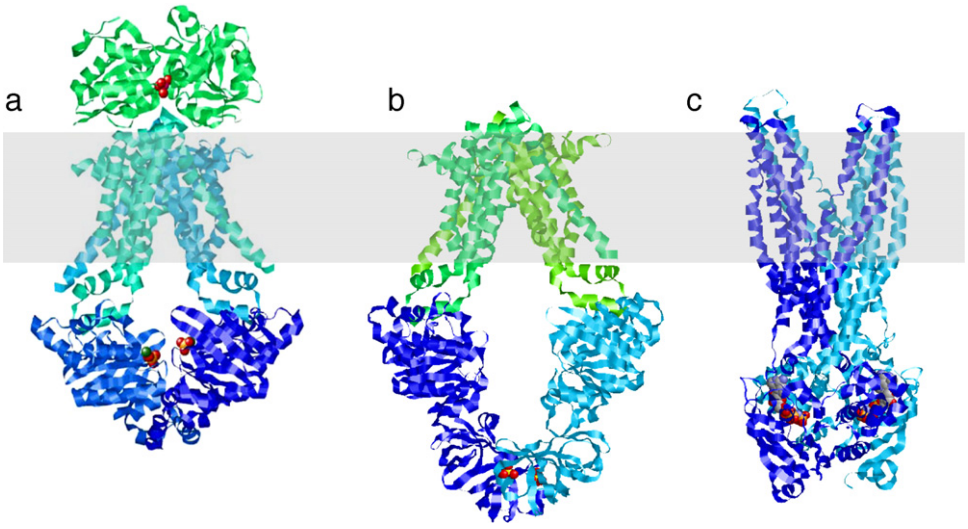

Fig. 5. Structures of transmembrane complexes in inward-facing and outward-facing conformations. (a) *A. fulgidus* $WtpAB_2C_2$ with tungstate bound to the A-protein and phosphates bound to the C-subunits, (b) *M. acetivorans* $WtpB_2C_2$ with two tungstates bound to the C-subunits, (c) *S. aureus* Sav1866 dimer with two AMP-PNPs bound to the cytoplasmic domain. The grey bar symbolizes the plasma mebrane. Arbitrary colors are used to discriminate different subunits. PDB files: 2ONK, 3D31, 2ONJ.

that blocks ATP hydrolysis and thus also transport of periplasmic tungstate. This regulatory mechanism has been named trans inhibition [16].

6. Working mechanism(s) of Mo/W transport

6.1. The minimal hypothesis: two states versus three states

For an evaluation of our present knowledge of the molybdate transporter as a molecular machine we start from an analogy with a macroscopic machine. Conceptually the simplest form of the internal combustion engine is the two-stroke variant. Although a two-state model may suffice to picture its primary mechanical movements, a minimum of three different events is required for even the most basic description of its mode of action: compression, power stroke, and exhaust. By the same token the working of a biological engine can be understood in basic terms as the cyclic sequence of minimally three events such as the three Boyer conformations (loose, tight, open) of the catalytic subunits in ATPsynthase [55,56] or the torque generation (proton binding, power stroke, proton release) that drives this synthase [57] (see also [58]). Clearly, the number of states (or intermediates) increases with the level of descriptional detail of these nano-engines in action, but the key question that we will address here is whether a proposed minimal model of two conformations [7,16,18,59–61] finds sufficient support in structural studies of the molybdate transporter.

6.2. Crystallographic studies of ABC transporters

ABC transporters are 'active', i.e. they exert work: they create a solute gradient, at the expense of ATP hydrolysis. The central event should be a (at least one) conformational change induced either by binding of ATP, or by its hydrolysis, or by release of ADP. Perhaps the simplest way to picture such a two-state protein conformer would be an open-ended funnel-like structure (such as indicated in the cartoon of Fig. 1) whose opening moves from one side of the membrane to the other.

This simple mechanics is indeed what has been suggested to occur at the molecular level in the molybdate transporting machine, as grasped from inspection of the two available 3D structures of Wtp transporters in comparison with structures from a bacterial export system (Fig. 5).

The left-hand structure (a) is that of *A. fulgidus* WtpABC with tungstate bound to the WtpA periplasmic binding protein and with two ADPs bound to the two WtpC subunits that in vivo stick out into the cytoplasm [7]. This is called the inward-facing orientation with reference to the conformation of the transmembrane WtpB subunits that are thus blocked for reception of the oxoanion. The middle structure is *M. acetivorans* WtpBC with two ATPs bound to the WtpC dimer, and in this complex the WtpA protein is absent [16]. Interestingly, this is also an inward-facing conformation. The complex has been proposed to be stable by virtue of the fact that this *M. acetivorans* variant of the Wtp system happens to have C-subunits with elongated primary structure, and these C-terminal extensions form two binding pockets for molybdate or tungstate, which, when occupied (as in Fig. 5b), result in inhibition of ATP hydrolysis and thus in preventing the conformation to change to outward-facing [16]. The right-hand structure (c) is the B_2C_2 complex of the multidrug transporter Sav1866 from *Staphylococcus aureus* in complex with the non-hydrolysable ATP analog adenosine-5′-(beta,gamma-imido)triphosphate, or AMP–PNP, in an outward-facing conformation [62].

From these conformational snapshots a two-state minimal model has been deduced in the literature for the working mechanism of these ABC transporters; binding of ATP affords an outward-facing conformation which, upon hydrolysis of ATP, returns to an inward-facing conformation [7,16,18]. A cartoon of this model is given in Fig. 6a.

6.3. The transport mechanism is insufficiently defined

In addition to the general objection that a machine in action cannot be described with two states only, there are several specific problematic issues, which are perhaps well illustrated by expanding the two-state cartoon into a complete minimum hypothesis scheme including the events of small-molecule binding and release in Fig. 6b. Note that the outward-facing conformations of the protein complex are color-coded in soft red to indicate that the proposal implies these conformations to be metastably induced. The following questions arise: (i) why is the *M. acetivorans* WtpBC complex with ATP bound not in the outward-facing conformation; (ii) why *is* the *S. aureus* Sav1866BC complex with ADP bound in the outward-facing conformation. Regarding the first question the crystallographers claim that the inward-facing conformation

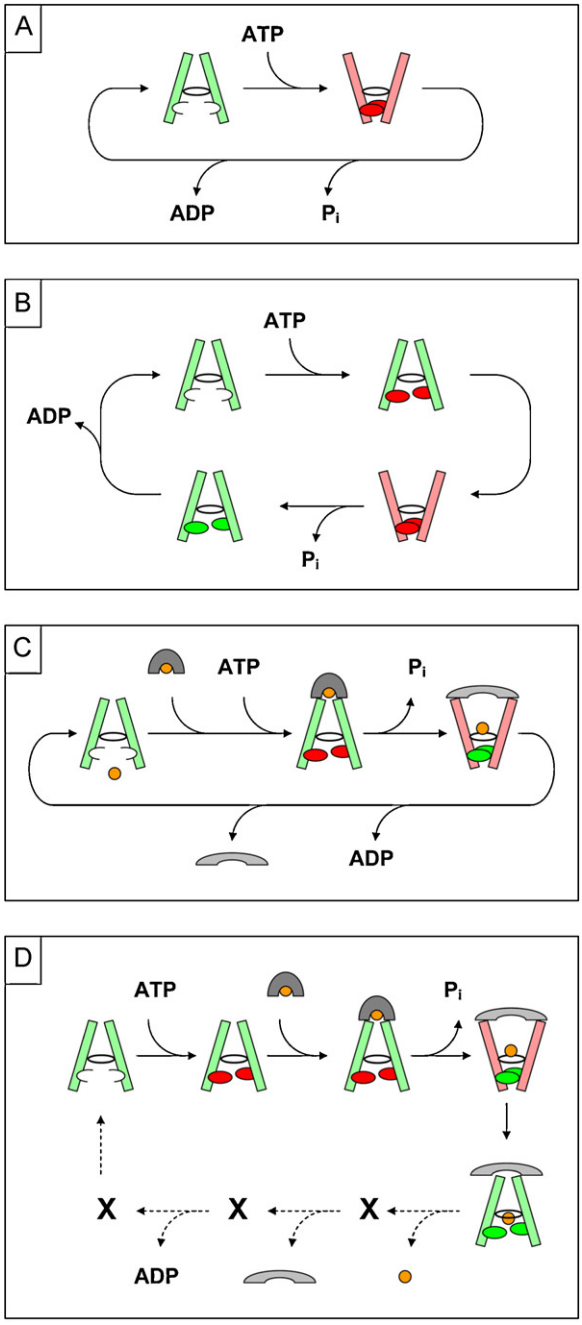

Fig. 6. Schematic models for the mechanism of action of (molybdate/tungstate) ABC transporters. The two colored bars with their middle connected by a ring symbolize two BC dimers separated by a space that exceeds the size of the oxoanion. Red bars are in an energized conformation; green bars are relaxed. Small ellipses are bound ATP (red) or ADP (green). In panels c and d the grey arc is the periplasmic binding protein, and the orange ball is the oxoanion. (a) A two-state model inferred from crystallographic studies in which the transmembrane complex switches between inward-facing and outward-facing conformations; (b) the same model but now including all primary binding events; (c) a model in which binding of the loaded periplasmic protein initiates pumping action; (d) the same model plus binding events; the X's indicate that the order of occurrence of these intermediates is unknown.

is retained because bound oxoanion prevents ATP hydrolysis [16], however, the model would require that bound molybdate prevents a change to the metastable conformation and how this could come about is unclear. In addressing the second question the crystallographers suggest that "purification and crystallization conditions, in particular the presence of detergent, have shifted the conformational equilibrium of Sav1866 to the ATP-bound state" [63]. This reviewer would venture the suggestion to be unsatisfactory, not only because there is no proof that the two states are in equilibrium, but also because it is a *de facto* declaration of defeat on the part of functional protein crystallography to solve the mechanism of these transporters. Indeed, in a subsequent study no conformational difference whatsoever was observed between the complex with ADP-bound versus that with the non-hydrolysable ATP analog AMP–PMP-bound [62,63].

Originally for the *E. coli* maltose ABC transporter it has been proposed that the periplasmic binding protein undergoes a conformational change upon binding of the substrate maltose and that subsequent binding of the loaded protein to the transmembrane complex induces a mechanistically essential conformational change, which triggers the pumping action [64]. Recently, this proposal has also been adapted to describe the action of *A. fulgidus* WtpABC tungstate transporter [65,66]. This signals a paradigm change from a model in which ATP binding is the initiator of action to one in which periplasmic protein binding in concert with (or in sequence with) ATP hydrolysis are the triggers. It would also be consistent with the recently reported conformational difference between *M. acetivorans* WtpA in its apo-form versus its oxoanion-bound form [19]. The published cartoons for this model [64–66] are redrawn in our format in Fig. 6c, and since they are once more incomplete, we also give an extended version in Fig. 6d that includes the primary binding and release events.

Inspection of the schemes in Fig. 6 (and possible alternatives) inevitably leads to the conclusion that crystallographic studies, in spite of the intrinsic beauty of the resolved 3D structures of these ABC transporters, have not yet led to consistent insight in their mechanism of action. Several important mechanistic issues still remain to be addressed, the most important one being the question of what exactly is the stroke/leverage event in these machines. Additional and related problems are: (a) is binding of the loaded A-protein preceding or following binding of ATP? (b) What is the order of events in the release of phosphate, molybdate/tungstate, and apo A-protein? (c) Are the inward-facing and outward-facing conformations largely different in total energy (i.e. a stable ground state and a metastable excited state) or are they rather in quasi equilibrium? (d) Is the oxoanion like a smooth ball rolling through a 'teflon-coated' tunnel [18], or are there yet to be discovered specific binding interactions? Experimentally addressing these difficult questions may well require combining the crystallography with multiple forms of time-resolved spectroscopy.

6.4. Multiple controls of cellular Mo/W levels

Mo and W homeostasis also depend on other factors than the functioning of their transporter systems. Since the subject transgresses the scope of this chapter, we give here only a succinct summary of the (limited) state of the art in terms of key factors involved and of the experimental basis for their operation.

Fig. 7 is a cartoon for a generalized prokaryotic cell. Each process appears to occur in some species, but all species may not accommodate all events. Possible processes are: (1) at least some of the molybdate ABC transporters exhibit 'selection at the gate', i.e. the WtpA and the TupA systems discriminate between molybdate versus tungstate by means of differential binding affinities [12,20,41]; (2) at least one system, the *M. acetivorans* WtpBC, exhibits trans inhibition, i.e. binding of molybdate or tungstate to the WtpC subunits blocks the pump presumably by preventing ATP hydrolysis [16]; (3) in some species (e.g., *Clostridium pasteurianum* Mop) hexameric 'molbindin' proteins are found that can bind 6–8 molybdates and they have been proposed to act as storage devices [67–71]. A different protein, thus far only studied in *A. vinelandii*, has a more substantial binding capacity of some 100 oxoanions per

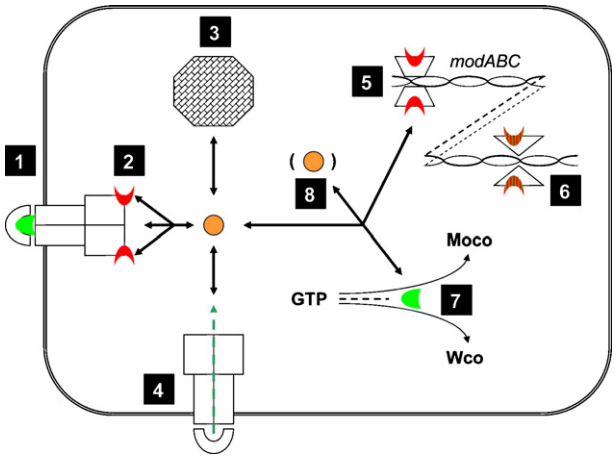

Fig. 7. Overview of regulatory events in the homeostasis of intracellular molybdate and/or tungstate. Not all events necessarily occur in a single species. The enclosure is a plasma membrane; the oxoanion is symbolized by an orange ball; green half-moon symbols indicate selection points; red quarter-moon symbols indicate inhibition points. Regulatory events: (1) selection between molybdate versus tungstate by A-protein binding; (2) trans inhibition by binding of oxoanion to regulatory sites on the C-subunits; (3) storage of oxoanions in/on specific storage proteins; (4) import of molybdate by non-specific pumps; (5) specific inhibition of transporter gene transcription by DNA-binding of oxoanion-activated inhibitor protein (6) up- or down-regulation of genes involved in molybdenum processing; (7) selection between molybdate versus tungstate at the level of cofactor synthesis; (8) changes in speciation of free oxoanion following changes in intracellular concentration of complexing solutes.

hexamer [72]; (4) molybdate can also enter through 'leakage' by parasitic use of other systems, in particular the sulfate ABC transporter [73]; (5) transcription of the *ModABC* operon is inhibited when the ModE dimer in complex with molybdate binds to the proper DNA regulatory site [9,27]. Equivalent systems outside the Mod family are yet to be discovered; (6) gene-expression relations can also be less specific than between molybdate/tungstate and synthesis of their ABC transporter mRNA. For example, the *mod* operon is upregulated by arsenite [74]. Also, production of the Mo/Se-enzyme *E. coli* formate hydrogenlyase is antagonized by tungstate [75]. And algal MoT1 synthesis is upregulated, and activity is stimulated, by nitrate [38]; (7) distinct but homologous sets of enzymes catalyze the biosynthesis of the molybdenum cofactor Moco versus the tungsten cofactor Wco, but, e.g., the tungstate-specific *P. furiosus* set can also incorporate Mo, although with lower overall yield [3]. Effectively these systems thus represent a second selection step for tungstate versus molybdate (or the other way around); (8) finally, the free oxoanion will be subject to speciation in the cytoplasm; cf. the specific complexation with the Mg^{2+} ion in vitro [76]. Little is known about molybdate speciation in vivo.

The model in Fig. 7 presumes that the oxoanions are available in the environment and are not hampered in their diffusive itinary to the plasma membrane. That this is not necessarily true for soil bacteria has been documented in a recent series of papers on Mo homeostasis in Mo-nitrogenase producing *A. vinelandii* by Kraepiel et al. (e.g., [77–80]). Siderophores (i.e. iron complexing agents) of the bis- and tris-catecholate type also have relatively high affinity (submicromolar K_D-values) for molybdate (they are also 'molybdophores'), and their release can compensate for the low free molybdate concentration in soil due to oxoanion adsorption to mineral surfaces and complexation by tannin-like compounds [80]. Coordination chemical information is thus far limited to XAS data comparison with model compounds of known structure [77]. The question whether sequestring of molybdate (and tungstate [78] and vanadate [79]) also occurs in other species (e.g., non-nitrogen-fixing, aquatic microorganisms) is yet to be addressed.

7. What remains to be addressed

The coordination chemical contours of the initial sequestring event by specific molybdate import systems are taking shape. The principle of selectivity for Mo versus W needs sharper definition. Apparently, at least four different modes of binding the oxoanion can be discerned. Is there an underlying unifying principle in these patterns, or do they represent multiple independent routes of converting evolution? With a present paucity of data on synthetic models for the binding interactions, it would seem that model-compound chemistry could develop an important clarifying role here.

The available X-ray data on the membrane-spanning Wtp complexes attest clearly to the bearing of protein crystallography to our understanding of molecular machines, however, their inspection is yet to lead to insight in the primary molecular steps of energy-consuming gradient building. Key features of the mechanism of action, such as, what is (are) the energy-transducing event(s) and what, if any, is the molecular interaction of oxoanions with the pump channel, are unclear or even undefined.

The subject of molybdenum homeostasis, or the regulation of intracellular Mo/W concentrations, has many loose ends waiting to be addressed. Are there ModE equivalents in the Tup and Wtp systems? Is the trans inhibition observed for the *M. acetivorans* Wtp system an exception? What happens upon release of the oxoanion from the pump, i.e. what is the intracellular (and in eukaryotic cells organellar) speciation? And how does the biosynthetic machinery contribute to Mo versus W selection while it converts the oxoanion into the Moco/Wco thiolato complex?

The single-subunit eukaryotic MOT1 system, recently identified in plants and algae, is still poorly characterized. There are no structural data yet. The *Arabidopsis* protein appears to function in mitochondrial import, which raises the question whether there is also a specific cytoplasmic importer. In addition to the *MoT1* gene *Chlamydomonas* carries another gene whose mutation appears to lead to impaired Mo import. How 'eukaryotic' is this system anyway, when homolog *MoT1* genes are also found in bacteria. And how do animals, including man, get essential molybdate into their cells?

Breakthroughs in searches for answers to these numerous questions are likely to come from an interplay between different disciplines, notably, physiological, biochemical, and protein crystallographical studies, and in this combination a strong coordination chemical component will have a notable presence.

References

[1] J.R.R. Fraústo da Silva, R.J.P. Williams, The Biological Chemistry of the Elements, Oxford University Press, 1991.
[2] L.E. Bevers, P.-L. Hagedoorn, W.R. Hagen, Coord. Chem. Rev. 253 (2009) 269.
[3] A.-M. Sevcenco, L.E. Bevers, M.W.H. Pinkse, G.C. Krijger, H.T. Wolterbeek, P.D.E.M. Verhaert, W.R. Hagen, P.-L. Hagedoorn, J. Bacteriol. 192 (2010) 4143.
[4] K. Osseo-Asare, Metall. Trans. B 13 (1982) 555.
[5] I.H. Warren, D.C. Reid, Metall. Trans. B 13 (1982) 565.
[6] T. Ozeki, H. Kihara, S. Ikeda, Anal. Chem. 60 (1988) 2055.
[7] K. Hollenstein, D.C. Frei, K.P. Locher, Nature 446 (2007) 213.
[8] S. Rech, U. Deppenmeier, R.P. Gunsalus, J. Bacteriol. 177 (1995) 1023.
[9] A.M. Grunden, R.M. Ray, J.K. Rosental, F.G. Healy, K.T. Shanmugam, J. Bacteriol. 178 (1996) 735.
[10] Y. Hu, S. Rech, R.P. Gunsalus, D.C. Rees, Nature Struct. Biol. 4 (1997) 703.
[11] K. Makdessi, J.R. Andreesen, A. Pich, J. Biol. Chem. 276 (2001) 24557.
[12] L.E. Bevers, P.-L. Hagedoorn, G.C. Krijger, W.R. Hagen, J. Bacteriol. 188 (2006) 6498.
[13] R.N. Schicho, L.J. Snowden, S. Mukund, J.B. Park, M.W.W. Adams, R.M. Kelly, Arch. Microbiol. 145 (1993) 380.
[14] S. Mukund, M.W.W. Adams, J. Bacteriol. 178 (1996) 163.
[15] K. Hollenstein, M. Comellas-Bigler, L.E. Bevers, M.C. Feiters, W. Meyer-Klaucke, P.-L. Hagedoorn, K.P. Locher, J. Biol. Inorg. Chem. 14 (2009) 663.
[16] S. Gerber, M. Comellas-Bigler, B.A. Goetz, K.P. Locher, Science 321 (2008) 246.
[17] B.A. Goetz, E. Perozo, K.P. Locher, FEBS Lett. 538 (2009) 266.
[18] K.P. Locher, Philos. Trans. R. Soc. B 364 (2009) 239.

[19] S. Chan, I. Giuroiu, I. Chernishof, M.R. Sawaya, J. Chiang, R.P. Gunsalus, M.A. Arbing, L.J. Perry, Acta Crystallogr. F 66 (2010) 242.
[20] J.R. Andreesen, K. Makdessi, Ann. N.Y. Acad. Sci. 1125 (2008) 215.
[21] Y. Zhang, V.N. Gladyshev, J. Biol. Chem. 285 (2010) 3393.
[22] D.M. Lawson, C.E.M. Williams, L.A. Mitchenall, R.N. Pau, Structure 6 (1998) 1529.
[23] A. Balan, C. Santacruz-Pérez, A. Moutran, L.C. Ferreira, G. Neshich, J.A.R. Barbosa, Biochim. Biophys. Acta 1784 (2008) 393.
[24] R. Zhang, L. Volkart, K. Buck, A. Joachim, (2010) http://www.pdb.org/pdb/explore/explore.do?structureId=3LR1.
[25] D.R. Hall, D.G. Gourley, G.A. Leonard, E.M.H. Duke, L.A. Anderson, D.H. Boxer, W.N. Hunter, EMBO J. 18 (1999) 1435.
[26] D.G. Gourley, A.W. Schüttelkopf, L.A. Anderson, N.C. Price, D.H. Boxer, W.N. Hunter, J. Biol. Chem. 276 (2001) 20641.
[27] A.W. Schüttelkopf, D.H. Boxer, W.N. Hunter, J. Mol. Biol. 326 (2003) 761.
[28] K. Pyrzynska, Anal. Chem. 590 (2007) 40.
[29] A.K. Das, R. Chakraborty, M.L. Cervera, M. de la Guardia, Talanta 71 (2007) 987.
[30] L.I. Hart, M.A. McGartoll, H.R. Chapman, R.C. Bray, Biochem. J. 116 (1970) 851.
[31] J. Cardenas, L.E. Mortenson, Anal. Biochem. 60 (1974) 372.
[32] P.-L. Hagedoorn, P. van't Slot, H.P. van Leeuwen, W.R. Hagen, Anal. Biochem. 297 (2001) 71.
[33] S. Rech, C. Wolin, R.P. Gunsalus, J. Biol. Chem. 271 (1996) 2557.
[34] J. Imperial, M. Hadi, N.K. Amy, Biochim. Biophys. Acta 1370 (1998) 337.
[35] M. Zahalak, B. Pratte, K.J. Werth, T. Thiel, Mol. Microbiol. 51 (2004) 539.
[36] A.-M. Sevcenco, M.W.H. Pinkse, E. Bol, G.C. Krijger, H.Th. Wolterbeek, P.D.E.M. Verhaert, P.-L. Hagedoorn, W.R. Hagen, Metallomics 1 (2009) 395.
[37] H. Tomatsu, J. Takano, H. Takahashi, A. Watanabe-Takahashi, N. Shibagaki, T. Fujiwara, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 18807.
[38] M. Tejada-Jiménez, Á. Llamas, E. Sanz-Luque, A. Galván, E. Fernández, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 20126.
[39] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.-F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.-M. Claverie, O. Gascuel, Nucleic Acids Res. 36 (2008) W465.
[40] A. Balan, C.P. Santacruz, A. Moutran, R.C. Ferreira, C.H.I. Ramos, J. Medrano, L.C. Ferreira, Protein Expr. Purif. 50 (2006) 215.
[41] J.P. Smart, M.J. Cliff, D.J. Kelly, Mol. Microbiol. 74 (2009) 742.
[42] L.A. Anderson, T. Palmer, N.C. Price, S. Bornemann, D.H. Boxer, R.N. Pau, Eur. J. Biochem. 246 (1997) 119.
[43] I. Baxter, B. Muthukumar, H.C. Park, P. Buchner, B. Lahner, J. Danku, K. Zhao, J. Lee, M.J. Hawkesford, M.L. Guerinot, D.E. Salt, PloS Genet. 4 (2008) e1000004.
[44] W. Li, D.R. Fingrut, D.P. Maxwell, Physiol. Plantarum 136 (2009) 336.
[45] E. Gürmen, E. Daniels, J.S. King, J. Chem. Phys. 55 (1971) 1093.
[46] R.D. Shannon, Acta Cryst. A 32 (1976) 751.
[47] K.-H. Tytko, Polyhedron 5 (1986) 497.
[48] G.C. Bond, J. Mol. Catal. A 156 (2000) 1.
[49] G.C. Tucci, J.P. Donahue, R.H. Holm, Inorg. Chem. 37 (1998) 1602.
[50] P.-L. Hagedoorn, W.R. Hagen, L.J. Stewart, A. Docrat, S. Bailey, C.D. Garner, FEBS Lett. 555 (2003) 606.
[51] L.E. Bevers, W.R. Hagen, Dalton Trans. (2009) 8168.
[52] M.E. Taveirne, M.L. Sikes, J.W. Olsen, Mol. Microbiol. 74 (2009) 758.
[53] D. Rauh, PhD Thesis, University of Halle, Germany, 2009 (http://nbn-resolving.de/urn:nbn:de:gbv:3:4-595).
[54] P.A. Cameron, G.J.P. Britovsek, V.C.D. Gibson, J. Williams, A.J.P. White, Chem. Commun. (1998) 737.
[55] P.D. Boyer, Annu. Rev. Biochem. 66 (1997) 717.
[56] Y. Rondelez, G. Tresset, T. Nakashima, Y. Kato-Yamada, H. Fujita, S. Takeuchi, H. Noji, Nature 433 (2005) 773.
[57] C. von Ballmoos, A. Wiedermann, P. Dimroth, Annu. Rev. Biochem. 78 (2009) 649.
[58] H.C. Berg, Annu. Rev. Biochem. 72 (2003) 19.
[59] K. Hollenstein, R.J.P. Dawson, K.P. Locher, Curr. Opin. Struct. Biol. 17 (2007) 412.
[60] H.W. Pinkett, A.T. Lee, K.P. Locher, D.C. Rees, Science 315 (2007) 373.
[61] M.L. Oldham, D. Khare, F.A. Quiocho, A.L. Davidson, J. Chen, Nature 450 (2007) 515.
[62] R.J.P. Dawson, K.P. Locher, FEBS Lett. 581 (2007) 935.
[63] R.J.P. Dawson, K.P. Locher, Nature 443 (2006) 180.
[64] J. Chen, S. Sharma, F.A. Quiocho, A.L. Davidson, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 1525.
[65] A.L. Davidson, E. Dassa, C. Orelle, J. Chen, Microbiol. Mol. Biol. Rev. 72 (2008) 317.
[66] O. Lewinson, A.T. Lee, K.P. Locher, D.C. Rees, Nat. Struct. Mol. Biol. 17 (2010) 332.
[67] U.G. Wagner, E. Stupperich, C. Kratky, Structure 8 (2000) 1127.
[68] L. Delarbre, C.E. Stevenson, D.J. White, L.A. Mitchenall, R.N. Pau, D.M. Lawson, J. Mol. Biol. 308 (2001) 1063.
[69] A.W. Schüttelkopf, J.A. Harrison, D.H. Boxer, W.N. Hunter, J. Biol. Chem. 277 (2002) 15013.
[70] K. Makdessi, K. Fritsche, A. Pich, J.R. Andreesen, Arch. Microbiol. 181 (2004) 45.
[71] S.L. Masters, G.J. Howlett, R.N. Pau, Arch. Biochem. Biophys. 439 (2005) 105.
[72] D. Fenske, M. Gnida, K. Schneider, W. Meyer-Klaucke, J. Schemberg, V. Henschel, A.-K. Meyer, A. Knöchel, A. Müller, ChemBioChem 6 (2005) 405.
[73] K.L. Fitzpatrick, S.D. Tyerman, B.N. Kaiser, FEBS Lett. 582 (2008) 1508.
[74] D.R. Kashyap, L.M. Botero, C. Lehr, D. Hassett, T.R. McDermott, J. Bacteriol. 188 (2006) 1577.
[75] A. Hasona, W.T. Self, R.M. Ray, K.T. Shanmugan, FEMS Microbiol. Lett. 169 (1998) 111.
[76] T. Ozeki, K. Murata, H. Kihara, S. Hikime, Bull. Chem. Soc. Jpn. 60 (1987) 3585.
[77] J.-P. Bellenger, F. Arnaud-Neu, Z. Asfari, S.C.B. Myneni, E.I. Stiefel, A.M.L. Kraepiel, J. Biol. Inorg. Chem. 12 (2007) 367.
[78] T. Wichard, J.-P. Bellenger, A. Loison, A.M.L. Kraepiel, Environ. Sci. Technol. 42 (2008) 2408.
[79] J.P. Bellenger, T. Wichard, A.B. Kustka, A.M.L. Kraepiel, Nat. Geosci. 1 (2008) 243.
[80] T. Wichard, B. Mishra, S.C.B. Myneni, J.-P. Bellenger, A.M.L. Kraepiel, Nat. Geosci. 2 (2009) 625.