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Azotobacter vinelandii Metal Storage Protein: "Classical" Inorganic Chemistry Involved in Mo/W Uptake and Release Processes

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Dedicated to Professor Dante Gatteschi

The release of Mo (as molybdate) from the Mo storage protein (MoSto), which is unique among all existing metalloproteins, is strongly influenced by temperature and pH value; other factors (incubation time, protein concentration, degree of purity) have minor, though significant effects. A detailed pH titration at 12 °C revealed that three different steps can be distinguished for the Mo-release process. A proportion of ~15% at pH 6.8–7.0, an additional 25% at pH 7.2–7.5 and ca. *50% (up to 90% in total) at pH 7.6–7.8. This triphasic process supports the assumption of the presence of different types of molybdenum-oxide-based clusters that exhibit different pH lability. The complete release of Mo was achieved by increasing the temperature to 30 °C and the pH value to > 7.5. The Mo-release process does not require ATP; on the contrary, ATP prevents, or at least reduces the degree of metal release, depending on the concentration of the nucleotide. From this point of view, the intracellular ATP concentration is suggested to play—in addition to the pH value—an indirect but crucial role in controlling the extent of Mo release in the cell. The binding of molybdenum to the apoprotein (reconstitution process) was confirmed to be directly dependent on the presence of a nucleotide (preferably ATP) and $MgCl_2$. Maximal reincorporation of Mo required 1 mM ATP, which could partly be replaced by GTP. When the storage protein was purified in the presence of ATP and $MgCl_2$ (1 mM each), the final preparation contained 80 Mo atoms per protein molecule. Maximal metal loading (110–115 atoms/MoSto molecule) was only achieved, if Mo was first completely released from the native protein and subsequently (re-) bound under optimal reconstitution conditions: 1 h incubation at pH 6.5 and 12 °C in the presence of ATP, $MgCl_2$ and excess molybdate. A corresponding tungsten-containing storage protein ("WSto") could not only be synthesized in vivo by growing cells, but could also be constructed in vitro by a metalate–ion exchange procedure by using the isolated MoSto protein. The high W content of the isolated cell-made WSto (~110 atoms/protein molecule) and the relatively low amount of tungstate that was released from the protein under optimal "release conditions", demonstrates that the W-oxide-based clusters are more stable inside the protein cavity than the Mo-oxide analogues, as expected from the corresponding findings in polyoxometalate chemistry. The optimized isolation of the W-loaded protein form allowed us to get single crystals, and to determine the crystal X-ray structure. This proved that the protein contains remarkably different types of polyoxotungstates, the formation of which is templated in an unprecedented process by the different protein pockets. (*Angew. Chem. Int. Ed. ***2007***, 46, *2408–2413).*

Introduction

Molybdenum is an essential transition metal to all organisms. It is specifically bound to a large number of enzymes and proteins that are involved in Mo uptake/transport, Mo processing for cofactor biosyntheses, enzyme catalysis, gene regulation, intracellular Mo homeostasis and metal storage.[1,2] Based on the function of molybdenum, the protein's structural characteristics, and the type of Mo species that are present in the protein, molybdoproteins can be divided into five classes (for a short overview, see ref. [2]). Besides the cofactor-containing enzymes (Moco enzymes, FeMoco nitrogenases),[3,4] the largest and best characterized group of Mo proteins comprises all proteins that are capable of binding molybdenum as single molybdate (Mod/Mop proteins, "molbindins"). MoO_4^{2-}-binding proteins are principally involved in Mo transport, gene regulation, intracellular Mo transfer and homeostasis, and contain between one and eight molybdate ions (e.g., ModA and ModG of *A. vinelandii*).[5,6,7]

The Mo storage protein (MoSto), which is subject of the present study, exhibits several outstanding properties which differ fundamentally from those of all other known Mo-containing proteins and even of all metalloproteins.[2,8] It is functionally very closely connected to nitrogen fixation, however—

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in contrast to all *nif* gene products—it is not repressed by ammonium ions.[2] This enables the storage protein, independently from the nitrogen source, to accumulate large amounts of Mo inside the cell, thus guaranteeing Mo-dependent nitrogen fixation even under conditions of extreme Mo deficiency. The high-capacity Mo storage protein in combination with high-affinity Mo-transport proteins (Mo uptake into the cell)[1] represent a system that scavenges Mo very effectively from outside the cell, and might thus even cause a self-produced Mo starvation in the surrounding environment.[9,10] In natural habitats, where organisms compete for molybdate, species like *A. vinelandii* therefore have a great competitive advantage. The MoSto protein, which was first isolated and described already in 1981[11] has recently been analyzed to contain 70–90 Mo atoms per protein molecule,[2] but its maximal Mo-storage capacity is still unknown. In a recent single crystal X-ray structure analysis of the storage protein,[8] where for stability reasons molybdate was replaced by tungstate, 14 different polynuclear tungsten-oxide clusters that were embedded into pockets inside a large cavity of the cage-like protein complex, have been detected. The clusters (cluster fragments) can be divided into different types: (one) W_3, (three) W_6, (three) W_7, (three) W_2(I), (three) W_2(II), and (one) putative W_{7+x}. In this context, it should be mentioned that the incorporation of a variety of different polynuclear clusters that are encapsulated in the cavity of a protein is unprecedented, especially the fact that the formation of the different species is templated by the different pocket functionalities. Furthermore, besides the iron-containing systems (ferritin, frataxin) MoSto is the only known metal storage protein that contains a larger number of metal atoms.

Recent results of the single crystal structure analysis of the protein also revealed that the subunit structure is an $\alpha_3\beta_3$ hexamer that is organized as a trimer of $\alpha\beta$ dimers.[8] MoSto appears therefore to be much more complex than conventional molybdate-binding proteins,[2] its amino acid sequence does not show any similarity to sequences of other molybdoproteins either. Further, the existence of MoSto appears not to be an exclusive feature of the genus *Azotobacter*: current protein database comparisons revealed that MoSto genes (designated as *mosA* and *mosB*) also occur in *Rhodopseudomonas palustris, Xanthobacter autotrophicus, Bradyrhizobium* sp. and two non-diazotrophic *Nitrobacter* species (*N. hamburgensis, N. winogradskyi*).

It was a surprise to find that MoSto exhibits an architecture that is similar to that of nucleoside monophosphate kinases; the most related member is the hexameric UMP kinase.[2,12] This is in full accordance with 1) the detection of the P-loop motif of an ATP-binding site,[2] 2) the fact that the crystallized W-containing form of the storage protein contains functional ATP,[8] and 3) the finding that the binding of Mo/W to the apoprotein is ATP dependent. In contrast, the Mo-release reaction does not require ATP, but has been described to be pH regulated, that is, it occurs only above pH 7.1.[2] Both processes, Mo binding and release, appear to follow unprecedented (biological) mechanisms. There are, however, a great number of open questions. For instance, under which preparation conditions can the yield and stability of MoSto be substantially improved, the Mo content increased, and the Mo loss decreased? What are the optimal experimental conditions that allow the determination of the maximal Mo-binding capacity of the storage protein? Is the Mo binding only affected by ATP, and the Mo release only by pH changes, or are these processes also influenced by other important factors, such as temperature, incubation time, protein concentration and the degree of protein purity? Does an increase of the pH to values >7.0 cause a continuously increasing Mo release, an "all or nothing" release (switch on/switch off mechanism) or rather a stepwise release? Can Mo be exchanged by W, and do the tungsten-release and re-binding processes function according to the mechanisms described for Mo?

The studies concerned with these questions and problems are subject of the present work. The results that were obtained confirmed that the Mo storage protein represents a new type of molybdoprotein and even a new type of metalloprotein, and they provide optimal prerequisites for future crystallization studies and structural analyses. Detailed information about the isolation and crystallization of the W-loaded storage protein, which allowed us to perform a single-crystal X-ray structure analysis, and to prove that a new type of assembly process occurs in the protein cavity,[8] is presented here.

Results and Discussion

pH-regulated release of molybdate from the Mo storage protein

In the preceding paper[2] we characterized the process of Mo release from the Mo storage protein (MoSto) as a pH-regulated mechanism. Based on the results of the current study this could be confirmed; on the other hand this has been complemented by further fundamental information. The key condition that triggers the process of Mo release is the shift from weakly acidic to weakly alkaline pH values; however, the Mo release is significantly influenced also by the temperature, the incubation time, the protein concentration and the quality of preparation (purity, stability). The MoSto preparation that was used in the present study was homogeneous, stable, and it contained ~1.5 mg protein mL^{-1}, ca. 60 atoms of molybdenum per protein molecule and a basic low level of free molybdate that corresponds to 5–8% of protein-bound molybdenum.

To characterize the real relationship between the pH value and the Mo-release process, a detailed pH-dependence study was performed at 12 °C (Figure 1), at which a temperature-based Mo release was absent or negligibly low. After 1 h standard incubation time at 12 °C, no Mo release between pH 6.5 and 6.8 was observed. Over the course of further pH titration, it turned out that the Mo-release process did not, as concluded in preceding work (which was based on experiments at 30 °C), follow a "switch on" mechanism within a narrow range above pH 7.1, but rather occurred in three steps: 1) at pH 6.8–7.0 (very weak increase of the release level), 2) at pH 7.2–7.5 (significant release, ca. 40%), and 3) at pH 7.6–7.8 (most rapid and largest amount of Mo release). In pH regions between the release steps (7.0–7.2; 7.5–7.6; >7.8) the MoSto protein was

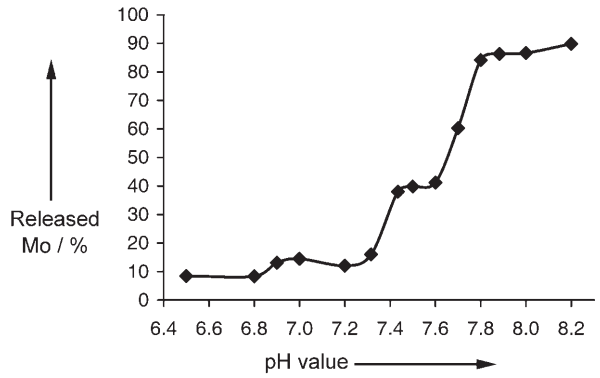

Figure 1. The pH dependence of the release of molybdenum at 12 °C. After adjustment of the selected pH value, the samples of the Mo storage protein (1.5 mg protein mL^{-1} each) were incubated for 1 h at 12 °C and subsequently applied to a Sephadex G-25 gelfiltration column. The resulting fractions were analyzed for protein-bound and released Mo.

stable and did not lose additional amounts of molybdenum (plateaus in the pH-dependence curve, see Figure 1). Within this physiologically acceptable pH range a complete (100%) release of Mo did not occur at 12 °C but required a temperature increase to 16 °C or higher (Figure 2, Mo-profile B).

How can it be explained that the pH-dependent Mo release neither occurs continuously nor abruptly (switch mechanism), but stepwise (triphasic) with increasing pH? Two processes presumably play crucial roles:

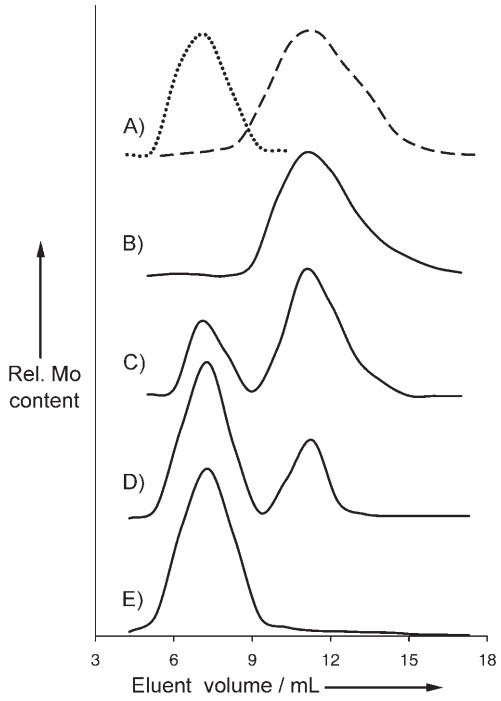

Figure 2. Mo elution profiles of Sephadex G-25 runs (pH 6.5) after quantitative Mo release and subsequent pre-treatment of MoSto with ATP at different concentrations. ATP was also present in the column elution buffers, its concentration each corresponded to that used in the preceding incubation step (1 h at 30 °C). 1 mM $MgCl_2$ was present in all solutions. A) Reference peaks of native MoSto (dotted line) and molybdate (dashed line); B apoprotein, Mo quantitatively released (after pretreatment at pH 7.6 and 30 °C for 1 h); C–E) re-binding of Mo at different ATP concentrations: C) 0.5 mM ATP; D) 0.7 mM ATP; E) 1 mM ATP.

1) It is known from basic chemistry that increasing the OH^- concentration in the polyoxometalate solutions leads to degradation of larger to smaller species and finally to single molybdate ions.[13] The most simple example would be the reaction of $Mo_2O_7^{2-}$ with OH^- to give two MoO_4^{2-} ions. The occurrence of several pH-dependent Mo-release steps could be explained by the presence of different kinds of metal–oxygen clusters, which are expected to have different affinities to protons and to react differently towards an OH^- increase.
2) It is also conceivable, on the other hand, that many of the hydrogen bonds, which have been proved to be involved in the binding of the Mo–O clusters within the MoSto protein,[8] might play a role in the Mo-release process. In the course of pH titration, amino acid residues that exhibit different pK_a values and are involved in cluster binding might become successively deprotonated; this could lead to a cleavage of hydrogen bonds thus triggering the described steps of Mo release. Histidine is the only ionic amino acid with a pK_a that is near neutral pH (6.1), and is therefore predestined to donate and accept protons, and thus to play a key role in the Mo-uptake and release process in the case of the storage protein. pK_a variations within a certain range might be caused by deviations in the internal protein shell forming the cavity. In fact, twelve histidine residues are located in the α-subunit[2] that might be involved in binding the metal clusters. In the case of the structurally analyzed tungstate-loaded storage protein, three histidines are located in the close neighborhood of both the W_6 and W_7 clusters, however, an unambiguous identification of H-bonds was not yet possible, because the potentially involved tungstate O atoms could not be localized due to low occupancy.[8] A clear identification was only possible in the case of the trinuclear W_3 cluster, where three direct and comparably strong W–N–His interactions were found to be (probably) responsible for the high occupancy/stability.[8]

From the available data from both the present and the crystallographic study, it can be postulated (definitely with the exception of the W3 and W_{7+x} clusters) that, upon increase of the pH value, both processes, the deprotonation of histidines and the polyoxometalate (POM) degradations are "working hand-in-hand" with regard to cluster destabilization. The clusters in the protein-bound form are certainly more pH stable than in the isolated form at comparably low concentration in solution; however, as soon as one or even more than one stabilizing bond to the protein pocket is broken, the degradation process is initiated and finally ends with the release of metalate. The stabilization/destabilization processes of the polyoxometalates that are responsible for their function in the cell protein, are of course one of the fascinating aspects of the work.

The sequence of cluster release and the events that trigger the destabilization and degradation of the clusters are not yet known in detail. Furthermore it has to be considered that the complete release or the partial decomposition of one or more cluster(s) might cause changes in the protein environment (conformational alterations, different structural flexibility),

which could also significantly influence neighboring protein–cluster interactions, and thus affect the further process of cluster degradation and the following molybdate/tungstate release from the protein.

Other factors that influence molybdate release are temperature, incubation time and protein concentration. As already mentioned, a further strong effect on the release of Mo from the storage protein was caused by the incubation temperature. In Table 1 the temperature influence on the release process at

Table 1. Influence of temperature and incubation time on the release of molybdenum from MoSto. Aliquots (0.4 mL each) of a storage protein solution (1.5 mg mL^{-1} each) were treated as indicated in the table and then subjected to Sephadex G-25 gel filtration followed by Mo analysis of the column fractions. The release of 100% Mo corresponds to 210 nmol of MoO_4^{2-}. Experimental error (standard deviation): ~15%.

Conditions		Mo release
Constant	Variable	[%]
pH 6.5,	12 °C	trace
1 h of incubation	16 °C	26
	30 °C	56
pH 7.6,	12 °C	41
1 h of incubation	16 °C	65
	30 °C	~100
pH 7.6,	15 min incubation time	55
30 °C	30 min incubation time	76
	60 min incubation time	~100

different pH values is compared. At pH 6.5, at which a pH-based Mo release does not take place or is hardly detectable, more than half of the MoSto-bound Mo (56%) becomes released if the sample is incubated at 30 °C for 1 h. Under the same incubation conditions, but at pH 7.6 (the standard pH in "release experiments") the Mo release is quantitative (Table 1 and Figure 2, Mo profile B).

Under which condition is the release of Mo more strongly affected, by pH or by temperature? If we compare two results of the experiments, where either the temperature or the pH value is practically without influence on the Mo release, that is, 1) pH 8.0, 12 °C (temperature without significant effect) leads to ~90% Mo release, 2) pH 6.5, 30 °C (pH without significant effect) leads to ~55% Mo release, we come to the conclusion that the crucial effect is caused by the pH shift; this confirms our earlier assumption.[2] The effect that is caused by increasing the temperature only plays an indirect role in this process in that it accelerates the release rates. From inspection of Figure 1 it becomes obvious that at 12 °C, even an increase of the pH value to 8.0–8.2 did not lead to a complete Mo release. The residual amount of protein-bound Mo (5–10%) is only released when the pH is higher than 7.5 and the temperature is increased up to 30 °C. It could be assumed that the Mo_3 cluster—provided that the molybdate-loaded storage protein contains such a cluster analogously to the comparably strongly bonded W_3 cluster in the WSto protein form—would be the most stabilized as well as the "last" cluster to be released from the protein.

Furthermore, under in vitro conditions, the rate of Mo release depends strongly on the incubation time (Table 1). At pH 7.6 and 30 °C a 1 h incubation is required for a complete release of Mo from MoSto without an accompanying inactivation/partial denaturation of the apoprotein (full reincorporation of Mo after ATP/$MgCl_2$ treatment).

The protein concentration was also found to play a role in holoprotein stability and the Mo release process, although its significance is less compared to that of temperature, incubation time, and especially of the pH value. While lowering the protein concentration supported Mo release, increasing the concentration had, vice versa, an obvious protecting effect (data not shown).

ATP influence on Mo release

Addition of ATP (1 mM) to a MoSto sample did not support or stimulate the release of Mo in any way; on the contrary, it inhibited the release process if it was present in a sufficiently high concentration. This was true, even when optimal "release conditions", with respect to pH value (>7.5) and incubation temperature (30 °C) were applied. Only at concentrations of 0.5 mM ATP or lower, did significant Mo release occur. From this aspect, the intracellular ATP concentration is suggested to play an indirect but also a crucial role in controlling the extent of Mo release in the cell.

ATP-dependent (re-)binding of molybdate to the storage protein

The pH-dependent release of Mo from the MoSto protein was found to be irreversible.[2] Upon lowering the pH to 6.5, regardless of the temperature and incubation time, molybdate was not re-bound to the protein. The binding of molybdate turned out to be absolutely dependent on the presence of a nucleotide (preferably ATP) and $MgCl_2$. The degree of Mo binding was strictly ATP-concentration dependent (illustrated in Figure 2). At concentrations <0.05 mM, only insignificant amounts of Mo were re-bound to the protein, at 0.5 mM ATP the proportion of re-bound Mo was about 40%, and at 0.8 mM or higher concentrations of ATP, the re-incorporation of Mo was quantitative. With respect to the other investigated nucleotides, GTP was as effective as ATP, however only if present at lower concentrations (45% Mo-binding at 0.5 mM GTP). At 1 mM and higher concentrations, GTP strongly inhibited Mo binding (data not shown).

In contrast to the Mo release, the overall Mo binding appeared almost to be pH-independent within the tested range 6.5–8.0. At first glance this seems to be an unexpected result, because the polyoxometalate cluster synthesis is certainly a pH-dependent process. We must consider, however that in our assay, the initial reaction rates were not determined, but only the Mo content of the final product after a 90 min procedure (see Experimental Section) was measured. Also, the influence of the temperature (16–30 °C) and incubation time (5–60 min) was found to be low under our conditions. For example, under the standard conditions (1 h at 30 °C), 100% of Mo became

incorporated, however, even under explicitly moderate conditions (15 min at room temperature) the Mo-incorporation process was almost complete as well (~90%). From these results it can be concluded that Mo-binding/cluster formation is a very rapid process that occurs within minutes or even less so that the influence of different experimental conditions on the reaction rates can not be followed by our long-term assay procedure.

The full and rapid re-incorporation of Mo into the apo-MoSto, that is, the complete reconstitution of the native holoprotein, suggests that during the whole procedure of Mo release and rebinding, the MoSto sample had not been subjected to any irreversible damage or denaturation. From this observation, it can be concluded that under the conditions applied (dilution by G-25 chromatography to $\approx 0.4\ mg\ mL^{-1}$ and subsequent incubation at 30 °C for 1 h) even the apoprotein is surprisingly stable.

The steps of the cluster syntheses in the protein are not yet known in detail either. But from the point of view of inorganic chemistry, the basic conditions that promote growth of even large polyoxometalate clusters,[14, 15] and that should also play a relevant role in the (bio)synthesis of the MoSto-associated clusters (high local MoO_4^{2-}concentration and the presence of template type species such as positively charged protein pockets), have already been referred to in our preceding paper.[2]

Studies on the determination of maximum molybdate-binding capacity

The maximum Mo storage capacity of MoSto was unknown until now. The Mo content of MoSto turned out to be variable, depending on the purification conditions (pH of buffers, temperature during preparation steps, protein concentration/degree of dilution in the course of column runs, duration of the whole procedure). Pienkos and Brill[11] reported a content of 14.5 Mo atoms/MoSto molecule, Fenske et al.[2] who purified the protein in a one-day procedure, determined a value of 70 Mo atoms but predicted an optimal content of approximately 90 Mo atoms/protein molecule considering the loss of about 20% of the protein-bound Mo during isolation despite the use of significantly improved conditions. Although the value of 90 Mo atoms is the multifold of the originally determined Mo content, the authors assumed that the actual storage capacity of MoSto might be even higher.

In the present study we therefore further optimized the purification conditions and storage protein stability by adding 1 mM each of ATP and $MgCl_2$ to the cell suspension prior to cell disruption as well as to each buffer that was used during the purification procedure. The final preparation then contained ca. 80 Mo atoms/protein molecule which is still a little less than the Mo content that was extrapolated in the preceding paper.[2] To either confirm that the value of 80 Mo atoms actually represents the maximal Mo content of MoSto, or to demonstrate that the Mo content can be increased still further, this high-quality MoSto preparation was subjected to two experimental approaches. While incubation (variable conditions) in the presence of ATP and $MgCl_2$ (each 1 mM) and excess molybdate (0.5–4 mM) did not lead to supplementary Mo binding, application of the well-established Mo release standard procedure (1 h at 30 °C, pH 7.6) followed by the Mo re-binding procedure (1 h at 12 °C, pH 6.5, presence of ATP and $MgCl_2$) but with the extra addition of molybdate (optimal concentration: 2 mM) resulted reproducibly in a value of 110–115 Mo atoms per MoSto molecule. This corresponds to an increase of the Mo content, compared to the value that was obtained from the originally prepared MoSto, of approximately 40% (Figure 3). Excess molybdate, that was not consumed in Mo–O cluster formation/incorporation, was separated by G-25 gel filtration according to the presence of an additional small elution peak (curve B of Figure 3). From these results we conclude that the content of about 110 Mo atoms actually represents the maximum possible "Mo-loading" of the storage protein.

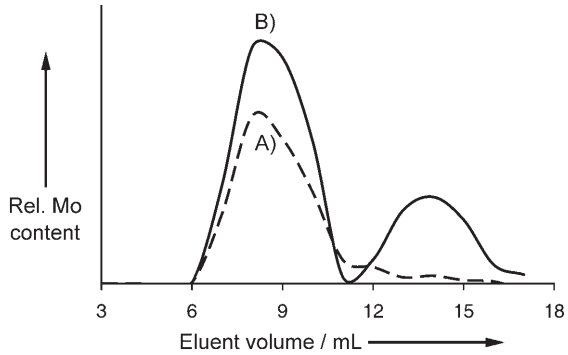

Figure 3. In vivo and in vitro Mo-binding capacity of MoSto. The G-25 gel filtration column was pre-equilibrated and eluted with 50 mM Mops buffer (pH 6.5) that contained ATP and $MgCl_2$ (1 mM each). The Mo-elution profile A (dashed line) represents the Mo content of untreated native MoSto (applied as isolated in the presence of 1 mM ATP and 1 mM $MgCl_2$). Elution profile B (solid line) represents the Mo distribution after subjecting MoSto to the Mo release standard procedure (1 h at 30 °C, pH 7.6) followed by a Mo re-binding procedure (1 h at 12 °C, pH 6.5, presence of ATP and $MgCl_2$ plus extra addition of 2 mM molybdate).

The question of what the mechanism of the supplementary Mo-binding to the native MoSto (as-isolated state) is, how it might be blocked, and why Mo must first completely be released to become able to bind the maximal amount of Mo in the subsequent reconstitution process, cannot be answered at present, though this might be due to the fact that complete growth of the different clusters is only optimal if this starts from the very beginning as partially degraded clusters cannot easily grow again.

Biosynthesis of a "W storage protein" in Mo-starved *Azotobacter* cells

As under diazotrophic growth conditions the conventional Mo nitrogenase is the most highly expressed protein in the cell, large amounts of Mo, provided by MoSto in *A. vinelandii*, are required to guarantee the biosynthesis of an adequate amount of FeMo-cofactor molecules. An enzyme system that requires W in comparable amounts does not exist in *A. vinelandii*, a tungsten storage protein is therefore assumed not to be needed by this organism. Nevertheless, because of the chemi-

cal similarity of tungsten and molybdenum a W-containing storage protein can be artificially constructed, either in vivo by *Azotobacter* cells themselves, or in vitro by a metal-ion-exchange procedure by using the isolated Mo storage protein. Both lines of experiment have successfully been applied in the present study.

When *Azotobacter* cells that were extensively Mo-starved by 2–3 precultures (see Experimental Section) were grown in a medium that lacks molybdate but that contains tungstate (0.1 mM), *A. vinelandii* synthesized the regular storage protein but with tungsten incorporated instead of molybdenum. The "W storage protein" (WSto) that was isolated according to the procedure as optimized for MoSto in the present work (in the presence of ATP/$MgCl_2$), contained in the "as isolated" state without any subsequent treatments, ca. 110 W atoms per protein molecule. This value corresponds in fact to that determined for the maximum possible Mo content. Not only the W-oxide-based cluster(s) seem to be tighter bound to the protein, also the clusters themselves are apparently more stable than the analogous Mo species. This statement is in full agreement with observations made in the field of polyoxometalate chemistry, where polyoxomolybdates are described as less stable than polyoxotungstates.[13] It is therefore concluded that the W content that was analyzed for the "artificial" WSto reflects the real storage capacity more realistic than the Mo content of the MoSto protein in the "as isolated" state (the highest value of which was determined to be only 80 atoms/molecule; see Table 2). On the other hand, we have observed that, although nearly no tungsten seems to get lost during protein isolation, the W-containing protein itself is less stable than the MoSto holoprotein, and it exhibits a pronounced tendency to precipitate, particularly after freeze/thaw treatments. This can be explained by the fact that the properties of the molybdate clusters and polyoxotungstates are different, and the polyoxotungstates do not necessarily fit as optimally as the natural molybdates to the metal-binding centers of the cavity pockets. This effect might lead to conformational constraints in the protein, and thus to an easier destabilization of the protein structure.

Table 2. Mo/W content of in vivo and in vitro preparations of the storage protein. The determinations are based on the molar mass of the apoprotein (172.2 kDa)

Preparation	Mo/W content (atoms/protein molecule)
MoSto as isolated (−ATP)	60 Mo
MoSto as isolated (+ATP)	80 Mo
MoSto reconstituted (Mo release→Mo rebinding)	115 Mo
WSto as isolated (−ATP)	80 W
WSto as isolated (+ATP)	110 W
WSto constructed in vitro (Mo release→W binding)	60 W

In vitro substitution of Mo for W in the isolated storage protein

Another elegant and easy method to generate WSto from MoSto is based on an in vitro metal-cluster exchange, which comprises two preparative steps. First, MoSto was subjected to conditions that lead to quantitative Mo release (1 h at 30 °C and pH 7.6). Then, after removing molybdate from the sample by G-25 and re-concentrating the protein solution (to 1.5-2 mg mL^{-1}) by ultrafiltration, the apoprotein was incubated at 30 °C for 1 h in the presence of 1 mM each of ATP and $MgCl_2$ and 2 mM tungstate (Na_2WO_4); for further details of the procedure, see Experimental Section.

In fact, a storage protein with incorporated polytungstates could be successfully constructed under the described in vitro conditions (Figure 4). On the other hand, it was rather surprising that the final product contained only ~60 W atoms per protein hexamer, which is less than the in-vivo-synthesized WSto. In the case of the Mo-containing storage protein, we determined that a maximum of 60 Mo atoms/MoSto molecule after standard purification conditions (in the absence of ATP) were present, but almost double the amount of Mo atoms were present after the application of Mo-release and subsequent Mo-rebinding conditions. In the case of WSto, the metal-binding behavior proved to be exactly the opposite (see the comparative data of Table 2). It is noteworthy in this context that the type of cluster formation does not need to be identical for the two elements, a fact that is known from inorganic chemistry.[13c]

W-release experiments yielded similar results as expected. Under the conditions that were routinely applied to release Mo quantitatively from the protein (1 h incubation at 30 °C and pH 7.6), only 34% of W was released, about $^2/_3$ of the W remained protein bound (Figure 5). This is in full accordance with our aforementioned interpretation, namely that the W species that are present in WSto are significantly more stable. In this context, it is pertinent to note that the elution peak of

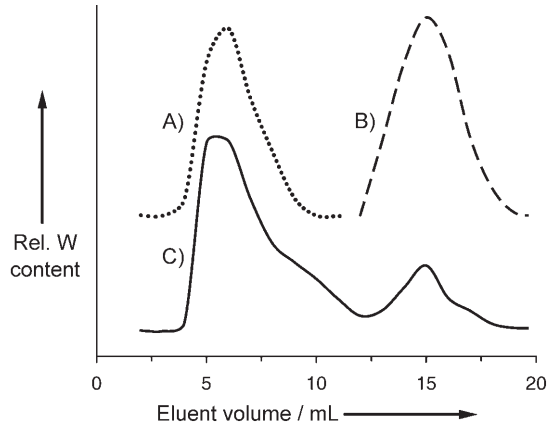

Figure 4. In vitro formation of WSto. Substitution of W for Mo by using the isolated MoSto holoprotein, was carried out as described in the text and in more detail in the Experimental Section. After quantitative release of Mo from the protein and the subsequent final incubation step (1 h at pH 6.5 and 12 °C in the presence of 1 mM ATP, 1 mM $MgCl_2$ and 2 mM tungstate), the protein sample was applied to the G-25 gel-filtration column pre-equilibrated and eluted with 50 mM Mops buffer (pH 6.5) that contained 1 mM each of ATP and $MgCl_2$. The elution profile A (dotted line) displays the apoprotein (OD280) as high-molecular-weight reference and B (dashed line) the mononuclear tungstate as low-molecular-weight reference. The profile C (solid line) represents the W elution profile of the sample that had been subjected to conditions of WSto generation in vitro.

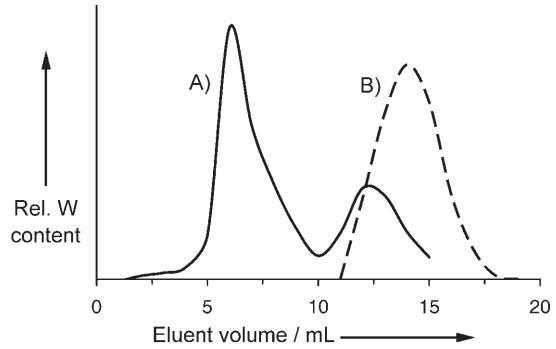

Figure 5. Partial release of tungsten from Wsto. The in-vivo-produced tungsten-containing storage protein was subjected to the "release conditions" (1 h incubation at 30 °C and pH 7.6) that were routinely applied in Mo-release experiments. After the incubation step, the protein sample was applied to the G25 gel-filtration column pre-equilibrated and eluted with 50 mM Mops buffer (pH 7.6). The elution profile A (solid line) displays the distribution of protein-bound and released tungsten and B (dashed line) represents the reference elution peak of mononuclear tungstate.

the low-molecular-weight W-compound, which was released as a minor portion from the protein does not completely coincide with the tungstate peak. It is conceivable that due to persistent interaction with the protein, even after incubation under "release conditions", dissociation of the W compound, and its simultaneous breaking down to mononuclear tungstate ions, occurs—if at all—only during gel filtration; this might be the reason for a slightly delayed W elution.

Outlook

After the discovery of an unprecedented type of molybdenum/tungsten-oxide-based uptake and release process in a storage protein, it is a challenge to perform similar release/uptake studies with protein preparations that contain other related metal oxides (like that of vanadium) as well as single-crystal X-ray structural analyses of protein forms that are either completely Mo/W-free (apoprotein) or display different degrees of Mo/W loading. This concerns, on the one hand, the investigation of the different polyoxometalate clusters that are present in the protein with different degrees of decomposition, and on the other hand with different degrees of growth. As the protein pockets obviously function as templates for cluster formations (different pocket functionalities lead to different clusters), such a study would fundamentally help to gain a deeper insight into the understanding of the present type of unprecedented assembly processes and especially of the template-directed syntheses of polyoxometalates (see, for example, ref. [16]).

Another aim of future studies will be the elucidation of the specific functions of ATP in the molybdate-release and binding processes. We have found that ATP stabilizes the storage protein, is directly involved in the Mo-binding process, and controls the Mo release indirectly. However, details of these functional mechanisms and, in particular, the crucial question, whether ATP has only conformational influence or functions as cosubstrate (complexed with Mg^{2+}) supporting somehow the cluster syntheses thereby being hydrolyzed, have yet to be answered. The observation that the ATP-binding sites are occupied in the α-subunit, while those in the β-subunit are not,[8] points to the possibility that ATP might even fulfill different functions in the two subunit types present in the storage protein.

Experimental Section

Bacterial strain and growth conditions: The bacterial strain that was used in this study was *A. vinelandii* wild-type strain OP (DSM 366; ATCC 13705). The growth medium and the conditions for culturing and harvesting the cells were as described previously.[2] The standard medium contained molybdate (10 μM), the medium used to favor the formation of a W storage protein, contained tungstate (100 μM).

Removal of molybdenum impurities from glassware, growth medium and *Azotobacter* cells: To remove Mo from potentially "contaminated" glass vessels, all tubes and culture flasks that were used in the experiments to produce a W-containing storage protein were soaked in a nitric acid solution (0.1 M) overnight. The vessels were then rinsed with bi-distilled water (H_2O_{bid}) several times, soaked in an EDTA solution (1 mM) overnight and rinsed again extensively with H_2O_{bid}.

Fe^{III}–citrate, the nutritient component, which turned out to be the one most strongly contaminated with Mo, was subjected to the activated-coal method in order to effectively remove Mo.[10]

To inoculate the main culture with cells that were as Mo-starved as possible, *A. vinelandii* was cultured first on Mo-free agar plates and then precultured three times in Mo-deficient liquid growth medium.

Procedure of cell extract preparation via alkaline cell lysis: To obtain cell-free extracts as material for protein purification, cells were routinely disrupted by French press treatment. For the purpose of determining the Mo content of Mo-deficient cells/extracts that served as material for construction of a W-containing storage protein, a much faster method that was based on protein precipitation was applied. In this procedure, NaOH (150 μL of a 40% solution) was added to a cell suspension (1 mL); this mixture was then boiled for 30 min in a water bath. The resulting extract was then cooled in an ice bath and subsequently neutralized with HCl. The neutralized sample was finally centrifuged for 10 min at 13 000 rpm, and the resulting supernatant was used for Mo and protein analysis.

Protein purification: The storage protein, expressed (without any tag) in the original bacterial wild-type strain of *Azotobacter vinelandii* (see above), was isolated and purified to homogeneity according to the basic procedures (French press treatment, ultracentrifugation, DEAE-Sephacel chromatography, ammonium sulfate fractionation and Superdex-200 gel filtration) that were described in the preceding paper.[2]

Mo and W determination: The content of Mo and W in extracts, column fractions and in the purified MoSto protein was determined according to the catalytic method of Pantaler,[17] which was based on the oxidation of dithiooxamide in the presence of H_2O_2. The experimental scale was reduced by a factor of 10 (total test volume: 2.5 mL) so that the reaction could be carried out in standard 3-mL cuvettes. Prior to the assay, sample aliquots were filled in gas-tight vessels, placed in a boiling water bath for 15 min, then

cooled to room temperature and centrifuged to remove precipitated protein. Depending on the amount of molybdate present, an appropriate amount of the protein samples (10–50 μL) were added to the assay mixture.

The described catalytic assay that was used as a routine assay could not discriminate between Mo and W. It was therefore ascertained by ICP-MS analyses that the basic chemicals that were used in our experiments, that is, Na_2MoO_4 and Na_2WO_4 (both p.a., from Merck), were not contaminated with each other.

Protein determination: The protein content was determined by using the bicinchoninic acid method.[18]

Experiments on molybdate-binding and release mechanisms: The procedures for Mo release and (re)binding that were described by Fenske et al.[2] have been successfully reproduced in the present work. For most of the experiments, the basic conditions and sequential steps of sample treatment were applied as described previously, except that only one parameter was varied (pH, temperature, incubation time, protein concentration, ATP/$MgCl_2$ concentration). For some experiments on the Mo-binding capacity and metal exchange, the following modified procedures were required.

To determine the maximum possible capacity of Mo-binding, the Mo storage protein was first purified according to the methods described,[2] however, in order to prevent the loss of significant amounts of Mo during purification, ATP and $MgCl_2$ (1 mM each) were added to the cell suspension prior to cell disruption and to all of the buffers that were used in the subsequent preparation steps. Samples (each ~1.5 mg mL^{-1}) of the final preparation were incubated in the presence of ATP, $MgCl_2$ and varying concentrations of MoO_4^{2-} (1–20 mM) between 12–30 °C and for 30–60 min.

The resulting preparations were gel-filtrated by Sephadex G-25 (4 °C, pH 6.5) to separate quantitatively the excess molybdate, which was not bound to MoSto, from the protein solution. The protein fractions were finally subjected to Mo analysis.

The samples that were used to substitute W for the protein-bound Mo (in vitro formation of a W storage protein), were treated as follows:

To produce the apoprotein, MoSto (~2 mg in a 1 mL sample) was first subjected to conditions that led to quantitative Mo release (1 h incubation at 30 °C and pH 7.6 followed by a Sephadex G-25 gel filtration to remove the released molybdate).

The resulting apoprotein was diluted to ~5 mL (≅0.4 mg mL^{-1}) by the preceding G-25 column run, was re-concentrated to 1 mL by ultrafiltration in a B15 Amicon chamber at 4 °C. To stabilize apo-MoSto, and to cause a partial incorporation of W into the protein, the ultrafiltration was performed in the presence of ATP and $MgCl_2$ (1 mM each) and Na_2WO_4 (2 mM).

To accomplish a quantitative W binding, the storage protein solution that included ATP, $MgCl_2$ and tungstate was subsequently incubated for 1 h at 30 °C.

The sample was then gel-filtrated by Sephadex G-25 at 4 °C. The elution buffer (MOPS, 50 mM, pH 6.5) contained ATP and $MgCl_2$ at the same concentration as the sample itself. The collected fractions (1 mL each) were finally subjected to W analysis to examine whether the incorporation of the alternative metal into the protein was successful.

Abbreviations: MoSto, molybdenum storage protein; EXAFS, Extended X-ray Absorption Fine Structure; SAXS, Small Angle X-ray Scattering; MOPS, 3-Morpholino-propanesulfonic acid.

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Keywords: *Azotobacter vinelandii* · bioinorganic chemistry · metal storage protein · metalloproteins · molybdenum · tungsten

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