VIP Metal-Storage Proteins

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Towards Biological Supramolecular Chemistry: A Variety of Pocket-Templated, Individual Metal Oxide Cluster Nucleations in the Cavity of a Mo/W-Storage Protein**

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Dedicated to Professor Jean-Marie Lehn

Since the time of Linus Pauling's famous book "*The Nature of the Chemical Bond*" (chapter: "The Sharing of Polyhedron Corners, Edges and Faces"), assembly/nucleation processes based on simple AB_n-type polyhedra have been important research subjects, not only in inorganic chemistry, but also in fields such as discrete mathematics, crystal chemistry (also discussed by Pauling in detail for silicates), materials science, and geoscience. Especially polyoxometalate (POM) chemistry,[1a] which is dominated by the elements Mo and W, should be mentioned, as it is based on this type of assembly/nucleation processes (see also a related paper by Pauling).[1b] This topic, which represents an extreme structural versatility and includes the largest inorganic clusters known to date, is directly related to the present investigation.[1c]

During the last 10 years, tremendous progress has been made in the understanding of the very versatile role of Mo in fundamental biological processes in which it becomes functional when in protein-associated form.[6] Herein, it is of relevance that, in some cases, Mo can be replaced by W in the biosphere, for example, in some hyperthermophilic organisms,[7] as the chemical behavior of the two elements, at least in the highest oxidation state, is similar. A rather unique situation was found for the Mo-storage (MoSto) protein from *Azotobacter vinelandii*. It is not only capable of binding the large number of approximately 100 biologically relevant Mo atoms,[8] but alternatively it can bind approximately 100 W atoms (it is therefore termed Mo/WSto) in the form of aggregates of the POM type that have not yet been structurally defined. The release of Mo and W depends on parameters such as the pH value and temperature,[10] and shows similarities with corresponding degradation processes of POMs under bulk conditions.[1a] (Until now, details of how nature controls important biological metal-storage/release processes have only been described for the protein ferritin, which contains a nanosized iron oxide fragment.[11,12]) In the present study, we refer to appropriately functionalized protein pockets within the cavity of the cage-type protein complex. These pockets harbor different individual polynuclear tungsten oxide aggregates, that is, polyoxotungstates which are, remarkably, separated from each other. Most importantly: different pocket functionalities can be specifically correlated with different directed-assembly processes. As the pockets in the protein-cavity shell act as a polytopic host for the noncovalently or weakly bonded polyoxotungstate guests, we can refer to a "biological supramolecular chemical system".[13]

To elucidate the basic principles of the structuring of the polynuclear metal oxides and their noncovalent binding to the protein with the aim of gaining insight into the nucleation/growth and degradation processes, the crystal structure of the metal-storage protein isolated from *A. vinelandii* and loaded with "tungstate" was investigated and determined with rather high resolution (Table 1). The reason for the tungstate-based procedure is that in solution the natural MoSto protein released molybdate almost quantitatively under the crystallization conditions and during long-term incubation at 18 °C. This situation is compatible with earlier results obtained with different purification methods.[8,9] In the W case, partial release can also not be completely avoided, and in principle, it might be impossible to keep all the polytungstate clusters intact during the crystallization process (partly owing to the weak interactions between the clusters and the pockets; see below). Nevertheless, this finding corresponds to the real biological situation in a bacterium where Mo is partially released from its storage protein to keep the nitrogen-fixation process running. The Mo/WSto protein is organized as an $(\alpha\beta)_3$ heterohexamer consisting of a trimer of $\alpha\beta$ dimers (Figure 1). (On the basis of gel filtration, Mo/WSto was preliminarily formulated as an octamer.[8]) The two structurally similar subunits, α and β, represent an open α,β structure whose architecture is similar to that of the amino acid kinase family,[14] the most closely related member being the hexameric uridine monophosphate (UMP) kinase.[15] The subunits are composed of an α,β core and two lobes attached

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Table 1: Data collection and refinement statistics for Se-methionine derivatives of the protein Mo/WSto.

	Se-methionine 1	Se-methionine 2, MAD[a]		
Data set	high-resolution	peak	inflection	remote
Data collection[b]				
Wavelength [Å]	0.9787	0.9784	0.9789	0.9762
Space group	$P6_322$	$P6_322$	$P6_322$	$P6_322$
Resolution [Å]	1.6 (1.6–1.7)	1.8 (1.8–2.0)	1.8 (1.8–2.0)	2.5 (2.5–2.6)
Cell axes *a*, *c* [Å]	114.0, 233.4	114.3, 234.3	114.7, 234.4	115.2, 234.1
Completeness [%]	95.4 (82.9)	97.0 (90.0)	96.7 (89.6)	98.9 (99.5)
R_{sym} [%]	6.5 (51.5)	7.2 (45.8)	7.3 (72.1)	5.2 (31.2)
$I/\sigma I$	10.3 (2.0)	15.9 (4.3)	12.5 (2.1)	15.1 (4.2)
Redundancy	3.1 (2.4)	6.2 (5.7)	3.8 (3.5)	2.9 (2.9)
Refinement statistics[c]				
No. of αβ units in the a.u.[d]	1			
No. of residues, solvent molecules, ATP molecules, Mg^{2+} ions, phosphate ions, and W atoms in the a.u.[d]	512, 391, 1, 1, 2, 21			
Resolution range [Å]	20.0–1.6			
$R_{working}$, R_{free} [%]	17.9, 20.0			
$B_{average}$ [Å^2]	40.5			
B_{Wilson} [Å^2]	28.6			
rms deviation from ideal values for bond lengths [Å], angles [°][e]	0.015, 1.69			

[a] MAD = multiple anomalous dispersion. [b] Data were collected under cryogenic conditions. Data for the last resolution shell are given in parentheses. [c] 5% of the data were set aside for the calculation of R_{free}. [d] a.u. = asymmetric unit. [e] rms = root mean square.

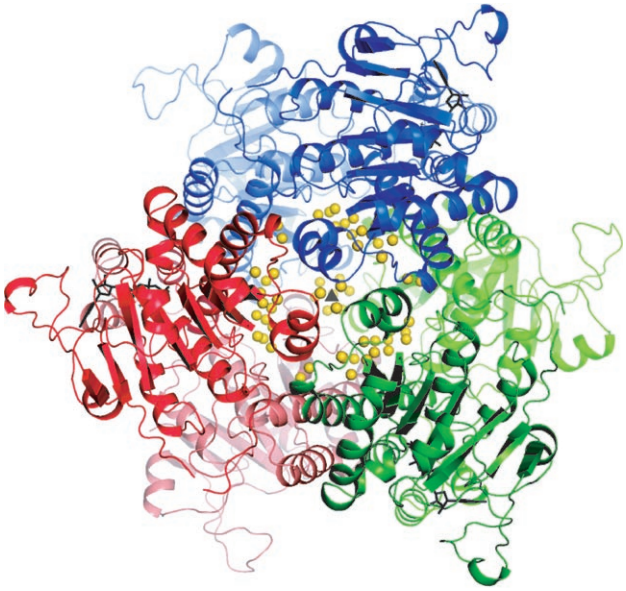

Figure 1. Structure of the Mo/WSto protein of *A. vinelandii*. The heterohexameric $(\alpha\beta)_3$ protein complex has a size of about $100\times100\times70$ Å^3 and has pseudo 32 (D_3) symmetry. The approximate twofold symmetry between subunits α and β is due to their close structural relationship, which is reflected in a sequence identity of 45%. The monomers α_1 (red), α_2 (blue), and α_3 (green), and β_1 (light red), β_2 (light blue), and β_3 (light green) assemble as trimers on either side (called interfaces α and β) of the heterohexamer. The three monomers of each trimer are related by the crystallographic threefold axis (▲). UMP kinase has a similar hexameric quaternary structure, in which two pores with diameters of 7 Å are formed along the threefold axis. The ATP molecules are shown as black stick models, and the W atoms of the polynuclear tungsten oxide clusters as yellow spheres.

above the edges of the central β sheet of the α,β core, thereby forming a groove which serves as an adenosine triphosphate (ATP) binding site in the α subunit. The binding mode of the ATP/Mg^{2+} complex and its role in the Mo/W-binding and release process is not the subject of the present study.[16]

Most important, the heterohexamer contains in its center a large cavity of about 7250 Å^3 (i.e., comparable in size to some of the functionalized cavities of the giant spherical polymolybdate clusters;[3–5] Figure 2), which is endowed with appropriate pockets that serve as binding sites for the 14 polynuclear tungsten oxide clusters; the rest is filled with solvent/water molecules. The clusters can be divided into the following different types: one W_3, three W_6, three W_7, three W_2(I), three W_2(II), and one $W_{7+x} \equiv (W)W_{6+x}$ (Figure 2). Owing to the molecular symmetry of Mo/WSto, each cluster occurs three times except for the two clusters located on the C_3 axis, which occur once. In total, 60–70 W-atom sites were identified on the basis of an anomalous-difference Fourier electron-density map that was calculated from data collected at the Se and W absorption edges. The W–W distances are in the range of 3.3 ± 0.4 Å, comparable to the situation in a large number of polyoxotungstates (see ref. [1a] and references therein). A complete structure description containing all the O atoms was only possible for the well-defined W_3 cluster (Figure 3). The other clusters show only fractional occupation because of partial tungstate release during the crystallization process.

The W_3 cluster, $[W_3O_{10}H_xN_3]^{(6-x)-}$, is composed of three edge-sharing octahedra that are related by the crystallographic threefold axis (Figures 2 and 3). Each of the W atoms is coordinated to five O atoms and to the imidazole $N_{\varepsilon 2}$ atom of Hisα139. The W_3O_{10} unit is present as a (formal) constituent of the well-known $[W_{12}O_{40}H_2]^{6-}$ species.[1a] As

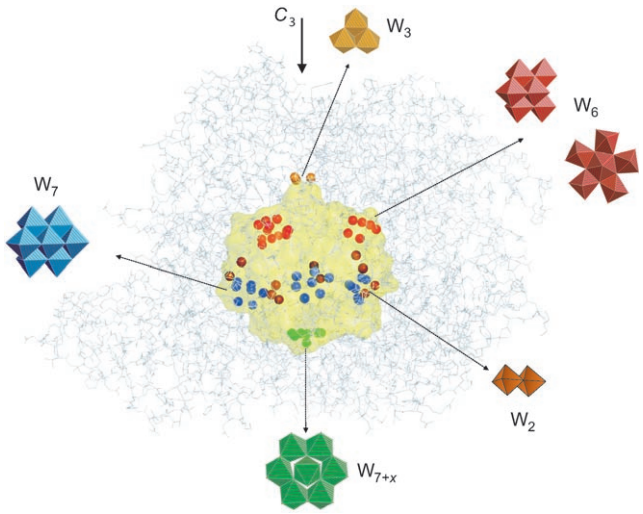

Figure 2. Surface representation of the Mo/WSto protein. The (roughly) ellipsoidal protein shell (gray) and the nanocavity (shown as a yellow surface) have a thickness or diameter, respectively, of 20–30 Å. The protein surface of the cavity forms several well-distributed pockets, which harbor the polynuclear tungsten oxide clusters. The W_3 (yellow) and $W_{7+x} \equiv (W)W_{6+x}$ (green) clusters are positioned on the threefold axis, in front of the interfaces α and β, respectively, and are embedded in a small hydrophilic and a larger hydrophobic pocket, respectively. The binding sites of the three W_6 clusters (red; two idealized structural options are shown; see text for details) are essentially built up by segments of the α subunit forming a flat and hydrophilic pit. The three W_7 clusters (blue) lie between subunits α and β in a deep pocket, while the W_2 clusters I and II (brown) are located in the vicinity of the W_7 cluster. The clusters, except W_3 for which all the O atoms were found, are represented as idealized polyhedra constructed by assuming the O positions on the basis of POM chemistry[1a] and the present results; the individual W centers are shown as spheres of the corresponding color in the cavity of the Mo/WSto protein.

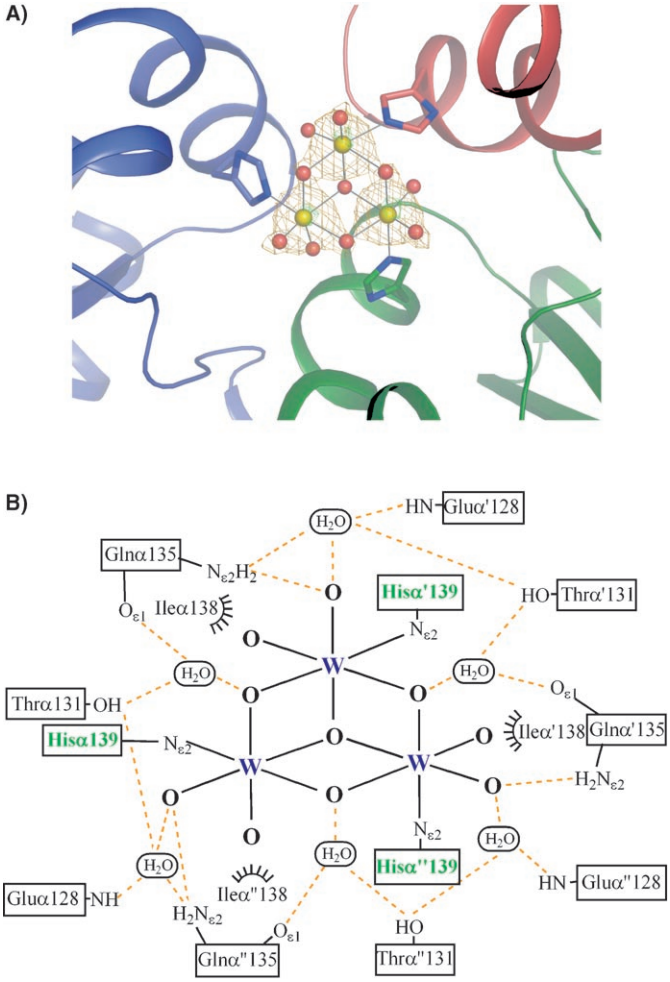

Figure 3. The structure of the W_3 cluster and its protein surroundings. A) The three triangularly arranged W atoms (yellow) are 3.4 Å from each other (a distance typical for polyoxotungstates without W–W bonds). Each is linked to five O atoms and the $N_{\varepsilon 2}$ atom of Hisα139 (green residue in B). The molecular symmetry of Mo/WSto seems to be exploited for the synthesis of the W_3 cluster. (Interestingly, Mo-binding proteins (Mop) harboring monotungstates also use the interface between three symmetry-related subunits as binding sites.[36]) The W site of the W_3 cluster is approximately 50% occupied, and the O sites were located in the $2F_{obs}-F_{calcd}$ electron-density map (contour level $\sigma = 1.0$) at 1.6-Å resolution. B) Scheme of the interactions between the protein matrix and the W_3 cluster. For each W center, hydrogen bonds (orange dashed lines) are formed between one terminal O atom and Glnα135 and through a water molecule to Glnα135, Thrα131, and Gluα128. The other terminal O atom points towards the side chain of Ileα138 and is probably protonated, which would lead to the formulation $[W_3O_{10}H_3N_3]^{3-}$. The three μ^2-bridging O atoms of the W_3 cluster also interact through a water molecule with Glnα135 and Thrα131. The apical O atom, which coordinates to all three W atoms, sits on the threefold axis and points towards the bottom of the cavity.

the O atoms can be clearly identified, an accurate description of hydrogen-bond type and van der Waals interactions between them and the protein matrix is feasible (Figure 3B).

The other cluster lying on the C_3 axis shows four arrays of W atoms whereby the first (formal) array consists of a single W atom at the vertex, the second of six W atoms and the next two can not be clearly described owing to their very low occupancy.[17] The major pattern of the W peaks is either interpreted as a $W_{7+x} \equiv (W)W_{6+x}$ cluster composed of a hexagon capped with an apical W atom (Figure 2) or—not considering the two weakest "arrays"—as (three) disordered W_3 clusters, each composed of the apical W atom and two W atoms from the hexagon. The protein pocket of the cluster is predominantly nonpolar, suggesting that the W-coordinating O atoms are protonated.

The W positions of the three W_7 clusters correspond to the related positions of the stable heptanuclear POMs $[M_7O_{24}]^{6-}$ (M = Mo, W)[1a] (Figure 2) that are generated with high formation tendency in aqueous solution at intermediate pH values. The $\{M_7O_{24}\}$ building block is also present in the largest polyoxomolybdate that can be obtained under non-reducing conditions, that is, $[Mo_{36}O_{112}]^{8-}$.[1a] Note that the twofold symmetry axis of each W_7 cluster does not coincide with the pseudo-twofold axis between subunits α and β. Interestingly, the W positions are homogeneously occupied to about 30%, in agreement with a homogeneous cluster removal/degradation during the release process. Furthermore, the low occupancy is reflected in the relatively high temperature factor found for the contacting side chains and in a largely disordered glycine-rich loop close to the β interface.

The most pronounced features of the protein matrix in the neighborhood are three sequentially adjacent histidine residues (Hisα155, Hisα156, Hisα157) that interact with six of the seven WO_6 units. The small W_2 clusters I and II (Figure 2) are located in the vicinity of the W_7 clusters and should correspond to two edge-sharing WO_6 octahedra.

The W_6 cluster can be correlated with a distorted pentagonal pyramidal $(W)W_5$ unit (Figure 2), the formation of which is directed by the asymmetrical arrangement of the adjacent polypeptides. The W-atom arrangement is comparable with the metal part of the fundamental (but flatter) building unit $(Mo)Mo_5$ which occurs 12 times in the unique spherical porous capsules/artificial cells of the type $\{(Mo)Mo_5\}_{12}\{linker\}_{30}$,[3,5] but also in the hedgehog-shaped Mo_{368},[2] as well as in different wheel-shaped clusters based on Mo and O atoms.[18] More important is that the peak heights of the W sites of the W_6 cluster are different: two peaks refine to an occupancy of approximately 50%, and the other peaks to an occupancy of approximately 15%. This outcome suggests the presence of superimposed clusters resulting from nonhomogeneous degradation processes. The W_6 cluster might therefore be a degradation product of the above-mentioned W_7 cluster, as it resembles the W_6 part of the latter (Figure 2). Again, three histidine residues (Hisα113, Hisα129, and Hisα155) interact with all six "WO" units (Hisα155 serves as linker between the W_6 and W_7 clusters).

Structural-comparison studies suggested that the related hexameric Mo/WSto and UMP kinase[15] should both exist in closed and open conformations. In the open state, pores across the protein shell are formed at the interfaces α and β along the threefold axis (Figure 1), which are, in the present case, sufficiently wide to facilitate the entrance of small tungstate ions, such as $[WO_4H]^-$ and $[WO_4]^{2-}$. Because of the small protein pores, the cluster synthesis can only proceed inside the cavity.

Note that the W_{7+x}, and W_6 clusters, which are not stable in bulk solution, are synthesized in a protein-assisted process whereby the protein serves not only as a template, but also as a protector for these clusters against hydrolysis.

The types of nucleation and growth processes differ considerably for the different clusters. The template-directed formation (see for example, refs. [19,20]) of the W_3 and $W_{7+x} \equiv (W)W_{6+x}$ species (in contrast, for example, to the W_6 species) follows primarily geometric constraints in agreement with the threefold molecular symmetry. More precisely, the comparably large $W_{7+x} \equiv (W)W_{6+x}$ cluster is characterized by multiple contacts to a complementary sized/shaped protein pocket and the W_3 cluster by weak bonds to the appropriately positioned Hisα139 (Figure 3 A). Selective binding of both of these clusters, which lie on the C_3 axis, is realized by different structures of a helical segment in subunits α and β, which give rise to smaller and larger pockets. The special growth mechanism of the W_{7+x} cluster might be correlated with a cascade-directed process postulated for the Mo_{37} cluster,[20] while the formation of the first hexanuclear layer of the cluster is directed by the (first-formed) mononuclear part at the cluster vertex together with the protein pocket. The further growth, which is essentially protein-independent, shows, because of a lack of protection, a (dramatic) decrease in the occupancy of the W sites. The mentioned cascade-type process proceeds in a way such that the "older" parts direct the formation of the next fragments and subsequently react with them. The formation of the three W_6 clusters appears to be predominantly driven by electrostatic forces as these clusters are specifically hydrogen-bonded to three positively charged histidine residues. Moreover, the electrophilic surface generated by the histidine residues can attract nucleophilic, small, tungstate ions that have been taken up by the protein. This process supports the assembly in that on the electrophilic surface the repulsion between the negatively charged small tungstate ions is decreased with the consequence that they can easily be positioned near to each other, a condition for the subsequent condensation process. It is important to note that the growth always starts at the protein pocket with the largest degree of protection and the cluster de-aggregation/release proceeds in the reverse direction. The remaining clusters, that is, the six W_2 and especially the three rather stable W_7 clusters should, according to our knowledge of bulk-type POM chemistry,[1a] form spontaneously in the cavity in a (nondirected) self-assembly process and subsequently get bound to the protein surface.

With its polynuclear tungsten oxide system, the protein investigated combines, in a unique manner, macromolecular biochemistry with an unprecedented type of inorganic chemistry, as well as supramolecular chemistry[21,22] under confined conditions. The term supramolecular is correct in this context because the protein cavity acts as polytopic host for several different inorganic clusters, that is, weakly or noncovalently bonded guests.[21,22] All the aggregates found exhibit structural details well-known from POM chemistry.[1a,23] Interestingly, some of the clusters have not been detected in bulk media, as they are instable under those conditions, but they could, in principle, serve as precursors for bulk-type species. Though nature has developed the Mo/WSto protein system for the storage of Mo in the form of compact polynuclear molybdates, the process for the formation and release of the tungstate species must be considered as analogous because the POM chemistry of Mo(VI) and W(VI) is very similar.[1a,23,24] In any case, from these observations we can learn much about cluster synthesis based on tailor-made protein-based templates, as well as about nucleation/condensation processes on a single-molecular level, which is an unprecedented aspect. The option to use the Mo/WSto protein as a suitable "nano test tube" can probably be explored: on the one hand, variation of the aqueous solutions should lead to different encapsulated polyoxotungstates, while on the other hand, modification of the relevant protein pockets—that is, of the template conditions—by the site-directed exchange of amino acids, can create new functionalities or patterns with directing function.

Experimental Section

Protein purification and crystallization: *A. vinelandii* was grown in a tungstate-containing medium[10] and the Mo/WSto protein was purified from *A. vinelandii* as described elsewhere.[8] A Se-methionine-labeled protein was produced by metabolic inhibition.[25] The most suitable crystals were obtained with the hanging-drop method at

18 °C with a drop volume of 1 μL protein solution (15 mg mL^{-1} Mo/WSto, 50 mM 3-(4-morpholinyl)-1-propanesulfonic acid (Mops), pH 6.5, and 50 mM NaCl) and 1 μL reservoir solution (1 M $(NH_4)_2HPO_4$ and 0.1 M sodium citrate, pH 5.6).

Structure determination and refinement: Data for native and Se-methionine-labeled Mo/WSto crystals were collected at the Swiss Light Source, beamlines X6SA and X10SA, Paul Scherrer Institute, Villigen, Switzerland and were processed with the HKL[26] and XDS[27] program suites. The positions of the Se atoms and many W atoms were determined with SHELXD[28] using the peak data of the MAD experiment performed at the Se absorption edge. The sites found were refined in SHARP,[29] and the subsequently calculated phases were improved by solvent flattening.[30] The resulting electron density at 2.5-Å resolution was of excellent quality. 80% of the polypeptide chain was automatically incorporated using RESOLVE,[31] and the residual segments, ATP, ions, solvents, and the polynuclear tungsten oxide clusters were manually incorporated using O.[32] The refinement was started with CNS[33] and brought to convergence with REFMAC.[34] The occupation factors of the W atoms were refined within SHELXL.[35] (Very small electron densities like those above the W_6 and (W)W_{6+x} clusters caused by the degradation process were not taken into account.) Refinement statistics are given in Table 1. The model includes residues 31–276 of subunit α, residues 3–270 of subunit β, one ATP molecule, and fourteen polynuclear tungsten oxide clusters of different types. Because of the rather low occupation of the cluster sites, the O-atom positions could not be found, except for those of the W_3 cluster. Structure coordinates for the Mo/WSto protein have been deposited in the Protein Data Bank under the accession number 2OGX.

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[17] The occupancy of the W sites decreases from the first array (30%), to the second (10%), to the next arrays ($\ll$10%), reflecting a degradation process starting far from the protecting protein pockets.

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