Multiple and variable binding of pharmacologically active bis(maltolato)oxidovanadium(IV) (BMOV) to lysozyme

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ABSTRACT

The interaction with proteins of metal-based drugs plays a crucial role in their transport, mechanism, and activity. For an active ML_n complex, where L is the organic carrier, various binding modes (covalent and non-covalent, single or multiple) may occur and several metal moieties (M, ML, ML_2, etc.) may interact with proteins. In this study, we have evaluated the interaction of $[V^{IV}O(malt)_2]$ (bis(maltolato)oxidovanadium(IV) or BMOV, where malt = maltolato is the common name for 3-hydroxy-2-methyl-4*H*-pyran-4-onato) with the model protein hen egg white lysozyme (HEWL) by electrospray ionization mass spectrometry (ESI-MS), electron paramagnetic resonance (EPR) and X-ray crystallography techniques. The multiple binding of different V-containing isomers and enantiomers to different sites of HEWL is observed. The data indicate both non-covalent binding of *cis*-$[VO(malt)_2(H_2O)]$ and $[VO(malt)(H_2O)_3]^+$ and covalent binding of $[VO(H_2O)_{3\text{-}4}]^{2+}$ and *cis*-$[VO(malt)_2]$ and other V-containing fragments to the side chains of Glu35, Asp48, Asn65, Asp87, Asp119 and to the C-terminal carboxylate. Our results suggest that the multiple and variable interaction of potential $V^{IV}OL_2$ drugs to proteins can help to better understand their solution chemistry and contribute to define the molecular basis of the mechanism of action of these intriguing molecules.

INTRODUCTION

The use of metal species in diseases' treatment is a field of extensive research. They were proposed as potential anticancer, antidiabetic, antimicrobial, antiviral and antiarthritis drugs,[1] with activity often higher than the organic compounds.[2] However, rather surprisingly, they attracted less the attention of the pharmaceutical companies compared to the organic compounds. One of the reasons is the lack of knowledge on their transformation in the organism that is related to the interaction with bioligands and, in particular, with proteins. In fact, in bloodstream and cellular environment, for a generic pharmacologically active ML_n complex, where L is an anionic organic carrier, the binding of several metal moieties (M, ML, ML_2, etc.) with various binding modes (covalent and non-covalent, single or multiple) may occur.[3] This appears to be particularly important for the first-row transition metals, whose labile complexes can lose one or more ligands depending on the conditions such as pH and concentration.

In the recent years, oxidovanadium complexes have attracted much attention for their medicinal potential and have been tested particularly as antidiabetic and anticancer drugs.[4] Bis(maltolato)oxidovanadium(IV) ($[V^{IV}O(malt)_2]$ or BMOV, where with malt is abbreviated maltolato or, according to the IUPAC nomenclature, 3-hydroxy-2-methyl-4*H*-pyran-4-onato) and bis(ethylmaltolato)oxidovanadium(IV) (BEOV) are among the most potent orally active insulin-mimetic agents (**Figure 1**).[5] They have undergone extensive pre-clinical testing and BEOV has been promoted to phase II clinical trials.[4c, 5b] Even though the experimentation as an antidiabetic drug was temporarily stopped due to renal problems of several patients and financial problems of Akesis Pharmaceuticals,[1a, 6] BMOV is usually considered the reference for the new molecules with insulin-mimetic action. Surprisingly, the tests on BMOV are continued by CFM Pharma (CFM10, Vanadis) and now it is arrived to the phase II for the treatment of patients with injuries on

secondary tissues caused by accidents or fire and with myocardial infraction.[1a, 7] In the solid state, $[V^{IV}O(malt)_2]$ has a square pyramidal geometry, but when it is dissolved in water it undergoes isomerization to the *cis*-octahedral species $[V^{IV}O(malt)_2(H_2O)]$,[8] that predominates at physiological pH and shows good membrane transport properties. At low V^{IV} concentration and/or at moderately acidic pH, the neutral complex transforms into $[VO(malt)(H_2O)_3]^+$, while at strongly acidic pH a significant part of V is in the free aqua ion form.[9]

Closely related to the absorption, transport and activity of biologically active vanadium species, the binding to bioligands plays a crucial role in the development of new potential V drugs. Among the bioligands, amino acids, small peptides and proteins have a special place. If on one hand the interaction with amino acids and oligopeptides appears now rather clear and has been reviewed few years ago,[10, 11] on the other much less progress has been made with proteins, both because of their intrinsic complexity and the difficulty in studying such large molecules with the instrumental and computational techniques.[12] Up to now, several evidences suggest that the pharmacologically active vanadium complexes bind to proteins;[13, 14] the same is true for $[V^{IV}O(malt)_2(H_2O)]$ which interact with several proteins and, particularly, with transferrin, albumin and immunoglobulins in blood serum,[15] and hemoglobin in erythrocytes.[16]

To study in detail the reactivity of V complexes with proteins, small models like hen egg white lysozyme (HEWL) have been also used.[17] It has been demonstrated through electrospray ionization mass spectrometry (ESI-MS), electron paramagnetic resonance (EPR) and computational studies (DFT, QM/MM) that potential $V^{IV}OL_2$ drugs react forming adducts not only with the intact $V^{IV}OL_2$ complex but also with $V^{IV}OL^+$ and $V^{IV}O^{2+}$ ion.[12b, 15h, 17] These studies revealed that V mainly coordinates to the side chains of Asp, Glu and His residues upon replacement of water ligand or the release of one or more ligands.[12, 15h] However, experimental structural data based on X-ray

diffraction (XRD) on the interaction between oxidovanadium(IV) species and proteins are still scarce.[12a] Up to date, the following five structures were reported: $V^{IV}O(pic)_2$, pic = picolinato ligand, with HEWL (Asp binding),[18] ribonuclease A (RNase A, Glu binding),[19] and trypsin (Ser binding);[20] $V^{IV}O$(bipy/phen), where bipy = 2,2'-bipyridine and phen = 1,10-phenathroline, with HEWL (simultaneous binding of Asn and Asp).[20]

To enrich the list of known structures and define on structural ground the type and number of V binding sites in these biologically relevant adducts, here we studied $[V^{IV}O(malt)_2]$ interaction with HEWL using ESI-MS and EPR techniques to determine the number and type of V moieties and donors bound to protein and X-ray crystallography to disclose the interacting sites and the three-dimensional structure.

The results can help to better understand the solution chemistry of $[V^{IV}O(malt)_2]$, and in general of $V^{IV}OL_2$ potential drugs, and define the molecular basis of their transport in the organism and action mode.

EXPERIMENTAL SECTION

Materials. Water was deionized through the Millipore Milli-Q Academic system or purchased from Sigma-Aldrich (LC-MS grade). $V^{IV}OSO_4 \cdot 3H_2O$, maltol (malt), 1-methylimidazole (MeIm), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), sodium formate, sodium acetate, sodium nitrate, and ethylene glycol were Sigma-Aldrich products of the highest grade available and used as received. HEWL was purchased from Sigma-Aldrich and used without further purification. BMOV was synthesized according to the procedure reported in literature.[21]

Spectrometric and spectroscopic measurements. The solutions for ESI-MS measurements were prepared dissolving in ultrapure water (LC-MS grade, Sigma-Aldrich) BMOV and HEWL

to obtain a metal-to-protein molar ratio of 2/1 and a metal concentration of 100 μM. The pH of the solution was 5.0 or 6.5. ESI-MS spectra w. Spectra in positive-ion mode (ESI-MS(+)), immediately recorded after the solution preparation, were obtained on a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ (Thermo Fisher Scientific) mass spectrometer in the *m/z* range 300-4500 with a resolution of 140000 and accumulated for at least 5 min to increase the ratio signal-to-noise. The experimental settings were: flow rate of infusion into the ESI chamber 5.00 μL/min; spray voltage 2300 V; capillary temperature 250 °C; sheath gas 10 (arbitrary units); auxiliary gas 3 (arbitrary units); sweep gas 0 (arbitrary units); probe heater temperature 50°C. ESI-MS spectra were analyzed with Thermo Xcalibur 3.0.63 software (Thermo Fisher Scientific) and the average deconvoluted monoisotopic masses were obtained with the software Unidec 4.4.0.[22]

The EPR spectra were recorded on solutions obtained dissolving in water at pH 7.4: i) BMOV alone, ii) BMOV and HEWL at molar ratio 2/1 and iii) BMOV and 1-methylimidazole at ratio 1/4. Hepes buffer (0.1 M) was also added to all the solutions. The spectra were recorded at 120 K with an X-band Bruker EMX spectrometer equipped with a HP 53150A microwave frequency counter. This was the instrumental setting: microwave frequency was 9.40 GHz; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 4.0 Gauss; time constant 81.9 ms; sweep time 335.5 s; resolution 4096 points. To increase the signal to noise ratio, signal averaging was used.[13a] The full spectra are reported in the Supporting Information (SI), but in the text only the high-field region of the EPR spectra is shown, being more sensitive than the low-field region to the identity of the equatorial donors and amount of the several species in solution.[15a, 15c, 23] The number of scans for the high-field region of the spectra was 5.

Crystallization. HEWL crystals were grown by hanging drop vapor diffusion method under two different experimental conditions: i) 2.0 M sodium formate and 0.1 M Hepes pH 7.5 (structures **A**

and **A'**), and ii) in 20% ethylene glycol, 0.1 M sodium acetate at pH 4.0 and 0.6 M sodium nitrate (structures **B** and **B'**). These crystals were then exposed to stabilizing solutions containing the mother liquors and a saturated solution of $[V^{IV}O(malt)_2]$ for a soaking time in the range 3-22 days.

Data collection and refinement. X-ray diffraction data were collected on four crystals of HEWL soaked with $[V^{IV}O(malt)_2]$ (two crystals for each condition). HEWL crystals diffract X-ray at a resolution in the range 1.13-1.31 Å. Data collections have been carried out on Beamline XRD2 at Elettra synchrotron (Trieste, Italy), using a wavelength of 1.00 Å and a cold nitrogen stream of 100 K. Before exposure to X-ray, crystals were cryoprotected using a solution of the reservoir with 25% glycerol. Data processing and scaling were performed using Global Phasing autoPROC pipeline.[24] Data collection statistics are reported in the Supporting Information. Since the structures of **B** and **B'** are basically identical to each other, only the structure **B** is reported.

Structure solution and refinement. The structures were solved by the molecular-replacement method using Phaser[25] with PDB entries 193L[26] as templates. Refmac was used for the refinement and Coot for manual model building.[27] The structures refine to R-factors and Rfree values within the range 0.168-0.199 and 0.198-0.256. V atom position was validated using anomalous difference electron density maps. Ligand positions were restraints to guide geometry optimization. The final models have good geometries and refinement statistics (SI). UCSF Chimera software[28] and Pymol (www.pymol.org) were used to generate molecular-graphics figures. During refinement, solvent molecules were added to the model when they had reasonable electron density levels in the 2Fo-Fc and Fo-Fc maps and were within hydrogen bonding distances to possible donors or acceptors. Coordinates and structure factors of the adduct were deposited in the Protein Data Bank[29] under the accession codes 8AJ3, 8AJ4 and 8AJ5.

RESULTS AND DISCUSSION

Behavior of BMOV in aqueous solution. Maltol is a naturally occurring compound able to form chelate complexes with hard metal ions through the 3-hydroxy-4-pyrone moiety. In water, it can undergo the deprotonation to maltolato ($malt^-$) with pK_a of 8.44.[9] The structure of the solid complex formed by $malt^-$ with the $V^{IV}O^{2+}$ ion has been solved by X-ray diffraction analysis and has a stoichiometry $[V^{IV}O(malt)_2]$ (BMOV) with a square pyramidal geometry and two anionic ligands coordinated in the equatorial position (**Figure 1**).[8a] The dissolution of BMOV in coordinating organic solvents such as pyridine and DMSO or in water results in the transformation

Once BMOV is dissolved in water the square pyramidal → *cis*-octahedral isomerization of $[V^{IV}O(malt)_2]$ to *cis*-$[V^{IV}O(malt)_2(H_2O)]$ (*SPY*-5 → *OC*-6) occurs.[8b, 9, 30] It must be noted that, when *OC*-6 is formed, all the eight possible isomers in equilibrium to each other exist: *OC*-6-34-Δ, *OC*-6-34-Λ, *OC*-6-24-Δ, *OC*-6-24-Λ, *OC*-6-32-Δ, *OC*-6-32-Λ, *OC*-6-23-Δ, *OC*-6-23-Λ (**Figure S1**). Moreover, depending on pH, the aqua ion $[V^{IV}O(H_2O)_4]^{2+}$ (very acidic values), and the complexes $[V^{IV}O(malt)(H_2O)_3]^+$ (acidic values) and $[V^{IV}O(malt)_2(H_2O)]$ (neutral values) are formed (**Figure 1**).[9] In principle, all these species could react with a protein.

In aqueous solution, the complexation is influenced, not only by the pH but also by vanadium concentration. When it is 1 mM, the 1:1 species exists between pH 3 and 4, the 1:2 complex *cis*-$[V^{IV}O(malt)_2(H_2O)]$ predominates between 4.5 and 8.5 and, subsequently, transforms to $[V^{IV}O(malt)_2(OH)]^-$ with a pK of 8.79 upon the deprotonation of the equatorial water ligand (the concentration distribution curves are shown in **Figure S2A**);[9] with lowering the V concentration to 10 μM, the hydrolysis becomes very important, for $[V^{IV}O(malt)(H_2O)_3]^+$ the maximum concentration shifts around pH 4.5, the pH range of existence of *cis*-$[V^{IV}O(malt)_2(H_2O)]$ narrows

and at pH higher than 8 $[(V^{IV}O)_2(OH)_5]^-$ and then $[(V^{IV}O)_2(OH)_5]^-$ become the major species in solution (**Figure S2B**).

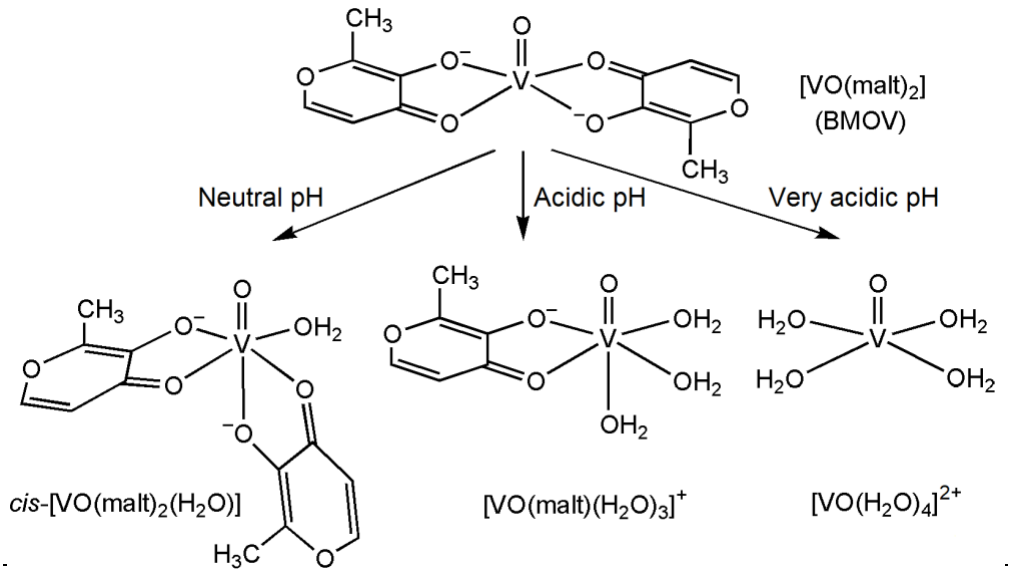

Figure 1. Structure of $[V^{IV}O(malt)_2]$ (BMOV) and its possible transformations in aqueous solution. The shown behavior corresponds to a V^{IV} concentration around 1 mM.

ESI-MS and EPR studies. To evaluate if the interaction of BMOV with HEWL takes place and establish the type and number of the possible adducts, ESI-MS(+) spectra were recorded at pH 5.0 and 6.5 with molar ratio 2/1 and V concentration of 100 μM. The reference raw spectrum of free HEWL shows a series of peaks with different charged states from +8 to +12 from m/z range of ca. 1800 to ca. 1200 (**Figure S3A**). In the deconvoluted spectrum, the central peak found at 14303.9 Da is surrounded by those of the adducts formed with Na^+ ions or H_2O molecules (**Figure S3B**). When BMOV is present in aqueous solution, in the raw spectrum, for each HEWL peak other signals at higher m/z values are detected, indicating the formation of HEWL–VO–malt adducts, in

agreement with the literature data.[17] However, the charge state distribution does not vary appreciably upon the V-containing fragments, suggesting that HEWL keeps its conformation. The deconvoluted spectra recorded at pH 5.0 and 6.5 (**Figure 2**) give important insights: i) at both the pH values the interaction with $V^{IV}O(malt)^{+}$ and $V^{IV}O(malt)_2$ is revealed and the multiple binding of these two moieties is observed; ii) at pH 5.0 the binding of $V^{IV}O^{2+}$ is also detected; iii) by increasing the pH to 6.5, the number of adducts with $V^{IV}O(malt)_2$ fragment (HEWL–$[VO(malt)_2]$ and HEWL–$[VO(malt)_2]_2$) increases. These experimental findings agree well with the results predicted through the thermodynamic stability constants[9] and with the distribution curves of the $V^{IV}O$ species shown in **Figure S2** which suggest that at acidic pH values aqua ion and 1:1 species exist and the amount of 1:2 complex is limited, while going toward the neutrality $[V^{IV}O(H_2O)_4]^{2+}$ disappears and the relative amount of $V^{IV}O(malt)_2$ compared to $V^{IV}O(malt)^{+}$ increases.

The final comment concerns the oxidation state of vanadium, for which ESI-MS results suggest +IV in the timescale used for the experiments. In fact, an oxidation to V^{V} should results in the formation of adducts based on the $V^{V}O_2$ moiety.

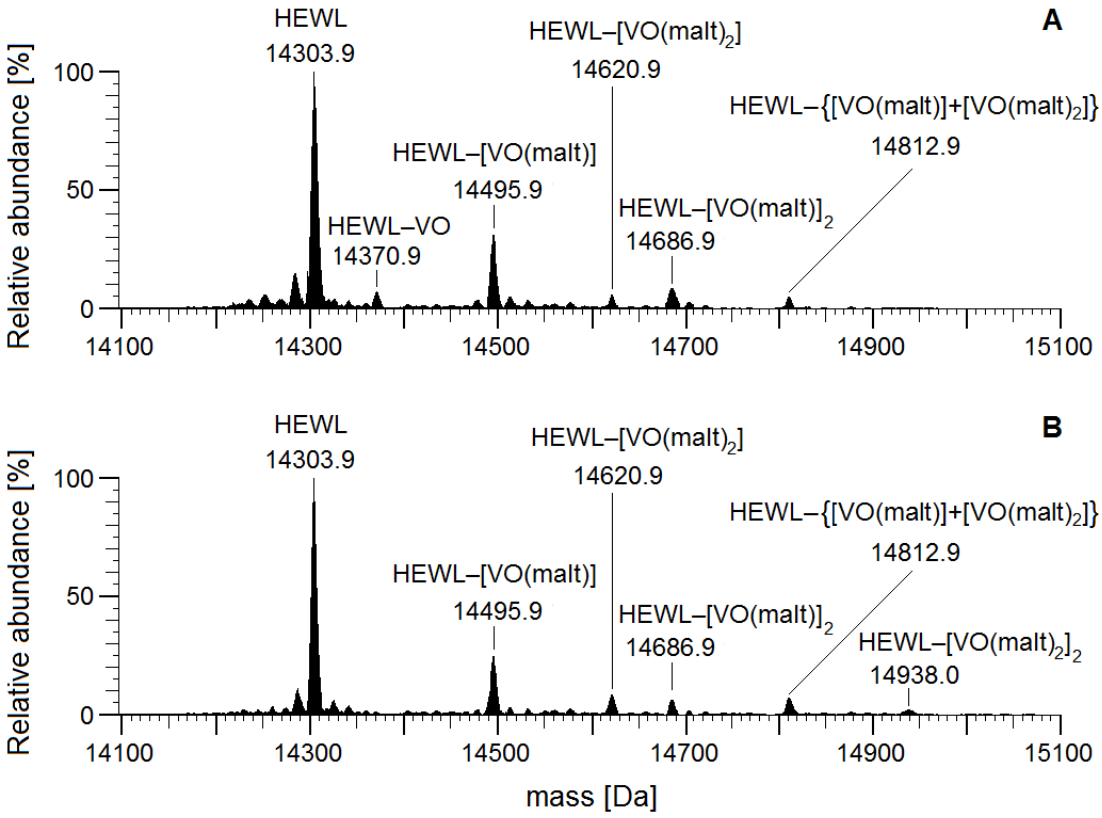

Figure 2. Deconvoluted ESI-MS(+) spectra recorded on the system containing BMOV and HEWL with a metal to protein molar ratio of 2/1 and a V concentration of 100 μM: A) pH 5.0 and B) pH 6.5.

Anisotropic EPR spectra, recorded at 120 K in the system BMOV/HEWL 2/1 at pH 7.4, are shown in **Figure 3**. All the spectra were simulated with the software WINEPR SimFonia.[31] The experimental and simulated signals of the full spectra are shown in **Figures S4-S7**, where the instrumental settings are also reported. The spin Hamiltonian parameters are listed in **Table S1**. The spectra were simulated assuming a tetragonal symmetry with $g_x = g_y$ and $A_x = A_y$; this agrees

well with the data in the literature, which suggest that the value of $|A_x - A_y|$, related to the x,y anisotropy, is very small for octahedral $V^{IV}O$ species (generally less than 2-3 × 10^{-4} cm^{-1} [32]).

The spectra in the traces A, C and D, measured at the same experimental conditions, have been added as a reference. In the spectrum of the solution containing BMOV (trace A) only the resonances of *cis*-[$V^{IV}O(malt)_2(H_2O)$] are detected (denoted with **I** in **Figure 3**) and the hyperfine coupling constant on z axis (A_z) is 170.8 × 10^{-4} cm^{-1}. The spectrum in the trace C has been measured on the system BMOV/MeIm and is characterized by the presence of *cis*-[$V^{IV}O(malt)_2(MeIm)$] (**IIIa** in **Figure 3**), where the equatorial water is replaced by an imidazole-N, with an A_z of 164.8 × 10^{-4} cm^{-1}. Finally, in the spectrum reported in the trace D the resonances of the adduct of $V^{IV}O(malt)_2$ with human serum albumin (HSA) have been observed with A_z 165.0 × 10^{-4} cm^{-1} (**IIIb** in **Figure 3**); in such an adduct the value of A_z as well as the coordination around V is similar to *cis*-[$V^{IV}O(malt)_2(MeIm)$] (indeed, it could be denoted with HSA–[$V^{IV}O(malt)_2$] or *cis*-$V^{IV}O(malt)_2$(HSA-His) with albumin binding to V with a His-N donor).

In contrast, in the system with BMOV and HEWL the resonances of two species (**I** and **II** in **Figure 3**) are observed. The resonances **I** coincide with those of *cis*-[$V^{IV}O(malt)_2(H_2O)$], while those indicated with **II** are intermediate between those of *cis*-[$V^{IV}O(malt)_2(H_2O)$] and *cis*-[$V^{IV}O(malt)_2(MeIm)$] / *cis*-[$V^{IV}O(malt)_2$(HSA-His)] with experimental A_z of 167.8 × 10^{-4} cm^{-1} (**II** in **Figure 7**). This suggests that the fourth donor in the equatorial plane of $V^{IV}O^{2+}$ ion is an O atom that – according to "additivity relationship", the empirical rule that allows to estimate A_z from the contribution of the four donors in the equatorial plane of $V^{IV}O^{2+}$ ion [33] – should give a value of A_z between those of an imidazole-N and a water-O. Globally, these data allowed us to assign the resonances **II** to an Asp/Glu-COO^- or an Asn/Gln-CO side chain and to exclude the binding of the unique histidine residue of HEWL, His15, that is the preferred donor for cisplatin and other Pt-

based drugs.[34] Compared to an equatorial water-O, the "additivity relationship" predicts a decrease of the value of A_z of ca. 3-4 × 10^{-4} cm^{-1} upon the coordination of a carboxylate-O or carbonyl-O binding and of ca. 5-6 × 10^{-4} cm^{-1} for a His-N coordination,[33] in agreement with what was experimentally observed.

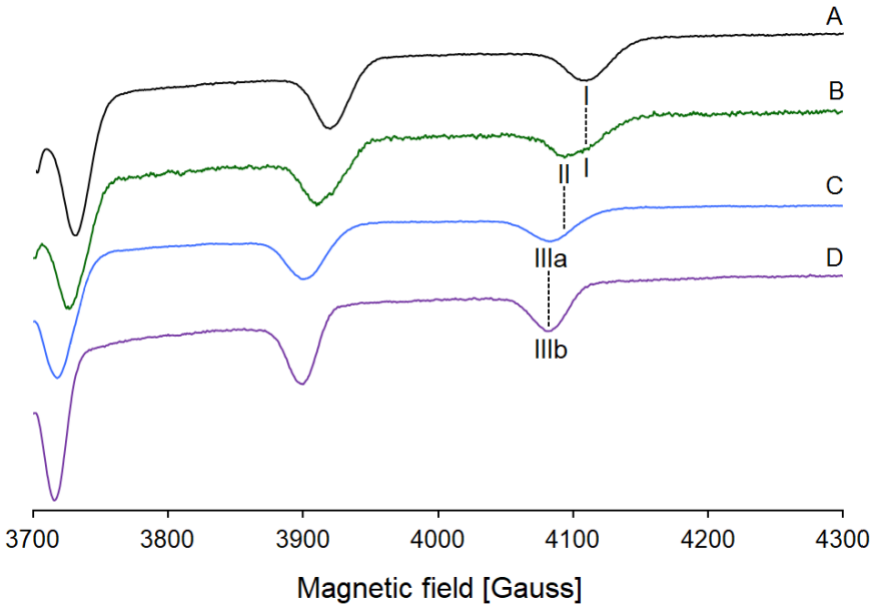

Figure 3. High-field region of anisotropic X-band EPR spectra recorded at pH and 120 K in the systems an aqueous solution containing: A) [$V^{IV}O(malt)_2(H_2O)$]; B) BMOV/HEWL 2/1; C) BMOV/HSA 2/1; D) BMOV/MeIm 1/4. V concentration is always 1.0 mM. The M_I = 7/2 resonance of [$V^{IV}O(malt)_2(H_2O)$] is indicated with **I**, of HEWL–[$V^{IV}O(malt)_2$] with **II**, of *cis*-[$V^{IV}O(malt)_2$(HSA-His)] with **IIIa** and of *cis*-[$V^{IV}O(malt)_2$(MeIm)] with **IIIb**. The coordination of the two maltolato ligands is (equatorial-equatorial) and (equatorial-axial) and the fourth equatorial donor is a water-O for **I**, an Asp/Glu-COO^- or an Asn/Gln-CO for **II**, and a His-N or an imidazole-N for **IIIa** and **IIIb**.

X-ray study: structures A and A'. X-ray diffraction data were collected on two crystals (structures **A** and **A**') of HEWL grown in 2.0 M sodium formate and 0.1 M Hepes, pH 7.5 and exposed to $[V^{IV}O(malt)_2]$ for 3 weeks. Crystals are isomorphous to each other and isomorphous with those of the ligand-free protein with minor differences in their unit cells (**Table S2** of the SI). The overall conformation of the protein in the two crystals is not significantly affected by these differences: Cα root mean square deviation (rmsd) between the two structures is as low as 0.07 Å. Furthermore, it appears that the protein structure is not significantly affected by the V compound binding, confirming the ESI-MS results. Indeed, rmsd from the metal-free protein structure (PDB code 193L[26]) is 0.20 Å. Concerning the oxidation state of V, we mentioned above that ESI-MS technique suggests the maintenance of +IV. Further experiments, evaluating the EPR intensity of the signal as a function of the time, confirm that the +IV state ($3d^1$, EPR-active) is rather stable during the time range explored for the growth of the crystals (**Figure S8**); therefore, even if a partial oxidation to V^V cannot be excluded, for all the adducts +IV has been considered.

In both structures **A** and **A'**, three sites were found, named **1-3** and distinguished with black, red, and blue colors in **Figures 4-6**. However, analysis of the diffraction data reveals significant differences between the two structures.

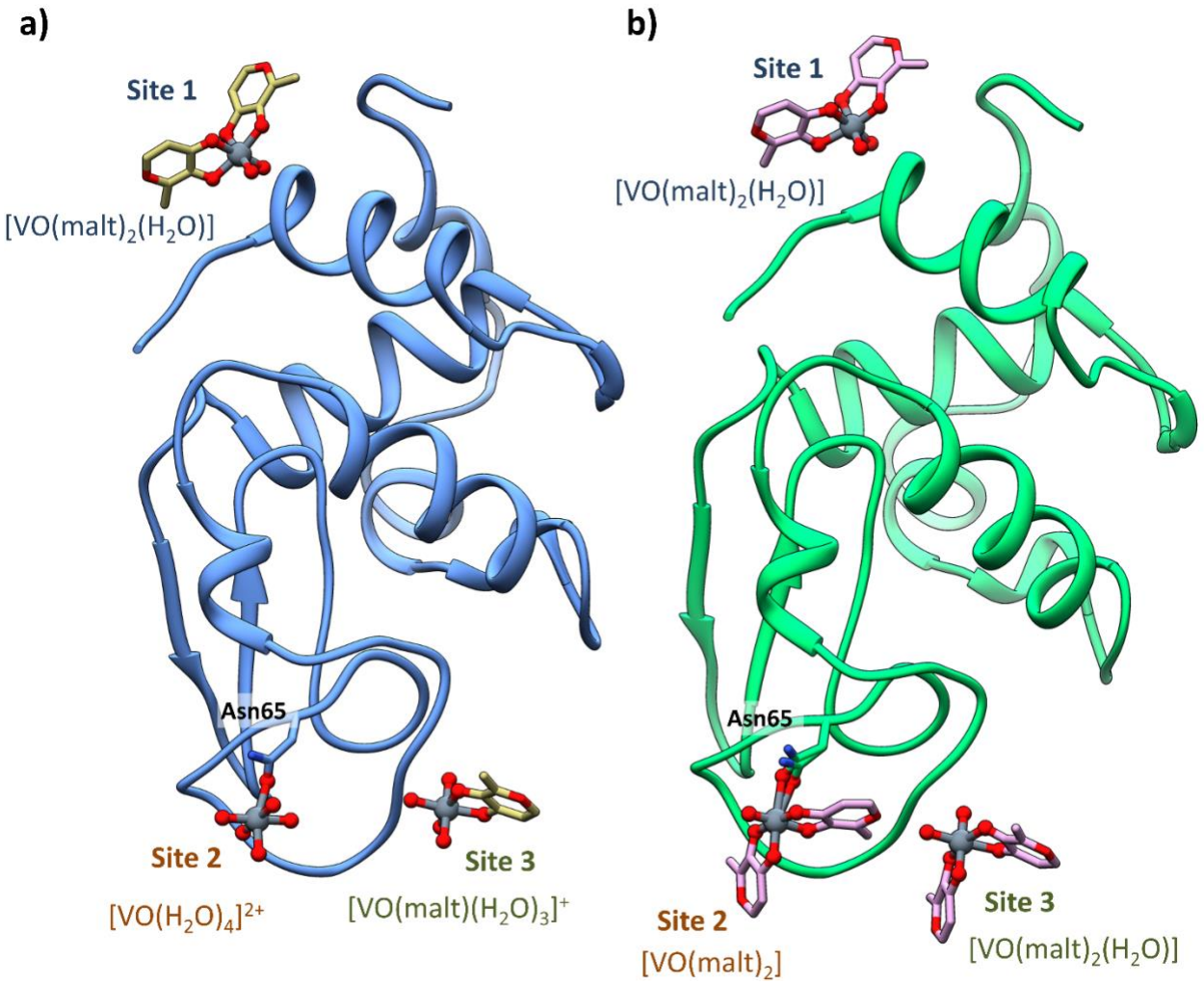

Figure 4. Overall structure of HEWL in the presence of $[V^{IV}O(malt)_2]$ in 2.0 M sodium formate and 0.1 M Hepes buffer pH 7.5 (structure **A** in panel A and structure **A'** in panel B). With **1**-**3** the three binding sites are indicated.

In structure **A**, refined at 1.13 Å resolution to Rfactor/Rfree values of 0.199/0.249, non-covalent binding of *cis*-$[VO(malt)_2(H_2O)]$ and $[VO(malt)(H_2O)_3]^+$ on the protein surface was found (**Figure 5a** and **5b**), together with covalent binding of a $[VO(H_2O)_4]^{2+}$ ion to the side chain of Asn65 (**Figure 5c**). 2Fo-Fc electron density map at these sites, reported at 1.0 σ, indicates a clear

definition of the V geometry and its ligands (**Figure 5a-c**). The electron density is very well defined for *cis*-[VO(malt)$_2$(H$_2$O)] bound at the site **1** with 0.70 occupancy. For the second site (site **2**), occupied by [VO(malt)(H$_2$O)$_3$]$^+$, the definition is a little bit low with 0.30 occupancy, while it is 0.50 for the site **3**, where [VO(H$_2$O)$_4$]$^{2+}$ is bound.

In structure **A'**, non-covalent binding of two molecules of *cis*-[VO(malt)$_2$(H$_2$O)] (**Figures 6a** and **6b**) on the protein surface and covalent binding of *cis*-[VO(malt)$_2$] to the side chain of Asn65 (**Figure 6c**) were observed. The 2Fo-Fc electron density maps of these V binding sites are reported at 1.0 σ in **Figure 6a-c**. The electron density is very well defined for the first *cis*-[VO(malt)$_2$(H$_2$O)] (site **1**), which refines with high occupancy (0.80), while it is less defined in the case of the second *cis*-[VO(malt)$_2$(H$_2$O)] (site **2**) and [VO(malt)$_2$] (site **3**), that have a 0.30 occupancy in the final model.

These data are in excellent agreement with the ESI-MS experiments that indicate the multiple binding of three different moieties, VO^{2+}, VO(malt)$^+$ and VO(malt)$_2$ (see **Figure 2**). Moreover, the fragment VO(malt)$_2$ covalently bound to HEWL in structure **A'** supports the existence in solution of the complex [V^{IV}O(malt)$_2$(H$_2$O)] which gives the adduct HEWL–V^{IV}O(malt)$_2$ (revealed also by ESI-MS technique) after the replacement of a weak water ligand by an O donor from a side-chain of Asn65 residue; in contrast, the oxidation of [V^{IV}O(malt)$_2$(H$_2$O)] to [V^{V}O$_2$(malt)$_2$]$^-$, possible in principle, would preclude the observation of HEWL–V^{V}O(malt)$_2$ because the break of one of the two V^{V}=O bonds by an amide-O of Asn65 is not plausible at all.

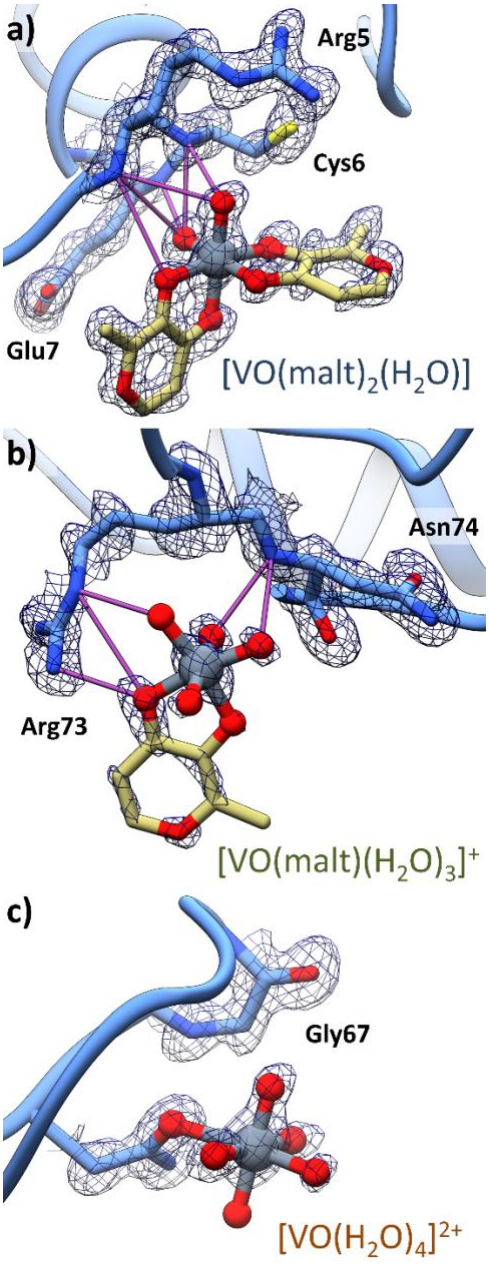

Figure 5. Details of the vanadium binding sites in structure **A**. Non-covalent binding of *cis*-$[VO(malt)_2(H_2O)]$ and $[VO(malt)(H_2O)_3]^+$ to the sites **1** and **2** are reported in panels a and b, respectively. The covalent binding of $[VO(H_2O)_4]^{2+}$ to Asn65 of the site **3** is reported in panel a. 2Fo-Fc electron density maps are shown at 1.0 σ level in blue.

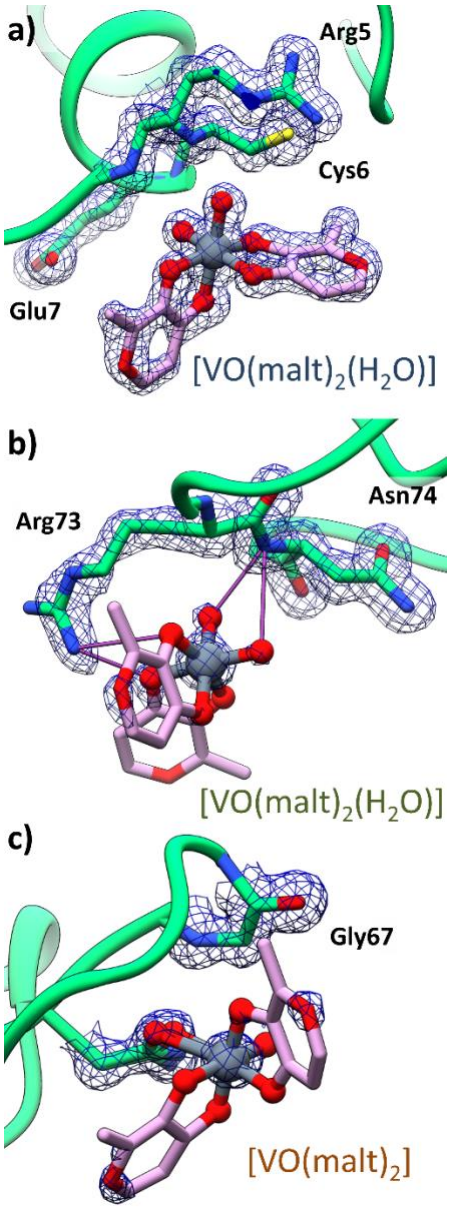

Figure 6. Vanadium binding site in structure **A'**. Non-covalent binding of the two *cis*-[VO(malt)$_2$(H$_2$O)] molecules to the sites **1** and **2** is reported in panels a and b, respectively. The covalent binding of [VO(malt)$_2$] molecule to Asn65 of the site **3** is reported in panel c. In this panel, the side chain of Asn65 adopts two different conformations. 2Fo-Fc electron density maps are shown at 1.0 σ level in blue.

In both structures **A** and **A'**, site **1** (in black) is occupied by *cis*-[VO(malt)$_2$(H$_2$O)], which is hydrogen bonded with the N atoms of the main chain of Arg5, Cys6, Glu7 (**Figure 5a** and **Figure 6a**), solvent water molecules, the side chain of Arg14 of a symmetry related molecule and is in close contact with a V-containing species from a symmetry related molecule (**Figure S9**). At the site **2** of structure **A** (in red), [VO(malt)(H$_2$O)$_3$]$^+$ ion forms hydrogen bonds with the main chain N atom of Asn74 and with the side chain of Arg73 (**Figure 5b**), with solvent water molecules and with the *cis*-[VO(malt)$_2$(H$_2$O)] molecule from a symmetry mate (**Figure S10**). The site **2** of the structure **A'** is occupied by a *cis*-[VO(malt)$_2$(H$_2$O)] complex (**Figure 6b**), which forms hydrogen bonds with [VO(malt)$_2$] (located at site **3**), with the side chain of Arg73, the main chain of Asn74, the *cis*-[VO(malt)$_2$(H$_2$O)] molecule from a symmetry mate and water molecules; the maltolato ligand forms additional stacking interaction with the [VO(malt)$_2$] from a symmetry related molecule (**Figure S11**). Finally, at site **3** of structure **A** (in blue), a [VO(H$_2$O)$_4$]$^{2+}$ cation is found coordinated to the side chain of Asn65 with the V-containing fragment held in its position by hydrogen bonds formed with the main chain of Gly67 (**Figure 5c**) and water molecules. In structure **A'**, in the same position, the [VO(malt)$_2$] has been modeled (**Figure 6c**), interacting with a covalent bond to Asn65 residue and forming hydrogen bonds with *cis*-[VO(malt)$_2$(H$_2$O)] and water molecules, plus additional stacking with *cis*-[VO(malt)$_2$(H$_2$O)] from a symmetry mate (**Figure S12**). Such results, which suggest the simultaneous presence of *cis*-[V^{IV}O(malt)$_2$(H$_2$O)] (with a non-covalent bond) and HEWL–[V^{IV}O(malt)$_2$] (with a covalent bond) in the system BMOV/HEWL and – in addition – the covalent binding of HEWL through an O donor side chain, are perfectly in line with the EPR data (see **Figure 3**).

Thus, in structures **A** and **A'**, all the possible fragments derived from the transformation of BMOV in aqueous solution (**Figure 1**) are observed. Concerning the interaction of bis-chelated

complex, the crystallographic results demonstrate that: i) the *SPY*-5 → *OC*-6 reaction takes place, as suggested by potentiometric and spectroscopic data,[8b, 9] but never supported by X-ray analysis up to now; ii) several V-containing isomers bind, covalently or non-covalently, to the protein having two equatorial phenolato-O^- (site **1** of the structures **A** and **A'**, and site **2** of the structure **A'**) or two equatorial keto-O (site **3** of the structure **A'**); iii) the interaction occurs with various enantiomers (*OC*-6-24-Λ at site **1** of both the structures, *OC*-6-24-Δ at site **2** of structure **A'**, and *OC*-6-34-Δ at site **3** of structure **A'**). These findings indicate that, in aqueous solution, the isomers and enantiomers derived by BMOV are in equilibrium and that the subtle energy and steric factors, such as hydrogen bonds, van der Waals contacts and the chiral specificity of the protein sites, stabilize the interaction with the metal moieties as suggested by our previous reports.[13g, 13i-l]

X-ray study: structure B. Structure **B** has been obtained exposing HEWL crystals grown in 20% ethylene glycol, 0.1 M sodium acetate at pH 4.0 and 0.6 M sodium nitrate to a reservoir solution saturated with $[V^{IV}O(malt)_2]$ for 3 weeks. The structure, refined at 1.31 Å resolution, up to Rfactor and Rfree values of 0.168 and 0.198. The overall structure of the protein in the adduct (**Figure S13**) closely resembles that of the metal-free protein and of structures **A** and **A'** (rmsd within the range 0.24 Å). In structure **B**, covalent binding of three $[VO(H_2O)_3]^{2+}$ ions together with two additional V atoms, whose geometry is not well defined, are found. The first $[VO(H_2O)_3]^{2+}$ ion (occupancy = 0.50) was found close to the side chain of Asp48 (**Figure 7a**). Here, the V atom adopts a square pyramid geometry, with the side chain of Asp48 on the plane and the V=O group at the apex of the pyramid. The V-containing fragment is held in its position by hydrogen bonds formed with the side chain of Asn46, Ser50, Asn59 and Arg61, with a water molecule and the main chain N atom of Asp48 (**Figure 7a**). The second $[VO(H_2O)_3]^{2+}$ ion (occupancy = 0.50) was observed close to the side chain of Asp87 (**Figure 7b**). Here, the interpretation of the map is

complicated by conformational disordered, as evidenced by the presence of alternative conformations of the side chain of Asp87 and by the presence of a nitrate ion. This V fragment is on the protein surface and in contact with the side chains of His15 and Arg14 and with water molecules. The final $[VO(H_2O)_3]^{2+}$ ion was found close to the side chain of Glu35 (**Figure 7c**). At this site, the electron density around the V center is not very well defined, probably also because of the low occupancy (0.35). The oxygen atoms of the V fragment interact with the main chains of Gln57, Ala107, Val109, and Ala110, and the side chain of Asp52 (**Figure 7c**). Notably, the residues Glu35 and Asp87 were proposed as possible candidates for V drugs binding.[13k]

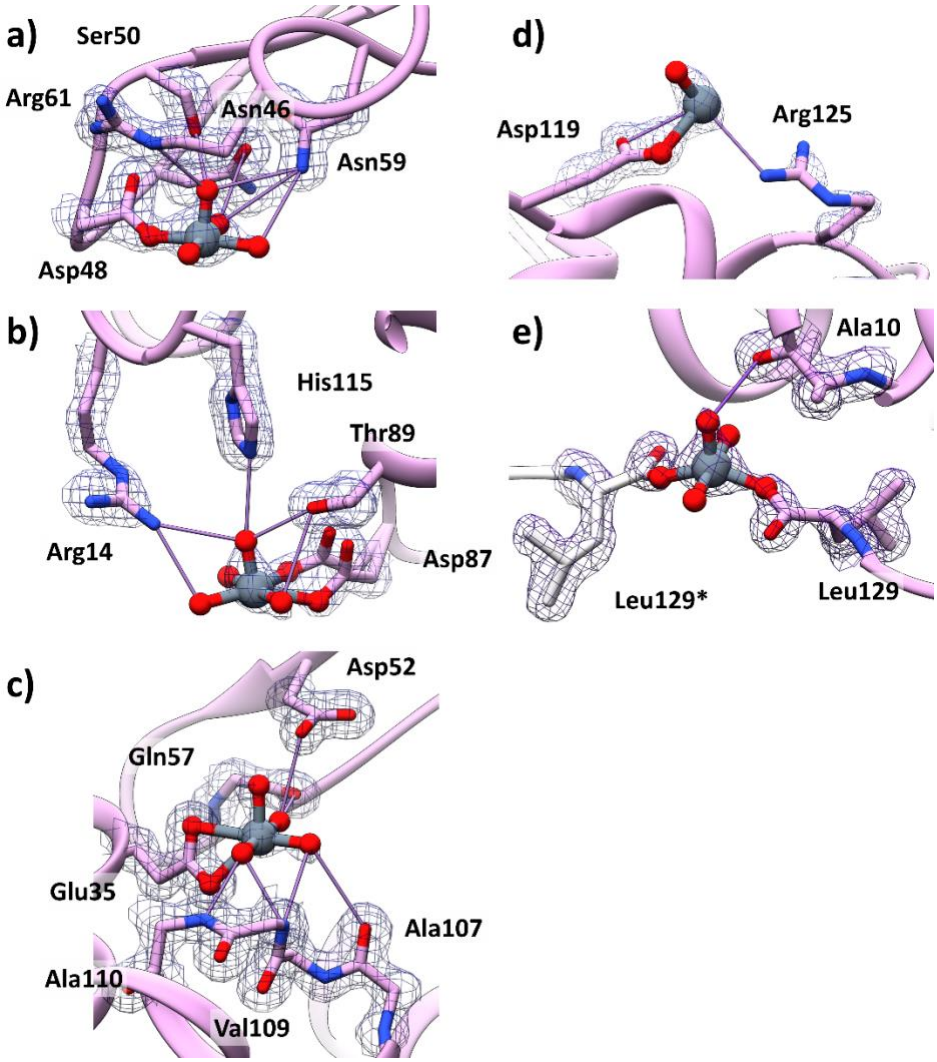

Figure 7. Details of the V compound binding sites in structure **B**. Covalent binding of $[VO(H_2O)_3]^{2+}$ to the side chains of Asp48 (panel a), Asp87 (panel b), Glu35 (panel c). Other V containing fragments are covalently bound to the side chain of Asp119 (panel d) and the C-terminal carboxylate (panel e). 2Fo-Fc electron density maps are reported at 1.0 σ level in grey. Water molecules and nitrate ions in contact with the V ligands are omitted for sake of clarity. Atoms from symmetry related molecules are highlighted as * and colored in grey; their density is represented in red.

Furthermore, covalent binding of V atoms to the side chain of Asp119 (**Figure 7D**) and to the C-terminal carboxylate (**Figure 7E**) was observed. Close to Asp199, the V center coordinates an oxygen and could be in contact with a N atom of the side chain of Arg125, whose electron density is not well defined. At this site, although the V center presents high occupancy (1.00), inspection of the electron density map does not allow a precise definition of missing ligands. The V atom found at the C-terminal carboxylate (Leu129) is at the interface between two symmetry-related molecules (**Figure 7E**). This V completes its coordination sphere with three water molecules. The precise identification of the V geometry in this site is complicated by the low definition of the electron density map and by the proximity of this site to the twofold axis. The V binding induces the formation of a covalently linked protein dimers, as occurs when HEWL reacts with dirhodium tetraacetate.[35]

Comparison with other $V^{IV}OL_n$–protein adducts. As pointed out in the Introduction, structural data between the interaction of $V^{IV}O^{2+}$ and its complexes $V^{IV}OL_n$, with n = 1-2, are still scarce.[12a] To the best of our knowledge, only five X-ray structures have been reported in the

literature: 1) HEWL–$V^{IV}O(pic)_2$ with the covalent binding of Asp52;[18] 2) RNase A–$V^{IV}O(pic)_2$ with the covalent binding of Glu111;[19] 3) trypsin–$V^{IV}O(pic)_2$ with the covalent binding of Ser195;[20] 4) HEWL–$V^{IV}O(H_2O)$(bipy) and 5) HEWL–$V^{IV}O(H_2O)$(phen) with the simultaneous covalent binding of Asn46 and Asp52.[20] The results obtained in this study provide further elements in the comprehension of the behavior of the systems containing $V^{IV}O$ species and proteins. Below, the most significant differences are highlighted.

First of all, the capability of binding of Asn (and Gln) side chain has been confirmed; these donors add to the list proposed for the $V^{IV}O$ binding, namely Asp, Glu, His and Ser.[12] Second, the multiple binding of $V^{IV}O$ adducts, never observed until now, has been demonstrated. Third, the binding can be covalent or non-covalent. Fourth, for a $V^{IV}OL_2$ complex, all the possible fragments, different for composition ($V^{IV}OL_2$, $V^{IV}OL$, $V^{IV}O^{2+}$), geometry (octahedral and square pyramidal), isomerism (*OC*-6 and *SPY*-5 species) and enantiomerism (Δ and Λ enantiomers), can interact with proteins.

Finally, it must be remembered that the possibility of oxidation of V^{IV} to V^{V}, depending on the crystallization conditions and type and stability of the $V^{IV}OL_2$ complex, cannot be excluded.

CONCLUSIONS

In conclusion, here we have reported the crystal structures of the adducts formed upon reaction of the potential drug [$V^{IV}O(malt)_2$] (abbreviated in the literature with BMOV) with HEWL, under two different experimental conditions. An analysis of the data reveals that: i) the protein does not interact with the dissolved square pyramidal compound, [$V^{IV}O(malt)_2$], but with its fragments in aqueous solution, i.e. *cis*-[$VO(malt)_2(H_2O)$], [$VO(malt)(H_2O)_3$]$^+$ and the aquaion [$VO(H_2O)_4$]$^{2+}$;

ii) the obtained fragments can bind the protein both covalently and non-covalently; iii) HEWL contains several solvent accessible binding sites on its surface, iv) a different number of adducts on such sites is observed and a multiple binding of V occurs; v) the interaction of the vanadium-containing moieties does not induce any significant structural variation of HEWL; vi) the complex $[V^{IV}O(malt)_2(H_2O)]$ exists in several isomers and enantiomers and many of them can interact with the protein, depending on the chiral specificity of the protein sites; vii) Asp/Glu side chains with the carboxylate (Glu35, Asp48, Asp87, Asp119 for HEWL) and Asn and possibly Gln with a carbonyl group (Asn65 for HEWL) are the residues mainly involved in the V binding; viii) ESI-MS and EPR techniques allow to confirm the crystallographic results, confirming themselves as very valuable tools for the study of V^{IV}–protein interaction; ix) the occupancies of V-containing fragments are often > 0.5 and sometimes very close to 1. Although it is not possible to derive a direct relation between the occupancy and the affinity of the metal-containing fragment for a protein, this latter result merits attention since it is a feature not found in the protein metalation by Pt,[36] Au,[37] Ru,[38] and Rh[39] drugs.

Overall, the results of the present structural and spectrometric/spectroscopic analysis fortify the concept that a biologically active $V^{IV}OL_2$ compound can lose its carrier ligand L before and upon interaction with proteins, also forming derivatives with water molecules replacing the carrier ligand. The simultaneous covalent and non-covalent interaction, each realized with variable strength, allows the multiple binding of various vanadium-containing fragments and the possibility that several metal moieties are transported in bloodstream and cellular environment towards the targets in the organism, amplifying the effect of the potential drug.

Finally, the reactivity of BMOV with HEWL elucidated in this paper could help in the understanding of the mechanisms at the basis of the formation of $V^{IV}O$–(carrier L)–protein adducts

that biologically active VOL_2 compounds form with transferrin, albumin, or other membrane or cytosolic proteins, promoting and boosting the development of new V complexes as putative therapeutic agents.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs. inorgchem.xxxxxxx. Tables with EPR parameters (**Table S1**) and X-ray diffraction data collection and refinement statistics (**Table S2**), figures with isomers of $[V^{IV}O(malt)_2(H_2O)]$ (**Figure S1**), concentration distribution curves of $V^{IV}O^{2+}$/malt system (**Figure S2**), ESI-MS spectra of HEWL (**Figure S3**), simulated EPR spectra (**Figures S4-S7**), time dependence of EPR intensity (**Figure S8**) and interactions of the metal fragments and HEWL in structures A, A' and B (**Figures S9-S13**).

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

There are no conflicts to declare.

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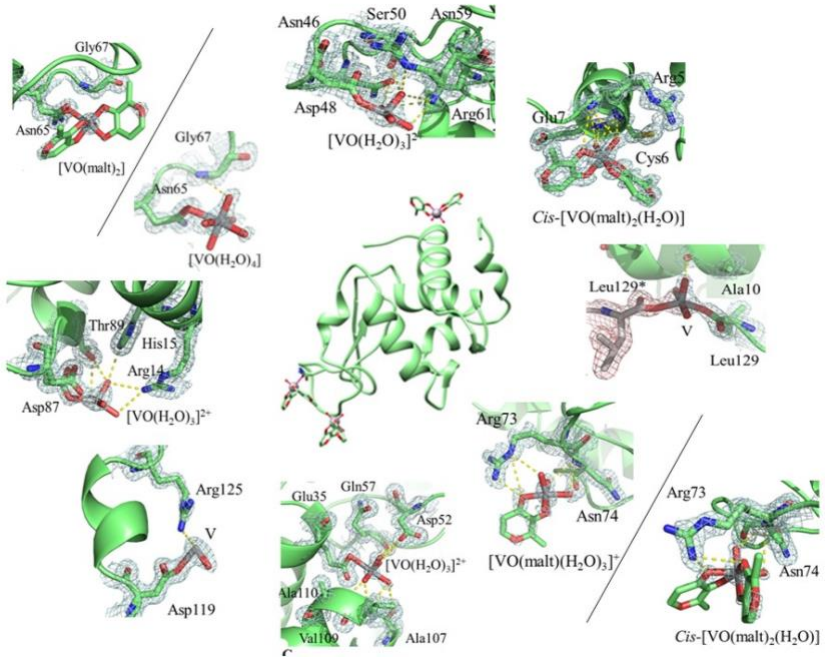
GRAPHICAL ABSTRACT



TEXT FOR GRAPHICAL ABSTRACT

The interaction of $[V^{IV}O(malt)_2]$ (BMOV, malt = maltolato) with hen egg white lysozyme (HEWL) reveals the multiple binding of different V-containing isomers and enantiomers to different sites and non-covalent and covalent binding of *cis*-$[VO(malt)_2(H_2O)]$, $[VO(malt)(H_2O)_3]^+$ $[VO(H_2O)_{3-4}]^{2+}$ and *cis*-$[VO(malt)_2]$ to Glu, Asp, and Asn residues.