# Protein-Assisted Formation of Molybdenum Heterometallic Clusters: Evidence for the Formation of S$_2$MoS$_2$−M−S$_2$MoS$_2$ Clusters with M = Fe, Co, Ni, Cu, or Cd within the Orange Protein

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S *Supporting Information*

ABSTRACT: The Orange Protein (ORP) is a small bacterial protein, of unknown function, that harbors a unique molybdenum/copper (Mo/Cu) heterometallic cluster, $[S_2Mo^{VI}S_2Cu^IS_2Mo^{VI}S_2]^{3-}$, noncovalently bound. The apo-ORP is able to promote the formation and stabilization of this cluster, using Cu^{II}- and $Mo^{VI}S_4^{2-}$ salts as starting metallic reagents, to yield a Mo/Cu-ORP that is virtually identical to the native ORP. In this work, we explored the ORP capability of promoting protein-assisted synthesis to prepare novel protein derivatives harboring molybdenum heterometallic clusters containing iron, cobalt, nickel, or cadmium in place of the "central" copper (Mo/Fe-ORP, Mo/Co-ORP, Mo/Ni-ORP, or Mo/Cd-ORP). For that, the previously described protein-assisted synthesis protocol was extended to other metals and the Mo/M-ORP derivatives (M = Cu, Fe, Co, Ni, or Cd) were spectroscopically (UV−visible and electron paramagnetic resonance (EPR)) characterized. The Mo/Cu-ORP and Mo/Cd-ORP derivatives are stable under oxic conditions, while the Mo/Fe-ORP, Mo/Co-ORP, and Mo/Ni-ORP derivatives are dioxygen-sensitive and stable only under anoxic conditions. The metal and protein quantification shows the formation of 2Mo:1M:1ORP derivatives, and the visible spectra suggest that the expected {S$_2$MoS$_2$MS$_2$MoS$_2$} complexes are formed. The Mo/Cu-ORP, Mo/Co-ORP, and Mo/Cd-ORP are EPR-silent. The Mo/Fe-ORP derivative shows an EPR $S = \frac{3}{2}$ signal ($E/D \approx$ 0.27, $g \approx$ 5.3, 2.5, and 1.7 for the lower $M = \pm\frac{1}{2}$ doublet, and $g \approx$ 5.7 and 1.7 (1.3 predicted) for the upper $M = \pm\frac{3}{2}$ doublet), consistent with the presence of either one $S = \frac{5}{2}$ Fe^{III} antiferromagnetically coupled to two $S = \frac{1}{2}$ Mo^V or one $S = \frac{3}{2}$ Fe^I and two $S = 0$ Mo^{VI} ions, in both cases in a tetrahedral geometry. The Mo/Ni-ORP shows an EPR axial $S = \frac{1}{2}$ signal consistent with either one $S = \frac{1}{2}$ Ni^I and two $S = 0$ Mo^{VI} or one $S = \frac{1}{2}$ Ni^{III} antiferromagnetically coupled to two $S = \frac{1}{2}$ Mo^V ions, in both cases in a square-planar geometry. The Mo/Cu-ORP and Mo/Cd-ORP are described as {Mo^{VI}−Cu^I−Mo^{VI}} and {Mo^{VI}−Cd^{II}− Mo^{VI}}, respectively, while the other derivatives are suggested to exist in at least two possible electronic structures, {Mo^{VI}−M^I− Mo^{VI}} ↔ {Mo^V−M^{III}−Mo^V}.
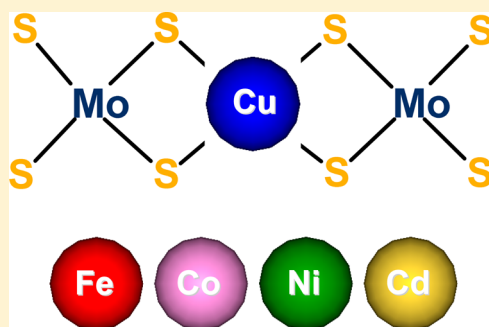
INTRODUCTION

The protein-assisted synthesis of metallic complexes is a highly valuable approach, extensively explored in the 1980s to study the properties of certain metalloproteins,[1−3] as was the case of ferredoxins,[4−6] whose iron/sulfur (Fe/S) center interconversions and heterometallic derivatives, harboring cobalt, nickel, zinc, or cadmium, were thoroughly studied. Besides the knowledge gathered about the target metalloprotein, the protein-assisted synthesis could also provide information on how the protein promotes the assembly and stabilization of the metallic cluster. In addition, the protein-assisted synthesis allows the preparation (for subsequent study) of metallic complexes that cannot be obtained in aqueous systems, or with adequate stability, in the absence of the protein. Moreover, the protein-assisted synthesis allows the introduction of metallic clusters in proteins that are heterologously produced and obtained in the apo form (because the host organism does not present the metabolic pathways to produce the holo forms or these are not expressed at the appropriate rate).

Inspired by the previous experience of our group on the thermodynamically driven Fe/S and heterometallic/sulfur center reconstitution in proteins,[7,8] we decided to use the bacterial Orange Protein (ORP) to promote the protein-assisted formation of novel protein derivatives harboring sulfur-coordinated molybdenum heterometallic clusters containing iron, cobalt, nickel, or cadmium of the type S$_2$MoS$_2$−M− S$_2$MoS$_2$ (M stands for the "central" metal ion of the cluster, iron, cobalt, nickel, or cadmium). The relevance of these sulfur-coordinated transition-metal complexes stems from their rich redox chemistry, which is thoroughly explored by nature and in the industry,[9] where the tetrahedral MoS$_4^{2-}$ units can act as reductants and as bidentate ligands, displaying both donor and acceptor character (through sulfur bonding and back-bonding to the metal, respectively).[10,11] Tetrathiometallate complexes are known for a variety of transition-metal ions.[12,13] Their
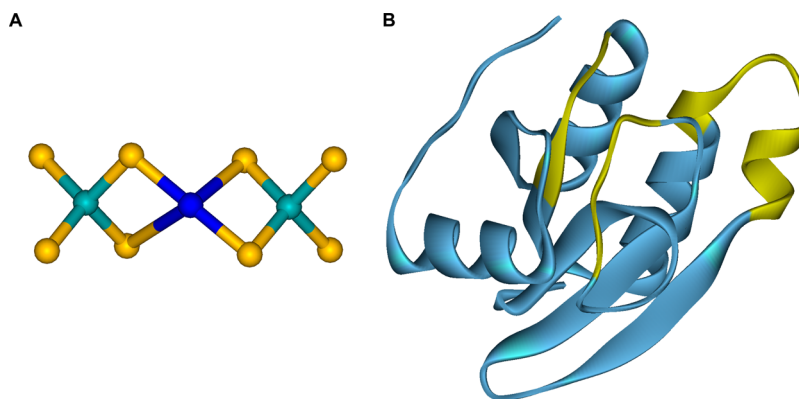
Figure 1. (A) Structure of the ORP Mo/Cu cluster, $[S_2MoS_2CuS_2MoS_2]^{3-}$, as determined by EXAFS.[14] (B) Backbone representation (solid ribbon) of the X-ray structure of the *D. gigas* apo-ORP (PDB ID 2WFB).[17] Regions including the residues 18−24, 46−55, and 72−77 are colored in yellow.

synthesis is often achieved using bulky counterions, in a nonaqueous system. However, their poor stability and solubility in aqueous buffers impose severe constraints to their study under "biological conditions" (under which the complexes undergo rearrangements and form high-nuclearity complexes, because of the MoS_4^{2-} tendency to bind metal ions through all of its sulfur atoms). In this scenario, we decided to revisit the protein-assisted synthesis methodology to synthesize a series of novel protein derivatives harboring sulfur-coordinated heterometallic complexes using the ORP.

ORP is a small monomeric protein (∼12 kDa, ∼120 residues), of still unknown physiological function, that is conserved in sulfate-reducing bacteria and in other anaerobic bacteria. It harbors a unique molybdenum/copper (Mo/Cu) heterometallic cluster, $[S_2Mo^{VI}S_2Cu^IS_2Mo^{VI}S_2]^{3-}$ (Figure 1A),[14−16] noncovalently bound, that was recently shown to be stabilized within the protein through both hydrophobic and electrostatic interactions, without direct interaction with the common metal-binding amino acid side chains, such as cysteine, histidine, or methionine residues.[16] The crystallographic three-dimensional structure of the *Desulfovibrio gigas* apo-ORP (beyond a 2.0 Å resolution; Figure 1B),[17] as well as the 98% NMR resonance assignment of the apo-ORP (using ^{13}C-detection experiments) and sequence assignment of reconstituted ORP, was reported.[16,18] The NMR data of this and other ORPs indicate the presence of a loop (48−54; Figure 1B, loop in yellow), whose amide proton resonances are not detected because of fast exchange with a bulk solvent.[19,20] The cluster binding region was identified in *D. gigas* ORP by NMR chemical shift perturbation to involve the residues 18−24, 46−55, and 72−77. Yet, the structure of the binding pocket of the holo-ORP remains elusive.

Heterologously produced ORP is purified in the apo form.[16,18,19,21] The apo-ORP can be, subsequently, reconstituted, using Cu^{II} and $Mo^{VI}S_4^{2-}$ salts as starting metallic reagents, to yield a reconstituted protein (Mo/Cu-ORP) that is virtually identical to the native ORP.[16,21] Hence, the apo-ORP is able to promote the formation and stabilization of the $[S_2Mo^{VI}S_2Cu^IS_2Mo^{VI}S_2]^{3-}$ cluster within its polypeptide chain. In this work, we explored this "protein-assisted synthesis skill" of the ORP to prepare novel protein derivatives harboring sulfur-coordinated molybdenum heterometallic clusters containing iron, cobalt, nickel, or cadmium in place of the "central" copper ion of the native ORP, {S₂MoS₂−M−S₂MoS₂} (Mo/Fe-ORP, Mo/Co-ORP, Mo/Ni-ORP, or Mo/Cd-ORP). For that, we modified the previously described protein-assisted

synthesis protocol[16,21] and spectroscopically (UV−visible and electron paramagnetic resonance (EPR)) characterized the Mo/M-ORP derivatives. The experimental evidence gathered supports that the Mo/M-ORP derivatives harbor the expected $\{S_2MoS_2FeS_2MoS_2\}$, $\{S_2MoS_2CoS_2MoS_2\}$, $\{S_2MoS_2NiS_2MoS_2\}$, and $\{S_2MoS_2CdS_2MoS_2\}$ complexes.

■ MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all reagents were of analytical or higher grade and were purchased from Sigma-Aldrich and Fluka.

Heterologous Expression and Purification of the ORP in the Apo Form. *D. gigas* ORP heterologous production and purification were performed as previously described.[18]

Apo-ORP-Assisted Formation of Molybdenum Heterometallic Clusters Containing Iron, Cobalt, Nickel, Copper, or Cadmium. The Mo/Cu-ORP was prepared as previously described (under oxic conditions).[16,21] The reconstitution of apo-ORP with clusters containing iron, cobalt, or nickel was performed under anoxic conditions, using Schlenk-line techniques, while the reconstitution with cadmium was carried out under oxic conditions. The procedure was as follows: 1 mM "central" metal ($FeSO_4·7H_2O$, $CoSO_4·5H_2O$, $NiSO_4·6H_2O$, or $CdSO_4·H_2O$) was added to 1 mM apo-ORP, in 50 mM HEPES, pH 7.5, followed by the addition of 10 mM dithiothreitol (DTT) and 2 mM $(NH_4)_2MoS_4$; the mixture was incubated for 2 h, in an ice bath (4 °C). The protein was, subsequently, separated from unbounded, small molecules (metal ions and DTT) by size-exclusion chromatography (PD-10 column; size exclusion of 5 kDa; GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.6 (for the copper derivative), or 50 mM HEPES, pH 7.5 (for the other metallic derivatives), and elution was carried out with the same buffer. The fraction containing the protein was collected and concentrated by ultrafiltration (centrifugation) using an YM5 membrane filter (size exclusion of 5 kDa; Sartorius).

Synthesis of Inorganic Mo/M Complexes in Aqueous Buffer Conditions. M^{II} and MoS_4^{2-} were also reacted, in a 2:1 Mo/M ratio, in the absence of the ORP, in aqueous buffers, aiming to obtain the respective inorganic Mo/M complexes in the same conditions as those of the Mo/M-ORP derivatives. For that, in the absence of apo-ORP, $CuCl_2$ (1 mM) was reacted with $(NH_4)_2MoS_4$ (2 mM), in 50 mM Tris-HCl, pH 7.6, for 15 min, under oxic conditions. $CdSO_4·H_2O$ (1 mM) was also reacted with $(NH_4)_2MoS_4$ (2 mM) for 15 min, under oxic conditions, but in 50 mM HEPES, pH 7.5. The other metals ($FeSO_4·7H_2O$, $CoSO_4·5H_2O$, or $NiSO_4·6H_2O$; 1 mM) were reacted with $(NH_4)_2MoS_4$ (2 mM), for 2 h, under an argon atmosphere, in 50 mM HEPES, pH 7.5.

Metal and Protein Quantification. The metal content of each Mo/M-ORP derivative (molybdenum, iron, cobalt, nickel, copper, and cadmium) was determined by inductively coupled plasma (ICP) emission analysis in a Jobin Yvon Ultima instrument using the Reagecom 23 ICP multielements, as a standard solution, in a

Table 1. Properties of Native ORP and of Reconstituted Mo/M-ORP Derivatives

"central" metal, M	Mo/M/ORP ratio (Mo/M ratio)[a]	visible spectral features of Mo/M-ORP:[b] λ, nm (ε, mM^{-1} cm^{-1})[c]	visible spectral features of an inorganic Mo/M cluster synthesized in a nonaqueous system: λ, nm (ε, mM^{-1} cm^{-1})[d]	visible spectral features of inorganic complex(es) synthesized in an aqueous buffer system:[e] λ, nm	EPR spectral features of Mo/M-ORP[b]	suggested formula for the Mo/M cluster in Mo/M-ORP
native ORP (copper)[15]	0.43:0.22:1.0 (1.95:1.0)	338 (10.7) 433 (shoulder) 480 (5.5)	(below)	(below)	EPR-silent; consistent with one Cu^{I} S = 0 (d^{10}) and two Mo^{VI} S = 0 (d^{0})	[Cu^{I}(Mo^{VI}S$_4$)$_2$]$^{3-}$ (Cu: tetrahedral)
reconstituted with copper[16]	1.9:0.96:1.0 (1.9:1.0)	338 (11.4 ± 0.8) 433 (shoulder) 480 (6.3 ± 0.4)	327 and 344 (29.5),[f] 430 (shoulder) 480 (20.0)[23]	316 360 (shoulder) 468 520 (shoulder)	EPR-silent; consistent with one Cu^{I} S = 0 (d^{10}) and two Mo^{VI} S = 0 (d^{0})	[Cu^{I}(Mo^{VI}S$_4$)$_2$]$^{3-}$ (Cu: tetrahedral)
reconstituted with iron	1.8:0.93:1.0 (1.9:1.0)	346 (19.5 ± 0.9) 420 (shoulder) 498 (10.2 ± 0.4) 564 (6.3 ± 0.1) 632 (shoulder)	348 (25.5) 375 (shoulder) 409 (9.8) 510 (18.3) 583 (8.4) 650 (shoulder)	319 346 470 505 568	S = $^3/_2$ signal consistent with one Fe^{I} S = $^3/_2$ (d^{7}) and two Mo^{VI} S = 0 (d^{0}) or one Fe^{III} S = $^5/_2$ (d^{5}) antiferromagnetically coupled to two Mo^{V} S = $^1/_2$ (d^{1})	[Fe^{I}(Mo^{VI}S$_4$)$_2$]$^{3-}$ ↔ [Fe^{III}(Mo^{V}S$_4$)$_2$]$^{3-}$ (Fe: tetrahedral)
reconstituted with cobalt	1.6:0.85:1.0 (1.9:1.0)	315 (18.4 ± 0.7) 385 (13.3 ± 0.6) 465 (10.1 ± 0.4) 540 (shoulder) 590 (4.6 ± 0.2) 774 (1.8 ± 0.1)	394 (19.5) 453 (shoulder) 545 (5.3) 645 (6.6) 825 (4.4)[25]	315 385 (shoulder) 466	EPR-silent; consistent with one Co^{I} S = 1 (d^{8}) and two Mo^{VI} S = 0 (d^{0}) or one Co^{III} S = 2 (d^{6}) antiferromagnetically coupled to two Mo^{V} S = $^1/_2$ (d^{1})	[Co^{I}(Mo^{VI}S$_4$)$_2$]$^{3-}$ ↔ [Co^{III}(Mo^{V}S$_4$)$_2$]$^{3-}$ (Co: tetrahedral)
reconstituted with nickel	1.5:0.80:1.0 (1.9:1.0)	306 (15.4 ± 0.9) 340 (shoulder) 401 (10.0 ± 0.5) 464 (shoulder) 536 (3.5 ± 0.2) 590 (3.1 ± 0.2)	not reported in literature	315 390 468 536 (shoulder)	S = $^1/_2$ signal, g$_{||}$ = 2.133 > g$_{\perp}$ = 2.037, consistent with one Ni^{I} S = $^1/_2$ (d^{9}) and two Mo^{VI} S = 0 (d^{0}) or one Ni^{III} S = $^1/_2$ (d^{7}) antiferromagnetically coupled to two Mo^{V} S = $^1/_2$ (d^{1})	[Ni^{I}(Mo^{VI}S$_4$)$_2$]$^{3-}$ ↔ [Ni^{III}(Mo^{V}S$_4$)$_2$]$^{3-}$ (Ni: square planar)
reconstituted with cadmium	1.7:1.1:1.0 (1.5:1.0)	316 (20.4 ± 0.7) 468 (10.8 ± 0.4) 550 (shoulder)	314 (28.3) 468 (13.7) 559 (3.0)[26]	315 391 (shoulder) 466 530 (shoulder)	EPR-silent; consistent with one Cd^{II} S = 0 (d^{10}) and two Mo^{VI} S = 0 (d^{0})	[Cd^{II}(Mo^{VI}S$_4$)$_2$]$^{2-}$ (Cd: tetrahedral)

[a]The values are mean values obtained from three independent syntheses; the associated standard deviation values are ≈15%. [b]Mo/M-ORP synthesized with the protein-assisted procedure (as described in the Materials and Methods section). [c]The values are mean ± standard deviation, obtained from three independent syntheses. The values are reported per molybdenum concentration. [d]Spectral features in DMF. [e]Inorganic complexes formed in aqueous buffer conditions, from the reaction of M^{II} with MoS$_4$$^{2-}$ in a 1:2 M/Mo proportion, in the absence of apo-ORP, as described in the Materials and Methods section. [f]The unique band splitting, as a doublet with equal intensity, may be related to the unequal bonding of the two MoS$_4$$^{2-}$ ligands toward the "central" copper ion.[23]

concentration range of 0.05−6 ppm. The protein concentration was determined by the modified Lowry method,[22] using bovine serum albumin as the standard protein.

Spectroscopic Characterization. UV−visible spectra were acquired using a Shimadzu UV-1800 spectrometer, at room temperature. X-band EPR (9.65 GHz) spectra were recorded using a Bruker EMX 6/1 spectrometer and a dual-mode ER4116DM rectangular cavity (Bruker); the samples were cooled with liquid helium in an Oxford Instruments ESR900 continuous-flow cryostat, fitted with a temperature controller. The spectra were acquired at different temperatures (as indicated in the figure captions) and with a modulation frequency of 100 kHz, a modulation amplitude of 0.1− 0.5 mT, and a microwave power of 0.633−2.01 mW. The Mo/M-ORP samples and inorganic Mo/M complex(es) prepared under anoxic conditions were transferred immediately after being synthesized to a septum-sealed UV−visible *cuvette* or to a septum-sealed EPR tube and frozen in liquid nitrogen (samples were prepared fresh before being measured).

■ **RESULTS AND DISCUSSION**

Procedure for Apo-ORP-Assisted Formation of Molybdenum Heterometallic Clusters. The metal and protein quantification showed that the Mo/M-ORP derivatives (with the exception of Mo/Cd-ORP; discussed below) contained a ratio of Mo/M very close to 2:1 (Table 1, second column, values in parentheses), as was found in the cluster of the native ORP.[14,15] The presence of ≈2 molybdenum atoms per metal atom is evidence that the clusters formed by our procedure have the same chemical composition as the native ORP cluster (Figure 1) but with the "central" copper ion substituted by an iron, a cobalt, or a nickel ion, as depicted in Figure 2. The M/
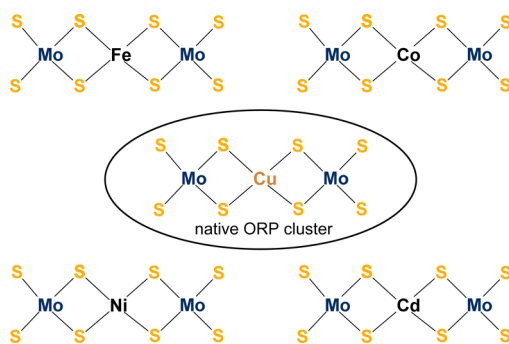

Figure 2. Schematic structure of the Mo/Cu cluster of native ORP (central structure) and proposed structures for the Mo/M clusters of the Mo/M-ORP derivatives, with M = Fe, Co, Ni, or Cd.

ORP ratios show that the self-assembly of the clusters within the protein is a very efficient process, with almost one cluster per protein molecule (0.80−0.93 cluster per ORP, depending on the "central" metal). Moreover, the procedure is reproducible (as judged by the standard deviation values of ≈15% obtained for the quantification of metals and proteins; Table 1).

In the case of Mo/Cd-ORP, the results obtained were not so clear relative to the structure of the complex formed. The ratio of Mo/Cd deviated from the expected 2:1 and was found to be 1.5:1.0, while the ratio of Cd/ORP was ≈1:1 (Table 1). Nevertheless, the Mo/Cd-ORP cluster is proposed to be isomorphous of the other clusters, as will be discussed below (and as is represented in Figure 2).

Mo/Cu-ORP Properties. The visible spectrum of the Mo/Cu-ORP shows characteristic S−Mo charge-transfer bands,[14] with maxima at 338 and 480 nm and a shoulder at 433 nm

(Figure 3 and Table 1, third column), in perfect agreement with the spectral features of the native ORP[14,15] and as had
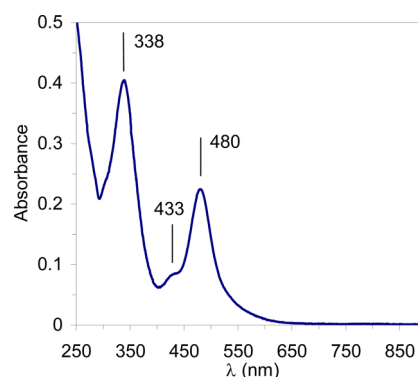

Figure 3. UV−visible spectrum of the Mo/Cu-ORP (35 μM), in 50 mM Tris-HCl, pH 7.6.

been previously described for the reconstituted ORP.[16] The inorganic tetrahedral Mo/Cu cluster synthesized in a non-aqueous system (no protein), $[S_2MoS_2CuS_2MoS_2]^{3-}$, displays a notably similar spectrum (in *N*,*N*-dimethylformamide (DMF); Table 1, fourth column),[23] suggesting that the assignment of the Mo/Cu-ORP cluster to a $[S_2MoS_2CuS_2MoS_2]^{3-}$ structure is correct (Figure 2). The inorganic Mo/Cu complex(es) synthesized from MoS_4^{2-} and Cu^{II} in an aqueous buffered system (as described in the Materials and Methods section) display(s) a clearly different absorption spectrum (Table 1, fifth column, and Figure S1), confirming that, under aqueous conditions, only the protein-assisted procedure gives rise to the targeted $\{S_2MoS_2-Cu-S_2MoS_2\}$ complex.[16]

The Mo/Cu-ORP is stable at +4 and −20 °C, at least for 4 and 20 days, respectively, as judged by its spectrum, which does not change during those time periods (there is no significant absorbance decrease or any wavelength shifting). Comparatively, the inorganic Mo/Cu cluster synthesized in a non-aqueous system is converted into polynuclear Mo/Cu complexes, involving three or four bridging MoS_4^{2-} ligands.[23] The Mo/Cu-ORP stability highlights the key role of the protein in the self-assembly and subsequent stabilization of the heterometallic Mo/Cu cluster within the protein pocket.

The Mo/Cu-ORP is EPR-silent and is suggested to hold one Cu^I (d^{10}, $S = 0$) and two Mo^{VI} (d^0, $S = 0$) ions, as was established for the native ORP, which is also EPR-silent.[14,15,21] This electronic structure indicates that the system was reduced during synthesis (because the starting metallic reagents were Cu^{II} and Mo^{VI} salts, as described in the Materials and Methods section). The reduction process will be discussed below (with the Mo/Fe-ORP derivative, which is not EPR-silent and provided more information about this process and also in the the Global Discussion section).

Overall, the Mo/Cu-ORP visible and EPR spectroscopic properties (which are similar to those of the native ORP and Mo/Cu cluster synthesized in a nonaqueous system) suggest that this derivative harbors a $[S_2Mo^{VI}S_2Cu^IS_2Mo^{VI}S_2]^{3-}$ cluster identical to the one found in the native ORP[14,15,21] (Table 1 and Figure 2).

Mo/Fe-ORP Properties. The visible spectrum of the Mo/ Fe-ORP displayed multiple absorption bands, with maxima at 346, 498, and 564 nm and shoulders at 420 and 632 nm. The 564 and 633 nm features are attributed to S−Fe charge-transfer processes[27] and the bands at 346 and 498 nm are assigned to
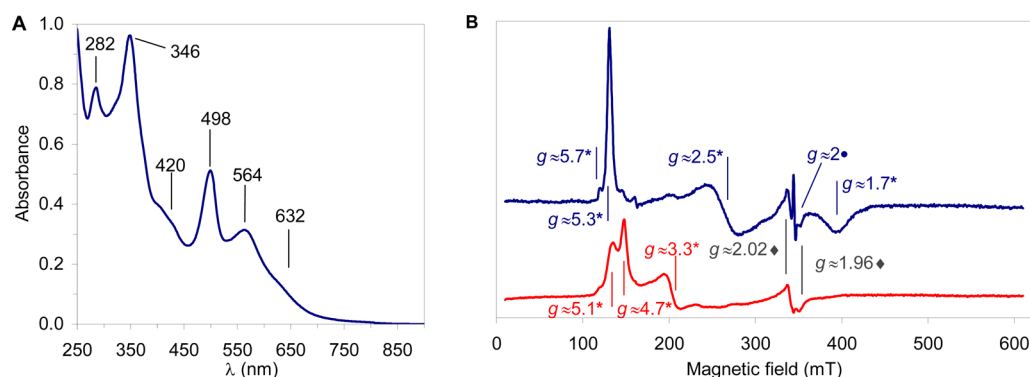
Figure 4. (A) UV−visible spectrum of the Mo/Fe-ORP (49 μM), in 50 mM HEPES, pH 7.5. (B) X-band EPR spectra at 4 K of the Mo/Fe-ORP (800 μM), in 50 mM HEPES, pH 7.5 (blue line), and of the inorganic Mo/Fe complex(es) (1 mM) formed in 50 mM HEPES, pH 7.5 (red line). The main g features are indicated in their respective colors; the values represented in gray are common to both spectra; the values marked with *, ◆, and ● are interpreted as arising from a $S = {}^3/_2$ species, from a sulfur-radical-containing species, and from an amino acid-radical-containing species, respectively.

the S−Mo ones (Figure 4A and Table 1). These spectral features are very similar to the ones displayed by the inorganic tetrahedral Mo/Fe cluster synthesized in a nonaqueous system (no protein; Table 1), $[S_2MoS_2FeS_2MoS_2]^{3-}$,[24,28] suggesting that the Mo/Fe-ORP harbors a similar cluster. Spectroscopic investigations of inorganic Mo/Fe complex(es) synthesized from MoS_4^{2-} and Fe^{II} in an aqueous buffered system (as described in the Materials and Methods section) indicate that other species may be present in solution (Table 1 and Figure S2). This difference shows that, under aqueous conditions, only the protein-assisted procedure enables the preparation of a sample whose visible spectrum is similar to that of a true tetrahedral $[S_2MoS_2FeS_2MoS_2]^{3-}$ cluster.

The Mo/Fe-ORP (prepared under anoxic conditions) is dioxygen-sensitive, at either +4 or −20 °C (as judged by striking changes in the visible spectrum; not further investigated). Under an argon atmosphere, at +4 and −20 °C, the Mo/Fe-ORP is stable for at least 3 and 7 days, respectively.

The Mo/Fe-ORP EPR spectra are very complex (Figures 4B (blue line) and S3 for spectra at 4−40 K) and somewhat unexpected, when one considers that the starting metallic reagents were Fe^{II} (d^6) and Mo^{VI} (d^0) (so an EPR-silent sample would be expected). The resonances at $g \approx 5.7, 5.3, 2.5,$ and 1.7 (Figure 4B, blue spectrum, values marked with *) can be interpreted in terms of a highly rhombic $S = {}^3/_2$ spin system, with an $E/D \approx 0.27$ and $D > 0$. The intensities of the $g \approx 5.3, 2.5,$ and 1.7 lines decrease as the temperature increases (from 4 to 40 K; Figure S1), and these resonances are suggested to belong to the lower $M = \pm{}^1/_2$ doublet (for which resonances at $g \approx 5.3, 2.4,$ and 1.6 are predicted). On the contrary, the intensity of the $g \approx 5.7$ line increases with increasing temperature (4−40 K), suggesting that $g \approx 5.7$ belongs to the upper $M = \pm{}^3/_2$ doublet (for which resonances at $g \approx 5.7, 1.7,$ and 1.3 are predicted).

The assignment of these resonances to a $S = {}^3/_2$ species finds support in the reported magnetic data of the inorganic Mo/Fe complex synthesized in a nonaqueous system. This $[S_2MoS_2FeS_2MoS_2]^{3-}$ complex is paramagnetic, with $\mu_{eff} = 3.9 \ \mu_B$[24,29] (consistent with a species with three unpaired electrons), and displays an EPR signal also interpretable as arising from a $S = {}^3/_2$ species. The EPR signal is solvent-dependent[30] and, in water, the g (5.15, 2.52, and 1.71) and E/D

$[\approx 0.23, D > 0 \ (4.2 \ K)^{29}]$ values are very similar to the ones described above for the Mo/Fe-ORP.

A $S = {}^3/_2$ signal in a {2Mo,Fe} complex could arise from several combinations of the formal oxidation states of the metals (which are discriminated in ref 31). The inorganic Mo/Fe complex synthesized in nonaqueous systems was suggested to have one $S = {}^5/_2 \ Fe^{III}$ (d^5) antiferromagnetically coupled to two $S = {}^1/_2 \ Mo^V$ (d^1) ions.[24,29,32−34] The rather short Fe−S bonds of the complex[32] suggested that the charge is delocalized into the MoS_4^{2-} units and the long terminal Mo−S bonds may reflect a higher electron density at the terminal sulfur atoms.[24,33] Also, the Mössbauer data (a low value of the isomer shift) suggested that the formal d-orbital occupancy of the iron is smaller than that expected for either a 1+ or even a 2+ oxidation state.[24,34] Moreover, assuming that the two Mo^V units are equivalent, Hoffman et al.[29] successfully explain both the Mo^V−Fe^{III}−Mo^V spin coupling and the absence of the expected ^{97,95}Mo hyperfine splitting (the molybdenum hyperfine splitting in the complex would be 5 times lower than that expected for the individual Mo^V and, thus, would not be visible within the relatively broad EPR lines). Yet, McDonald et al.,[35] on the basis of theoretical and magnetic studies, concluded that this complex could be better described as one $S = {}^3/_2 \ Fe^I$ (d^7) plus two $S = 0 \ Mo^{VI}$ (d^0) ions.

Regarding the Mo/Fe-ORP, in the absence of further experimental evidence, (at least) two situations are possible: (i) one $S = {}^5/_2 \ Fe^{III}$ (d^5) antiferromagnetically coupled to two $S = {}^1/_2 \ Mo^V$ (d^1) ions or (ii) one $S = {}^3/_2 \ Fe^I$ (d^7) plus two $S = 0 \ Mo^{VI}$ (d^0) ions. Moreover, given the ability of the MoS_4^{2-} units to delocalize charge, it is possible that both electronic structures could exist (eq 1); yet, for simplicity, from here on, only the {Mo^{VI}−M^I−Mo^{VI}} structure will be indicated.

$$\{Mo^{VI}-Fe^I-Mo^{VI}\} \leftrightarrow \{Mo^V-Fe^{III}-Mo^V\} \qquad (1)$$
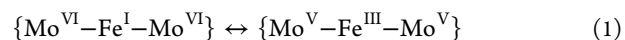
Of note, the assignment of the Mo/Fe-ORP cluster to a {Mo^{VI}−Fe^I−Mo^{VI}} complex indicates that the system was reduced (since the starting metallic reagents were M^{II} and Mo^{VI} salts), and this complex will be from here on designated as a "reduced" complex.[36]

Besides the $S = {}^3/_2$ signal, the Mo/Fe-ORP EPR spectra also display two resonances at $g = 2.02$ and 1.96 (Figure 4B, blue spectrum, values marked with ◆). Their temperature and power dependences suggest that both lines belong to a single, $S = {}^1/_2$, axial signal, with $g_\perp = 2.02$ and $g_\parallel = 1.96$ (Figure S3). A

somewhat similar signal, with $g_\parallel = 2.09$ and $g_\perp = 2.03$, was described for iron dithiolate compounds and interpreted as arising from one $S = 0$ Fe^{II} (d^6) plus a sulfur-centered $S = {}^1/_2$ radical.[37] The small g anisotropy of the Mo/Fe-ORP axial signal, which is centered at $g \approx 2$, is also in excellent agreement with a sulfur-centered $S = {}^1/_2$ (π) radical in the presence of one $S = 0$ Fe^{II} (d^6) and two $S = 0$ Mo^{VI} (d^0).[38] Such sulfur-radical-containing species could be formed by the intermolecular oxidation/reduction of a hypothetical $\{Mo^{VI}-Fe^{II}-Mo^{VI}\}$-ORP: the $\{Mo^{VI}-Fe^{II}-Mo^{VI}\}$ cluster of one ORP molecule would be oxidized by one electron, to yield an "oxidized" sulfur-radical-containing species, $(\{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)\}$-ORP), while the cluster from another ORP molecule would be reduced by one electron, to yield the above-described "reduced" Mo/Fe-complex-containing species (eq 2). In accordance with this hypothesis, the intermolecular oxidation/reduction process (eq 2) could contribute to the formation of the "reduced" Mo/Fe complex, $\{Mo^{VI}-Fe^{I}-$

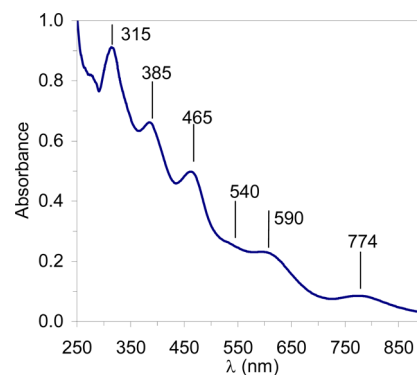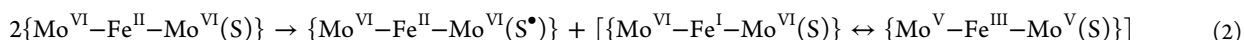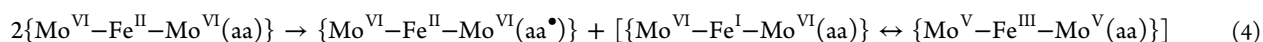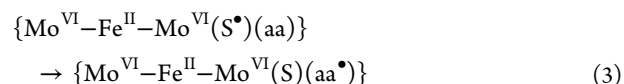**Figure 5.** UV–visible spectrum of the Mo/Co-ORP (50 μM), in 50 mM HEPES, pH 7.5.

$Mo^{VI}\}$ (even though, certainly, other reductants are in place, as will be discussed in the Global Discussion section).

$$2\{Mo^{VI}-Fe^{II}-Mo^{VI}(S)\} \rightarrow \{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)\} + [\{Mo^{VI}-Fe^{I}-Mo^{VI}(S)\} \leftrightarrow \{Mo^{V}-Fe^{III}-Mo^{V}(S)\}] \qquad (2)$$

If this hypothesis is correct and the intermolecular oxidation/reduction is feasible, then it would be expected that also the inorganic Mo/Fe complex can exist in both forms, $\{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)\}$ and $\{Mo^{VI}-Fe^{I}-Mo^{VI}(S)\}$, and display both the axial $S = {}^1/_2$ and ${}^3/_2$ signals. While the inorganic Mo/Fe complex synthesized in a nonaqueous system was described as not having the $S = {}^1/_2$ axial signal,[39] a different scenario was found in the Mo/Fe complex(es) formed in an aqueous buffer [Figures 4B (red line) and S4 for spectra at 4 to 20 K]. The spectra of the Mo/Fe complex(es) formed in a HEPES buffer display several resonances, at $g \approx 5.1$, 4.7, and 3.3 (Figure 4B, red spectrum, values marked with *), which could be interpreted as arising from a mixture of $S = {}^3/_2$ species (one species with $E/D = 0.23$, for which g values of 5.1, 2.6, and 1.7 are expected, and another with $E/D = 0.13$, for which g values of 4.7, 3.2, and 1.9 are expected). The spectral differences between the $S = {}^3/_2$ species of Mo/Fe-ORP and inorganic Mo/Fe complex(es) (both in a HEPES buffer) must reflect structural distortions imposed by the protein environment in the heterometallic cluster (a similar situation is observed in the inorganic Mo/Fe complex synthesized in a nonaqueous system, whose EPR spectrum is solvent-dependent, as described above[30]). In addition, the spectra of Mo/Fe complex(es) formed in a HEPES buffer also show the two resonances at $g_\perp = 2.02$ and $g_\parallel = 1.96$ (Figure 4B, red spectrum, values marked with ◆; Figure S4). The observation of the same $S = {}^1/_2$ axial signal with the Mo/Fe center either within the ORP or without the protein provides evidence that the formation of the

respective signal-giving species through intermolecular oxidation/reduction (eq 2) is reasonable.

Finally, the Mo/Fe-ORP spectra show a sharp resonance ($\Delta_{pp} = 1.5$ mT) at $g \approx 2$ (Figure 4B, blue spectrum, value marked with ●), which is consistent with the presence of an organic "free" radical. The absence of this sharp "free"-radical signal in the EPR spectra of the inorganic Mo/Fe complexes (synthesized in both buffered aqueous and nonaqueous systems) suggests that it arises from an amino acid radical from the ORP. The identity of the suggested amino acid radical is, however, presently unknown (the radical signal did not show superhyperfine interactions with protons or nitrogen atoms; site-directed mutagenesis studies are being performed to address the identity of the radical). The origin of the amino acid radical is also elusive, but it is highly probable that the protein oxidation is promoted by the metals, as well as by the suggested sulfur-radical-containing species, $\{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)\}$ (eq 3).[40] However, the possibility that it is the protein that is responsible for the reduction of the hypothetical initial $\{Mo^{VI}-Fe^{II}-Mo^{VI}\}$-ORP to the observed "reduced" $\{Mo^{VI}-Fe^{I}-Mo^{VI}\}$ complex (eq 4) can be ruled out because the "reduced" complex is also formed in the absence of the ORP (that is, because the inorganic Mo/Fe complex(es) also displays the $S = {}^3/_2$ and axial $S = {}^1/_2$ signals, even though it is synthesized in the absence of ORP).

$$\{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)(aa)\}$$
$$\rightarrow \{Mo^{VI}-Fe^{II}-Mo^{VI}(S)(aa^\bullet)\} \qquad (3)$$

$$2\{Mo^{VI}-Fe^{II}-Mo^{VI}(aa)\} \rightarrow \{Mo^{VI}-Fe^{II}-Mo^{VI}(aa^\bullet)\} + [\{Mo^{VI}-Fe^{I}-Mo^{VI}(aa)\} \leftrightarrow \{Mo^{V}-Fe^{III}-Mo^{V}(aa)\}] \qquad (4)$$

Overall, the Mo/Fe-ORP visible and EPR spectra suggest that this derivative harbors a tetrahedral "reduced" Mo/Fe complex, which can be envisaged as a $[S_2Mo^{VI}S_2Fe^{I}S_2Mo^{VI}S_2]^{3-}$ or a $[S_2Mo^{V}S_2Fe^{III}S_2Mo^{V}S_2]^{3-}$ complex (Table 1 and Figure 2). Two "oxidized" species, a sulfur-radical-containing species ($\{Mo^{VI}-Fe^{II}-Mo^{VI}(S^\bullet)\}$) and an amino acid-radical-containing species ($\{Mo^{VI}-Fe^{II}-Mo^{VI}(S)(aa^\bullet)\}$), are also suggested to be present in the samples of Mo/Fe-ORP.

Mo/Co-ORP Properties. The visible spectrum of the Mo/Co-ORP is characterized by the presence of multiple absorption bands, with maxima at 315, 385, 465, 590, and 774 nm and with a small shoulder at 540 nm (Figure 5 and Table 1), with the 590 and 774 nm features being attributed to S–Co charge transfer and the bands at 315 and 465 nm to S–Mo charge transfer.[41,42] These spectral features resemble those of the inorganic tetrahedral Mo/Co cluster synthesized in a
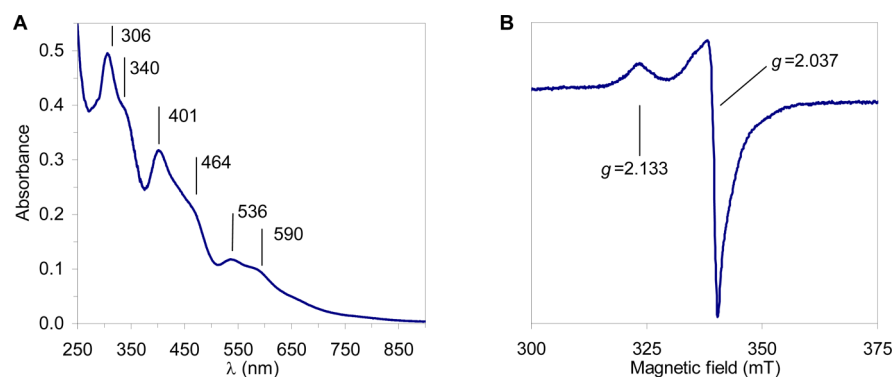
Figure 6. (A) UV−visible spectrum of the Mo/Ni-ORP (32 μM), in 50 mM HEPES, pH 7.5. (B) X-band EPR spectrum of the Mo/Ni-ORP (700 μM), in 50 mM HEPES, pH 7.5, at 80 K. The g features are indicated (see the text for details).

nonaqueous system, $[S_2MoS_2CoS_2MoS_2]^{3-}$,[25] although with the higher-wavelength bands hypsochromically shifted (590 and 774 nm in Mo/Co-ORP and 645 and 825 nm in an inorganic cluster in DMF; Table 1).

The inorganic Mo/Co complex(es) synthesized from MoS_4^{2-} and Co^{II} in an aqueous buffered system (as described in the Materials and Methods section) display(s) a markedly distinct visible spectrum (Table 1 and Figure S5), clearly demonstrating that, under aqueous conditions, only the protein-assisted procedure enables the preparation of a protein sample whose visible spectrum is similar to that of a tetrahedral $[S_2MoS_2CoS_2MoS_2]^{3-}$ cluster.

As the inorganic Mo/Co cluster synthesized in a nonaqueous system was described to be extremely air-sensitive,[25] also the Mo/Co-ORP (that is prepared under anoxic conditions) is dioxygen-sensitive, at either +4 or −20 °C (as judged by the striking changes in the visible spectrum; not further investigated). Under argon atmosphere, at +4 and −20 °C, the Mo/Co-ORP is stable for at least 24 h and 3 days, respectively.

The Mo/Co-ORP is EPR-silent (from 4 and 200 K, in both perpendicular and parallel EPR modes), suggesting that the cobalt is not present in a 2+ oxidation state (a d^7 ion). The inorganic tetrahedral Mo/Co cluster synthesized in a nonaqueous system is paramagnetic, with an μ_{eff} = 3.10 μ_B (consistent with a species with two unpaired electrons).[25] In accordance with the magnetic data and the tetrahedral geometry,[25,43] the cobalt ion of the inorganic complex was suggested to have been reduced during synthesis to the 1+ oxidation state, Co^I (d^8; S = 1).[44] Hence, it is probable that also Mo/Co-ORP holds one "reduced" S = 1 Co^I (d^8) ion in a tetrahedral $[S_2Mo^{VI}S_2Co^IS_2Mo^{VI}S_2]^{3-}$ cluster, with two S = 0 Mo^{VI} (d^0). Yet, as described above for the Mo/Fe-ORP, in the absence of further experimental evidence, other combinations of the formal oxidation states of the metals cannot be disregarded, including one S = 2 Co^{III} (d^6) antiferromagnetically coupled to two S = $^1/_2$ Mo^V (d^1) ions.[45] The formation of "oxidized", EPR-silent, species is also possible.[46]

Overall, the Mo/Co-ORP is suggested to hold a tetrahedral cluster that can be envisaged as a $[S_2Mo^{VI}S_2Co^IS_2Mo^{VI}S_2]^{3-}$ or a $[S_2Mo^VS_2Co^{III}S_2Mo^VS_2]^{3-}$ complex (Table 1 and Figure 2).

Mo/Ni-ORP Properties. The visible spectrum of the Mo/Ni-ORP shows absorption maxima at 306, 401, 536, and 590 nm and two shoulders at 340 and 464 nm, with the 536 and 590 nm absorptions being attributed to S−Ni charge-transfer bands and the 306 and 401 nm bands to S−Mo charge transfer[47] (Figure 6A and Table 1). The inorganic Mo/Ni

complex(es) synthesized from MoS_4^{2-} and Ni^{II} in an aqueous buffered system (as described in the Materials and Methods section) display(s) a featureless visible spectrum at higher wavelengths (Table 1 and Figure S6), clearly demonstrating that, under aqueous conditions, only the protein-assisted procedure enables the preparation of a protein sample whose visible spectrum is similar to that of a true $[S_2MoS_2NiS_2MoS_2]^{3-}$ cluster.

Like the other Mo/M-ORP derivatives that are prepared under anoxic conditions, the Mo/Ni-ORP is also not stable under an oxygen atmosphere, at either +4 or −20 °C (as judged by changes in the visible spectrum; not further investigated). Under an argon atmosphere, at +4 and −20 °C, the Mo/Ni-ORP is stable for at least 24 h and 3 days, respectively.

The Mo/Ni-ORP displayed an axial EPR signal, characteristic of a S = $^1/_2$ species, with $g_\parallel$ = 2.133 > $g_\perp$ = 2.037 (Figure 6B). The inorganic square-planar $[Ni(MoS_4)_2]^{2-}$ complex synthesized in a nonaqueous system is diamagnetic, consistent with the presence of one S = 0 Ni^{II} (d^8) plus two S = 0 Mo^{VI} (d^0) ions.[12,48,49] This inorganic Mo/Ni complex can undergo electrochemical reversible one-electron reduction, and its EPR spectrum consists of a strong, but very broad, S = $^1/_2$ signal with unexpected[50] "reversed" g values, $g_\perp$ = 2.12 > $g_\parallel$ = 2.035.[49] On the contrary, the anisotropy of the Mo/Ni-ORP EPR signal is consistent with the presence of one S = $^1/_2$ Ni^I (d^9) and two S = 0 Mo^{VI} (d^0) ions, in a square-planar geometry (and not in a tetrahedral arrangement, for which a $g_\perp$ > $g_\parallel$ signal would be expected). The Mo/Ni-ORP EPR signal is also in agreement with the presence of one square-planar S = $^1/_2$ Ni^{III} (d^7) ion antiferromagnetically coupled to two S = $^1/_2$ Mo^V (d^1) ions. These alternative assignments suggest that also this system could exist in different electronic structures[52] and that the nickel derivative was also "reduced" during its synthesis. The formation of "oxidized" species is also probable. An "oxidized" system containing one square-planar S = $^1/_2$ Ni^{III} (d^7) and two S = 0 Mo^{VI} (d^0) ions would give rise to a similar axial signal, with $g_\parallel$ > $g_\perp$, while the signals from the hypothetical radical species, either {Mo^{VI}−Ni^{II}−Mo^{VI}($S^\bullet$)} (eq 2) or {Mo^{VI}−Ni^{II}−Mo^{VI}(aa$^\bullet$)} (eq 3), would be obscured underneath the main axial signal.

Overall, the Mo/Ni-ORP is suggested to hold a square-planar cluster that can be envisaged as a $[S_2Mo^{VI}S_2Ni^IS_2Mo^{VI}S_2]^{3-}$ or a $[S_2Mo^VS_2Ni^{III}S_2Mo^VS_2]^{3-}$ complex (Table 1 and Figure 2).

Mo/Cd-ORP Properties. The visible spectrum of the Mo/Cd-ORP shows strong absorption bands at 316 and 468 nm, along with a small shoulder at 550 nm (Figure 7 and Table 1). The inorganic tetrahedral Mo/Cd cluster synthesized in a
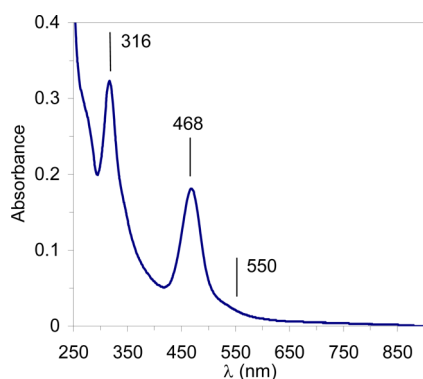
Figure 7. UV−visible spectrum of the Mo/Cd-ORP (17 μM), in 50 mM HEPES, pH 7.5.

nonaqueous system (no protein), $[S_2MoS_2CdS_2MoS_2]^{2-}$, displays a notably similar spectrum (in DMF), although with a clearly visible absorption band at 559 nm (Table 1), which was assigned to S−Cd charge transfer.[26] Also, the spectrum of the inorganic Mo/Cd complex(es) synthesized in an aqueous system is notoriously similar, with absorption bands at 315 and 466 nm (Figure S7 and Table 1). Moreover, also the $Abs_{\approx316}/Abs_{\approx468}$ ratio is quite similar in all three cases. Clearly, the Mo/Cd-ORP visible spectrum is dominated by the MoS_4^{2-} spectroscopic properties.

The Mo/Cd-ORP is stable at −20 °C, under oxic conditions, for at least 3 days, but it slowly decays at 4 °C. After 24 h, at 4 °C, under oxic conditions, the absorbance at all wavelength values decreases by ≈15%.

The Mo/Cd-ORP is EPR-silent, suggesting the presence of one $S = 0$ Cd^{II} (d^{10}) and two $S = 0$ Mo^{VI} (d^0) ions. Hence, in the cadmium derivative, there is no reduction of the system during synthesis (no evidence for the formation of a "reduced" complex or of an "oxidized" radical species) and the observed Mo/Cd-ORP cluster is consistent with the starting metallic reagents (Cd^{II} and Mo^{VI} salts). The absence of reduction in this derivative is discussed below, in the Global Discussion section.

Overall, the Mo/Cd-ORP visible spectrum (which is similar to the one of the inorganic Mo/Cd complex synthesized in either a nonaqueous[26] or aqueous system) and the absence of an EPR signal suggest that this derivative holds a $[S_2Mo^{VI}S_2Cd^{II}S_2Mo^{VI}S_2]^{2-}$ complex (Table 1 and Figure 2). Therefore, the Mo/Cd-ORP is suggested to harbor a doubly negatively charged cluster, instead of the 3− charged complexes suggested for the other derivatives. This charge difference could be responsible for the dissimilar Mo/Cd/ORP ratio observed (Table 1) and the slow decay observed at 4 °C (compared to the Mo/Cu-ORP, which is also synthesized under oxic conditions but is more stable). It is plausible that a 2− charged cluster is not adequately stabilized in the protein pocket "designed" to hold a 3− charged complex.

■ **GLOBAL DISCUSSION**

The ORP is able to promote the formation and stabilization of the $[Cu^I(Mo^{VI}S_4)_2]^{3-}$ cluster, using Cu^{II} and $Mo^{VI}S_4^{2-}$ salts as starting metallic reagents to yield a Mo/Cu-ORP that is virtually identical to the native ORP.[16,21] In this work, we explored the "protein-assisted synthesis skill" of the ORP to prepare novel protein derivatives, harboring molybdenum heterometallic clusters, containing iron, cobalt, nickel, or cadmium in place of the native "central" copper. Overall, the ORP scaffold tends to stabilize 3− over 2− charged clusters and tetrahedral rather than square-planar clusters.

Although these new Mo/M-ORP derivatives were not structurally characterized (as the native ORP was, using EXAFS[14]), the metal and protein quantification showed that they contain ≈2Mo per 1 M and the spectroscopic data (absorption and EPR) made us highly confident that we successfully produced a series of $\{S_2MoS_2−M−S_2MoS_2\}$ complexes with a structure similar to that of the native ORP cluster (Figure 2). Moreover, a comparison with the absorption spectra of the inorganic Mo/M complexes synthesized in an aqueous buffered system clearly demonstrated that ORP has the perfect scaffold to synthesize linear clusters of the type $\{Mo−M−Mo\}$, with bridging sulfur atoms. In fact, although the ORP biological function is not yet known, it might be related to this characteristic.

It is worth noting that, as the Mo/Cu-ORP contains a "reduced" $\{Mo^{VI}−Cu^I−Mo^{VI}\}$ cluster, also the other Mo/M-ORP derivatives (M = Fe, Co, or Ni) are suggested to harbor a "reduced" complex, $\{Mo^{VI}−M^I−Mo^{VI}\}$. In the absence of further experimental evidence, these "reduced" complexes are proposed to exist in (at least) two possible electronic structures (eq 5). The Mo/Cd-ORP is an exception, discussed below.

$$\{Mo^{VI}−M^I−Mo^{VI}\} \leftrightarrow \{Mo^V−M^{III}−Mo^V\}$$

$$(\text{with } M = Fe, \; Co, \text{ and } Ni) \tag{5}$$
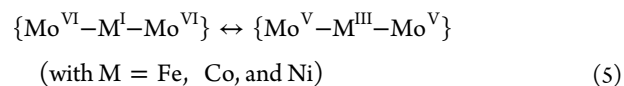
The presence of "reduced" complexes indicates that reduction of the system has occurred (the starting metallic reagents were M^{II} and Mo^{VI} salts, so a $\{Mo^{VI}−M^{II}−Mo^{VI}\}$ would be anticipated). Hence, we turned our attention to the identification of possible species responsible for this reduction process. A significant contribution to the system reduction must come from the MoS_4^{2-} itself, as previously suggested,[16] as well as from DTT. Similar reductant suggestions (the Fe^{II} complex itself and thiol compounds) were envisaged by McDonald et al.[28] and Hoffman et al.[29] for the reduction of the inorganic Mo/Fe complex (see ref 39) and by Stiefel et al.[25] for the reduction of the inorganic Mo/Co complex (see ref 44). Additionally, it is very tempting to suggest (based on the EPR data of the Mo/Fe-ORP) that an intermolecular oxidation/reduction of a hypothetical initial $\{Mo^{VI}−M^{II}−Mo^{VI}\}$-ORP species could contribute to the formation of the "reduced" complex, with the concomitant formation of an "oxidized" sulfur-radical-containing species (eq 6).[53] The ORP is also suggested to become oxidized and to give rise to a species with an amino acid radical ($\{Mo^{VI}−M^{II}−Mo^{VI}(aa^\bullet)\}$). Being in the presence of different transition-metal complexes and of the proposed sulfur-radical species, it is highly probable that the protein becomes oxidized. However, the hypothesis that the protein oxidation could contribute to the system reduction was ruled out (based on the discussion of the Mo/Fe-ORP derivative).

$$2\{Mo^{VI}−M^{II}−Mo^{VI}(S)\} \rightarrow \{Mo^{VI}−M^{II}−Mo^{VI}(S^\bullet)\} + [\{Mo^{VI}−M^I−Mo^{VI}(S)\} \leftrightarrow \{Mo^V−M^{III}−Mo^V(S)\}] \tag{6}$$

The reduction process does not need to be centered on the

"central" M ion. The molybdenum of the MoS_4^{2-} units has unoccupied d orbitals of energy comparable to that of the valence orbitals of the "central" M metal ion, which may allow delocalization of the electron density from M to Mo. Hence, as suggested previously,[33,54] the overlap between the d orbitals of M and Mo could result in M → Mo^{VI} charge delocalization. In accordance, the MoS_4^{2-} units are thought of as noninnocent redox ligands that can act to delocalize the charge over the molybdenum ions, thus giving rise to different possible electronic structures, as the EPR data clearly support. This charge delocalization can be thought of as an internal oxidation/reduction process, where M^{I} is oxidized to M^{III}, with the simultaneous reduction of two Mo^{VI} to Mo^{V} (eq 5). The Mo/S chemistry is certainly well suited for such internal oxidation/reduction reactions, as was elegantly shown, e.g., by Stiefel et al.,[11] with Mo^{VI}S$_4^{2-}$ oxidation to Mo$^{V}_2$S$_8^{2-}$, or, latter, by Young et al.,[55] with the generation of Mo^{V} species upon either the reduction or oxidation of Mo/S complexes. In addition, this charge delocalization over the MoS_4^{2-} units can significantly contribute to stabilization of the $\{S_2MoS_2–M–S_2MoS_2\}$ complexes (in particular in the inorganic clusters, where the additional stabilization provided by the protein environment is absent).

In this scenario, the native ORP, with a single $\{Mo^{VI}–Cu^{I}–Mo^{VI}\}$ electronic structure ($[S_2Mo^{VI}S_2Cu^{I}S_2Mo^{VI}S_2]^{3-}$), appears to be an exception. This protein is EPR-silent, and the formal oxidation states of the metals were assigned based on the EXAFS data.[14] The presence of thio-coordinating Mo^{VI} units, with approximately tetrahedral geometry, was suggested based on the Mo K-near-edge spectrum, which strongly resembles the one of MoS_4^{2-}, while the Cu K-near-edge spectrum, lacking the 8979 eV $1s → 3d$ peak characteristic of Cu^{II}, was taken as evidence for the presence of Cu^{I}.[14] Another possible electronic structure for an EPR-silent Mo/Cu complex is to have one Cu^{III} (d^8; $S = 1$ or 0) plus two Mo^{V} (d^1; $S = {}^1/_2$) ($\{Mo^{V}–Cu^{III}–Mo^{V}\}$; that would be in accordance with eq 5), but such an arrangement is not supported by the EXAFS data. Moreover, given the stability of the filled d shell of the Cu^{I} (d^{10}) ion, the $\{Mo^{VI}–Cu^{I}–Mo^{VI}\}$ must be the most favorable electronic structure. Hence, the Mo/Cu cluster is suggested to exist only in the $\{Mo^{VI}–Cu^{I}–Mo^{VI}\}$ form ($[S_2Mo^{VI}S_2Cu^{I}S_2Mo^{VI}S_2]^{3-}$).

Regarding the other derivatives, also the Mo/Cd-ORP, with only a single electronic structure, $\{Mo^{VI}–Cd^{II}–Mo^{VI}\}$, appears in this series of complexes as an exception. Yet, in view of the stability of its filled d shell, cadmium (electronic configuration $[Kr]4d^{10}5s^2$; Cd^{II}, d^{10}) shows few of the characteristic properties of transition metals (in spite of its position in the d block of the periodic table).[51] In this context, the otherwise expected reduction of the $\{Mo^{VI}–Cd^{II}–Mo^{VI}\}$ complex is not favorable and was not observed (no evidence for the formation of a "reduced" or an "oxidized" species).

Overall, it can be suggested that, because of the presence of the MoS_4^{2-} units, this series of heterometallic complexes could exist in different electronic structures (at least two; eq 5), dictated by possible internal oxidation/reduction reactions. Yet, when one of the possible electronic structures is more favorable than the other(s), then only that electronic structure would be observed. This is the case of the Cu^{I} and Cd^{II} (d^{10})-containing complexes, in opposition to the Fe^{III}/Fe^{I} (d^5/d^7)-, Co^{III}/Co^{I} (d^6/d^8)-, and Ni^{III}/Ni^{I} (d^7/d^9)-containing complexes.

In conclusion, the modified protein-assisted synthesis procedure described here allows the simple, efficient, and reproducible preparation of ORP derivatives harboring "reduced" molybdenum heterometallic clusters containing copper, iron, cobalt, or nickel, in aqueous, "biological", conditions (Table 1 and Figure 2). The "reduced" Mo/M clusters would exist in different electronic structures (eq 5) as long as there is no arrangement whose electronic structure is more favorable than the other(s), as is the case of the Mo/Cu-ORP, with a single $\{Mo^{VI}–Cu^{I}–Mo^{VI}\}$ electronic structure proposed. Also a single electronic structure is suggested for the "not-reduced" Mo/Cd-ORP, $\{Mo^{VI}–Cd^{II}–Mo^{VI}\}$. The ORP plays a key role not only in the self-assembly of the heterometallic complexes but also in their subsequent stabilization within the protein pocket, as can be appreciated when the (low) stability of the inorganic complexes is compared with that of the Mo/M-ORP derivatives.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorg-chem.6b02906.

> UV−visible spectra of the inorganic Mo/Cu, Mo/Fe, Mo/Co, Mo/Ni, and Mo/Cd complex(es) formed in the absence of protein and X-band EPR spectra of the Mo/Fe-ORP at 4−40 K and of the inorganic Mo/Fe complex(es) at 4−20 K (PDF)

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Notes

The authors declare no competing financial interest.

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(31) Considering Fe^{III}, a $S = {}^3/_2$ signal could arise from one $S = {}^1/_2$ Fe^{III} (d^5) ferromagnetically coupled to two $S = {}^1/_2$ Mo^V (d^1) ions, but a tetrahedral FeS$_4$ is unlikely to be low-spin, and ferromagnetic coupling in such clusters is improbable.[29] A $S = {}^3/_2$ signal could also arise from one $S = {}^3/_2$ Fe^{III} (d^5) plus two $S = 0$ Mo^{VI} (d^0) ions, as well as from one $S = {}^5/_2$ Fe^{III} (d^5) antiferromagnetically coupled to two $S = {}^1/_2$ Mo^V (d^1) ions. Regarding Fe^{II}, a $S = {}^3/_2$ signal could also arise from one $S = 1$ Fe^{II} (d^6) ferromagnetically coupled to one $S = {}^1/_2$ Mo^V (d^1) ion or one $S = 2$ Fe^{II} (d^6) antiferromagnetically coupled to one $S = {}^1/_2$ Mo^V (d^1) ion. Considering Fe^I, other possible combinations are one $S = {}^3/_2$ Fe^I (d^7) plus two $S = 0$ Mo^{VI} (d^0) ions or one $S = {}^1/_2$ Fe^I (d^7) ferromagnetically coupled to two $S = {}^1/_2$ Mo^V (d^1) ions.

(32) The Mo/Fe complex was shown to hold a tetrahedrally coordinated iron atom, bound by two MoS$_4^{2-}$ bidentate chelates, with rather short Fe−S bonds; the FeS$_2$Mo unit is essentially planar, with a slightly bent Mo−Fe−Mo (173° angle).[24]

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(38) One $S = 1$ Fe^{II} antiferromagnetically coupled to one sulfur-centered (π) radical would also result in a total spin of $^1/_2$, but the unpaired electron would then reside in the metal d orbital, giving rise to a signal with a large g anisotropy. The same situation (unpaired electron in the metal d orbital and large g anisotropy) would be found with (i) one $S = ^1/_2$ Fe^{III} alone, (ii) one $S = ^3/_2$ Fe^{III} antiferromagnetically coupled to two sulfur-centered (π) radicals, (iii) one $S = ^5/_2$ Fe^{III} antiferromagnetically coupled to two sulfur-centered (π) radicals plus two Mo^V, or (iv) Fe^I.

(39) The synthesis of an inorganic Mo/Fe complex in a nonaqueous system was made from Fe^{II}(S$_2$CN(CH$_2$)$_5$)$_2$ plus 2 equiv of (Et$_4$N)$_2$Mo^{VI}S$_4$, in CH$_2$Cl$_2$. The species responsible for the reduction that yields the final complex, [Fe^{III}(Mo^{V}S$_4$)$_2$]$^{3-}$, was not identified but was suggested to be the dithiocarbamate ligands or the Fe^{II} complex itself.[28,29] In this scenario, it can be suggested that the concomitantly formed oxidized species is EPR-silent, as would be the case if a persulfide, R−S−S−R, is formed.

(40) Some of the {Mo^{VI}−Fe^{II}−Mo^{VI}(S$^\bullet$)} molecules could have been rereduced at the expense of the oxidation of a neighboring amino acid (eq 3). Because of its sharp form, the radical signal is easily observed, even if its signal-giving species is present in a small concentration. Hence, it would be sufficient that only some molecules had reacted to produce the observed EPR spectra that show both the axial signal (originating from the sulfur radical) and the sharp "free"-radical signal (originating from the amino acid radical).

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(44) As with the inorganic Mo/Fe complex (see ref 39), also the inorganic cobalt system was found to be reduced. Stiefel et al.[25] used a thiol (C$_6$H$_5$S$^-$) as the reductant and suggested the formation of the respective persulfide (C$_6$H$_5$S−SC$_6$H$_5$).

(45) Other combinations of the metal formal oxidation states can give rise to a "reduced", EPR-silent, system, including one $S = 1$ Co^{III} (d^6) coupled to two $S = ^1/_2$ Mo^V (d^1) ions, with $S_{total} = 0$ or 2. As long as the total spin of the system is an integer, the respective EPR spectrum would be very difficult to observe.

(46) The formation of an "oxidized", EPR-silent, species could also occur in the Mo/Co-ORP derivative. For example, one $S = 1$ Co^{III} (d^6) with two $S = 0$ Mo^{VI} (d^0) ions give rise to an "oxidized" system with a $S_{total} = 1$. In addition, also the hypothetical radical species, {Mo^{VI}−Co^{II}−Mo^{VI}(S$^\bullet$)} (eq 2) and {Mo^{VI}−Co^{II}−Mo^{VI}(S)(aa$^\bullet$)} (eq 3), would probably be EPR-silent and not observable. The sulfur-radical-containing species would harbor one $S = ^3/_2$ Co^{II} (d^7), which, if coupled to a sulfur-centered $S = ^1/_2$ (π) radical, would result in $S_{total} = 1$ or 2. Even if we consider a square-planar $S = ^1/_2$ Co^{II}, S_{total} would be 0 or 1. For the same reason, the homologous organic "free"-radical signal would also not be observable.

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(50) The factors that determine the geometry of a particular complex of a d^8 metal ion are multiple, and both square-planar and tetrahedral geometries can be observed.[51] Providing that the ligand field is sufficiently strong (or of sufficiently low symmetry) to split the d orbitals as much as needed to offset the energy required to pair-up two electrons, then the square-planar geometry can be energetically preferable to the tetrahedral. Regarding the d^9 redox pair, if the ligand field imposes a square-planar geometry on the d^8 complex, it would be expected that, upon reduction, the complex does not undergo a drastic structural alteration and does not change from a square-planar to a tetrahedral geometry and vice versa for the tetrahedral complexes. In accordance, it would be expected that the inorganic Ni^I (d^9) complex retained the square-planar geometry of the Ni^{II} (d^8) pair and, thus, a $S = ^1/_2$ EPR signal with $g_\parallel > g_\perp$ would be expected.

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(53) The absence of an "oxidized" sulfur-radical-containing species in the Mo/Cu-ORP EPR spectra is expected. Such a complex ({Mo^{VI}−Cu^{II}−Mo^{VI}(S$^\bullet$)}) would have one $S = ^1/_2$ Cu^{II} (d^9) plus one sulfur-centered $S = ^1/_2$ (π) radical and thus would be EPR-silent ($S_{total} = 0$ or 1 if the two are antiferromagnetically or ferromagnetically coupled).

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