Molybdenum- and Tungsten-Substituted Hemoproteins. Models for the High-Valent Iron Porphyrin in Myoglobin and Horseradish Peroxidase

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Abstract: Molybdenum (Mo) and tungsten (W) substituted myoglobin (Mb) and horseradish peroxidase (HRP) (where the heme iron is replaced by Mo=O or W=O) were prepared. The relative stabilities of their +4 and +5 oxidation states were studied in relation to the high-valent iron porphyrin compounds of these hemoproteins. Mb favors the reduced state (+4), while HRP stabilizes in the oxidized state (+5). This parallels the relative stabilities of the corresponding iron-porphyrin compounds in higher oxidation states; the oxoiron(IV) porphyrin compound of Mb (the ferryl compound II) is fairly stable, while compound I with an iron(IV) π-cation radical (formally Fe(V) state) is formed only for HRP. This specific feature (the stabilization of the high-valent metalloporphyrin in HRP) is discussed in relation to the unique anionic character of its heme-bound proximal imidazole. HRP-Mo^{IV}=O exhibits pH-dependent UV-visible spectral changes with pK 9, suggesting the presence of two interconvertible conformers having the Mo-bound and -unbound proximal base. Mb-Mo^{IV}=O did not experience such a pH-dependent structural alteration. The substrate-induced structural changes were also found in HRP-Mo^{V}=O and HRP-W^{V}=O but not in the Mo(IV) state. To gain insight into substrate binding to HRP compounds I and II, we obtained the dissociation constants of substrate (benzohydroxamic acid) binding to HRP-Mo^{V}=O and HRP-W^{V}=O. From these results, Mo- and W-substituted Mb and HRP are suggested to serve as stable, model proteins to mimic the heme environmental structures of compounds I and II, for hemoproteins with high-valent iron porphyrin moieties.

The high-valent iron porphyrin complexes Fe^{IV}=O and Fe^{IV}=O-$Por^{\bullet+}$ are currently the subject of intensive study, since they are the active species in the reaction cycle of heme enzymes such as peroxidases, catalase, and possibly cytochrome P-450.[1,2] Ferric horseradish peroxidase (HRP), for example, reacts with H_2O_2 to form a green compound I (formulated formerly as an Fe(V) state but now as an oxoferryl (Fe^{IV}=O) porphyrin π-cation radical[1,4,5] followed by one-electron reduction caused by the substrate to form a red compound II having the oxoferryl-porphyrin.[1,6-8] In contrast to HRP, metmyoglobin (met-Mb) or methemoglobin (met-Hb) reacts with H_2O_2 to yield only the compound II (oxoferry-Mb or oxoferryl-Hb).[9-11]

The question then arises as to why HRP stabilizes the high-valent compound I while Mb and Hb do not, in spite of the common prosthetic group (protoporphyrin IX iron complex) and common proximal histidyl ligand in these hemoproteins. One possible explanation for this is that the iron-bound imidazole of the proximal histidine in HRP has a unique anionic character.[36,37,39,48] This results from the strong hydrogen bond between its N_1H and the nearby amino acid residue, favorable for stabilization of the high-valent state in HRP compound I. In this connection, we note that the Fe(III) state of HRP is much more stable than the Fe(II) state, as seeen in its unusually low redox potential. However, there has been no direct evidence for the structural characteristics of peroxidases that are responsible for stabilization in their higher oxidation states.

It then seems interesting to examine whether or not a high-valent metalloporphyrin other than iron porphyrin is also stabilized upon its incorporation into HRP or Mb. As such a high-valent metalloporphyrin, we have chosen oxomolybdenum (Mo^{IV}=O, Mo^{V}=O) and oxotungsten (W^{V}=O, W^{IV}=O) porphyrin complexes. These complexes are interconvertible between M(IV) and M(V) oxidation states[13-23] and thus are structurally more relevant to compounds I and II than the oxovanadium (V^{IV}=O) complex having a single +4 oxidation state.[24] Furthermore, they are more stable than other oxo transition metal porphyrin complexes such as oxoiron,[25] oxochromium,[26] and oxomanganese[27] derivatives, which undergo the oxygen-transfer reaction.

We report here the first trial of preparation and structural characterization of Mo- and W-substituted Mb and HRP. The differences in relative stabilities between the two oxidation states of these metal-substituted proteins are discussed in relation to their

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Table I. Electronic and ESR Spectral Data for Mo- and W-Substituted Porphyrin Complexes and Hemoproteins

compound	λ_{max}, nm	ESR (77 K) $g_\parallel$	ESR (77 K) $g_\perp$	A_{Mo}, G	solvent	ref
OEP-Mo^V=O·X^-						
X^- = OCH_3	342, 443, 562, 595				*a*	12
X^- = OPh	343, 454, 573, 606				*c*	12
TPP-Mo^V=O·X^-						
X^- = OCH_3	367, 393, 455, 583, 623	1.961		45	*a*	19
X^- = OAc	379, 411, 485, 610, 653	1.961		48	*d*	19
X^- = OH	408, 446, 585, 615, 667	1.987		50	*c*	13
MPDME-Mo^V=O·X^-						
X^- = OCH_3	339, 447, 559, 596	1.95	1.96	49	*a*	*g*
X^- = Cl	345, 471, 585, 618				*a*	*g*
X^- = Im(*N*-Me)[i]	345, 480, 592	1.96	1.97	63, 27[j]	*b*	*g*
MPDME-Mo^{IV}=O	412, 433, 559, 573, 592				*h*	*g*
Mb-Mo^V=O	349, 489, 592	1.97		*f*	*e*	*g*
Mb-Mo^{IV}=O	436, 551, 592, 600				*e*	*g*
HRP-Mo^V=O	351, 489, 590	1.96	1.97	78, 33[j]	*e*	*g*
HRP-Mo^{IV}=O	410, 448, 543, 581				*e*	*g*

compound	λ_{max}, nm	ESR (77 K) g_x	ESR (77 K) g_y	ESR (77 K) g_z	A_{Mo}, G	solvent	ref
OEP-W^V=O·X^-							
X^- = OCH_3	360, 432, 557, 594	1.87	1.84	1.92	87	*a*	12, *g*
X^- = OPh	356, 433, 562, 598					*c*	12
MPDME-W^V=O·X^-							
X^- = OCH_3	322, 355, 431, 557, 593	1.87	1.84	1.92	*f*	*a*	*g*
X^- = Cl	328, 365, 455, 572, 610					*a*	*g*
X^- = Im(*N*-Me)[i]	431, 557, 593	1.87	1.85	1.91	*f*	*b*	*g*
Mb-W^V=O	452, 571, 608	1.86	1.83	1.90	102	*d*	*g*
Mb-W^{IV}=O	431					*d*	*g*
HRP-W^V=O	451, 570, 608	1.86	1.83	1.90	93	*d*	*g*

[a] CH_2Cl_2 + CH_3OH (9:1). [b] *N*-Methylimidazole (neat). [c] Benzene. [d] CH_2Cl_2 + AcOH (50:1). [e] 0.1 M phosphate buffer, pH 7. [f] Unresolved. [g] This work. [h] Reduced by hydrazine in CH_2Cl_2. [i] *N*-Methylimidazole. [j] $A_\parallel$, $A_\perp$.

oxoferryl counterparts. With Mo- and W-substituted HRPs, we also studied substrate binding to the protein in order to determine the substrate-induced conformational changes and substrate binding constants. This was compared to the interaction of very active native HRP compounds I and II with substrates.

Materials and Methods

Sperm whale myoglobin (type II) and horseradish peroxidase (G-I-C) were obtained from Sigma and Toyobo, respectively. The mesoporphyrin IX Mo^V=O complex (MP-Mo^V=O) was prepared by the insertion of oxometal into mesoporphyrin IX dimethyl ester (MPDME), by the literature method used for the octaethylporphyrin (OEP) analogue,[12] followed by the hydrolysis of its ester parts in 1% KOH-CH_3OH. For the oxotungsten complex, mesoporphyrin IX was decomposed under the conditions used for the synthesis of OEP analogue.[12] Therefore, the oxotungsten complex (MP-W^V=O) was obtained by a slightly modified method. MPDME (250 mg, 0.43 mmol) and H_2WO_4 (150 mg, 0.60 mmol) were dissolved in phenol (1.5 g), and the resultant mixture was heated at 170 °C for 168 h. Supplementary H_2WO_4 (150 mg, 0.60 mmol) was added at three times (at 20, 60, and 100 h). The reaction mixture was extracted with chloroform, followed by drying and evaporation. The product was esterified with 5% H_2SO_4-dry methanol. The crude product was purified by Al_2O_3 (grade III) column chromatography (CH_2Cl_2:CH_3OH = 98:2) and TLC (silica gel, CH_2Cl_2:CH_3OH = 9:1). The purified product was crystallized from CH_2Cl_2-*n*-hexane (73 mg, 19%). Under these difficult conditions, it was impossible to prepare M^V=O protoporphyrin complex (M = Mo, W).

The electronic absorption and ESR spectral data for MPDME-Mo^V=O·X^- and MPDME-W^V=O·X^- (X = CH_3O, Cl) are compiled in Table I. Incorporation of MP-M=O (M = Mo, W) into apohemoproteins was made by the procedure normally used for reconstitution with iron porphyrins.[50] Reconstituted proteins were purified with Sephadex G-25 (0.1 M phosphate buffer, pH 7). Embedding of W=O and Mo=O porphyrin complexes in the heme pocket of proteins was checked by ^1H NMR and the Styer method using 1-anilino-8-naphthalenesulfonate (ANS), which binds to apomyoglobin and exhibits a characteristic fluorescence.[35]

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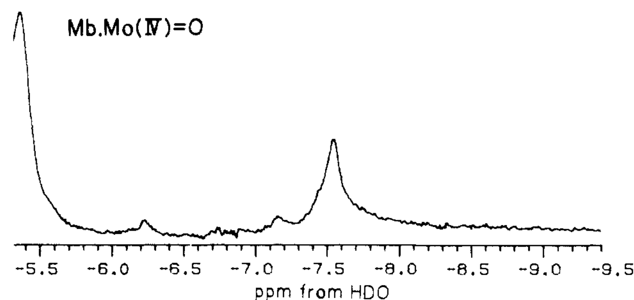

Figure 1. Ring current shifted ^1H NMR (300-MHz) resonance of the Val E11 γ-methyl group of Mb-Mo^{IV}=O in D_2O, 50 mM phosphate buffer, pD 6.8, at 23 °C.

MP-W^V=O was not stable in the presence of oxidant or strong binding ligand (e.g., H_2O_2, imidazole), giving free-base (nonmetallo) porphyrin.

Electronic absorption spectral measurements were made on a Hitachi 330 spectrometer. ESR spectra were recorded with a JEOL JES-3X spectrometer operating with 100-MHz field modulation. Proton NMR spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1280 computer system.

Results and Discussion

We first tried to incorporate mesoporphyrin IX Mo^V=O (MP-Mo^V=O·CH_3O^-) into apo-Mb at pH 7. However, the yield of this reconstitution reaction was very low (<5%) and the reconstituted protein (**1**) was so unstable that it was denatured to form a precipitate after standing for several hours. Then, we reconstituted Mb with MP-Mo^V=O in the presence of a reducing agent, $Na_2S_2O_4$. Under these conditions the reaction proceeded stoichiometrically to yield stable Mb-Mo^{IV}=O (**2**) without any precipitate. The electronic absorption spectrum observed (Table I) was similar to that for the oxomolybdenum(IV) porphyrin complex.[13-21]

Illustrated in Figure 1 is the ^1H NMR spectrum of **2** in the upfield region. The ring current shifted Val E11 γ-methyl proton resonance was observed at −7.6 ppm from HDO resonance. Since this amino acid residue is located close to the heme sixth coor-

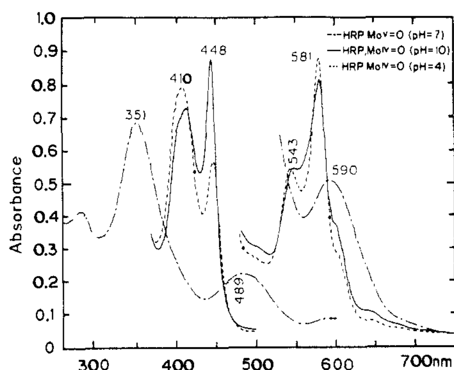

Figure 2. Electronic absorption spectra of Mo-substituted HRP at room temperature: HRP-Mo^{V}=O at pH 7 (–··–); HRP-Mo^{IV}=O at pH 10 (—); HRP-Mo^{IV}=O at pH 4 (---).

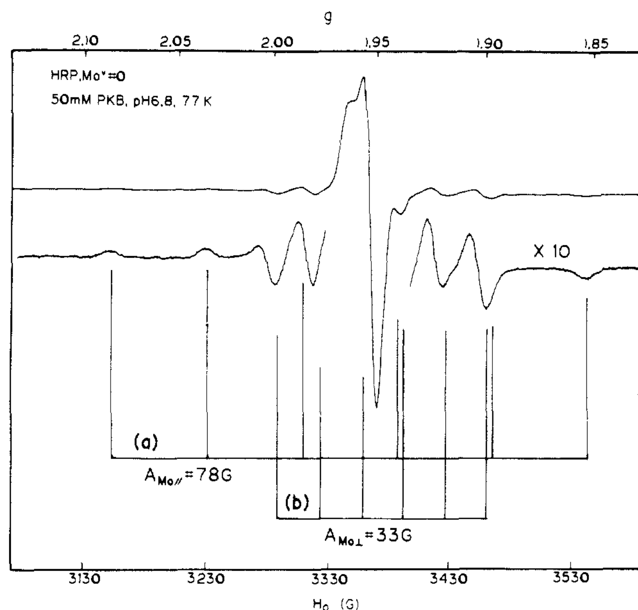

Figure 3. ESR spectrum of HRP-Mo^{V}=O in 50 mM phosphate buffer, pH 6.8, at 77 K. Vertical lines indicate the parallel (a) and perpendicular (b) components of the hyperfine structure.

dination site, the NMR signal has been utilized for characterization of the heme environment of Mb. The signal position is comparable to those for diamagnetic Mb-CO (−7.2 ppm from HDO)[28] and Mg- (−7.1 ppm)[29] and Zn-substituted Mb's (−8.5 ppm).[30] This indicates that the oxomolybdenum porphyrin complex is properly incorporated into a heme pocket and the heme environment of Mo-substituted Mb is similar to that of the native one.

In contrast to Mb-Mo^{V}=O (**1**), Mb-Mo^{IV}=O (**2**) was extremely stable and its electronic absorption spectrum remained unchanged even after removal of excess $Na_2S_2O_4$ with Sephadex G-25. The spectrum was, however, reversibly changed upon oxidation by a variety of oxidizing reagents such as H_2O_2 and K_2IrCl_6. This yielded four isosbestic points at 407, 454, 547, and 574 nm and eventually three broad bands at 349, 489, 592 nm. This was identical with that of **1**. This spectrum was plausibly simulated by MPDME-Mo^{V}=O in *N*-methylimidazole (**5**) (Table I). The Mo(V) species thus obtained was also unstable and was readily denatured at room temperature.

Reconstitution of HRP with MP-Mo=O likewise resulted in a high yield (>90%) of HRP-Mo^{IV}=O (**4**) or HRP-Mo^{V}=O (**3**), depending on the presence or absence of $Na_2S_2O_4$, respectively. Both reconstituted proteins exhibited characteristic electronic

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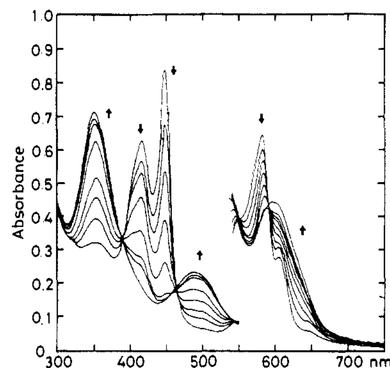
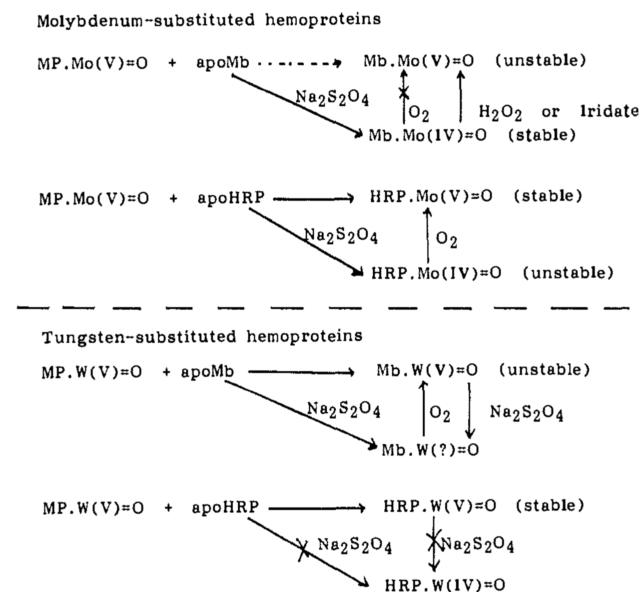


Figure 4. Time-resolved optical absorption spectra of Mb-Mo^{IV}=O obtained during exposing to air in 0.1 M phosphate buffer, pH 7.0, at room temperature.

Scheme I



absorption spectra, as shown in Figure 2. HRP-Mo^{V}=O (**3**) exhibited an ESR spectrum at 77 K (Figure 3), where an intense central signal arising from ^{96}Mo(V) ($S = {}^1/_2$, $I = 0$) is observed at $g = 1.96$ with unresolved porphyrin pyrrole nitrogen hyperfine coupling. This is most probably due to mesoporphyrin asymmetry and to the proximal histidyl imidazole nitrogen ligated to Mo(V). Another set of six lines due to superhyperfine interactions ($g = 1.956$, $g = 1.974$, $A_{Mo} = 78$ G, $A_{Mo} = 33$ G) with isotopic molybdenum-95 and -97 ($I = {}^5/_2$, natural abundance 15.7 and 9.6%, respectively) is partly overlapped. These characteristic electronic and ESR spectral data are compiled in Table I, together with those for some Mo=O porphyrin complexes. Inspection of this table shows that the spectra for HRP-Mo^{V}=O (**3**) are quite similar to those for the model compound MPDME-Mo^{V}=O(*N*-MeIm) (**5**).

HRP-Mo^{V}=O (**3**) was much more stable than Mb-Mo^{V}=O (**1**) and did not form a precipitate even after standing for several days, in sharp contrast to Mb-Mo^{V}=O (**1**). HRP-Mo^{IV}=O (**4**) was also stable in the presence of excess $Na_2S_2O_4$, but it is rapidly converted to the Mo(V) species when excess $Na_2S_2O_4$ was removed with G-25. However, further oxidation of HRP-Mo^{V}=O (**3**) caused no significant spectral change, although the molybdenum (V) porphyrin complexes undergo electrochemical one-electron oxidation to form their π-cation radicals.[19] The reconstitution reactions of molybdenum porphyrin into apo-Mb and apo-HRP are summarized in Scheme I.

Relative stabilities of the Mo=O-substituted hemoproteins are expected to reflect their characteristic heme environmental structures. In order to gain further insight into this point, we also attempted to reconstitute Mb and HRP with tungsten porphyrin complexes, which can be in +4 and +5 oxidation states like

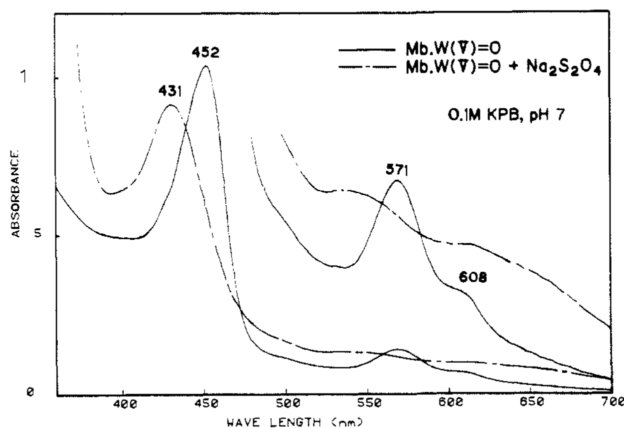


Figure 5. Electronic absorption spectra of Mb-W^V=O in the absence (—) and presence (-··-) of $Na_2S_2O_4$ in 0.1 M phosphate buffer, pH 7.0.

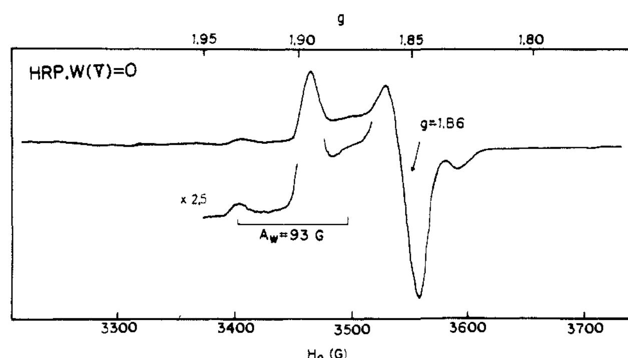


Figure 6. ESR spectra of HRP-W^V=O in 0.1 M phosphate buffer, pH 7.0, at 77 K.

molybdenum analogues. We carried out reconstitution of Mb and HRP with mesoporphyrin IX W^V=O (MP-W^V=O·CH_3O^-) by the same procedure employed for the molybdenum systems.

Upon reconstitution in the absence of a reducing agent, oxotungsten(V)-reconstituted myoglobin [Mb-W^V=O (**6**)] was obtained in a low yield (<30%). This protein was relatively unstable and formed a precipitate after standing for several hours. Reduction of Mb-W^V=O (**6**) with $Na_2S_2O_4$, or incorporation of MP-W^V=O into apo-Mb in the presence of $Na_2S_2O_4$, yielded a new species (**7**) giving an electronic absorption spectrum different from that of Mb-W^V=O (**6**) (Table I; Figure 5). This reduced species (**7**) was stable under anaerobic conditions but reversibly turned into Mb-W^V=O (**6**) upon removal of excess $Na_2S_2O_4$ with Sephadex G-25, which was monitored by the electronic absorption spectral change.

The reduced species (**7**) gave several well-resolved hyperfine-shifted proton resonances in the upfield and downfield regions, suggesting that it is in a paramagnetic state. Its ESR was silent, probably due to enhanced electron spin relaxation. The electronic absorption spectrum of **7** was not a π-anion radical type. The Soret band was significantly decreased, and the absorption in a visible region (>600 nm) was increased (Figure 5). The CO gas bubbling into the protein solution of **7** did not cause any changes in the electronic and ^1H NMR spectra, implying that the tungsten sixth coordination site remains occupied with the oxo ligand. Although the electronic structure of this reduced species (**7**) cannot be identified at this moment,[31] we tentatively assign it as a W^{IV}=O porphyrin complex, which has not been reported so far.

HRP-W^V=O (**8**) obtained by incorporation of MP-W^V=O into apo-HRP was extremely stable and exhibited characteristic ESR and electronic absorption spectra, as shown in Figure 6 and Table I. In the ESR spectrum of **8**, rhombic-type signals due to

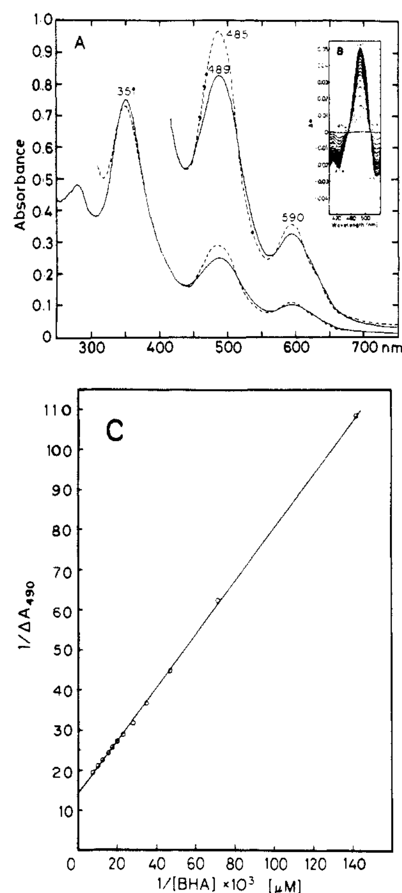


Figure 7. (A) Electronic absorption spectra of HRP-Mo^V=O in the absence (—) and presence (---) of benzohydroxamic acid in 0.1 M phosphate buffer, pH 7.0, at room temperature. (B) Difference spectra obtained upon titrating 4 μM HRP-Mo^V=O with benzohydroxamic acid (4 mM, each 5 μL) in 50 mM phosphate buffer, pH 7.0, at 25 °C. (C) Analysis of the data in (B) to obtain the substrate dissociation constant K_1 on the basis of the equation[42] $1/L = [[HRP]/K_1](\Delta\epsilon l/\Delta A - 1/K_1)$, where L is the BHA concentration, [HRP] the total concentration of HRP, $\Delta\epsilon$ the change in molar absorptivities, l the solution light path, and ΔA the observed absorbance.

$^{184}W(V)$ ($S = {}^1/_2$, $I = 0$) were observed at $g_x = 1.86$, $g_y = 1.83$, and $g_z = 1.90$, with unresolved nitrogen hyperfine coupling. Another set of doublet lines arising from tungsten-183 ($I = {}^1/_2$, natural abundance 14%) was also observed with a hyperfine coupling constant of $A_W = 98.4$ G (HRP-W^V=O). HRP-W^V=O (**8**) was not reduced by $Na_2S_2O_4$ and was further oxidized by H_2O_2. The reconstitution reactions of tungsten porphyrins into apo-Mb and apo-HRP are also summarized in Scheme I.

It was found here that Mo=O and W=O mesoporphyrins can be incorporated into apo-Mb and apo-HRP to form the corresponding protein complexes under appropriate conditions (Scheme I). There have been some studies on Mo=O and W=O complexes of synthetic porphyrins (TPP, OEP) including X-ray crystallography.[12-25,32] It was suggested that these metal oxo porphyrin complexes are usually in the 5+ oxidation state, which is presumably stabilized by the oxo ligand, and that the reduction of Por-Mo^V=O needs a high potential (−0.8 eV).[19]

It is shown here that oxomolybdenum(V) and oxotungsten(V) states are more favorably stabilized in HRP than in Mb. This finding appears to parallel the well-established features of the high-valent iron porphyrin in HRP and Mb; the formal "oxometal(V)" state in compound I is stable in HRP, while it is unstable or not formed in Mb. These results evidently support the previous suggestion that the heme environment of HRP is characterized by its specific protein structure, which is favorable

(31) It was difficult to determine the exact concentration of the purified reduced species (**7**) in solution because the reconstitution with MP-W^V=O to apo-Mb was not a stoichiometrical reaction and the pure reduced species was not isolated. Consequently, the magnetic susceptibility of the reduced species was not feasible.

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for stabilization of the high-valent metalloporphyrins. Bearing in mind that the Mo(V) state in TPP–Mo^{V}=O is stabilized by the coordination of an anionic axial ligand (Cl^-, CH_3O^-),[33] an anionic character of the proximal imidazole in HRP could also be responsible for this stabilization. The anionic proximal imidazole of HRP resulting from its strong hydrogen-bonded N_1H or its deprotonated form has been suggested by several investigators for ferrous and ferric enzymes, on the basis of spectroscopic studies.[36,47] The present results may provide further evidence that the anionic nature of the proximal ligand could be responsible for the stabilization of the high-valent iron porphyrin in compound I.

Recent NMR study by de Ropp et al.[47] showed that in HRP the proximal histidyl imidazole N_1H hydrogen-bonding strength is altered on going from a ferric resting enzyme to a low-spin ferric cyanide complex, as monitored by the N_1H proton NMR signal.[48] This result seems to suggest that the nature of the proximal histidyl imidazole in HRP differs under various conditions such as a sixth-coordinated ligand, pH difference, and state of oxidation. Therefore, it is of interest to study the effect of pH on the visible and ESR spectra of Mo-substituted HRP.

Both adsorption and ESR spectra of HRP-Mo^{V}=O (**3**) were insensitive to pH. HRP-W^{V}=O (**8**) also showed no pH dependence in the optical absorption and ESR spectra. On the other hand, the absorption spectrum of HRP-Mo^{IV}=O (**4**), in the presence of dithionite, exhibited a pH-dependent change (Figure 2) with pK 9, indicating a mixture of alkaline species with the 448-nm Soret band and the acid species with the 410-nm band. Above pH 11, only the 448-nm species was present. Similar spectral changes were reported for vanadium(IV)-substituted Mb (Mb-V^{IV}=O) and were attributed to interconvertible species with and without a coordinated proximal histidyl imidazole.[24] It is therefore likely that the alkaline and the acid species of HRP-Mo^{IV}=O are due to the base-bound and the base-free forms, respectively. This was further confirmed by the finding that the denatured enzyme of HRP-Mo^{IV}=O (**4**) by guanidine hydrochloride exhibited a strong 410-nm absorption while addition of imidazole caused a decrease of this base-free band, accompanied by a concomitant appearance and increase of a base-bound band at 442 nm. The protein conformational change is induced by the ionization of a group with pK 9, thereby affecting the heme proximal side and causing cleavage of the Mo(IV)–proximal histidyl imidazole bond in an acidic region. The metal–proximal histidyl imidazole bond cleavage induced by the protein conformational change has been observed for the α-chain of Hb–NO + IHP.[40] The 410-nm band, which was attributed to the acid species, was still strong at pH 10, while it disappeared above pH 12. In contrast to HRP-Mo^{IV}=O (**4**), the optical absorption and ^1H NMR (Figure 1) spectra of Mb-Mo^{IV}=O (**2**) were pH independent. This suggests that the Mo(IV)–histidyl imidazole bond in Mb may be more resistant to the pH-induced cleavage than that in HRP. The same trend has also been found for V(IV)-substituted hemoproteins, Mb-V^{IV}=O (**2**) being more stable than HRP-V^{IV}=O (**4**). These results may allow us to suggest that the coordination of the imidazole to oxometal(IV) is much more sensitive to the protein conformational change in HRP than in Mb. It may be also suggested that the metal–histidyl imidazole bonds in Mb and HRP are weaker for the V^{IV}=O complex than for the Mo^{IV}=O (**2**) complex.

In the catalytic reaction of HRP, an oxygen atom (coordinated to ferryl iron in compound II) is believed to be released as a water molecule upon one-electron reduction by a substrate.[48] The kinetic study revealed that this rate is affected by the ionization of the group with pK 8.6 located in close proximity to the active site.[48] Recent resonance Raman studies further suggested[6,49] that the ionization of this group is reflected in the Fe^{IV}=O stretching frequency, which could be associated with changes in the exchange rate of the coordinated oxygen atom with the bulk water oxygen. Combining our present results with these, it seems likely that the structural change induced by the ionization of the group with pK 9, most probably the distal histidine, could be translated through the peptide onto the heme proximal side, which consequently modulates the reactivity of oxygen in compound II.

The Mo- and W-substituted HRP and Mb have now been demonstrated to serve as stable model proteins to mimic the high-valent heme environmental structure of native HRP compounds I (Fe^{IV}=O–$Por^{\bullet+}$) and II (Fe^{IV}=O). In relation to the reaction of compounds I and II with a substrate, the redox properties of HRP-Mo=O (**3**) and HRP-W^{V}=O (**8**) and their substrate binding features are of considerable interest. Indolepropionic acid (IPA) and benzohydroxamic acid (BHA), which are potential substrates of native HRP, have been shown to bind to the resting enzyme by the NMR technique.[45,46] BHA binding causes substantial conformational changes of protein in the heme vicinity.[41] However, it has not yet been clarified that such a substrate-induced structural change is also induced for HRP compounds I and II. This is because these reaction intermediates are readily reduced with substrates to the resting enzyme upon substrate binding. Thus, with the present model protein compounds, we further examined substrate bindings to HRP in the higher oxidation state.

We followed the electronic and ESR spectral changes of HRP-Mo=O and HRP-W=O when the substrate was added to the enzyme solution. Addition of BHA to HRP-Mo^{V}=O (**3**) and HRP-W^{V}=O (**8**) caused no ESR spectral changes but did cause slight electronic absorption spectral alterations, with several isosbestic points at 341, 450, 515, 570, and 613 nm (Figure 7A). From the electronic absorption spectral changes of HRP-Mo^{V}=O (**3**) (Figure 7B) and HRP-W^{V}=O (**8**) in the BHA titration, the dissociation constants of BHA from HRP-Mo^{V}=O (**3**) and HRP-W^{V}=O (**8**) were estimated as 47 and 103 μM, respectively. These are comparable to 150 μM for the native enzyme in the low-spin ferric cyanide complex,[41] while quite different from 2.4 μM for the ferric high-spin resting state.[42] This result implies that the BHA binding affinity is dependent on whether or not the sixth coordinating site of the heme is occupied and may suggest that native HRP compounds I and II have BHA binding affinity as low as for its cyanide complex. It is also suggested that BHA can bind to the protein moiety of HRP-Mo^{V}=O (**3**) in such a way that the conformational change is not so drastic as to affect the electronic structure of the central metal(V), as sensed by the ESR spectral change. Unlike HRP-Mo^{V}=O (**3**), HRP-Mo^{IV}=O (**4**) did not experience BHA-induced electronic absorption spectral changes. This could be due to substantial conformational differences between these HRP compounds at the BHA binding site.

When IPA was used as a substrate, the electronic absorption spectra of all of Mo- and W-substituted HRPs were nearly un-

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changed, indicating that IPA binds weakly to these HRP compounds, as has been found for native HRP.[41]

In summary, the present study on the incorporation of Mo=O- and W=O-substituted porphyrin complexes into apo-Mb and apo-HRP has revealed that Mb and HRP favor the lower and higher metal oxidation states, respectively. This appears to parallel the relative stabilities of higher oxidation states of native Mb and HRP. These oxometal-substituted hemoproteins are stable and structurally similar to compounds I and II in their heme environments, which allows us to study the substrate binding features of reactive intermediates of native hemoproteins.

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Communications to the Editor

The Biosynthesis of Furanomycin: On the Mechanism of Formation of the Ether Linkage

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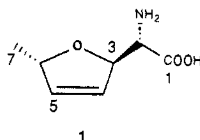
The novel antibiotic furanomycin (**1**) was isolated in 1967 from *Streptomyces threomyceticus* (ATCC 15795).[1] The compound is a competitive antagonist of L-isoleucine, and it inhibits the growth of T-even coliphage. Furanomycin was synthesized in



1980 by Joullie and co-workers, who also revised the stereochemistry to that shown in **1**.[2] Previous experiments carried out in our laboratory have demonstrated that **1** is derived from two acetate units and one propionate unit, with the latter serving as the starter unit.[3] The specific incorporation of propionate into **1** requires the oxidation of C-2 of the propionate skeleton and formation of an ether linkage between C-2 of propionate and C-1 of the adjacent acetate unit. Our prior studies revealed that one hydrogen atom is removed from C-2 of propionate as the result of this oxidation and that lactate is not an intermediate. Experiments will now be outlined that provide additional insight into the mechanism of formation of the ether linkage.

The stereochemistry of hydrogen loss from C-2 of propionate was examined by means of incorporation experiments with (2*R*)- and (2*S*)-[2-^{3}H]propionate. Attempts to prepare the stereospecifically tritiated forms of propionic acid from D- and L-alanine utilizing a route reported for the synthesis of the chirally deuteriated compounds[4] failed due to the capricious nature of [^{3}H]lithium aluminum hydride. Consequently, we developed a new synthesis of chirally tritiated propionate in which the introduction of the isotopic label could be carried out with [^{3}H]-borohydride. The route devised (Scheme I) employs the vanillyl moiety as a carboxyl protecting group[5] and provides stereospecifically tritiated sodium propionate in ca. 10% overall yield. The optical purity and configuration of the labeled propionic acid obtained from this synthesis was determined by a Parker analysis[6] of the [2-^{2}H$_1$]propionic acid obtained from reduction of [*formyl*-^{2}H$_1$]-3-methoxy-4-mesyloxybenzaldehyde with R-Alpine Borane. The deuteriated propionate had the expected configuration (*S*) and an optical purity of ca. 90%. Administration of the (2*R*)- and (2*S*)-[2-^{3}H]propionate to *S. threomyceticus* in conjunction with [1-^{14}C]propionate yielded samples of radioactive furanomycin whose tritium to carbon-14 ratios revealed that propionic acid is converted into the antibiotic with loss of the 2 *pro*-R hydrogen atom (Table I, expt 1 and 2). Since the absolute stereochemistry at C-6 of furanomycin is *S*, it follows that the introduction of the ether oxygen atom at C-2 of propionate occurs with overall *inversion* of configuration.

Scheme I

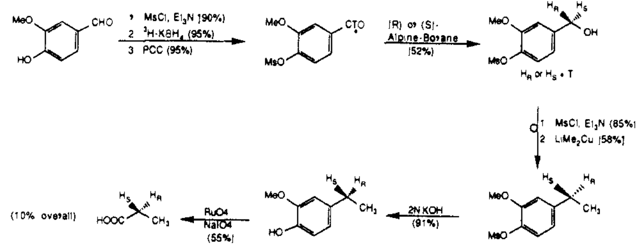


Table I. Incorporation of Labeled Propionic Acid into Furanomycin

expt no.	precursor (^{3}H/^{14}C)	^{3}H/^{14}C furanomycin	% ^{3}H retn
1	sodium [1-^{14}C,2(*S*)-2-^{3}H]propionate (4.80)	4.37	91[a]
2	sodium [1-^{14}C,2(*R*)-2-^{3}H]propionate (4.88)	0.97	21[b]

[a] The optical purity of the precursor was ca. 90%. [b] The optical purity of the precursor was ca. 80%.

The stereochemistry of carbon oxygen bond formation having been elucidated, the question of the origin of the ether oxygen atom was addressed. A priori, it appeared that the oxygen atom could be derived either from the carbonyl oxygen of the adjacent acetate unit or from molecular oxygen. Administration of sodium [^{18}O$_2$, 1-^{13}C]acetate[7] to the fermentation yielded furanomycin exhibiting ^{13}C enrichment at C-1 and C-3, but no oxygen-18 induced shift[8] was apparent for either of these carbon atoms. Appropriate conditions for the administration of ^{18}O-labeled molecular oxygen to the *S. threomyceticus* fermentation were therefore sought. The experiments utilized a closed system containing 1-L shake flasks through which molecular oxygen was circulated with a modified aquarium pump. The system also contained a concentrated aqueous potassium hydroxide solution to trap the expired carbon dioxide. All of the initial attempts to utilize this setup were unsuccessful due to an increase in the pH of the medium from 7 to ca. 9 during the course of the fermentation. Eventually, we discovered that this behavior was the consequence of the production of volatile, basic compounds (ammonia?), and so a 2 N sulfuric acid trap was introduced into the

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