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Preparation of Ruthenium(II) and Ruthenium(III) Myoglobin and the Reaction of Dioxygen, and Carbon Monoxide, with Ruthenium(II) Myoglobin*

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Ruthenium myoglobins have been prepared by the reconstitution of horse heart apomyoglobin with either ruthenium(II) or ruthenium(III) mesoporphyrin IX (MpIX) derivatives. The ruthenium(II) and -(III) myoglobins (RuMb and $RuMb^+$, respectively) contain one ruthenium porphyrin/heme binding site; the species are readily interconverted using dithionite for reduction and bromine for oxidation. RuMb binds carbon monoxide to give the known carbonyl complex. Reversible oxygenation occurs readily with protein-free Ru^{II}(MpIX) species in dimethylformamide, but RuMb in phosphate buffer is irreversibly oxidized by dioxygen to give $RuMb^+$ via an outer sphere electron transfer mechanism.

The discovery by Hoffman and Petering (1) of reversible binding of dioxygen to cobalt-substituted myoglobin and hemoglobin has provided a new method for the study of the structure/function relationship in heme proteins, and extensive studies have now been carried out on CoMb[1] and CoHb using various physical and chemical techniques (2-5).

Ruthenium, the second row transition metal analogue of iron, is an obvious choice for further studies on metal ion substitution in heme proteins. Furthermore, work from this laboratory (6) has shown that ruthenium(II) *m*-tetraphenylporphyrin and octaethylporphyrins in dimethylformamide bind dioxygen reversibly. Srivastava (7) has reported the synthesis of Ru(CO)Mb by reconstitution of sperm whale myoglobin with Ru^{II}(CO)MpIX. We describe here the preparation of RuMb and $RuMb^+$ by reconstitution of horse heart apomyoglobin with Ru^{II}(MpIX) and Ru^{III}(MpIX) species. In addition, data on the interaction of RuMb with carbon monoxide and dioxygen are also presented.

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[1] The abbreviations used are: CoMb, cobaltomyoglobin; Mb^+, ferrimyoglobin (metmyoglobin); Mb, ferromyoglobin (deoxymyoglobin); $CoMb^+$, cobaltimyoglobin; CoHb, cobaltohemoglobin; RuMb, ruthenium(II) myoglobin; $RuMb^+$, ruthenium(III) myoglobin; MpIX, mesoporphyrin IX; DMF, dimethylformamide; py, pyridine; Im, imidazole; TMS, trimethylsilane.

EXPERIMENTAL PROCEDURES

Preparation of Ruthenium Porphyrins—Equal weights of ruthenium dodecacarbonyl ($Ru_3(CO)_{12}$, Strem Chemical Co.) and mesoporphyrin IX di-*t*-butyl ester (8) were refluxed, under N_2, in toluene for 24 h (9). The toluene was removed by rotary evaporation, and the residue dissolved in 50:50 C_2H_5OH/CH_2Cl_2 and refluxed under N_2 for an additional 30 min. Neutral alumina was added to make a slurry and the solvent removed by rotary evaporation followed by drying *in vacuo* overnight. The solid residue was chromatographed over Woelm neutral alumina using CH_2Cl_2. The product was recrystallized from 50:50 C_2H_5OH/CH_2Cl_2 by gently blowing N_2 over the solution to remove most of the CH_2Cl_2. The crystals were washed with 30-60°C petroleum ether and dried *in vacuo* to give ruthenium(II)(CO)mesoporphyrin IX di-*t*-butyl ester·(C_2H_5OH), **I**. (m.p. 234-237°C. NMR (pyridine-d_5) δ_{TMS}: 1.10 (*s*, 9, $-C(CH_3)_3$), 1.12 (*s*, 9, $-C(CH_3)_3$), 1.64 (*t*, 6, J = 7 Hz, ethyl-CH_3), 3.19 (*t*, 4, J = 7 Hz, propionate-CH_2—), 3.30 (*s*, 6, $-CH_3$), 3.35 (*s*, 6, $-CH_3$), 3.80 (*q*, 4, J = 7 Hz, ethyl-CH_2-), 4.25 (*t*, 4, J = 7 Hz, propionate-CH_2—), 9.97, 9.99, 10.00, 10.22 (*s*, 1 each, methine-H). IR (CH_2Cl_2): 1920 (*s*), 1926 (*sh*) cm^{-1}. Visible spectrum[2] (CH_2Cl_2): λ_{max} = 546 (ϵ = 32.6), 514 (18.6), 393 (258).)

$C_{45}H_{58}N_4O_6Ru$

Calculated: C 63.4, H 6.9, N 6.6
Found: C 63.1, H 6.8, N 6.75

Ru^{II}(CO)MpIX dicarboxylic acid derivatives were prepared by first stirring Complex **I** in CH_2Cl_2 saturated with dry HCl gas to remove the *t*-butyl groups. After 2 h the solvent was removed by rotary evaporation and the residue chromatographed over silica gel using 10:10:1 py/CH_2Cl_2/H_2O. The product was crystallized from 6:1 ethyl acetate/acetic acid to which an equal volume of cyclohexane had been added. The crystals were washed with hexane and dried *in vacuo* over P_2O_5 for 8 h to give Ru^{II}(CO)(MpIX)py, **II**. (m.p. > 285°C: NMR (pyridine-d_5) δ_{TMS}: 1.87 (*t*, 4, J = 7 Hz, ethyl-CH_3)$_3$, 3.56 (*s*, 6H, $-CH_3$), 3.60 (*t*, 4, J = 7 Hz, propionate-CH_2—), 3.62 (*s*, 6H, $-CH_3$), 4.06 (*q*, 4, J = 7 Hz, ethyl-CH_2—), 4.62 (*t*, 4, J = 7 Hz, propionate-CH_2—), 8.18, 8.20, 8.26, 8.70 (*s*, 1 each, methine-H). Visible spectrum (DMF): λ_{max} = 548 (ϵ = 23.6), 516 (13.5), 393 (212).) The chemical analysis indicates the presence of 2 water molecules of crystallization:

$C_{40}H_{45}N_5O_7Ru$

Calculated: C 59.35, H 5.6, N 8.65
Found: C 58.9, H 5.5; N 8.75

Ru^{II}(CO)MpIX·n H_2O (**III**) was prepared as described for the pyridine complex except that the sample was crystallized from acetone/H_2O. (Visible spectrum (DMF): λ_{max} = 548 (ϵ = 21.9), 516 (14.8), 393 (225).) Much better reconstitution results with DMF solutions were obtained using complex **III** rather than **II**.

The bis(DMF) species Ru^{II}(MpIX)(DMF)$_2$, **IV**, was prepared *in situ* by degassing a DMF solution of Complex **II** or **III** through several freeze-thaw cycles and irradiating *in vacuo* with a 600-watt tungsten lamp. (Visible spectrum (DMF): λ_{max} = 519 (ϵ = 32.6), 496 (10.3), 390 (156).) A Ru^{III}(MpIX) species was prepared by titrating a DMF

[2] All extinction coefficients ϵ are expressed in units of mM^{-1} cm^{-1}; wavelengths are given in nanometers.

solution of **IV** with Br_2 in DMF. (Visible spectrum (DMF): λ_{max} = 520 (ϵ = 10.7), 391 (134).)

Preparation of Ruthenium Myoglobins—Mb^+ (horse heart, type III), was obtained from Sigma Chemical Co. An apomyoglobin solution (0.1 to 1.0 mM in heme site in 0.01 M phosphate buffer, pH 8.0) was prepared from the metmyoglobin using a modified acid/butanone procedure (10). A stoichiometric amount of $Ru^{II}(MpIX)(DMF)_2$ in 0.5 ml of DMF was added slowly at 0°C to 3 to 4 ml of apomyoglobin solution that was gently stirred. Immediately after mixing, the solution was passed through a column (3 × 50 cm) of Sephadex G-25 at 4°C, equilibrated with a 0.01 M phosphate buffer at pH 6.3, and the column then was eluted with the same buffer. The reconstituted myoglobin eluted in the void volume as $RuMb^+$. This was adsorbed immediately onto a CM-cellulose ion exchange column equilibrated with the pH 6.3 buffer, and then eluted with 0.1 M phosphate buffer (pH 7.0) to give purified $RuMb^+$. (Visible spectrum (pH 8.0, phosphate): λ_{max} ~ 500 (ϵ = 10.0) and 396 (95.7).) The fact that identical visible spectral data were obtained for $RuMb^+$ samples prepared from $Ru^{II}(MpIX)(DMF)_2$ and the apomyoglobin mixed in mole ratios of 1:1, 10:1, and 1:10 shows that there is only one ruthenium moiety bound/apomyoglobin, and this is considered to be at the heme site. RuMb was generated by mixing under argon, 2 to 4 ml of degassed 0.05 M phosphate buffer (pH 8.0) with 50 to 500 μl of $RuMb^+$ solution in the same buffer, followed by titration with 0.1 to 1.0 mM sodium dithionite again in the degassed pH 8.0 buffer. (Visible spectrum (pH 8.0, phosphate): λ_{max} = 520 (ϵ = 24.8), 495 (11.3), and 394 (130).)

$RuMb^+$ could also be generated by titration of degassed buffer solutions of RuMb with Br_2 in degassed DMF solution, as well as by reconstitution of the apomyoglobin using $Ru^{III}(MpIX)$ species in DMF.

The carbonyl derivative $Ru^{II}(CO)Mb$ could be formed in aqueous buffer by the reconstitution procedure but using the carbonyl complex **III**. The visible spectrum (λ_{max} = 553 (ϵ = 14.0), 547 (12.3), 519 (13.4) and 398 (198)) is identical with that reported by Srivastava (7).

The protein concentration of apomyoglobin samples was determined by absorbance measurements at 280 nm (ϵ = 15.5), while that in the ruthenomyoglobin samples was determined by the Lowry method (11) using native myoglobin as the standard.

Kinetic Experiments—The rates of reactions of RuMb with O_2 to give $RuMb^+$, and with CO to give Ru(CO)Mb, were monitored by following the decay of the RuMb 520 nm band in the case of oxidation or by following the increase in the 398 nm band of the carbonyl complex. Both reactions usually went to completion at the 1-atm pressure of O_2 (or CO) used; their solubility, of the order of 10^{-3} M atm^{-1} (12), maintains an effective constant concentration of the gases throughout the reaction at the dilute RuMb concentrations used (10^{-6} to 10^{-5} M). The data were analyzed in terms of a first order loss of the initial [RuMb], using a standard log *versus* time plot resulting from integration of $-d[RuMb]/dt = k[RuMb]$. If n is the mole fraction of RuMb present at any time t, $\ln(1/n) = kt$ (or $n = e^{-kt}$), where n is given by $(A_{obs} - A_\infty)/(A_0 - A_\infty)$; A_0, initial absorbance (t = 0); A_{obs}, absorbance at time t; and A_∞, absorbance at completion of the reaction.

RESULTS AND DISCUSSION

Protein-free $Ru^{II}(MpIX)$ Species—The mesoporphyrin species $Ru^{II}(MpIX)(DMF)_2$ **IV** is generated in DMF solution by photolysis of the monocarbonyl precursor **I** as described and illustrated previously for the *m*-tetraphenyl- and octaethylporphyrin derivatives (6). Bubbling CO through the final solution of **IV** regenerates completely the monocarbonyl complex as $Ru^{II}(CO)MpIX(DMF)$. (Visible spectrum as given for the aquo complex **III** when dissolved in DMF.) Quantitative anaerobic titration of **IV** with bromine/DMF solution leads to ruthenium(III) species; the resulting spectral changes (Fig. 1) generate several clean isosbestic points. The ruthenium(II) species **IV** can also be converted quantitatively and reversibly to ruthenium(III) by electrochemical methods; the interconversion has been carried out through several cycles. The electrochemical oxidation product, presumably $Ru(MpIX)(DMF)_n^+$ (n = 1 or 2), has a different absorption spectrum (λ_{max} ~ 520 (ϵ ~ 14), 386 (160)) to that of bromine oxidation product which, thus, likely contains coordinated bromide.

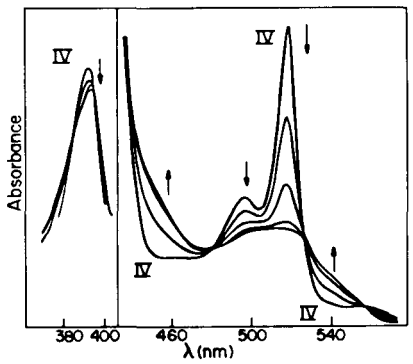


FIG. 1. Titration of $Ru^{II}(MpIX)(DMF)_2$, **IV**, with aliquots of Br_2/DMF to give Ru(III) species (directions of *arrows* indicate changes in spectra as Ru(II) is consumed).

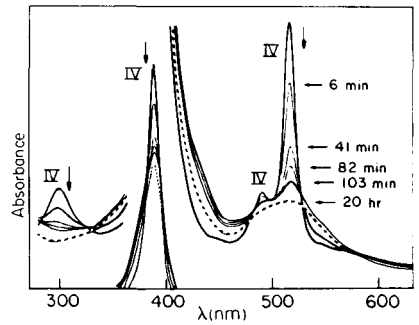


FIG. 2. Oxygenation (1 atm of O_2) of $Ru^{II}(MpIX)(DMF)_2$, **IV**, in DMF at 0°C to give $Ru^{II}(O_2)(MpIX)(DMF)$. The spectrum at 20 h (- - -) shows irreversible oxidation to Ru(III).

In a manner analogous to that reported previously for the corresponding octaethylporphyrin system (6), $Ru^{II}(MpIX)(DMF)_2$ binds O_2 reversibly in DMF solution. Fig. 2 shows the visible spectral changes accompanying the oxygenation at 0°C. During the time that the isosbestic system is maintained (up to ~2 h), the reaction can be reversed, albeit slowly (6); the spectrum after 20 h shows irreversible oxidation to Ru(III). At 22°C, the O_2 treatment leads to more rapid oxidation (autoxidation), and reversible binding cannot be demonstrated. The spectrum of the autoxidation product differs, especially in the Soret region (λ_{max} = 390 (ϵ = 120)), from those of the Ru(III) species produced electrochemically and by bromine oxidation. The autoxidation product may contain an oxo-bridged Ru(III)-O-Ru(III) moiety (13, 14); reduction using either borohydride in DMF or dithionite in phosphate buffer/DMF regenerates $Ru^{II}(MpIX)(DMF)_2$ *in situ*.

Ruthenium Myoglobins—The absorption spectra for RuMb and $RuMb^+$ are given in Fig. 3. The Soret band in RuMb is shifted ~4 nm to higher wavelength compared to that in $Ru(MpIX)(DMF)_2$, and the $RuMb^+$ system shows similar bathochromic shifts (4 to 10 nm) compared to the various $Ru^{III}(MpIX)$ species. The RuMb spectrum shows α and β bands of intensity ratio 3:1, very similar to those observed for the six-coordinate, low spin $Ru(porphyrin)L_2$ complexes (L = CH_3CN, DMF, py) reported here and elsewhere (6, 15), suggesting that the ruthenium(II)-substituted myoglobin, unlike Mb itself, is six-coordinate, low spin. A number of high spin "bare" hemes in noncoordinating solvents are reported to give two well defined bands in the visible region (16), but the ruthenium systems discussed here are in more strongly coordinating environments and the spectral data together with their reactivity toward CO and O_2 (see below) are best accommodated in terms of low spin, six-coordinate geometry. In $RuMb^+$, the visible region is broad with the α band appearing as a shoulder on the β band (α:β intensity ratio ~0.8:

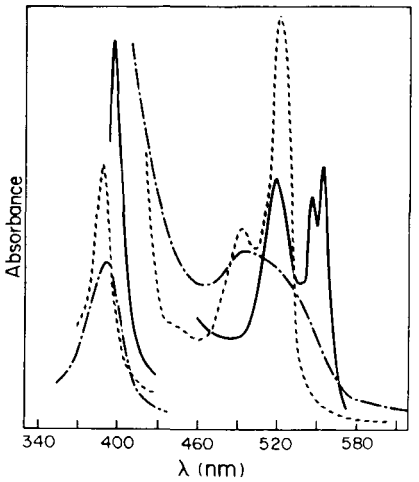


FIG. 3. Absorption spectra of ruthenium myoglobins in aqueous phosphate buffer, pH 8.0. $Ru^{III}Mb$, -·-·-; $Ru^{II}Mb$, - - -; $Ru^{II}(CO)Mb$, ——

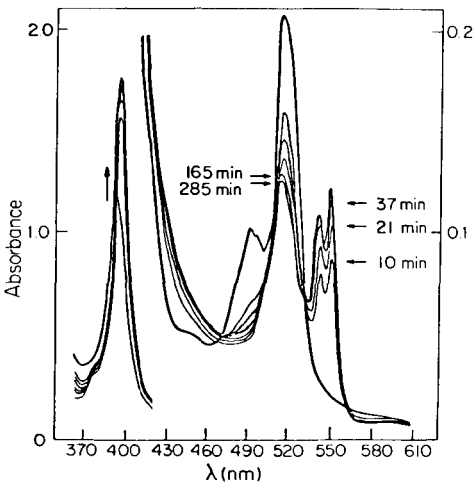


FIG. 4. Reaction of $Ru^{II}Mb$ with 1 atm of CO at 0°C to form $Ru^{II}(CO)Mb$; spectra as a function of time (*cf.* Fig. 3).

1); there is a similar broad region in the protein-free Ru^{III}(MpIX) complexes, although the intensity ratio is now reversed.

Reaction with Carbon Monoxide—The unusual splitting of the α-band in the spectrum of Ru(CO)Mb (Fig. 3) was noted also for the sperm whale myoglobin (7). Our reconstituted Ru(CO)Mb sample was indistinguishable spectrally from that formed by carbonylation of RuMb. Fig. 4 shows spectral changes during such a reaction at 5°C. It is immediately evident qualitatively that the loss of RuMb is not a first order process. Fig. 5, the attempted first order $\ln(1/n)$ *versus* time plot, is readily interpreted as resulting from two simultaneous first order reactions involving two different initial RuMb species, **1** and **2**, reacting with rate constants k_1^{CO} and k_2^{CO}, respectively; k_2^{CO} (0.033 min^{-1}) is determined directly from the slope of the final linear region of the log plot. The initial mole ratio of **1** and **2** is estimated, by extrapolation of the "k_2^{CO} line" to $t = 0$, to be 1.8:1; a calculation of $k_1^{CO} = 0.57$ min^{-1} gives an excellent fit for the overall experimental data (see Fig. 5). Thus, about 65% of the RuMb sample reacts some 20 times faster than the remaining 35%. In addition, the spectral changes usually indicated that a small amount (≤5%) of starting RuMb remained unreacted. It then was found that the amount of this species (**3**) increased on allowing the protein sample to stand in phosphate buffer at 20°C and that, by gentle anaerobic heating of such solutions, the RuMb could

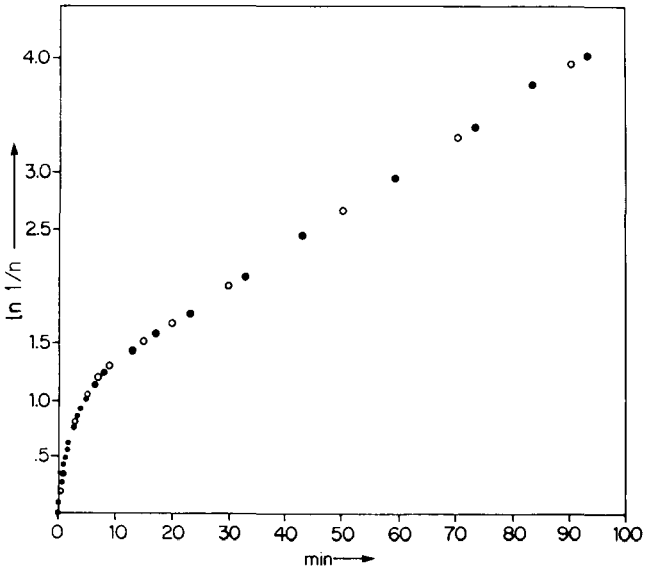


FIG. 5. Analysis of kinetic data for $Ru^{II}Mb$ + CO reaction: ●, experimental data (398 nm); ○, calculated (see text).

be converted entirely into species **3** that was unreactive toward CO. Species **1**, **2**, and **3** must have essentially identical absorption spectra, and they are all considered to be six-coordinate and low spin (see below).

Compared to $Ru^{II}(CO)$MpIX derivatives, the Ru(CO)Mb species is extremely resistant to photodecarbonylation. Photolysis at ≥40°C was necessary to generate any RuMb; species **3** is produced mainly since less than 50% of the RuMb so formed would rebind CO. Srivastava (7) has proposed that the observed splitting of the α band of Ru(CO)Mb is due to a bent Ru—C(=O) system, unlike the linear arrangement found in the protein-free environment (9). Whether this structural difference can lead to differences in photolability is unclear. Hoffman and Gibson (17) have suggested that linear systems such as Fe(II)—C—O should be relatively labile compared to bent systems such as Fe(II)—O(=O), but this is based on electronic arguments involving total count of metal *d* electrons plus ligand π^* electrons, in this example 6 (linear *versus* 8 (bent).

Photolysis of Ru(CO)Mb in the presence of trace amounts of dioxygen (initially accidentally) leads to a dramatic decrease in the intensity of the Soret band, a disappearance of the α- and β-bands, and the production of a new intense band at 590 nm ($\epsilon \sim 30$). The spectrum is similar to that attributed to an iron verdohemochrome obtained by hydrogen peroxide oxidation of Fe(octaethylporphyrin)py_2 (18), in which the porphyrin ring has been oxidized. Neither RuMb nor $Ru^{II}(CO)$MpIX derivatives underwent the photooxidation.

Reaction with Dioxygen—The exposure of a phosphate buffer solution of RuMb at 0°C to 1 atm of O_2, either in the absence or presence of excess dithionite, resulted in rapid formation of $RuMb^+$. (The RuMb can be regenerated, of course, with excess dithionite once all the dioxygen in the system has been consumed via the Ru(II) to Ru(III) conversion.) It is possible that $Ru(O_2)Mb$ and $RuMb^+$ have indistinguishable absorption spectra, as in the case of some $Co(O_2)Hb$ and $CoHb^+$ systems that have remarkably similar spectra (2, 3). However, since we can readily distinguish between $Ru(O_2)$MpIX and Ru^{III}(MpIX) in the protein-free systems, it is very unlikely that we have produced any $Ru(O_2)Mb$. The kinetic data on the O_2 reaction (see below) also favor oxidation rather than oxygenation.

As anticipated from the findings on the carbonylation re-

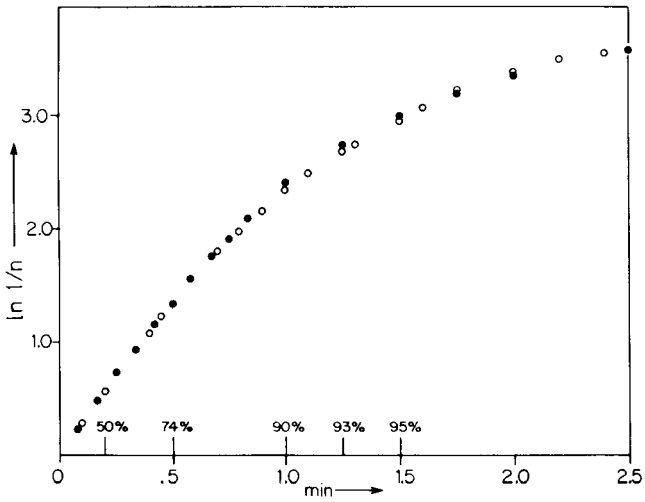
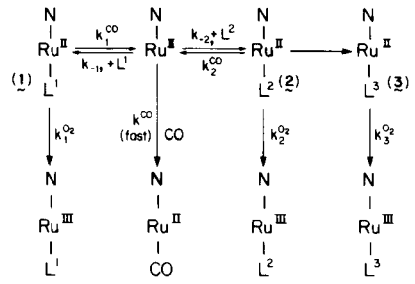


FIG. 6. Analysis of kinetic data for $Ru^{II}Mb + O_2$ reaction: ●, experimental data (520 nm); ○, calculated (see text).

action, the O_2 reaction (in the absence of excess dithionite) did not analyze for a simple first order process. Fig. 6 shows the log $(1/n)$ *versus* time plot; this experimental curve can be matched by assuming simultaneous reactions of the O_2 with the three RuMb species, **1**, **2**, and **3**, whose concentrations were calculated from a CO uptake experiment on a sample from the same reconstituted batch. The data of Fig. 6 pertain to a sample with 60% **1**, 34% **2**, and 6% **3**, and analyze to give $k_1^{O_2} = 3.7\ min^{-1}$, $k_2^{O_2} = 2.1\ min^{-1}$, and $k_3^{O_2} = 0.35\ min^{-1}$ at 0°C.

The kinetic findings lead us to propose tentatively Scheme 1 for the reactions of RuMb. The six-coordinate low spin species **1** and **2** react with CO via a dissociative process with rate constants k_1^{CO} and k_2^{CO} to give a five-coordinate intermediate which then reacts rapidly (k^{CO}) with the CO. Such a mechanism is well established for corresponding iron(II) porphyrin complexes (19), although more generally, depending on the ratio of the CO concentration to that of the displaced axial ligand, the $k_{-1}(k_{-2})$ reaction may compete with the k^{CO} step. This is not the case in the present system since pseudo-first order kinetics, even of the two component variety shown in Fig. 5, would not be evident. A bis(histidine) formulation for **2** and aquohistidine structure for **1** could explain the relative reactivities toward CO. The CO inactive species **3** must be formed irreversibly (possibly as the first step in protein denaturation). Formation from **2** could involve conversion from a nitrogen- to a carbon-bound imidazole axial ligand since such a rearrangement has been observed in $(Ru(NH_3)_5(Im))^{2+}$ (20). Reversible formation of internal hemichromes formed by deprotonation of N-1 in coordinated imidazoles has been suggested for the denaturation of ferric hemoglobin A (21, 22).

The oxidation of RuMb by dioxygen (Scheme 1, $k_n^{O_2}$, $n = 1, 2, 3$) is proposed to occur via an outer sphere mechanism possibly involving electron transfer from the porphyrin periphery to the dioxygen, followed by an internal electron transfer from the metal to the porphyrin. The rate constants are significantly larger than k_1^{CO} or k_2^{CO}, thus eliminating the possible formation of a dioxygen adduct via the five-coordinate intermediate. The O_2 oxidation of low spin Fe^{II}-(porphyrin)$(amine)_2$ may occur via such an outer sphere mechanism as well as by inner sphere pathways involving metal-coordinated O_2 (13). In order to model species **2**, the $Ru^{II}(MpIX)(Im)_2$ complex was formed *in situ* by adding imidazole ($\sim 10^{-2}$ M) to DMF solutions containing Ru^{II}-$(MpIX)(DMF)_2$. Although there is no reaction with 1 atm of



SCHEME 1. L^1, L^2, L^3 are various axial ligands (see text).

CO at 22°C over 1 day (the k_{-2} reaction with the excess Im effectively competing with k^{CO}), there is a very rapid oxidation with 1 atm of O_2 to give a $Ru^{III}(MpIX)$ species and this can only occur via an outer sphere mechanism which is consistent with Scheme 1.

Reconstitution of Apomyoglobin with Ru(O$_2$)MpIX—Since the O_2 reaction with RuMb did not lead to $Ru(O_2)Mb$, attempts were made to reconstitute the apomyoglobin (pH 8, phosphate buffer) at 0°C using DMF solutions of $Ru(O_2)MpIX$. When the resulting mixture was immediately chromatographed over Sephadex G-25 almost all of the porphyrin separated from the protein fraction, indicating little incorporation of the metalloporphyrin. However, a very slow reconstitution does take place to give $RuMb^+$, $t_{1/2}$ being several days at 0°C, and about 1.5 h at 22°C. In contrast, reconstitution using $Ru^{III}(MpIX)$, $Ru^{II}(MpIX)$, or Ru^{II}-(CO)MpIX species is very much faster, and indeed is essentially instantaneous with the ruthenium(II) systems. It is likely that in the protic buffer, the $Ru(O_2)MpIX$ is rapidly oxidized; corresponding iron(II) systems readily yield μ-oxo-bridged species Fe(III)—O—Fe(III) under such conditions (13, 14, 23), and such "dimers" are unlikely to enter the heme site. The slow reconstitution observed with the ruthenium system could be due to slow formation from the dimer of some monomeric species that is capable of entering the protein binding site.

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