Preparation of Ruthenium(II) and Ruthenium(III) Myoglobin and the Reaction of Dioxylene, 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 (MIX) 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 RuMb(MIX) species in dimethylformamide, but RuMb in phosphate buffer is irreversibly oxidized by dioxygen to give RuMb+ via an outer electron transfer mechanism.

EXPERIMENTAL PROCEDURES

Preparation of Ruthenium Porphyrins—Equal weights of ruthenium dodecacarbonyl (Ru(II)CO)12, Strem Chemical Co.) and mesoporphyrin IX di-t-butyl ester (8) were refluxed, under Nz, in toluene for 24 h (9). The toluene was removed by rotary evaporation, and the residue dissolved in 50:50 C2H5OH/CH2Cl2 and refluxed under Nz 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 CH2Cl2. The product was recrystallized from 50:50 C2H5OH/CH2Cl2 by gently blowing Nz over the solution to remove most of the CH2Cl2. 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-(CH2)=OH, I. (m.p. 234–237°C. NMR (pyridine-d5) δH) 1.10 (s, 9, —C(CH3)3), 1.12 (s, 9, —C(CH3)3), 1.64 (t, 6, J = 7 Hz, ethyl-CH2), 3.19 (t, 4, J = 7 Hz, propionate-CH—), 3.30 (s, 6, —CH2), 3.35 (s, 6, —CH2), 3.80 (q, 4, J = 7 Hz, ethyl-CH2—), 4.25 (t, 4, J = 7 Hz, propionate-CH—), 9.97, 9.99, 10.00, 10.22 (s, 1 each, methine-H). IR (CH2Cl2): 3195 (w), 3196 (w), 3196 (w).) Much better reconstitution results with DMF solutions were obtained using complex III rather than II. The bis(DMF) species Ru(II)(MIX)(DMF)2, IV, (m.p. &gt; 285°C, m.p. &gt; 295°C, NMR (pyridine-d5) δH) 1.87 (t, 4, J = 7 Hz, ethyl-CH2), 3.50 (s, 6H, —CH3), 3.60 (t, 4, J = 7 Hz, propionate-CH—), 3.96 (s, 6H, —CH3), 4.06 (q, 4, J = 7 Hz, ethyl-CH2—), 6.15 (t, 4, J = 7 Hz, propionate-CH—), 8.18, 8.20, 8.26, 8.70 (s, 1 each, methine-H). Visible spectrum (DMF): λmax = 546 (ε = 23.6), 516 (13.5), 393 (213).) The chemical analysis indicates the presence of 2 water molecules of crystallization.

Ru(II)(MIX) dicarboxylic acid derivatives were prepared by first stirring Complex I in CH2Cl2 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 10:1:1 py/CH2Cl2/H2O. The product was crystallized from 6:1 ethyl acetate/acetonic acid to which an equal volume of cyclohexane had been added. The crystals were washed with hexane and dried in vacuo over P2O5 for 8 h to give Ru(II)(MIX)(py), II. (m.p. &gt; 285°C, m.p. &gt; 295°C, NMR (pyridine-d5) δH) 1.87 (t, 4, J = 7 Hz, ethyl-CH2), 3.50 (s, 6H, —CH3), 3.60 (t, 4, J = 7 Hz, propionate-CH—), 3.96 (s, 6H, —CH3), 4.06 (q, 4, J = 7 Hz, ethyl-CH2—), 6.15 (t, 4, J = 7 Hz, propionate-CH—), 8.18, 8.20, 8.26, 8.70 (s, 1 each, methine-H). Visible spectrum (DMF): λmax = 546 (ε = 23.6), 516 (13.5), 393 (213).) The chemical analysis indicates the presence of 3 water molecules of crystallization.

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 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 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 apomyoglobin with Ru(II)(MIX) species. We describe here the preparation and stabilization of RuMb and RuMb+ by reconstitution of horse heart apomyoglobin with Ru(II)(MIX) species. In addition, data on the interaction of RuMb with carbon monoxide and dioxygen are also presented.

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The abbreviations used are: CoMb, cobaltomyoglobin; Mb+, ferrimyoglobin (metmyoglobin); Mb, ferromyoglobin (deoxymyoglobin); CoMb+, cobaltimyoglobin; CoHb, cobaltohemoglobin; RuMb, ruthenium(II) myoglobin; RuMb+, ruthenium(III) myoglobin; MIX, mesoporphyrin IX, DMF, dimethylformamide, py, pyridine, Im, imidazole; TLS, trimethylsilane.

* All extinction coefficients ε are expressed in units of mM–1 cm–1; wavelengths are given in nanometers.
solution of IV with Br₂ in DMF. (Visible spectrum (DMF): \( \lambda_{\text{max}} = 590 \) (\( e = 10.7 \)), 591 (1341.).)

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/butane procedure (10). A stoichiometric amount of Ru³⁺(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 was then 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): \( \lambda_{\text{max}} \approx 500 \) (\( e = 10.0 \)) and 396 (95.7).) The fact that identical visible spectral data were obtained for RuMb⁺ samples prepared from Ru³⁺(MpIX)(DMF)₂ and the apomyoglobin mixed in mole ratios of 1:1, 1:10, and 1:10 shows that there is only one ruthenium moiety bound/apomyoglobin, and this is considered to be at the heme site.

Protein-free Ru³⁺(CO)Mb could also be generated in aqueous buffer by the reconstitution procedure but using the carbonyl complex III (pH 6.3) to give purified RuMb⁺. (Visible spectrum (pH 8.0, phosphate): \( \lambda_{\text{max}} = 520 \) (\( e = 24.8 \)), 495 (11.3), and 394 (130.).)

RuMb⁺ could also be generated by titration of degassed buffer solutions of RuMb with Br₂ in degassed DMF solution, as well as by reconstitution of the apomyoglobin using Ru³⁺(MpIX) species in DMF.

The carboxyl derivative Ru³⁺(CO)Mb could be formed in aqueous buffer by the reconstitution procedure but using the carbonyl complex III. The visible spectrum \( \lambda_{\text{max}} = 553 \) (\( e = 14.0 \)), 547 (12.3), 519 (13.4) and 398 (198)) is identical with that reported by Srivastava (7).

The carbonyl derivative Ru³⁺(CO)Mb could be formed in aqueous buffer by the reconstitution procedure but using the carbonyl complex III. The visible spectrum \( \lambda_{\text{max}} = 553 \) (\( e = 14.0 \)), 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 (\( e = 15.5 \)), while that in the ruthenomyoglobin samples was determined by the Lowry method (11) using native myoglobin as the standard. Kinetic Experiments—The rate of reactions of RuMb with O₂ 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 308 nm band of the carbonyl complex. Both reactions usually went to completion at the 1-atm pressure of O₂ (or CO) used; their solubility, of the order of 10⁻³ M atm⁻¹ (12), maintain an effective constant concentration of the gases throughout the reaction at the dilute RuMb concentrations used (10⁻⁴ to 10⁻⁵ M). The data were analyzed in terms of a first order 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 \( t \), then \( n = \frac{A(t) - A_0}{A(t) - A_{\infty}} \), where \( A(t) \) is given by \( A(t) - A_0 \). If \( A(t) - A_0 \) absorbance at time \( t \), and \( A_0 \) absorbance at completion of the reaction.

RESULTS AND DISCUSSION

Protein-free Ru³⁺(MpIX) Species—The mesoporphyrin species Ru³⁺(MpIX)(DMF)₂ IV is generated in DMF solution by photolysis of the monocarboxyl 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 monocarboxyl complex as Ru³⁺(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(III) species IV can also be converted quantitatively and reversibly to ruthenium(III) by electrochemical methods; the interconversion has been carried out under several cycles. The electrochemical oxidation product, presumably Ru³⁺(MpIX)(DMF)₃⁺⁺, has a different absorption spectrum (\( \lambda_{\text{max}} \approx 520 \) (\( e = 14 \)), 386 (160)) to that of bromine oxidation product which, thus, likely contains coordinated bromide.
Studies on Ruthenium Myoglobin

FIG. 3. Absorption spectra of ruthenium myoglobins in aqueous phosphate buffer, pH 8.0. Ru^{II}Mb, ---; Ru^{III}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^{II}(Mpx) 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; $k_1^{CO}$ (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 $k_1^{CO} = 0.5$/min 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 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}(Mpx) derivatives, the Ru(CO)Mb species is extremely resistant to photodecarbonylation. Photoysis 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$\equiv$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)$\equiv$C—O should be relatively labile compared to bent systems such as Fe(II)$\equiv$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}(Mpx) 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(I1) to Ru(II1) 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$_2$)MbIX and Ru^{II}(Mpx) 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-
action, the O₂ 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 O₂ 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 

\[ k_{1} = 3.7 \text{ min}^{-1}, \quad k_{2} = 2.1 \text{ min}^{-1}, \quad \text{and} \quad k_{3} = 0.35 \text{ min}^{-1} \text{ at } 0°C. \]

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 \( k_{1}^{\text{CO}} \) and \( k_{2}^{\text{CO}} \) to give a five-coordinate intermediate which then reacts rapidly (\( k^{\text{CO}} \)) 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 \( k^{\text{CO}} \) 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 of 2 could involve conversion from a nitrogen- to a carbon-bound imidazole axial ligand since such a rearrangement has been observed in (Ru(NH₃)₅(Im))²⁺ (20). Reversible formation of internal hemes from 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, \( k_{n}^{\text{O₂}} \), \( n = 1, 2, 3 \)) 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 \( k_{1}^{\text{O₂}} \) or \( k_{2}^{\text{O₂}} \), thus eliminating the possible formation of a dioxygen adduct via the five-coordinate intermediate. The O₂ oxidation of low spin Fe(II)-porphyrin(amine) may occur via such an outer sphere mechanism as well as by inner sphere pathways involving metal-coordinated O₂ (13). In order to model species 2, the Ru(II)(MpIX)(Im)₂ complex was formed in situ by adding imidazole (\( 10^{-2} \text{ m} \)) to DMF solutions containing Ru(II)(MpIX)(DMF)₂. Although there is no reaction with 1 atm of CO at 22°C over 1 day (the \( k_{-2} \) reaction with the excess Im effectively competing with \( k^{\text{O₂}} \)), there is a very rapid oxidation with 1 atm of O₂ to give a Ru(II)(MpIX) species and this can only occur via an outer sphere mechanism which is consistent with Scheme 1.

Reconstitution of Apomyoglobin with Ru(O₂)MpIX—Since the O₂ reaction with RuMb did not lead to Ru(O₂)Mb, attempts were made to reconstitute the apomyoglobin (pH 8, phosphate buffer) at 0°C using DMF solutions of Ru(O₂)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(O₂)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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Preparation of ruthenium(II) and ruthenium(III) myoglobin and the reaction of dioxygen, and carbon monoxide, with ruthenium(II) myoglobin.

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