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# Conformation of Bound Carbon Monoxide in Carbon Monoxide Complex of Ruthenium(II) Myoglobin

T. S. SRIVASTAVA

*Department of Chemistry, Indian Institute of Technology, Powai, Bombay-400 076, India*

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*The Ru(II)Mb(CO) complex shows visible bands at 554, 546.5 (split α bands), 519 (broad β band) and a Soret band at 398.5 nm. The separation between two α bands is 7.5 nm which is very sensitive to changes in tertiary structure of the protein. The decrease in separation of α bands is mediated by protonation of distal histidine at acid pH. The low concentrations of guanidine hydrochloride is also able to reduce the separation of α bands. This protein complex is denatured by acid and guanidine hydrochloride respectively. The helix-coil transition of each is sharp and indicates the presence of one complex. The splitting of α band and broadening of β band in the protein complex have been interpreted in terms of lowering of square planar symmetry of metal porphyrin. This symmetry lowering is a result of constraint of distal histidine on linear RuCO grouping forcing it off the axis normal to the porphyrin plane.*

## Introduction

Recently M. F. Perutz [1] has suggested on the basis of X-ray diffraction analysis that the Fe–C–O grouping is straight in the carbon monoxide complex of hemoglobin and it is bent with respect to the axis perpendicular to molecular plane of the porphyrin. L. Pauling [2] has supported the above conformation of bound carbon monoxide in the carbon monoxide complex of hemoglobin on the basis of valence bond theory. Similar conformation of bound carbon monoxide in its complex with myoglobin has also been suggested [1]. The above structural information is only obtained in solid state on the carbon monoxide complex of hemoglobin. Recently the first experimental evidence [3] has been presented in solution that the carbon monoxide complex of ruthenium(II) myoglobin has conformation similar to carbon monoxide complex of myoglobin. In this paper further spectroscopic evidences of the above formulation of bound carbon monoxide in the carbon monoxide complex of ruthenium(II) myoglobin in solution are presented.

## Experimental

Guanidine hydrochloride was ultrapure from Mann Biochemical Co., U.S.A.. All other chemicals were of standard reagent grade.

The carbon monoxide complex of ruthenium(II) mesoporphyrin IX (Ru(II)MPIX(CO)) was prepared and purified as described elsewhere [4]. The apomyoglobin was prepared from sperm whale skeletal muscle myoglobin (Sigma Chemical Co., U.S.A., Type III) using the acid-butanone method of Teale [5]. The carbon monoxide complex of ruthenium(II) myoglobin (Ru(II)Mb(CO)) was prepared as described earlier [3]. The ruthenium porphyrin content in a carbon monoxide complex of ruthenium(II) myoglobin solution was determined spectrophotometrically using the pyridine-hemochromogen method [3, 6].

A pH meter model 25, Radiometer, Copenhagen, was used. Perkin-Elmer 124 and 323 spectrophotometers equipped with a cuvette compartment thermostatically controlled at 25 °C were used to record the optical absorption spectra. Fluorescence emission spectra were recorded at 25 °C with Perkin-Elmer Hitachi MPF-2A fluorescence spectrophotometer operating in the ratio mode with a R 106 HTV photomultiplier tube. The fluorescence spectra were not corrected for phototube response. The infrared spectra of Ru(II)MPIX(CO) were recorded on a Perkin-Elmer 521 infrared spectrophotometer. The Ru(II)MPIX(CO) shows C–O stretches at 1928 and 1938 $cm^{-1}$ in nujol and pyridine respectively.

## Results and Discussion

The optical absorption spectrum of Ru(II)Mb(CO) in 0.1 *M* potassium phosphate buffer of pH 7 shows three visible bands at 554, 546.5 (split α bands), 519 (broad β band) and a Soret band at 398.5 nm, and is shown in Fig. 1. The separation of α bands, 7.5 nm, is sensitive to changes of pH from 8 to 4.6 [3], concentrations of urea from 4 to 8 *M* [7], and concen-

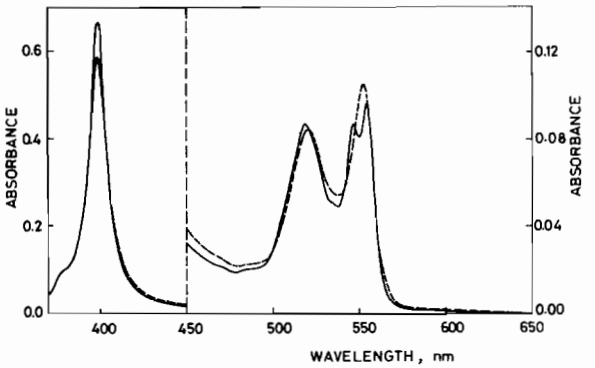


Fig. 1. Absorption spectra of Ru(II)Mb(CO) in 0.05 *M* Bis-Tris acetate buffers of pH 7 (———), and pH 4 (– – –). Concentration of Ru(II)Mb(CO) = 6.9 $\mu M$.

trated sodium chloride solution [3], and these observations are interpreted in terms of changes in tertiary structure of Ru(II)Mb(CO).

In order to confirm the 1:1 stoichiometry of binding between Ru(II)MPIX(CO) and apomyoglobin, the difference spectral titration of Ru(II)-MPIX(CO) with various concentrations of apomyoglobin against same concentration of Ru(II)MPIX(CO) was monitored at 398.5 nm. The spectral increases at 396.5 nm with various concentrations of apomyoglobin showed that one mole of apomyoglobin combined with one mole of Ru(II)MPIX(CO). In order to check further that there is a single complex of the protein, the reaction mixture of Ru(II)MPIX(CO) with apomyoglobin in 1:1 molar ratio was passed through a long Sephadex G-25 column. The fractions were identified by absorption spectroscopy. The absorption spectra of the protein fractions point to an existence of only one protein complex.

The separation between α bands in the Ru(II)Mb-(CO) complex is sensitive to variation of pH [3]. Therefore a systematic study on changes in separation of α bands in the protein complex with pH in the range 8 to 3.5 was undertaken to relate these changes with the heme-linked protonation group(s). When the separation between maxima of two α bands of the protein complex is plotted as a function of pH in the range of pH 8 to 4.6, a S-shaped curve is obtained as shown in Fig. 2. The $pK_a$ value of 5.6 (50% of total change in separation between two α bands) is obtained for the Ru(II)Mb(CO) and this value is very close to $pK_a$ value of 5.67 for the Fe(II)Mb(CO) [8, 9]. This $pK_a$ value has been assigned to the distal histidine which is, in fact, involved in the change of separation of two bands in the protein complex. The Fe(II)Mb(CO) at pH 9.36 shows a shoulder at two wavelength side of the main maxima of α band [9]. The absence of well resolved two bands in Fe(II)Mb-(CO) may be due to short life time of the excited state of porphyrin because of presence of low lying (d, d) transitions of the metal [10].

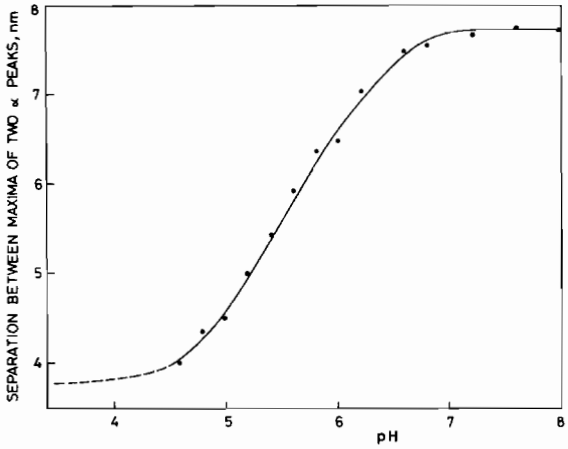


Fig. 2. Plot of separation between maxima of two α bands in the absorption spectra (.) of Ru(II)Mb(CO) as a function of pH 4.5 to 8, in 0.01 *M* KCl, 0.01 *M* potassium citrate-potassium phosphate buffers. Concentration of Ru(II)Mb(CO) = 7 $\mu M$. The samples were incubated half an hour at 25 °C before spectral measurements.

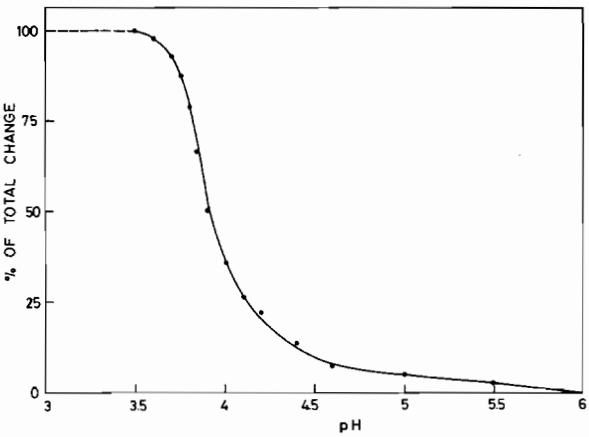


Fig. 3. The changes in absorption (.) of Ru(II)Mb(CO) at 398.5 nm as a function of pH in 0.1 *M* KCl, 0.01 *M* potassium phosphate buffers. Values at each pH are expressed as a percentage of the maximum intensity found at pH 6. The readings were taken after one hour incubation of the samples at 25 °C. Concentration of Ru(II)Mb(CO) = 3.6 $\mu M$.

The separation between two α bands in the protein complex starts closing to one peak below pH 4.5, and at pH 4 there is one α band as shown in Fig. 1. The absorbance of the Soret band in the protein complex decreases below pH 4.5 for the same concentration of the protein complex. This decrease in absorbance was used to study the acid transition of Ru(II)Mb(CO) to verify the presence of one species in the protein complex. A plot of changes in percent absorbance as a function of pH (6 to 3.5) at 398.5 showed a helix-coil transition (or cooperative transition) as shown in Fig. 3. The pH of 50 percent of total change of

absorbance is 3.9 whereas 50 percent transition point for the metmyoglobin is 4.4 [11]. This indicates that the low-spin Ru(II)Mb(CO) is more stable than high-spin sperm whale myoglobin to acid denaturation. Further the helix-coil transition of Ru(II)Mb-(CO) is sharp and indicates one species only. The acid denaturation of Ru(II)Mb(CO) is completely reversible on passing the denatured sample of protein complex at pH 3.4 through the Sephadex G-25 column equilibrated with 0.1 *M* potassium phosphate buffer of pH 7, the spectrum of the protein complex from the column comes back to original conformation at pH 7. In order to confirm that the changes in absorbance of the Soret band of Ru(II)Mb(CO) follow the protein denaturation at low pH (3.9) with exposure of the indole rings in aqueous environment [12, 13], the fluorescence spectra of Ru(II)Mb(CO) at four different pH 6, 4.6, 4.2 and 3.6 were measured at 290 nm excitation as shown in Fig. 4. The

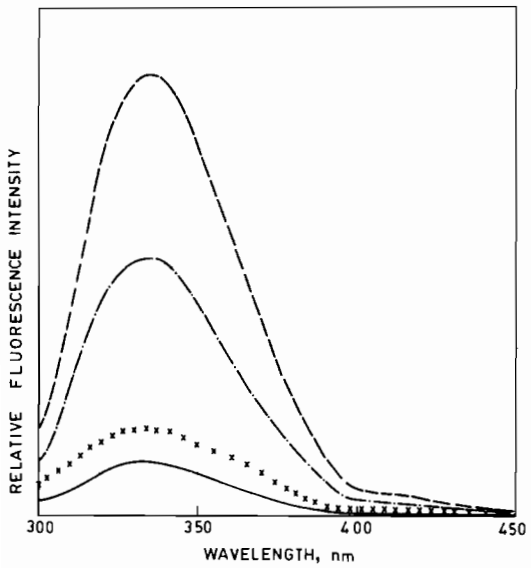


Fig. 4. Fluorescence emission spectra of Ru(II)Mb(CO) at pH 6 (———), pH 4.6 (xxxxx), pH 4.2 (–•–•–), pH 3.6 (– – –) in 0.1 *M* KCl, 0.01 *M* potassium phosphate buffers. The spectra were taken after one hour incubation of the samples at 22 °C. Excitation wavelength was at 290 nm. Concentration of Ru(II)Mb(CO) = 3.6 μM.

Ru(II)Mb(CO) has very small fluorescence maximum at pH 6. The intensity of emission maximum is several fold increased at pH 4.2 which is just before transition point. The fluorescence intensity is very much further increased at pH 3.6 which is after transition point (pH 3.9). These observations further support the above conclusion that the decrease in absorbance of the Soret band in the Ru(II)Mb(CO) complex follows unfolding of protein complex at low acid pH.

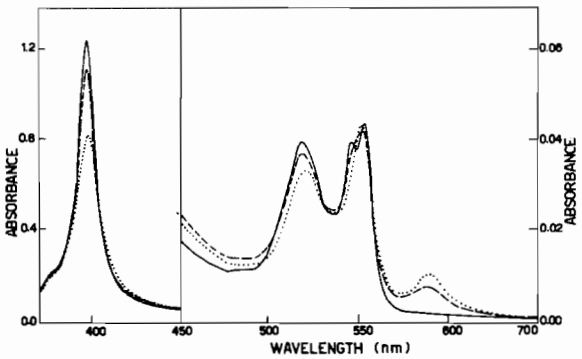


Fig. 5. Absorption spectra of Ru(II)Mb(CO) in 0 *M* (———), 1 *M* (– – –) and 3 *M* (• • •) guanidine hydrochloride in 0.1 *M* KCl, 0.01 *M* potassium phosphate buffer of pH 7. The spectra were recorded after one hour incubation of samples at 22 °C. Concentration of Ru(II)Mb(CO) = 6.4 μ*M*.

In order to check further that the separation of α bands is very sensitive to the changes in tertiary structure of the protein complex, a different type of denaturing agents namely, guanidine hydrochloride was used. This compound interacts with the protein by breaking up of hydrophobic nonpolar interactions and rupture of hydrogen bonds. These interactions are very different than the $H^+$ ions' unfolding of the protein [14]. The absorption spectra of Ru(II)-Mb(CO) in presence of 0, 1, and 3 *M* guanidine hydrochloride are shown in Fig. 5. In less than 1 *M* guanidine hydrochloride, the spectra have been affected to some extents both in the Soret and visible regions. There is substantial reductions in separation of α bands with increase in concentrations of guanidine hydrochloride. There are also reductions in absorbances of the visible and Soret bands with increase in concentrations of guanidine hydrochloride. In 1 to 2 *M* guanidine hydrochloride, there is a single α band. There are also reductions of absorbances of visible and Soret bands with increase in concentrations of guanidine hydrochloride. At still higher concentrations of 2 to 3.5 *M*, there is very substantial reductions of absorbances of the visible and Soret bands. The reductions in absorbance of the Soret band with increasing concentrations of guanidine hydrochloride were used to study the denaturation of the protein complex. A plot of percent absorbance change at the Soret maximum (398.5 nm) as a function of concentration of guanidine hydrochloride showed a helix-coil transition as shown in Fig. 6. The concentration of guanidine hydrochloride for 50 percent change of total absorbance is 2.2 whereas 50% transition point for metmyoglobin is 2.36 [15]. This shows again that there is only one species present in the protein complex. In order to verify that the changes in absorbance of Ru(II)Mb(CO) in presence of higher concentrations of guanidine hydrochloride are due to the unfolding of the protein, the fluorescence emission

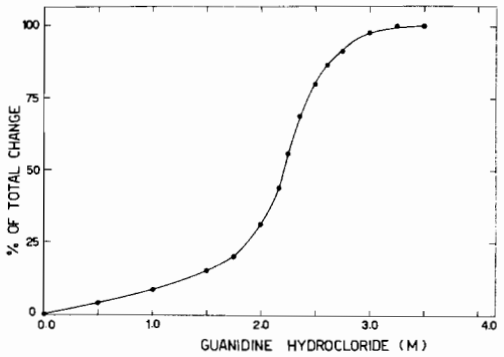


Fig. 6. The changes in absorption (.) of Ru(II)Mb(CO) at 398.5 nm as a function of molarity of guanidine hydrochloride in 0.1 *M* KCl, 0.01 *M* potassium phosphate buffer of pH 7. The spectra were taken after one hour incubation of samples at 22 °C. Concentration of Ru(II)Mb(CO) = 3.4 $\mu M$.

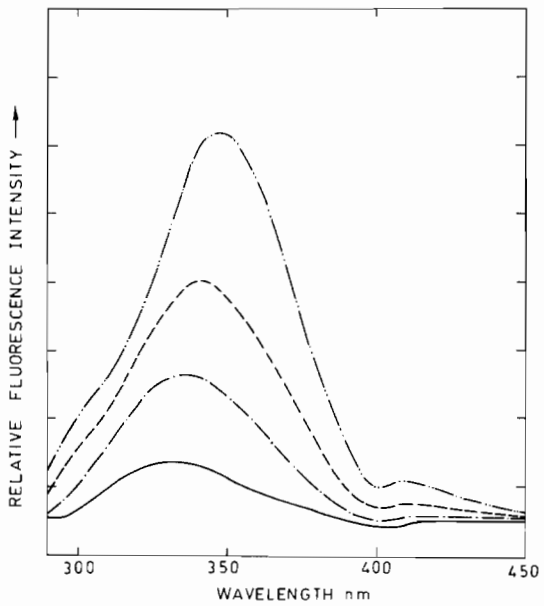


Fig. 7. Fluorescence emission spectra of Ru(II)Mb(CO) in presence of 0 *M* (———), 1 *M* (–•–•), 2 *M* (– – –) and 3 *M* (–••–••) guanidine hydrochloride in 0.1 *M* KCl, 0.01 *M* potassium phosphate buffer of pH 7. The spectra were recorded after one hour incubation of the samples at 22 °C. Excitation wavelength was at 280 nm. Concentration of Ru(II)Mb(CO) = 6.4 $\mu M$.

spectra of the protein complex in 0, 1, 2 and 3 *M* guanidine hydrochloride were measured as shown in Fig. 7. The positions of fluorescence emission maxima moves from about 332 to 348 nm on increasing the concentrations of guanidine hydrochloride from 0 to 3 *M*. The intensities of maxima are also increased with increasing concentrations of 0 to 3 *M* guanidine hydrochloride. These results can be interpreted in terms of exposure of trytophan and tyrosine to an aqueous environment due to unfolding of protein complex [12, 13]. Thus the above changes in absorbance of Ru(II)Mb(CO) at Soret region in presence of higher concentrations of guanidine hydrochloride are due to unfolding of the protein complex.

Recent X-ray data on carbon monoxide complex of horse hemoglobin have been interpreted by Perutz [1, 16] in terms linear Fe–C–O grouping making an angle with the axis normal to the heme plane in both $\alpha$- and $\beta$-chains of hemoglobin. In the protein complex, the heme pocket is designed to accomodate the oxygen which forms bent Fe–O–O angle of 136°. The distal histidine and valine are at the mouth of the pocket. The bound carbon monoxide complex of myoglobin has conformation similar to CO·hemoglobin [16]. The Ru(II)Mb(CO) is expected to have the structure similar to carbon monoxide complex of myoglobin, $\alpha$- and $\beta$-chains of hemoglobin. The bending of linear Ru–C–O grouping in the Ru(II)-Mb(CO) complex might lower the metal porphyrin symmetry from $D_{4h}$ [3, 17] due to constraint of tertiary structure of the protein. The lowering of metal porphyrin symmetry from $D_{4h}$ gives rise to four bands in the visible region. The resolution of four visible bands depends upon how much effective symmetry of metal porphyrin is lowered from $D_{4h}$. The above model explains the presence of two $\alpha$ bands and $\beta$ band broadening in the visible spectrum of protein complex because of bending of linear Ru–C–O grouping away from the axis perpendicular to the heme plane due to constraint of tertiary structure of the protein [3].

The changes in separation of two $\alpha$ bands in the visible spectrum of the protein complex with pH can be explained by using above model. The separation between two $\alpha$ bands is constant (7.5 nm) in pH range of 7 to 8, and the close ligand pocket is maintained in this pH range. The separation between two $\alpha$ bands is smaller as pH is lowered from 7 to 4.5 [3]. This decrease in separation of bands in the protein complex from 7 to 4.5 can be accounted by opening of the ligand pocket which is possibly mediated by protonation of heme linked distal histidine group. The opening of ligand pocket in the protein complex with lowering of pH allows the bending of linear Ru–C–O grouping to move towards the axis perpendicular to the porphyrin plane. The lowering of pH of the protein complex is expected to protonate the distal histidine. This positively charged histidine present at the mouth of the heme pocket in the hydrophobic region [16] tries to move away from the heme pocket towards hydrophillic region and this releases the constraint due to tertiary structure on the linear Ru–C–O grouping. Thus the absence of splitting of $\alpha$ band in the protein complex around pH 4 and below can be explained in terms of linear Ru–C–O grouping coinciding with the axis perpendicular to the porphyrin plane because of unconstraint of distal histidine. The constraint of distal

histidine is lost in the unfolded protein complex because of destruction of tertiary structure of the protein in the acid medium.

In presence of 0.25 to 0.5 *M* guanidine hydrochloride, the protein complex shows changes in $\alpha$ bands. These changes are interpreted in terms of some relaxation of constraint of distal histidine. The protein complex is substantially unfolded around 1 *M* guanidine hydrochloride and completely unfolded at 1.5 to 3.5 *M* guanidine hydrochloride. Thus the linear Ru–C–O grouping coincides with the axis perpendicular to the porphyrin plane due to absence of constraint of distal histidine in the unfolded protein complex. The separation of $\alpha$ bands of Ru(II)Mb(CO) is again very sensitive to changes of tertiary structure of the protein. The changes in tertiary structure of the protein have been mediated by guanidine hydrochloride which acts on the protein by a mechanism different from $H^+$ ions [14]. The bending of bound CO in the Ru(II)Mb(CO) at the metal atom is further supported by comparing its visible absorption spectrum with that of the dinitrosyl complex of the ruthenium(II) mesoporphyrin IX dimethyl ester [18]. This shows that two NO vibrations at 1838 and 1786 $cm^{-1}$ which are assigned to a linear Ru(II)$NO^+$ and a bent Ru(II)$NO^-$ units respectively [19]. The visible absorption spectrum of this dinitrosyl complex shows only one $\alpha$ and a $\beta$ bands even in presence of a bent Ru(II)$NO^-$ unit at nitrogen atom. Therefore the bending at the nitrogen atom in the Ru(II)$NO^-$ unit does not lower the effective symmetry of metal porphyrin from $D_{4h}$. Thus the bending of bound CO in the Ru(II)Mb(CO) is at the metal atom rather than at the carbon atom.

This work has demonstrated that the Ru(II)MPIX-(CO) is an excellent optical probe for the changes in tertiary structure of myoglobin. There is a bent heme pocket in the Ru(II)Mb(CO) in solution, which is responsible for bending of bound CO in the protein complex at the metal atom.

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