# RAMAN STUDY OF THE ACID–BASE TRANSITION OF FERRIC MYOGLOBIN; DIRECT EVIDENCE FOR THE EXISTENCE OF TWO MOLECULAR SPECIES AT ALKALINE pH

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## 1. Introduction

The acidic and alkaline forms of ferric myoglobin (Mb) which have been studied extensively [1–6], are characterized by the sixth ligand of the heme iron which is $H_2O$ in the former ($MbH_2O$) [1] and $OH^-$ in the latter (MbOH) [2]. An apparently anomalous magnetic moment of MbOH was explained earlier in terms of three unpaired electrons on the heme iron [3,4], but could also be due to the coexistence of both high ($S = 5/2$) and low spin ($S = 1/2$) forms in thermal equilibrium at alkaline pH [2]. Using Resonance Raman spectroscopy (RRS) we present here direct experimental evidence to support the existence of two distinct forms of MbOH and we identify the predominant form as the high spin species. We also show that high spin form of MbOH is different from $MbH_2O$. Previous applications of RRS have demonstrated the sensitivity of the method to the identity of the sixth ligand [7,8], and to the oxidation and spin states of the heme iron [9–11].

## 2. Materials and methods

Equine skeletal muscle myoglobin (Sigma type I) was purified on CM-cellulose column just before the measurement of the Raman spectra and its pH values were adjusted with HCl and NaOH. MbF, MbSCN, $MbN_3$, MbIm (Im; imidazole), and MbCN were obtained by adding 50-fold excesses of NaF, KSCN, $NaN_3$, imidazole, and KCN, to 0.7 mM solution of Mb, respectively.

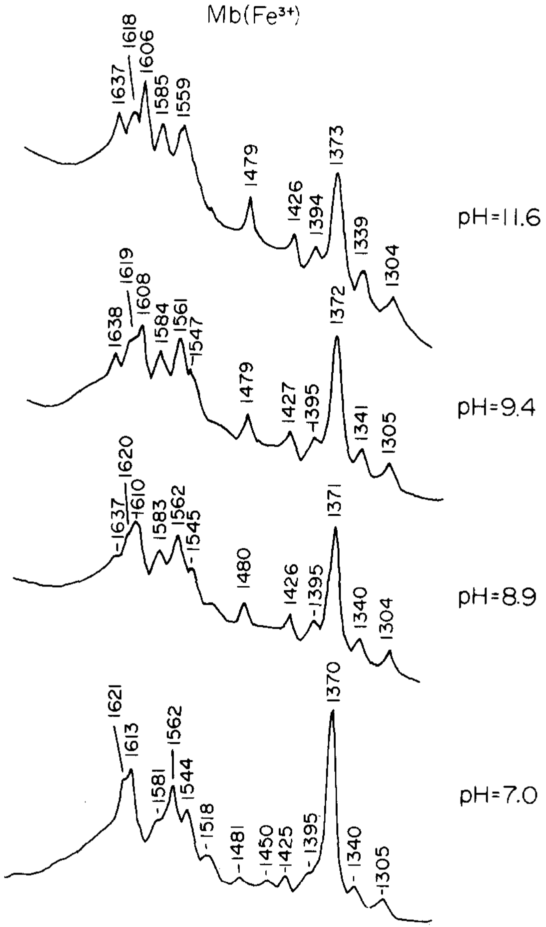

Fig.1. Resonance Raman spectra (488.0 nm excitation) of ferric myoglobin (equine) at several pH values. Instrumental conditions: power, 200 mW; time constant, 0.5 sec; sensitivity, 1000 counts full scale; rate, 0.25 sec; slit width, 5 $cm^{-1}$; scanning speed, 20 $cm^{-1}$/min.

Raman spectra were measured with the use of a JEOL-02AS Raman spectrometer and an $Ar^+$–$Kr^+$ mixed laser (Spectra physics model 164-02). Absorption spectra were recorded with a Hitachi 124 recording spectrophotometer.

## 3. Results and discussion

The RRS of Mb, as shown in fig.1, varies appreciably with pH. An intensity increase of the Raman line at 1637 cm$^{-1}$, which is the band characteristic of the ferric low spin state of hemoproteins [12] and is mainly associated with methine-bridge stretching vibrations [13,14], clearly indicates an increase in the concentration of ferric low spin molecules with increasing pH. However, the Raman lines at 1618, 1606, 1559 and 1479 cm$^{-1}$ at pH 11.6 imply simultaneously the presence of ferric high spin species [12].

The pH dependence of absorbance at 580 nm, as shown in fig.2a, indicates a p$K$ of 8.9 for the equilibrium between equine $MbH_2O$ and MbOH, in good agreement with the p$K$ for sperm whale Mb [6]. The fraction of MbOH [c] at an arbitrary pH is given therefore by the expression:

$$[c] = 1/(1 + 10^{8.9-\mathrm{pH}}) \tag{1}$$

A plot of [c] against pH is given in fig.2b. At pH 11.6, we calculate that the equilibrium mixture should contain 99.8% of Mb in the alkaline form even though Raman lines characteristic of both high and low spin states coexist in the RRS (fig.1).

Previously Yamamoto et al. [9] pointed out that

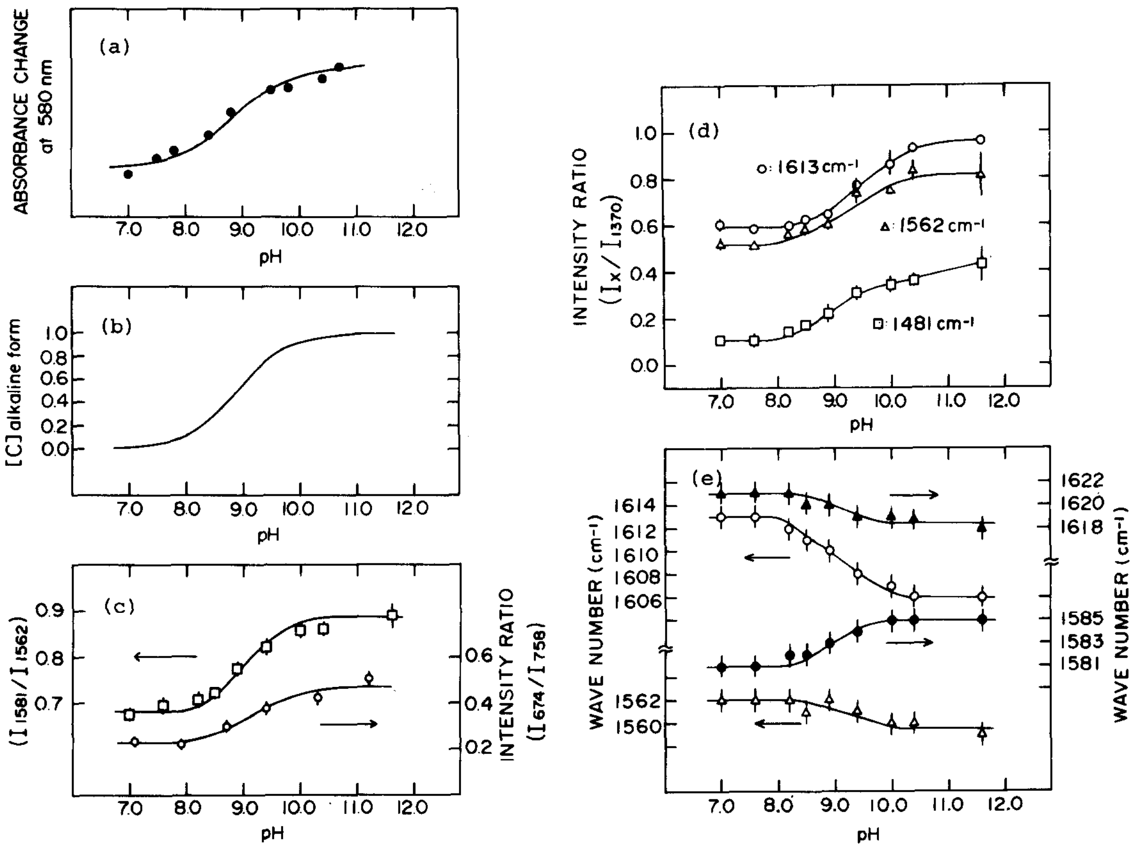

Fig.2. The pH dependence of equine ferric myoglobin. (a) Absorbance at 580 nm. (b) Concentration of the alkaline form (see text). (c) Intensity ratios of Raman lines: (□) $I$(1581 cm$^{-1}$)/$I$(1562 cm$^{-1}$); (○) $I$(674 cm$^{-1}$)/$I$(758 cm$^{-1}$) (d) Raman peak intensities relative to the 1370 cm$^{-1}$ band. (e) Frequency shifts of Raman lines.

the intensity ratio of the two Raman lines [$R_s = I(1585\ \mathrm{cm}^{-1})/I(1560\ \mathrm{cm}^{-1})$] reflects the relative concentration of low and high spin states of the heme iron. This ratio was confirmed to be larger for Mb derivatives in the ferric low spin state, such as MbCN, $MbN_3$ and MbIm, while it is smaller for ferric high spin derivatives such as MbF, MbOCN, and $MbH_2O$, when all RRS are excited by the 488.0 nm line. The pH dependence of this ratio for Mb, as shown in fig.2c, is consistent with the expected increase of the low spin species at higher pH, deduced from NMR [5,6] and magnetic susceptibility measurements [15]. Therefore it is clear that the change in $R_s$ with pH is due to the same factors governing the changes observed in n.m.r. [6] and absorption spectra.

The intensity ratio [$R_s$ (x)] at pH x can be approximated in terms of a sum of contribution from low ($R_s^l$) and high spin ($R_s^h$) species as:

$$R_s\,(x) = \alpha R_s^l + (1\text{-}\alpha)R_s^h \qquad (2)$$

where $\alpha$ is the mol fraction of low spin species. Assuming $R_s^l = 1.45$ and $R_s^h = 0.64$*, as the limiting values for purely low and high spin states, respectively, we obtain $\alpha = 0.3$ at pH 11.6 and $\alpha = 0.06$ at pH 7.0 for Mb. These results agree closely with the values deduced from magnetic susceptibility measurements, namely $\alpha = 0.31$ ($MbH_2O$) and $\alpha = 0.08$ (MbOH) [15]. This implies that 69% of Mb is in the high spin state at pH 11.6 although 99.8% of Mb is in the alkaline form.

It is emphasized here that the high spin species at pH 11.6 (MbOH, h.s.) is evidently different from the high spin species at pH 7.0 ($MbH_2O$). Figs.2d and 2e illustrate the pH dependence of selected Raman frequencies and relative intensities of Mb. The relative intensities of the Raman lines at 1613, 1562, and 1481 cm$^{-1}$, characteristic of the high spin state increase as pH is raised, in spite of the expected decrease in concentration of $MbH_2O$ (Eq.1). The frequency shifts of ring stretching vibrations shown in fig.2e, suggest a slight difference between MbOH, h.s. and $MbH_2O$ in bond strength and/or structure of the heme groups. The existence of two kinds of high spin species in the alkaline solution was not taken into consideration in the recent analysis of the pH dependence of the n.m.r. hyperfine shift of Mb [5,6].

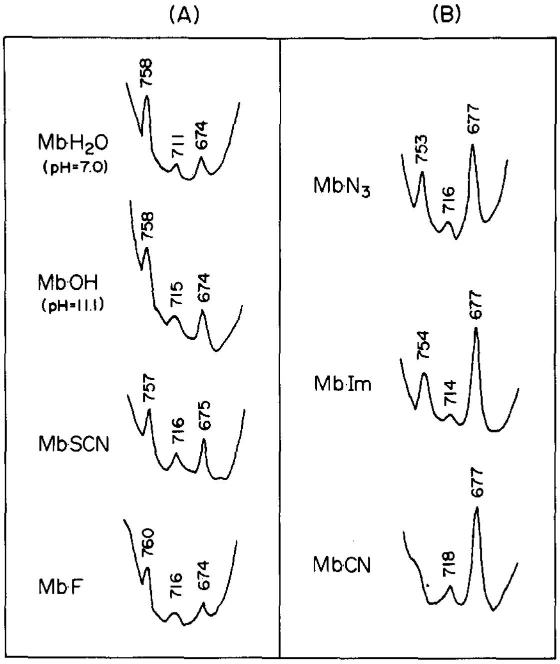

Fig.3. Resonance Raman spectra in the region of 800–600 cm$^{-1}$ of several high spin (A) and low spin (B) derivatives of ferric myoglobin.

It was also found in the present study that the intensity ratio of the Raman lines in the lower frequency region [$R = I(674\ \mathrm{cm}^{-1})/I(758\ \mathrm{cm}^{-1})$] is spin state dependent. Raman spectra in this frequency region for various derivatives of Mb are shown in fig.3, where (A) and (B) include those derivatives known to be mainly in the high and low spin states, respectively. This intensity ratio for Mb varies also with pH as illustrated in fig.2c, yielding larger values as the fraction of low spin species is increased. Since this ratio is small in square planar metallooctaethylporphyrin [M(OEP)] and low spin Fe(OEP) but large for high spin Fe(OEP), irrespective of the axial ligand [16], it may also be dependent upon the planarity of the heme group.

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* These values were derived from RRS observed for MbF ($R_s^h$) and MbCN ($R_s^l$), respectively. One may note that these values are applicable only to the Raman spectra excited by the 488.0 nm line because of resonance effect.

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