Axial Coordination of Ferric Aplysia Myoglobin*

Denis L. Rousseau and Yuan-chin Ching
From the AT&T Bell Laboratories, Murray Hill, New Jersey 07974

Maurizio Brunori
From the Dipartimento di Biochimica, Università di Roma, La Sapienza, Piazzale Aldo Moro, 00100 Roma, Italy

Giorgio M. Giacometti
From the Dipartimento di Biologia, Via Trieste 75, 35100 Padova, Italy

Resonance Raman spectra of ferric Aplysia myoglobin in the ligand-free and the azide-bound forms have been studied over a wide pH range to determine the coordination states of the heme iron atom. In the hydroxide form at high pH (~9) the iron is six-coordinate and is in a high/low spin equilibrium. As the pH is lowered below the acid/alkaline transition (pH = 7.5), the heme becomes five-coordinate. When the pH is lowered even further no other changes in the resonance Raman spectrum are detected; thus, the heme remains five-coordinate down to pH 4, the lowest value studied. For ferric azide-bound Aplysia myoglobin, the iron is six-coordinate in a high/low spin equilibrium at all pH values (4.8–9). These data indicate (i) that the unusual reactivity toward azide previously observed at neutral pH is indeed related to the absence of a coordinated water molecule, and (ii) that causes other than the heme coordination are responsible for the spectral differences and the ligand-binding kinetics differences observed below pH 6.

Although hemoglobins and myoglobins have been extensively studied for many years, the control of the reactivity of the heme by the amino acids forming its pocket is still not completely understood. Aplysia myoglobin is an important hemoprotein to study in this regard because, unlike most other hemoglobins and myoglobins, it lacks a distal histidine, instead having a valine (Val-63(E7)) at this important position (1, 2). Studies of the optical spectrum and the ligand-binding properties of the ferric form of this protein have revealed a very strong dependence on pH (3), which in part is a consequence of the absence of histidine at the distal position. Based on functional and spectroscopic measurements, Giacometti et al. (4, 5) inferred that there are several pH-dependent coordination changes which could account for the variation in the kinetics of azide binding. They stated that for pH >7.5 the iron atom of the ferric form of Aplysia myoglobin is six-coordinate, with an OH⁻ ion as the sixth axial ligand; at pH values between 6.0 and 7.0, the iron becomes five-coordinate because water is not bound to the metal; and at pH <5 it is four-coordinate, due to rupture of the proximal histidine-iron bond.

Resonance Raman scattering has been applied to the study of hemoproteins extensively over the past several years (see Ref. 6). Furthermore, the spectra of many porphyrin model compounds have been reported and used to establish empirical markers of heme characteristics (7–10). Lines sensitive to the spin state of the central iron atom, sensitive to the size of the porphyrin macrocycle core, sensitive to the iron coordination, and sensitive to the σ-electron density in the heme have been reported. Therefore, the resonance Raman spectrum of hemoproteins is a powerful tool to determine the spin state and the coordination state of the iron atom. To test the hypothesis that a series of changes in iron coordination occur in ferric Aplysia myoglobin, we have obtained the resonance Raman spectra of the met and met-azide derivatives of this protein at several different pH values.

MATERIALS AND METHODS

Aplysia myoglobin was prepared from the buccal muscle of the mollusc Aplysia limacina as reported by Rossi Fanelli and Antonini (11). The purified protein was stored under liquid nitrogen until ready for use. Horse heart myoglobin was purchased from Sigma, solubilized in buffer, and filtered just before obtaining the Raman data. The protein concentration used to obtain the resonance Raman spectra was approximately 100 μM. For pH 4 and 6, sodium acetate buffers (90 mM) were used; for pH 7.5 and 11, sodium phosphate buffers (180 and 90 mM, respectively) were used; and at pH 9, the buffer was sodium borate (90 mM). The Raman spectra were obtained on previously described Raman difference instrumentation (12).

RESULTS

In Fig. 1 we present the high frequency region of the resonance Raman spectra of Aplysia metmyoglobin, in the absence of exogenous ligands, at four different pH values. The frequencies of the major peaks are labeled. Some very clear changes occur in the 1470–1525 cm⁻¹ region as well as in the 1550–1600 cm⁻¹ region. At high pH a line is found at 1478 cm⁻¹ which is replaced as the pH is lowered by a line at 1494 cm⁻¹. In all of the data lines are present at ~1563 and ~1585 cm⁻¹. At high pH these two lines have approximately equal intensity but the intensity changes significantly as the pH is lowered. At pH 7.5 and lower the intensity remains fixed with the component at 1563 cm⁻¹ being much stronger than that at 1588 cm⁻¹. It also should be stressed that a comparison of the spectra at pH 3.9 and 5.9 shows essentially no differences, in spite of the fact that there are clear differences in the optical absorption spectrum as previously reported (4).

For comparison, we have also obtained data on the met form of horse heart myoglobin. As shown in Fig. 2, in the 1550–1600 cm⁻¹ region the resonance Raman spectrum of the horse protein behaves similarly to that of the Aplysia myoglobin. At the highest pH (pH 11) the lines at 1562 and 1584

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**FIG. 1.** Resonance Raman spectra of ferric Aplysia myoglobin obtained at several different pH values. The laser excitation wavelength was 413.1 nm at ~50 milliwatts of power. The spectral slit width was 5 cm⁻¹.

**FIG. 2.** Resonance Raman spectra of ferric horse heart myoglobin obtained at several different pH values. Same conditions as described in the legend to Fig. 1.

**FIG. 3.** Resonance Raman spectra of azide-bound ferric Aplysia myoglobin at two different pH values. Same conditions as described in the legend to Fig. 1.

The absorption spectrum of the azide adduct of ferric Aplysia myoglobin has been shown (4) to change as a function of pH with a pK of 6.1. Thus, we have also obtained the resonance Raman spectrum of the azide-bound form of the protein at two different pH values (4.8 and 9). As is apparent from the data in Fig. 3, no differences are detectable between these two spectra. At both of the pH values there are two lines in the 1475-1520 cm⁻¹ region, one at 1490 and one at 1508 cm⁻¹. In the 1550-1600 cm⁻¹ region, the 1564 cm⁻¹ line is weaker than that at 1585 cm⁻¹.

**DISCUSSION**

Resonance Raman spectra in the high frequency region of the porphyrin macrocycle have been shown to be very sensitive indicators of the iron coordination and spin state (7–10, 13–15). The 1475-1520 cm⁻¹ region has been assigned as originating from $v_3$, a mode that is sensitive to both the coordination and the spin of the iron. This empirical sensitivity results from several influences: π-back donation, the porphyrin core size, and the heme doming. Each of these factors, which affect the frequencies of many vibrational modes, changes as a function of axial coordination and spin state (9, 10). Consequently, for five-coordinate high spin, six-coordination high spin, and six-coordinate low spin ferric heme groups, $v_3$ appears at 1490–1500, 1475–1485, and 1500–1510 cm⁻¹, respectively. Because of this sensitivity, the frequencies and intensities of the modes in this region have been used in other heme-containing proteins to assess the spin equilibrium and the coordination state of the iron. In addition, the region between 1550 and 1600 cm⁻¹ ($v_2$) has been shown to be very sensitive to the ferric iron spin state. A mode at ~1565 cm⁻¹ is characteristic of high spin and one at ~1585 cm⁻¹ is characteristic of low spin.

At high pH, $v_2$ in Aplysia metmyoglobin has components at 1478 and at 1506 cm⁻¹. These frequencies are consistent with six-coordinated high spin and six-coordinated low spin species, respectively. In horse heart myoglobin the same lines are detected in the spectra at high pH. In the case of Aplysia metmyoglobin when the pH is lowered to 5.9, a clear change takes place: the six-coordinated high spin marker line disappears and a broad line appears in the spectrum at 1495 cm⁻¹ indicating the presence of a five-coordinate high spin species.
In horse heart myoglobin there is no evidence for a five-coordinate species at pH 5.9 (where the line is at 1483 cm\(^{-1}\); see Fig. 2). Upon further lowering of the pH from 5.9 to 3.9, the spectrum of the *Aplysia* metmyoglobin is little changed, remaining characteristic of a five-coordinated species. The \(v_2\) region of *Aplysia* metmyoglobin is also consistent with this analysis. At high pH the equal intensity of the lines at 1562 and 1582 cm\(^{-1}\) indicates a high spin/low spin mixture. We presume (in accord with optical spectroscopy) that this is due to the presence of an OH-bound species which is known to exist in a spin equilibrium (16, 17). As the pH is lowered, the iron becomes predominantly high spin, fully consistent with five-coordination.

In *Aplysia* metmyoglobin the changes in the resonance Raman spectra upon going from pH 5.9 to 3.9 are very small, although there is a slight increase in intensity at 1519 cm\(^{-1}\). If a change from five- to four-coordination did occur would such a spectrum be anticipated? Although there are no reported spectra of ferric four-coordinate model compounds from a large amount of literature that is available on other model compounds, the anticipated properties of such a species may be considered. First, whether or not the iron atom moves out of the heme plane is known to be a consequence of steric hinderance from the coordinated axial ligands (18). The iron d-orbital population does not give rise to any out-of-plane forces. Thus in a four-coordinate species, a planar heme is expected. Second, the frequency of \(v_3\) correlates with the porphyrin core size (9, 10). This size is large for planar hemes with occupation of the \(d_{x^2-y^2}\) orbital which points along the iron-nitrogen (pyrrole) bond; but it is small when the \(d_{x^2-y^2}\) orbital is unoccupied. Consequently, for a high spin planar configuration of a four-coordinate species with an occupied \(d_{x^2-y^2}\) orbital, \(v_3\) would be expected to have a frequency similar to that of six-coordinate high spin hemes (\(-1480\) cm\(^{-1}\)). For intermediate or low spin configurations of a four-coordinate species with an unoccupied \(d_{x^2-y^2}\) orbital, the frequency would lie in the range of six-coordinate low spin compounds (\(-1505\) cm\(^{-1}\)). However, this coordination-sensitive region of the spectrum in *Aplysia* metmyoglobin at pH 3.9 is quite characteristic of a five-coordinate high spin (out-of-plane iron atom) configuration (\(v_2 = 1495\) cm\(^{-1}\)), thus not supporting the formation of a four-coordinate species.

From inspection of the coordination-sensitive region (\(v_2\)) in ferric horse heart myoglobin it is clear that this protein is six-coordinate from pH 6 to 11. At low pH (pH 4) a contribution from a five-coordinated high spin species is present in the spectrum. From the \(v_2\) region the pH dependence of the changes in spin are similar to those of *Aplysia* metmyoglobin although the transition to low spin is shifted by about 2 pH units, i.e. the spectrum of the horse myoglobin at pH 11, with roughly equal contributions from both high and low spin components, is similar to that of *Aplysia* myoglobin at pH 9. Thus, in the case of *Aplysia* metmyoglobin a lower pH is sufficient to convert from high to low spin as compared to horse myoglobin (in agreement with a lower \(pK_a\) for the acid-alkaline transition in *Aplysia* metmyoglobin (19)). At the other pH extremes, the horse heart myoglobin must be brought all the way down to pH 4 in order to detect a five-coordinated species, which is detected already at pH 7.5 in *Aplysia* metmyoglobin.

The azide-bound protein has a resonance Raman spectrum which is invariant with changes in pH over the range examined in this study. For both extremes of pH only two lines are detected in the coordination-sensitive region. These lines are at \(-1480\) and \(-1507\) cm\(^{-1}\), frequencies characteristic of six-coordinated high and low spin species, respectively. The relative intensities of the two lines in the \(v_2\) region of the azide-bound *Aplysia* myoglobin indicate that the spin equilibrium is poised toward low spin. We conclude that when azide is bound to the protein, it behaves in a way similar to that of hemoglobin or myoglobin in which azide also confers a spin equilibrium favoring the low spin form (16, 17).

Within the reliability and limitations of the Raman marker lines, the results of these experiments are clear. At high pH *Aplysia* metmyoglobin has the same coordination as does horse heart metmyoglobin, i.e. it is six-coordinate in high spin/low spin mixture. When the pH is lowered to six, a change in the coordination state of *Aplysia* metmyoglobin occurs, yielding a spectrum characteristic of a five-coordinated high spin species. Upon further reduction of pH to 4, the spectrum of the *Aplysia* metmyoglobin is unchanged, remaining characteristic of a five-coordinate high spin structure. Upon binding of azide, the protein becomes six-coordinate over the pH 4-9 range. Furthermore, although azide confers a spin equilibrium, there is no apparent change in the position of this equilibrium as a function of pH.

The change in the spectrum of *Aplysia* metmyoglobin from six- (at pH 9) to five-coordinate near pH 6 is consistent with the change in ligand-binding kinetics and thereby confirms the conclusions drawn by Giacometti et al. (3-5). They have shown that there is a change in the exogenous ligand binding rate with a \(pK\) of 7.5. Above this pH exogenous ligands must replace a sixth ligand on the heme iron (an \(\text{OH}^-\) ion) prior to binding. Our data confirm that above the \(pK\) of the acid/alkaline transition (7.5), the heme of ferric *Aplysia* myoglobin is six-coordinate and below the transition it becomes five-coordinate. The absence of a coordinated water molecule, consistent with crystallographic observations (2, 20), may be related to the absence of a distal histidine, which is replaced by Val-63(E7). The importance of hydrogen bonding from the distal histidine on the stabilization of exogenous ligands has also been noted in a study of model compounds by Mitchell et al. (21). We expect that other hemeproteins lacking a distal histidine may also fail to show a coordinated \(\text{H}_2\text{O}\) molecule in the high spin form (at pH 6-7) and have an out-of-plane iron geometry. Accordingly, the transition from five- to six-coordination is not observed in horse myoglobin since in this protein, as well as in other mammalian myoglobins, a water molecule is bound at the sixth coordination position below the \(pK\) of the acid/alkaline transition which is approximately 9 in horse and sperm whale myoglobin (19).

In the acidic region, we find no change in the structural properties of the heme of ferric *Aplysia* myoglobin as the pH is lowered from 6 to 4, thereby passing through the \(pK\) values which Giacometti et al. (4) proposed to be associated with a conversion from five- to four-coordination. Likewise, and in spite of the fact that changes in the absorption spectrum were found in the azide-bound protein with a \(pK\) of 5.8, we found no structural changes in the resonance Raman spectrum for this derivative. The present data do not confirm a proton-induced breakage of the iron-proximal histidine bond in either the ligand-free or azide-bound form of ferric *Aplysia* myoglobin.

Any molecular description of the pH dependence of *Aplysia* metmyoglobin must be able to account for the origin of the pH-induced changes observed (4) in the optical absorption spectra of *Aplysia* metmyoglobin below pH 6 in contrast to the absence of any changes detectable in the resonance Raman spectra over the pH range 4-6. The resonance Raman data not only rule out changes in coordination state, but also other changes. For example, changes in the heme conjugation due to reorientation of the porphyrin ring substituents would be
expected to change the frequencies of the heme vibrational modes. Similarly, changes in porphyrin structure such as doming would also cause frequency shifts in vibrational modes. Thus, since we can exclude coordination and heme structural changes, in order to account for the optical, Raman, and kinetic data at low pH in \textit{Aplysia} metmyoglobin, changes in the structure of the heme pocket, linked to protonation (or deprotonation) of amino acid residues, may be proposed. Examination of the three-dimensional structure (2, 20) of \textit{Aplysia} myoglobin (see “Discussion” in Ref. 4) suggests some specific groups which are near the heme and may influence its spectroscopic and kinetic properties. These include protonation of Asp-62(E6) which is adjacent to the distal position (E7) and as such could control ligand accessibility to the heme; and protonation of 6-propionate on the heme. Recent NMR data indicate that this carboxyl becomes protonated in the cyanomet derivative of \textit{Aplysia} myoglobin in the 4–5 pH range. Additional experiments to explore further the origin of the binding rate differences and the qualitative differences in behavior between the absorption spectra and the resonance Raman spectra will be useful.

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\textbf{REFERENCES}