Variations of Electrostatic Interactions in Myoglobin Probed by UVRR Spectroscopy. Effect of Iron Ligand and pH on Tryptophan and Tyrosine Vibrations

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ABSTRACT: The present work investigates the variations of electrostatic interactions within the myoglobin molecule associated with azide heme binding and pH variations. Far ultraviolet (223 nm) resonance Raman spectroscopy of the tryptophan and tyrosine residues, along with acid–base titration measurements, have been used to monitor variations in the protein matrix. With previously determined mode assignments, it is shown that the Trp and Tyr residues of the globin moiety are influenced by the charge spatial distribution. Upon ligand binding or under various pH conditions, the polar interactions inside the protein appear to be modulated by the electric field generated by the charge array. It is concluded that the binding site properties of myoglobin can be modulated by the charge spatial distribution within the protein, even in the absence of measurable conformational changes of the bulk.

Myoglobin is a monomeric hemoprotein whose crystallization structure depends neither on the iron oxidation state (II or III) nor on the iron ligand nature (Nobbs et al., 1966). Only the heme macrocycle of the protein in solution seems to exhibit slight changes subsequent to ligand binding (Scheler et al., 1957; Rousseau et al., 1989). In fact, no direct information is available at present on the inner behavior of this globular protein in solution. Thus, the goal of the present study was to explore whether or not the iron-ligand nature alters the interactions inside the matrix of the protein in solution, whereas the crystallographic structure remains unaltered.

Proteins are such complex systems that the investigation of their properties requires different techniques, each of them revealing only particular features. X-ray crystallographic studies of the myoglobin molecule provided the determination of the average relative positions of every amino acid residue and showed the great stability of the protein conformation (Takano, 1977; Evans & Brayer, 1990). NMR data allowed the determination of the pK values of some histidyl residues and of the terminal amino group of myoglobin (Hayes et al., 1975; Botelho et al., 1978). Ultraviolet resonance Raman spectroscopy (UVRR)[1] of the aromatic residues should bring relevant complementary informations to X-ray and NMR techniques, in probing the modulation of interactions between residues whose identification had come from the latter techniques. Indeed, owing to their polarizability properties, aromatic amino acids are sensitive to any variation of the electrostatic fields (possibly due to either local reorganization or transmission throughout the whole molecule) inside the protein matrix, and consequently, their vibrational modes can be used to monitor such variations.

As shown in a previous paper, the UVRR spectra of the hemoglobin molecule excited at 223.6 nm are dominated by the vibrational modes of the tryptophanyl and tyrosyl residues (El Antri et al., 1989). Thus, the effects of the iron-ligand nature on the apoprotein moiety have been studied by using the vibrational modes of Trp and Tyr of the azido- and aquometmyoglobin derivatives. Ferrimyoglobin was preferred to the ferrous form since it prevents changes of the iron oxidation state under different experimental conditions. In addition, to understand the role played by the ionization of the amino acids residues on the intramolecular interactions in myoglobins, we have also investigated the vibrational modes of Trp and Tyr in relation to the acid–base titration of the protein.

Materials and Methods

Samples. Metmyoglobin solutions (Mb^+) were prepared from lyophilized horse heart metmyoglobin (Sigma M-1882), followed by a sample centrifugation (18 000 rpm, 10 min, 4 °C). The azido form of metmyoglobin ($Mb^+N_3^-$) was obtained by the addition of a known amount of sodium azide powder (Prolabo) to the protein solution. In the present study, the final protein and sodium azide concentrations were ca. 0.4–0.5 mM and 11 mM, respectively, to allow an almost complete iron-ligand saturation.

For acid–base titration of the protein, the samples were used without further salt addition. The initial pH of the protein solutions (0.5 mM) was equal to 7. For UVRR measurements, the $Mb^+N_3^-$ and Mb^+H_2O metmyoglobin derivatives (0.4 mM) were dialyzed against 20 mM $Na/K/K_2$ phosphate buffer, at pH values 5.5, 7.0, and 8.5. The protein solutions were allowed to settle for 48 h at 5 °C, the pH values were then adjusted when necessary. The protein solutions were centrifuged before each experimental measurement.

Assays. Protein concentrations were determined spectroscopically by using a model 219 Varian Cary spectrophotometer equipped with a thermostated cell holder to measure the optical density of the metmyoglobin solutions at 5 °C. Metmyoglobin final concentration was determined by absor-

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[1] Abbreviations: Mb, myoglobin; UVRR, ultraviolet resonance Raman spectroscopy.

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bance at 540 nm (ϵ = 11 300 M^{-1} cm^{-1}) after potassium cyanide (Merck) addition (Scheler et al., 1957). Azidometmyoglobin concentration was determined directly by using at 540 nm an extinction coefficient of ϵ = 11 200 M^{-1} cm^{-1} (Scheler et al., 1957).

Acid–Base Titration. The potentiometric H^+ titrations of metmyoglobin at 5 °C were carried out by adding aliquots of metmyoglobin solutions (0.5 mM) in separate vials containing various amounts of 50 mM HCl or NaOH. These titrating reagents were prepared by diluting standard solutions purchased from Prolabo (1 N NaOH) or Merck (1 N HCl) into freshly boiled water in order to remove all traces of carbon dioxide. In order to allow the complete proton equilibration between protein and solvent, the pH measurements were performed 48 h after the addition of titrating aliquots. During this delay, the sealed samples were kept at 5 °C and submitted to gentle stirring. The pH measurements were made with a combined electrode (GK2321C Radiometer) under a smooth moistened nitrogen flux, by using a PHM62 pH meter (Radiometer). The pH values were also recorded on an *XY* recorder to check the complete stability of the signal. The accuracy of every measurement was within 1.5%. The titration curves were obtained by symmetrical smoothing of about 80 experimental points.

Ultraviolet Raman Measurements. The Raman excitation was made by a Datachrom 5000 (Quantel Corp.) dye laser that was pumped by a Nd–YAG laser (repetition rate 10 Hz, pulse duration 20 ns). The dye used was Rhodamine 590 perchlorate (Exciton Co.). The 223.6-nm excitation was obtained by frequency doubling the emission at 565 nm of the dye, through a nonlinear crystal, and mixing the 282.5-nm line with the 1.06-μm fundamental of the Nd-YAG laser through a second nonlinear crystal. The Raman analogue signal obtained for each laser pulse through a Jobin-Yvon HG2S double monochromator (working in the second order of the gratings) and a solar-blind photomultiplier was digitized in an AD converter and registered in a computer. For each pulse of excitation, a ratio of the Raman signal to the excitation energy was obtained by using a beam splitter and a monitoring photomultiplier. The sample was contained in a quartz cuvette that was placed in a thermostated cell holder. All measurements were performed at 5 °C. The sample was continuously stirred with a little magnet to avoid local photodecomposition during the acquisition. The samples were also checked by UV–visible absorption spectroscopy for laser-induced damage after the Raman spectra collection; no evidence for laser damage was observed. Each Raman spectrum derives from the accumulation of five computerized scans and was normalized on the large water O–H stretching band located around 3400 cm^{-1}. The contributions of water, salts, or azide to the Raman spectra were removed by subtraction of the appropriate spectra.

UVRR Spectral Assignments. With the 223–nm wavelength excitation, the fractional absorption arising from the aromatic side chains is intense; this is particularly true for the tryptophanyl residues, which have their $B_{a,b}$ electronic transition located at 218 nm (ϵ = 34 000 M^{-1} cm^{-1}) (Platt, 1949; Hirakawa et al., 1978), while for the tyrosyl residues (below the pK_a of 10.1) the 223.6-nm excitation corresponds to the L_a transition located at 222 nm (ϵ = 7000 M^{-1} cm^{-1}). The 223 nm wavelength also corresponds to the red limb of the L_a transition at 207 nm (ϵ = 7000 M^{-1} cm^{-1}) of the phenylalanyl residues.

It is known that the first π–π^* ($B_{a,b}$) transition of benzene is strongly allowed: the same holds for Trp, although its ($B_{a,b}$) transition is largely red-shifted from the benzene one. In resonance with allowed electronic transitions, the dominant Raman scattering factor is the so-called A (Franck–Condon) term (Tang & Albrecht, 1970): some totally symmetric modes of vibration are resonance enhanced, roughly proportionally to the square of the allowed electronic transition moment. In contrast, the L_a and L_b are two symmetry-forbidden transitions for the benzene molecule. However, in substituted benzenes like Phe and Tyr, the symmetry is lowered, which introduces some allowed character into the symmetry-forbidden transitions. In addition, the so-called B Raman scattering term (vibronic scattering) may become large if the weakly allowed resonant state is vibronically coupled to a nearby strongly allowed state. This is the case for the Tyr and Phe molecules: some vibronic modes are active in coupling the electronic states, the L_a transition couples to the $B_{a,b}$ transtion via the ν_{8a} and ν_{9a} normal coordinates.

The histidyl residues of the myoglobin molecule do not contribute to the 223-nm RR spectra: the molar absorptivity is 5000 for His at its 207-nm maximum, thus the scattering cross section for Trp is more than one order of magnitude higher than that of His for the 223-nm excitation. Caswell and Spiro (1986) already mentioned that the utility of the His RR spectrum for protein studies is severely limited by low sensitivity (see the molar enhancement factors of the aromatic amino acids in Table IV of their paper).

Thus, the 223-nm RR spectra of the myoglobin should be maintained dominated by the Trp bands, in good agreement with the data of Johnson et al. (1984) and El Antri et al. (1989). There are yet some important contributions of the tyrosyl residues (ν_{8a} line) due to the coupling between the Tyr L_a and $B_{a,b}$ transitions (Rava & Spiro, 1985). The participation of phenylalanyl residues to the myoglobin spectra will be much weaker, since at 223 nm the resonance conditions through the L_a band are much less favorable for Phe than they are for Tyr. In fact, we used a least-squares fit method to have a rough estimation of the aromatic amino acid contributions to the myoglobin spectra, taking as references the spectra of Trp, Tyr, and Phe, isolated in aqueous solutions (pH 7) and obtained with the same excitation: the Phe contribution was found in all cases very weak. Although it is unlikely that the RR participation of the aromatic amino acids to the protein spectrum is a mere combination of the spectra of the isolated constitutive residues, this estimation gives an idea of such relative intensity contributions. The Phe ν_{8a} participation to the protein spectrum is, at most, 10% of that of the Tyr ν_{8a} line intensity. The preceding considerations will help us to assign the myoglobin RR spectral features.

Results

Metmyoglobin UV RR Spectra. Figure 1 shows the UVRR spectra of the Mb^+H_2O obtained at pH 5.5 and 7.0. The UVRR spectra of the azidometmyoglobin derivative was also observed at pHs ranging from 5.5 to 8.5 (Figure 2).

At pH 5.5, the native aquometmyoglobin has a water molecule as a Fe^{3+} sixth axial ligand; since the heme acid transition has a pK value of 9.0 (George & Hanania, 1952), the H_2O molecule still represents 99% of the iron ligand at pH 7.0. Hence one can consider that the hydroxy iron-ligand (1%) is negligible at the latter pH value. It is not possible to explore a wider pH range for this derivative, since there would be a competition between a pure pH effect and a ligand effect ($Mb^+H_2O \leftrightarrow Mb^+OH^-$) at pH higher than 7.0.

Horse myoglobin contains two Trp (at 7 and 14 positions) and two Tyr residues (at 103 and 146 positions). The band frequencies and assignments of the myoglobin spectra from

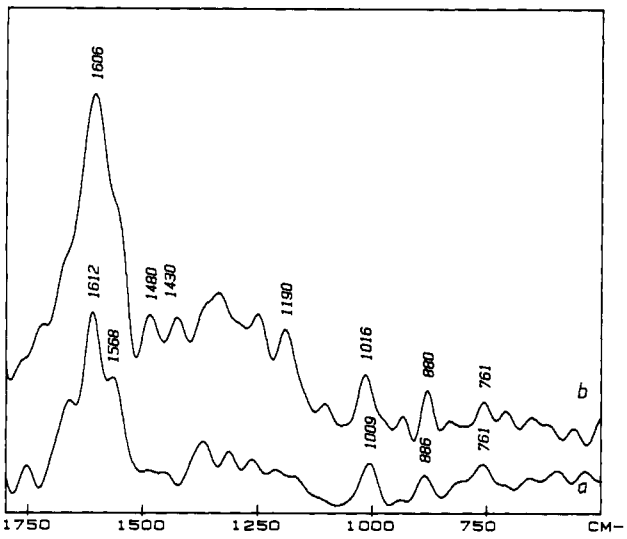

FIGURE 1: Evolution with pH of the UVRR spectrum of horse heart aquometmyoglobin at 5 °C in 20 mM phosphate buffer; (a) pH 5.5; (b) pH 7.0. The spectra were vertically shifted for easier comparison between the different samples.

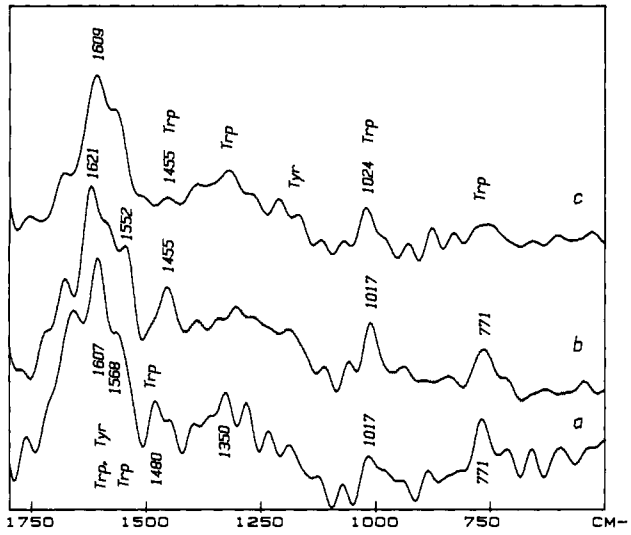

FIGURE 2: pH dependence of the UVRR spectrum of horse heart azidometmyoglobin at 5 °C in 20 mM phosphate buffer; (a) pH 5.5; (b) pH 7.0; (c) pH 8.5. The spectra were vertically shifted for easier comparison between the different samples.

Figures 1 and 2 are displayed in Table I. The Trp bands are found at ≈760, ≈880, 1010, ≈1350 cm^{-1}, and an important shoulder is seen at 1560 cm^{-1}; they were found at 760, 875, 1009, 1349, and 1552 cm^{-1}, respectively, in the RR spectrum of a Trp aqueous solution (pH 8.5) excited at the same wavelength (El Antri et al., 1989). A doublet of Trp lines in the 1455–1490-cm^{-1} region, which likely corresponds to the ν_{19b} and ν_{19a} vibrations (with a possible coupling to the ≈ 1450-cm^{-1} CH_2 bending mode), seems to be very sensitive to the nature of the heme ligand and to pH conditions. The most prominent band of the spectra is found in the 1605–1612-cm^{-1} range: it corresponds to the ν_{8a} vibration of Tyr (it is also the most important band at 1611 cm^{-1} in the spectrum of Tyr aqueous solution at pH 8.5), with a possible contributions of the Trp ν_{8a} vibration at 1619 cm^{-1}. Another contribution of the Tyr residues is seen in the 1170–1180-cm^{-1} range, which likely corresponds to the ν_{9a} Tyr mode of vibration. At this point, it is to be noted that the so-called "tyrosine Fermi doublet" in the 830–850-cm^{-1} region is barely seen in the spectra of Figures 1 and 2; the band intensity ratio of this

Table I: Myoglobin Vibrational Frequencies Assigned to the Main Modes of Vibration of Trp and Tyr Residues (See Figures 1 and 2)[a]

myoglobin freq (cm^{-1})	amino acid assign.	vib. assign.	description
≈760	Trp		sym. benzene/pyrrole in-phase breath. vib.
≈880	Trp	ν_{10a}	indole ring breath. + NH bend
≈1010	Trp		benzene/pyrrole out-of-phase breath. vib.
≈1175	Tyr	ν_{9a}	in plane CH bend, C_6H_5–C stretch
≈1350	Trp	ν_{14}	pyrrole ring vib.
≈1455	Trp	ν_{19b}	
1490	Trp	ν_{19a}	
1560	Trp	ν_{8a}	sym. phenyl ring mode
≈1605–1612	Tyr	ν_{8a}	in plane ring stretch
1619	Trp	ν_{8a}	phenyl ring vib.

[a] λ_{ex} = 223.4 nm. Assignments derive from Dollish et al. (1974).

Fermi doublet is often used, in classical Raman spectroscopy of non-hemic proteins with visible excitation wavelength, as a marker of Tyr hydroxyl group interactions with other parts of the molecule (or solvent) through H bonding. Unfortunately, this cannot be done here since this doublet does not show up with the 223.6-nm excitation, in agreement with what was already mentioned by Rava and Spiro (1985) when they used a 218-nm excitation.

Let us focus on the spectral changes observed in the myoglobin molecule spectra in varying the heme-ligand nature and the pH conditions.

Iron-Ligand Nature Effect. With an excitation wavelength lying in the visible region, heme RR spectroscopy of the myoglobin molecule in solution has been found to show slight changes on the heme macrocycle upon iron–azide binding (Scheler et al., 1957; Rousseau et al., 1989). Here, with UV excitation (UVRR) of the protein, we show that the neutralization of the positive charge at the heme iron (Friend et al., 1980) is also sensed throughout the whole protein. Figures 1a and 2a give the UVRR spectra of Mb^+H_2O and $Mb^+N_3^-$ at pH 5.5, respectively. The observed changes must only be related to a heme ligand effect. In going from H_2O to the N_3^- ligand, the most evident changes are (i) the Trp 761-cm^{-1} frequency is upshifted by 10 wavenumbers at 771 cm^{-1}, with an intensity increase, (ii) the Trp 1009-cm^{-1} frequency is shifted in the same direction, to 1017 cm^{-1}, (iii) the Trp ≈ 1480-cm^{-1} line is drastically increased in intensity, the side component at ≈1455 cm^{-1} remaining almost unchanged, and (iv) the Tyr + Trp mixed contribution at 1612 cm^{-1} downshifts and decreases in intensity toward 1607 cm^{-1}; this may correspond to a weakening of the Trp ν_{8a} intensity contribution at 1619 cm^{-1}, to the benefit of the contribution of a Tyr residue involved in a strong OH interaction, whose effect is to lower the ν_{8a} Tyr frequency.

It is evident from Figures 1a and 2a that many other spectral changes occurred in the 1100–1400-cm^{-1} region, but the lines are far too much entangled in this region to allow any reasonable tentative of explanation.

pH Effect. Whatever the iron-ligand nature, it has been earlier assumed that, for every pH value, the protein charge array confers a particular electrostatic field at each charged site (Matthew & Gurd, 1986). Let us now compare the UVRR spectra of the $Mb^+N_3^-$ molecule obtained at pH 5.5 (Figure 2a) and 8.5 (Figure 2c). In increasing the pH from 5.5 to 8.5, the major changes are (i) a broadening toward lower frequencies and an intensity decrease of the 771-cm^{-1} Trp line, the line area remaining about the same, (ii) an upshift of the

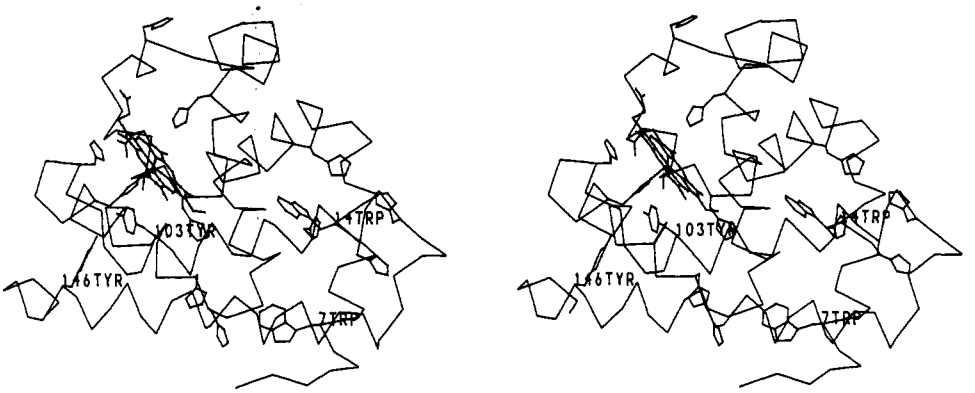

FIGURE 3: Location of His, Trp, and Tyr side chains in sperm whale metmyoglobin in the X-ray crystallographic structure on the basis of the atomic coordinates of "1MBN" in the Brookhaven Protein Data Bank. The stereographic view of the protein was achieved by using the MANOSK (Cherfils et al., 1988) graphics program.

1017-cm^{-1} Trp line to 1024 cm^{-1}), (iii) the intensity of the 1455–1480-cm^{-1} Trp doublet drastically decreases, (iv) a decreasing of the Tyr ν_{8a} intensity, the observed upshift from 1607 to 1609 cm^{-1} being within the limits of band position accuracy: it probably does not correspond to an increase of the Trp contribution in this region, since the Trp shoulder at 1568 cm^{-1} remains unchanged.

It is also evident from the comparison of these two spectra that many other slight changes occurred in the 1100–1400-cm^{-1} region, but, for the same reasons as above, any interpretation would be doubtful. However the line features of Figures 2a and 2c are roughly alike in this frequency range, and we have seen above that ligand changes induce much more drastic spectral variations.

Once the RR spectra of $Mb^+N_3^-$ at pH 5.5 and 8.5 have been compared, one would expect that the RR spectrum of the same species observed at the intermediate pH 7.0 (Figure 1b) would be intermediate in its evolution. If it is actually the case for the 771- and 1017-cm^{-1} bands, it is indeed not the case for some other lines. For the 1455–1481-cm^{-1} doublet, the 1481-cm^{-1} component decreases much faster than the 1455-cm^{-1} component in increasing the pH. In addition, for pH 7.0 a new feature appears at 1621 cm^{-1} at the expense of the 1607-cm^{-1} band, the Trp 1552-cm^{-1} shoulder remaining almost unchanged. These observations mean that the aromatic amino acid vibrational features are likely not directly correlated to the amount of protons exchanges with the aqueous solvent. This behavior should hence result from a specific property of the protein charge array organization.

Now considering the spectra of the Mb^+H_2O molecule obtained at pH 5.5 (Figure 1a) and 7.0 (Figure 1b), the comparison also shows drastic changes in the aromatic acid vibrational features. This again means that the protein matrix interactions are indeed highly pH dependent. Thus, whatever the iron-ligand nature, every ionizable group contributes in a specific manner to the modulation of the electrostatic fields throughout the protein.

Acid–Base Titration of the Different Metmyoglobin Forms. For $Mb^+H_2O \leftrightarrow Mb^+OH^-$, the total amount of exchanged H^+ in equilibrium is directly equal to the amount of added titrating reagent, whereas for $Mb^+N_3^-$ the exchanged H^+ have been calculated by using a formula accounting for the azide ionization, using a pK value of 4.6 (Burns & Chang, 1958). The circulation of proton equivalents/protein (ΔH^+ values) was

$$\Delta H^+ \times [Mb^+N_3^-] = [R] - \frac{10^{(4.6-pH)} \times ([N_3]_0 - [Mb^+N_3^-])}{1 + 10^{(4.6-pH)}}$$

where [R], $[N_3]_0$, and $[Mb^+N_3^-]$ are the concentration of added titrating reagent (HCl or NaOH), the total azide concentration ($[N_3]_0 \approx 11$ mM), and the protein concentration, respectively. Above pH 7, the second term of the equation becomes negligible.

For both aquo- and azido- derivatives, the amount of protons exchanged between pH 5.5 and 8.5 is the same and equal to 8 (data not shown). This result agrees well with the one observed by Botelho et al. (1978). Indeed, these authors have reported that the pK of the observable His residues (there are a total of 11 His residues in horse metmyoglobin) are located in the 7.1–8.6 pH range. The N-terminal group also shows a pK value in this pH range (Friend & Gurd, 1979). Besides, His 64 (distal) and 93 (proximal) are not titratable in this pH range, since their pK values are 4.4 and 4.6, respectively (Wilbur & Allerhand, 1976). Thus, our data along with the previous observations allow to assume that the 5.5–8.5 pH region is clearly dominated in myoglobin by the hydrogen ion equilibria of imidazole and α-amino groups. From this titration it can be assumed that the His residues are titrated continuously, the lack of sharp transition in the titration curves arising from the overlapping of individual His titrations with pK values widespread in this pH range.

Discussion

For hemoproteins, the intense heme resonance Raman intensities that occur with a visible wavelength of excitation have prevented the observation of the Raman vibrations of the protein moiety. Here, UVRR spectra of the protein matrix have been obtained with excitation at 223.6 nm, which enhances the Raman cross section of aromatic residues. The UVRR spectra of Mb^+H_2O and $Mb^+N_3^-$ do not exhibit the same patterns under various pH conditions. Thus it is important first to consider the relative positions of the aromatic residues to the titratable His residues. X-ray data of the horse and sperm whale metmyoglobin, whose structures are very similar (Crumpton & Polson, 1965; Dautrevaux et al., 1969; Takano, 1977; Evans & Brayer, 1988, 1990) give the locations of the 11 histidyl residues inside the myoglobin molecule (Figure 3) along with those of the Trp and Tyr residues. No direct molecular contacts (at 3 Å) are observed between His and Trp or Tyr residues. This point is relevant for the analysis of the feature changes in the RR spectra of aromatic residues, which cannot therefore be interpreted in terms of particular contacts between atomatic cycles and more or less protonated His residues.

On the other hand, the influence of pH on the distal pocket structure seems well-established, particularly on the myoglobin CO derivative (Caughey et al., 1981; Choc & Caughey, 1981; Ansari et al., 1987; Ramsden & Spiro, 1989; Hong et al.,

1990). For the azidometmyoglobin derivative, the relative position of azide and distal histidine as seen in the crystal (Stryer et al., 1964) has also driven to suggest the occurrence of an H bond between azide and this distal histidine. However, infrared data obtained from pH 3 to 11.6 do not support this speculation (McCoy & Caughey, 1970). The lack of H bonding is then consistent with the histidine's more remote nitrogen atom retaining a proton over this pH range and the closer nitrogen atom behaving as an electron donor to azide. This supports a n–σ donor–acceptor interaction with histidine as a donor and azide as an acceptor. Dipole–dipole-type interactions between azide and the distal histidine are also possible (McCoy & Caughey, 1970).

The different steric hindrance of the sixth ligand could produce small conformational changes in the heme region and slightly remodulate the protein bulk organization. Then, the slight differences between the proteic structures of the aquo- and azidometmyoglobins could be detected by UVRR spectroscopy. However, Copeland and Spiro (1985) have reported the UVRR spectra of distinct conformational states of oxidized cytochrome *c*. Their results indicate little effect of the protein conformation upon Raman frequencies and intensities of the aromatic residues. Another study dealing with the R–T quaternary transition in methemoglobin F by UVRR technique has shown, again, little change in the Trp and Tyr vibrational bands (Copeland et al., 1985). These studies indicate that UVRR spectroscopy would be poorly sensitive to any conformational reorganization in metmyoglobin.

Here, it is assumed that iron-ligand binding can influence the charge distribution at substantial distances and produce attractive or repulsive forces within the protein matrix (Gurd & Rothgeb, 1979). Indeed, it has been reported (Ho & Russo, 1987) that the presence of a ligand can induce a shift of the p*K* value up to 2 pH units for a particular histidyl residue in hemoglobin. In the case of anion binding in metmyoglobin, the positive charge at the heme iron is neutralized, so that there is a p*K* increase of all the electrostatically heme-linked ionizable groups when the electric field becomes more negative (Hayes et al., 1975). These heme-linked ionizable groups were found spread over the whole protein (Friend et al., 1980). Thus, the Trp residues can be sensitive to the charge cancellation of the heme iron, even if they are located far away from the binding site; that is what is actually seen on the Raman spectra.

Considering together the UVRR spectra of azidometmyoglobin at various pH values and the acid–base titration curve of this species, it arises that the UVRR spectra evolve with pH in an nonmonotonic way, despite the fact that the titration curve does not exhibit a sharp transition. Thus, these data imply that the aromatic residues are less sensitive to the net protein charge than to the electrostatic field in the protein. This agrees well with the role played by the directionality of the dipoles in the electrostatic interactions (Murray-Rust & Glusker, 1984; Burley & Petsko, 1988). In fact, interactions between aromatic residues and charged groups should occur in the Mb molecule. For example, Figure 4 shows a drawing of the relative positions of Phe 106, Tyr 103, Glu 38, and His 36. It is clear that such electric dipoles are involved in the interactions that may stabilize or destabilize the hemeprotein. It is not yet well understood, however, how electrostatic interactions warrant the modulation of the biological properties of the proteins without inducing measurable structural changes (Friend et al., 1980).

Hence, variations in the electrostatic network would confer a molecular basis to the "interactivity" of the protein matrix;

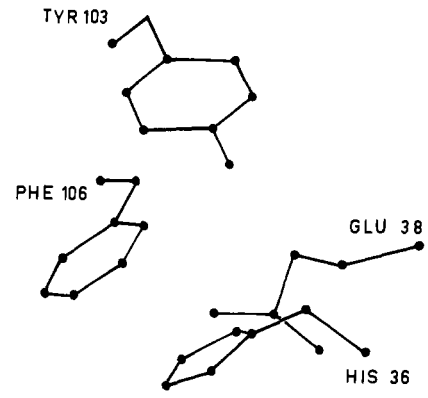

FIGURE 4: Example of proximity between aromatic residues (Tyr 103, Phe 106) and charged groups (His 36, Glu 38). Data are from the same source as in Figure 3. The distance between the N^+ atom of His 36 and the centroid of Phe 106 is 3.7 Å; that between the O^- atom of Glu 38 and the centroid of Tyr 103 is 5.2 Å.

this has been earlier mentioned (Ho & Russu, 1987; El Antri et al., 1989). An interesting consequence of this assumption is that proteins, which have been chemically modified or transformed by site-directed mutagenesis, would differ from the wild-type in the whole protein bulk rather than in the region surrounding the modified locus only. It is important to emphasize that the trigger mechanism of the electrostatic network modulation may originate either from the iron-binding site or from the physicochemical properties of the solvent. On one hand, a change in the ligand nature leads to a reorganization of the domains electrostatically linked to the heme, via a charge cancellation. On the other hand, a pH variation must alter this network, inducing occupancy modifications in most of the charged sites. Thus, the myoglobin affinity for the azide ligand should depend on pH (Friend et al., 1980). Hence, even such a monomeric protein as the myoglobin molecule, which exhibits a very stable structural organization, has the potentiality for a "two-ways" energy transduction. This specific property of the protein macromolecules relies on the modulation of the charge spatial distribution in the bulk, namely the proteic electrostatic network. These effects are likely helped by the fact that the ionization constant of a given group is influenced by the combined effects of electrostatic fields from the close surroundings (Matthew & Gurd, 1986) as well as from more remote domains. Hence, every ionizable group, due to its particular location, influences and is influenced in a proper manner by the ionization state of the other groups.

The key role played by the electrostatic interactions has been emphasized since they contribute to the net free energy of stabilization of the protein structure (Perutz, 1978; Burley & Petsko, 1988). It is the modulation of electrostatic interactions that may supply, when a structural change is permitted, the free energy available for driving changes in the structure (Friend et al., 1980). This electrostatic free energy is very sensitive to the relative orientation of segments in the secondary structure of which the peptide dipoles are regularly arranged, and the alignment of secondary structure dipoles is in turn significant for determining the tertiary structure of globular proteins (Sheridan et al., 1982). So, every ionizable group contributes in a specific manner to the protein conformation in response to solvent conditions. For each pH value, the protein charge array confers an electrostatic potential at each particular site (Matthew & Gurd, 1986). Although the myoglobin molecule can keep a tertiary structure roughly unaltered, it appears that, depending on pH conditions or on iron-ligand nature, a different set of electrostatic interactions occurs inside the protein matrix. Hence, any attempt to elu-

cidate the intimate mechanisms underlying the protein activity will have to take into account the electrostatic network of the macromolecule.

Registry No. Try, 73-22-3; Tyr, 60-18-4; iron, 7439-89-6.

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