UV RESONANCE RAMAN DETERMINATION OF α-HELIX MELTING DURING THE ACID DENATURATION OF MYOGLOBIN

Sanford A. Asher and Zhenhuan Chi
Department of Chemistry
University of Pittsburgh
Pittsburgh, PA 15260, USA

Abstract

We have studied the absorption spectrum and the 206.5 and 229 nm excited resonance Raman spectrum of aquometmyoglobin (Mb) between pH 7.5 and pH 1.5. The acid denaturation of Mb below pH 3.5 results in small absorption increases below 220 nm due to α-helix melting, and an absorption decrease at ~230 nm due to environmental changes of the trp and/or tyr residues. Dramatic heme Soret band changes indicate major alterations of the heme pocket. We examined the 206.5 nm resonance Raman spectra, which selectively enhances amide backbone vibrations and compared these results to the 229 nm excited resonance Raman spectra, which selectively enhances tyr and trp vibrations. We calculate that the Mb α-helical composition decreases from ~80% at neutral pH to ~19% below pH 3.5. The trp Raman cross sections dramatically decrease at low pH which indicates that the A helix melts. The tyr Raman bands are pH independent which indicates that the G and H helices do not melt. This result indicates that helices A, B, C, D and E melt in a concerted fashion, while the antiparallel G and H helices only partially melt. This melting of the helices framing the heme pocket is responsible for the change in heme binding; the diffuseness of the unfolded Mb heme Soret band suggests that the heme no longer has a single defined binding site.

Introduction

One central unsolved problems in the understanding of protein structure and dynamics is the mechanism by which a protein self assembles from a primary sequence of amino acids to form a three dimensional functional enzyme with its well defined, and crucial secondary tertiary and quaternary protein structure [1]. Numerous studies of protein folding, as well as the protein unfolding have utilized a host of techniques such as NMR, CD, absorption and fluorescence spectroscopy etc. [2-4]. These studies, which attempt to elucidate the rules for protein folding (and unfolding), utilize highly sensitive and selective techniques to examine protein structural changes.

G. Vergoten and T. Theophanides (eds.), Biomolecular Structure and Dynamics, 263–270.

Myoglobin has often been used in these folding and unfolding studies as a model system because of the detailed structural information available from x-ray measurements [5]. In this report we apply UV resonance Raman spectroscopy to determine myoglobin secondary structural changes during acid denaturation. We developed a new methodology to assign secondary structural changes to particular α-helical segment. We accomplish this by monitoring the UV Raman spectra of both the amide vibrations and the tyr and trp aromatic amino acid vibrations, as well as the heme Soret band. We utilized our recent PSCS methodology to determine secondary structure by fitting pure secondary component spectra obtained from 206.5 nm excited protein amide band spectra [6]. These data are combined with 229 nm excited resonance Raman spectra that selectively enhance the tyr and trp aromatic amino acids. Secondary structure changes are correlated with changes in particular aromatic amino acid environments and hydrogen bonding, to localize the secondary structural changes to particular regions of the protein. In this study we directly monitored the melting of α-helical segments of aquometmyoglobin during its reversible acid denaturation. We determine which helices melted and which were stable at low pH.

Experimental

Horse heart myoglobin (Mb) was purchased from Sigma Co. (St. Louis, MO) and was used as received; the protein was in the oxidized met form. For Raman spectra excited at 206.5 nm we utilized 18 μM Mb solutions, and for 229 nm excitation we used 91 μM Mb solutions. The pH value of the sample solutions were adjusted by adding small amounts of concentrated hydrochloric acid and potassium hydroxide solutions with rapid stirring.

The UV Raman measurements were obtained by using instrumentation described in detail previously [7,8]. 206.5 nm (2.5 mW) and 229 nm (9 mW) CW laser excitation was obtained from intracavity frequency-doubled krypton and argon ion lasers, respectively [8].

Results

Figure 1 shows the absorption spectra of aquomet Mb at various pH values between pH=7.5 and pH=1.5. The absorption spectra show the strong Mb heme Soret band between 330-450 nm, and modest tyr and trp absorption bands in the region between 260-300 nm [9]. Strong tyr and trp absorption bands occur between 210 and 230 nm [9,10]. The region below 220 nm is dominated by the $\pi \rightarrow \pi^*$ absorption of the amide backbone linkages [6].

The absorption spectra are independent of pH for pH values between pH 7.5 and 3.5. However, a pH decrease below 3.5 results in a Mb absorption increase between 200-220 nm and a small absorption decrease around 230 nm. These spectral changes are correlated with the known unfolding of the dominantly Mb α-helical structure (81%); the absorption increase below 220 nm results from hyperchromism of

the amide π–π^* electronic transition moments due to the decreased excitonic interactions in the unfolded random coil conformations compared to that in the α-helix conformation [11].

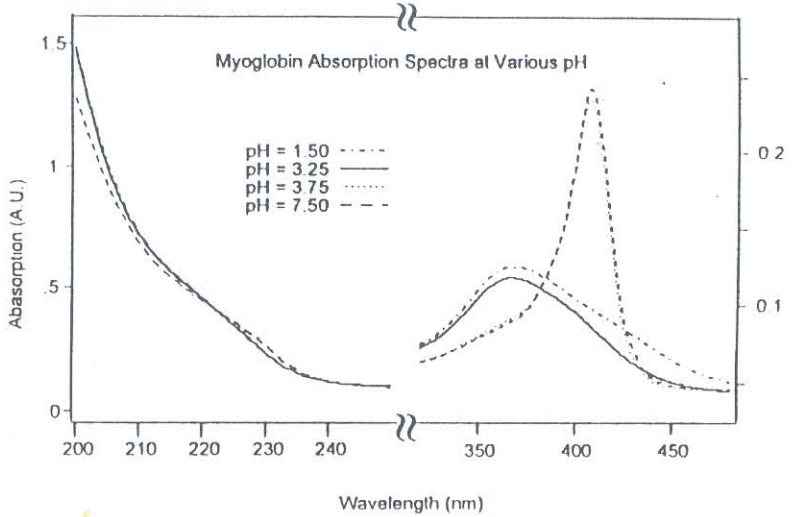

Figure 1. Aquometmyoglobin (30 μM, 0.05 cm pathlength) absorption spectra at various pH values.

The ca. 230 nm Mb absorption decrease indicates that the environment of tyr and trp residues are changing during the acid denaturation. The large 413 nm Mb Soret absorption band decrease and the replacement with a much more diffuse 363 nm Soret band indicates a major changes in binding of the heme at low pH.

Figure 2 shows the 206.5 nm excited resonance Raman spectra of Mb at pH values between 7.50 and 1.50. The pH 7.27 sample was obtained by adjusting the sample pH from 7.50 to 1.50, then returning the pH to 7.27; the fact that the pH 7.27 Raman spectrum is identical to that at pH 7.50 indicates that the protein structural changes that give rise to the low pH spectral changes are reversible.

The 206.5 nm excited Raman are dominated by the amide vibrations [6] since this excitation wavelength is resonant with the amide $\pi \rightarrow \pi^*$ transitions. The spectra show strong enhancement of the amide I vibration (~1660 cm^{-1}), which is predominantly carbonyl stretching; some enhancement of the ring stretching vibrations of the tyr and trp aromatic amino acid side chains (~1610 cm^{-1}); strong enhancement of the amide II vibration (1555 cm^{-1}), which involves all the atoms of the amide group, but contains a large contribution of C-N stretching and C-H bending; some intensity for the numerous CH_2 side-chain bending vibrations (1455cm^{-1}); a variable intensity of the amide C_α-H bending vibration (1386 cm^{-1}); and strong enhancement of the amide III vibration (1240-1300 cm^{-1}), whose composition is similar to that of the amide II vibration, except that the opposite phasing occurs between the C-N stretching motion and the N-H bending motion [9, 10].

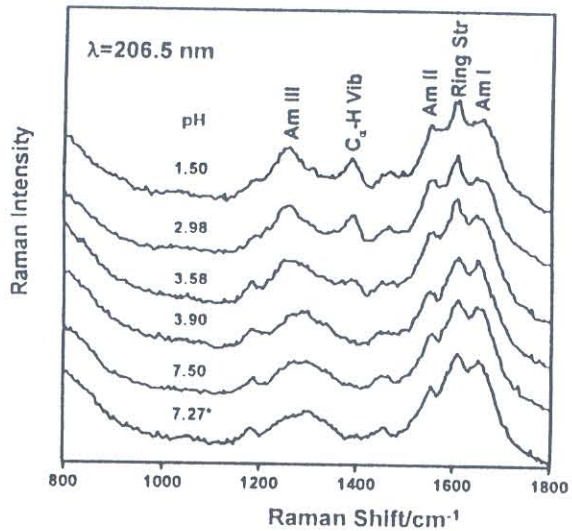

Figure 2. 206.5 nm excited Raman spectra of aquo-metmyoglobin (18 μM) at various pH values.

Between pH 7.50 and 3.90 the Mb amide Raman bands are essentially ntical; they show a strong amide I (1655 cm^{-1}) band, and medium intensity amide II 55 cm^{-1}) and III (1290 cm^{-1}) bands. No band near 1386 cm^{-1} is evident, which licates that the Mb secondary structure is mainly α-helical [6, 11]. The invariance the Raman frequencies, intensities and band shapes indicates little change of the otein secondary structure between pH 7.50 and 3.90.

Below pH 3.9, the 1386 cm^{-1} amide C$_\alpha$-H bending band appears, which licates a significant unraveling of the Mb α-helical structure. The amide III band quency shifts from 1290 to 1268 cm^{-1} between pH 3.9 and 3.58, and further shifts to 61 cm^{-1} at pH 2.98. The amide III band Raman cross section increases from mbarn/molc str at pH 3.98 to 39 mbarn/molc str at pH 2.98. The amide I band fts 7 cm^{-1} to lower frequency, and its Raman cross sections slightly decreases. The ide II band frequency remains constant, while its Raman cross section increases m 27 to 46 mbarn/molc-str. A further pH decrease to 1.50 causes little additional man spectral changes, which indicates little further change of the Mb secondary ucture.

We have previous determined the amide band resonance Raman spectra of re α-helix, β-sheet and random coil secondary structures for excitation at 206.5 nm. also demonstrated that we can use these pure secondary structure spectra (PSCS) a linear fit of the spectra of proteins to reliably determine protein secondary ucture composition. This is a robust and sensitive determination since we

simultaneously utilize the amide I, II, III bands and the C$_\alpha$-H amide bending vibrations of these PSCS to determine secondary structure [6].

We used this methodology to determine the Mb secondary structure for the different pH value spectra shown in Figure 2. Table I lists our calculated Mb secondary structural compositions at the different pH values. The calculated α-helical composition remains relatively constant at ca. 75% from pH 7.50 to 3.90, but it decreases rapidly to 19% α-helix at pH 3. An abrupt transition between the folded and unfolded states occurs at pH ~3.50.

TABLE I. Myoglobin Secondary Structural Composition at Various pH Value

pH	α-Helix %	β-Sheet %	Random Coil
1.50	19	0	81
2.98	19	0	81
3.58	44	9	46
3.90	78	0	22
4.52	77	0	23
5.05	73	0	27
7.50	73	0	27
7.27r	78	0	22

The Figure 2 bottom Mb spectrum, which is for a sample where the pH was first adjusted down from neutrality to pH = 1.50 and then adjusted back to pH = 7.27, is identical to that at pH 7.50, this clearly indicates that this acid induced unfolding of Mb is reversible.

Figure 3 shows 229 nm excited Raman spectra of Mb at various pH. This excitation occurs in resonance with the tyr and trp ring $\pi \rightarrow \pi^*$ transitions. Thus, the 229 nm Mb Raman spectra are dominated by Raman bands of tyr and trp. The 759, 857, 879, 1012, 1339, 1359, 1453, 1516 and 1557 cm^{-1} bands are assigned to trp, while the 1179, 1207 and 1258 cm^{-1} bands are assigned to tyr. The 1613 cm^{-1} bands derive mainly from the tyr Y8a band but also have a small contribution from the trp W1 band [10].

The Raman cross sections of the strongly enhanced trp 759, 879, 1012, 1360 and 1551 cm^{-1} bands decrease as the pH decreases below 4.0; the cross section decrease levels off by pH 2.50. In contrast, the strongly enhanced tyr 1129, 1207, 1258 and 1613 cm^{-1} bands show no significant frequency or Raman cross section changes.

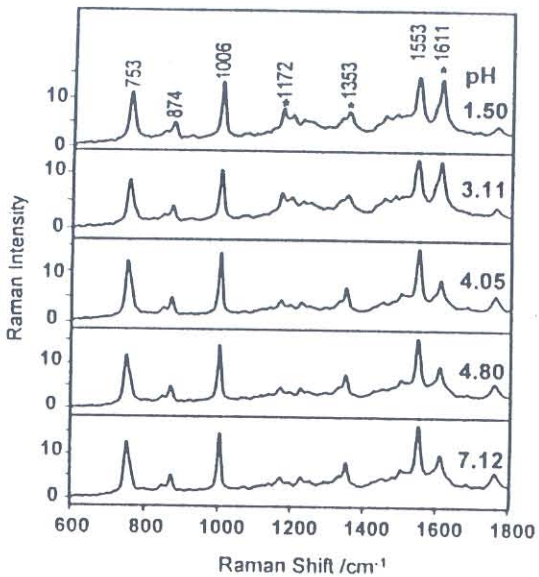

Figure 3. 229 nm excited Raman spectra of aquo-metmyoglobin (91 μM) at various pH values.

Discussion

Horse heart Mb is a globular, single-domain protein of 153 amino acids (Fig. 4) which possesses eight rod-like α-helix segments of variable length (labeled as A-H) [5]. Horse heart Mb contains two trp (trp7 and trp14 localized in helix A) and two tyr residues (tyr103 and tyr146 localized in helices G and H, respectively). The B, C, E, and F helices frame the heme pocket.

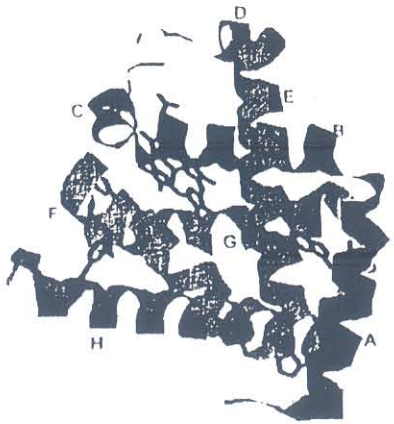

Figure 4 Structure of horse heart myoglobin showing the helical segments, which was adapted from ref 5.

We can partially determine which helices melt by correlating changes in the trp and tyr Raman spectra with changes in the amide band Raman spectra, and with the collapse of the heme pocket which results in a change in heme binding evident from the heme Soret absorption spectral changes upon acid denaturation. The full acid denaturation indicates that the calculated ~75% α-helical structure melts to give 19% α-helical structure containing an 81% random coil structure (Table I).

The decreased trp Raman cross sections indicates a change in the trp environment from hydrophobic to hydrophilic. This indicates that the A helix melts. The invariance of tyr Raman frequencies and cross sections indicates little change in the α-helical structure of helices G and H around the tyr residues. The G and H helices alone give rise to a 29% α-helical structure which is significantly greater than the residual α-helix abundance we calculate at low pH.. Our previous determinations of α-helix abundance was accurate to within 10% for highly helical peptides. In fact, we generally overestimated the α-helical content [6]. Thus, we can conclude that parts of the G and H helices are melted and that these are localized away from the tyr residues. Finally, we can conclude the A, B, C, D, E and F helices melt. This is consistent with the destruction of the heme crevice that alters the heme binding. The breadth of the Soret band may indicate the existence of numerous modes of heme binding.

Our results indicate that there is an almost simultaneous melting of most of the α-helical segments of Mb at pH ~3.5. The origin of this concerted melting may result from destabilization of the α-helix internal hydrogen bonding at low pH values. In contrast, Helices G and H are strongly coupled, lie antiparallel to one another and the resulting helix contacts may stabilize each other to the extent that they do not completely melt at these low pH values.

Conclusions

We have demonstrated a methodology to follow the unfolding of specific helical segments by examining the alterations in the environment of specific aromatic amino acids localized within different α-helical segment. We assign the spectral changes to unfolding of helices A-F at pH ~3.5 in aquo metMb, while the helices G and H still remain some α-helical structure, especially around the tyr residues. We presume that this melting of the helices framing the heme pocket is responsible for the observed change in heme-protein binding. The diffuseness of the unfolded Mb heme Soret band indicates that the heme no longer has a single defined binding site.

Acknowledgment

We gratefully acknowledge financial support from the National Institute of Healtl through Grant No. R01GM30741-14 to S.A.A.

References

1. Kim, P.S. and Baldwin, R.L. (1990) *Annu. Rev. Biochem.* **59**, 631-660.
2. Johnson, W.C., Jr. (1996) In *Circular Dichroism and the Conformational Analysis of Biomolecules*; Fasman, G.D., Ed., Plenum Press, New York and London, p. 635-654.
3. Ballew, R., Sabelko, J., and Gruebele, M. (1996) Proc. *Natl. Acad. Sci. USA*, **93**, 5759-5764.
4. Hughson, F.M., Wright, P.E., and Baldwin, R.L. (1990) *Science* **249**, 1544-1548.
5. Evans, S.V. and Brayer, G.D. (1988) *J. Biol. Chem.*, **263**, 4263-4268.
6. Chi, Z., Chen, X.G., Holtz, J.S.W., and Asher, S.A., *Biochemistry*, submitted.
7. Asher, S.A., Johnson, C.R., and Murtaugh, J. (1983) *Rev. Sci. Instrum.* **54**, 1657-1662.
8. (a) Asher, S.A., Bormett, R.W., Chen, X.G., Lemmon, D.H., Cho, N., Peterson, P., Arrigoni, M., Spinelli, and Cannon, J. (1993) *Appl. Spectrosc.* **47**, 628-633; (b) Holtz, J.S.W., Bormett, R.W., Chi, Z., Cho, N., Chen, X.G., Pajcini, V., Asher, S.A., Arrogoni, M., Owen, P., and Spinelli, L. (1996) *Appl. Spectrosc.* **50**, 1459-1468.
9. Asher S.A., Larkin, P.J., and Teraoka, J. (1991) *Biochemistry*, 30, 5944-5954.
10. Cho, N., Song, S., and Asher, S.A. (1994) *Biochemistry*, **33**, 5932-5941.
11. Song, S. and Asher, S.A. (1989) *J. Am. Chem. Soc.* **111**, 4295-4305.