BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 48 (5), 1394—1396 (1975)

Resonance Raman Spectra of Methylorange Bound to Proteins and Cationic Surfactants

Bak-Kwang Kim, Akira Kagayama, Yutaka Saito, Katsunosuke Machida, and Toyozo Uno

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606

(Received December 9, 1974)

Resonance Raman spectra of methylorange were recorded for aqueous solutions containing various proteins and cationic surfactants. The base form of methylorange was found to retain its trans-azo structure on the interaction with human serum albumin and with cetyltrimethylammonium bromide. The relative intensities of the Raman bands at 1420 cm^{-1} and at 1155 cm^{-1} of methylorange change appreciably along with the shift of the absorption maximum in the visible and ultraviolet region on addition of proteins and surfactants.

Proteins and surface active agents give rise often to the anomaly in visible and ultraviolet absorption spectra and the deviation of the color-change interval of various acid-base indicators.[1–4] This phenomenon has long been the subject of extensive studies with special attention on the intermolecular interaction involved. Structure changes of indicator molecules associated with this color change may be reflected, if any, in resonance Raman spectra of the indicators in aqueous solutions in the presence of proteins and surfactants.

The absorption maximum, $\lambda_{\max}$, in the visible absorption spectrum of methylorange (MO) in neutral aqueous solutions undergoes a blue shift by 20—30 nm on addition of bovine serum albumin (BSA).[2,3] Various postulates such as the change in the molecular environment[5] or the twisting around the N=N bond[6] have been forwarded to account for this shift. Carey *et al.* have recently found, based on rather small changes in the resonance Raman spectrum, that no appreciable twisting around the N=N bond occurs for MO bound to BSA.[7] Change of the $\lambda_{\max}$ in the visible and ultraviolet absorption spectra of MO is also known for the solutions containing human serum albumin (HSA),[5] poly-L-lysine hydrobromide (PLL)[8] and cetyltrimethylammonium bromide (CTB).[4,8] We have investigated the effects of these substances on the resonance Raman spectrum of MO with particular interest in the change of the relative band intensities.

Experimental

Reagent grade methylorange (Tokyo Kasei Co.) was recrystallized several times from water. Crystallized and lyophilized bovine serum albumin (Sigma Chem. Corp.), poly-L-lysine hydrobromide (Protein Research Foundation, Osaka) and crystallized human serum albumin (Miles Lab.) were used without further purification. Cetyltrimethylammonium bromide (Tokyo Kasei Co.) was recrystallized from ethanolic solution. The concentration of MO in the sample solutions was 1×10^{-4} M and those of proteins and surfactants were between 5% and 0.5%. The pH of these solutions was controlled by using 0.1 M HCl and the phosphate buffers, and was determined by Radiometer titrator TTT2 and combined electrode GK 2302C.

Resonance Raman spectra were recorded on a JEOL S-1 laser Raman spectrophotometer with the spectral slitwidth 12 to 15 cm^{-1}. The excitation lines at 514.5 nm, 488.0 nm and 476.5 nm were obtained from a Coherent 52G Ar$^+$ laser. The sample solutions were sealed in 1 mm glass capillary tubes. Band intensities were measured as the peak heights on appropriate base lines. The visible and ultraviolet spectra were recorded on a Shimadzu W-40 spectrophotometer.

Results and Discussion

In the previous work,[9] we could not detect any noticeable change in relative intensities of resonance Raman bands of the base form of MO on changing the excitation wavelength from 488.0 nm to 514.5 nm. The 1420 cm^{-1} band due to the N=N stretching vibration is always much stronger than the other bands. We found in this work, however, that the intensity ratio of the two bands at 1420 and 1155 cm^{-1}, I_{1420}/I_{1155}, in the spectra excited by the 514.5 nm line decreases appreciably on addition of BSA. It has been known that the $\lambda_{\max}$ of the base form of MO is at 464 nm in the neutral aqueous solution and shifts to 440—430 nm on addition of BSA up to 0.2%.[2,3] In the case of the presently used 3% BSA solution (pH 3.9—5.5), the $\lambda_{\max}$ of MO appears at 430 nm. Thus the change in I_{1420}/I_{1155} may be attributed to the different sensitivities of the band intensities I_{1420} and I_{1155} on the separation between the $\lambda_{\max}$ and the excitation wavelength. According to Ito *et al.*, the intensity of the N=N stretching band of *p*-aminoazobenzene at 1422 cm^{-1} increases much more quickly than those of two bands around 1150 cm^{-1} on changing the excitation wavelength from 632.8 to 337.1 nm.[10] In this work, a similar dependence of the relative band intensities on the excitation wavelength was observed for MO bound to BSA. As shown in Fig. 1, the ratio I_{1420}/I_{1155} increases when the excitation wavelength is

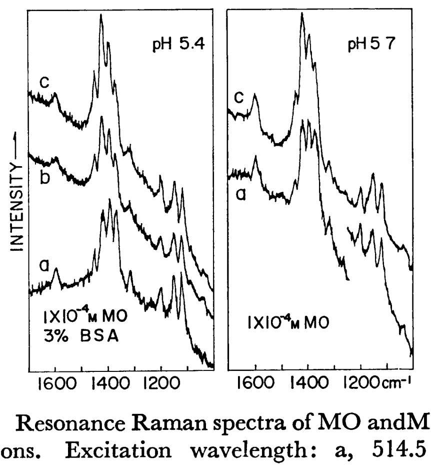

Fig. 1. Resonance Raman spectra of MO andMO–BSA solutions. Excitation wavelength: a, 514.5 nm; b, 488.0 nm; c, 476.5 nm.

shortened in the order 514.5, 488.0, and 476.5 nm. The BSA-bound molecules of MO excited by these Ar$^+$ lines seem therefore to be in the state of preresonance, during which the resonance effect on the stretching vibration of the N=N bond increases as the excitation line approaches the λ_{max} 430 nm. On the other hand, the unbound MO with the λ_{max} 464 nm shows no appreciable change in I_{1420}/I_{1155} on the change of the excitation line from 476.5 to 514.5 nm, probably because the band intensity I_{1420} is saturated under a rigorous resonance and is almost insensitive to the change of the excitation wavelength within the visible absorption band.

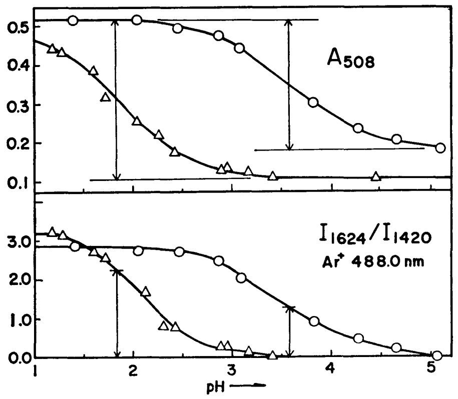

Fig. 2. The pH dependence of the absorbance (A_{508}) and the Raman intensity ratio (I_{1624}/I_{1420}): circle, 1×10^{-4} M MO; triangle, 1×10^{-4} M MO+3% BSA. The arrows indicate the pH at which the concentrations of the acid and the base forms become equal to each other.

The decrease of I_{1420} on the shift of λ_{max} from 464 nm to 430 nm is manifested in the change of the resonance Raman spectra of the bound MO and the unbound MO on the change of pH across the color-change interval. In the acidic solutions, MO is not bound to BSA, and the absorption spectrum of the acid form of MO ($\lambda_{max}=$ 508 nm) dose not change on addition of BSA. The Raman band at 1624 cm^{-1} of the acid form of MO may thus be used, in the middle of the color change interval, as an internal standard to estimate the difference in I_{1420} between the bound MO and the unbound MO in the base form. In Fig. 2, the pH dependence of the ratio I_{1624}/I_{1420} is compared with that of the absorbance A_{508} for both the MO and the MO–BSA solutions. The pH's at which the concentrations of the base and the acid forms in these solutions become equal are easily determined from the sigmoid curves for A_{508}. Comparing the ratio I_{1624}/I_{1420} at these pH's, we can conclude that I_{1420} of MO bound to BSA is about 3/5 of that of the unbound MO.

Klotz *et al.* have found that the solution of MO containing HSA shows a complicated shift of the λ_{max} on the change of pH.[5] Figure 3 shows the resonance Raman spectra of the MO-HSA solution excited by the 488.0 nm and the 514.5 nm lines. The shifts of the λ_{max} on the addition of HSA and on the subsequent change of pH are not accompanied by the shift of the Raman bands, and the resonance Raman spectra of the MO–HSA solutions are entirely different from the previously reported spectrum of the acidic solution of MO.[9] This result indicates that there is no proton donation from protein residues to MO bound to HSA. In the resonance Raman spectra of the MO-BSA solutions excited by the 514.5 nm line, the ratio I_{1420}/I_{1155} increases appreciably as the approach of the λ_{max} to the excitation wavelength. On the contrary, there seems to be no appreciable change in I_{1420}/I_{1155} on the change of the λ_{max} for the spectra recorded for the 488.0 nm excitation.

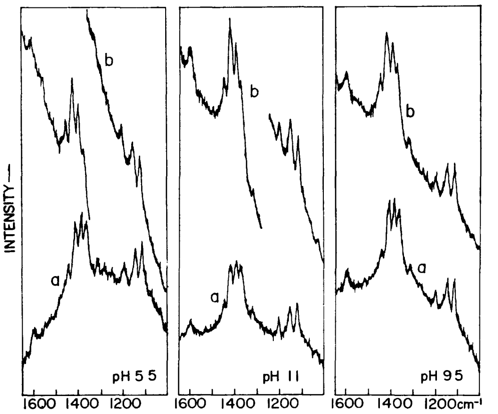

Fig. 3. Resonance Raman spectra of MO–HSA solutions (1×10^{-4} M MO+1.5% HSA) at various pH. Excitation wavelength: a, 514.5 nm; b, 488.0 nm.

According to Quadrifoglio and Crescenzi, the λ_{max} of MO in the visible region undergoes a marked blue-shift to 370 nm on addition of poly-L-lysine hydrobromide (PLL).[8] These authors obtained, by using the 1×10^{-5}M solution, the absorption spectra in which no peaks were observed above 400 nm. Unfortunately, the limitation in the Raman sensitivity compelled us to use the solution 10 times as concentrated as these authors' solution, and with this concentration, we could not find the condition for eliminating a peak around 450 nm. The resonance Raman spectrum of this solution (Fig. 4) taken by the 488.0 nm excitation is the same as that of MO bound to HSA, and is thought to arise from the species responsible for the absorption at 450 nm.

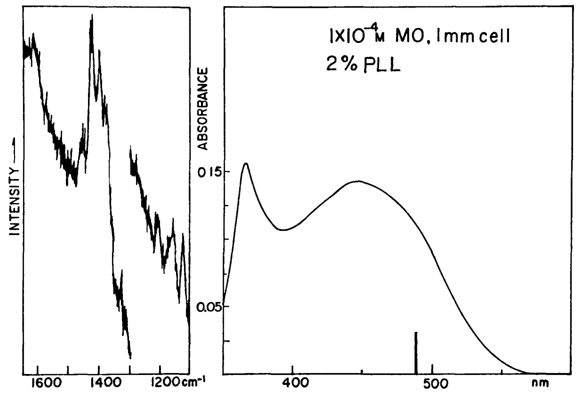

Fig. 4. Resonance Raman and visible absorption spectra of MO–PLL solution. The bar indicates the excitation wavelength (488.0 nm).

The absorption spectrum of the base form of MO changes in two steps on addition of the cationic surfactant cetyltrimethylammonium bromide (CTB). When the concentration of this surfactant is slightly lower than

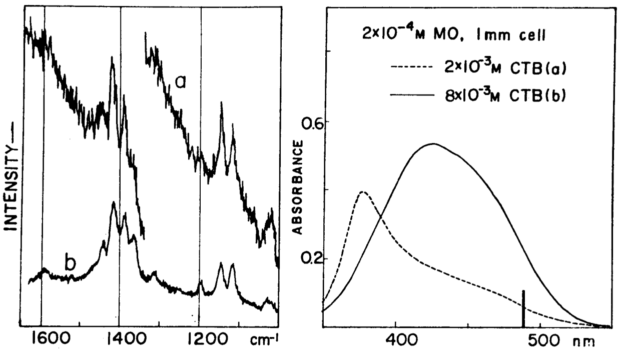

Fig. 5. Resonance Raman and visible absorption spectra of MO–CTB solutions. The bar indicates the excitation wavelength (488.0 nm).

the critical micelle concentration (3×10^{-3} M), a peak appears at 370 nm as in case of MO bound to PLL.[8] As shown in Fig. 5a, the noise level of the resonance Raman spectrum of the MO solution containing 2×10^{-3} M of CTB is very high because the resonance effect is diminished according to the large separation between the $\lambda_{\max}$ and the excitation wavelength. It is recognized, however, that the intensity ratio I_{1420}/I_{1155} in Fig. 5a is appreciably smaller than that in the spectrum of the solution containing 8×10^{-3} M of CTB (Fig. 5b). In contrast to the case of the MO–PLL solution, the Raman bands shown in Fig. 5a and the $\lambda_{\max}$ at 370 nm are due certainly to the same species. A conformational change of the azo group from the *trans* form to the *cis* form has been postulated in order to explain the large blue-shift of the $\lambda_{\max}$ of MO on being bound to PLL and CTB.[8] However, the resonance Raman spectrum shows clearly that there is no possibility for such a conformational change; the *cis*-azo group is known to give the stretching frequency around 1500 cm^{-1}, about 100 cm^{-1} higher than that of the *trans*-azo group.[11] This conclusion has been drawn by Carey *et al.* for the BSA-bound MO showing the $\lambda_{\max}$ 430 nm.[7] When the concentration of CTB is much higher than the critical micelle concentration, the absorption spectrum of MO is similar to the case when BSA is added.[4] The corresponding Raman spectrum is identical to that of MO bound to BSA.

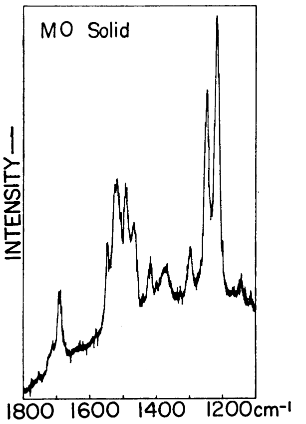

Fig. 6. Raman spectrum of solid MO excited by the He–Ne 632.8 nm line.

The difference in the sensitivities of the Raman band intensities to the excitation wavelength is reflected also on the spectra of MO in the crystalline state. As shown in Fig. 6, the Raman spectrum of the crystalline MO excited by the He–Ne 632.8 nm line gives the ratio I_{1420}/I_{1155} less than unity. According to the spectrum reported by Carey *et al.*, this ratio is much larger than unity when excited by the Ar$^+$ 488.0 nm line.[7] Presumably, the $\lambda_{\max}$ of the crystalline MO is at the shorter wavelength side of the 488.0 nm line, giving rise to the above difference in I_{1420}/I_{1155} between the 488.0 nm and the 632.8 nm excitations.

We wish to thank Professor Hisashi Tanaka for the use of the W-40 spectrophotometer in his laboraroty.

References

1) G. S. Hartley, *Trans. Faraday Soc.*, **30**, 444 (1934).
2) I. M. Klotz, *J. Am. Chem. Soc.*, **68**, 2299 (1946).
3) J. R. Colvin, *Can. J. Chem.*, **31**, 734 (1953).
4) C. F. Hiskey and T. A. Downey, *J. Phys. Chem.*, **58**, 835 (1954).
5) I. M. Klotz, R. K. Burkhard, and J. M. Urquhart, *J. Phys. Chem.*, **56**, 77 (1952).
6) W. F. Forbes and B. Milligan, *Australian J. Chem.*, **15**, 841 (1962).
7) P. R. Carey, H. Schneider, and H. J. Bernstein, *Biochem. Biophys. Res. Commun.*, **47**, 588 (1972).
8) F. Quadrifoglio and V. Crescenzi, *J. Colloid Interface Sci.*, **35**, 447 (1971).
9) K. Machida, B.-K. Kim, Y. Saito, K. Igarashi, and T. Uno, This Bulletin, **47**, 78 (1974).
10) S. Koide, Y. Udagawa, N. Mikami, K. Kaya, and M. Ito, This Bulletin, **45**, 3542 (1972).
11) R. Kübler, W. Lüttke, and S. Weckherlin, *Z. Elektrochem.*, **64**, 650 (1960).