RESONANCE RAMAN SCATTERING FROM HEMOPROTEINS: pH-DEPENDENCE OF RAMAN SPECTRA OF FERROUS DICARBOXYMETHYL–METHIONYL–CYTOCHROME *C*

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1. Introduction

The pH-dependent change of absorption spectra of ferrous alkylated cytochrome *c* (dicarboxymethyl–methionyl–cytochrome *c*) was reported to be due to the transition between low spin and high spin states [1]. Since the ferrous cytochrome *c* gives strong Raman lines [2] and Raman spectra are sensitive to the spin and oxidation states [3] of hemoproteins, we attempted to measure the resonance Raman spectra as well as optical absorption spectra of ferrous alkylated cytochrome *c* in the region between pH 3.0 and 11.0. General patterns of Raman spectra of ferrous alkylated cytochrome *c* resemble to that of ferrous native cytochrome *c* and most of Raman lines do not show pH-dependent frequency shift. However, a pH-sensitive Raman line was found around 1540 cm^{-1} and it was concluded that the pH-dependent change is due to the transition between two kinds of low spin state with a p*K* of 7.9. In alkylated cytochrome *c* two methionyl residues (at the 65th and 80th positions) were alkylated with bromoacetate in the presence of KCN [4], and lysine-79 is supposed to be the 6th ligand of the heme iron [5]. Since ferrous native cytochrome *c* did not show any pH-dependence of Raman spectra, the spectral change of alkylated cytochrome *c* is deduced to be associated with the coordination of lysine-79. The spin state and structure of ferrous alkylated cytochrome *c* will be discussed in relation with the results of kinetic measurements by Brunori et al. [1].

2. Materials and methods

Horse heart cytochrome *c* (Sigma type VI) was purified as described previously [6]. Alkylated cytochrome *c* was prepared by slightly modified method of Schejter and Aviram [4]. Ferric cytochrome *c* was incubated with 0.15 M bromoacetate in the presence of 0.1 M potassium cyanide in 0.1 M Tris–Cl buffer at pH 7.0 for 36 hr at 25°C. After the dialysis of the above solution against 0.01 M phosphate buffer at pH 7.0 for 24 hr at 5°C, sodium dithionite was added to dissociate the bound cyanide. Gel filtration was performed on Sephadex G-25 column equilibrated with 0.01 M phosphate buffer at pH 7.0 to remove the cyanide. The filtrate was oxidized by potassium ferricyanide, and then passed through Sephadex G-25 column equilibrated with the same buffer. The elute was charged on CM-cellulose column equilibrated with 0.01 M phosphate buffer at pH 6.8 and then developed with 0.08 M phosphate buffer at pH 6.8. The main fraction of the elute was used in the present investigation.

Resonance Raman spectra were recorded with the use of a JEOL-02AS spectrometer equipped with cooled HTV-R374 photomultiplier and photon counting detection.

Absorption spectra were measured with a HITACHI 124 recording spectrophotometer at 25°C.

Reduction of alkylated cytochrome *c* was performed in anaerobic condition (approx. 0.05 mmHg) with sodium dithionite. Concentrations of the cytochrome were 0.2 mM and 25 μM for the measurements of resonance Raman and absorption spectra, respectively.

3. Results

Resonance Raman spectra of ferrous alkylated cytochrome *c* between pH 3.0 and 11.0 exhibit the almost similar features to those of ferrous native cytochrome *c* [2] except for the highly pH-dependent band around 1540 cm^{-1}, and are typical low spin type of ferrous hemoproteins [3].

For elucidation of this pH-dependent band, parallel and perpendicular components of the Raman scattering near 1540 cm^{-1} of ferrous alkylated cytochrome *c* in 0.1 M phosphate buffer at pH 3.9, 7.0 and 9.7 are illustrated in fig.1. Although the inverse polarized band at 1583 cm^{-1} and polarized band at 1488 cm^{-1} do not exhibit pH-dependence, the depolarized band at 1545 cm^{-1} disappears and new depolarized band appears at 1533 cm^{-1} by increasing the pH value from 3.9 to 9.7. At pH 7.0, a broad band is observed at 1542 cm^{-1}. It may be due to the coexistence of two forms which give Raman lines at 1533 cm^{-1} and 1545 cm^{-1}. The 1545 cm^{-1} and 1533 cm^{-1} bands are thought to be characteristic of the acid and alkaline forms [1] of ferrous alkylated cytochrome *c*, respectively. This pH-dependent transition shown in resonance Raman spectra was found to be reversible.

In fig.2, the apparently observed frequency* of the pH sensitive Raman line is plotted against pH, together with the change of optical absorbance of α-band at 550 nm of ferrous alkylated cytochrome *c*. The pH-dependence of optical absorbance of at 550 nm are essentially the same as that previously reported by Schejter and Aviram [4]. The pH-dependence of the frequency of Raman line corresponds quite well to that of absorption spectra, giving rise to the p*K* value of this acid-alkaline transition to be 7.9.

4. Discussion

From resonance Raman spectroscopy, ferrous alkylated cytochrome *c* was found to be in low spin

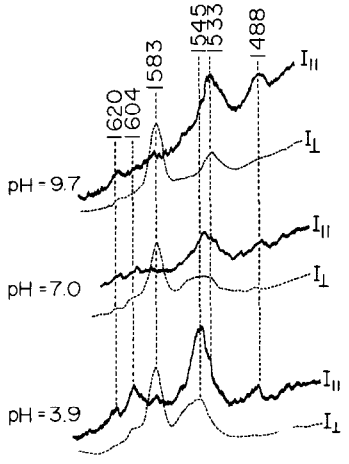

Fig.1. Polarized Raman spectra of ferrous alkylated cytochrome *c* near 1540 cm^{-1} band at pH 3.9, 7.0 and 9.7. Instrumental conditions : excitation, 514.5 nm line of Ar^+/Kr^+ laser (Spectra Physics model 164–02); power, 200 mW; time constant, 0.5 sec; sensitivity; 4000 counts full scale, rate; 0.25 sec, slit width; 6 cm^{-1}. Solid line: electric vector of the scattered radiation is parallel to that of the incident radiation. Broken line: electric vector of the scattered radiation is perpendicular to that of incident radiation.

* We have plotted the wave number of the band instead of the ratio of relative intensities ($I_{1533\ cm^{-1}}/I_{1545\ cm^{-1}}$) because of large uncertainties in estimation of the intensities.

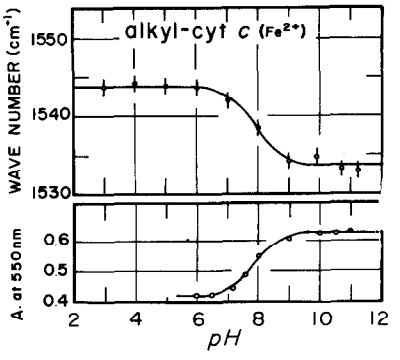

Fig.2. pH-dependence of the apparently observed wave number of the ligand sensitive Raman band (top) and that of optical absorbance at 550 nm (bottom) of ferrous alkylated cytochrome *c*.

state in the pH region between 3.0 and 11.0, because the Raman line characteristic of high spin state [3] never appears. Also the Raman spectra of alkylated cytochrome *c* between pH 3.0 and 11.0 and of native cytochrome *c* which is known to be in low spin state are alike to each other. The pH-dependence of resonance Raman and optical absorption spectra is, therefore, thought to correspond not to the transition between low spin and high spin states but to that between two kinds of low spin states.

The p*K* value of the transition in Raman spectra, 7.9 is in good agreement with the previously reported value, 8.0, determined by optical absorption spectra by Schejter and Aviram [4]. Brunori et al. [1] observed a similar transition by kinetic measurement and interpreted it as structural change associated with a change in coordination state of the heme iron between penta (high spin) and hexa (low spin) coordinations, coupled with protonation at the nitrogen atom of the ϵ-amino group of lysine-79. Dickerson et al. [5] proposed that the 6th coordination position of the heme iron of this modified cytochrome may be occupied by internal ligand, lysine-79 from the results of X-ray crystallographic studies, although Keller et al. [7] claimed that the position is vacant by NMR studies. As interpreted above, this transition has become clear to be between two kinds of low spin state, therefore lysine-79 is thought to be coordinated to the heme iron of this modified cytochrome in low spin state between pH 3.0 and 11.0. The transition observed for Raman bands at 1533 cm^{-1} and 1545 cm^{-1} may possibly be due to the change of the binding nature of lysine-79 followed by pH-dependent structural change of the protein. Similar transition from low spin to another type of low spin states without substitution for the 6th ligand was observed in ferric cytochrome b_5 in alkaline pH region [8]. It may be concluded that this pH sensitive band reflects a delicate change of the interaction between ligand and heme iron, though the origin of the band is not yet clear at the present stage.

Acknowledgements

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References

[1] Brunori, M., Wilson, M. T. and Antonini, E. (1972) J. Biol. Chem. 247, 6076–6081.
[2] Strekas, T. C. and Spiro, T. G. (1972) Biochim. Biophys. Acta 278, 188–192.
[3] Yamamoto, T., Palmer, G., Gill, D., Salmeen, I. T. and Rimai, L. (1973) J. Biol. Chem. 248, 5211–5213.
[4] Schejter, A. and Aviram, I. (1970) J. Biol. Chem. 245, 1552–1557.
[5] Dickerson, R. E., Takano, T., Kallai, O. B. and Samson, L. (1972) in: Structure and Function of Oxidation Reduction Enzymes (Akeson, A. and Ehrenberg, A. eds.) pp. 69–83, Pergamon Press, Oxford.
[6] Ikeda-Saito, M. and Iizuka, T. Anal. Biochem. in press.
[7] Keller, R. M., Aviram, I., Schejter, A. and Wüthrich, K. (1972) FEBS Lett. 20, 90–92.
[8] Ikeda, M., Iizuka, T., Takao, H. and Hagihara, B. (1974) Biochim. Biophys. Acta 336, 15–24.