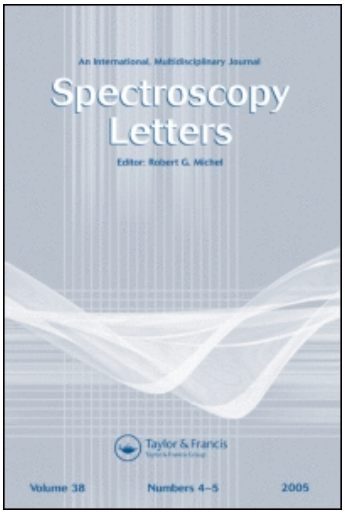

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DETECTION OF HEME-DISTORTIONS IN FERRI- AND FERROCYTOCHROME C BY RESONANCE RAMAN SCATTERING

Key words: Resonance Raman scattering, cytochrome c, distortions of the heme group

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ABSTRACT

We have measured the depolarization ratio and polarized intensities of the oxidation marker A_{1g}-Raman line at 1363 cm^{-1} and 1375 cm^{-1} in ferri- and ferrocytochrome c respectively. From these data we derive symmetry classified distortions of the heme group from its ideal D_{4h}-symmetry. In ferrocytochrome c these distortions are independent of the pH-value of the solution, indicating that the heme environment is stable with respect to pH. In ferricytochrome c we observe pH-dependent changes of the distortions, indicating the well known conformational change with pK ▫ 9.05, where the methionine 80 ligand is replaced by lysine-79. The data indicate that in this conformation due to opening of the heme crevice the heme group has a higher effective symmetry.

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INTRODUCTION

The investigation of the dependence of polarized Raman intensities (excitation profiles, EPs) and depolarization ratio (DPR) on the frequency of the exciting laser light has recently been proven as a suitable tool to obtain information on symmetry lowering distortions introduced into the porphyrin ring embedded into hemeproteins by heme-apoprotein interaction[1,2]. DPR and EPs of the oxyhaemoglobin A_{1g}-Raman line at 1375^{-1} and the B_{1g}-line at 1638 cm^{-1} show a complicated structure in the preresonant region between the Q- and B-absorption bands, which depends on the pH-value of the solution[1]. If the heme group were in its ideal D_{4h}-symmetry a wavelength independent DPR ($\varrho_{A_{1g}} = 0.125$, $\varrho_{B_{1g}} = 0.75$) is expected. The reason for the DPR-dispersion are symmetry lowering distortions on the porphyrin due to the asymmetric side chains, and due to interactions with the surrounding apoprotein matrix. By analyzing DPR and EPs using Loudon's time dependent perturbation theory one can obtain vibronic parameters, which are linearly related to symmetry classified distortions δQ^{Γ}($\Gamma = A_{1g}$, B_{1g}, A_{2g}, B_{2g}) of the heme. From the pH-dependence of these parameters conclusions on heme-apoprotein interaction can be drawn.

We have applied this method to cytochrome c in both the ferro- and ferri state, to investigate structural changes of the heme group. The heme and the hememoiety in cytochrome c can be regarded as one rigid structural unit, in which the existence of the heme determines the folding of the protein (even in the case when the porphytin iron is missing)[3]. The heme is embedded within a closed hydrophobic crevice by covalent bonds to cysteine-14 and -17 and axial ligand bonds to histidine-18 and the sulfur of methionine-80. The hydrophilic proprionic groups are compensated by several hydrogen bonds.

This is in contrast to hemoglobin, where the apoprotein folding is not determined by the existence of the heme and the heme is loosely embedded by only one covalent bond into the open crevice and its O_2-binding properties are modulated by heme-apoprotein interaction.

MATERIALS AND METHODS

Horse heart cytochrome c (type 6) was obtained from Sigma and used without further purification. The material was dissolved in 0.1 M buffer solutions (citrate for pH between 4.3 to 5.3, bis-tris pH = 5.5 to 7.3, tris pH = 7.5 to 9, and glycol pH = 9.1 to 11.3). The concentration was determined by absorption spectroscopy to be in the range 1.1 $\pm$ 0.1 mM. The samples were kept at a constant temperature 4° C $\pm$ 0.5° C. Ferrocytochrome c was prepared by addition of $Na_2S_2O_4$ and kept under N_2 atmosphere. The ferristate was obtained by addition of potassium ferricyanide. The Raman spectrometer has been described elsewhere[1].

RESULTS

Fig. 1 shows the DPR $\varrho = I_{\parallel}/I_{\perp}$ and the two polarized excitation profiles $I_{\parallel}$, $I_{\perp}$ of the ferrocytochrome c A_{1g}-line at 1363 cm^{-1} (oxidation marker line). The pH of the solution is 7.5. The measurements were repeated at the same pH for three freshly prepared samples. Within the error of measurement there is no pH-dependence between pH = 4.5 to 11. The full lines in Fig. 1 have been obtained by fitting simultaneously theoretical expressions of $I_{\parallel}$, $I_{\perp}$ and ϱ in terms of the Raman-tensor-components to the experimental data. The Raman-tensor is formulated by fifth-order time dependent perturbation theory. The deviation from the ideal D_{4h}-symmetry is incorporated into this formulation by symmetry lowering distortions δQ in terms of normal distortions $\delta Q^{\Gamma j}$,

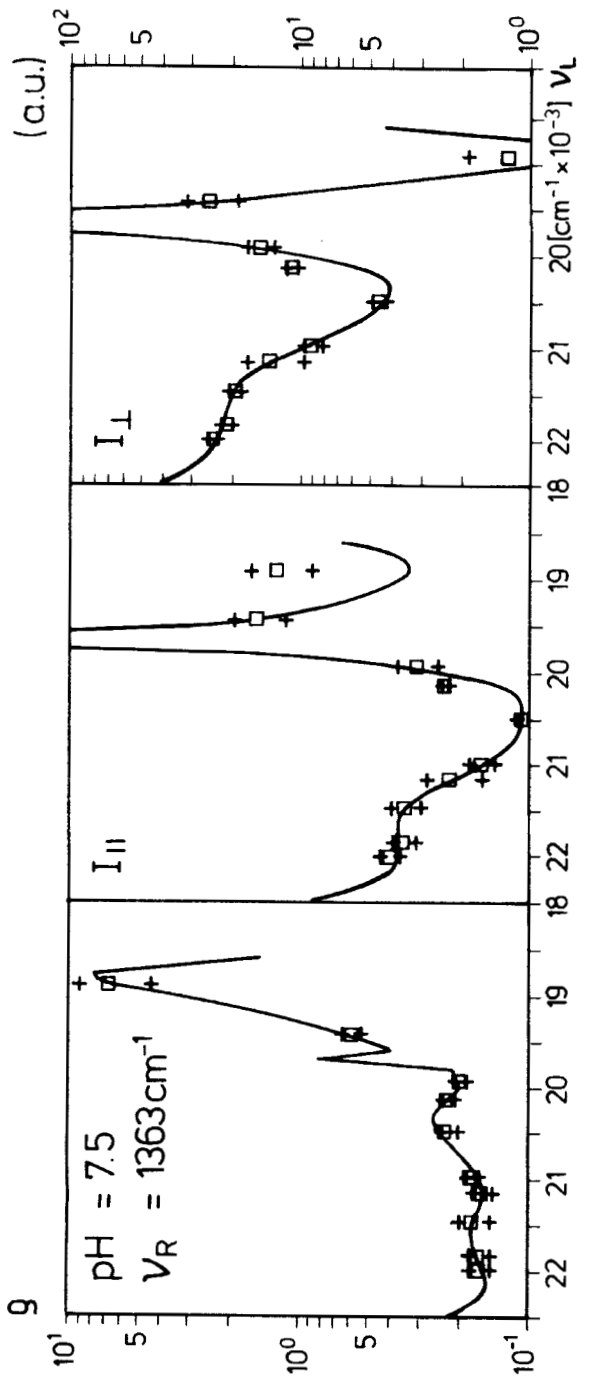

Fig. 1. Depolarization ratio and polarized intensities $I_{\parallel}$ and $I_{\perp}$ as a function of the frequency ν_L of the exciting radiation for the line at 1363 cm^{-1} (A_{1g}) in ferrocytochrome c at pH = 7.5. The full lines are obtained by fitting the experimental data. The intensities are given in arbitrary units. Note the logarithmic scales for 9 and I.

($\Gamma_j = A_{1g}, A_{2g}, B_{1g}, B_{2g}$) (Note that the distortions $\delta Q^{\Gamma j}$ in this case are not normalized)

$$\delta Q = \sum_{\Gamma_j} \delta Q^{\Gamma_j} \qquad (1)$$

The vibronic coupling operator is expanded about its ideal D_{4h}-symmetry in terms of the normal distortions $\delta Q^{\Gamma j}$

$$\frac{\partial H}{\partial Q^{\Gamma_R}} = \frac{\partial H}{\partial Q^{\Gamma_R}}_{/\delta Q = 0} + \sum_{\Gamma_j} \frac{\partial^2 H}{\partial Q^{\Gamma_R} \partial Q^{\Gamma_j}}_{/\delta Q = 0} \cdot \delta Q^{\Gamma_j} \qquad 2)$$

Γ_R: representation of the Raman-active vibration. Introducing this into the perturbation formalism leads to constants $C_{e,s}^{j}$, which are linearly related to symmetry classified distortions $\delta Q^{\Gamma j}$ if $\Gamma_R = A_{1g}$ (as in the case here), and represent the vibronic coupling elements of eq. (2) between the intermediate electronic heme-states e = Q, B and s = Q, B causing absorption in the B- and Q-bands.

These parameters are used as free fitting parameters. The fits are predictive as has been shown in [2], since in oxyHb the knowledge of the data obtained by use of 10 Ar^+-laser lines is sufficient to predict the experimentally observed DPR of three Raman lines (1375 cm^{-1}, 1583 cm^{-1}, and 1638 cm^{-1}) for the region of the Q-bands.

Fig. 2 shows the pH-dependence of the sums of these parameters

$$\sum A_{1g} = \left(\sum_{e,s} |C_{e,s}^{A1g}|^2 \right)^{1/2} \qquad 3)$$

$$\sum B = \left(\sum_{e,s} |C_{e,s}^{B1g}|^2 + \sum_{e,s} |C_{e,s}^{B1g}|^2 \right)^{1/2}$$

$$\sum A_{2g} = 2 \cdot |C_{Q,B}^{A2g}|$$

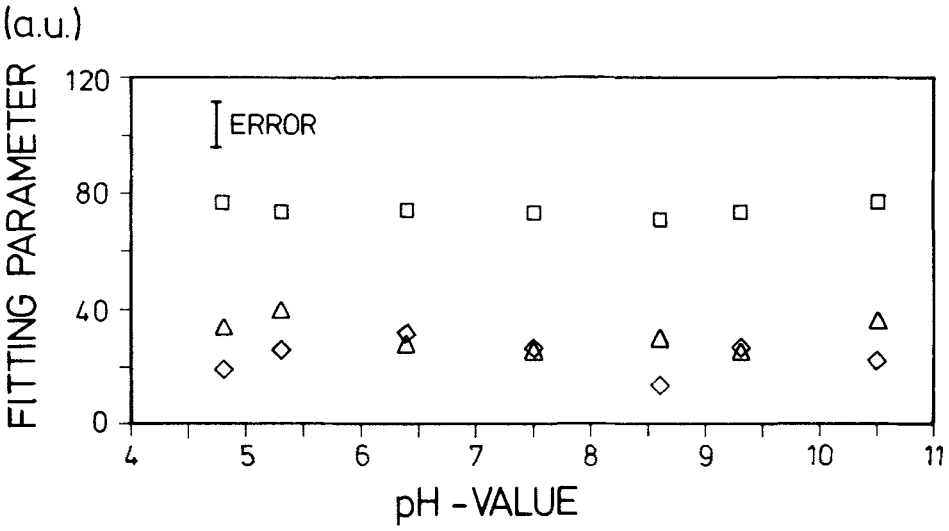

Fig. 2. Fitting parameter sums as defined by eq. (3) for ferrocytochrome c. These sums are linearly related to perturbing symmetry classified distortions of representation $\Gamma(A_{1g}, B_{1g} + B_{2g}, A_{2g})$. The units are arbitrary. □ A_{1g}, Δ $B_{1g} + B_{2g}$, ◇ A_{2g}. The error is indicated by the bar.

The presence of A_{1g}, B_{1g}, A_{2g}, B_{2g} distortions shows that the heme symmetry is lowered from D_{4h} to C_s. The independence of the distortions on pH confirms prior findings showing that ferrocytochrome does not show structural changes between pH= 4.5 to 11. This is not the case for ferricytochrome c, where a pH-dependent conformational transition occurs with pK = 9.05 [3,4]. In this conformational change the sulfur-iron bond between the heme and methionine-80 is broken and most likely replaced by a bond to the ε-amino group of lysine-79. We therefore have measured the DPR and EPs of the oxidation marker A_{1g}-line at 1375 cm^{-1} for ferricytochrome c in the range of pH = 5.0 to 11. Fig. 3 shows two representative examples. Although at a first glance there

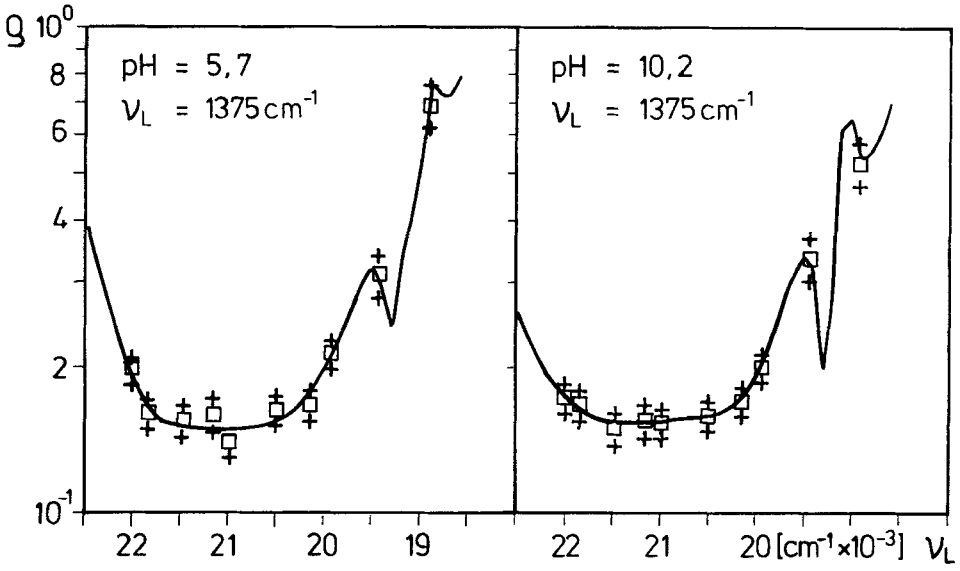

Fig. 3. Depolarization ratio dispersion ϱ for the line at 1375 cm^{-1} in ferricytochrome c for pH = 5.7 and pH = 10.2. The full lines are obtained by fitting the experimental data. Note the logarithmic scale for ϱ .

seems to be no significant difference in the DPR, this is not so. The DPR at the highest-energy laser line is different beyond the error of measurement in the two extreme pH-values and the same is the case for the line at lowest energy.

As the resulting fits show (full lines) this effect becomes larger in the region towards the B-band absorption (ν_L ▫ 22000 cm^{-1}). Measurements with a tunable laser are planned to ascertain this. Fig. 4 shows the resulting sums of the fitting parameters as a function of pH. Clearly a variation of the distortions in the region of pH = 9 is seen, reflecting the structural transition.

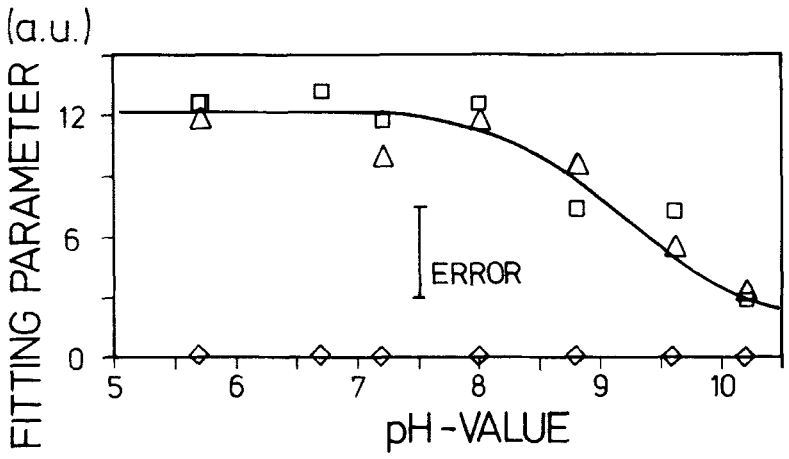

Fig. 4. pH-dependence of the fitting parameter sums as defined in eq. (3) for ferricytochrome c. These sums are linearly related to perturbing symmetry classified distortions of representation (A_{1g}, $B_{1g} + B_{2g}$, A_{2g}). The units are arbitrary. □ A_{1g}, △ $B_{1g} + B_{2g}$, ◇ A_{2g}. The error is indicated by the bar. The full line is calculated from eq. (4) and (5), with pK = 9.0.

To interpret these data, we have to note that two differently distorted conformations of the porphyrin are present now, which are in equilibrium with each other by

$$X_1 \cdot [H^+] = K \cdot X_2 \qquad (4)$$

where x_1, x_2 are the mole fractions of the conformations stable at very high and very low pH, respectively. In this case the Raman intensity is due to incoherent superposition of the two scattered intensities and a pH-dependent effective $C_{e,s}(pH)$ can be formulated[1] by

$$\left(C^{\Gamma}_{e,s}(pH)\right)^2 = X_1 \cdot \left(C^{\Gamma,1}_{e,s}\right)^2 + X_2 \cdot \left(C^{\Gamma,2}_{e,s}\right)^2 \qquad (5)$$

The full line in Fig. 4 thus represents a "Raman titration" curve using the sums of $C^{\Gamma,\nu}_{e,s}$, ν = 1, 2 and K as fit-

ting parameters. The pK-value obtained is 9.0, in excellent agreement to that of 9.05 obtained for horse ferricytochrome c by titration of the absorption band at 695 nm[4].

DISCUSSION

From the data in Figs. 2 and 4 we conclude that the heme-environment in both forms of cytochrome c remains unaltered in the physiological region, maintaining thus a constant reduction potential. In the ferristate reduction of the perturbing distortions occurs for state 1 stable at high pH, indicating that this conformation is the more relaxed one with a higher effective symmetry. In this state the heme is more exposed to the exterior, thus relaxing the constraints at the propionic acid groups. A similar effect was found by observation of the Raman spectra in the conformational transition with pK = 12 for ferrocytochrome[5], where Raman spectra indicate a higher effective symmetry for the conformation stable at pH ◻ 13. In this state the heme crevice opens and ferrocytochrome becomes reactive to external ligands[3].

The results show that to maintain the high reduction potential, needed for the synthesis of ATP, the protective hydrophobic environment exerts a symmetry lowering constraint onto the heme group.

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