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Spectroscopic Studies of Hemes in Electron Carrier c-Type Cytochromes and Its Model Compounds

Miriam Abe[1], Toshihiko Nagamura[1,3], Hideyuki Itagaki[2] and Tatsuhiko Yagi[2]

[1]*Graduate School of Electronic Science and Engineering, Shizuoka University, 3-5-1 Johoku, Hamamatsu 432-8011, Japan*

[2]*Department of Chemistry, Faculty of Education, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*

[3]*Crystalline Films Laboratory, Research Institute of Electronics, Shizuoka University, 3-5-1 Johoku, Hamamatsu 432-8011, Japan*

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1. Introduction

Recently, the photophysical processes of iron-free cytochromes had attracted our interest because they can be expected as an opto-electronic protein device for novel optical memory by means of photochemical hole burning [1,2]. Throughout the previous works, c-type cytochromes were found to behave like iron-free cytochromes in acidic solutions from the standpoint of photophysics. It would be more efficient if acid solution of c-type cytochromes could be used as a device. Among c-type cytochromes, cytochrome c is the one extensively studied with several spectroscopic techniques such as absorption, fluorescence and circular dichroism (CD) measurements [3-10]. However, the photophysical properties of the heme of other c-type cytochromes have not been clarified so much compared to those of cytochrome c. The purpose of this research is to clarify photophysical processes of the c-type cytochromes (c, c-553 and c_3), their model compounds, microperoxidase-9, and hemin in acid solutions.

2. Experimental

2.1. Sample

Cytochrome c (type IV from horse heart) is monohemic and contains 104 amino acid residues, possessing a molecular weight (MW) of 11,700. The axial ligands of the iron atom are methionine and histidine. Cytochromes c-553 and c_3 are electron carrier proteins extracted from the sulfate-reducing bacterium *Desulfovibrio vulgaris*, Miyazaki. Cytochrome c-553 is monohemic and has a polypeptide chain with 79 amino acids, possessing a MW of 9,000. The iron atom of the cytochrome c-553 is coordinated with methionine and histidine [11,12]. Cytochrome c_3 has four hemes and contains 107 amino acid residues, possessing the MW of 14,000 [13]. The heme distances range from 11.0 Å to 17.8 Å [14]. The fifth and sixth ligands of the iron atoms are both histidines [14]. Hemin does not have amino acid residues, therefore there is no axial ligands of amino acid to the iron heme. Microperoxidase-9 (MP-9) has one heme and contains 9 amino acid residues, possessing a MW of 1,630 and one histidine ligand to the iron atom [15]. The solvent used for all the measurements was 10 mM Tris-HCl pH 7.2. The pH of solutions was adjusted by addition of negligible volume of 6 M HCl and 10 M NaOH.

2.2. Measurements

Uv and visible absorption spectra of the samples were measured with a Shimadzu UV-2200 spectrophotometer. Fluorescence spectra and fluorescence excitation spectra were measured

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Table I: Uv and visible absorption peak wavelengths for cytochromes, MP-9 and hemin. The wavelengths underlined are the values for the Soret band.

	pH = 7.2	pH < 2.0	pH > 12 .0
cytochrome c	406, 530	393, 494, 620	406, 530
cytochrome c-553	409, 525	394, 499, 620	409, 537
cytochrome c_3	408, 530	370, 497, 637	406, 537
MP-9	395, 507, 622	392, 493, 619	404, 535
hemin	383, 613	374, 646	385, 605

with a Hitachi F-4500 spectrofluorometer at room temperature (25°C).

3. Results and Discussion

Table I shows the wavelengths at maximum absorbance of the Soret band and the Q-band observed for the samples studied in this work. The peak wavelengths of the Soret band, for the cytochromes c, c-553 and c_3 at pH=7.2 are almost identical with one another (at around 408 nm). However the absorption spectra of MP-9 and hemin at neutral pH were found to be different from those of the above c-type cytochromes: the maxima for the Soret bands were blue-shifted compared to them and other absorption peaks appeared at 613 nm for hemin and at 622 nm for MP-9. According to earlier works [4,8-10], histidine and methionine, strong-field ligands of the heme iron, of the cytochrome c were assumed to be replaced in acid solution by weak-field ligands such as oxygen or halogens supplied by the solvent, because the acidification induces spectral change in uv and visible absorption. In fact, the conformational transition of cytochrome c due to the acidification was ascertained by the measurements of viscosity and CD [4,8,10]. Coordination with two weak-field ligands was considered to produce a high-spin complex having the Soret maximum between 390 and 395 nm [4,8-10]. The high-spin complex and the equilibrium mixture of spin configurations, one strong-field ligand and one weak-field ligand complex, were presented to be characterized by a maximum at 620 nm [8,10]. Thus, the appearance of 620-nm band is quite reasonable in the present absorption spectra of hemin and MP-9 at neutral pH.

First, we tried to prove this assumption [4,8-10] by using MP-9 which has only one axial ligand: if histidine or methionine is added to MP-9, the axial ligands to the iron heme would be the same as cytochrome c_3 (histidine, histidine) or c-553 (histidine, methionine).

Fig. 1 shows the influence of L-histidine on the absorption maximum of Soret band when added to oxidized form of MP-9. The addition of histidine

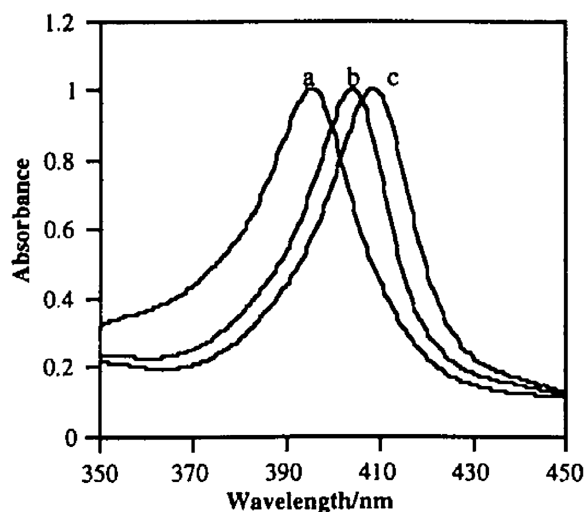

Fig. 1: Uv-visible spectra of Soret maxima of MP-9 (a), MP-9 upon addition of histidine (b), and cytochrome c_3 (c). Addition of histidine is about four times as much as the molar concentration of the heme.

shifted the absorption spectrum of MP-9 to the red and made it close to that of cytochrome c3, which has two histidine ligands: the Soret peaks are 404 nm for MP-9 with histidine and 408 nm for cytochrome c_3, respectively. At lower pHs where histidine must be detached from iron of heme, the spectra of MP-9 did not change so much even when histidine was added. Methionine was also

added to the aqueous solutions of MP-9 at various pHs, however, there was no change observable in the uv and visible absorption spectra.

Fig. 2 shows the influence on the Q-bands of the reduced and oxidized MP-9 upon addition of L-histidine and L-methionine. The samples were reduced by addition of very small amount of sodium hydrosulfite. The ferro form of c-type cytochromes has typical absorption peaks at 553 or 552 nm (α peak) and 524 nm (β peak). It is worthwhile to mention that the purity of cytochrome c's can be assessed by the appearance

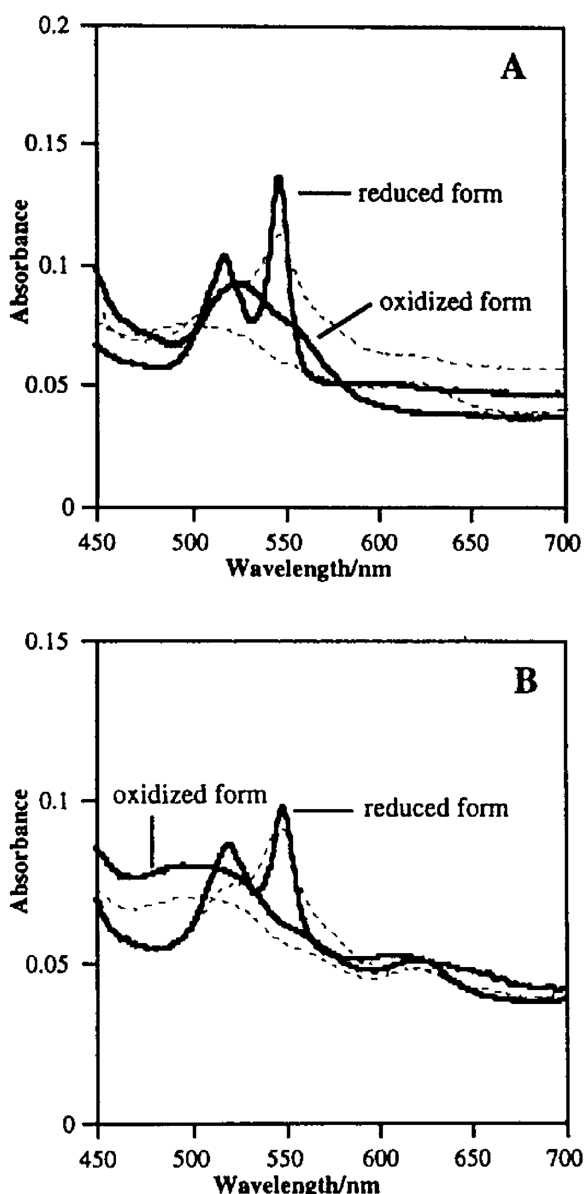

Fig. 2: Q-bands absorption in spectral changes upon addition of histidine (A) and methionine (B) to the oxidized and reduced form of MP-9. The solid lines are for the spectral changes upon addition of histidine and methionine.

of these sharp peaks in visible spectrum when they are reduced. Fig. 2 indicates that the absorption of ferro form is not so clear as that when histidine and methionine are added. The 549 and 518 nm bands of the reduced form of the MP-9 became sharper and increased more when histidine was added: actually the ferro form of MP-9 with histidine almost agrees with that of cytochromes c, c-553 and c_3. Thus, the spectral change is shown to be explicable by what the ligands of the heme iron are. This reveals the importance of the presence of the two ligands to the heme-iron to show a very defined reduced form spectrum, which is the characteristic of the cytochromes. In the case of methionine, the ferro form of MP-9 has slightly broad peaks, although the absorption maxima of the reduced form are 549 and 520 nm.

Thus far, most spectral changes observed in absorption of c-type cytochrome have been able to be reproduced by using a model compound, MP-9, and histidine. For example, the absorption spectrum of MP-9 at low pH is almost identical with the spectra of cytochrome c and c-553 at low pH. It can be explained by the formation of a high-spin complex from which histidine is detached. However, one big question is why the absorption spectrum of cytochrome c_3 at low pH is different from those of other cytochrome c's and rather similar to the spectrum of hemin (see Table I): the Soret band appears at 370 nm, which is shifted by 24 nm in comparison with the Soret peaks of other c-type cytochromes.

This can be attributed to the influence of the environment around the heme. Degree of hydrophobicity around a heme can be estimated by using hydropathy value for each amino acid given by Kyte and Doolittle [16]. The average hydropathy values of 11-13 amino acids near the heme for cytochromes c, c-553 and MP-9 were +0.25, +0.29, and +0.22. These positive values indicate that the microenvironments around the heme of these compounds are less polar and hydrophobic. On the other hand, the average hydropathy values of 11-13 amino acids near each heme for cytochrome c_3 are -0.34, -0.73, +0.42, and -0.48. Although one heme is under less polar condition, the average value is -0.28, indicating that the hemes of cytochrome c_3 are under polar condition and are anticipated to have higher interaction with water. Thus the absorption

spectrum of cytochrome c_3 in acid solution is considered to agree with that of hemin, which has no peptide chain and is directly in contact with water molecules in acid solution.

4. Conclusions

It was clarified in this work the influence of the axial ligands (methionine and histidine) and pH to the photophysical properties of cytochromes c, c-553 and c_3, MP-9 and hemin. Besides the influence of the axial ligands and pH on the cytochrome, the polarity around the heme produced by the amino acid residues is also very important. Now, by comparing the photophysical properties of the cytochromes here studied and the photophysical properties of iron-free cytochromes c-553 and c_3, we can have a better comprehension of the latter ones which are hopeful proteins to be used as novel optoelectronic devices and they will be reported in near future.

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