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Photophysical properties of cytochromes *c*-553 and c_3 extracted from *Desulfovibrio vulgaris* Miyazaki

Miriam Abe[a], Tatsuhiko Yagi[b], Hideyuki Itagaki[b], Toshihiko Nagamura[a,c,*]

[a]*Graduate School of Electronic Science and Technology, Shizuoka University, 3-5-1 Johoku, Hamamatsu 432-8011, Japan*
[b]*Faculty of Education, Department of Chemistry, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan*
[c]*Crystalline Films Laboratory, Research Institute of Electronics, Shizuoka University, 3-5-1 Johoku, Hamamatsu 432-8011, Japan*

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Abstract

Fluorescence and UV/visible absorption properties were studied on electron carrier proteins cytochromes *c*-553 and c_3 and their Fe-free forms (Fe-free cytochromes) over wide pH and temperature ranges in comparison with cytochrome *c*, hemin and microperoxidase-9 (MP-9). Although cytochromes *c*-553 and c_3 were extracted from the same sulfate-reducing bacterium, their absorption spectra at acidic pH and pH dependence of fluorescence spectra were different. Cytochrome *c*-553 showed photophysical characteristics similar to cytochrome *c*. This is attributed to the influence of the ligands of the fifth and sixth positions of the heme iron in cytochromes *c*-553 and c_3, which was confirmed by the experiments on MP-9. Through the temperature-dependent fluorescence spectrum and time profile, the coexistence of protonated and deprotonated forms of Fe-free cytochromes at neutral pH was observed. The fluorescence in native cytochromes was observed at acidic pH, whereas MP-9 and hemin showed no fluorescence at the same condition.

Keywords: Cytochrome *c*-553 and c_3; Microperoxidase-9; Porphyrin; Heme; Fluorescence spectrum; UV/visible spectrum

1. Introduction

Among *c*-type cytochromes, cytochrome *c* is the one extensively studied with several spectroscopic techniques such as absorption, fluorescence, circular dichroism (CD), etc. [1–8]. Cytochrome *c* is monohemic and contains 104 amino acid residues, possessing a molecular weight of 11 700. The axial ligands of the iron atom are methionine and histidine in cytochrome *c* [9]. The conformation of the protein and the ligation state of the heme are the main interests in such studies, but the photophysical properties of the heme of the cytochrome have not been clarified so much.

Cytochrome *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 molecular weight of 9000. Its standard redox potential is +26 mV at pH 7.0 [10]. 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 a molecular weight of 14 000 [13]. The heme distances in the cytochrome c_3 range from 11.0 to 17.8 Å [14]. The standard redox potential ranges from −230 to −360 mV [15], which is much lower than other ordinary *c*-type cytochromes. Both the fifth and the sixth ligands of the iron atoms are histidines in cytochrome c_3 [14]. The electronic states of the iron heme in the cytochromes studied here are in the Fe(III) state (ferric) unless otherwise mentioned.

The Fe-free cytochromes may be used as one of the promising materials to develop an optoelectronic protein device such as novel optical memory by means of photochemical hole burning (PHB). In order to use cytochromes *c*-553 and c_3 as a component of optoelectronic devices, it is essential to know their spectroscopic properties and also those of Fe-free forms under various conditions. Specially, the study of protonation of the porphyrin moiety of cytochromes is very important due to the occurrence of the tautomerism inside the porphyrin, which is the basis for the PHB method. We recently reported excited state properties of cytochrome *c*-553 and its Fe-free form studied with an fs laser [16]. No transient absorption was observed upon fs laser excitation of cytochrome *c*-553. Removal of an iron

*Corresponding author. Fax: +81-53-478-1306; e-mail: nagamura@rie.shizuoka.ac.jp

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atom resulted in a transient absorption with a peak at 450 nm of ps–ns lifetimes [16]. The cytochromes c_3 and *c*-553 do not show fluorescence at neutral pH. However, by removing the iron atom of the hemes [17] or acidifying the medium [1,2,6–8], they come to show fluorescence.

The present paper deals with the study of fluorescence and absorption behavior of the Fe-free and acidified cytochromes *c*-553 and c_3 by comparison with other porphyrins or hemes: cytochrome *c*, hemin and microperoxidase-9 (MP-9) covalently bound to 9 amino acid residues, including histidine, from 14th to 22nd of cytochrome *c*, possessing a molecular weight of 1630 [18].

2. Experimental

2.1. Materials

The structures of the hemes and porphyrins of the proteins employed in this work are shown in Fig. 1.

Desulfovibrio vulgaris Miyazaki was cultured as described before [13]. Wet cells of *Desulfovibrio vulgaris* Miyazaki were suspended in 5–6 volumes of H_2O and disintegrated with an ultrasonic disintegrator (UR-200P, Tomy Seiko, Tokyo) at 20 kHz, 180 W, for 12 min to produce a bacterial sonicate, by which cytochromes *c*-553 and c_3 were purified as described before [10,19].

Fe-free cytochromes c_3 and *c*-553 were prepared as described before [17,20]. A 15 mg sample was placed in an open Teflon beaker and cooled by suspending in a Dewar flask containing liquid nitrogen. Anhydrous HF (pressure of 0.2 kg/cm^2) was passed into the beaker for 3 min, and the cytochromes turned purple. Excess HF was removed under a stream of nitrogen at room temperature. The Fe-free cytochrome was dried under reduced pressure for 24 h. Then it was dissolved in a 5 ml of 10 mM Tris–HCl pH 7.2, and purified through a Sephadex G-50 chromatographic column. Tris–HCl (50 mM) buffer containing 0.2 M NaCl was used as an eluent. The purified Fe-free cytochromes were eluted a little earlier than the native cytochromes, which indicate that the molecule is somewhat unfolded. The fractions of the Fe-free cytochromes were collected, dialyzed against distilled water and were frozen dried.

The Fe-free cytochrome *c* (type IV from horse heart) and Fe-free MP-9 were prepared by the same method as described above. Hemin was purchased from Wako and MP-9 from Sigma. The sample solutions were acidified with 1 M or 6 M HCl aqueous solutions and alkalized with 1 M or 12 M NaOH aqueous solutions.

2.2. Measurements

The UV/visible absorption spectra were measured with a Shimadzu UV-2000 spectrophotometer. The fluorescence spectra and fluorescence excitation spectra were measured with a Hitachi F-4500 spectrofluorometer at room temperature (25°C). Fluorescence decay measurements were carried out with a Horiba NAES550 single-photon counting machine. The emission of Fe-free cytochrome *c*-553 in degassed Tris–HCl solution (pH 7.2) was measured at 25°C through an interference filter (580, 630, 652, 680 nm) and that of Fe-free cytochrome c_3 was measured

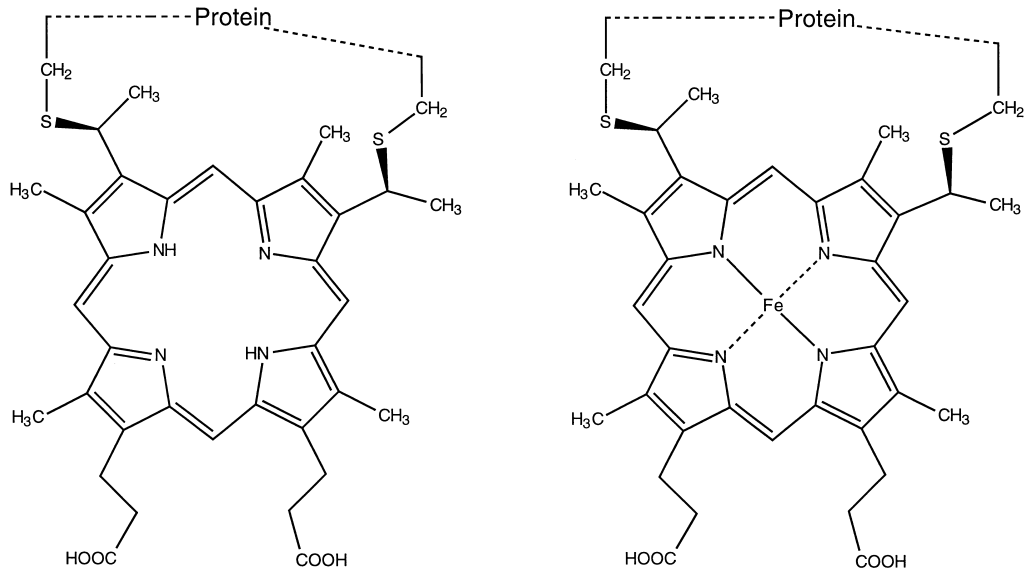

Fig. 1. Structures of (A) a porphyrin and (B) a heme.

through an interference filter (580, 618, 652 nm). The excitation light source is a nanosecond lamp (NFL-111A) which is separated through a B-390 filter with the transmittance peak at 390 nm. The decay curves were analyzed by the deconvolution method after O'Connor and Phillips [21]. To assess the validity of the trial fitting function, Durbin–Watson factor (DW) was used [22]. It approached the value of 2.0 for the best fit. When it was necessary the sample solutions for the fluorescence measurements were degassed by freeze-pump-thaw cycles under high vacuum. The concentration of the sample solutions was confined to less than 0.09 mM.

3. Results

Fig. 2 shows the Soret bands of the cytochromes (A) *c*-553 and (B) c_3 at neutral, acidic and basic media. The Soret band absorbance of cytochrome c_3 decreased and broadened at low pH, while one of the cytochrome *c*-553 was very similar and increased when compared with, at neutral pH. In basic media the absorbance of the Soret band of cytochrome c_3 decreased, while the absorbance of the cytochrome *c*-553 slightly increased.

Table 1 summarizes the wavelength at maximum absorbance of the Soret band and the Q-band observed for native and Fe-free forms of cytochromes, MP-9 and hemin at several pHs.

All cytochromes in the oxidized form at neutral pH showed similar wavelengths at maximum absorbance of the Soret band, 406–409 nm. The MP-9 and hemin showed a peak at shorter wavelengths, 395 and 383 nm, respectively. At the acidic conditions, the Soret band of the cytochromes considerably blue-shifted. The cytochrome c_3 showed the largest shift among cytochromes, from 408 to 370 nm. The shift of the Soret band of the MP-9 from neutral pH to acidic pH was very small. The absorption at around 530 nm for the cytochromes at neutral pH disappeared at very low pHs and new absorption at around 620 nm appeared. However, MP-9 and hemin showed such a peak at 622 and 613 nm, respectively.

One interesting feature of the spectra of the Fe-free cytochromes *c*-553 and c_3 at low pH is the appearance of a clear shoulder at around 390 nm of the Soret band in addition to a peak at 400 nm as shown in Fig. 3. It overlapped the Soret band at neutral pH which suggested the coexistence of two species, the protonated form and deprotonated form, respectively. The Soret band of the Fe-free MP-9 at neutral pH was remarkably blue-shifted as compared with Fe-free cytochromes. The Soret band peak at 378 nm is similar to that of hemin at acidic pH, 374 pm. The

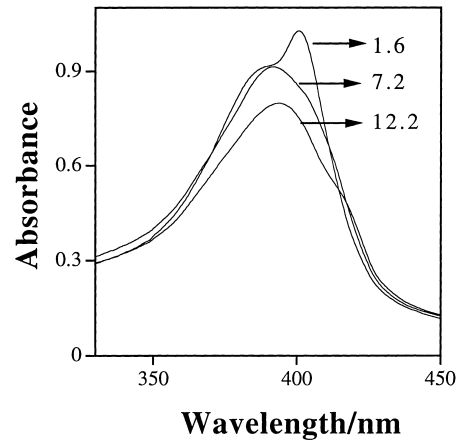

Fig. 3. Soret band absorption spectra of the Fe-free cytochrome c_3 at several pHs. The pH is shown in the figure. Samples in Tris–HCl 10 mM at 25°C.

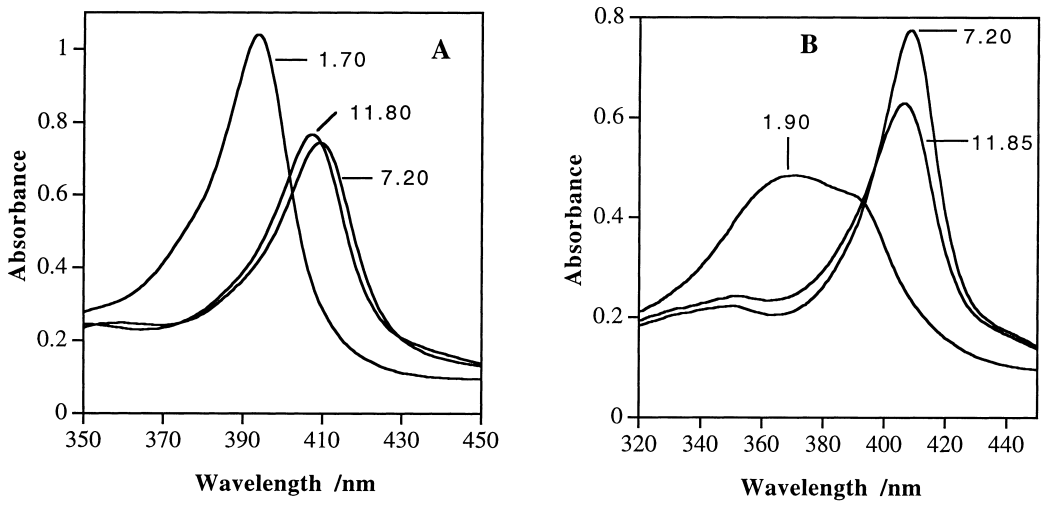

Fig. 2. Soret band absorption spectra of the iron porphyrins: (A) cytochrome *c*-553; (B) cytochrome c_3 at several pHs. The pH is shown in the figure. Samples in Tris–HCl 10 mM, at 25°C.

Table 1
UV/visible maximum absorbances wavelengths for porphyrins and hemes

	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
Fe-free cytochrome *c*	*402*, 503, 540, 568, 620	*404*, 551, 600	*398*, 502, 537, 565, 621
Fe-free cytochrome *c*-553	*398*, 504, 539, 568, 620	*405*, 550, 555	*398*, 503, 539, 565, 617
Fe-free cytochrome c_3	*391*, 500, 540, 573, 624	*400*, 498, 545	*394*, 499, 541, 568, 617
Fe-free MP-9	*378*, 503, 536, 568, 622	*405*, 551, 592	*396*, 502, 538, 562, 614

Samples in Tris–HCl 10 mM at 25°C. Acidification upon addition of HCl 6 M and alkalinization upon addition of NaOH 10 M. The italicized wavelengths are the maximum absorbances of the Soret band wavelengths.

Q-bands characteristic to the free base porphyrins, i.e., 503, 540, 568, and 620 nm [17,23], also were observed for the Fe-free cytochromes *c*-553, c_3 and MP-9. However, at acidic pH, these bands disappeared and a single band with a peak at around 550 nm was observed for cytochromes *c* and *c*-553. The Soret bands' wavelengths of the Fe-free cytochromes and Fe-free MP-9 at acidic pH were very similar, 400–405 nm. At basic pH, the Fe-free cytochromes and Fe-free MP-9 showed almost the same absorption spectra for the Soret and Q-bands as those at neutral pH. Fe-free MP-9 showed the largest red-shift of the Soret band from 378 to 396 nm.

L-histidine and L-methionine were added to MP-9 in order to study the influence of ligands on the spectroscopic properties of cytochromes and their pH dependences. Fig. 4 shows the changes of the Soret band by the addition of L-histidine to MP-9. The addition of L-histidine resulted in a red-shift of the Soret band from 395 to 404 nm at neutral pH, but there were no shifts at acidic and basic conditions as shown in Fig. 4. The addition of L-methionine hardly affected the absorption spectra of the MP-9, except a slight increase of the Soret band without a shift at basic pH.

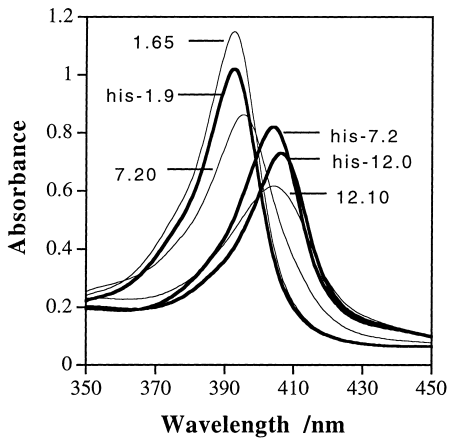

Fig. 4. Spectral changes upon addition of histidine to MP-9 at various pHs. The pH is shown in the figure. Samples in Tris–HCl 10 mM, at 25°C.

The cytochromes, MP-9 and hemin showed no fluorescence upon excitation at 400 nm at neutral pH. However, upon acidification to pH lower than 2.0, the cytochromes revealed weak fluorescence. Once this fluorescence appeared, it was observed even when the pH of the solution was adjusted to be basic. The Fe-free cytochromes and Fe-free MP-9 showed fluorescence in the whole pH range as shown in Table 2.

The protonation of the free base porphyrin (PH_2) at acidic pH will occur with the addition of two protons as follows:

$$PH_2 \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} PH_3^+ \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} PH_4^{2+}$$

The Q-band absorption spectra of Fe-free cytochromes and Fe-free MP-9 showed only two peaks at pH<2.0 as shown in Table 1. These results indicate that the protonated forms are all PH_4^{2+}. The fluorescence spectra of Fe-free cytochromes showed two characteristic bands at neutral pH, 620 and 680 nm and showed two more bands at acidic pH, about 590 and 650 nm, showing the existence of only two species (PH_2 and PH_4^{2+}). Thus, the protonated form of the porphyrin (PH_4^{2+}) is expressed by the 590 and 650 nm peaks. The neutral form (PH_2) is expressed by the 620 and 680 nm peaks. In Fig. 5, no other peaks were found indicating that the protonation to two free nitrogen atoms in porphyrin occurred at the same time. Therefore, the protonation tendency of porphyrins was best monitored by the fluorescence spectrum. The pH dependences of fluorescence spectra for the Fe-free cytochromes at the same excitation wavelength shown in Fig. 5 give us the pK_a of the Fe-free cytochromes. The pK_a for the Fe-free cytochrome c_3 is higher than those of the cytochromes *c* and *c*-553 as the 590 and 650 "acidic" peaks are formed at higher pHs (around pH 3.4) than the other two cytochromes (around pH 2.3). Taking into account that the polarity of microenvironment around the porphyrin of Fe-free cytochrome c_3 is higher than the Fe-free cytochrome *c*-553 and MP-9, it leads to a higher protonation tendency than the other two ones. The fluorescence spectrum is dependent on the excitation wavelength as shown in Fig. 5

Table 2
Wavelengths of maximum fluorescence intensity for porphyrins and hemes

	pH=7.2	pH<2.0	pH>12.0
Cytochrome *c*	No fluorescence	Weak fluorescence	Weak fluorescence
Cytochrome *c*-553	No fluorescence	Weak fluorescence	Weak fluorescence
Cytochrome c_3	No fluorescence	Weak fluorescence	Weak fluorescence
Hemin	No fluorescence	No fluorescence	No fluorescence
MP-9	No fluorescence	No fluorescence	No fluorescence
Fe-free cytochrome *c*	(584), 620, 683	596, (616), 653	618, 680
Fe-free cytochrome *c*-553	(580), 619, 683	596, (617), 653	(586), 611, 680
Fe-freecytochrome c_3	(578), 615, 676	594, (614), 651	(582), 616, 677
Fe-free MP-9	617, 680	597, (619), 655	616, 678

Solvent used was Tris–HCl 10 mM, at 25°C. Acidification upon addition of HCl 6 M and alkalinization upon addition of NaOH 10 M. Excitation at 400 nm. The values in parenthesis correspond to a very small peak or a shoulder.

at pH 7.2, which strongly suggested the coexistence of at least two species.

The fluorescence spectra of all Fe-free cytochromes and Fe-free MP-9 were reproducibly observed upon repeated changes of pH between acidic and basic pHs. One interesting feature of the fluorescence spectra of Fe-free cytochromes *c*-553 and c_3 is the appearance of a third peak at 580 nm and a shoulder around 650 nm at neutral and basic pHs as shown in Fig. 6(A) and (B), respectively. The relative intensity of the third fluorescence band clearly depended on the excitation wavelength as shown in Fig. 6. The fluorescence excitation spectra for Fe-free cytochromes and Fe-free MP-9 at acidic and basic pHs corresponded to the absorption spectra at each condition.

Fig. 7 shows fluorescence spectra of Fe-free cytochromes: (A) c_3; (B) *c*-553; and (C) MP-9 in glycerol–water as a function of temperature. They showed two strong peaks at 620 and 680 nm at room temperature. However, the fluorescence spectra showed a blue-shift and increase in the peak intensity at 580 and 650 nm, and also showed the decrease in the peak intensity at 620 and 680 nm during cooling. After warming up to room temperature, all fluorescence spectra recovered to the original ones observed at the beginning of the experiment. These results strongly suggest the coexistence of at least two fluorescent species and reversible changes dependent on temperature. The fluorescence spectra of Fe-free MP-9 in an aprotic solvent, a-picoline (2-methylpyridine), are shown in Fig. 8 at 296.9 and 210.0 K. In contrast to Fig. 7(C), no increase in the 580 and 650 nm peaks and no decrease in 620 and 680 nm were observed even at 210.0 K.

The fluorescence decay curves also revealed the existence of two components. They were well fitted by a doubly exponential equation for Fe-free cytochromes *c*-553 and c_3 at pH 7.2 in Tris–HCl solution,

$$I(t) = A_1 \exp(-t/t_1) + A_2 \exp(-t/t_2),$$

where $A_1+A_2=1$, t_1 and t_2 are the lifetimes of a fast and a slow component, respectively. The observed data are summarized in Table 3.

The fluorescence decay at 580 nm of the Fe-free cytochrome *c*-553 showed only one component with the lifetime 2.3 ns, whereas the Fe-free cytochrome c_3 showed two components at the same wavelength. From the fluorescence lifetime of the cytochrome c_3 and *c*-553 the peaks at 580 and 650 nm were assigned to the same species, which is the protonated form. The fluorescence decay at 620 or 629 nm, which is characteristic to the neutral form, showed two components for both cytochromes. The decay at 680 nm, which is assigned to the same species as the 620 nm, also showed two components. In all cases the fast component was a major one, $A_1=0.76\sim0.99$.

Table 3
Results of fluorescence decay measurements for iron-free cytochromes *c*-553 and c_3 at several wavelengths

Wavelength per nm	A_1	τ_1 (ns)	A_2	τ_2 (ns)
Iron-free cytochrome *c*-553				
580	1.00	2.3	–	–
630	0.95	2.2	0.053	14.0
652	0.89	2.4	0.11	13.0
680	0.76	2.2	0.24	13.0
Iron-free cytochrome c_3				
580	0.99	2.3	0.008	10.0
618	0.98	2.1	0.025	13.0
652	0.98	2.3	0.020	10.0

The samples were in 10 mM Tris–HCl at 25°C, under vacuum.

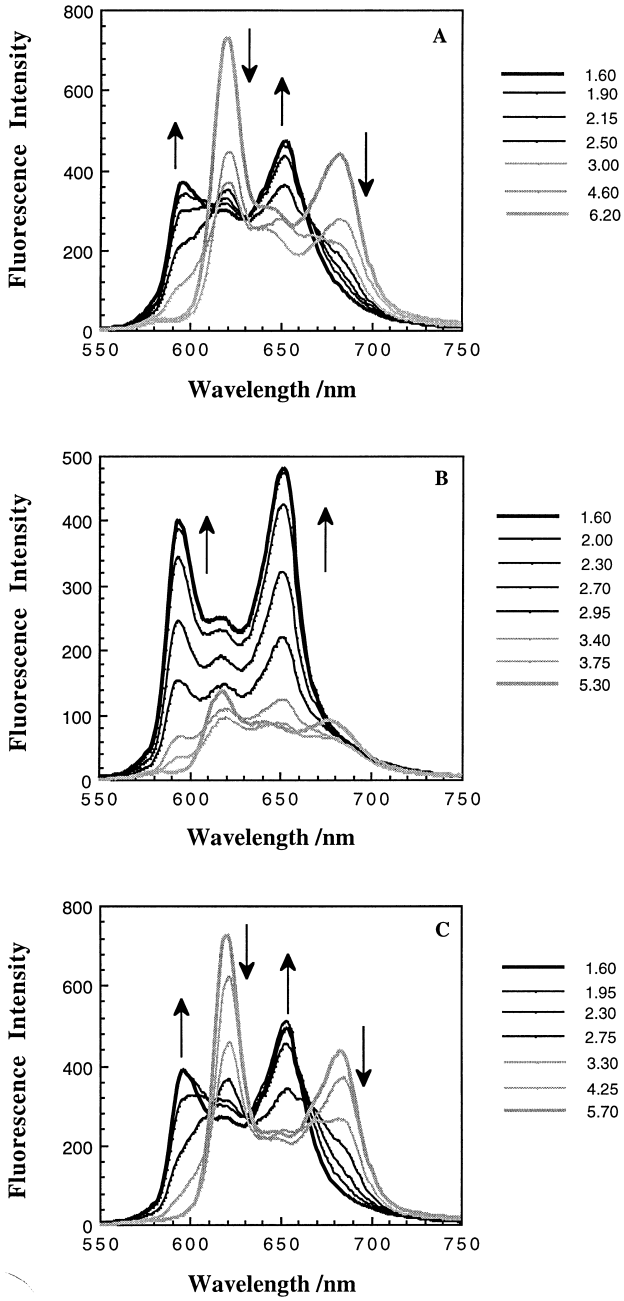

Fig. 5. Fluorescence spectra of Fe-free cytochromes: (A) *c*-553; (B) c_3; (C) *c* in Tris–HCl 10 mM at different pHs below 7.0, excited at 400 nm.

4. Discussion

In order to use cytochromes *c*-553 and c_3 as components of optoelectronic devices, it is essential to know their spectroscopic properties and those of Fe-free forms at various conditions. Their spectroscopic properties in the native and Fe-free forms will be discussed below in relation to cytochrome *c*, MP-9, and hemin.

As reported by earlier workers [6–8], coordination of the heme iron of the cytochrome *c* by strong-field ligands (sulfur of the methionine or nitrogen of the histidine) produces a low-spin complex having a Soret maximum above 400 nm. The replacement of these strong-field ligands by weak-field ligands such as oxygen or halogens supplied by the solvent produces a high-spin complex having a Soret maximum between 390 and 395 nm, while coordination of one strong-field ligand and one weak-field ligand produces a mixed equilibrium complex with a Soret maximum between 396 and 400 nm [23,24]. The high-spin complex and the equilibrium mixture of spin is characterized by the band at around 620 nm (Table 1). Protonation of the imidazole nitrogens of histidyl residues is expected to prevent coordination of these residues with the heme iron [6]. At pH<2.0, cytochrome *c* showed the shift of Soret band indicating the replacement of the two strong-field ligands. Such changes will induce conformational changes of cytochromes. In the case of cytochrome *c*, three conformational states were proposed [2,7], the native state (N), the unfolded state (U), and the molten globule state (M). The replacement of the methionine is responsible to the transition from N to M. Cytochrome c_3 has two histidine ligands on the fifth and sixth positions of the heme iron, the replacement of which resulted in the largest Soret band shift at pH<2.0. MP-9 has only nine amino acids including one histidine ligand. Hemin does not have amino acid residues and the fifth and sixth positions of the iron are chloride ions provided by the solvent, weak ligands. These two hemes showed the smallest shifts.

The low-spin complex was confirmed for the cytochromes *c*-553 and c_3 at pH 7.2 showing the Soret maximum above 400 nm. The MP-9 at pH 7.2 has a equilibrium mixture of spin states with a Soret maximum at 395 nm (Table 1). Moreover, the result that the maximum absorptions of hemin at 613 nm and of MP-9 at 622 nm at neutral pH are close to the value (620 nm) of the cytochrome *c* at pH<2.0 also supports the high spin state of the iron heme, which is related to the replacement or lack of the ligands. However, at acidic pH (<2.0), cytochrome c_3 behaved differently and the Soret maximum was at 370 nm. It should have the Soret maximum around 390 and 395 nm, as in the case of the cytochromes *c* and *c*-553, shown in Fig. 2(B). In order to explain such a large blue-shift for the Soret maximum of the cytochrome c_3 at acidic pH, the polarity of the amino acid residues around the iron heme should be taken into account in addition to the influence of the axial ligands to the iron heme. The degree of polarity can be estimated by using hydropathy values, which are given by Kyte and Doolittle [25], for each amino acid around the heme. The average hydropathy values of 11–13 amino acids around the heme for cytochromes *c*, *c*-553 and MP-9 were +0.25, +0.29 and +0.22, respectively, i.e., the microenvironment around the heme of these compounds are less polar and rather hydrophobic. In the case of cytochrome c_3, the average hydropathy values of 11–13 amino acid residues around each heme were −0.34, −0.73, +0.42 and −0.48. Although one heme with +0.42 shows a less polar microenvironment, the average value −0.28 indicates that the hemes of cytochrome c_3 are under polar conditions and are anticipated to have higher interaction with water.

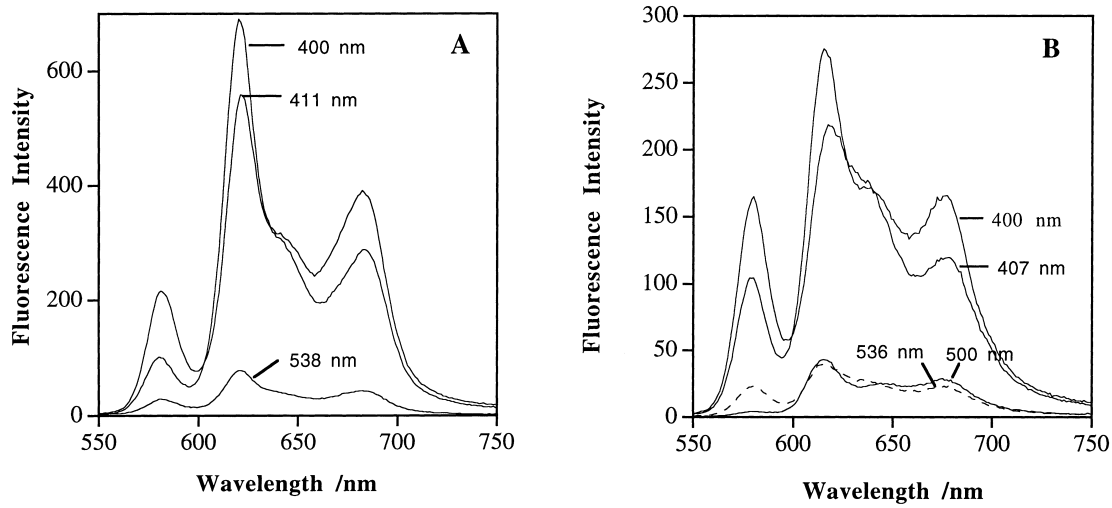

Fig. 6. Excitation wavelength dependences of the fluorescence spectra from Fe-free cytochromes: (A) *c*-553; (B) c_3 in Tris–HCl 10 mM pH 7.2, at 25°C. Excitation wavelengths are shown in the figure.

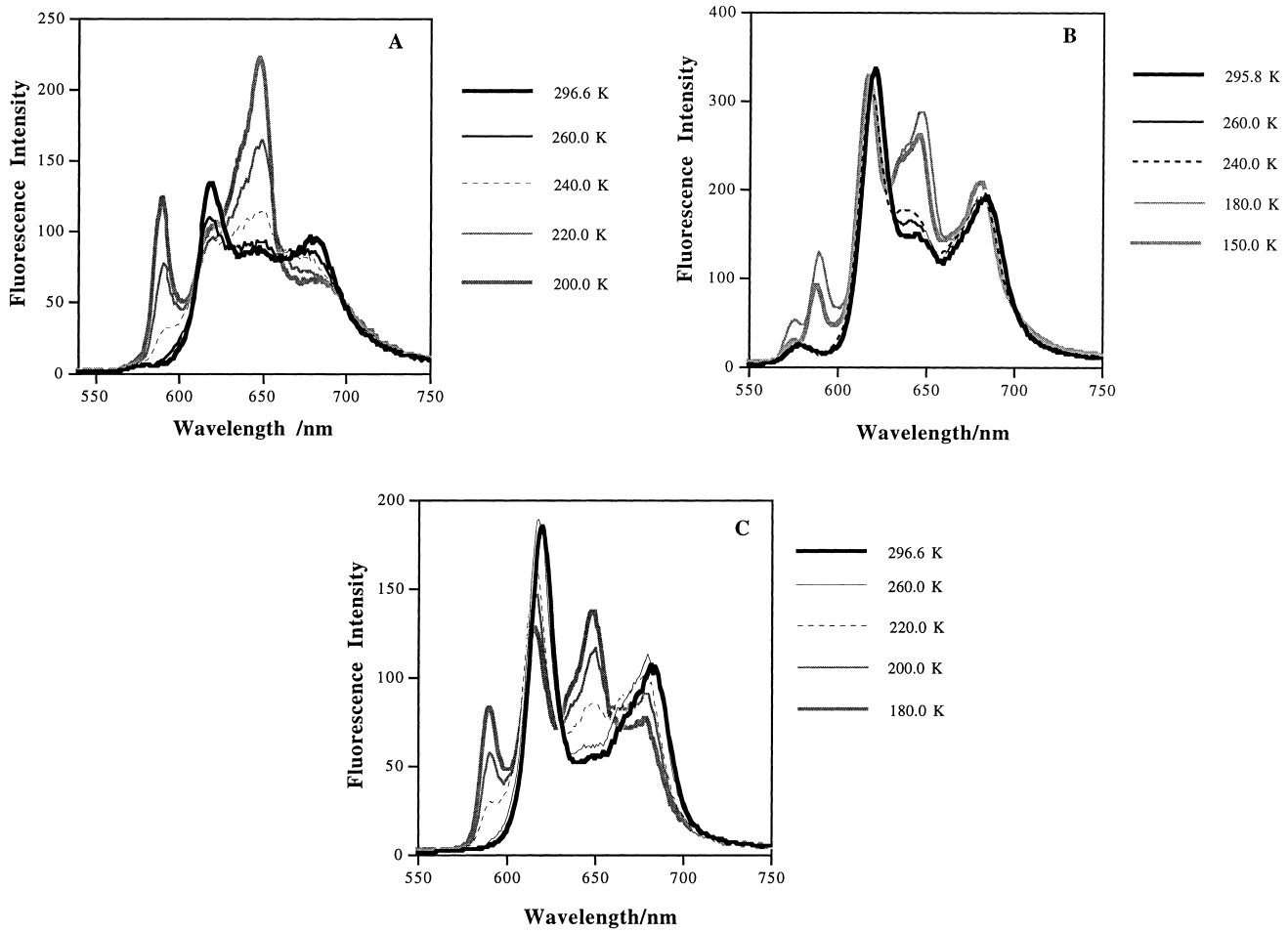

Fig. 7. Fluorescence spectra of (A) Fe-free cytochromes c_3, (B) Fe-free cytochrome *c*-553, and (C) Fe-free MP-9 excited at 400 nm in glycerol–water (3:1 v/v) at different temperatures.

The similar wavelength of the Soret bands for cytochrome c_3 and hemin at acidic pH can be explained as follows. Hemin has no amino acids and is directly contacted with water molecules in solutions. The hemes of cytochrome c_3 are also presumed to be directly in contact with water to result in higher degree of protonation due to polar microenvironment.

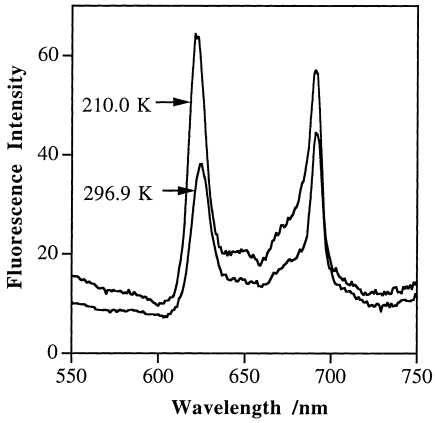

Fig. 8. Fluorescence spectra of Fe-free MP-9 in α-picoline, excited at 400 nm. The temperatures are shown in the figure.

The tendency of protonation of the imidazole nitrogens of the histidine is very high, preventing the coordination of this residue to the iron heme, leading to a higher unfolding degree of the cytochrome c_3 and therefore, larger blue shift than that of cytochromes *c* and *c*-553, at acidic pH.

On the other hand, the Soret band of the Fe-free cytochromes at neutral pH showed a blue-shift as compared to the native ones at pH 7.2. Since it cannot be considered the low-spin complex of the heme iron at pH 7.2, this shift is attributed to the unfolding of the protein, which was confirmed by their earlier elution from the Sephadex G-50 column than native cytochromes, because the size of the unfolded cytochrome is higher than the native one. All four imidazole groups of the porphyrin ring in Fe-free cytochromes are expected to become protonated (PH_4^{2+}) at pH 2.0, which caused the red-shift of the Soret band as indicated in Table 1.

As shown in Table 2 and Fig. 5, the fluorescence spectra of Fe-free cytochromes and Fe-free MP-9 at acidic pH are very distinct from those at the neutral and basic pHs. There are two main peaks at 615–620 nm and at 676–783 nm in the fluorescence spectrum at acidic pH and they are coincident for the Fe-free cytochromes, Fe-free MP-9, and cytochromes at acidic pH. The peak wavelengths at the basic pH also show the same characteristic for both cytochromes, Fe-free cytochromes and Fe-free MP-9. The fluorescence excitation spectra for Fe-free cytochromes and Fe-free MP-9 at the acidic and basic pH corresponded to the absorption spectra at each condition.

It was observed in the temperature dependence of fluorescence spectra that the 590 and 650 nm bands appeared and increased their intensities while the bands at 620 and 680 nm decreased their intensities during cooling process as shown in Fig. 7(A)–(C). After warming up to room temperature, all the fluorescence spectra showed again the original patterns at neutral pH with peaks at 620 and 680 nm. These results strongly suggested that the 590 and 650 nm bands correspond to the protonated form of porphyrins in Fe-free cytochromes. This hypothesis was supported by the temperature dependent fluorescence measurements of Fe-free MP-9 in an aprotic solvent, a-picoline shown in Fig. 8, where the peak at 590 nm and the shoulder at 650 nm did not exist at room temperature, nor they appear even at 210.0 K. The 620 and 680 nm peaks, on the contrary, increased showing that the protonation did not take part, the fluorescence spectrum did not change as the ones in the Fig. 7. It was also observed that longer the period the Fe-free cytochromes samples are kept in 10 mM Tris–HCl pH 7.2, the higher tendency they have to be protonated.

On the study of the pH dependence of the native cytochromes, it was observed at very low pHs such as 1.6 that the cytochromes showed weak fluorescence as shown in Table 2. This fluorescence might be caused by several mechanisms such as unfolding of cytochromes and the partial removal of iron atom suggesting the protonation of the porphyrin, etc. First we checked the possibility of the partial removal of iron. Although the removal of the iron atom is expected to be much easier in MP-9 and hemin with no or less amino acid residues, these hemes did not show fluorescence property even at very low pH. This result clearly excluded the first possibility. Then we checked the solvent mechanism. If we compare the native cytochromes to the Fe-free cytochromes and Fe-free MP-9 at neutral pH, the latter are unfolded and the porphyrin is more directly in contact with the solvent. However, in our work, MP-9 and hemin did not show fluorescence at any pH, even in the acidic pH. Thus this second possibility that the hemes of the cytochromes could show fluorescence because of a direct contact with the solvent must be excluded. The only possibility that it is still acceptable is the conformational change. The great difference between the cytochromes and the MP-9 or hemin is the amino acid residues' number around the heme. The conformation of the amino acid residues around the heme could contribute to the appearance of the fluorescence in the cytochromes. However, even with the appearance of the fluorescence, the native cytochromes could not have protonation in the porphyrin ring, because protonation occurs in the imidazole nitrogen of the histidyl group.

5. Conclusion

The fifth and sixth ligands of the heme-iron were found to greatly influence the UV/visible absorption characteristics of the cytochromes *c*, *c*-553 and c_3. After removing iron atoms from the cytochromes c_3 and *c*-553, most of the photophysical characteristics became to be the same. The different pH dependent fluorescence spectra for cytochromes *c*-553 and c_3 were attributed to the fact that the microenvironment polarity around the heme is different. The cytochrome c_3 showed higher tendency of protonation than *c*-553.

It was clarified that spectroscopic properties of cytochromes *c*-553 and c_3 together with their Fe-free forms

were controlled by several factors such as the spin state, polarity of microenvironment, conformation, and protonation mainly through ligands. The present results may contribute a great deal to develop novel optoelectronic protein devices such as photochemical hole burning.

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