Fluorescence Properties of Cytochromes c-553 and c3

Miriam Abe*, Tatsuhiko Yagi**, Hideyuki Itagaki**, and Toshihiko Nagamura*,***

(Received Nov. 20, 1997)

Abstract

Photophysical properties of cytochrome c3 and c-553 from the same sulfate-reducing bacteria, *Desulfovibrio vulgaris*, Miyazaki, and thier iron-free forms were studied over a wide pH range in comparison with cytochrome c, hemin and TTMAPP. Although some photophysical characteristics are the same between cytochrome c-553 and c3, as UV/visible absorption spectrum and fluorescence spectrum, several very different properties such as UV absorption spectrum at pH 1.6 and pKa were observed. Removal of the iron atom from the hemes of cytochrome c-553 and c3 revealed that the photophysical properties of both porphyrins become very similar. It can be attributed to the fact that the ligands of the fifth and sixth positions of the iron are different for both cytochromes.

It was shown in this work that the fluorescence of the porphyrin or its quenching in cytochromes c-553 and c3 is not related to the triptophan, which was the case in cytochrome c. At very low pHs, the cytochromes c-553 and c3 showed fluorescence, even in the absence of denaturing solvent such as urea or guanidine. Iron-free cytochromes c-553 and c3 showed fluorescence even at neutral pH. Moreover, at pHs higher than pKa, the evidence of the coexistence of two components was obtained, suggesting species with different protonation or aggregation.

Introduction

Monoheme proteins like cytochrome c have been studied extensively by using the photophysical techniques such as fluorescence or uv absorption measurements [1-8]. However, most of the authors were interested in their structure or conformational transition. Systematic studies of pH effects on photophysical properties of cytochromes have not been clarified so much. The present paper deals with the photophysical behavior of novel cytchromes such as c-553 and c3 at various pH values and temperatures.

The hemoproteins cytochrome c3 and c-553 from *Desulfovibrio vulgaris* Miyazaki are reported to be c-type cytochromes that act as electron carrier proteins. Cytochrome c-553 has only one heme, bound to a polypeptide chain of a molecular weight of 8,500, and its standard redox potential is + 0.026 V at pH 7.0 [9]. The heme iron of cytochrome c-553 is coordinated with methionine and histidine [10,11]. Cytochrome c3 has four hemes, and it contains 107 amino acid residues and its molecular weight is 14,000 [12]. The distances between the heme irons are of the order of 1 nm [13] and the fifth and sixth coordinate ligands of the iron atom of the heme are histidines. The standard redox potential are different for the four hemes (-226, -278, -298, and -339 mV). [14]

Recently, the photophysical processes of iron-free cytochromes had attracted our interest because they can be used as a promising material to develop an opto-electronic protein device such as novel optical memory by means of photochemical hole burning (PHB). In order to use cytochrome c3 and c-553 as a component of optoelectronic devices, it is essential to know the spectroscopic properties of them and also of their iron-free forms at various conditions. The hemoproteins cytochrome c3 and c-553 do not show fluorescence at neutral pH. However, by removing the iron of the hemes [15] or acidifying the medium [1,2,6-8], they come to show fluorescence. The present experiment is designed to study the fluorescence and absorption behaviour of the iron-free and acidified cytochromes c3 and c-553 by comparative studies with other iron-free porphyrins as: iron-free cytochrome c, 5,10,15,20-tetrakis(4-N-trimethylamino-phenyl)porphine (TTMAPP) and as well as with iron porphyrins as cytochromes c, c3 and c-553 , and hemin.

Experimental

Materials

The general structures of the hemes in the cytochromes are shown in **Fig. 1**.

Desulfovibrio vulgaris Miyazaki, was cultured as described before [16]. Wet cells of *Desulfovibrio vulgaris* were disintegrated and suspended in 5-6 volumes of H_2O with an ultrasonic disintegrator (UR-200P, Tomy Seiko Co., Tokyo) at 20 kHz, 180 W, for 12 min producing a bacterial sonicate which was treated with $(NH_4)_2SO_4$. Cytochrome c-553 was concentrated from the treated bacterial sonicate using a

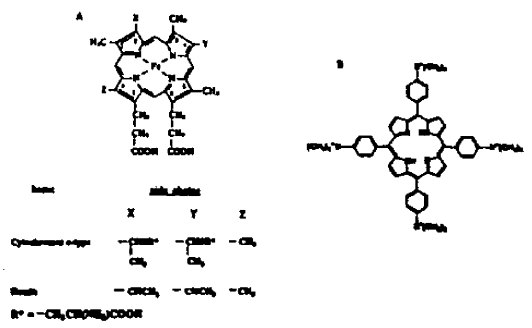

Fig.1: Structures of hemes.
A: General structure for cytochromes and hemin.
B: Structure of 5,10,15,20-tetrakis (4-N-trimethylaminophenyl) porphine (TTMAPP).

* Graduate School of Electronic Science and Technology, Shizuoka University 3-5-1 Johoku, Hamamatsu 432-8011
** Department of Chemistry, Faculty of Education, Shizuoka University 836 Ohya, Shizuoka 422
*** Crystalline Films Laboratory, Research Institute of Electronics, Shizuoka University3-5-1 Johoku, Hamamatsu 432-8011

DEAE-cellulose (Whatman) chromatographic column. Sephadex G-50 chromatographic column was used to separate high molecular weight cytochrome and cytochrome c-553.

Iron-free cytochrome c3 and c-553 were prepared by cooling fifteen milligrams of each in an open Teflon beaker suspended in a Dewar flask containing liquid nitrogen. Anhydrous HF (pressure of 0.2 kg/cm^2) was passed into the beaker for 3 minutes and the cytochromes turned purple [15,17]. After the reaction occured, HF was removed under a stream of nitrogen at room temperature. The iron−free cytochrome was dried under reduced pressure for 24 hours. Then it was dissolved in 5 ml of Tris-HCl 10 mM and was purified through a Sephadex G-50 chromatographic column. Tris-HCl buffer was used as eluent. The purified iron-free cytochromes eluted a little earlier than the normal cytochromes, which indicates that the molecular sizes of the formers are slightly larger than the latters. The fractions of the iron-free cytochromes were pooled, dialyzed against distilled water and were frozen dried.

The iron-free porphyrin of cytochrome c (type IV from horse heart) was prepared by the same method as described above. Hemin was purchased from Wako and TTMAPP from Dojindo Laboratories. The sample solutions were acidified with 1 or 6 M HCl or 3 M H_2SO_4 aqueous solutions and alkalized with 1 or 12 M NaOH aqueous solutions.

Measurements

The U.V. absorption measurements of cytochrome c-553 and other iron-porphyrins in tris-HCl solution were carried out on a Shimadzu UV-2200. The fluorescence spectra and fluorescence excitation spectra were measured on a Hitachi F-4500 spectrofluorometer at ca. 25°C. Fluorescence decay measurements were carried out on a Horiba NAES550 single-photon counting machine. The emission of iron-free cytochrome c-553 in degassed tris-HCl solution (pH 7.2) was measured at ca. 25°C through an interference filter (630, 652 and 682 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 [19]. To assess the validity of the trial fitting function, Durbin-Watson factor (DW) was used [20]. 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 were linearly proportional to the absorbance of the Soret peak, which was confined in the range of less than 2.0 giving a concentration of less than 90 μ M.

Results

UV/visible absorption spectra

Table I summarizes the wavelength at maximum absorbance of the Soret band and the Q-band found for each of iron-free porphyrins and iron porphyrins.

The first four samples have iron atoms of a ferri-form in the porphyrin ring while the last four samples do not have iron atoms. Acidification occured by addition of 6 M HCl (20 ml) or 3 M H_2SO_4, and alkalinization was made by addition of 10 M NaOH (0.5-1.0 ml).

Table I: UV maximum absorbances wavelengths for porphyrins and iron porphyrins. Conditions for the measurements: samples in Tris-HCl 10 mM at 25℃. Acidification upon addition of 20 μ l of HCl 6 M and slow alkalinization was upon NaOH 1 M and 10 M (0.7-1.0 ml). The wavelengths in the square are the values for the Soret band wavelength of each porphyrin.

	pH 7.20	pH < 2.00	pH > 12.00
cytochrome c	409, 530	695, 494, 620	409, 530
cytochrome c-553	408, 525	694, 499, 620	408, 537
cytochrome c3	408, 530	370, 497, 637	408, 537
hemin	383, 613	374, 646	383, 605
Fe-free cyt. c	409, 503,540, 568, 620	409, 551	694, 502, 537, 565, 621
Fe-free cyt. c-553	694, 504, 539, 568, 620	408, 550	694, 503, 539, 565, 617
Fe-free cyt. c3	651, 500, 540, 573, 624	409, 498, 545	694, 499, 541, 568, 617
TTMAPP	411, 500, 513, 549, 578, 630	411, 589, 639	411, 462, 513, 550, 578, 632

All the iron porphyrins showed a blue shift of the Soret band at acidic pHs (**Table I**). The degree of Soret band shifts for cytochrome c and c-553 were almost the same. Specially for the cytochrome c3 the observed shift was from 408 to 370 nm. It was a remarkable shift compared with other hemoproteins. The absorption at around 530 nm for the cytochromes at neutral pH disappeared at very low pHs. A new band was formed at around 620 nm for cytochrome c and c-553 or 637 nm in the case of cytochrome c3, while hemin did not show such a new band. Alkalinization of the iron-porphyrins showed absorption spectra very similar to those at neutral pH, basically without any shift of the Soret band for the cytochromes, but a small red shift for hemin.

The iron-free proteins showed different properties from those of iron porphyrins. The Soret band at neutral pH has the tendency of red shift by acidification of the protein (**Table I**). The other Q bands which are characteristic to the iron-free cytochrome c, i.e. 503, 540, 568 and 620 nm [21] disappeared at very low pH and a single band with a peak at around 550 nm was observed. Iron-free cytochrome c-553 showed identical behavior as iron-free cytochrome c: the shift of the Soret band and the formation of a new band agreed with the case of the iron-free cytochrome c reported by Vanderkooi and Erecinska [15]. However, the wavelengths of the shifted spectrum of iron-free cytochrome c3 showed to be different from the other cytochromes, at longer wavelenghts (**Table I**). The alkalinization of the iron-free protein does not give any great influence on the Soret band. Most of the Soret bands basically were not shifted, although the absorbance values were slightly increased or decreased depending on the protein.

Fig.2 shows the acidification of each iron porphyrin resulted in different absorbance values for the Soret band.

The Soret band absorbance of both cytochrome c3 and hemin decreased and broadened, while the absorbance of the Soret band of cytochrome c and c-553 increased if compared with their Soret band absorbance at neutral pH. The alkalinization resulted in a decrease of the absorbance of the Soret band of both cytochrome c3 and hemin, while for the others the absorbance were slightly increased, even with dilution of the protein by addition of 10 M sodium hydroxide solution. For the iron-free porphyrins, the acidification showed enhancement of the absorbance of the Soret band, while at high pH the absorbance of the Soret band was different for each protein. One interesting feature of the spectrum of the iron-free cytochrome c3 at low pH is the appearance of a clear shoulder at around 390 nm on the Soret band, overlaping the Soret band at neutral pH (**Fig.2**).

Fig.3 shows the effect of pH on the protonation of the porphyrin ring of iron-free cytochrome c3, c and c-553, monitored at the Soret band, respectively. The absorbance values at Soret band were plotted against pH and the value of the middle of the curve between neutral pH and protonated form was taken as a pKa. The ferri form and iron-free forms of the cytochromes showed the same pKa; 2.5 for c and c-553 and 3.0 for cytochrome c3.

Fluorescence results

The iron porphyrins showed no fluorescence upon excitation at 400 nm at neutral pH. The gradual alkalinization of the iron porhyrins with 1 M NaOH solution from neutral to pHs higher than 12.0 also did not show any changes of the fluorescence behavior. However, upon acidification to pH lower than 2 with HCl or H_2SO_4, the iron porphyrins

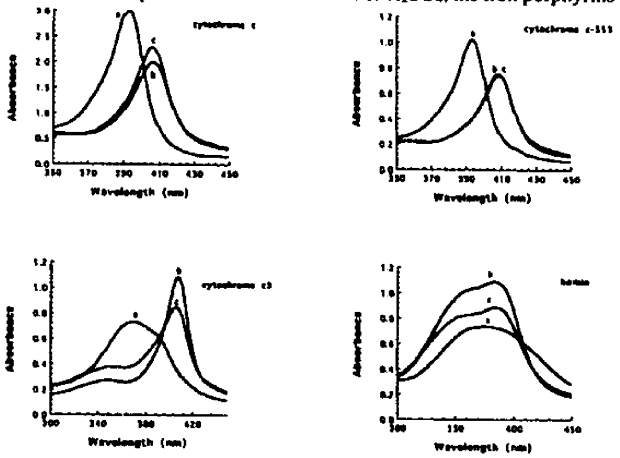

Fig.2 Soret absorption spectra of the native cytochromes and hemin. (a) pH<2.0, (b) neutral pH and (c) pH>12.0. Conditions of the measurement: iron porphyrins in Tris-HCl mM at 25℃. Acidification was upon addition of 20 μ l of HCl 6 M and alkalinization was upon NaOH 1 M and 10 M (0.7-1.0 ml). Concentrations of the iron-porphyrins were less than 90 μ M.

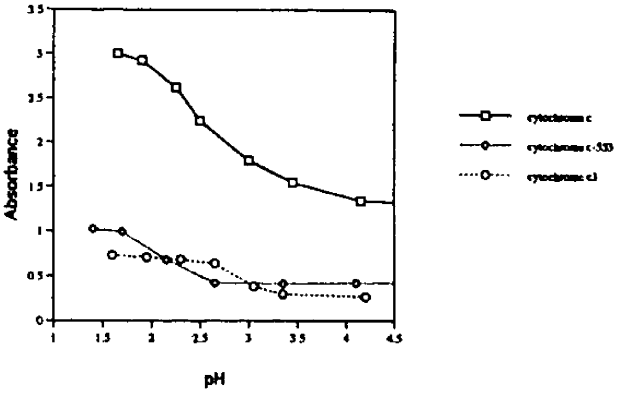

Fig.3 Effect of pH on the porphyrin ring of cytochromes, at Soret band. Conditions of the measurements: iron-free porphyrins in Tris-HCl mM at 25 ℃. Acidification upon addition of 20 μ l of HCl 6 M and alkalinization was upon NaOH 1 M and 10 M (0.7-1.0 ml). The absorbance values at Soret band of the iron-free porphyrins were in the range of 1.0 -1.5.

except for hemin reveal fluorescence, although with a very low intensity. Once this fluorescence appeared, it never disappeared even when the pH of the solution was adjusted again to be neutral. In contrast to the iron porphyrins, the iron-free porphyrins showed fluorescence in the whole pH range.

Table II summarizes the peak wavelengths of the fluorescence for all the samples employed in this work when excited at 400 nm.

The fluorescence spectra of the acidic form of porphyrins are quite different from those of the basic form. The maximum intensity wavelengths for the acidic form are the same for iron-free porphyrins are shown in **Fig.4**, and iron porphyrins and the maximum intensity wavelengths for the basic form show the same characteristic for both

Table II: Wavelengths of maximum fluorescence intensity for porphyrins and iron porphyrins.(*) iron-free cytochromes. Solvent used was Tris-HCl 10 mM. Conditions for the measurements: samples in Tris-HCl at 25 ℃. Acidification was upon addition of 20 μ l of HCl 6 M and alkalinization was upon NaOH 1 M and 10 M (0.7-1.0 ml). The fluorescence intensity of the iron porphyrins at pH>12 were obtained after gradual alkalinization from the acidic solution of the protein.

	neutral pH	pH < 2	pH > 12
cytochrome c	no fluorescence	596, 652	620, 675
cytochrome c-553	no fluorescence	593, 694	613, 674
cytochrome c3	no fluorescence	592, 652	611, 673
hemin	no fluorescence	595, 651	615, 674
*cytochrome c	584, 620, 683	596, 616, 653	618, 680
*cytochrome c-553	580, 619, 683	596, 617, 653	586, 611, 680
*cytochrome c3	578, 615, 676	594, 614, 651	582, 616, 677
TTMAPP	642, 700	664	642, 700

iron and iron-free porphyrins. It is shown in **fig.5** that the fluorescence excitation spectra for the iron-free porphyrins is identical with the absorption spectra for the basic pH, although those for the iron porphyrins are not so clear except for the peaks corresponding to the Soret bands.

The acidic and basic forms of all the iron-free porphyrins were reversible even by changing the pH of the solution repeatedly. The fluorescence spectra of both iron and iron-free porphyrins excited at 400 nm, showed two strong bands at around 600 and 650 nm for the acidic form and at around 620 and 680 nm for the basic form. TTMAPP showed only one band at 664 nm in acid solution and two bands at 640 and 700 nm in basic solution. The appearance of only one band for the acidic form of the TTMAPP is most probably attributed to four phenylene rings (**Fig.1**) attached to the porphyrin: it will modify the electronic state of the porphyrin ring especially at its protonated form.

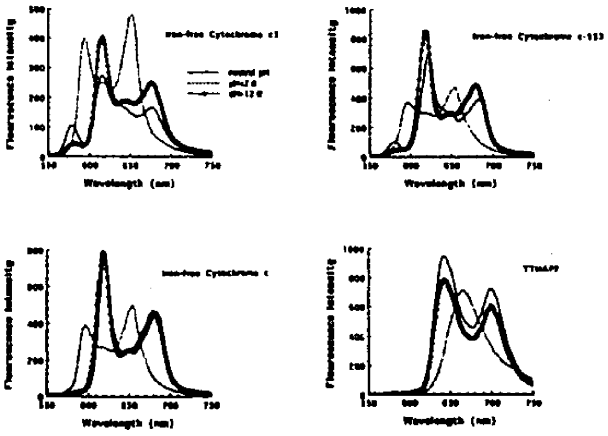

Fig.4 Fluorescence emission spectra of iron-free porphyrins. Excitation at 400 nm. Slit widths: 5 nm. Samples in Tris-HCl mM at 25℃, pH<2.0: addition of HCl 6 M, pH>12.0: addition of NaOH 10 M.

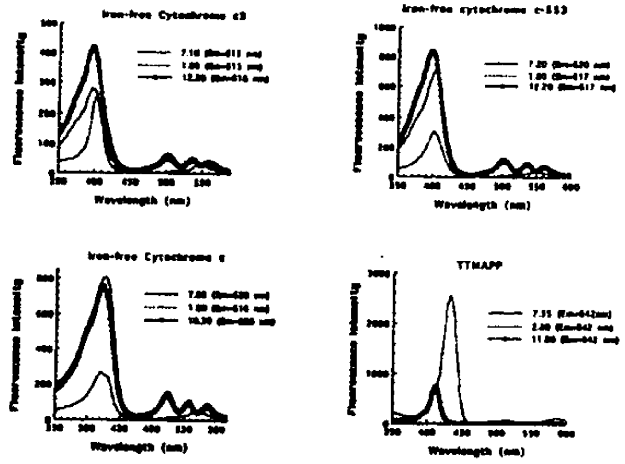

Fig.5 Fluorescence excitation spectra of iron-free porphyrins. Emission wavelengths are shown in the Figure. Samples in Tris-HCl mM at 25 ℃, pH<2.0: addition of HCl 6 M, pH>12.0: addition of NaOH 10 M.

One interesting feature of the fluorescence spectra of iron-free cytochrome c3 and c-553 is the appearance of a third band at around 580 nm and a shoulder at around 650 nm for neutral and alkaline pHs. Especially in the case of iron-free cytochrome c3 and c-553, this third fluorescence bands are clearly dependent on excitation wavelengths which is shown in **Fig.6**, and on temperature, which is shown in **Fig.7**. These results strongly suggest the presence of more than one fluorescent species in these iron-free cytochromes probably due to the different extent of aggregation.

The evidence of more than one fluorescent species,shown in **Fig.8**, was obtained from the time profile of fluorescence of iron-free cytochrome c-553. It showed the presence of two fluorescence decay components at 630 and 650 nm upon excitation at the Soret band. The time dependence of fluorescence intensity was well fitted to the next equation:

$$I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \qquad (A_1 + A_2 = 1)$$

The decay curves of iron-free cytochrome c-553, at pH 7.20 in tris-HCl solution, gave following results.

fluorescence at	A_1	τ_1 (ns)	A_2	τ_2 (ns)
630 nm	0.93	3.3	0.07	14.6
650 nm	0.93	12.6	0.07	3.2

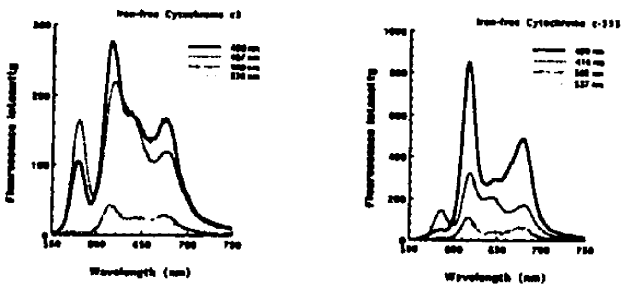

Fig6 Dependence on excitation wavelength of the fluorescence emission of iron-free cytochromes 3 and c-553. Excitation wavelengths are shown in the Figure. Conditions of the measurements: cytochromes in Tris-HCl 10 mM . The pH for both cytochromes is 7.10.

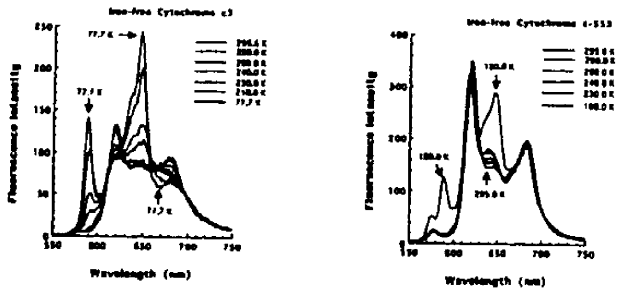

Fig.7 Fluorescence emission spectra of iron-free cytochromes c3 and c-553 in glycerol-water (3:1 v/v) at different temperatures. Excitation wavelength is 400 nm.

580 nm 1.0 2.2

The fluorescence decay at 580 nm showed only one component with a time constant of 2.2 ns. The 580 nm fluorescence is a characteristic band for the acidic form. The time decay at 630 nm, which is characteristic to the basic form, showed two components. The shorter lifetime component contributes the fluorescence spectrum by a fraction of 75% ($A_1 \tau_1 / (A_1 \tau_1 + A_2 \tau_2)$). The fluorescence decay at 650 nm, wich is characteristic to the acidic form, showed also two components. The longer lifetime component contributes to the fluorescence

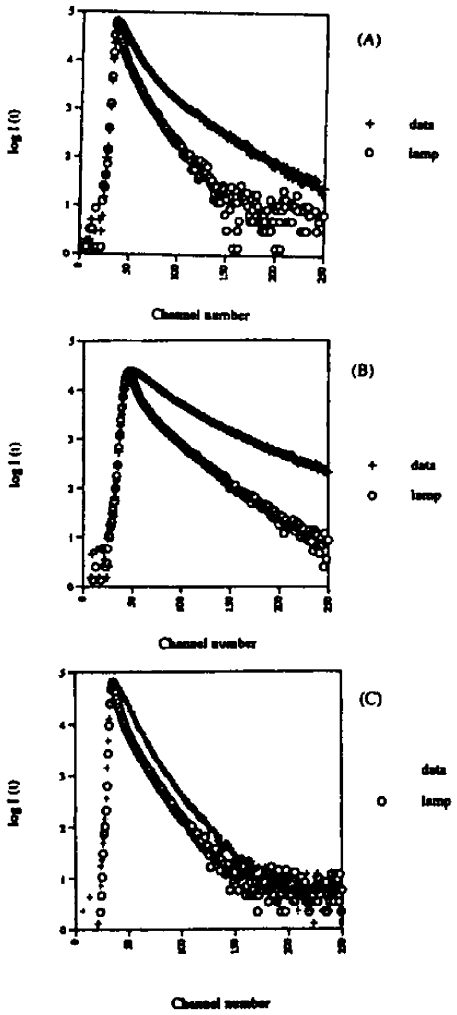

Fig.8 Fluorescence emission spectra of iron-free cytochromes c3 and c-553 in glycerol-water (A)fluorescence at 630 nm(0.2 ns/channel); (B)fluorescence at 650 nm(0.4 ns/channel).The condition of the experiment is described in the text.

spectrum by a fraction of 98%.

Discussion

As reported somewhere else [6-8], upon acidification of cytochrome c, the strong ligands such as methionine and histidine are replaced by weak ligand such as oxygen or halogens supplied by the solvent, which is accompanied by conformational transition. 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 ligation with the heme iron is responsible for the N to M transition, and protonation of the histidine residue is involved in the M to U transition. Furthermore, at pH 2.0, when both methionine and hystidine are replaced, the iron atom becomes a high-spin complex, which causes a red shift of the Soret band (**Table I**) and an appearance of a band at around 620 nm with the unfolding of the protein. However cytochrome c3 has two histidine ligands and its UV/visible absorption spectrum at pH 1.60 is more similar to the hemin than the other cytochromes. Hemin does not have amino acid residues, and the fifth and sixth ligand positions of the iron are chloride ions, meaning the weak ligands. This explains the appearance of a Soret band at 383 nm, which is blue shifted as compared with other cytochromes at neutral pH. The result that maximum absorpion of hemin at 613 nm at neutral pH is close to the value (620 nm) of the cytochrome c at pH 2.0 also supports the mechanism mentioned above. In the case of cytochrome c3, at neutral pH, the absorption peaks at 408 and 530 nm agree with those of cytochrome c, showing a low-spin complex of the iron atom. However, upon acidification the absorbance of the Soret band of both cytochrome c3 and hemin decreased and broadened, which revealed a "disordered" conformation. It suggests that the unfolding process of cytochrome c3 is "disordered" due to the lack of methionine, and consequently the lack of M state.

Another point to be considered is that at very low pHs such as 1.6, a very small fraction of iron atoms were removed from the hemes. This fraction is small enough so that could not be detected by UV/visible absorption spectrum, but it was observed by fluorescence measurements. Once this fluorescent form is produced, the iron porphyrins seemed to show fluorescence even after increasing the pHs of the solution again to neutral.

The pKa values for iron-free porphyrins and iron porphyrins (**Fig.3**) were the same, showing that the presence of the iron in the porphyrin or the polypeptide chain do not contribute to the pK.

All the references about fluorescence phenomenon of cytochrome c until now [1,3-5,15] proposed that the excitation of tryptophan, at 280 nm, located at the 59th position on the polypeptide chain is responsible for the fluorescence behavior of cytochrome c. Moreover, Foster-type energy transfer between tryptophan 59 and the iron heme is also reported to be responsible for the quenching of the fluorescence of the porphyrin. According to Vanderkooi and Erecinska [15], the fluorescence emission of the iron-free porphyrin cytochrome c is shown only at basic and acidic solutions. However, in our work iron-free porphyrin cytochrome c showed fluorescence even at neutral pH. It is interesting also to note that there is no tryptophan residue in the polypeptide chain of both cytochrome c-553 and c3. Therefore, it can be concluded that tryptophan is not responsible for the quenching of fluorescence in cytochromes c, c-553 and c3 nor responsible for fluorescence of the porphyrins.

All the iron-free porphyrins used in this work showed fluorescence at any pH, while the native iron porphyrins showed fluorescence only after acidification.

The pH dependence of fluorescence spectra for the iron-free porphyrins at the same excitation wavelength shown in **fig.9** indicates a higher tendency of protonation of cytochrome c3 than other cytochromes (c and c-553). This could be related to the absence of methionine residue in cytochrome c3, and consequently the absence of the M state, leading to an unfolding process much faster.

The iron-free porphyrins showed two bands with high intensity at pH below 2.0 (**Fig.4**). At higher pHs, a third band at 580 nm with low intensity and a small shoulder at 650 nm were observed in addition to

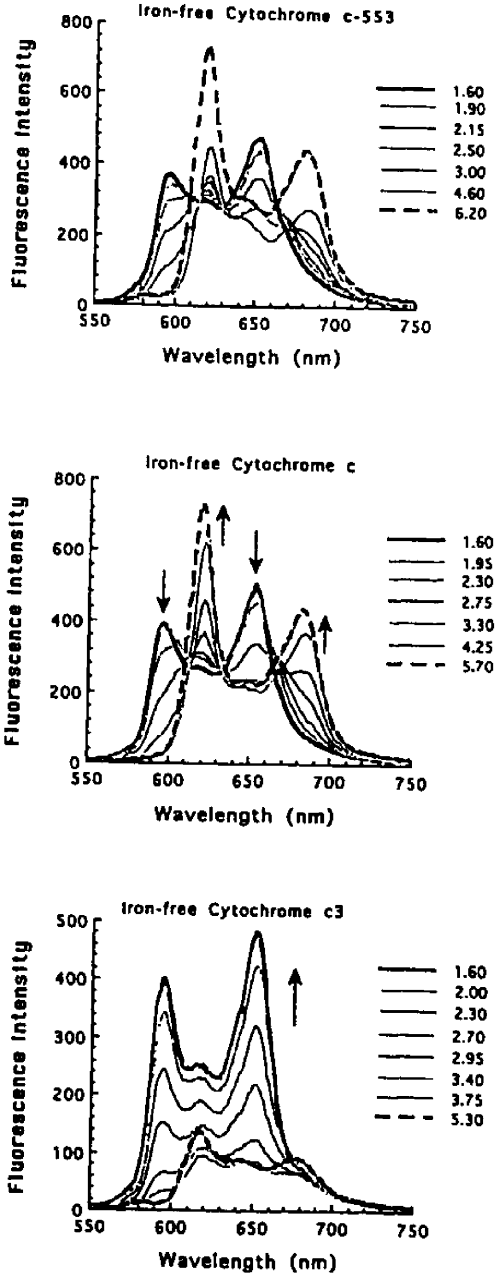

Fig.9 Fluorescence emission spectra of iron-free cytochromes c, c3 and c-553 in Tris-HCl 10 mM at different pHs below 7.0. Acidification upon HCl 6 M and gradual alkaliniation upon NaOH 1 M. Excitation wavelength was 400 nm.

the red shift of the main two bands. These results reveal the existence of another fluorescent species, which is considered to be the acidic form or aggregated form. This spectrum is dependent on the excitation wavelength. Moreover, this phenomenon is very evident in the case of iron-free cytochrome c3 and c-553. The temperature dependence of the fluorescence spectra of cytochrome c3 and c-553 showed a strong tendency of such species to be formed at very low temperatures (Fig. 7) due most probably to the "freezing" of the protein. The confirmation of the existence of two species or components at neutral pHs for cytochrome c-553 was derived from the results of fluorescence lifetime decay. The time profile for the cytochrome c-553, at pH 7.20, show the existence of only one component with short lifetime (2.2 ns) at emission wavelength of 580 nm, characteristic band for the acidic form. At the emission wavelengths of 630 and 650 nm, the results show two distinct components, one with long lifetime (12.6 and 14.6 ns), and another with short lifetime (3.3 and 3.2 ns). It is considered that the acidic form is present with a small fraction at neutral pH. The minor componet at 650 nm with shorter lifetime is thus attributed to the acidic form. The major component at 650 nm and a minor componet at 630 nm with long lifetime can be attributed to the neutral form. At 630 nm which is characteristic band for the basic form, the main component showed again short lifetime, 3.3 ns.

Conclusion

Photophysical properties of cytochrome c3 and c-553 from the same sulfate-reducing bacteria, *Desulfovibrio vulgaris*, Miyazaki, and thier iron-free forms were studied over a wide pH range in comparison with cytochrome c, hemin and TTMAPP. Although some photophysical characteristics are the same between cytochrome c-553 and c3, as UV/visible absorption spectrum and fluorescence spectrum, several very different properties such as UV absorption spectrum at pH 1.6 and pKa were observed. Removal of the iron atom from the hemes of cytochrome c-553 and c3 revealed that the photophysical properties of both porphyrins become very similar. It can be attributed to the fact that the ligands of the fifth and sixth positions of the iron are different for both cytochromes.

It was shown in this work that the fluorescence of the porphyrin or its quenching in cytochromes c-553 and c3 is not related to the triptophan, which was the case in cytochrome c. At very low pHs, the cytochromes c-553 and c3 showed fluorescence, even in the absence of denaturing solvent such as urea or guanidine. Iron-free cytochromes c-553 and c3 showed fluorescence even at neutral pH. Moreover, at pHs higher than pKa, the evidence of the coexistence of two components was obtained, suggesting species with different protonation or aggregation.

REFERENCES

[1] Tsong T. Y., Biochemistry, **14**, 1542-1547 (1975)

[2] Goto Y., Hagihara Y., Hamada D., Hoshino M., Nishii I., Biochemistry, **32**, 11878-11885 (1993)

[3] Tsong T. Y., J. Biol. chem., **249**, 1988-1990 (1974)

[4] Tsong T. Y., Biochemistry, **15**, 5467-5473 (1976)

[5] Elove G. A., Bhuyan A. K., Roder H., Biochemistry, **33**, 6925-6935 (1994)

[6] Babul J., Stellwagen E., Biochemistry, **11**, 1195-1200 (1972)

[7] Dyson J., Beattie J. K., J. Biol. Chem., **257**, 5, 2267-2273 (1982)

[8] Stellwagen E., Babul J., Biochemistry, **14**, 5135-5140 (1975)

[9] Yagi T., Methods Enzymol., **243**, 109-112 (1994)

[10] Nakagawa A., Higuchi Y., Yasuoka N., Katsube Y., Yagi T., J. Biochem., **108**, 701-703 (1990)

[11] Ono K., Kimura K., Yagi T., and Inokuchi H., J. Chem. Phys., **63**, 1640 (1975)

[12] Niki K., Yagi R., and Inokuchi H., Adv. Chem. Ser., 201 (1982)

[13] Higuchi Y., Kusunoki M., Yasuoka N., Kakudo M., Yagi T., J. Biochem., **90**, 1715-1723 (1981)

[14] Yagi T., Inokuchi I., Kimura K., Acc. Chem. Res., **16**, 2-7 (1983)

[15] Vanderkooi J. M., Erecinska M. (1975), Eur. J. Biochem., **60**, 199-207 (1975)

[16] Yagi T., Honya M., Tamiya N., Biochim. Biphys. Acta, **153**, 699 (1968)

[17] Fisher W. R., Taniuchi H., Anfinson C. B., J. Biol. Chem., **248**, 3188 (1973)

[18] Falk, J.E. "Porphyrins and Metalloporphyrins"; (Elsevier, Amsterdam, 1964), p. 244

[19] O'Connor, D.V. and Phillips D., Time-correlated single-photon counting (Academic Press, New York, 1985)

[20] Durbin J. and Watson G.S., Biometrika, **37**, 409 (1950); **38**, 159 (1951)

[21] Gouterman M., J. Mol. Spectrosc., **6**, 138 (1961)