STUDIES ON WATER-SOLUBLE ALGAL

B- AND C-TYPE CYTOCHROMES

A Doctoral Thesis

By

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CONTENTS

I. GENERAL CONSIDERATION

All living organisms on the earth require energy for their growth and maintenance. The ultimate source of energy is the solar electromagnetic radiation. Algae as well as higher plants obtain the energy for their lives directly from the solar radiation to drive the reductive synthesis of carbohydrate with water from carbon dioxide, with the molecular oxygen evolution,

$$CO_2 + 2H_2O \longrightarrow (CH_2O) + O_2 + H_2O.$$

In the autotrophic process of carbohydrate synthesis, viz., photosynthesis, the reducing equivalents are transfered from water to $NADP^+$ through the photosynthetic electron transport potentiated by light energy at photosystems I and II as shown in Fig. 1 (1). Photophosphorylation of ADP to ATP is coupled to the "downhill" electron flow via primary electron acceptor (Q, Em = -0.05 volt) of photosystem II to deexcited P700 (Em = +0.45 volt) of photosystem I. The resulting ATP and NADPH are used for the reductive assimilation of carbon dioxide in dark processes.

Four cytochrome components, viz., cytochromes b_6, b-559, c-553 and f have been found to participate in algal photosynthetic electron transport, but the position of b-559 is not clear at present (1). Cytochrome c-553 which is thought to function as an electron carrier between f and P700 has been isolated from all the algal cells examined in this thesis and other paper (2), but not from higher plants. On the other hand, plastocyanin is found in higher plants (3),

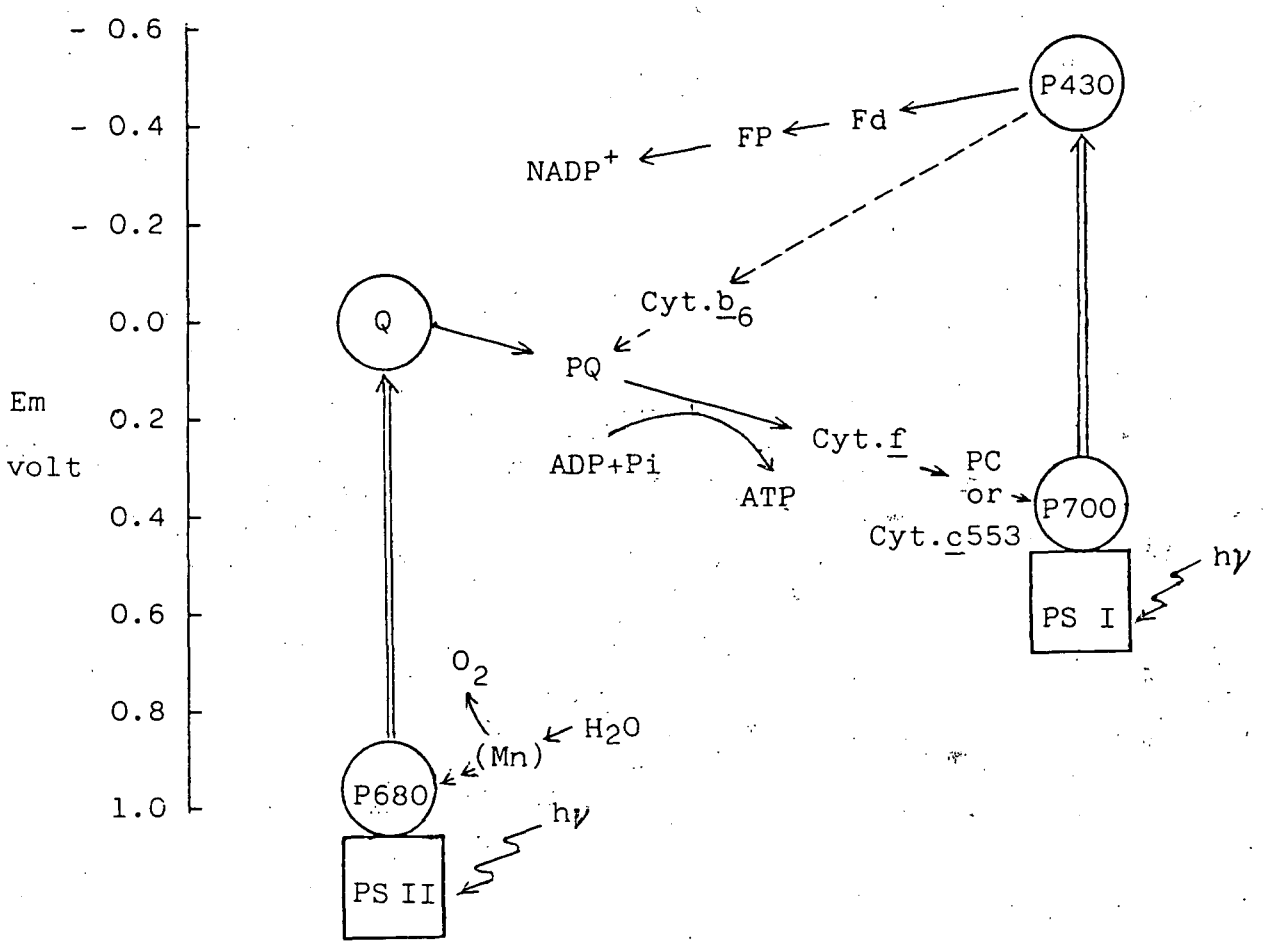

Fig. 1. Energy diagram of photosynthetic electron transport via photosystems I (PS I) and II (PS II), plotted in terms of midpoint redox potentials (Em,volt). The symbols for the electron carriers are defined as followings: P430, pigment 430 = primary electron acceptor of photosystem I; Q, quinone = primary electron acceptor of photosystem II; Fd, ferredoxin ; FP, ferredoxin-NADP+ reductase; Cyt.b_6, cytochrome b_6; PQ, plastoquinone; Cyt.f, cytochrome f; Cyt.c553, algal cytochrome c-553; (Mn), a possible Mn-protein. Arrows, (⟹), (⟶) and (- - →) show, respectively, the photochemical boosting of electrons, dark electron flow and postulated path of cyclic electron flow.

and also in blue-green and green algae accompanied with cytochrome c-553 (4-8), but it is not found in yellow-green (9), red, brown, Euglena (10) algae or a green alga, Bryopsis maxima (11). In the blue-green and green algal cells, cytochrome c-553 and plastocyanin are reciprocally formed in dependence on copper salt concentration in culture medium (12-14).

Cytochromes b_6, b-559 and f are tightly bound to chloroplast membrane but can be solubilized with a detergent Triton X-100, ammoniacal ethanol or methyl ethyl ketone (15-18). In contrast to these components cytochrome c-553 and plastocyanin are loosely bound to the membrane and so can be extracted with usual salt buffer. Therefore, the two proteins were easily purified from many algal and plant sources; their physico-chemical properties and structural features were able to be investigated. In this investigation a wide distribution of the cytochrome among algae was confirmed by its isolation from all of 37 species of algae including 19, 6, 1, 10 and 1 species of red, brown, yellow-green, green and chara algae, respectively. However, plastocyanin could be isolated from only 2 species of green algae, Ulva pertusa and Enteromorpha prolifera, among them. From the two green algae were Ulva cytochrome b-562.5 and Enteromorpha cytochrome b-561 extracted with usual salt buffer, partially purified and characterized. Neither Ulva b-562.5 nor Enteromorpha b-561 is thought to be b_6 from their positive midpoint potentials; they are not conceivable to be b-559 from their long-wavelength α-peak.

The various cell materials derived originally by

photosynthesis are degraded via the heterotrophic metabolic process, viz., digestion, glycolysis and TCA cycle to yield carbon dioxide, NADH, and also ATP via substrate-level phosphorylation. This NADH was oxidized with molecular oxygen to form water via respiratory electron transport which couple to oxidative phosphorylation of ADP to ATP. Higher plant mitochondria have cytochromes b-556, b-558, b-560, b-565, c-552, c-550, a and a_3. They are sequenced in the respiratory electron transport as shown in Fig. 2. The b-565 component is not among the chain and its rôle in mitochondria is unknown (19). A green-algal respiratory electron transport chain has been briefly reported to be as follows: NADH ---> b-562 --> c-551 --> a --> a_3 --> O_2 (20). The b-562 component as well as a-type cytochromes are firmly bound to mitochondrial membrane and its detailed properties are unknown. Therefore, neither of the b-type cytochromes, viz., Ulva b-562.5 and Enteromorpha b-561 in this thesis, can be identified as an algal respiratory chain component.

In order to elucidate the electron transfer mechanism between various electron carriers, it is necessary to investigate their physico-chemical properties, viz.,absorption spectra, isosbestic points, midpoint redox potentials, molecular size and so forth, and also to determine the amino acid sequences and three dimensional structure. For several kinds of electron carrier proteins viz., cytochromes c, ferredoxins, plastocyanins and cytochrome c_2 have been studied the sequences and three dimensional structures to clarify their structure-function relationship and also their

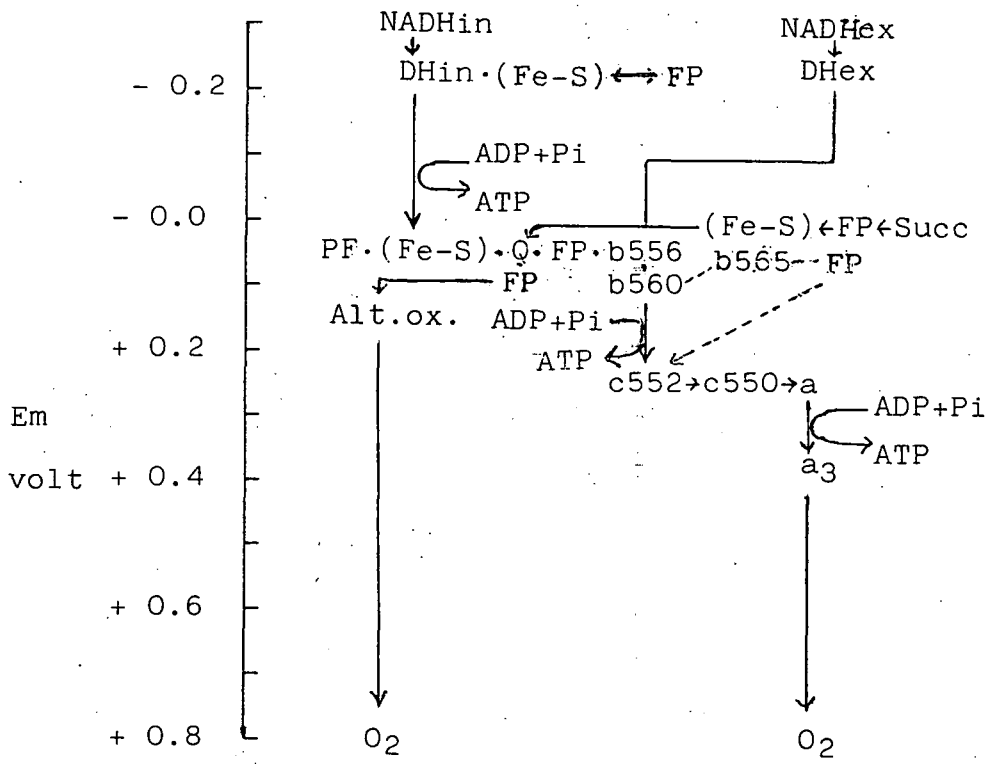

Fig. 2. Energy diagram of higher plant respiratory chain, plotted in terms of midpoint redox potentials (Em, volt). The symbols for the electron carriers are defined as follows : NADHin, endogenous NADH; NADHex, exogenous NADH; DHin, internal dehydrogenase; DHex, external dehydrogenase; (Fe-S), iron-sulfur protein; FP, flavoprotein; Succ, succinate; a, cytochrome a; b, cytochrome b; c, cytochrome c; Alt.ox., alternative oxidase; Q, ubiquinone. Arrow (⟶) shows electron flow. Dotted line indicates association with the respiratory chain by links as yet unknown (19).

molecular evolution (21-27).

In this thesis the author describes on water-soluble algal cytochromes: cytochromes c-553: their distribution among algae, purification, crystallization, physico-chemical properties, heme and heme ligand, amino acid sequence; cytochrome b-562.5 (*Ulva pertusa*) and b-561 (*Enteromorpha prolifera*) of green algae: their purification procedures and physico-chemical properties.

II. PURIFICATION AND PROPERTIES OF C-TYPE CYTOCHROMES

1. Introcuction

Water-soluble algal c-type cytochrome was first isolated from a red alga, Porphyra tenera, by Yakushiji in 1935 (28). This cytochrome was at first believed to be respiratory cytochrome c (28) or cytochrome c_1 (29). Later Katoh (2,30-32) and Yakushiji et al.(33) have obtained it in crystalline form and studied its physico-chemical properties. Katoh confirmed its localized existence in plastids of the Porphyra tenera cells. Nishimura and Takamiya observed the light-induced redox reactions of this cytochrome in the intact cells and also in isolated particles of three species of Porphyra (34). On the other hand Yamanaka and Okunuki have found that the cytochrome of P. tenera could not be oxidized by cytochrome a (35). Based on these findings this cytochrome was evacuated as one of the components in photosynthetic system. From this point of view, a great number of similar cytochromes have been purified from several divisions of algae and their properties reported (36-47).

During the past eighteen years, the soluble c-type cytochromes, cytochromes c-553, had been considered to be cytochrome f which was discovered and isolated by Hill and coworkers (17, 48), because of their similarity to one another in several properties: the asymmetric α-band, the sharp γ-band, the high midpoint potential, and the acidic nature. In 1977, however, Wood extracted a membrane-bound

c-type cytochrome with a mixture of ethyl acetate, ethanol and ammonia from three divisions of algae, Chlamydomonas reinhardtii (Chlorophyta), Euglena gracilis (Euglenophyta) and Anacystis nidulans (Cyanophyta), and identified them as cytochrome f (49), indicating that the algal cytochrome f is distinguishable from the soluble cytochrome c-553 also in the absorption spectrum. A similar comparison was also carried out for Bryopsis maxima (Chlorophyta) (18). Cytochrome c-553 is thought to function as an electron transfer protein between cytochrome f and P700 in the photosynthetic electron transport system (14), at which plastocyanin has been located in higher plant photosynthesis (50).

The sequence -Cys-X-Y-Cys-His- was common in all the five cytochromes sequenced so far (51-56), and methionie-62 was one of the invariant residues, assumed to be the sixth ligand to the heme iron (44, 55).

This chapter describes the preparation procedure, some physico-chemical properties and amino acid composition of crystalline cytochrome c-553 from a brown alga, Petalonia fascia, and also its wide distribution among algae.

2. Materials and Methods

Thalli: The thalli of Petalonia fascia were harvested from Matsushima and Shizugawa Bays at Matsushima and Shizugawa, Miyagi, and from Sagami Bay at Shichirigahama Beach, Kanagawa, Japan. The other marine algae were collected from the Pacific coast at Chōshi, Awaamatsu and Tateyame Beaches,

Chiba, Japan, from Tokyo Bay at Tsudanuma Beach, Chiba and at Kannonzaki Beach, Kanagawa, and from Sagami Bay at Shimoda and Shichirigahama Beaches, Kanagawa, Japan. Two species of freshwater algae, Vaucheria sp. and Chara sp. were collected from a pond at Futtsu-Misaki, Chiba.

Chemicals: DEAE-cellulose was obtained from Brown Co., U.S.A. Sephadex, DEAE-Sephadex and Pharmalyte were obtained from Pharmacia Fine Chemicals, Sweden. Standard molecular weight markers were obtained from either Pharmacia Fine Chemicals, Sweden, or Sigma Chemical Co., U.S.A. All other reagents were of analytical grade obtained from Koso Chemical Co., Tokyo, Wako Pure Chemicals, Osaka, and Nakarai Chemicals Co., Kyoto.

Measurement of Absorption Spectra: Absorption spectra at room temperature were measured in a Hitachi-Perkin-Elmer UV-VIS 139, a Hitachi 124 or a Hitachi 200 spectrophotometer. Low temperature absorption spectra at liquid nitrogen temperature (77°K) were measured in a Hitachi 356 spectrophotometer. Ferrocytochrome solution was prepared by adding a minute amount of solid ascorbic acid to the cytochrome dissolved in 5 mM phosphate buffer, pH 7.0 and by removing ascorbate by adsorption on a small DEAE-cellulose column and elution with 0.1 M same buffer from it as mentioned later under "Extraction and Purification" of 3-1. Ferricytochrome was obtained by dialysis against 50 μM potassium ferricyanide solution containing 20 mM phosphate buffer, pH 7.0. In this case, the measurement was carried out against the dialyzate. About 5-10 μM cytochrome solution was used. For the

measurement of 695-nm region was used 50 μM cytochrome solution. The ferrocytochrome in 0.5 mM phosphate buffer, pH 7.0 was used for the low-temperature spectrum.

Preparation of Pyridine Hemochrome: The pyridine hemochrome was prepared according to the method of Shichi and Hackett (56). The measurement of spectra was carried out in anaerobic condition. The sample in a Thumberg-type cuvette, with 1 mg of sodium hydrosulfite in the side arm, was evacuated at 1 mm Hg at 2°C for 3 min and then warmed at 25°C for 5 min. The evacuation was performed once more. The absorption spectra of the oxidized and reduced pigment were measured before and after hydrosulfite was mixed with the sample.

Redox Property and Midpoint Redox Potential ($E_{m,7}$): The redox reactions of the algal cytochrome c-553 with several redox reagents were tested in the α-band region before and after about 0.5 mg of each reagent to 3 ml of about 10 μM cytochrome dissolved in 50 mM phosphate buffer, pH 7.0. The concentrated solution of the cytochrome was dialyzed against 40 mM phosphate buffer, pH 7.0, and diluted with the same buffer to about 28 μM of the cytochrome or about 0.8 of the absorbance at α-peak when reduced with hydrosulfite. The diluted sample was subjected to titration by the method of Davenport and Hill (17) with ferri-ferrocyanide system of which midpoint redox potential was 0.416 volt in 40 mM phosphate buffer, pH 7.0 at 20°C (57). The reduction and oxidation reactions of the cytochrome at various potentials were followed by reading the absorbances at α-peak, that is

$$(\text{ferrocytochrome})/(\text{ferricytochrome}) = (A-A_o)/(A_r-A)$$
$$= (A-A_o)/(3.31A_o-A)$$

where A is the absorbance corrected for dilutions at each titration step, A_o is the absorbance after complete oxidation by adding 10 mM potassium ferricyanide solution and A_r represents the absorbance after full reduction by adding a minute amount of sodium ascorbate (about 0.5 mg). A_r/A_o was measured to be 3.31. The titration was started by adding 50 µl of 10 mM ferricyanide solution to 2.7 ml of the sample in a 1-cm cuvette (A_o) and then 30-340 µl of 50 mM ferrocyanide solution were added step by step to bring the reaction mixture to potential between 0.388-0.327 volt (A) and finally ascorbate was added (A_r). $Em,_7$ of the cytochrome was determined by plotting log(ferrocytochrome)/(ferricytochrome) versus log(ferrocyanide)/(ferricyanide). At the half reduction of the cytochrome, the $Em,_7$ (volt) was calculated using Nernst's equation as follows:

$$Em,_7 = 0.416 - 0.0581\log(\text{ferrocyanide})/(\text{ferricyanide})$$

at 20°C and pH 7.0.

Isoelectric Points: The isoelectric points of algal c-type cytochromes were determined by a density gradient isoelectric focusing method (58) using Pharmalyte carrier ampholyte of the pH range of 2.5 to 5.0 in a 110-ml column (59). A linear density gradient was prepared in a column by adding a thin solution (2.5 g of glycerol, 51 ml of water and 1.4 ml of Pharmalyte) to a more dense solution (27 g of glycerol, 34 ml of water and 1.4 ml of Pharmalyte) with constant mixing. The cytochrome c-553 (about 0.02 µmol)

dissolved in 1.5 ml of a mixture of the dense and the thin solutions (1:1) was 50% oxidized by the addition of 1mM potassium ferricyanide solution (about 10 μl). The cytochrome solution was applied to the middle of the column. Electrophoresis was performed at 800 volts (10-1.5 mA) and 0°C for 30 hr. The solution was collected in 1-ml fractions after the electrophoresis. The pH of each fraction was measured at 0°C. The absorbances at 410 and 415.5 nm were monitored at 20°C for ferri- and ferrocytochromes, respectively.

Analysis of Iron Content and Specific Absorbance: Iron analysis was performed by the o-phenanthroline method of Morrison et al.(60) with a slight modification. Cytochrome crystals collected by centrifugation were dissolved in a small quantity of ion-free water, dialyzed against distilled water for 80 hr at 5°C with several changes of outside water, and centrifuged at 14 x 10^4 x g for 1 hr. The supernatant solution was used for the determination of iron content, dry weight, and specific absorbancy. An aliquot sample containing 10-20 mg of cytochrome was subjected to wet combustion with a mixture of 5 ml of 9.5% sulfuric acid solution and 5.0 ml of 6.5% nitric acid solution in a micro-Kjeldahl flask by flame for 10 hr. The sample was decolorized clearly during evaporation of water and nitric acid. To the cooled sample were added 6.0 ml of 50% sodium acetate solution, 0.4 ml of 1% ascorbic acid solution freshly prepared, 2.0 ml of 0.25% o-phenathroline solution, and water to 25 ml in total. The reaction mixture was allowed to stand at 36°C for 4 hr.

Iron was determined spectrophotometrically at 512.5 nm using the millimolar extinction coefficient of "ferroin" i.e., 11.1 $mM^{-1}cm^{-1}$. The dry weight was determined by air-drying an equal volume of the sample at 105°C to constant weight for about 12 hr. The absorbance of the same sample was measured after quantitative dilution.

Amino Acid Composition: The amino acid composition of Petalonia cytochrome c-553 was determined with a Beckman Model 120 B amino acid analyzer according to the method of Spackman et al. (61). The sample was hydrolyzed in an evacuated and sealed glass tube for 24 or 48 hr at 110°C with 6 N HCl. Tryptophan was determined by thioglycollic acid procedure (62). Cyteine content was determined using a performic acid-oxidized sample (63).

Molecular Weights: The molecular weight of Petalonia cytochrome c-553 was estimated by gel filtration on a Sephadex G-75 column by the method of Andrews (64) with modifications and by polyacrylamide gel electrophoresis in the presence of SDS (65) using rabbit muscle phosphorylase b (molecular weight of 94,000), bovine serum albumin (67,000), egg white ovalbumin (43,000), bovine erythrocyte carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), bovine milk α-lactoalbumin (14,400), and bovine pancreatic insulin (5,700) as molecular weight markers. The cytochrome (0.02 μmol) and these marker proteins (0.02 μmol each) were pretreated at 100°C for 5 min in 60 μl of the mixture of 25% glycerol, 5% β-mercaptoethanol and 1% SDS, and 10-μl portions of the resulting mixture were applied to the 15% polyacryl-

amide gels (0.5 x 9 cm) containing 0.1% SDS and 0.1 M phosphate buffer, pH 7.2, The electrophoresis was carried out at 7 mA and 25°C for 6 hr. Its molecular weight was also calculated from the iron-content and the amino acid composition.

3. Results

3-1. Cytochrome c-553 of a Brown Alga, *Petalonia fascia*

Extraction and Purification: The *Petalonia fascia* materials used were fresh thalli or stocked thalli frozen at - 20°C, which contained some 88% water.

Step 1. About 2.5 kg of *Petalonia* thalli were immersed, without any mechanical disruption, in 10 liters of ammonia water making the final concentration 20 mM NH_4OH and allowed to stand at 20°C for 24 hr with occasional stirring. The slimy extract was filtered through absorbent cotton.

Step 2. To the 10-liter solution was added 1 liter of 3% acrinol solution, stirred, and again filtered through absorbent cotton to remove yellow flocking. The filtrate was passed through a cellulose powder column on which excess acrinol was adsorbed.

Step 3. The yellowish pink filtrate was half saturated with ammonium sulfate (380 g per 1 liter), and filtered on a Buchner funnel through a thin layer of Celite. The precipitate was discarded and the brownish pink filtrate obtained was fully saturated with ammonium sulfate. Pink precipitate was collected on a Buchner funnel and was

dissolved in 200 ml of 5 mM phosphate buffer, pH 7.0. The crude preparation contained 4.1 μmol (40 mg) of cytochrome c-553, 85% of which was found to be reduced. The purity index (A553/A273) was 0.42 in this stage.

Step 4. The crude preparation was dialyzed against 5 mM phosphate buffer, pH 7.0. The dialyzed solution was reduced with 1 mg of sodium ascorbate and then applied to a DEAE-Sephadex A-25 column (2.64 x 45 cm) equilibrated with the same buffer. After washing with 200 ml of 10 mM phosphate buffer, pH 7.0, the column was eluted with a linear gradient of 0 to 0.25 M NaCl in 1.2 liters of the same buffer. The eluate was collected in 5-ml fractions. The fractions with a purity index of above 0.9 were combined and saturated with ammonium sulfate. The precipitate was collected by centrifugation at 15,000 x g for 10 min and dissolved in 3 ml of 0.125 M NaCl containing 10 mM phosphate buffer, pH 7.0.

Step 5. This solution was applied to a Sephadex G-75 column (2.64 x 45 cm) equilibrated with same buffer and the cytochrome was developed with the same buffer. The fractions with a purity index of above 1.0 were combined, saturated with ammonium sulfate, centrifuged, and dissolved in 3 ml of 10 mM phosphate buffer, pH 7.0.

Step 6. After centrifugation of the solution at 2,300 x g for 10 min, the supernatant was dialyzed against 0.2 M Na_2HPO_4 saturated with ammonium sulfate. Crystallization of the cytochrome was almost completed within a few days. After recrystallization twice under the same conditions, about 20 mg of crystalline cytochrome c-553 was obtained with a

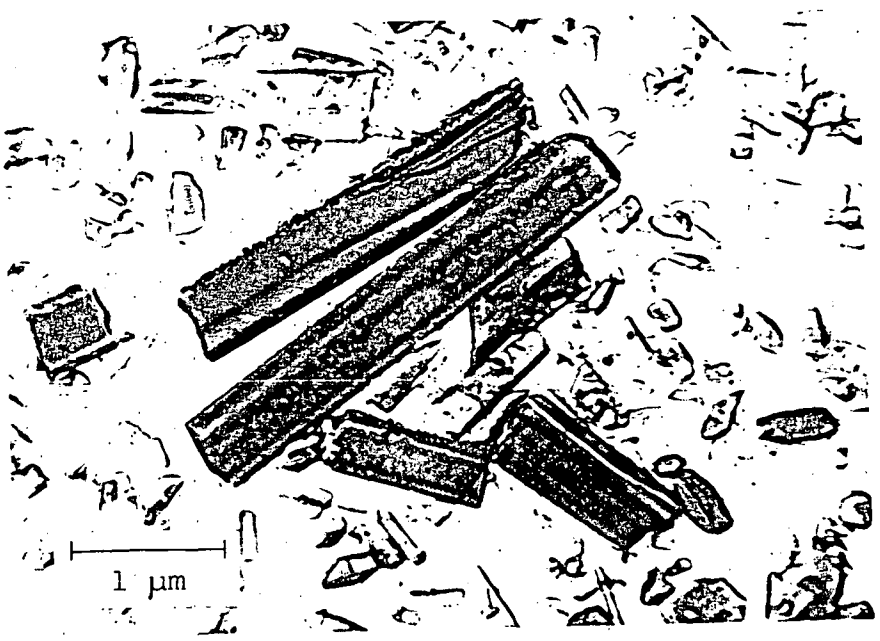

Fig. 3. Crystals of Petalonia cytochrome c-553 in saturated ammonium sulfate solution containing 0.2 M Na_2HPO_4.

purity-index of 1.10 (Fig. 3). The polyacrylamide gel electrophoresis in the presence of SDS showed a single band.

Absorption Spectra: The absorption spectra of ferro- and ferricytochrome c-553 are shown in Fig. 4. The difference spectrum (ferrocytochrome-ferricytochrome) is shown in Fig. 5. The ferrocytochrome showed absorption maxima at 273, 293, 317.5 (δ-max), 415.5 (γ-max, shoulder at 390 nm), 471, 521,5 (β-max, shoulder at 511 and 528 nm) and 553 nm (α-max, shoulder at 549 nm); the absorption minima were found at 256, 289, 295.5, 368, 488 and 538 nm. The ferricytochrome showed absorption maxima at 264, 292, 360, 409.8 (γ-max) and 528 nm (shoulder at 560 nm); the absorption minima were found at 262, 279, 306, 370 and 495 nm. The isosbestic points were located at 268, 280, 292.5, 336, 410, 433.5, 506, 529.5, 541.5 and 560.5 nm. The difference spectrum showed maxima at 273.5, 317.5, 370, 387, 417, 521 (shoulder at 511 and 528 nm) and 553 nm (shoulder at 549 nm). Their millimolar extinction coefficients are listed in Table 1. The low temperature absorption spectrum at 77°K of the ferrocytochrome is shown in Fig. 6. The peaks of α-, β- and γ-bands shifted 1 to 2 nm to the shorter wavelength compared with those observed at room temperature. The α-band splits into two peaks at 551.5 (major band) and 546.5 nm (minor band). The β-band showed a small peak and three shoulders at 562.5, 512, 510 and 504 nm, respectively.

Pyridine Hemochrome: The absorption spectra and the difference spectrum (reduced-oxidized) of the pyridine hemochrome derived from Petalonia cytochrome c-553 are shown in Figs. 7 and 8. Absorption maxima of oxidized form lie at

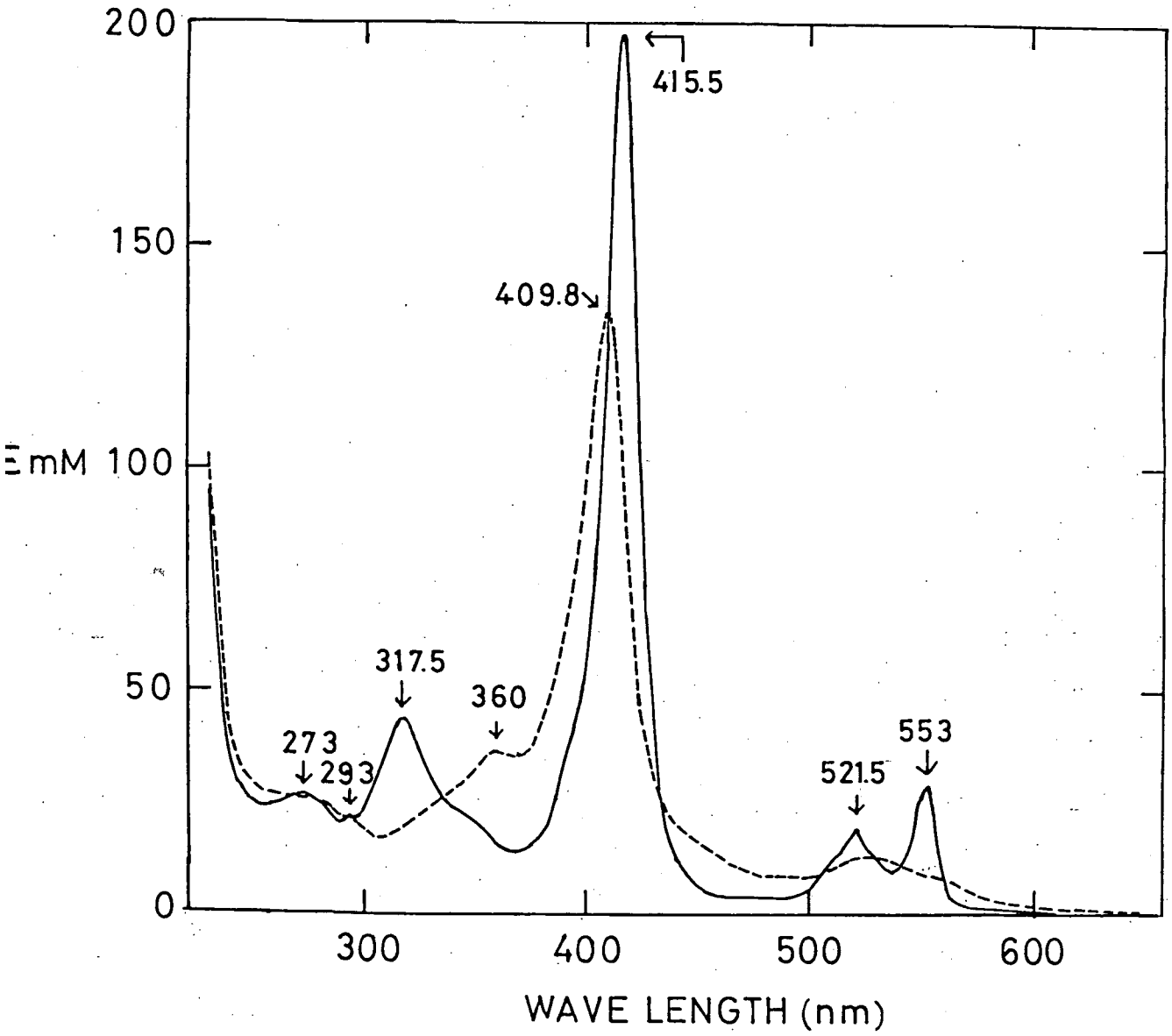

Fig. 4. Absorption spectra of ferro-(——) and ferricytochrome c-553 (----) purified from Petalonia fascia at room temperature in 0.1 M phosphate buffer, pH 7.0 and in 0.020 M phosphate buffer, pH 7.0 containing 0.05 mM ferricyanide, respectively. Millimolar extinction coefficients (EmM) were plotted from absorbances of about 5 or 10-μM cytochrome solution.

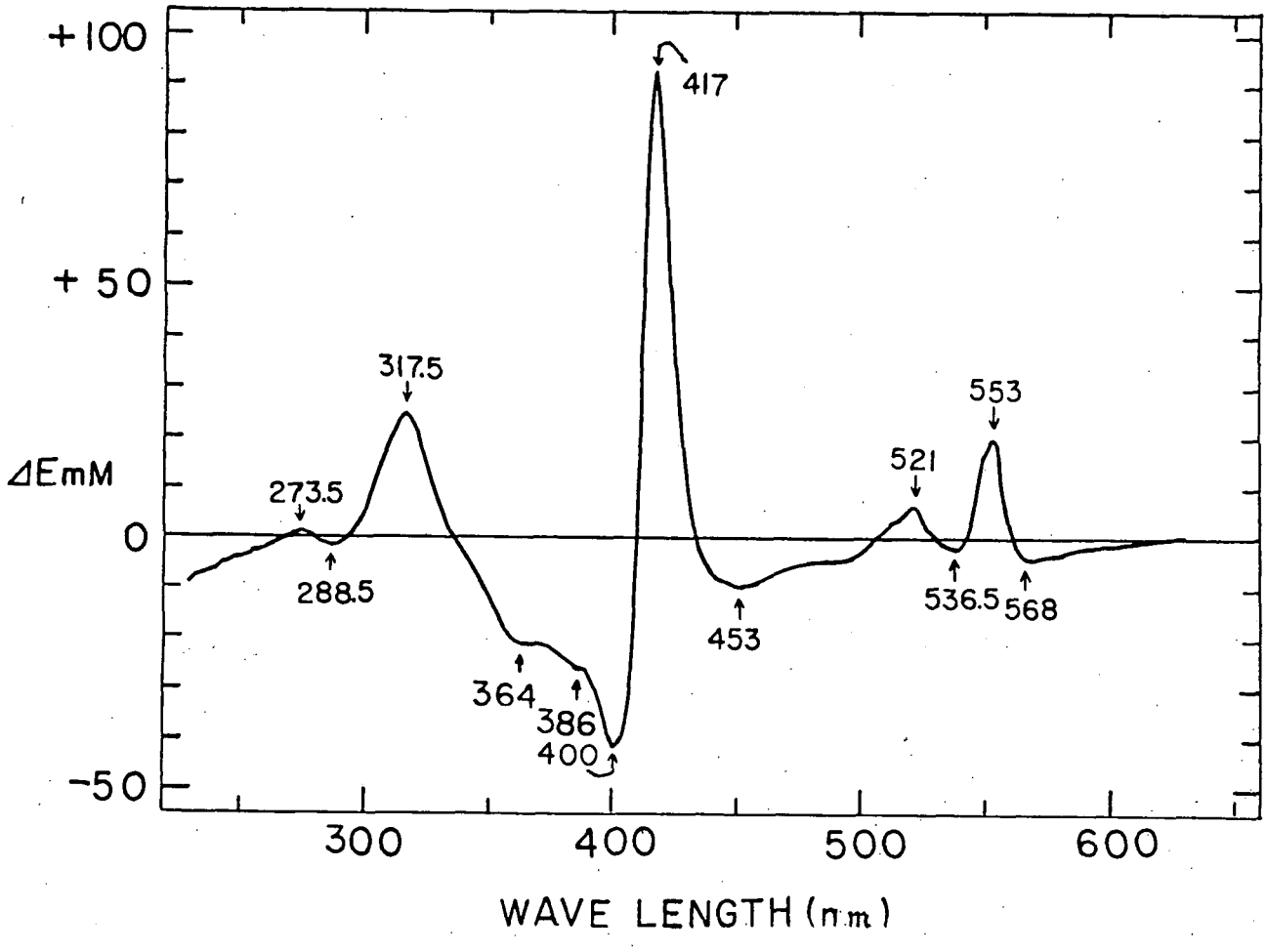

Fig. 5. Reduced-minus-oxidized spectrum of Petalonia cytochrome c-553. Differences of millimolar extinction coefficients (ΔEmM) were plotted from the data in Fig. 2.

Table 1. Millimolar Extinction Coefficients of Petalonia cytochrome c-553

The positions (nm) and millimolar extinction coefficients (E mM) of maxima (max), minima (min), isosbestic points (iso) and shoulders (shou) of ferro-(Cyt-Fe^{++}) and ferricytochrome c 553 (Cyt-Fe^{+++}) from Petalonia fascia at pH 7.0 and 20°C.

Wavelength (nm)	E mM				ΔE mM	
	Cyt-Fe^{++}		Cyt-Fe^{+++}		(Cyt-Fe^{++})−(Cyt-Fe^{+++})	
256.0	min	24.2		27.2		− 3.0
262.0		24.8	min	26.5		− 1.7
264.0		25.4	max	26.8		− 1.4
268.0	*iso*	26.5	*iso*	26.5		0.0
273.0	max	26.8		25.7		+ 1.1
273.5		26.7		25.6	max	+ 1.1
279.0		25.0	min	24.7		+ 0.3
280.0	*iso*	24.8	*iso*	24.8		0.0
288.5		20.6		22.1	min	− 1.5
289.0	min	20.5		22.0		− 1.5
292.0		21.7	max	22.1		− 0.4
292.5	*iso*	21.8	*iso*	21.8		0.0
293.0	max	21.9		21.7		+ 0.2
295.5	min	21.4		20.0		+ 1.4
306.0		30.1	min	17.0		+13.1
317.5	max	44.0		19.1	max	+24.9
336.0	*iso*	25.9	*iso*	25.9		0.0
360.0		15.8	max	36.2		−20.4
364.0		14.2		36.0	min	−21.8
368.0	min	13.9		35.6		−21.7
370.0		14.0	min	35.4	max	−21.4
386.0		25.7		52.0	min	−26.3
387.0		27.6		53.7	max	−26.1
390	shou	33.7		60.8		−27.1
400.0		56.8		98.2	min	−41.4
409.75		131.8	max	135.3		− 3.5
410.0	*iso*	135.3	*iso*	135.3		0.0
415.5	max	197.1		109.6		+87.5
417.0		190.2		97.9	max	+92.3
433.5	*iso*	26.5	*iso*	26.5		0.0
453.0		5.1		15.0	min	− 9.9
471	max	3.8		9.8		− 6.0
488.0	min	3.5		8.3		− 4.8
495.0		3.7	min	8.2		− 4.5
506.0	*iso*	8.7	*iso*	8.7		0.0
511.0	shou	12.0		9.6		+ 2.4
521.0		18.6		12.2	max	+ 6.4
521.5	max	18.6		12.3		+ 6.3
528.0	shou	14.0	max	12.9		+ 1.1
529.5	*iso*	13.0	*iso*	13.0		0.0
536.5		9.6		11.9	min	− 2.3
538.0	min	9.4		11.5		− 2.1
541.5	*iso*	10.8	*iso*	10.8		0.0
549.0	shou	24.2		9.2		+15.0
553.0	max	28.5		8.6	max	+19.9
560.0		9.2	shou	8.1		+ 1.1
560.5	*iso*	7.9	*iso*	7.9		0.0
568.0		2.0		6.5	min	− 4.5

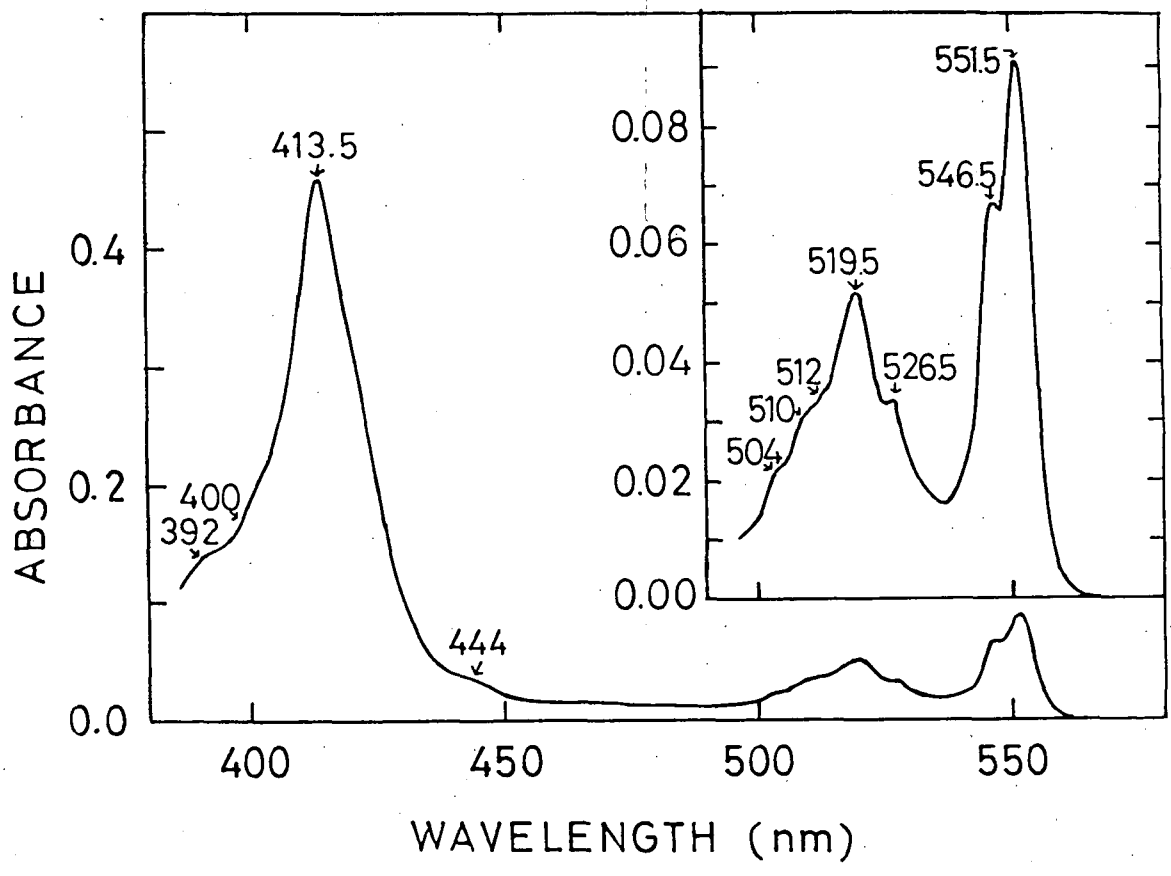

Fig. 6. Low temperature spectrum of Petalonia cytochrome c-553. The spectrum was taken at the temperature of liquid N_2 (77°K) in 0.5 mM K-phosphate buffer, pH 7.0. Inset: Enlargement of the spectrum at α- and β-regions.

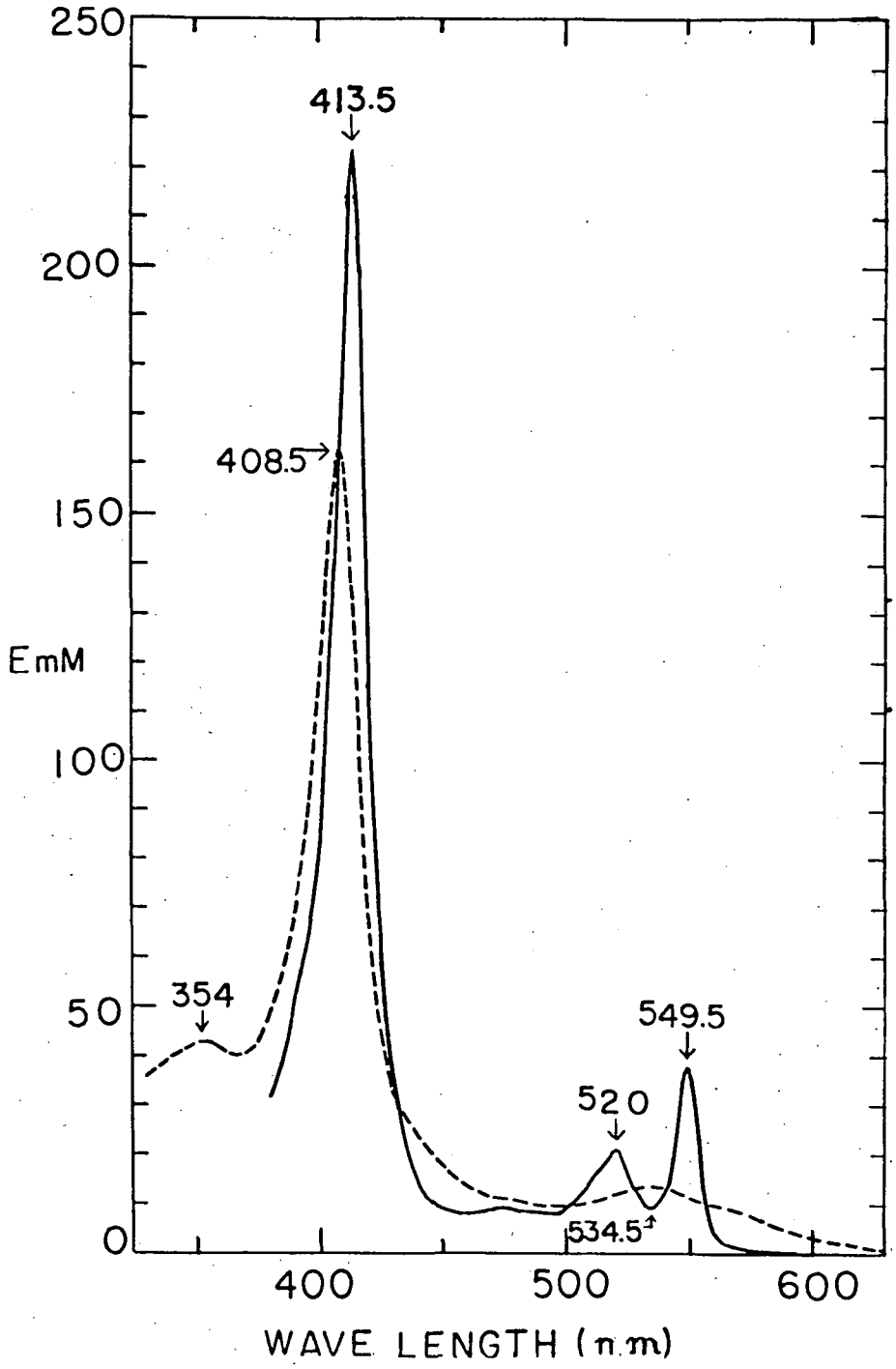

Fig. 7. Absorption spectra of reduced (——) and oxidized (----) pyridine hemochrome from Petalonia cytochrome c-553. Millimolar extinction coefficients (EmM) were plotted from the absorbances of about 5 or 10 μM solution.

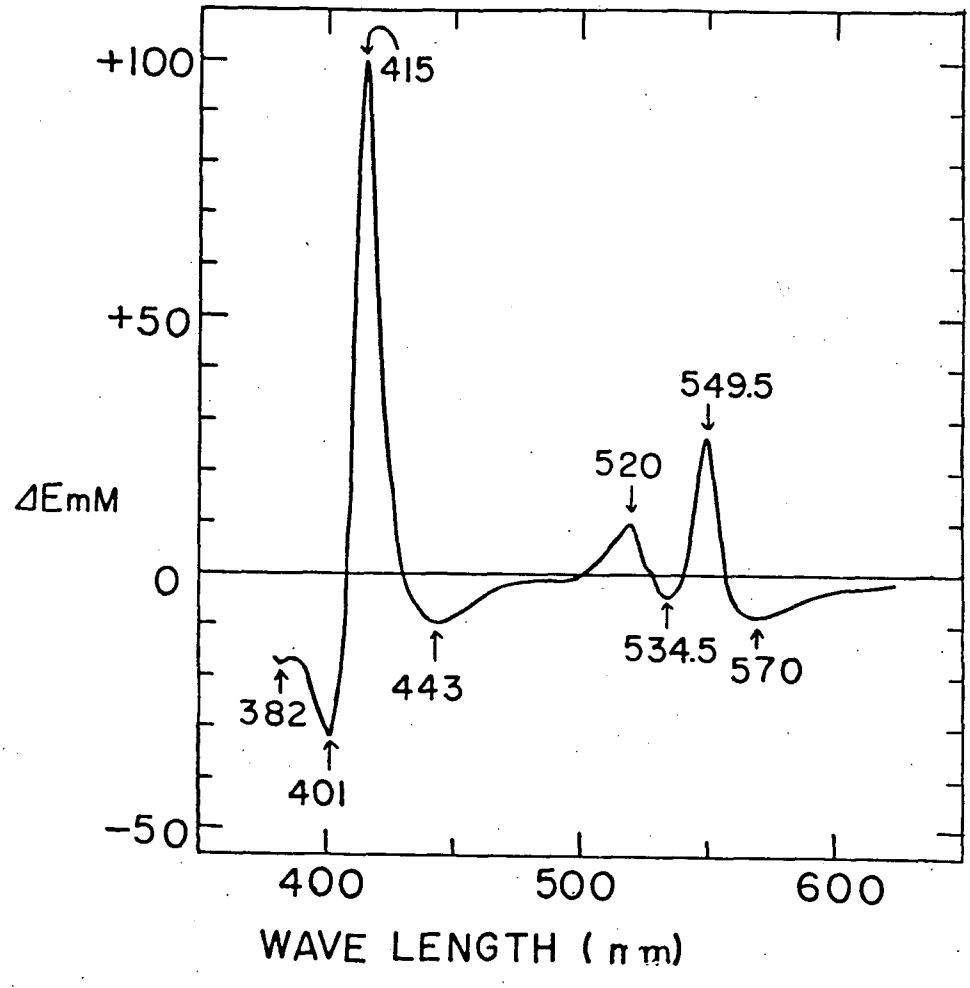

Fig. 8. Reduced-minus-oxidized difference spectrum of pyridine hemochrome from Petalonia cytochrome c-553. Differences of millimolar extinction coefficients (ΔEmM) were plotted from the data in Fig. 5.

354, 408.5 (γ-max) and 534 nm and a shoulder was found at 572 nm. The reduced form shows maxima at 413.5 (γ-max, shoulder at 390 nm), 475, 520 (β-max, shoulders at 510 and 528 nm) and 549.5 nm (α-max). The α-band of the pyridine derivative shows a symmetric feature without any shoulder. The heme cannot be split from the protein moiety by the usual acid-acetone method (1 ml of 20% HCl + 100 ml of acetone). From these results it appears that the heme of the cytochrome belongs to heme c.

Sixth Ligand of Heme Iron: The absorption spectra of concentrated solution of *Petalonia* cytochrome c-553 in the region from 600 to 800 nm is shown in Fig. 9. The ferricytochrome had two shoulders at around 640 and 695 nm, and the later has been suggested to be due to the heme-methionine coordination (66). Upon reduction with ascorbate, this shoulder disappeared but the 640-nm shoulder remained.

Redox Property and Midpoint Redox Potential: The *Petalonia* ferrocytochrome was not autoxidizable and was readily oxidized with ferricyanide. The ferricytochrome was fully reduced with ferrocyanide, ascorbate, cysteine-HCl, or hydrosulfite. The results of the titration of the cytochrome in ferricyanide solution with ferrocyanide are shown in Fig. 10. The 45° slope of the points plotted in the figure with the scale of log(ferrocytochrome)/(ferricytochrome) *versus* log(ferrocyanide)/(ferricyanide) indicates that one electron change was involved in the redox changes of the cytochrome. At half reduction of the cytochrome, the value of log(ferrocyanide)/(ferricyanide) was found to be 1.20;

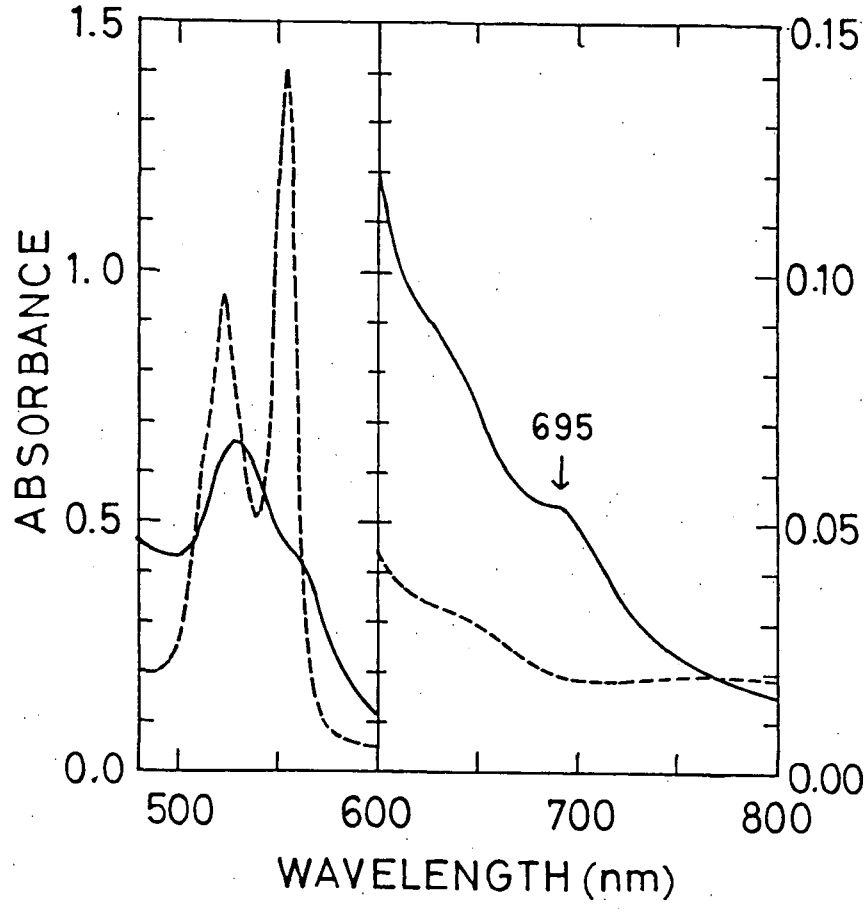

Fig. 9. Absorption spectra of Petalonia ferri- and ferro-cytochrome c-553 at 695-nm region. The cytochrome (0.5 mM) was in 2.5 ml of 50 mM K-phosphate buffer, pH 7.0, and the spectra were taken at 25°C. Ferricytochrome (——) prepared by adding 0.3 mg of solid K-ferricyanide and ferrocytochrome (---)by adding 0.5 mg of sodium ascorbate.

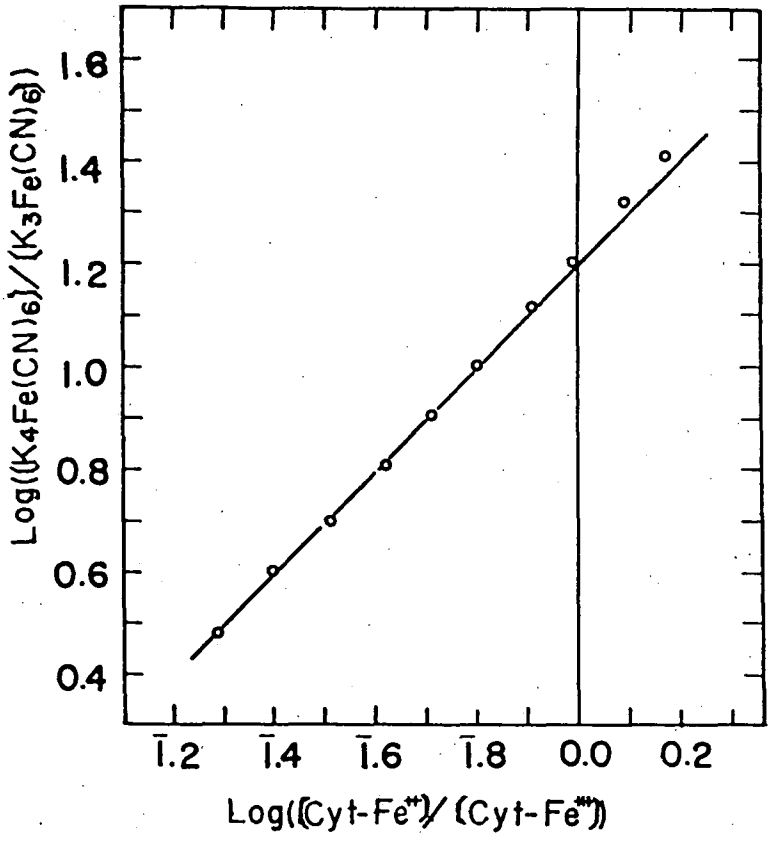

Fig. 10. Determination of the midpoint potential (Em) of <u>Petalonia</u> cytochrome <u>c</u>-553 at pH 7.0 and 20° C with mixture of ferri- and ferrocyanide of various ratios.

therefore the midpoint redox potential of the cytochrome was determined to be +0.346 volt at pH 7.0 and 20°C.

Isoelectric Points: Figure 11 shows the elution profile of *Petalonia* ferri- and ferrocytochrome *c*-553 using a pH gradient of isoelectric focusing at 0°C. The ferri- (brownish red band) and ferrocytochrome (fresh pink band) were clearly separated on the column, and their isoelectric points were at pH 4.3 and 4.1, respectively, at 0°C.

Iron Content and Specific Absorbancies: Concentrated solution of crystalline *Petalonia* cytochrome *c*-553 in ion-free water was sujected to iron analysis and determination of specific absorbancies. It was found that this solution showed 4.45 cm^{-1} of the absorbance at 553 nm and pH 7.0 when reduced with ascorbate, and a 10-ml aliquot contained 16.4 mg of the cytochrome protein and 0.0870 mg iron (0.53%). Assuming that one molecule of the cytochrome contains one heme, the molecular weight, the gram specfic absorbancy and the millimolar extinction coefficient at 553 nm were calculated to be 105 x 10^2, 2.71 liter $g^{-1}cm^{-1}$ and 28.5 $mM^{-1}cm^{-1}$, respectively.

Amino Acid Composition: The amino acid composition of the *Petalonia* cytochrome is shown in Table 2. The acidic amino acids are prominent, one residue each of histidine, arginine, tyrosine and tryptphan are present. This result presented the number of glutamic acid to be 11 in the cytochrome but the value was corrected to be 10 from the sequence analysis showing the residue numbers of 7 and 3 for glutamic acid and glutamine, respectively, as described

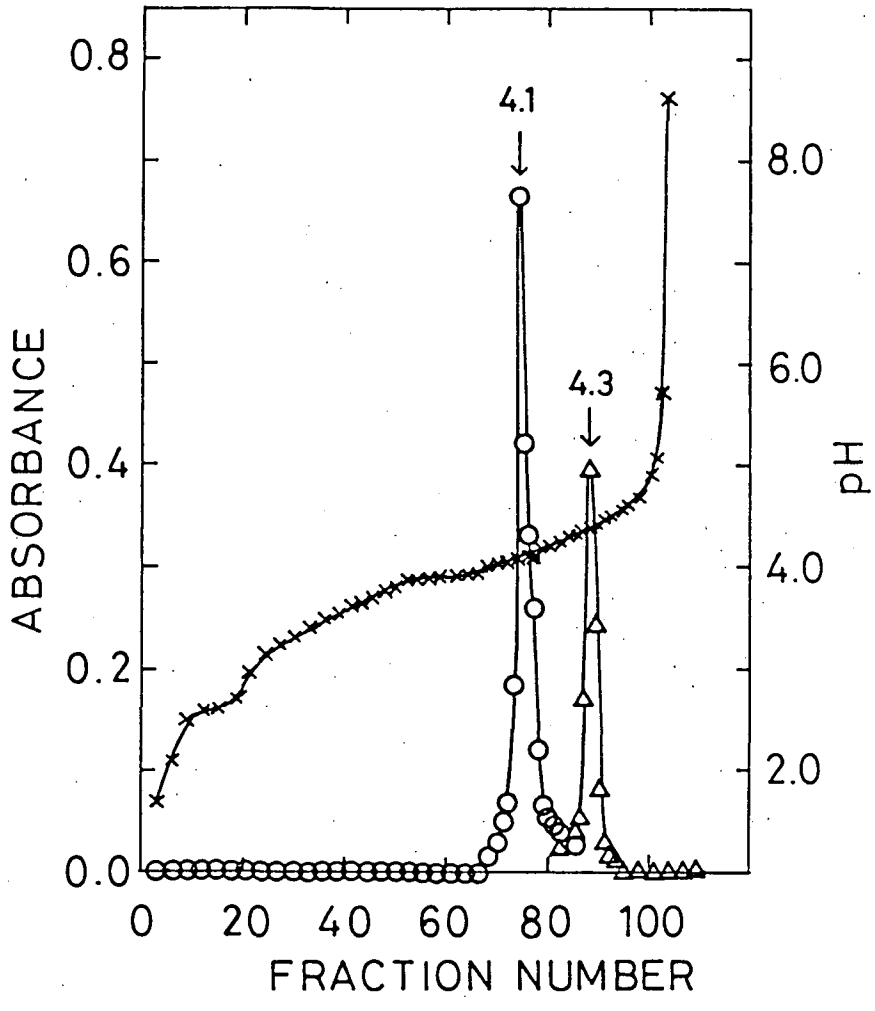

Fig. 11. Determination of the isoelectric points of Petalonia cytochrome c-553 in its oxidized and reduced forms. The isoelectric points were measured by the density gradient (49.1-4.54% glycerol) isoelectric focusing method using Pharmalyte (2.55%) of the pH range of 2.5-5.0 in a 110-cm column at 800 volt and 0°C for 30 hr. The solution was collected in 1-ml fractions. The absorbances at 410 nm (-Δ-) for ferricytochrome and 415.5 nm (-o-) for ferrocytochrome were measured at 20°C, and the pH (-x-) of each fraction was measured at 0°C.

Table 2. Amino acid compositions of Petalonia cytochr c-553, several other algal c-type cytochromes and rice-mitochondria tochrome c

Amino acid	Values after hydrolysis[a] for 24 hr	48 hr	Average	Nearest integer	Sp	Sm	Pt	Ml	Bf	Ae	Eg	Cyt. c
Lys	5.38	6.01	5.70	6	6	7	6		3	6	4	13
His	1.01	1.03	1.02	1	1	1	1		1	1	1	2
Arg	0.97	1.04	1.01	1	2	2	1		2	1	1	2
Asp	15.2	15.1	15.2	15	14	13	17	1	10	18	10	10
Thr	4.90	5.10	5.10	5	2	2	3		3	6	5	8
Ser	5.45	5.21	5.69[b]	6	6	5	4		4	4	5	7
Glu	10.9	11.7	11.3	11	7	5	8	1	7	6	9	10
Pro	1.97	2.13	2.05	2	2	2	2		1	2	2	9
Gly	7.34	7.61	7.48	7	7	9	7		6	6	12	11
Ala	7.23	7.72	7.46	7	17	16	12	1	9	10	10	9
Cys	1.63	1.84	1.74[c]	2	2	2	2		2	2	2	2
Val	5.60	6.33	6.33[d]	6	10	10	5		4	6	10	3
Met	2.60	3.34	2.97	3	2	2	3		1	3	1	2
Ile	5.63	6.46	6.46[d]	6	2	2	4		3	6	4	4
Leu	3.22	3.45	3.34	3	4	4	5		3	4	3	8
Tyr	1.10	1.27	1.19	1	3	3	2		2	1	5	6
Phe	3.15	3.45	3.30	3	3	3	2		2	3	1	4
Trp	0.85[e]	—	0.85	1	1	1	1		1	1	2	1
Total				86	91	89	85		63	86	87	111

Sp, Spirulina platensis(44); Sm, S. maxima(51); Pt, yra tenera(51); Ml, Monochrysis lutheri(52); Bf, Bumilleriopsis filifor 7); Ae, Alaria esculenta(53); Eg, Euglena gracilis(54); Cyt.c, rice chrome c(68), which includes two trimethyllysines.

[a] The average of the values per one mole of heme by determinations. [b] Value extrapolated to zero time of hydrolysis. [c] as cystei id. [d] Value of 48-hr hydrolysis. [e] Based on acid hydrolysis with thioglyc c acid.

in the next chapter. The total number of amino acid residues was 85.

Molecular Weight: The elution position of the *Petalonia* cytochrome from Sephadex G-75 gel filtration was plotted against the logarithm of molecular weight of various marker proteins as shown in Fig. 12. The polyacrylamide gel electrophoresis in the presence of SDS showed a single band as shown in Fig. 13. The molecular weight obtained from the various analyses carried out in the present study are as follows: 10.5×10^3 from the iron content; 10.2×10^3 from the gel filtration; 11×10^3 from the polyacrylamide gel electrophoresis in the presence of SDS; 9,803 from the amino acid composition and sequence. These results showed that *Petalonia* cytochrome *c*-553 was composed of a single polypeptide chain with a molecular weight of 9,803 containing 85 amino acid residues and one heme *c*.

3.2. Other Algal *C*-type Cytochromes

Thirty-seven species of wild-grown algae including 19 species of red algae, 6 species of brown algae, 1 species of yellow-green alga, 10 species of green algae and 1 species of stone-work were examined and *c*-type cytochromes were easily extracted from all of these algae shown in Table 3. The fundamental extraction procedure of cytochrome was by immersing fresh, dried or frozen-stocked thalli in water without mechanical disruption of algal cells. In the case of brown algae, the extraction was carried out exceptionally with dilute ammonia water. Sometimes it is convenient

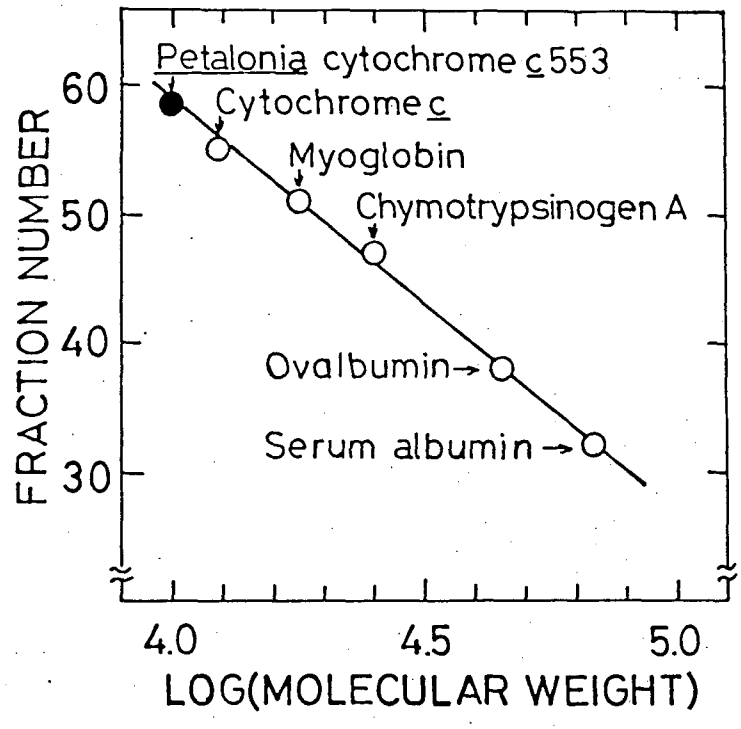

Fig. 12. Estimation of the molecular weight of Petalonia cytochrome c-553 by gel filtration. Gel filtration was carried out with a Sephadex G-75 column (2.64 x 45 cm) using 0.125 M NaCl in 0.02 M K-phosphate buffer, pH 7.0, at 5°C. The flow rate was 35 ml per hr and the eluate was collected in 3.2-ml fractions.

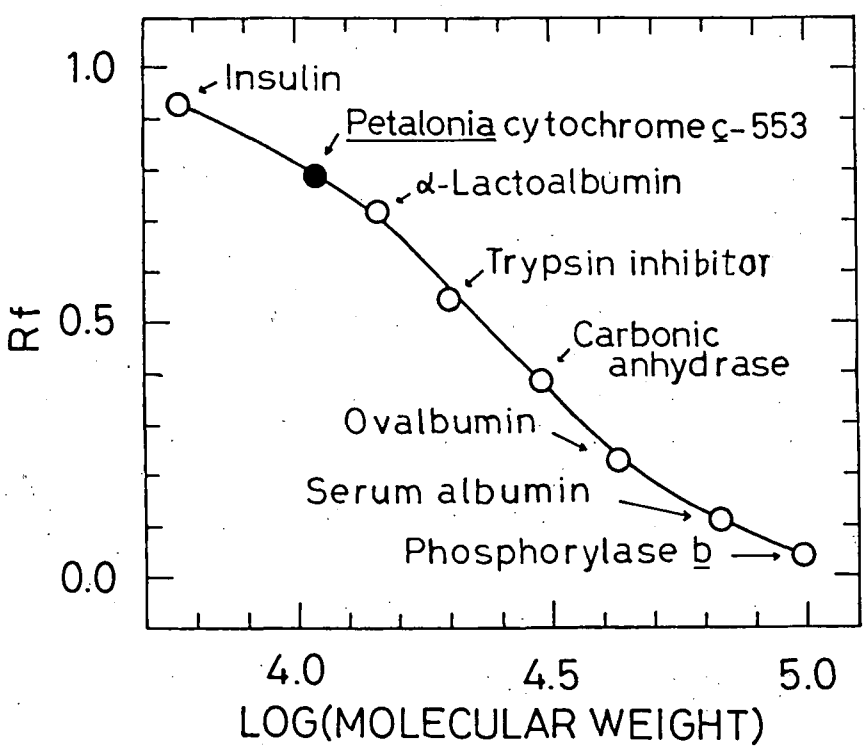

Fig. 13. Estimation of the molecular weight of Petalonia cytochrome c-553 by SDS gel electrophoresis. The polyacrylamide gel electrophoresis was carried out in 15% gel in the presence of 0.1% SDS and 0.1 M phosphate buffer, pH 7.2, at 7 mA per gel (0.5 x 10 cm) for 6 hr.

to plasmolyze the fresh material by the addition of solid ammonium sulfate which corresponds to about a quarter of the water of the material. A considerable amount of cytochrome is brought into solution by this procedure and the remainder in the thalli can be extracted by adding sufficient water. Interfering slimy substances were removed by acrinol treatment which is almost invariably essential for the preparation of algal cytochromes. By ammonium sulfate fractionation, the precipitate between 60 and 100% saturation was collected. Red-algal phycobilinoproteins could be removed by precipitating them at 0.35-0.5 saturation of ammonium sulfate. Ion exchange column chromatography with DEAE-cellulose or DEAE-Sephadex and the successive gel filtration chromatography with Sephadex G-75 were found to be most effective for purification. The value of the purity-index ($A\alpha$/Aprotein) was always near one before success in crystallization was attained. The nine cytochromes marked with asterisks in Table 3 were able to be crystallized according to the same procedure as mentioned above for the Petalonia cytochrome.

The absorption maxima of reduced forms and midpoint redox potentials of the cytochromes are listed in Table 3. These algal cytochromes predominantly have their α-peak at 553 nm accompanied by a shoulder at 549 nm. Exceptions are Polysiphonia cytochrome c-551.5 and Caulerpa cytochrome c-552.5 which have symmetrical bands. The Codium cytochrome shows the absorption peaks at higher wavelength, viz., α-peak at 554.5 nm with a shoulder at 550.5 nm, β-peak at 523 nm and

Table 3. Absorption peaks (λ) at reduced state and midpoint redox potentials (Em, volt) of water-soluble c-type cytochromes

Source	λ (nm)			γ/α	Em at pH 7.0
	α	β	γ		
Red algae					
*Bangia fusco-purpurea	553.5	521.5	415.5	6.7	-
*Porphyra tenera	553.0	522.0	416.0	7.0	-
*Porphyra yezoensis	553.0	522.0	416.0	7.7	0.328
*Porphyra pseudolinealis	553.0	522.0	416.0	-	0.326
Nemalion vermiculare	553.0	522.0	416.0	-	0.349
Scinaia japonica	553.0	521.5	415.5	6.7	-
Gloiophloea okamurai	553.0	521.0	416.0	5.7	-
Pterocladia tenuis	552.5	521.5	415.5	5.6	-
Grateloupia filicina	552.5	521.8	416.0	6.6	-
Grateloupia elliptica					
Pachymeniopsis lanceolata	552.5	521.5	415.3	7.2	0.357
*Gloiopeltis complanata	553.0	521.0	415.0	-	0.340
Schizymenia dubyi	553.5	522.5	416.0	6.8	0.340
Gracilaria verrucosa	552.8	521.5	415.5	6.4	-
Gracilaria textorii	553.0	522.0	416.0	6.8	-
Chondrus giganteus	553.0	521.5	415.5	6.3	-
Rhodoglossum pulcherum	553.5	521.5	415.5	5.9	-
*Polysiphonia urceolata	551.5	522.5	416.3	5.7	-
Chondria crassicaulis	553.0	521.5	415.5	5.9	-
Brown algae					
Spatoglossum pacificum	553	521.	416	5.7	0.336
Ishige okamurai	553.0	521.5	415.5	6.7	-
Scytosiphon lomentaria	553.0	521.5	415.5	-	-
*Endarachne binghamiae	553.0	521.5	415.5	6.6	0.347
*Petalonia fascia	553.0	521.5	415.5	6.9	0.346
Undaria pinnatifida	553.0	522.0	415.5	6.9	0.339
Yellow-green algae					
Vaucheria sp.	553.0	521.5	415.3	5.7	-
Green algae					
Ulva pertusa	553.0	522.3	416.3	6.7	0.376
*Enteromorpha prolifera	552.5	522.3	416.3	6.7	0.350
Chaetomorpha spiralis	553.0	521.5	416.0	6.5	0.368
Chaetomorpha crassa	552.8	522.5	416.0	6.5	0.367
Cladophora sp.	553.0	522.3	416.0	6.0	0.364
Caulerpa brachypus	552.5	523.0	416.0	6.6	0.371
Bryopsis maxima	554.0	523.5	417.3	-	-
Bryopsis sp.	553.0	522.5	-	-	0.371
Codium latum	554.5	523.0	417.0	7.0	0.376
Codium fragile	554.5	523.0	417.0	-	-
Stone-work(Chara)					
Chara sp.	552.3	522.3	416.3	6.8	-

* Obtained in crystalline form. - Not determined.

γ-peak at 417 nm in the reduced state. The midpoint redox potential of the red- and brown-algal cytochromes was found to be 0.34 volt on the average. For the green-algal cytochromes was obtained the more positive value of 0.368 volt. Isoelectric points of the cytochromes purified from 4 species of red, brown and green algae are listed in Table 4. A concentrated solution of ferricytochrome c-554 of *Bryopsis maxima* showed a shoulder around at 695 nm in the same manner as *Petalonia* cytochrome c-553 mentioned above.

Table 4. Isoelectric points (pH) of algal ferro- ($Cyt.Fe^{++}$) and ferricytochrome ($Cyt.Fe^{+++}$)

Source	Isoelectric points (pH)	
	$Cyt.Fe^{++}$	$Cyt.Fe^{+++}$
Red alga		
Pachymeniopsis lanceolata	3.70	3.95
Brown algae		
Spatoglossum pacificum	4.26	——
Undaria pinnatifida	4.13	4.39
Green alga		
Bryopsis maxima	3.91	4.02

—— not determined

4. Discussion

A wide distribution of water-soluble cytochromes among algae were confirmed by extraction and purification of the

proteins from each of the examined 37 species belonging to 10 families of 6 orders of Rhodophyceae, 4 families of 4 orders of Phaeophyceae, 1 family of Xanthophyceae, 5 families of 3 orders of Chlorophyceae and 1 family of Charophyceae as shown in Table 3. Similar cytochromes have been isolated by other authors also from algae of Cyanophyceae (2, 38, 44, 45, 69, 70), Chrysophyceae (52, 67), Bacillariophyceae (43, 46) and Euglenophyceae (36, 39, 42, 71).

In this investigation were the cytochromes extracted by immersing thalli in water without mechanical disruption of algal cells, except that dilute ammonia water was used for brown-algal cytochromes. In the most cases slime created difficulties when material was homogenized or acetone-treated. The best yield of the cytochromes was obtained when each cytochrome was extracted by immersing the intact or frozen-stocked thalli in water (or in 20 mM ammonia water for brown-algal cytochromes). The acrinol treatment was also found to be effective procedure for removing phycoerythrin from red-algal extract as well as for removing the slimy substance from most of algal crude extracts.

The behavior of the algal cytochromes on a DEAE-cellulose column was similar each other, but slight differences were observed. For example, Bryopsis maxima cytochrome c-554 can be adsorbed on the column from its solution in 50 mM phosphate buffer, pH 7.0, whereas in the case of Petalonia cytochrome c-553 or Endarachne cytochrome c-553, the buffer concentration must be lowered to 5 mM to adsorb the cytochrome on the same column. These behaviors are due to

the more positive isoelectric point of the Petalonia cytochrome than that of the Bryopsis cytochrome. Isoelectric focusing separated the oxidized and reduced forms of each of the cytochromes. The about 0.2 more positive isoelectric point of the oxidized form than that of the reduced form accounts for the observation that the cytochrome moved more rapidly on a DEAE-cellulose column in the oxidized state than in the reduced one. The charge change of the cytochrome may be due to the heme-iron valency change $Fe^{++} \rightleftharpoons Fe^{+++}$ of the protein. The algal cytochrome in oxidized form can be adsorbed on an Amberlite CG-50 cation-exchanger column at pH 5.0 (33). The method, however, is not suitable, as the adsorption is not very distinct and, moreover, the cytochrome are sometimes denatured in these acidic media. Endarachne and Petalonia cytochromes c-553 moved more rapidly on a CG-50 column in reduced state. Similar modes of behavior of heart-muscle cytochrome c on the same column have been observed by Margoliash (72). The isoelectric points of other four algal soluble cytochromes have been reported to be all acidic at pH 3.5-5.5 in reduced state, but the values of their oxidized forms are not yet determined (32, 40, 42, 44).

In c-type cytochromes of many kinds of algae were some slightly different cytochromes found besides typical cytochromes, e. g., Petalonia cytochrome c-553, showing about 553-nm α-peak accompanied by a shoulder at 549 nm. Namely, Polysiphonia cytochrome c-551.5 and Caulerpa cytochrome c-552.5 have symmetric α-bands without any shoulder. Euglena cytochrome c-552 has also a symmetric α-band (42). In

contrast to that, all of the cytochromes f so far studied have α-band at 554-555 nm with a shoulder at 551 nm (18, 49, 73, 74). The α-band of Codium cytochrome c-554.5 resembles that of cytochrome f. γ-Peak at 415-417 nm of the water-soluble algal cytochromes in reduced state is 5-7 nm lower than that of cytochromes f. The absorbance-ratio of γ- to α-peak of the soluble cytochromes was found to be 6-7, a value that is almost identical with that of cytochrome f. These values are distinguishable from the value of absorbance-ratio of about 5 of cytochrome c. The millimolar extincion coefficient of 28.5 at α-peak of Petalonia ferrocytochrome c-553 is comparable to the value of 29 of cytochrome c (75, 76), but it is considerably higher than the value of 22-26 obtained for the other four algal soluble cytochromes (32, 40, 42, 46).

The average value (0.368 volt) of midpoint potentials of the soluble cytochromes of 8 species of green algae is higher than the potential (0.35-0.36 volt) of green-algal cytochromes f (7, 18, 49), and these values are lower than that (0.43-0.53 volt) of P700 (77). These facts support the view of a electron flow sequence cytochrome f → c-553 → P700 between the photosystems II and I in algal photosynthesis (49). The midpoint redox potential of red- and brown-algal cytochrome c-553 is about 0.02 volt lower than that of the green-algal cytochrome, suggesting the redox property also of other components in red- or brown-algal photosynthetic electron transport system to be different from that in the green-algal system.

From the results of iron content, gel filtration, SDS polyacrylamide gel electrophoresis, and amino acid composition, Petalonia cytochrome c-553 was confirmed to be composed of a single polypeptide chain with a molecular weight of about 10,000 containing one mole of heme c. Both of the concentrated solutions of Petalonia and Bryopsis cytochromes in the oxidized form showed a absorption shoulder at 695 nm in the red region. This suggested that the sixth ligand of heme iron of these cytochromes is a methionine sulfur as suggested for the mitochondrial cytochrome c (66). Similar observations were reported for Euglena cytochrome c-552 (55) and Spirulina cytochrome c-554 (44). However, no cytochrome f shows such absorption in the red region (78). The amino acid sequences of five algal soluble c-type cytochromes have been studied, indicating that they have only one invarant methionine residue at a position 62 (51). Therefore, the sulfur of Met-62 is probably the sixth ligand for heme iron.

The amino acid compositions of seven algal cytochromes c-553 and one higher plant cytochrome c (68) are listed together with Petalonia cytochrome c-553 in Table 2. The Petalonia cytochrome most closely resembles the Alaria cytochrome; incidentally, these two algae belong to the same division. In general, algal cytochrome c-553 has fewer lysine, proline and leucine residues. The aspartic acid in the cytochrome of S. platensis, S. maxima, P. tenera, P. fascia and A. esculenta is high but that of E. gracilis, M. lutheri and B. filiformis is nearly the same as that of higher

plant cytochrome c. The amino acid sequence analysis of Petalonia cytochrome c-553 revealed that the cytochrome contained 14 amide residues of Asn_{11} and Gln_3 (Chapter III). Therefore, the 11 acidic residues of Glu_7 and Asp_4 may contribute to the acidic nature of the cytochrome protein against the 8 basic residues of Lys_6, His_1 and Arg_1. The amino acid analysis showed that the cytochrome had 11 Glu residues in a total of 86 residues as shown in Table 2. The sequence analysis mentioned later gave 7 Glu and 3 Gln in a total of 85 residues. A reexamination of HCl hydrolysis for 24 hr of the cytochrome revealed 10 Glu residues.

III. AMINO ACID SEQUENCE OF CYTOCHROME C-553 OF *Petalonia fascia*

1. Introduction

Algal cytochrome *c*-553 is thought to transport electron from cytochrome *f* to P700 between photosystems I and II (14). The preparation, distribution among algae and physico-chemical properties of this cytochrome were described in chapter II. The cytochrome protein consists of a single polypeptide chain of 63-91 amino acid residues with 1 mol of heme covalently bonded to the sequence Cys-X-Y-Cys-His. The sixth ligand of the heme iron is thought to be a sulfur of Met-62. Its characteristic properties are a water solubility, an α-absorption band at 551.5-554.5 nm, a sharp γ-band at 415.3-417.7 nm, a high midpoint redox potential of 0.33-0.38 volt, a low molecular weight of about 9,000, and an acidic nature of isoelectric point at pH 3.5-5.5.

Several workers have determined the amino acid sequences of the cytochromes isolated from the five divisions of algae; *Spirulina maxima* (Cyanophyta) (51), *Porphyra tenera* (Rhodophyta) (51), *Monochrysis lutheri* (Chrysophyta) (52), *Alaria esculenta* (Phaeophyta) (53), and *Euglena gracilis* (Euglenophyta) (54). They were homologous to one another, also to mitochondrial cytochrome *c* and bacterial photosynthetic cytochrome c_2.

The crystallization of cytochrome *c*-553 from a brown alga, *Petalonia fascia*, as well as its molecular properties

and amino acid composition were described in chapter II. It is now important to elucidate the complete amino acid sequence of this cytochrome in order to understand the relationship between structure and function and also the position of the alga in the evolutionary phyletic system relative to various other algae.

This chapter describes the amino acid sequences of the four chymotryptic peptides and the four BrCN peptides obtained from Petalonia cytochrome c-553 and the deduction of the complete sequence of the cytochrome by overlapping of those of the BrCN peptides and the chymotryptic peptides. The sequence is compared with those of five algal cytochromes reported so far, and shows highest and lowest homology to Alaria (Phaeophyta) cytochrome c-553 and Euglena cytochrome c-552, respectively. The possible secondary structures also are predicted from the complete sequences of Petalonia cytochrome c-553 as well as of other four algal cytochromes by the computerized Chou and Fasman method (79).

2. Materials and Methods

Material: The thalli of Petalonia fascia were harvested from Shizugawa Bay at Shizugawa, Miyagi and from Sagami Bay at Shichirigahama, Kanagawa, Japan. Petalonia cytochrome c-553 was prepared as described in chapter II to a purity index (A553/A273) of above 1.10. The crystallization procedure was omitted. The purified cytochrome was extensively dialyzed against pure water. A precipitate formed during dialysis was

removed by centrifugation at 5,000 rpm for 15 min.

Enzymes and Chemicals: Carboxypeptidase A was purchased from Worthington, Bio-Gel P-6 from Bio-Rad, Sephadex G-50 and SP-Sephadex from Pharmacia, Aminopropylglass and p-phenylene-diisothiocyanate from LKB Biochrome, and thin-layer plates (Silica Gel) from Merk. All other chemicals were of analytical or sequenal grade from Nakarai Chemical Co., Kyoto, or from Wako Pure Chemical Industries, Osaka.

Chymotryptic Digestion and Separation of the Resulting Peptides: About 60 mg (6.1 μmol) of *Petalonia* cytochrome *c*-553 was denatured with 90% ethanol and dried in vacuum at 45°C. The denatured protein was digested with chymotrypsin (2 mg) in 5 ml of 0.02 M Tris-HCl buffer, pH 8.0, at 40°C overnight. The hydrolyzate was fractionated on an anion exchange column of Dowex AG1-X2 (200-400 mesh, 1.5 x 90 cm) using the following buffer systems (80): (a) 0.124 M pyridine, pH 9.0 (160 ml), (b) a linear gradient from 0.124 M pyridine, pH 9.0 (200 ml), to 0.124 M pyridine/3 mM acetic acid, pH 6.8 (200 ml), (c) 0.124 M pyridine/3 mM acetic acid, pH 6.8 (200 ml), (d) a linear gradient from 0.124 M pyridine/3 mM acetic acid, pH 6.8 (250 ml) to 1 M acetic acid (250 ml), (e) 1 M acetic acid (100 ml), (f) a linear gradient from 1 M acetic acid (200 ml) to 5 M acetic acid (200 ml) and (g) 5 M acetic acid (100 ml). Each fraction (3.1 ml) was monitored by following the absorbances at 570 nm after the ninhydrin reaction (81) of the 0.1 ml aliquot. Further purification of the peptide was carried out by preparative paper chromatography and electrophoresis (82). Paper chromatography was carried out

with n-butanol/pyridine/acetic acid/water (15:10:3:12, v/v, BPAW). Paper electrophoresis was carried out at pH 3.6 (pyridine/acetic acid/water, 1:10:189, v/v) or at pH 6.5 (pyridine/acetic acid/water, 10:0.4:180, v/v) and at 2,000 volts. Location of peptides separated on paper was detected by the ninhydrin method (83) followed by specific staining for tryptophan (84), histidine (85), tyrosine (85) and arginine (86). Peptide were eluted from paper with 0.5 N NH_4OH.

Further Digestion of Chymotryptic Peptides with Thermolysin or Trypsin: Thermolysin digestion was carried out in 5 mM Tris-HCl buffer, pH 7.5 containing 2.5 mM $CaCl_2$ at room temperature (87). Trypsin digestion was carried out in 7 mM Tris-HCl buffer, pH 8.0 adjusted with 1 N NaOH, at room temperature. The peptides obtained by thermolysin digestion and by trypsin digestion were separated by paper electrophoresis at pH 6.5 and 3.5 as mentioned above for chymotryptic peptides, respectively.

Cleavage with BrCN and Separation of the Resulting Peptides: Cleavage of *Petalonia* cytochrome *c*-553 with BrCN was performed by the method of Steers *et al*. (88). The native cytochrome (2 μmol) was treated with BrCN (570 μmol) in 0.3 ml of 70% formic acid solution at room temperature for 14 hr. The reaction mixture was diluted with 2.7 ml of water and lyophilized. The dried sample was dissolved in 3 ml of 8 M urea-phosphoric acid, pH 2.6, and applied to an SP-Sephadex column (1.2 x 40 cm) equilibrated with the same urea-phosphoric acid solution. The column was eluted with a linear gradient of 0 to 0.25 M NaCl in 600 ml of 8 M urea-

phosphoric acid, pH 2.6 at a flow rate of 20 ml per hr and room temperature. The eluates were monitored in term of absorbance at 206 nm and collected in 4.4-ml fractions. Three BrCN peptides (CN-1, -3 and -4) were obtained as described later.

Peptide CN-2 was one of the peptides obtained by another BrCN cleavage experiment as follows. The cytochrome (3 μmol) was cleaved with BrCN (2.6 mmol) in 2 ml of 70% formic acid at 20°C for 20 hr. The resulting peptides were separated by SP-Sephadex column (1.5 x 30 cm) chromatography with a linear gradient of 0.1 to 0.4 M NaCl in 8 M urea-phosphoric acid, pH 2.6 at a flow rate of 30 ml per hr. The eluate were monitored in terms of absorbance at 206 nm and collected in 5-ml fractions. The third peak fractions (Nos. 44-66) (data not presented) were combined and desalted by gel filtration on a Sephadex G-10 column (4 x 20 cm) in 10% formic acid solution. The peptides were further purified by paper electrophoresis at pH 3.6 and 2,500 volts for 1 hr or by gel filtration on a Sephadex G-50 column with 10% formic acid.

Heme Cleavage and Carboxymethylation of Heme Peptide: The heme was removed by the method of Fontana *et al.*(89) from a heme peptide (0.5 μmol) obtained by BrCN cleavage of *Petalonia* cytochrome *c*-553. The heme-free peptide thus obtained was reduced with 2-mercaptoethanol and carboxymethylated with iodoacetate according to Crestfield *et al.* (90) to obtain Cm-peptide.

Amino Acid Analysis: *Petalonia* cytochrome *c*-553 and its peptides were hydrolyzed for 24 hr as menthioned in chapter II.

The amino acid compositions were determined with an amino acid analyzer, Irica model A-3300, according to the method of Spackman et al. (61).

N-Terminal Sequence Analysis: Sequential degradation from N-terminus was performed by a manual Edman procedure (91) or by solid-phase analysis (92) using an LKB model 4020 sequencer. In the solid phase method Petalonia cytochrome (0.15 μmol) was coupled to aminopropyl-glass (250-300 mg) through the ε-amino group of lysine residues and the BrCN peptides (0.1 μmol each) through the C-terminal homoserine. PTH derivatives were identified by TLC on Merk Silica-gel plates using mainly two solvent systems, II; methanol/chloroform (10:90,v/v) and V; n-propanol/ethylene dichloride/propionic acid (58:25:17, v/v)(93). High performance liquid chromatography also was applied to identify PTH-amino acids using acetonitrile/0.01 M acetate buffer pH 4.5 (42:58 or 48:52,v/v) (94). PTH-histidine and PTH-arginine were identified by paper electrophoresis at pH 6.5 (95).

C-Terminal Sequence Analysis: The C-terminal sequence of Petalonia cytochrome c-553 denatured with ethanol or the peptides were determined by digestion with carboxypeptidase A (96). The sample (22-nmol protein or 28-nmol peptide) was digested by carboxypeptidase A (40 μg) in 0.1 M Tris-HCl buffer (50 μl), pH 8.0 at 40°C for 0.5-18.5 hr. After various periods of time 10-μl aliquots of the digest were inactivated by the addition of 0.22 M sodium citrate-HCl buffer (50 μl), pH 2.2, and subjected to amino acid analyses.

Prediction of Secondary Structure from Amino Acid

Sequence: Secondary structures, viz., α-helix, β-sheet, β-turn, and coil, for Petalonia cytochrome c-553 and other four algal c-type cytochromes sequenced so far (51, 53, 54) were estimated from their complete amino acid sequences according to the empirical method of Chou and Fasman (79).

Construction of Phylogenic Tree of Algal Cytochromes: A phylogenic tree of algal cytochromes c-553 was constructed by comparing the numbers of different amino acids among six cytochromes, according to the method of Fitch and Margoliash (97).

Nomenclature of the Peptides: The prefixes C-, T- and Th- refer to chymotryptic, tryptic and thermolytic peptides, respectively. Peptides obtained from BrCN cleavage were referred to CN-. The numbers of BrCN peptides show their positions in the polypeptide chain of Petalonia cytochrome c-553 from the N-terminus. All analytical values of amino acids are expressed in moles per mole of peptide or protein.

Abbreviations: N-, amino-; C-, carboxyl-; Cm-, carboxymethyl-; Cmc, carboxymethylcysteine; Hse, homoserine; PTH-, phenylthiohydantoin-; TLC, thin layer chromatography; CPase, carboxypeptidase.

3. Results

Terminal Sequence of Cytochrome c-553: A manual Edman degradation of 0.13 μmol of the Petalonia cytochrome c-553 up to 3 residues and a solid-phase sequencing of 0.15 μmol of the cytochrome up to 34 residues revealed the N-terminus

to be as follows: Val-Asp-Ile-Asn-Asn-Gly-Glu-Ser-Val-Phe-Thr-Ala-Asn(Cys)Ser-Ala(Cys)His-Ala-Gly-Gly-Asn-Asn-Val-Ile-Met-Pro-Glu(Lys)Thr-Leu(Lys)(Lys)Asp. No PTH-amino acis were detected at the steps corresponding to lysine and cysteine residues, which were not released from the glass and the heme moiety, respectively. This result up to 10 residues agree with that of a chymotryptic peptide (C-17) from the cytochrome described later, except for Asp-5.

CPase A released from the ethanol-denatured cytochrome (3.46 nmol) only tryptophan (3.13 nmol) at 30 min, tryptophan (2.92 nmol) and glycine (0.45 nmol) at 2.5 hr and tryptophan (3.22 nmol) and glycine (1.92 nmol) at 18.5 hr, respectively, indicating the C-terminal sequence of the protein to be -Gly-Trp.

Separation and Amino Acid Compositions of Chymotryptic Peptides: The chymotryptic peptides were separated on an anion exchange column, Dowex AGL-X2, and the elution patterns shown in Fig. 14. Five fractions were purified by paper chromatography and paper electrophoresis, and only four peptides, C-9, C-14, C-15 and C-17 were studied for the sequence determination. Purification methods, amino acid compositions and properties of these peptides are summarized in Table 5.

Amino Acid Sequences of Four Chymotryptic Peptides: The sequencing of four chymotryptic peptides is given below.

Peptide C-9. This peptide was further purified by paper electrophoresis at pH 3.6 and 2,000 volt for 90 min using Whatman 3MM paper. Six steps of manual Edman degradation of 0.138 μmol of the peptide revealed th N-terminal sequence,

Gly-Lys-Asx-Ala-Met-Pro. Asx was identified only by subtractive analysis. The remainder (0.056 µmol) was digested with CPase A to release phenylalanine and alanine

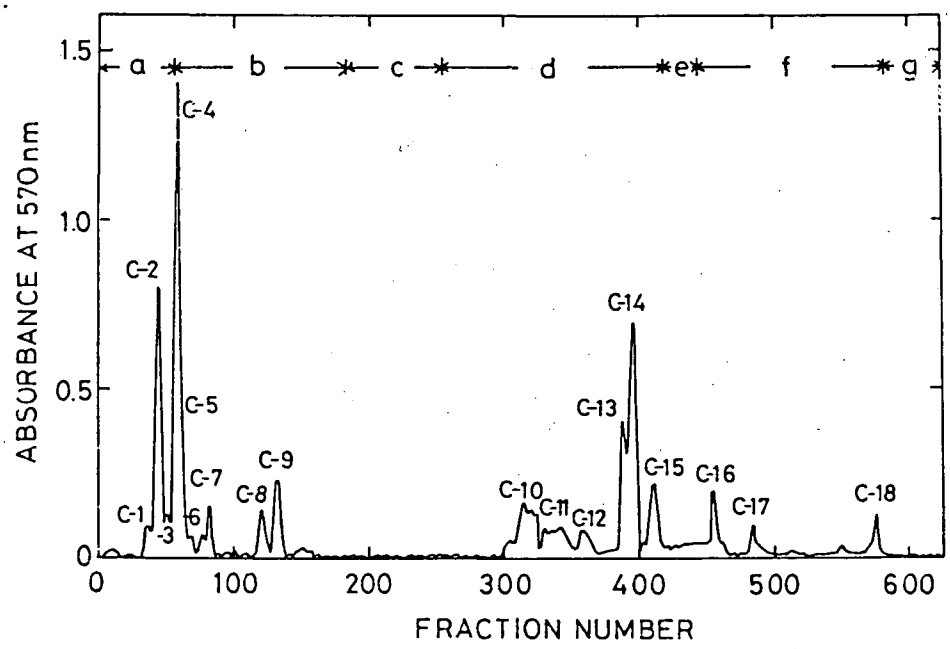

Fig. 14. Elution pattern of chymotryptic peptides of Petalonia cytochrome c-553. The chymotryptic peptides were chromatographed on a Dowex AG 1-X2 column (1.5 x 90 cm) and developed with the following buffer system: (a) 0.124 M pyridine, pH 9.0 (160 ml), (b) a linear gradient from 0.124 M pyridine (200 ml) to 0.124 M pyridine/3 mM acetic acid, pH 6.8(200 ml), (c) 0.124 M pyridine/3 mM acetic acid, pH 6.8 (200 ml), (d) a linear gradient from 0.124 M pyridine/3 mM acetic acid, pH 6.8 (250 ml) to 1 M acetic acid (250 ml), (e) 1 M acetic acid (100 ml), (f) a linear gradient from 1 M acetic acid (200 ml) to 5 M acetic acid (200 ml). Each fraction was monitored at 570 nm after the ninhydrin reaction (83).

successively. From these results and the amino acid composition, the peptide was concluded to have the sequence, Gly-Lys-Asx-Ala-Met-Pro-Ala-Phe.

Peptide C-14. Purification was carried out by paper chromatography. The manual Edman degradation of 0.27 μmol of

Table 5. Amino acid compositions of BrCN peptides of *Petalonia* cytochrome *c*-553

Amino acid	C-9	C-14	C-15	C-17
Lys	1.10(1)	1.90(2)	1.00(1)	
Asp	1.47(1)	2.13(2)		2.81(3)
Ser			1.73(2)	0.90(1)
Glu		3.12(3)	2.32(2)	1.16(1)
Pro	0.97(1)			
Gly	1.04(1)			1.10(1)
Ala	1.97(2)	0.98(1)		
Val			0.93(1)	1.82(2)
Met	0.73(1)	0.68(1)		
Ile			0.92(1)	1.00(1)
Leu		0.94(1)		
Phe	0.93(1)			0.94(1)
Trp			+ (1)*	
Total	8	10	8	10
Purification	PE	PC		

* Ehrlich reaction positive, suggesting of the presence of one tryptophan residue. PE, paper electrophoresis at pH 3.6. PC, paper chromatography using butanol/pyridine/acetic acid/water = 15:10:3:12, v/v.

the peptide revealed the N-terminal sequence, Lys-Lys-Asp-Ala. The digestion of 41 nmol of the peptide with CPase A (60 μg) in 3 ml of Tris-HCl buffer released two amino acids: 33 nmol of methionine and 12.6 nmol of glutamic acid after 2 hr; 31 nmol of methionine and 41 nmol of glutamic acid after 23 hr, showing the C-terminal sequence to be Glu-Met. The peptide (0.8 μmol) was digested with thermolysin (10 μg) in 0.2 ml of the buffer for 4 hr as described under "Further Digestion of Chymotryptic Peptides with Thermolysin or Trypsin". The resulting peptides were separated by paper electrophoresis at pH 6.5 for 1 hr to give three peptides, C-14-Th-1, C-14-Th-2 and C-14-Th-3. Their composition were estimated from the paper chromatography of each hydrolyzate to be Lys_2, Asp_1, Ala_1 for Th-1, and Asp_1, Glu_3, Met_1, Leu_1 for both Th-2 and Th-3. The sequence of Th-3 was found to be Leu-Glx-Glx by manual Edman degradation. These results suggested the total sequence of Peptide C-14 to be Lys-Lys-Asp-Ala-Leu-Glx-Glx-Asx-Glu-Met.

Peptide C-15. This peptide was positive to the Ehrlich reaction, suggesting the presence of tryptophan. The N-terminal sequence was Val-Ile as revealed by manual Edman cleavage of 0.22 μmol of the peptide. The peptide (0.78 μmol) was digested with trypsin (100 μg) in 0.7 ml buffer for 17.5 hr to give two peptides, Ehrlich-negative C-15-T-1 and Ehrlich-positive C-15-T-2 which were purified by paper electrophoresis at pH 3.6 and 2,000 volt for 110 min. The compositions were Lys_1, Ser_2, Glu_2, Val_1, Ile_1 for T-1 and and Gly_1, Trp_1 for T-2. Six steps of manual Edman degradation

and trypsin specificity suggested the sequence of T-1 to be Val-Ile-Ser-Gln-Ser-Gln-Lys. Chymotrypsin specificity suggested the sequence of the Peptide T-2 to be Gly-Trp. Therefore, the sequence of Peptide C-15 was concluded to be Val-Ile-Ser-Gln-Ser-Gln-Lys-Gly-Trp which was also identified as the C-terminal peptide of this cytochrome bcause of the presence of the sole tryptophan residue in this protein.

Peptide C-17. This peptide (0.5 μmol) was subjected to seven steps of manual Edman degradation and 60 nmol of it was digested with CPase A for 16 hr to release 63 nmol of valine and 50 nmol of phenylalanine. These results and chymotrypsin specificity suggested the sequence of Peptide C-17 to be Val-Asp-Ile-Asn-Asp-Gly-Glu-Ser-Val-Phe.

Peptide C-18 was more anionic than C-17, but both peptides showed the same composition. The N- and C-terminal sequences of this peptide were identical with those of Peptide C-17, except for the fourth residue of Asx. Therefore, Peptide C-18 was assumed to be derived from the cytochrome partially deamidated at the fourth position of Peptide C-17.

Separation and Amino Acid Compositions of BrCN Peptides: The fragments obtained from *Petalonia* cytochrome *c*-553 (2 μmol) by treatment with BrCN (570 μmol) were separated into three major peptides (CN-1, CN-3, CN-4) on an SP-Sephadex column (1.2 x 40 cm) (Fig. 15). Each of these peptides was dialyzed against water and lyophilized. The peptide CN-1 containing heme was purified by gel filtration on a Sephadex G-50 column equilibrated with 10% formic acid. The peptides CN-3 and CN-4 were not further purified. Peptide CN-2 was

separately purified as mentioned in methods. The amino acid compositions of the isolated BrCN peptides are shown in Table 6.

Amino Acid Sequences of BrCN Peptides: Figure 16 summarizes the sequence studies of *Petalonia* cytochrome *c*-553

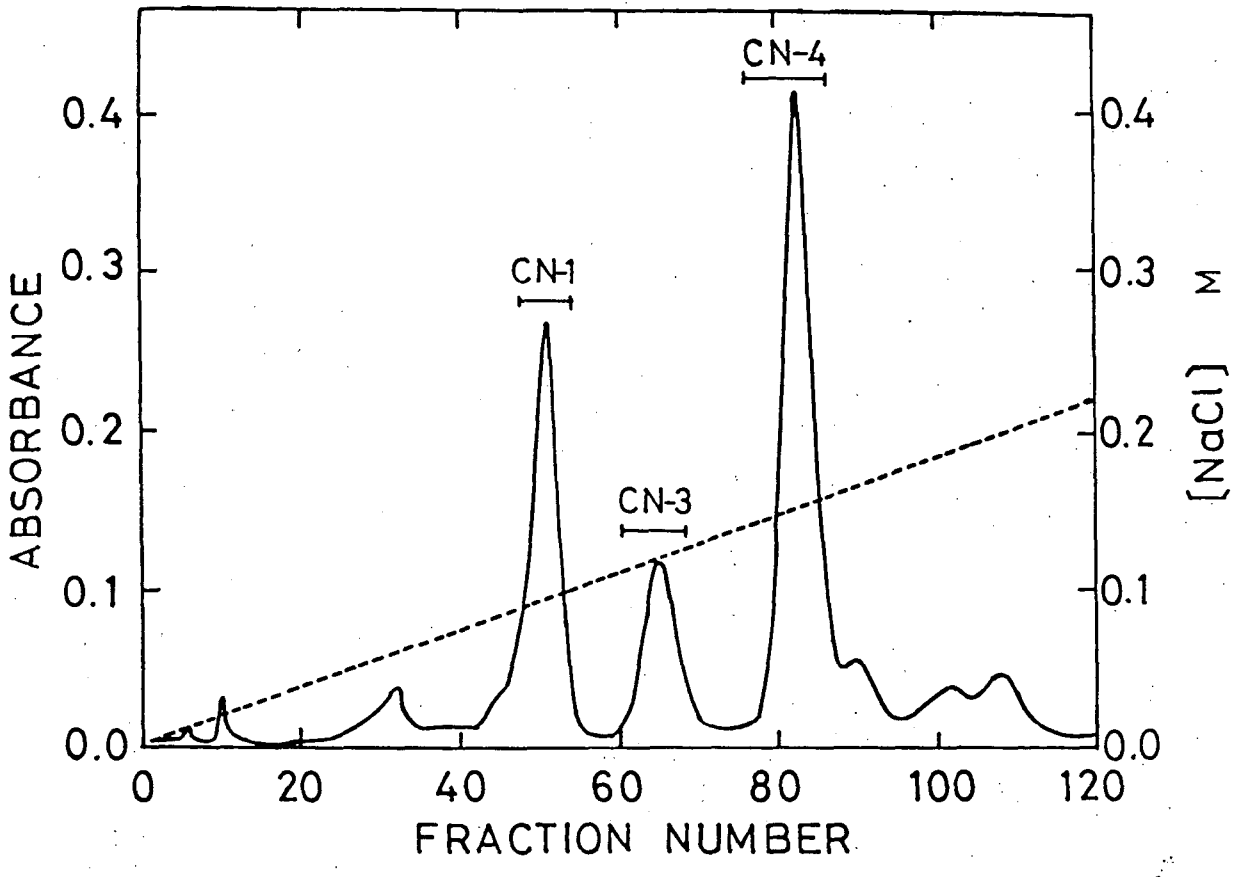

Fig. 15. Elution pattern of BrCN fragments. The fragments were chromatographed on an SP-Sephadex column (1.2 x 40 cm) using a linear gradient system from 0 to 0.25 M NaCl in 8 M urea-phosphoric acid, pH 2.6, at a flow rate of 20 ml per hr. Fractions (4.4 ml) were monitored by following the absorbance at 206 nm.

Table 6. Amino acid compositions of BrCN peptides of *Petalonia* cytochrome *c*-553

Amino acid	CN-1	CN-2	CN-3	CN-4
Aspartic acid	8.01 (8)	2.20 (2)	3.66 (4)	3.07 (3)
Threonine	1.95 (2)	0.99 (1)	1.62 (2)	0.96 (1)
Serine	1.78 (2)		0.86 (1)	2.53 (3)
Glutamic acid	5.28 (5)	3.83 (4)	1.12 (1)	3.88 (4)
Proline	0.82 (1)	0.77 (1)		0.88 (1)
Glycine	2.98 (3)		1.01 (1)	3.08 (3)
Alanine	4.06 (4)	1.05 (1)	1.01 (1)	2.03 (2)
Cysteine	1.60 (2)[a]			
Valine	3.07 (3)		1.19 (1)	1.92 (2)
Methionine	0.87 (1)			
Isoleucine	1.86 (2)		1.67 (2)	1.72 (2)
Leucine	1.83 (2)	1.76 (2)		0.96 (1)
Tyrosine			0.86 (1)	
Phenylalanine	0.92 (1)			1.72 (2)
Lysine	3.05 (3)	2.72 (3)	1.89 (2)	1.15 (1)
Histidine	1.15 (1)			
Arginine				0.94 (1)
Tryptophan				(1)[b]
Homoserine	0.09 } (1)	0.28 } (1)	0.15 } (1)	
Homoserine lactone	0.66 }	0.45 }	0.45 }	
Total	41	15	17	27
Yield (%)	10	1	70	72

[a] Determined as carboxymethylated cysteine.

[b] From the presence of one residue in the cytochrome and carboxypeptidase A digestion of CN-4 peptide.

and the details are given below.

Peptide CN-1 (Residues 1-41). The N-terminal sequence Val-Asp- was determined by the manual Edman procedure. The solid-phase sequence analysis of the heme-free peptide Cm-CN-1 gave its N-terminal sequence up to 20 residues: Val-Asp-Ile-Asn-Asn-Gly-Glu-Ser-Val-Phe-Thr-Ala-Asn-Cmc-Ser-Ala-Cmc-X-Ala-Gly. The result indicates that CN-1 is the N-terminal heme peptide.

Peptide CN-2 (Residues 27-41). All steps of the solid-phase sequence analysis established the sequence, Pro-Glu-Lys-Thr-Leu-Lys-Lys-Asp-Ala-Leu-Glu-Glu-Asn-Glu(Hse). The C-terminal homoserine was assumed from BrCN cleavage specificity and the amino acid composition of the peptide, and was also confirmed by overlapping of Peptide C-14 and this peptide.

Peptide CN-3 (Residues 42-58). The solid-phase sequence analysis of the peptide CN-3 gave the sequence Asn-Asn-Ile-Lys-Ser-Ile-Thr-Tyr-Gln-Val-Thr-Asn-Gly-Lys-Asn-Ala-Hse.

Peptide CN-4 (Residues 59-85). Twenty-step solid-phase sequence analysis of the peptide CN-4 revealed its partial sequence to be Pro-Ala-Phe-Gly-Gly-Arg-Leu-Ser-Glu-Thr-Asp-Ile-Glu-Asp-Val-Ala-Asn-Phe-Val-Ile-. CPase A released only tryptophan from the peptide at 30 min and tryptophan and glycine at 2.5 hr and 19 hr, respectively. The chymotryptic peptide C-15, whose sequence was Val-Ile-Ser-Gln-Lys-Gly-Trp, was considered to be located at the C-terminal region of Peptide CN-4 from the comparisons and C-terminal sequences of the two peptides. These results gave the following sequence for CN-4: Pro-Ala-Phe-Gly-Gly-Arg-Leu-Ser-Glu-Thr-Asp-Ile-Glu-Asp-Val-Ala-Asn-Phe-Val-Ile-Ser-Gln-Ser-Gln-Lys-Gly-Trp.

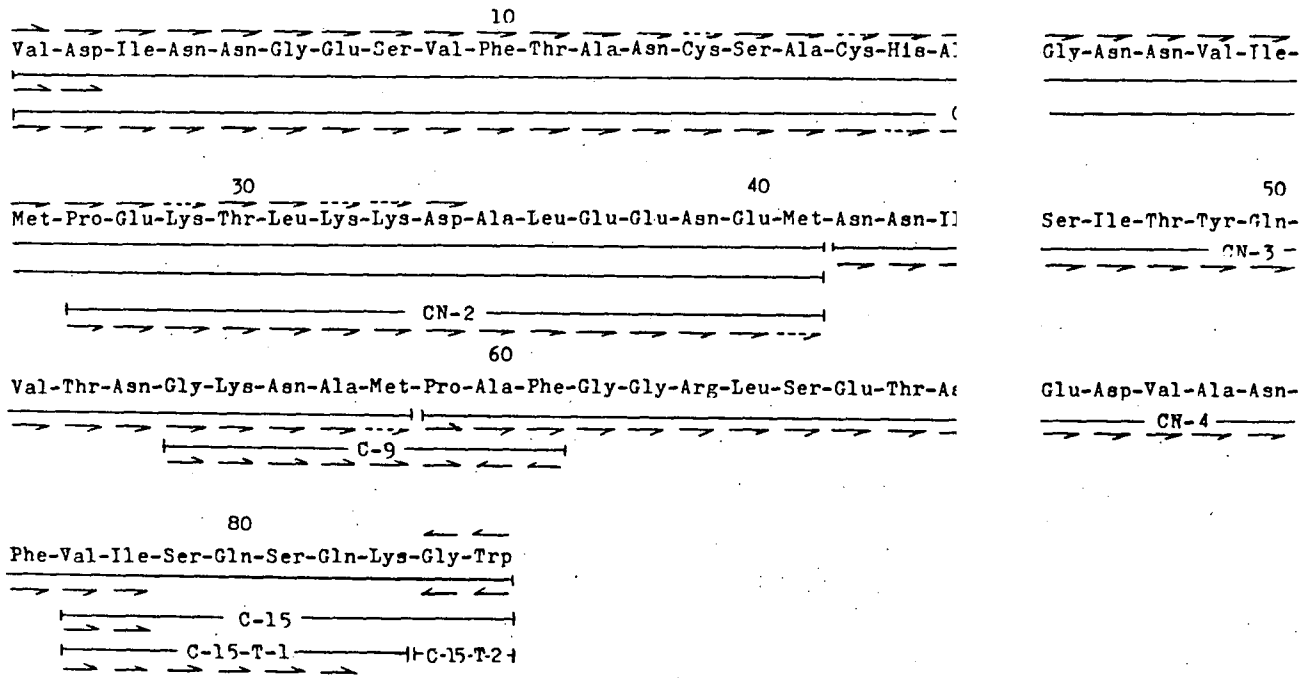

Fig. 16. Summary of the sequence studies of Petalonia cyt me c-553. CN- and Cm-CN- refer to BrCN peptides and carboxymethylated BrCN p de, respectively. C- and T- refer to peptides by chymotryptic and tryptic diges , respectively. Arrows, (⟶), (⟶), and (⟵) indicate amino acid resid dentified by solid- phase sequencing, manual Edman degradation, and digestion carboxypeptidase A, respectively. Arrows above and below the sequence represe he results for cytochrome and peptides, respectively. Dotted arrows show iguous identification. Cys was identified as PTH-Cm-cysteine.

Complete Amino Acid Sequence. The complete amino acid sequence of *Petalonia* cytochrome *c*-553 is shown in Fig. 16. The N-terminal 8 residues of CN-2 overlapped the sequence from 27 to 34 determined for the cytochrome, indicating that CN-2 was the C-terminal fragment of CN-1. The sequence of the chymotryptic peptide C-9, Gly-Lys-Asn-Ala-Met-Pro-Ala-Phe, overlapped the BrCN peptides CN-3 and CN-4. The sequences of Val-Ile and the C-terminal Gly-Trp of CN-4 were found in that of a chymotryptic peptide C-15, Val-Ile-Ser-Gln-Ser-Gln-Lys-Gly-Trp. The sequence of Gly-Trp was revealed by the CPase A digestion of the cytochrome, as well as of the peptide CN-4, indicating that CN-4 and C-15 are the C-terminal peptides of the cytochrome.

Although there was no overlap between CN-1 or CN-2 and CN-3, these peptides could be aligned by comparison of the amino acid composition of the original protein and the sum of those of BrCN peptides.

Prediction of Secondary Structures on Algal C-Type Cytochromes. The possible secondary structures in *Petalonia* cytochrome *c*-553 and other four algal *c*-type cytochromes so far sequenced (51, 53, 54) were estimated from their amino acid sequences by the procedure of Chou and Fasman (79) (Fig. 17).

Phylogenic Tree of Algal C-type Cytochromes. A phylogenic tree (Fig. 18) of *Petalonia* cytochrome *c*-553 and other four algal cytochromes (51, 53, 54) was constructed from the sequence difference shown in Table 7.

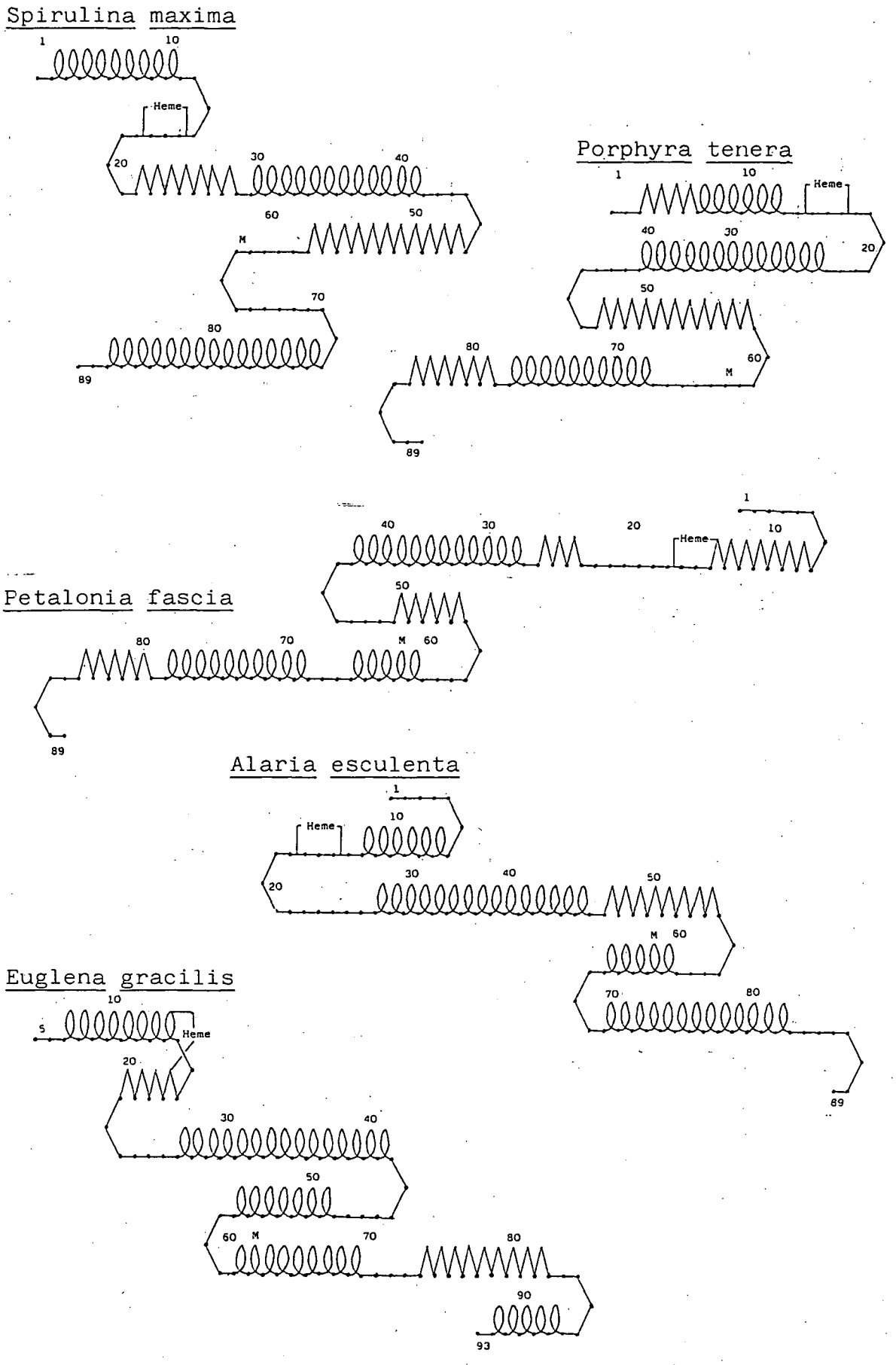

Fig. 17. Schematic diagrams of predicted secondary structures in the water-soluble algal c-type (continued on the next page)

4. Discussion

The complete amino acid sequence of a water-soluble c-type cytochrome, cytochrome c-553 of a brown alga, Petalonia fascia, was determined by Edman degradation of four BrCN peptides and two chymotryptic peptides from the cytochrome.

The sequence Asn-Gly is known to be susceptible to deamidation at alkaline pH (51). However, in the present study CN peptide fractionations were carried out at acidic pH and the Asn-Gly bonds at position 5-6 and 53-54 were determined without any ambiguity. The Asp-5 of the chymotryptic peptide C-17 is considered as a deamidation product. Although the BrCN cleavage occurred readily at Met-41 and -58 of the cytochrome to form the three peptides, CN-1, -3 and -4, the peptide bond (Met-Pro) at 26-27 was rather resistant under conditions employed in the present experiment (Fig. 16).

The four algal cytochromes were predicted to contain α-helix in their N-terminal sequence around an invariant Phe-10 residue. Both of respiratory cytochrome c and

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cytochromes. The structures were predicted from each of the complete amino acid sequences of Petalonia cytochrome c-553 and the other four algal cytochromes studied so far (51, 53, 54) by procedure of Chou and Fasman (79). Residues are represented in their respective conformation: α-helical (ℓ), β-sheet (Λ), coil (⟷). Chain reversals denote β-turn tetrapeptides. Numbers and "M" on residues represent sequence numbers according to Ambler's alignment (51) and the sixth ligand Met-62 of heme iron of the cytochromes, respectively.

Table 7. Amino acid differences among six algal cytochromes c-553. Calculations are based on the alignment of Fig.6. Z (Glx) was assumed to be acid or amide in accordance with acid or amide in a corresponding position of other sequences. A gap or insertion is counted as one difference.

	(a)	(b)	(c)	(d)	(e)	(f)
(a) Spirulina maxima	0	43	49	42	46	54
(b) Porphyra tenera	43	0	46	22	26	60
(c) Monochrysis lutheri	49	46	0	47	47	60
(d) Petalonia fascia	42	22	47	0	17	58
(e) Alaria esculenta	46	26	47	17	0	58
(f) Euglena gracilis	54	60	60	58	58	0

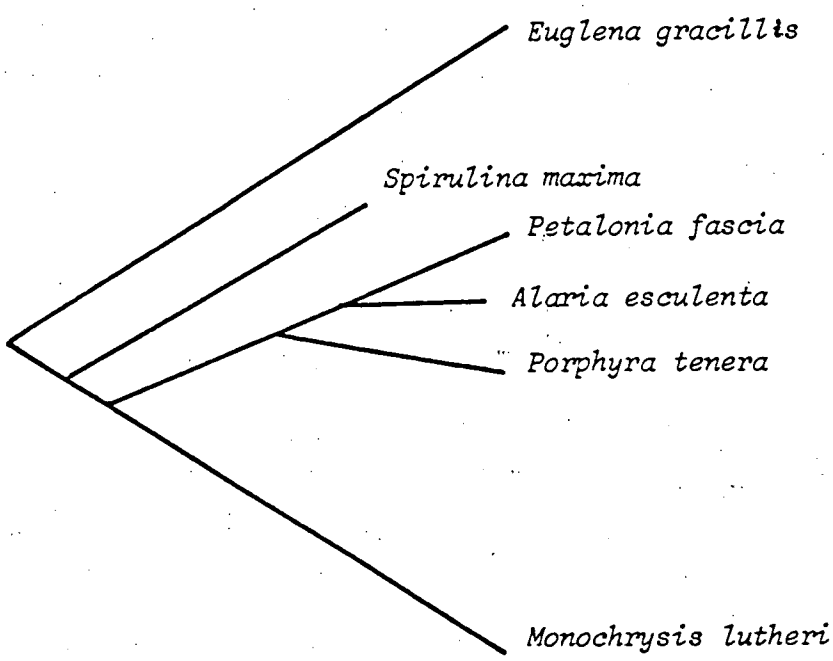

Fig. 18. A phylogenetic tree of the six algal cytochromes c-553 constructed by comparing the numbers of different amino acids among their sequences according to the method of Fitch and Margoliash (97).

photosynthetic cytochrome c_2 have the similar helical segment (98), though the Petalonia cytochrome was predicted to have β-sheet rather than α-helix in the corresponding N-terminal site. The heme binding site was estimated to be in coil for all of the five algal cytochromes. Segment around the sixth ligand Met-62 for heme-iron was predicted to be in coil for the Spirulina and Porphyra cytochromes, and to be in α-helix for the Petalonia, Alaria and Euglena cytochromes.

Figure 19 shows a sequence comparison among the six algal cytochromes. The 19 positions boxed in Fig. 19 are common among these cytochromes. A distinct change is found at position 49, where the other five algal cytochromes including Alaria (Phaeophyta) cytochrome c-553 an alanine residue, though Petalonia cytochrome c-553 has a serine residue. The five residues boxed near the heme-binding region, Gly-6, Phe-10, Cys-14, Cys-17, and His-18, are also conserved in respiratory cytochrome c. The algal cytochromes have an invariant Asn residue at position 13 in contrast to a basic residue, Arg or Lys, of respiratory cytochrome c. Three positions, Asn-4, Thr-72, and Phe-80, are common in the cytochromes of A. esculenta and P. fascia, suggesting that the three positions are conserved in the algal of Phaeophyta. It is interesting that nine positions, Ala-19, Asn-22, Met-26, Pro-27, Met-44, Thr-51, Asp-73, Asn-79, and Ser-85 are characteristically invariant among P. fascia, A. esculenta, and P. tenera, which are all thallus algae. The Pro-27 residue corresponds to an invariant Pro-30 residue in respiratory cytochrome c and cytochrome c_2. However, this

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                                                  10
(a) Spirulina maxima          G D V A A G A S V F S A N C A
(b) Porphyra tenera           A D L D N G E K V F S A N C A
(c) Monochrysis lutheri       G D I A N G E Q V F T G N C A
(d) Petalonia fascia          V D I N N G E S V F T A N C S
(e) Alaria esculenta          I D I N N G E N I F T A N C S
(f) Euglena gracilis                  G G A D V F A D N C S

                20                      30                      40
(a) A C H M G G R N V I V A N K T L S K S D L A K Y L K G F D D
(b) A C H A G G N N A I M P D K T L K K - D V - - L E A N S M N
(c) A C H S V Z Z Z X T L E L S S L W K - - A K S Y L A N F N G
(d) A C H A G G N N V I M P E K T L K K - D A - - L E E N E M N
(e) A C H A G G N N V I M P E K T L K K - D A - - L A D N K M V
(f) T C H V N G G N V I S A G K V L S K T A I E E Y L D G G Y -

                50                      60                      70
(a) D A V A A V A Y Q V T N G K N A M P G F N G R L S P K Q I E
(b) T I D A I T - Y Q V Q N G K N A M P A F G G R L V D E D I E
(c) D E S A I V - Y Q V T N G K N A M P A F G G R L E D D E I A
(d) N I K S I T - Y Q V T N G K N A M P A F G G R L S E T D I E
(e) S V N A I T - Y Q V T N G K N A M P A F G S R L A E T D I E
(f) T K E A I E - Y Q V R N G K G P M P A W E G V L S E D E I V

                80                      90
(a) D V A A Y V V D Q A E K G W
(b) D A A N Y V L S Q S E K G W
(c) N V A S Y V L S K A G
(d) D V A N F V I S Q S Q K G W
(e) D V A N F V L T Q S D K G W D
(f) A V T D Y V Y T Q A G G A W A N V S
```

Fig. 19. Sequence comparison of algal cytochromes c-553 from (a) Spirulina maxima (51), (b) Porphyra tenera (51), (c) Monochrysis lutheri (52), (d) Petalonia fascia, (e) Alaria esculenta (53), and (f) Euglena gracilis (54). The alignment is based on that of Ambler and Bartsch (51). The common amino acid residues of the six cytochromes are boxed. One-letter notation is used for each amino acid residue. — indicates a delition.

Pro residue is not found in the three cytochromes of unicellular algae (M. lutheri and E. gracilis) and a blue-green alga, S. maxima. A macro-umnicellular alga, Bryopsis maxima has only one Met residue, possibly invariant Met-62, in its cytochrome c-554 (data not presented) as well as other unicellular algae (M. lutheri and E. gracilis). Spirulina cytocharome c-554 has one more Met residue at position 19 than the invariant Met-62.

The amino acid differences calculated from Fig. 19 are shown in Table 7. The highest sequence homology was obtained between the cytochromes of P. fascia and A. esculenta, which belong to the same phylum. The next highest homology was found between the cytochromes of P. fascia and P. tenera (Rhodophyta) rather than that of M. lutheri (Chrysophyta). The Petalonia cytochrome, like the other four algal cytochromes, shows the poorest homology to the Euglena cytochrome, and the next poorest homology to the Monochrysis cytochrome. The structural difference among the algal cytochromes may reflect that of their reactivity with the Pseudomonas nitrite reductase (45). Although the algal cytochromes are completely unreactive with mitochondrial cytochrome c oxidase, they react approximately one third as rapidly with the nitrite reductase as does Pseudomonas cytochrome c, with the exception of the Euglena cytochrome, which reacts somewhat more slowly (45).

A phylogenetic tree (Fig.18) of the algal cytochromes constructed from the sequence differences suggested that the the thallus algae, P. tenera, A. esculenta and P. fascia,

are the descendants evolved from a unicellular ancestor common also to a unicellular alga, *M. lutheri*.

IV. PURIFICATION AND PROPERTIES OF B-TYPE CYTOCHROMES FROM GREEN ALGAE, *Ulva pertusa* AND *Enteromorpha prolifera*

1. Introduction

Without the aid of any detergent, b-type cytochromes were isolated and partially purified from five genera of green algae, *Monostroma* sp. (99), *Scenedesmus obliquus* D_3 (100), *Bryopsis maxima* (11), *Enteromorpha prolifera*, and *Ulva pertusa*. Recently Shimazaki *et al*. extracted cytochrome b-560 as a water-soluble form from a marine diatom *Phaeodactylum tricornutum* (43). A similar cytochrome has been obtained from spinach leaves (101). These cytochromes are characterized by an α-band at 562-563 nm, weak autoxidizability, insensitivity to carbon monoxide and cyanide, and anionic behavior on an ion exchanger. The function and localization of these cytochromes in algal cells are still unknown.

In this chapter the author describes the purification and some physico-chemical properties of the water-soluble b-type cytochromes from two species of green algae, *viz*., cytochrome b-562.5 from *Ulva pertusa* and cytochrome b-561 from *Enteromorpha prolifera*. Comparisons of the two cytochrome with other algal b-type cytochromes are described. Similar purification procedure and similar redox properties point to this cytochrome being homologous with the other of green algae and a diatom *Phaeodactylum tricornutum* mentioned above.

2. Materials and Methods

Thalli: Both *Ulva pertusa* and *Enteromorpha prolifera* belong to the group of Chlorophyceae. The *Ulva* thalli were collected from Tokyo Bay at Tsudanuma and Kisarazu, Chiba, Japan. The *Enteromorpha* thalli were cultivated for food at the mouth of the river Hitotsumatsu which empties into the Pacific Ocean, Kujukuri, Chiba, Japan.

Chemicals: DEAE-cellulose was obtained from Brown Co. (U.S.A.). Sephadex and DEAE-Sephadex were obtained from Pharmacia Fine Chemicals (Sweden). Molecular weight marker proteins were from Dickinson and Co. (U.S.A.). Bio-Gel was from Bio-Rad Laboratory (U.S.A.). Hydroxyapatite was prepared as described by Main *et al*. (102). All other reagents were of reagent grade.

Determination of B-type Cytochromes: The amount of cytochrome in the crude preparation was determined by measurement of the reduced-minus-oxidized absorbance at α-peak. Reduction was achieved by the addition of small amount of crystalline ascorbic acid (about 0.5 mg) and oxidation by the addition of potassium ferricyanide (about 0.5 mg) using 1-cm cuvettes. The ferro- and ferricytochromes were determined from the untreated-minus-oxidized and ascorbate-minus-untreated absorbance differences, respectively. A difference extinction coefficient, reduced-minus-oxidized, of 31.1 $mM^{-1} \cdot cm^{-1}$ at the α-peak obtained from the pyridine hemochrome (103) was adopted for the crude preparation. An extinction coefficient of 43.2 $mM^{-1} \cdot cm^{-1}$ at the α-peak of

reduced form was adopted for the pure preparation.

Absorption Spectra: Absorption spectra were measured in a Hitachi 124 spectrophotometer, using standard 1-cm light-path cuvettes. Ferricytochrome solutions were prepared by autoxidation or by dialysis against 10 μM-potassium ferricyanide solution containing 10 mM phosphate buffer, pH 7.0. The measurement was carried out against the outside solution. The spectra of ferrocytochromes were determined after the addition of about 0.5 mg of sodium ascorbate to the ferricytochrome solutions and the outside solutions as references. The low temperature absorption spectra were measured at liquid nitrogen temperature (77°K) in a Hitachi 356 spectrophotometer with a low temperature cell (3 mm) attachment. The cytochromes were dissolved in 1 ml of 50% glycerol containing 2.5 mM potassium phosphate buffer, pH 7.0, and 0.5 mg of fresh sodium ascorbate. In order to detect any denatured cytochrome or peroxidase, the effect of carbon monoxide on the absorption spectra was examined. A stream of commercial carbon monoxide was bubbled through the samples for 1 min.

Oxidants and Reductants: The oxidation-reduction reactions of cytochromes were detected by spectrophotometric measurements in the α-band region before and after adding about 0.5 mg of each redox reagent to 3 ml of about 0.2 μM cytochrome containing 0.1 M phosphate buffer, pH 7.0 in a cuvette. As redox reagents were used ferricyanide, ferrocyanide, hydroquinone, ascorbate, cysteine-HCl, and hydrosulfite.

Midpoint Redox Potential. The midpoint potential was determined by equilibration with a ferro- and ferri-EDTA system.

The absorbance at α-peak and redox potential were measured with a Hitachi 124 spectrophotometer and a Tōa Dempa HM-6A glass pH meter, respectively, in a 1-cm cuvette shown in Fig. 20. The titration was started by adding 25 mM ferric ammonium sulfate solution, pH 2.2, to 5.0 ml of reaction mixture consisting of 0.1-2.5 μM cytochrome and 0.1 M EDTA, pH 7.4, in the cuvette, resulting in pH decrease to 7.0, and then 2.5 mM ferrous ammonium sulfate containing 0.1 M EDTA, pH 7.0, freshly prepared, were added step by step using a microsyringe and finally solid ascorbate (about 1 mg) was added. For anaerobic titration, additions of 2.5 mM ferrous ammonium-0.1 M EDTA, pH 7.0, were made with a 10-cm No.23 needle inserted and left in position in a rubber injection port, and then nitrogen was bubbled with the same needle. Another No.23 needle (5 cm) was inserted in the port to permit air to escape. Redox potentials were measured with a Tōa Dempa PS-115C Ag/AgCl electrode (E= 0.199 volt at 25°C) inserted in the port.

Pyridine Hemochrome: The pyridine hemochrome was prepared from each cytochrome according to the method of Shichi and Hackett (56).

Peroxidase Activity: Peroxidase activity of each cytochrome preparation was determined as described by Shichi and Hackett using o-dianisidine (104). To 6.0 ml of 0.003% H_2O_2 containing 10 mM phosphate buffer, pH 6.0, was added 0.05 ml of 1% o-dianisidine in methanol. A 2.9 ml aliquot of this mixture was transfered to the cuvette, and the remainder was used as reference. The reaction was started by adding 0.1 ml of cytochrome solution, and the absorbance increase was

measured at 460 nm.

Molecular Weght: Molecular weight of each cytochrome was

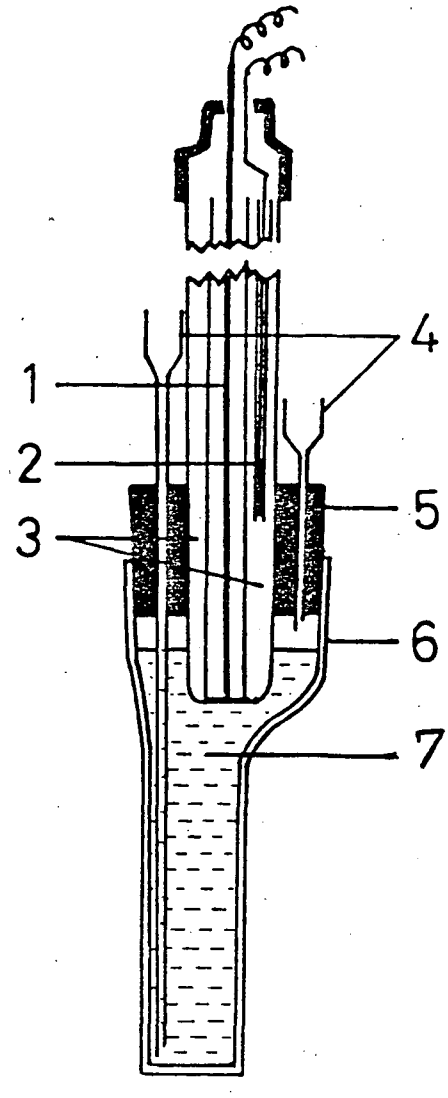

Fig. 20. Cuvette and upper assembly used in spectrophotometric titrations under anaerobic condition. 1, Pt electrode; 2, Ag/AgCl electrode; 3, saturated KCl solution; 4, No. 23 needle; 5, rubber injection port; 6, 1-cm cuvette, 12 ml capacity; 7, reaction mixture.

estimated by Sephadex G-75 gel filtration in 0.125 M NaCl containing 0.02 or 0.05 M phosphate buffer, pH 7.0, at 5°C. The column was 2.64 cm in diameter and 40 cm in height. The flow rate was adjusted to 15-30 ml/hr. As molecular weight markers, horse cytochrome c (molecular weight, 12,400), sperm whale myoglobin (17,800), beef pancreas chymotrypsinogen A (25,100), ovalbumin (45,000), and serum albumin (67,000) were used (64). The eluate was checked for absorbance at 280 nm for all marker proteins and at γ-peak for each ferricytochrome.

3. Results

3-1. Cytochrome b-562.5 of *Ulva pertusa*

Extraction and Purification: The *Ulva pertusa* thalli were stored frozen at -20°C.

Step 1. The frozen thalli (500 g) were ground with 1 liter of 0.1 M phosphate buffer, pH 7.0 in an electric mill and centrifuged at 3,600 x g for 15 min. The supernatant suspension was centrifuged at 11,000 x g for 15 min. The supernatant (1.4 liter) combined with the washings contained 0.28 μmol of cytochrome b-562.5, 75% of which was found to be reduced.

Step 2. To the extract was added 0.8 liter of 3% acrinol solution, and the resulting suspension was filtered on a Buchner funnel through a thin layer of Celite. The filtrate contained c-type cytochrome and plastocyanin. The resulting yellowish precipitates were suspended in 200 ml of 0.2 saturated ammonium sulfate solution containing 0.05 M phosphate,

pH 7.0, and centrifuged at 11,000 x g for 15 min. The supernatant was passed through a cellulose powder column on which excess acrinol in the solution was adsorbed.

Step 3. The pale yellow effluent (600 ml) combined with the washings was fully saturated with ammonium sulfate and filtered. The precipitate was dissolved in 20 ml of 0.1 M phosphate buffer, pH 7.0. The resulting yellowish solution (20 ml) contained oxidized cytochrome b-562.5, which was fully reduced with ascorbate to show an absorbance of 0.23 at 562.5 nm.

Step 4. The crude preparation was dialyzed overnight against 2 liters of 20 mM phosphate buffer, pH 7.0, at 4°C. The dialyzed solution was applied to a column of DEAE-cellulose (2.64 cm in diameter and 45 cm in height) previously equilibrated with same buffer. The flow rate was adjusted to 45 ml/hr. The cytochrome was adsorbed on top of the column and was contaminated with a brown substance(s). The charged column was washed with 300 ml of 20 mM phosphate buffer, pH 7.0, and then the cytochrome was eluted, leaving the brown substance(s), by gradient elution with NaCl in 20 mM phosphate buffer, pH 7.0. For gradient elution, 0.25 M NaCl-20 mM phosphate buffer, pH 7.0, was added with constant mixing to 500 ml of 20 mM phosphate buffer, pH 7.0. The eluate was collected in 10 ml fractions and the absorbance of each fractions and the absorbance of each fraction was determined at 275 and 415 nm. The fractions (Nos. 51-88) with absorbance at 415 nm were combined and saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation at 10,000 x g for

15 min and dissolved in 10 ml of 0.02 M phosphate buffer, pH 7.0.

Step 5. The solution was applied to a column of DEAE-Sephadex A-25 (1.0 cm in diameter and 30 cm in height) in the same buffer. Gradient elution was carried out by adding 0.4 M NaCl-20 mM phosphate buffer, pH 7.0, with constant mixing to 600 ml of 20 mM phosphate buffer, pH 7.0. The flow rate was adjusted to 50 ml/hr. The fractionation and concentration of the cytochrome were carried out in the same manner as for DEAE-cellulose chromatography. The fractions (Nos. 98-103) containing cytochrome b-562.5 were combined and concentrated by salting out with ammonium sulfate. This yielded a solution containing about 0.04 μmol of ferricytochrome b-562.5 with A562.5/A275 = 0.31. The absorbance at 562.5 nm was determined after reduction by adding a small amount of crystalline ascorbic acid.

Absorption Spectra: The absorption spectra are shown in Fig. 21. The ferricytochrome showed absorption maxima at 275, 415 (γ-max), and 537 nm (shoulder around 570 nm); the absorption minima were found at 251, 330, and 504 nm. The ferrocytochrome showed absorption maxima at 326 (δ-max), 429 (γ-max, shoulder at 406 nm), 530.5 (β-max, shoulder around 520 nm), and 562.5 nm (α-max, shoulder at 560 nm); the absorption minima were found at 312, 386, 496, and 543.5 nm. The isosbestic points of the ferro- and ferricytochrome were located at 359, 421, 433, 519, 537, and 571 nm. The absorption spectrum of the ferrocytochrome at liquid nitrogen temperature (77°K) is shown in Fig. 22. The α-band of the ferrocytochrome was splits into

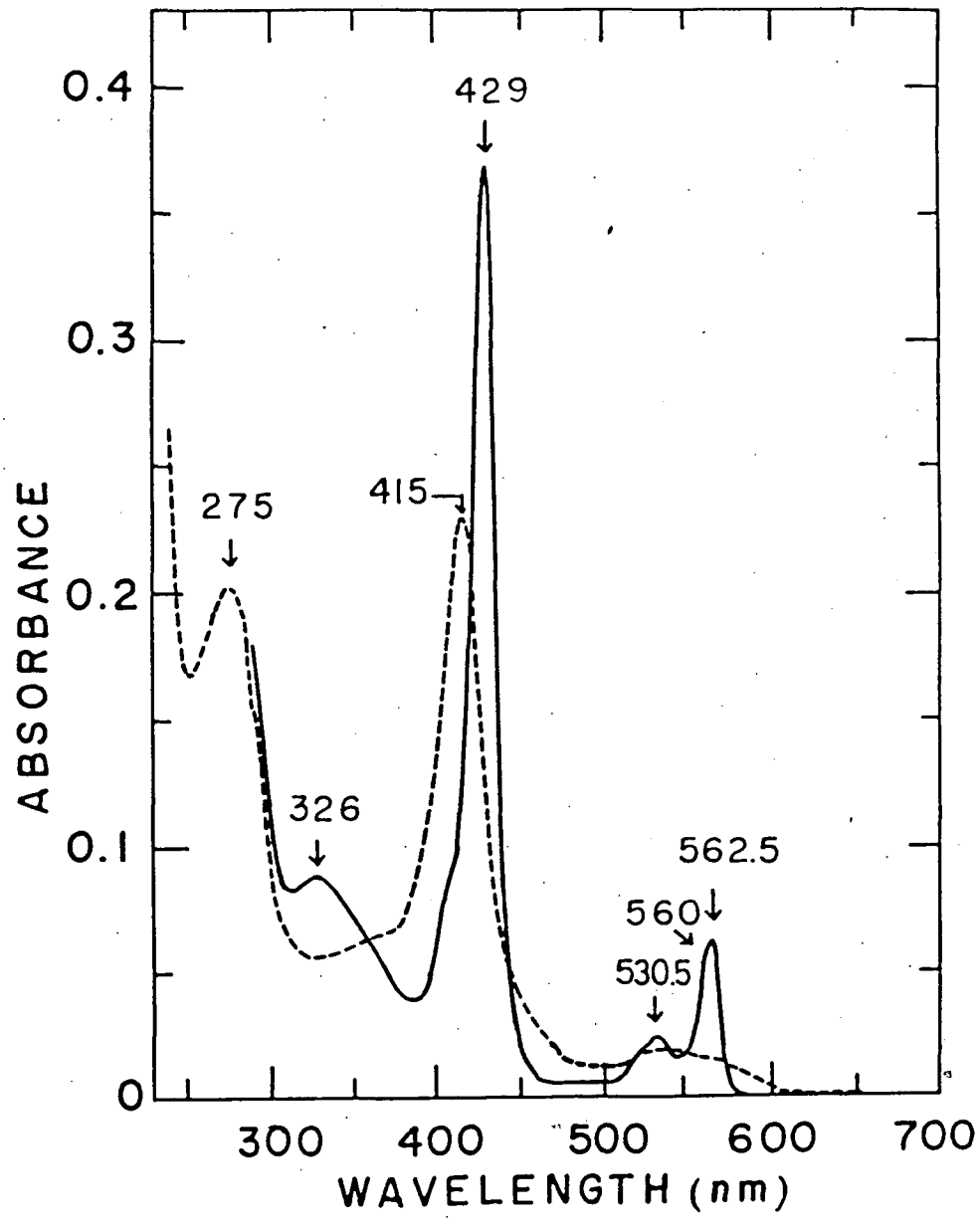

Fig. 21. Absorption spectra of Ulva cytochrome b-562.5. Oxidized(----) and reduced(——) forms were prepared by air bubbling and by addition of crystalline cysteine-HCl, respectively. The spectra were recorded at pH 7.0 in 0.4 M phosphate buffer at 20°C.

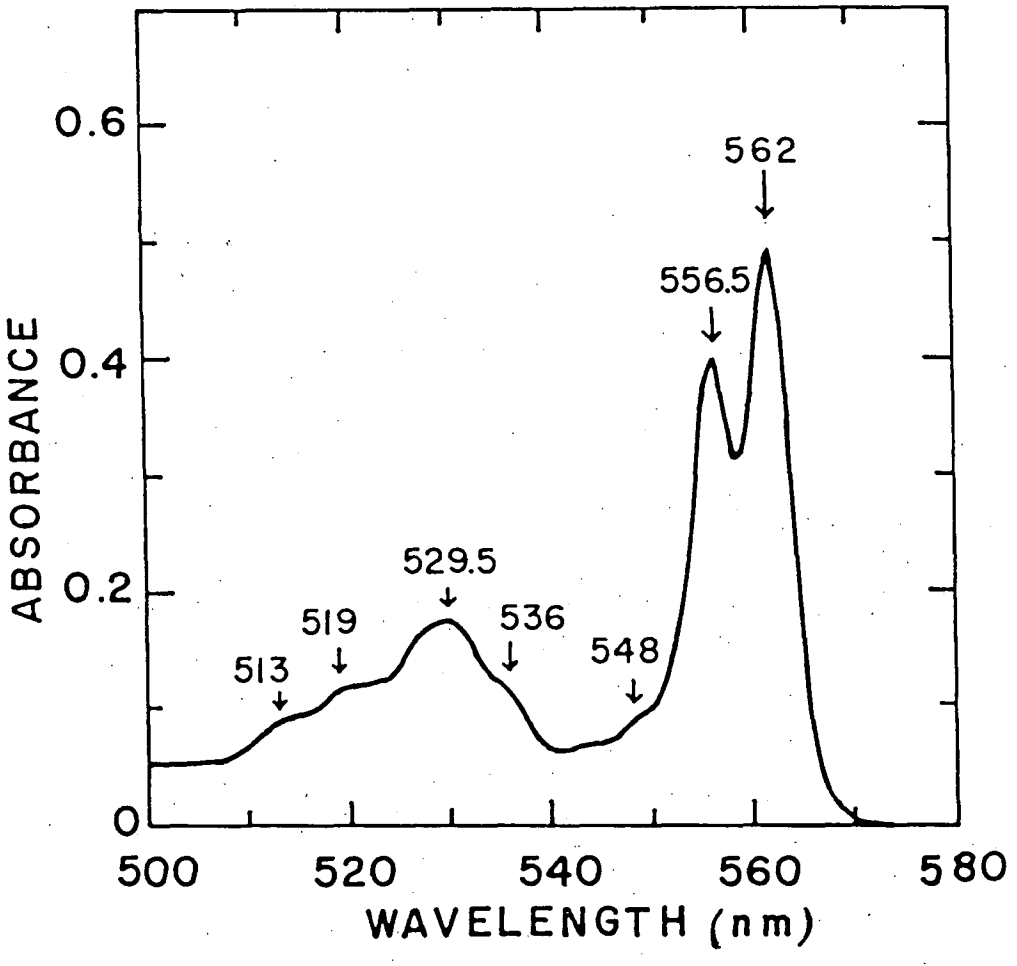

Fig. 22. Absorption spectrum of reduced Ulva cytochrome b-562.5 at the temperature of liquid N_2 (77°K). The spectrum was recorded in 50% (v/v) glycerol containing 2.5 mM K-phosphate buffer, pH 7.0.

two peaks at 562 (major band) and 556.5 nm. The β-band shifted 1.0 nm to shorter wavelength, and three shoulders appeared at 513, 519, and 536 nm. The spectra of the ferro- and ferricytochrome were not altered by CO or cyanide, indicating that the cytochrome does not combine with these agents.

Midpoint Redox Potential: The cytochrome was readily oxidized by ferricyanide and reduced by ascorbate, cysteine, or hydrosulfite. Ferrocyanide and hydroquinone could reduce it only 30 and 55%, respectively. The reduced form was autoxidized after thorough dialysis to remove excess reductant. The results of titration of the cytochrome in ferri-EDTA solution with ferro-EDTA are shown in Fig. 23. The 45° slope of the points plotted in the figure as log (ferricytochrome/ferrocytochrome) *versus* log (ferri-EDTA/ferro-EDTA) indicated that a one-electron change was involved in the oxidation-reduction of the cytochrome. At half-reduction of the cytochrome, the value of log (ferri-EDTA/ferro-EDTA) was found to be 1.39; thus the midpoint potential of the cytochrome was determined to be +0.24 volt at 20°C and pH 7.0 by use of a midpoint redox potential of 0.15 volt for ferri- and ferro-EDTA redox system (103).

Heme Group: The absorption spectra of the pyridine hemochrome derived from cytochrome *b*-562.5 are shown in Fig. 24. The ferrihemochrome showed a γ-absorption maximum at 398 nm and a broad band from 540 to 650 nm. The reduced form, obtained with ascorbate, had absorption maxima at 556 (α), 523 (β), and 418 nm (γ). The spectral feature suggest the heme moiety to be protoheme IX. The α-band of the pyridine derivative was

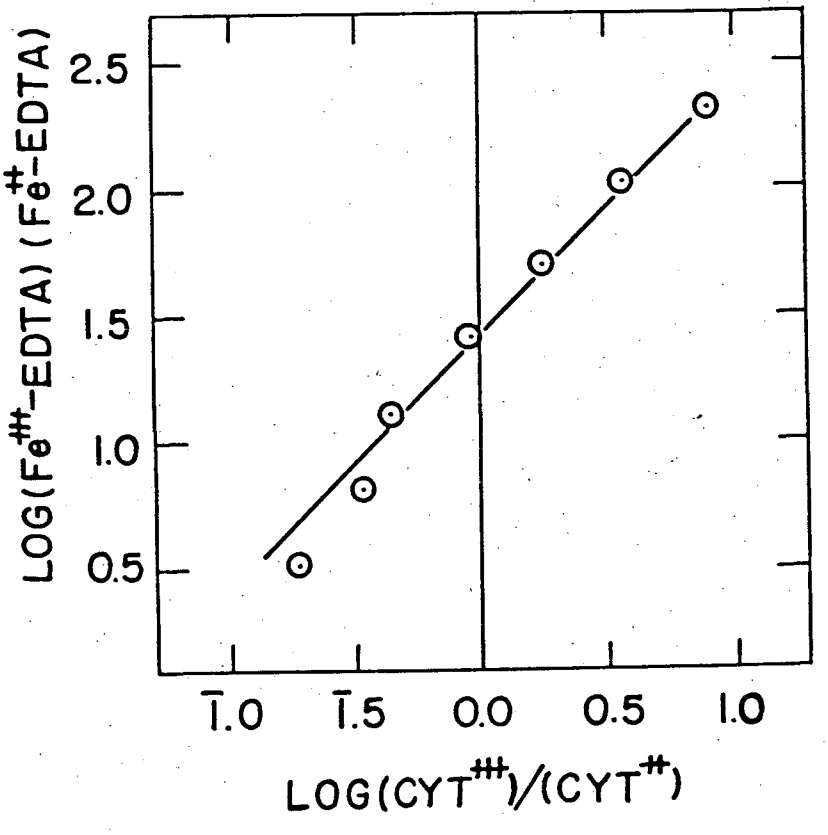

Fig. 23. Determination of the midpoint redox potential (Em) of Ulva cytochrome b-562.5 with mixtures of ferri- and ferro-EDTA in various ratios in 0.1 M K-phosphate buffer, pH 7.0, at 20°C. Reduction-oxidation reacion of the cytochrome was determined spectrophotometrically at 562.5 nm.

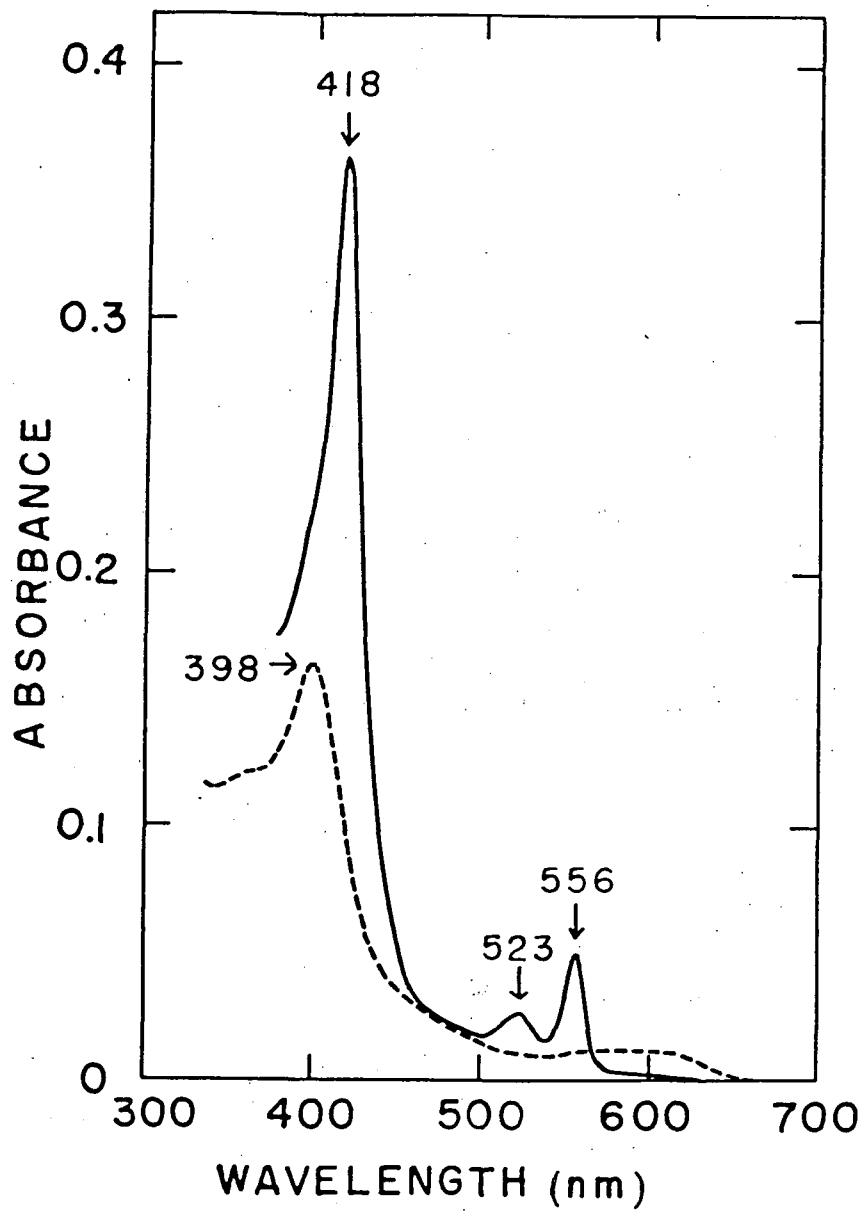

Fig. 24. Absorption spectra of pyridine hemochrome from Ulva cytochrome b-562.5. Oxidixed (----) and reduced (——) forms were prepared by air bubbling and by addition of crystalline ascorbic acid, respectively.

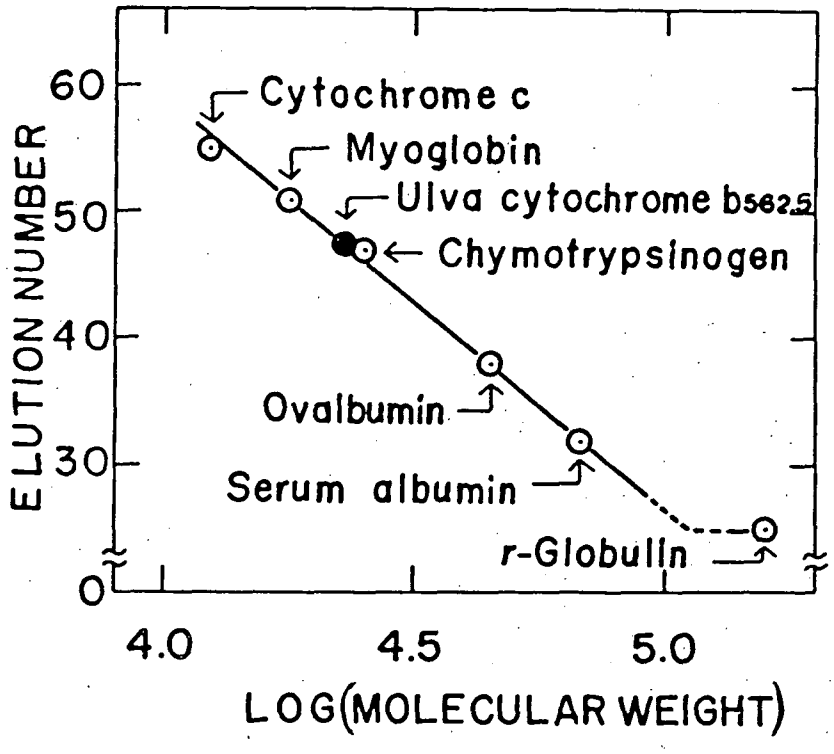

Fig. 25. Estimation of the molecular weight of Ulva cytochrome b-562.5 by Sephadex G-75 gel filtration in 0.125 M NaCl-0.05 M K-phosphate buffer, pH 7.0, at 5°C. The column was 2.64 cm in diameter and 50 cm in height. The flow rate was 30 ml per hr.

symmetrical, without any shoulder. The millimolar extinction coefficient (EmM) of the cytochrome at 562.5 nm was calculated to be 41.6 $mM^{-1}.cm^{-1}$ using an extinction coefficient of 34.4 $mM^{-1}.cm^{-1}$ for the α-peak of the pyridine protohemochrome (103)

Molecular Weight: A plot of the elution position against the logarithm of the molecular weights of the marker proteins is shown in Fig. 25. A molecular weight of 23 x 10^3 was calculated for the cytochrome.

Peroxidase Activity: Peroxidase activity was measured using 14.7 µg of cytochrome b-562.5 dissolved in 0.1 ml of 5 mM phosphate buffer, pH 7.0. The linear increase in absorbance at 460 nm was found to be 0.0185 in 9 min at 25°C, corresponding to 0.14 per mg protein. This value is only 0.017% of the specific activity of purified horseradish peroxidase (104).

3-2. Cytochrome b-561 of *Enteromorpha prolifera*

Extraction and Purification: The *Enteromorpha prolifera* thalli were air-dried at 0-5°C and stored at -20°C.

Step 1. The dried thalli (500 g) were immersed in 8 liters of 10 mM potassium phosphate buffer, pH 7, at 4°C and allowed to stand at 4°C with occasional stirring. The yellow extract was obtained by filtration through absorbent cotton.

Step 2. The extract was 0.3 saturated with ammonium sulfate and filtered on a Buchner funnel through a thin layer of Celite. The filtrate was 0.7 saturated with ammonium sulfate and filtered as described above. The filtrate was discarded and the pale yellow precipitate was dissolved in 1 liter of 10 mM phosphate buffer, pH 7.0.

Step 3. The resulting solution was dialyzed overnight against 8 liters of 10 mM phosphate buffer, pH 7.0, at 4°C and applied to a column of DEAE-cellulose (5 x 50 cm) previously equilibrated with the same buffer. A yellowish substance(s) on the column was washed with 1 liter of the same buffer and 1 liter of 0.2 M NaCl-0.02 M phosphate buffer, pH 7.0, leaving cytochrome b-561. The cytochrome was eluted by 550 ml of 0.45 M NaCl-0.045 M phosphate buffer, pH 7.0. By salting out at 0.3-0.7 saturation of ammonium sulfate was concentrated the eluate to 100 ml solution containing 0.83 μmol of cytochrome b-561.

Step 4. The cytochrome solution was dialyzed against 2 liters of 0.2 M NaCl-0.01 M phophate buffer, pH 7.0, and applied to a column of DEAE-Sephadex (2.5 x 40 cm) previously equilibrated with 0.2 M phosphate buffer, pH 7.0. The flow rate was adjusted to 72 ml/hr with a pump. The charged column was washed with 1 liter of the same NaCl-phosphate buffer and 1 liter of 0.24 M NaCl-0.01 M phosphate buffer, pH 7.0. The yellowish washings were discarded. Linear gradient elution was carried out by adding 0.8 M NaCl-0.01 M phosphate buffer, pH 7.0, with constant mixing to 600 ml of 0.24 M NaCl-0.01 M phosphate buffer, pH 7.0. The eluate was collected in 10-ml fractions and absorbance of each fracion was determined at 275 and 417 nm. The fractions (Nos. 30-100) with absorbance at 417 nm, eluted at 0.35-0.68 M of NaCl concentration in the eluant, were combined and saturated with ammonium sulfate. The resulting precipitate was collected by suction filtration and dissolved in 160 ml of 0.125 M NaCl-0.02 M phosphate buffer,

pH 7.0.

Step 5. The solution containing 0.8 μmol of cytochrome b-561 with A417/A275 = 0.18 was applied to a column of Bio-Gel P-150 (100-200 mesh, 5 x 70 cm) equilibrated with 0.125 M NaCl-0.02 M phosphate buffer, pH 7.0. The flow rate was adjusted to 72 ml/hr. The fractionation and concentration of the cytochrome were carried out as for DEAE-Sephadex chromatography (Step 4). The purest fractions (Nos. 48-52) were combined and concentrated to 25 ml solution containing 0.23 μmol of cytochrome b-561 with A417/A275 = 0.23.

Step 6. The cytochrome solution was dialyzed overnight against 1 liter of 5 mM phosphate buffer, pH 6.8, and applied to a hydroxyapatite column (1.5 x 23.5 cm) previously equilibrated with the same buffer. The charged column was washed with 100 ml of the same buffer, and then eluted by linear gradient from 5 mM to 100 mM of phosphate buffer, pH 6.8 (1.2 liters in total). The flow rate was adjusted to 72 ml/hr. The washings and eluate were collected in 10 ml fracions. The washings (Nos. 3-12) also contained 0.07 μmol of the cytochrome with A417/A275 = 0.73 (Form 1). The fractions (Nos. 18-21) eluted around 7 mM phosphate contained 0.05 μmol of the cytochrome with A417/A275 = 0.76 (Form 2). The elution pattern from hydroxyapatite column is shown in Fig. 26. No differences were seen between the absorption spectra, molecular weights, and the redox properties of two forms. Analytical polyacrylamide disc gel electrophoresis of cytochrome b-561 preparation (Form 1) at pH 9.5 (106,107) showed at least four bands, indicating that the preparation is still impure.

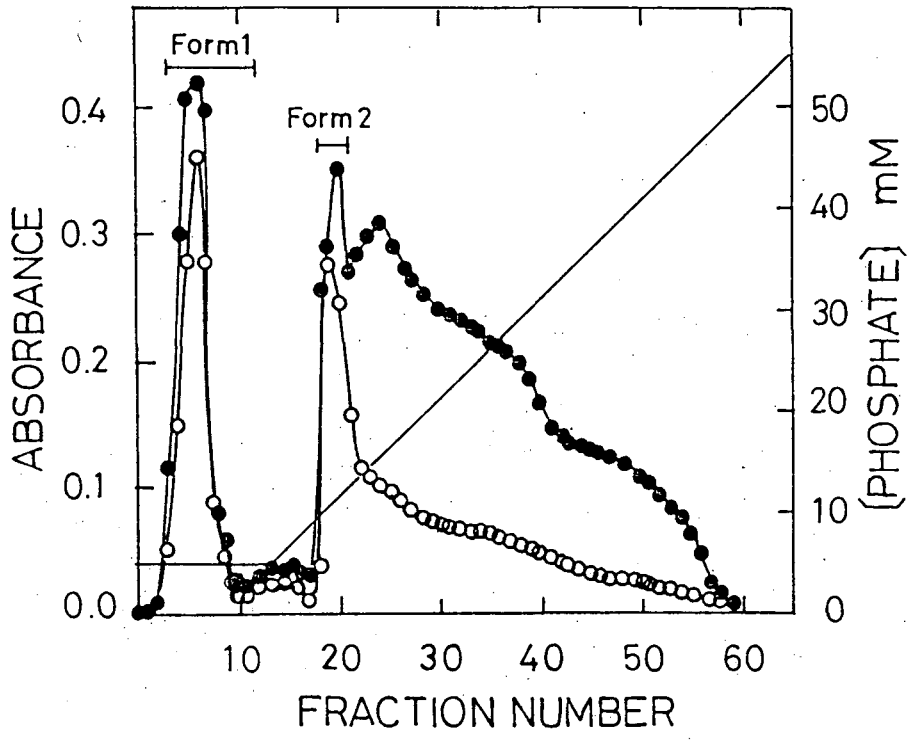

Fig. 26. Elution pattern obtained from the hydroxyapatite column chromatography of Enteromorpha cytochrome b-561 (Forms 1 and 2) by linear gradient from 5 to 100 mM of K-phosphate buffer, pH 6.8. The column was 1.5 X 23.5 cm. The flow rate was 72 ml per hr. The eluate was collected in 10-ml fractions. o , 417 nm; •, 275 nm; —, (phosphate) mM.

Absorption Spectra: The absorption spectra and the difference spectrum (reduced-oxidized) of cytochrome b-561 are shown in Figs. 27 and 28, respectively. The ferrocytochrome showed absorption maxima at 275, 324 (δ-max), 428.5 (γ-max, shoulder at 405 nm), 530.5 (β-max, shoulder at 520 nm), and 561 nm (α-max). The α-band shows a symmetrical feature without any shoulder. The ferricytochrome showed absorption maxima at 275, 417 (γ-max), and 530 nm (shoulder at 570 nm). The isosbestic points of the absorption spectra of the ferro- and ferricytochrome were located at 358, 422, 446, 518, 536, 548, and 569 nm. The difference spectrum showed maxima at 324, 432, 530.5, and 561 nm. The millimolar extinction coefficients at absorption maxima of the ferro- and ferricytochrome were calculated using an extinction coefficient of 34.4 $mM^{-1}.cm^{-1}$ for the α-peak of the pyridine hemochrome (103) on the assumption that the cytochrome contains one protoheme per molecule. These values are listed in Table 8. The spectra of ferro- and ferricytochrome were not altered by carbon monoxide or cyanide, indicating the cytochrome does not combine with these reagents. The low-temperature spectrum at 77°K had an α-peak at 557.5 nm and showed no splitting as shown in Fig. 29.

Midpoint Redox Potential. The cytochrome was readily oxidized by ferricyanide and reduced by ascorbate, cysteine, or hydrosulfite. Ferrocyanide and hydroquinone were able to reduce it only 23% and 50%, respectively. The reduced form was autoxidized after thorough dialysis to remove excess reductant. The results of the titration of the cytochrome

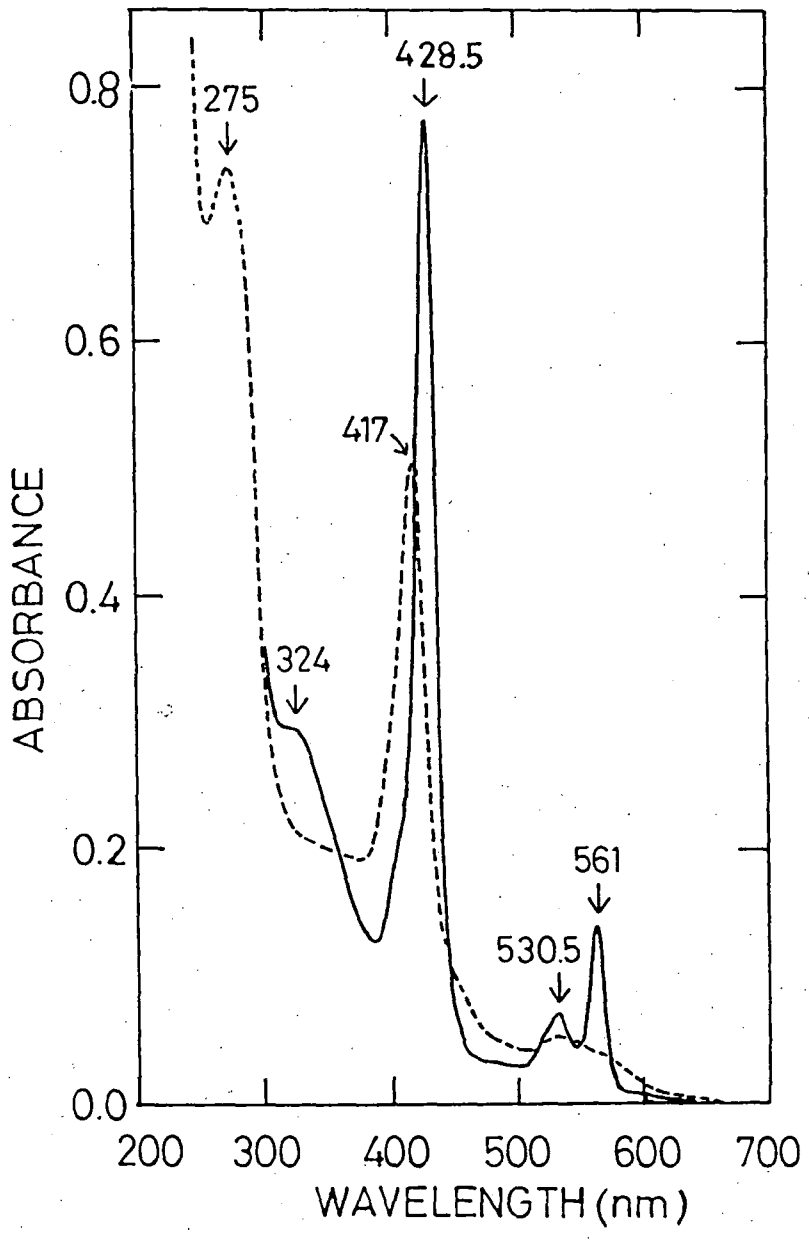

Fig. 27. Absorption spectra of Enteromorpha cytochrome b-561 (Form 1) at 25°C. The oxidized form (---) was prepared by dialysis against 10 μM ferricyanide-10 mM K-phosphate buffer, pH 7.0. The reduced form (——) by addition of crystalline ascorbate (about 0.5 mg) to the oxidized form.

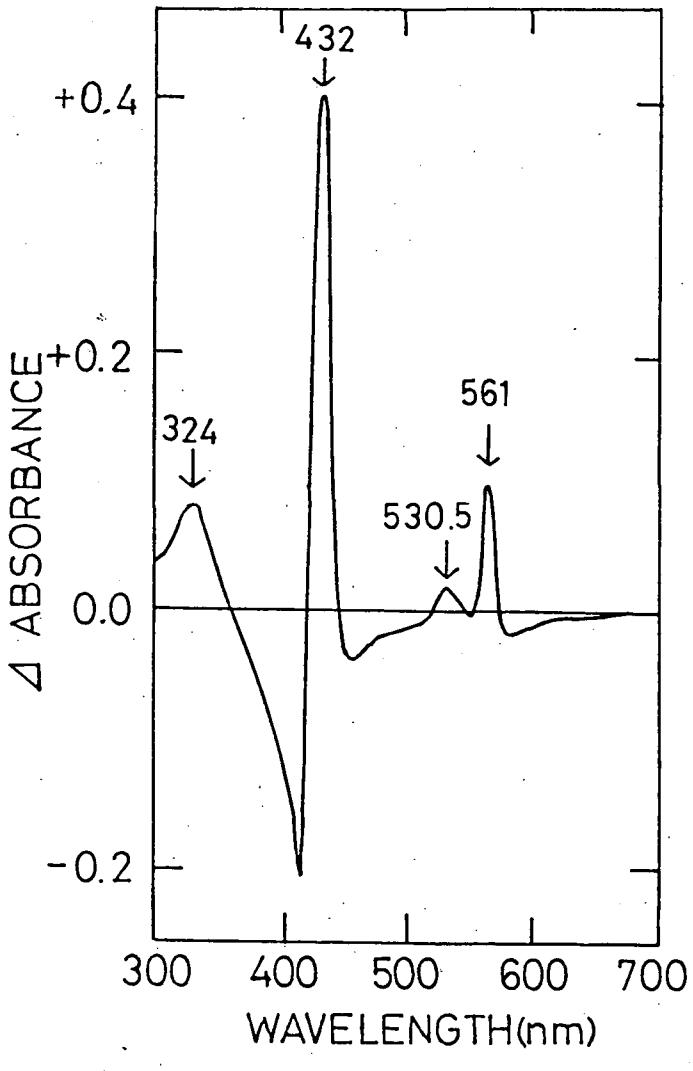

Fig. 28. Reduced-minus-oxidized difference spectrum of <u>Enteromorpha</u> cytochrome <u>b</u>-561 in 10 mM K-phosphate buffer, pH 7.0, at 25°C. Differences of absorbances were plotted from the data in Fig. 8.

Table 8

The positions (nm) and millimolar extinction coefficients (E mM) of absorption maxima (max) and isosbestic points (iso) of ferro- (Cyt^{2+}) and ferricytochrome b-561 (Cyt^{3+}) at pH 7.0 and 25°C

Position (nm)	EmM		ΔEmM
	Cyt^{2+}	Cyt^{3+}	Cyt^{2+} - Cyt^{3+}
358	iso 63.5		
417		γ-max 160	
422	iso 156		
428.5	γ-max 243		
432			γ-max 124
446	iso 38.1		
518	iso 15.2		
530.5	β-max 22.9	max 16.5	β-max 6.4
546	iso 16.5		
554	iso 15.2		
561	α-max 43.2		α-max 30.8
572	iso 11.4		

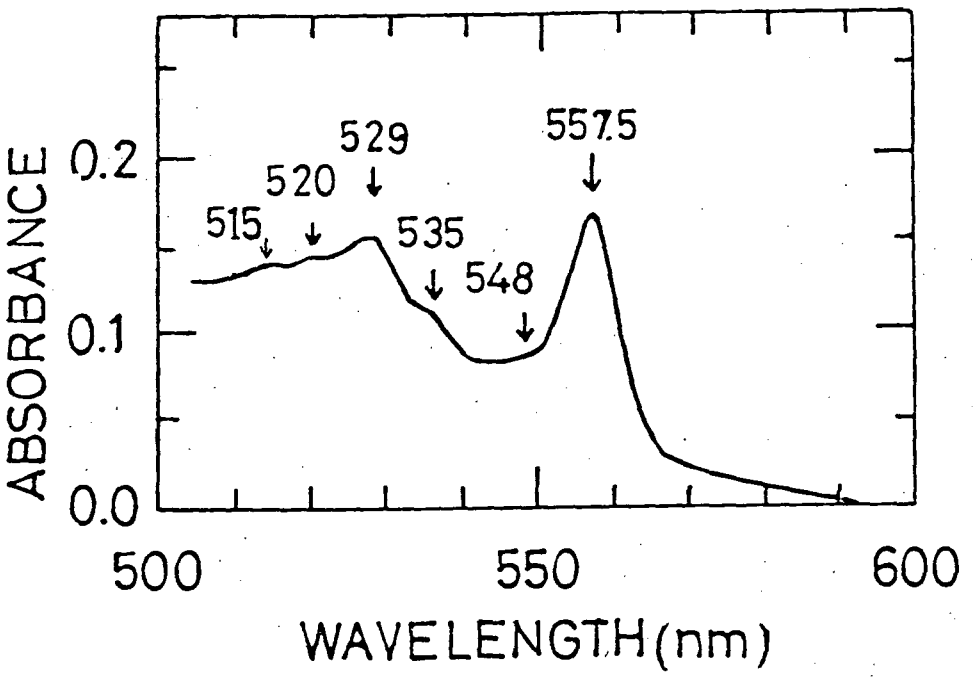

Fig. 29. Absorption spectrum of reduced Enteromorpha cytochrome b-561(Form 1) at the temperature of liquid N_2 (77°K) in 50% (v/v) glycerol containing 2.5 mM K-phosphate buffer, pH 7.0.

in ferri-EDTA solution with ferro-EDTA are shown in Fig. 30. Similar results were obtained for Form 1 in anaerobic or aerobic titration and for Form 2 in anaerobic titration (and for Form 2 in the aerobic titration although it is not shown in the figure). The 0.06 slope of the points plotted in the figure as log(ferricytochrome/ferrocytochrome) versus the redox potential adjusted by ferri- and ferro-EDTA system indicates that a one-electron change was involved in the redox reaction of cytochrome b-561. The value of the redox potential at half reduction of the cytochrome, namely the midpoint potential, was found to be 0.23 volt at pH 7.0 and 25°C.

Pyridine hemochrome. The reduced form of the pyridine hemochrome from the cytochrome had absorption maxima at 418, 524, and 556 nm. The spectral feature suggests the heme moiety to be protoheme IV.

Peroxidase Activity. Peroxidase activity was measured using 3.88 μg of cytochrome b-561 dissolved in 0.1 ml of 5 mM phosphate buffer, pH 7.0. The linear increase in absorbance at 460 nm was found to be 0.23 per min per mg protein. This value is only 0.028% of the specific activity of purified horseradish peroxidase (104).

Molecular Weight. A plot of the elution position against the logarithm of the molecular weight of the marker proteins is shown in Fig. 31. A molecular weight of 67 x 10^3 was estimated for Forms 1 and 2 of the cytochrome. The same value was obtained also by the use of 0.1 mM 1,4-dithiothreitol contained in the same buffer.

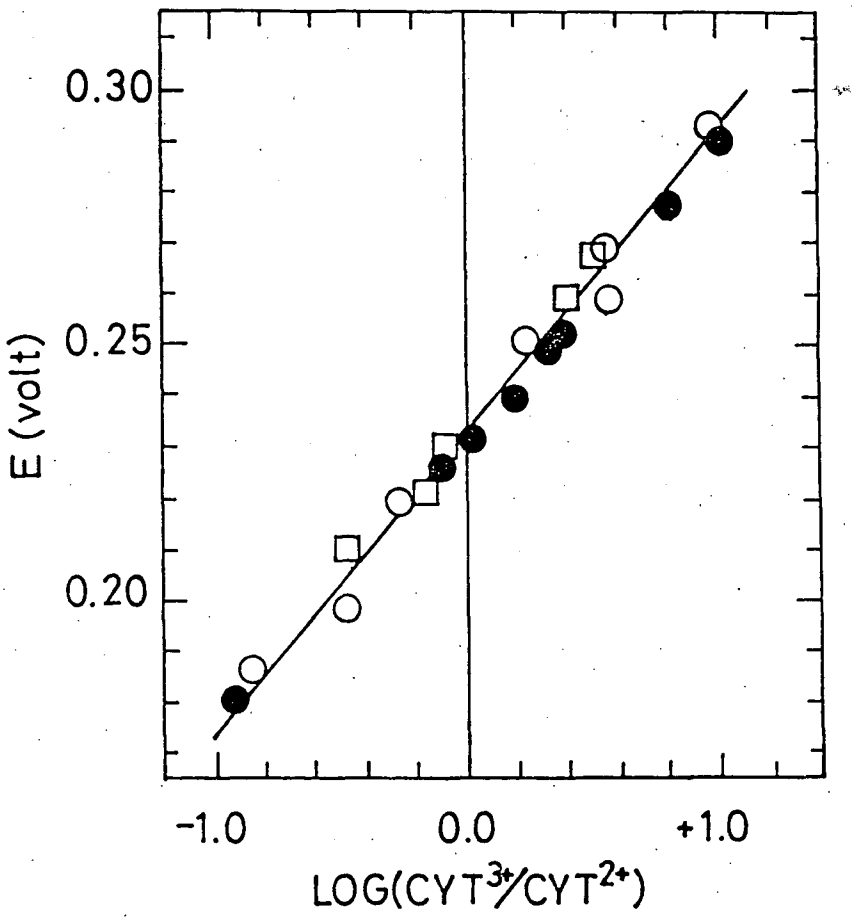

Fig. 30. Determination of the midpoint potential (Em) of Enteromorpha cytochrome b-561 (Forms 1 and 2). After 0.5 or 0.6 ml of 25 mM ferric ammonium sulfate, pH 2.2, was added to 5.0 ml of 0.1 or 2.5 μM cytochrome-0.1 M EDTA, pH 7.4, resulting in pH decrease to 7.0, a reductive titration was carried out with 2.5 mM ferrous ammonium sulfate-0.1 M EDTA, pH 7.0, at 25°C in air or N_2 gas. Redox change of the cytochrome and potential (volt) of the reaction mixture were measured spectrophotometrically at 561 nm and by a Ag/AgCl electrode, respectively, in each titration. ○, Form 1(0.1 μM) in N_2 gas; ●, Form 1(2.5 μM) in air; □, Form 2(0.1 μM) in N_2 gas.

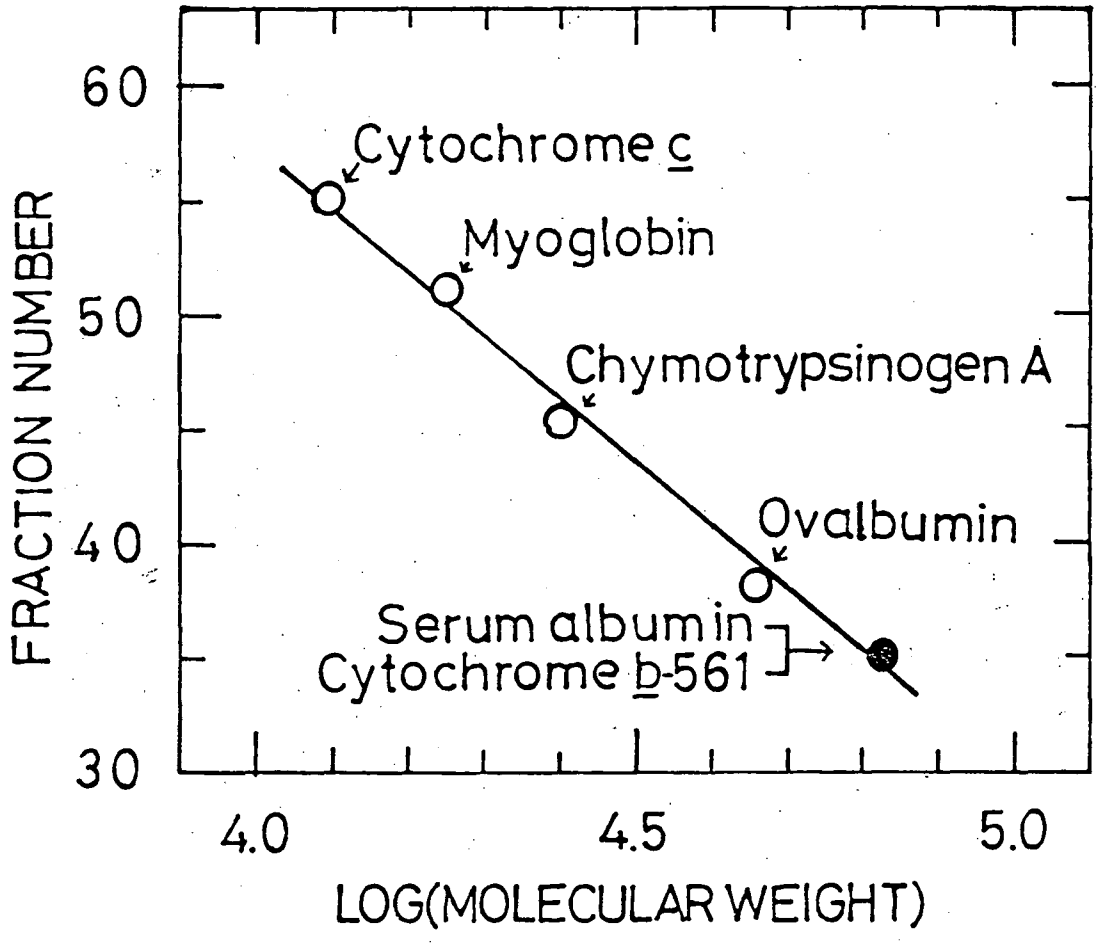

Fig. 31. Estimation of the molecular weight of Enteromorpha cytochrome b-561 (Forms 1 and 2) by Sephadex G-75 gel filtration in 125 mM NaCl-20 mM K-phosphate buffer,pH 7.0, at 5°C. The column was 2.64 x 40 cm. The flow rate was 15 ml per hr. The eluate was collected in 3.0 ml fractions.

4. Discussion

Two types of hemoproteins were extracted and partly purified from two species of green algae, Ulva pertusa and Enteromorpha prolifera as a water-soluble form and were studied some of its physico-chemical properties. Both of the pigments showed the absorption spectra characteristic of b-type cytochromes, weak autoxidizability, insensitivity to carbon monoxide and cyanide, and considerably positive midpoint redox potential, and had protoheme but little peroxidase activity. Thus the proteins were tentatively named cytochrome b-562.5 (Ulva pertusa) and cytochrome b-561 (Enteromorpha prolifera), respectively.

Enteromorpha cytochrome b-561 and Ulva cytochrome b-562.5 were extracted from the air-dried and the frozen thalli, respectively. The Enteromorpha cytochrome was also extracted from the frozen thalli, but its amount was nearly 7-fold lower than that from the air-dried thalli. There is no difference in several properties, viz., molecular weight, absorption spectra, and midpoint redox potential, betweeen the cytochromes extracted from the frozen thalli and from the air-dried thalli. The attempt to extract the Ulva cytochrome from the air-dried thalli has not yet been made. The acrinol treatment was found to be effective for the removal of the slimy substances in the crude extract of Ulva thalli. Ulva cytochrome b-562.5 was precipitated as a complex with acrinol from dilute salt solution; the cytochrome was eluted with 0.2 saturated ammonium sulfate solution, leaving the slime

in the precipitates. On the other hand, Enteromorpha cytochrome b-561 was irreversibly precipitated by acrinol and was unable to be eluted with any concentrated salt solution. By hydroxyapatite chromatography the Enteromorpha cytochrome extracted from either air-dried thalli or frozen thalli was found to be separated into two forms. There is no obvious explanation for this observation. The two forms could not be distinguished in the properties examined in this work.

The absorption spectra of the two b-type cytochromes resemble those of the purified b-type cytochromes from three species of green algae, Monostroma sp. (99), Scenedesmus obliquus (100) and Bryopsis maxima (11). But slight differences were found in the location of the maxima; in particular, the α-peak of Enteromorpha cytochrome was shifted to 1-1.5 nm lower than those of four green algae. Ulva cytochrome b-562.5 has an asymmetric α-band which splits into two peaks at liquid nitrogen temperature. The asymmetric α-band is characteristic of b_5-type cytochromes (11, 104, 108-111). The Enteromorpha cytochrome, on the contrary, has a symmetrical α-band, a property which shares with the soluble b-type cytochromes of other three algae and a marine diatom (43). Although water-soluble c-type cytochromes purified from numerous algae have an asymmetric α-band, the homologous cytochromes purified from Polysiphonia (in chapter II), Monochrysis (40), and Euglena (36, 39, 42) have a symmetrical α-band. Thus, Ulva cytochrome b-562.5 cannot be excluded from the group of b-type cytochromes of the green algae described above. The absorbance of the Enteromorpha cytochrome at α-

peak is about 26% stronger than that of its pyridine ferrohemochrome, hence the millimolar extinction coefficient of the ferrocytochrome at α-peak is a characteristic large value. This is true also for Ulva cytochrome b-562.5.

The molecular weight (67×10^3) of the Enteromorpha cytochrome differs greately from the 23×10^3 for the Ulva cytochrome and 30×10^3 for Bryopsis cytochrome b-562 (11). The Enteromorpha cytochrome might exist as an oligomer, although the possibility that the protein associates by disulfide bonds is excluded on the basis of the gel-filtration experiments in the presence and absence of dithiothreitol.

The redox properties of Ulva cytochrome b-562.5 and Enteromorpha cytochrome b-561 are similar to those of the b-type cytochromes of the three genera of green algae. The midpoint redox potentials obtained for the Ulva cytochrome (0.24 volt) and the Enteromorpha cytochrome (0.23 volt) are higher than those of Bryopsis cytochrome b-562 (0.175 volt). But the value of the Bryopsis cytochrome was obtained by using a midpoint potential of 0.110 volt for iron-EDTA redox system (105), and in this investigation was used the value of 0.15 volt obtained by the author for the iron-EDTA system, indicating that the three cytochrome possess a similar midpoint redox potentials.

Cytochrome b_6 and b-559 are known to be photosynthetic components (112, 113). Compared to the positions of α-peak and the redox potentials of the water-soluble green-algal b-type cytochromes, cytochrome b_6 isolated with a detergent has a similar α-peak at 563 nm but a lower potential of -0.08 volt

(114); cytochrome b-559 isolated with a detergent is ascorbate-reducible but a lower-wavelength α-peak at 559 nm (15, 115). Based on these findings, the water-soluble algal cytochrome seems to be distinct from the photosynthetic b-type cytochromes. Higher plant mitochondria contain three membrane-bound b-type cytochromes, viz., cytochromes b-553, b-557 and b-562, which have α-peaks at around 556, 560 and 565 nm at room temperature and midpoint potentials of 75, 42 and -77 millivolt, respectively (116-118). The mitochondria of several colorless algae also have multiple b-type cytochromes similar to those of the higher plants (119, 120). However, Euglena gracilis and Chlorella protothecoides contain only one respiratory b-type cytochrome, viz., cytochromes b-561 and b-562, respectively (20, 121).

In general, respiratory and photosynthetic b-type cytochromes are firmly bound to mitochondria and chloroplasts, respectively, but several b-type components with α-peaks at 561-563 nm have been extracted together with b_5-type components without the aid of a detergent from both photosynthetic and nonphotosynthetic organisms, including the higher plant Phaseolus aureus (104), the algae mentioned above, the fungus Neurospora sp. (108), and the insect Musca domestica (109). Phaseolus cytochrome b-561 and b-555 were extracted from the seedlings; Musca cytochrome b-563 and b-555 were from the larvae and pupae, not from the adults. Yamanaka and Okunuki have pointed out that solubilization of the b-type cytochrome may be related to the degree of tissue development (108). Neurospora cytochromes b-563 and b-556

were obtained from air-dried powder (108), while Screlotinia cytochrome b-554,561 was solubilized by endogenous lipase (122). It is therefore possible that the soluble algal b-type cytochromes may also be bound to some particulates in the cells, possibly to mitochondria, and may have been solubilized by unknown reasons.

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PUBLICATIONS

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