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ISOLATION AND CHARACTERIZATION OF SOLUBLE CYTOCHROME *c*-553 AND MEMBRANE-BOUND CYTOCHROME *f*-553 FROM THYLAKOIDS OF THE GREEN ALGA *SCENEDESMUS ACUTUS* *

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Summary

Soluble cytochrome *c*-553 and membrane-bound cytochrome *f*-553 from the alga *Scenedesmus acutus* were purifed to apparent homogeneity. The properties of cytochrome *c*-553 are comparable to preparations obtained from other eukaryotic algae, whereas the thylakoid-bound species resembles higher-plant cytochrome *f*.

Common characteristics are:

1. An asymmetrical α-band at 553 nm.
2. A midpoint redox potential of +380 mV (pH 7.0), with a pH dependency above pH 8.0 of —60 mV/pH unit.
3. Formation of a pyridine hemochromogen with a maximum at 550 nm; no adducts with CN^- or CO are observed.

Distinguishing features are:

1. Cytochrome *f*-553 has a more complicated β-band, with maxima at 531.5 and 524 nm, and hence a more complex low-temperature spectrum. Also the positions of the γ- and δ-band at 421.5 and 331 nm, respectively, distinguish cytochrome *f*-553 from cytochrome *c*-553, with γ- and δ-bands at 416 and 318 nm.
2. The ferricytochrome *c*-553 spectrum exhibits a weak band at 692 nm, which is not observed with cytochrome *f*.

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Abbreviations: SDS, sodium dodecyl sulfate.

3. The molecular weight of cytochrome *f* is about 33 000, that of cytochrome *c* about 10 000.

4. The isoelectric point of cytochrome *c* was determined at pH 3.7, that of cytochrome *f* at pH 5.1.

Introduction

As was shown by Wood [1] several algae contain a membrane-bound cytochrome *f* (mol. wt. 33 000, Soret band at 422 nm) in addition to soluble cytochrome *c* (mol. wt. 10 000, Soret band at 416 nm). Moreover, the kinetic and immunological properties differed markedly for both the soluble and membrane-bound cytochrome. Soluble *c*-type cytochromes may replace plastocyanin in its function, whereas the membrane-bound form is equivalent to cytochrome *f*, originally isolated from higher-plant chloroplasts [2—9]. In algae, soluble cytochrome *c*-553 is present in several-fold excess over cytochrome *f* [8—10]. Its pool size might be varied by different growth conditions, which leave the amount of cytochrome *f* constant [10,11].

Numerous reports on the isolation of so-called 'soluble' algal *c*-type cytochromes have appeared (e.g. reviews in Ref. 12), however, only a few were extensively purified and characterized. Much less information is available on the properties of algal cytochromes *f*. The purpose of this communication is to give a comprehensive biochemical characterization of both cytochromes and compare their properties.

Materials and Methods

Isolation and purification of cytochrome c-553. Cells of the green alga *Scenedesmus acutus* (= *Sc. obliquus* (Turp.), Kütz, strain 276.3a, Algal Culture Collection, Göttingen) were obtained from Professor Soeder, Dortmund. 250—300 g of wet algal cell paste was washed, suspended in 150 ml 50 mM phosphate buffer, pH 7.2, containing 0.4 M NaCl, mixed with 1200—1500 g glass beads, and homogenized with a 'Vibrogen Zellmühle' (Bühler, Tübingen) for 5 min. The glass beads were separated from broken cells by filtering through a coarse fritted glass funnel (G-1, Schott) into a suction flask. The homogenate was then centrifuged at 27 500 × *g* for 30 min. The green pellet was used for isolation of cytochrome *f* (see below). The yellow-green supernatant was subjected to ammonium-sulfate fractionation; the 40% to 90% precipitate contained cytochrome *c*-553, which was extracted from the pellet with 20 mM Tris-HCl, pH 8.0. After dialysis, this crude extract was placed on a DE-52 column (3 cm × 7 cm) and the cytochrome fraction was eluted by a linear NaCl gradient (0.1—0.25 M) at about 0.18 M NaCl. This procedure was repeated up to three times until a absorbance ratio (A_{318nm}/A_{274nm}) of 0.85 was reached (the δ-peak/protein-peak ratio was used throughout this study to monitor the proceeding purification of the protein, since both absorbance bands can be measured in the ultraviolet). The cytochrome fraction was then dialyzed, concentrated by lyophilization and placed on a Sephadex G-75 (superfine) column (2.5 cm × 90 cm). For the purest cytochrome fractions a

ratio of A_{318}/A_{274} of 1.3 was measured. The yield is, of course, dependent on the algal material, since its cytochrome *c*-553 content may vary substantially, depending on the growth conditions [10]. About 2 μmol of cytochrome *c* were present in the crude extract from 300 g of algae. The final yield of pure protein was about 0.5 μmol, with cytochrome *c*-553 in the reduced form. The cytochrome preparation gave a single band when subjected to gel electrophoresis (15% gel [13]).

Occasionally, a second band of lower electrophoretic mobility was observed, which was due to oxidized cytochrome *c*-553; it could be avoided by electrophoresis in the presence of 1 mM sodium ascorbate. For preliminary reports on isolation of cytochrome *c*-553 from *Scenedesmus acutus* see Refs. 7 and 14.

Isolation and purification of cytochrome f-553. The thylakoid membranes (see above) were freed from soluble proteins by three successive washing steps in about 3 l of the phosphate buffer. The chlorophyll content of the last suspension was adjusted to 1 mg chlorophyll/ml. 2 l of a mixture (−20° C) consisting of 1.5 l ethyl acetate, 0.5 l ethanol and 10 ml ammonia (25%) [15] was added to 500 ml of the membrane suspension (0° C) containing 1 mg/ml of sodium dithionite, and mixed in a 1-gallon Waring Blendor for 30 s at full speed. The emulsion was allowed to settle at −20° C, the organic phase was discarded and the residual aqueous/organic mixture centrifuged for 10 min at 20 000 × *g* (−20° C). This results in a green precipitate, an aqueous interphase and a dark-green organic phase on top. After separation, 1.1 volumes of acetone (−20° C) were added to the reddish aqueous phase, stirred for 15 min, and centrifuged for 10 min at 20 000 × *g*. The resulting pellet was extracted two times with 10 ml of 0.2 M Tris-HCl, pH 8.0; the extract was clarified by centrifugation and the supernatant brought to 70% saturation by ammonium sulfate. The solution was allowed to settle for 1 h at 0° C, centrifuged for 10 min at 28 000 × *g*, and cytochrome *f* extracted from the pellet by 2 ml of 20 mM Tris-HCl, pH 8.0. The reddish solution was dialyzed against 2 mM Tris-HCl, pH 8.0, overnight, concentrated by lyophilization, and applied to a Sephacryl G-200 column (diameter 2.5 × 90 cm) equilibrated with 0.1 M NaCl including 20 mM Tris-HCl, pH 8.0. For the main fractions a maximum absorbance ratio of 1.05 (A_{331nm}/A_{276nm}) was measured; the side fractions were purified on a second Sephacryl G-200 column. Other column materials were less effective, specially DE-52 cellulose or DE-Sephadex.

Homogenization of the algae, extraction of the cytochrome and purification by gel filtration can be easily accomplished within three days. The organic solvent extract contains about 0.5 μmol, from which about 0.1 μmol of pure cytochrome *f* can be obtained. The cytochrome is isolated in its reduced state.

Polyacrylamide electrophoresis (10% gel [13]) yielded two red bands, measured densitometrically at 421 nm (Shimadzu UV 300, gel-scan accessory). Spectral analysis of each band showed the characteristic cytochrome *f*-553 spectrum. After staining with Coomassie Brilliant Blue (R 250) no additional protein bands became visible. It is, therefore, concluded that the isolation procedure yields an essentially pure protein (see Fig. 6).

Physical and chemical measurements. Spectral properties of the cytochromes were determined by an Aminco-DW2 spectrophotometer; a special low-temper-

ature accessory with twin cuvettes (2 mm optical path length) was used for the 77 K spectra. The millimolar extinction coefficient (ϵ_{mM}) was calculated on the basis of formation of a pyridine hemochromogen [16,17] and compared to the spectral data obtained with horse-heart cytochrome [18].

Polyacrylamide-gel electrophoresis was performed according to standard procedures [13,19]. For determination of the molecular weight a 15% gel was used [19]. The samples (2—10 μg protein) were incubated with 2% sodium dodecyl sulfate (SDS) and 1% dithiothreitol at 60° C for 1 h [20].

The isoelectric point was determined by isoelectric focusing in flat-bed gels (Sephadex G-75, superfine) with carrier ampholytes (LKB, pH 3.5—5.0 and 4.0—6.0) according to [21]. The cytochrome band could be identified by its red colour and a 'paper print' technique [21].

The midpoint redox potential was obtained by a combined spectrophotometric/platinum-calomel electrode method as described [22]. Potassium ferricyanide was used as oxidant and redox buffer, sodium ascorbate as reductant.

Molecular weight was determined by Sephadex gel filtration [23] (Sephadex G. 75), SDS-gel electrophoresis (see above), ultracentrifugation and amino-acid analysis.

Analytical ultracentrifugation was run on a Beckman-Spinco, Mod. E, centrifuge equipped with a photoelectric scanner (ANH-Th2 rotor, 52 000 rev./min). The molecular weight was calculated using the meniscus-depletion method [24].

Amino-acid analysis according to Spackman et al. [25] was performed on a Biotronik Chromatography System 4010. The protein was hydrolyzed for 24 h in 6 N HCl at 110° C in sealed evacuated Kontess tubes. Tryptophan was determined separately by hydrolysis in toluene sulfonic acid and tryptamine [26], cysteine as cysteine sulfonic acid after oxidation with performic acid [27].

Protease activity was checked with *N*-α-benzoyl-L-arginine-4-nitroanilide (Merck) as substrate: the mixture contained 20 mM of the nitroanilide, 30 mM Tris-HCl, pH 8.0, and the protein to be tested (for cytochromes: 10—20 mg of protein); activity was measured as absorbance increase at 405 nm and compared to trypsin (Sigma) which gave about 3.5 μmol per mg protein per min.

Protein concentration was determined by the method of Lowry et al. [26], after precipitation with 10% trichloroacetic acid, by the method of Bradford [27], and according to Schaffner/Weissmann [28], with cytochrome *c* (horse-heart, Sigma) as standard. As a second method the desalted and lyophilized cytochrome sample was weighted in directly on a Mettler-ME22 balance, which allows for accurate determinations in the μg range. Due to the hygroscopic properties of the lyophilized sample, a correction for water uptake during manipulation had to be made. The weighed-in protein was then dissolved in an appropriate amount of phosphate buffer and cytochrome concentration was determined spectroscopically. Dialysis or gel filtration (G-25, coarse) did not effectively remove the salts and led to denaturation of the protein. Effective salt removal, as measured by an ion conductivity meter, could be achieved without denaturation of the protein by ultrafiltration only (Amicon, Mod. 8 MC, UM-05 membrane filter).

Results and Discussion

Absorption spectra

The absorption spectrum of cytochrome *c*-553 is shown in Fig. 1A, with peaks at 553 (α), 523 (β), 416 (γ), 318 (δ) and 272 (protein) nm in the reduced form; shoulders at 393, 355 and 289 nm are observable. Upon oxidation the

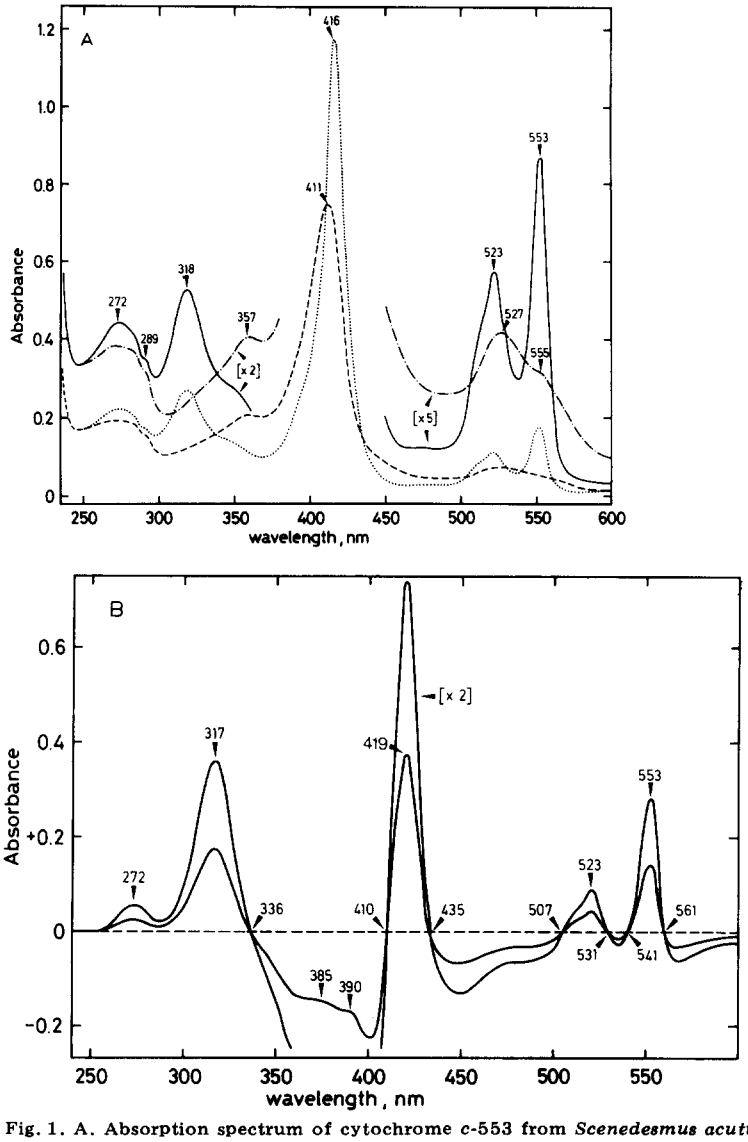

Fig. 1. A. Absorption spectrum of cytochrome *c*-553 from *Scenedesmus acutus*. Reduced (——) with borohydride after oxidation (– – –) with ferricyanide and removal of excess oxidant by Sephadex (G-25, coarse)-gel filtration. The solution contained cytochrome *c*-553 in 20 mM phosphate buffer, pH 7.0, and 80 mM NaCl. The α/β region of the spectrum was run with a five-fold, the ultraviolet region with a two-fold scale expansion. B. Difference absorption spectrum (reduced minus oxidized) of cytochrome *c*-553 from *Scenedesmus*. Conditions as in (A); a two-fold scale expansion was used to demonstrate isosbestic points.

α-β region is replaced by a more diffuse spectrum (weak bands at 555 and 527 nm) and the γ-band shifts to 411 nm; the 318-nm band disappears and a new band at 357 nm is observed. Since the cytochrome is isolated in the reduced form, the problem arises of an oxidant not interfering with the spectrum. Ammonium peroxodisulfate- or H_2O_2-oxidation proved not to be fully reversible. Oxidation by excess of ferricyanide and removal of the oxidant by dialysis or gel filtration (Sephadex G-25, coarse) led to autoreduction of the cytochrome stored in Tris buffer, pH 8.0 [29]; a more or less constant amount of 12—18% of the total cytochrome remained reduced. Systematic investigation of this effect led to the discovery that autoreduction depends on the buffer system and the pH used. Especially the zwitterionic buffers, originally introduced by Good [30], with a pH above 8.0 catalyzed autoreduction. Ferricyanide oxidation of cytochrome *c*-553 stored in 50 mM phosphate buffer, pH 7.2, and removal of excess oxidant by gel filtration produced a perfectly stable oxidized cytochrome; borohydride ($NaBH_4$) could then be used as reductant. The difference spectrum exhibited isosbestic points at 336, 410, 435, 507, 531, 541 and 561 nm (Fig. 1B); it should be noted that the Soret maximum shifts to 419 nm.

The asymmetrical α-band at 553 nm is split into two peaks at 552 and 548 nm at 77 K; the β-maximum shifts to 522 nm, with an additional band at 528 nm and shoulders at 514, 511 and 505 nm (Fig. 3). The γ/α ratio (A_{416nm}/A_{533nm}) of the reduced form gave a value of 6.7. Further examination of the absorption spectrum of ferricytochrome *c*-553 demonstrates the presence of a weak 692-nm band, which has originally been used as indicator of molecular integrity of the protein, but is presumably due to a methionine residue as sixth ligand of the heme iron (Fig. 4, [31,32]).

The covalent bonds of the heme group to the protein, characteristic for *c*-type cytochromes, could not be split by treatment with acid ethylmethyl ketone or alkaline KCN solution [17,33]. No spectral change is observed after treatment with carbon monoxide. The results show that carbon monoxide or cyanide are not able to displace the histidine/methionine ligands, indicating a protein in its native state [31]. Formation of the reduced pyridine hemochromogen produced by excess pyridine in the presence of dithionite shows absorption peaks at 413, 520 and 550 nm, characteristic of a heme-*c* as prosthetic group. The millimolar extinction coefficient ϵ at 553 and 523 nm was 25.3 and 16.2 ($mM^{-1} \cdot cm^{-1}$), respectively. From the difference spectrum (reduced minus oxidized) a $\Delta\epsilon$ at 553 of 17.3 ($mM^{-1} \cdot cm^{-1}$) is obtained.

The absorption spectrum of reduced cytochrome *f*-553 also shows the typical four absorption bands in the visible and two in the ultraviolet region of the spectrum: at 553, 524, 421.5, 331 and 276 nm. Distinctive spectral features (as compared to the soluble *c*-cytochrome) are (1) a Soret maximum at 421 nm, (2) a more complicated β-band with two maxima at 531.5 and 524 nm (there are shoulders at 543.5, 513, 508.5, 400 and 365 nm), (3) a δ-band at 331 nm. The γ (Soret)/α-absorbance ratio is about 7.5. The maximum of the oxidized form is at 411 nm, whereas the α-β region and the 331-nm band is replaced by a more diffuse spectrum (weak band at 530 and shoulder at 565 nm). In contrast to the soluble species cytochrome *f*-553 could be oxidized by ammonium peroxodisulfate and reduced by sodium boro-

hydride without denaturation. The absolute and difference spectra are shown in Figs. 2A, B. Isosbestic points are at 560, 541, 535, 508, 434, 415 and 350 nm. A further spectral characteristic is the absence of a 695-nm band of the oxidized cytochrome *f*-553 when compared to cytochrome *c*-553 (Fig. 4). The low-temperature difference spectrum (77 K) shows a split α-band at 551 and 549 nm and a shoulder at 541 nm; the β-region splits into seven bands at 529.5, 523.5, 521, 514.5, 510.5 and 506 nm (Fig. 3). A similar fine structure was found for spinach cytochrome *f* [34]. The data indicate in addition a less asymmetric α-band as compared to cytochrome *c*-553.

As expected for a *c*-type cytochrome, treatment with carbon monoxide

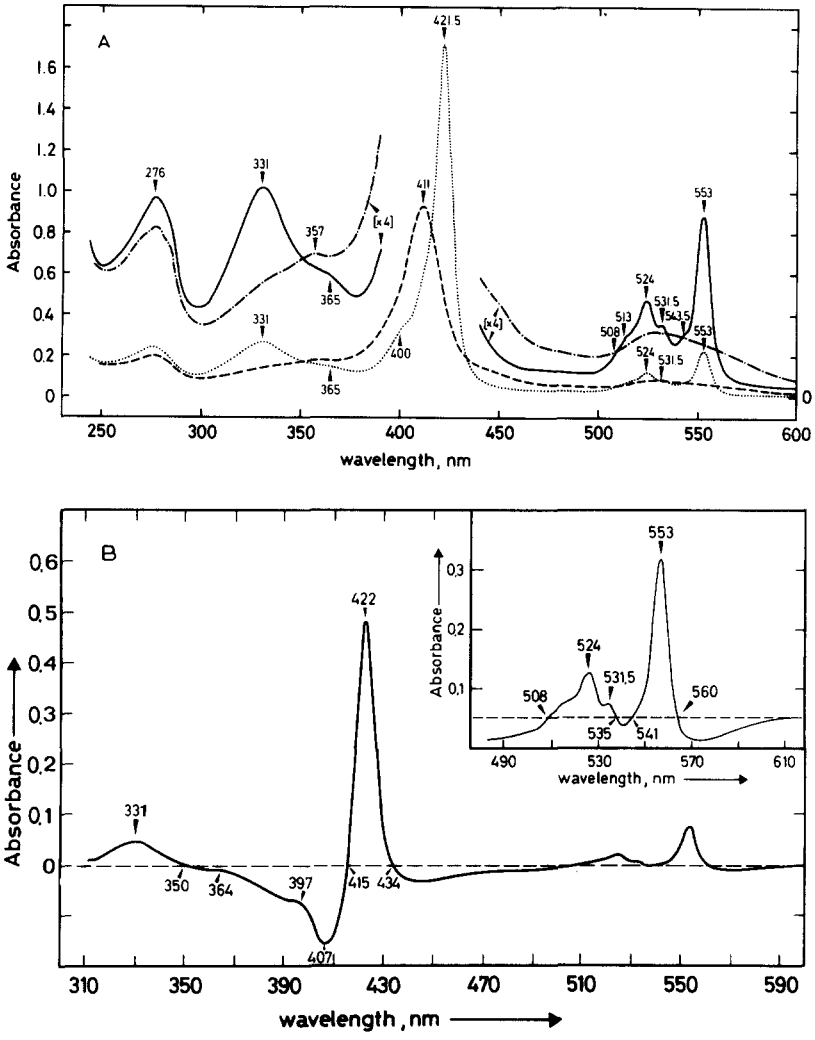

Fig. 2. A. Absorption spectrum of cytochrome *f*-553 from *Scenedesmus acutus*. Reduced (——) with borohydride; oxidized (— · — · —) with ammonium persulfate; upper trace is four-fold scale expansion; half band with: 1 nm. B. Difference absorption spectrum (reduced minus oxidized) of cytochrome *f*-553 from *Scenedesmus*. Conditions as in (A); inset shows a four-fold scale expansion of the α/β region of the spectrum; isosbestic points are indicated.

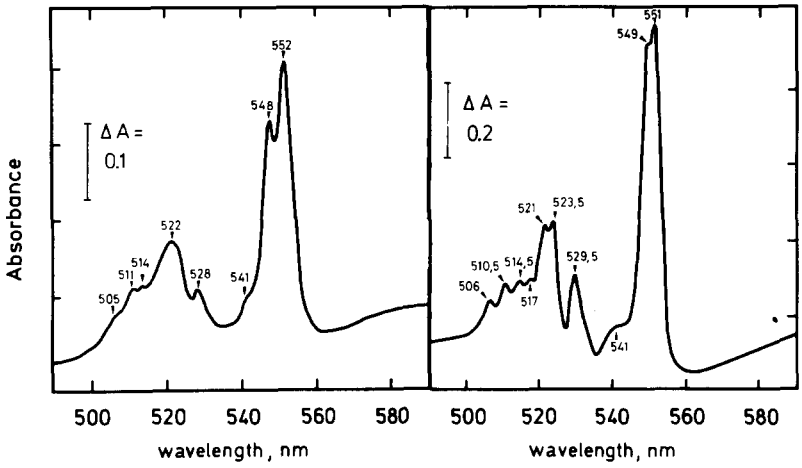

Fig. 3. Absorption spectrum of cytochrome *c*-553 (on the left) and cytochrome *f*-553 (on the right) from *Scenedesmus* at 77 K. Conditions as in Materials and Methods; half band width 0.7 nm.

did not change the spectrum, indicating a native protein. The heme could not be split off the protein by acid ethylmethyl ketone. The α-band of the pyridine hemochromogen was at 550 nm.

The extinction coefficients for the α- and β-band were 29.5 and 14.6 ($mM^{-1} \cdot cm^{-1}$), respectively; the $\Delta\epsilon$ at 553 nm was 21 ($mM^{-1} \cdot cm^{-1}$).

The midpoint potential was determined by plotting the absorbance change (553—541 nm, dual-wavelength mode) of the α-band versus the redox potential of the solution measured simultaneously with a platinum-calomel combination electrode. For both cytochromes we obtained an $E_{m\ 7.0}$ value of 383 ± 5 mV (Fig. 5A). The midpoint potential is pH-dependent in the pH region above 8.0; a slope of −60 mV/pH indicates the uptake of one proton per

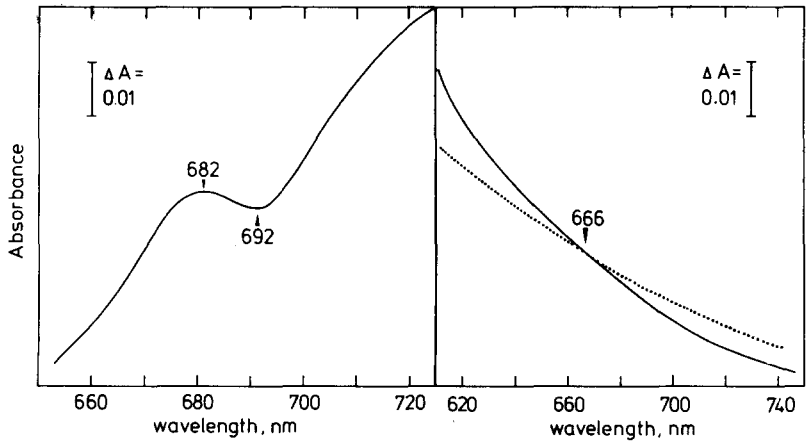

Fig. 4. Absorption spectrum of cytochrome *c*-553 (on the left) and cytochrome *f*-553 (on the right) in the far-red region of the spectrum. The trace on the left represents a difference spectrum reduced minus oxidized of cytochrome *c*-553, 70 μM. The trace on the right shows the ferricyanide-oxidized (- - - - - -) and the ascorbate-reduced (· · · · · ·) spectrum of cytochrome *f*-553, concentration 30 μM.

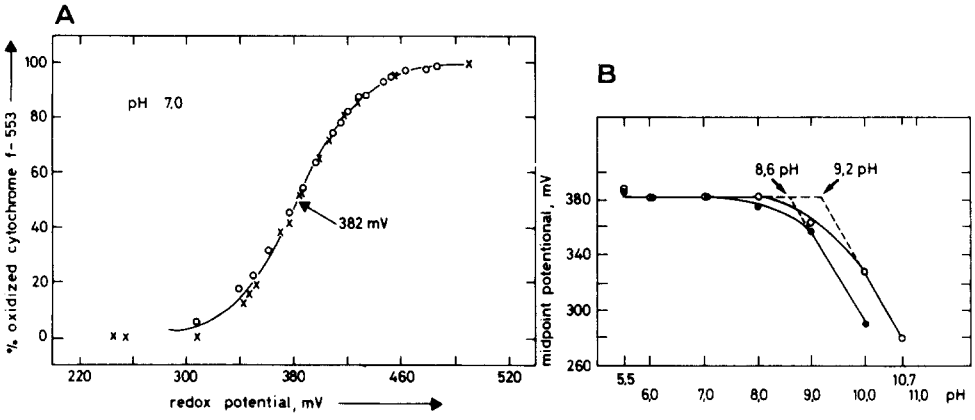

Fig. 5. A. Redox titration curve of cytochrome *c*-553 and cytochrome *f*-553 from *Scenedesmus* at pH 7.0 and 20°C. The buffer was 0.2 M morpholinoproprane sulfonic acid/NaOH, pH 7.0; wavelength pair used was 553—541 nm (dual wavelength); (x———x), reduction by sodium ascorbate; (○———○), oxidation by ferricyanide. B. Dependence of midpoint redox potential on pH of cytochrome *c*-553 (●———●) and cytochrome *f*-553 (○———○). The buffer systems were (0.2 M); morpholinoethane sulfonic acid, pH 5.5 and 6.0; morpholinopropane sulfonic acid, pH 7.0; *N*-tris(hydroxymethyl)-methylglycine, pH 8.0; *N,N*-bis(2-hydroxyethyl)glycine, pH 9.0; cyclohexylaminopropane sulfonic acid, pH 10.0 and 10.7; cytochrome concentration was 50 μM.

electron (Fig. 5B). This could be due to the replacement of methionine as heme-iron ligand by lysine which becomes deprotonated at higher pH [31]. The p*K* of this proton uptake is at pH 8.6 for cytochrome *c*-553 and at pH 9.2 for cytochrome *f*-553. As discussed above, both cytochromes have a tendency towards autoreduction during titration, which is especially pronounced at higher pH.

Gel filtration of cytochrome *c*-553 with Sephadex G-75 and ultracentrifugation showed apparent molecular weights of 10 000 and 8600, respectively. Slab-gel electrophoresis according to Laemmli (15% gel) with protein markers from 3500 to 45 000 gave a value of 7500 (Fig. 7). The reason for these discrepancies is not known. Comparing protein content [26] and heme content of one preparation gave a value of 8500 per heme. Also with cytochrome *c*-553 from *Bumilleriopsis filiformis* gel electrophoresis showed a molecular weight of 7500 [35] whereas amino-acid determination gave a figure of 9700 (Ambler, R., personal communication, Table I).

The molecular weight of cytochrome *f* determined by gel filtration (Sephadex G-75) was at 33 000. SDS-polyacrylamide gel electrophoresis (15% gel [19]) showed two bands at 33 000 and 31 000, respectively (Figs. 6 and 7). In the course of this investigation we also observed lower molecular weight components on SDS polyacrylamide gels, down to 8000, even with pure cytochrome *f*-553 preparations; this was, however, dependent on the algal material used and the individual preparation. We therefore checked for proteinase activity associated with those cytochrome fractions. A very low, but still measurable protease activity could be measured, by a factor of 10^{-5} to 10^{-6} less than pure trypsin on a protein basis. This low activity, however, could be stimulated considerably by the presence of 0.1—2% SDS, whereas trypsin itself was completely inactivated under these conditions. Incubation of the protein

TABLE I

AMINO-ACID COMPOSITION OF CYTOCHROME *c*-553 FROM *SCENEDESMUS ACUTUS* AS COMPARED TO CYTOCHROMES FROM OTHER EUKARYOTIC ALGAE

(1) *Euglena gracilis* cytochrome *c*-553 [39], (2) *Monochrysis lutheri* cytochrome *c*-553 [40], (3) *Bumilleriopsis filiformis* cytochrome *c*-553 (Ambler, R.P., personal communication, 1978, comp. also [35]).

Amino-acid residue	*Scenedesmus-acutus* cyt *c*-553	(1) *	(2) *	(3) *
Asx	11	10	11	15
Thr	5	5	3	4
Ser	3	5	7	5
Glx	10	9	10	9
Pro	3	2	1	2
Gly	9	12	8	8
Ala	15	10	11	12
Val	5	10	6	5
Met	2	1	1	3
Ile	3	4	3	5
Leu	6	3	6	4
Tyr	2	5	3	2
Phe	3	1	3	2
Lys	5 **	4	4	4
His	2	1	1	1
Trp	1	2	1	1
Arg	1	1	1	2
Cys	2	2	2	2
Number of residues	88	87	82	86
Calculated molecular weight ***	10 280	9734	9308	9743

* From sequence data.

** Additional elution peak, probably due to methylated lysine.

*** Includes the heme prosthetic group.

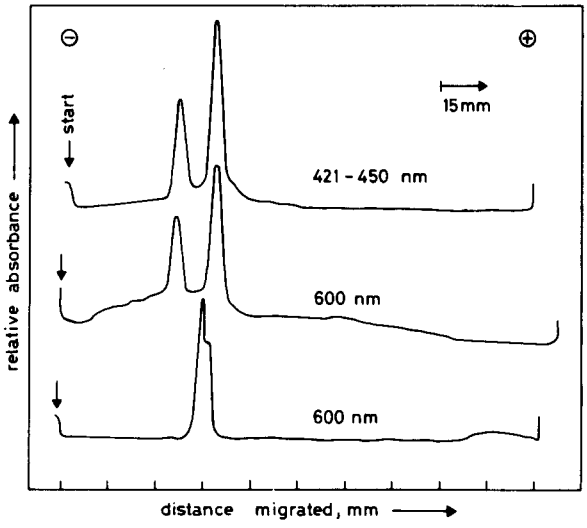

Fig. 6. Densitometric recordings of cytochrome *f*-553 bands after polyacrylamide electrophoresis in the absence (upper two traces) and presence (lower trace) of sodium dodecylsulfate. The upper trace shows a dual-wavelength densitometric scan at 421—450 nm, which records the cytochrome only. The middle trace shows the same gel after protein-staining with Coomassie Brillant Blue (RG 250); no additional bands are visible. The lower trace shows the same cytochrome sample after denaturation with SDS in the presence of dithiothreitol; after staining, two bands become visible with apparent molecular weights of 33 000 and 31 000.

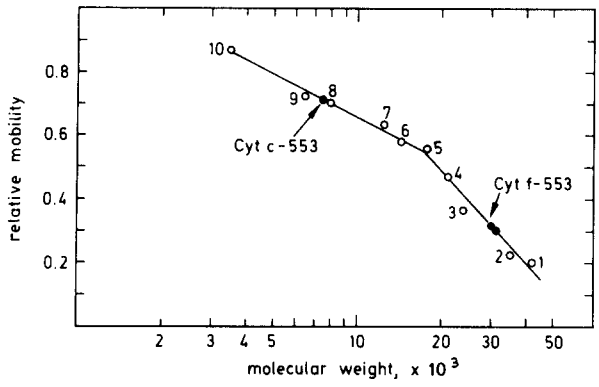

Fig. 7. Determination of molecular weight of cytochrome (Cyt) *c*-553 and cytochrome *f*-553 by polyacrylamide electrophoresis in the presence of SDS in a 15% gel. The protein markers were: (1) ovalbumin (45 000); (2) pepsin (34 700); (3) chymotrypsinogen A (25 000); (4) trypsin inhibitor (soybean, 21 500); (5) myoglobin (17 800); (6) lysozyme (14 300); (7) cytochrome *c*-550 (horse-heart, 12 400); (8) cytochrome *c*-551 (*Pseudomonas viridis*, 8000); (9) aprotinin (6500); (10) glucagon (3500). Molecular weights of cytochrome *f*-553 (•) and of soluble plastidic cytochrome *c*-553 (•) from *Scenedesmus* are indicated in the figure.

at room temperature for 24 h did not change the specific SDS-gel pattern, whereas incubation in the presence of SDS (2%) resulted in the production of the low-molecular weight species, with 2—3 bands between 7500 and 8500. This suggests that the native protein is probably not digestible by the associated protease, but its denaturation by SDS might stimulate protease activity quite considerably and lead to the production of smaller fragments. Spectral examination of the low-molecular weight species showed that a heme group remained still bound to the protein. For comparison, we checked protease activity of pure cytochrome *f*-554 from spinach (prepared with the same extraction method) and (soluble) cytochrome *c*-553 from *Scenedesmus*. In both cases a protease activity was not measurable. It should be noted that the presence of low-molecular weight components has been occasionally mentioned for spinach cytochrome *f*, but no further data were provided [36,37].

Isoelectric focusing of cytochrome *c*-553 in thin-layer gels gave a single band with an isoelectric point at pH 3.7, measured directly in the focused cytochrome band. With cytochrome *f*-553 multiple bands were observed with isoelectric points ranging from 5.2 to 4.8. Multiple bands do not necessarily indicate a heterogenous preparation, since they may result from interaction with carrier ampholytes [38]. In addition, the proteolytic activity of an individual cytochrome-f preparation might complicate the picture. However, when the main band at pH 5.1 was eluted from the Sephadex gel and subjected again to SDS-gel electrophoresis, a single band with an apparent molecular weight of 33 000 was obtained.

Comparison of the protein concentration determined according to Lowry [26], Schaffner and Weissmann [27] and Bradford [28], with cytochrome *c* (horse-heart) as standard protein and heme concentration, using an ϵ of 29.5 ($mM^{-1} \cdot cm^{-1}$) at 553 nm resulted in three different values: 20 300, 14 300 and 12 200. This shows that both the protein-determination method and the standard protein used do not give reliable values in the case of cytochrome *f*.

Therefore, the weight of a desalted and lyophilized cytochrome *f* sample was determined. The sample was dissolved in buffer and the heme concentration measured spectroscopically. This method yielded a molecular weight of 39 000 per heme, which is in the range of other determinations.

The amino-acid composition of cytochrome *c*-553 is given in Table I. Comparison of the amino-acid content with other eukaryotic algae shows a close similarity in composition and number of amino-acid residues. Protein concentration determined by weighing of the lyophilized protein or according to Lowry et al. [26] with cytochrome *c* (horse-heart) as standard, gave quite similar values and a minimum molecular weight of 10 200, inluding the iron-porphyrin ring.

The data presented clearly show features distinguishing between membrane-bound and soluble *c*-type cytochromes mainly in the spectroscopic properties and molecular weight. The analysis of molecular weight, however, might be complicated by associated proteinase activities.

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References

1 Wood, P.M. (1977) Eur. J. Biochem. 72, 605—612
2 Lightbody, J.J. and Krogmann, D.W. (1967) Biochim. Biophys. Acta 131, 508—515
3 Elstner, E., Pistorius, E., Böger, P. and Trebst, A. (1968) Planta (Berl.) 79, 146—161
4 Anderson, M.M. and McCarty, R. (1969) Biochim. Biophys. Acta 189, 193—206
5 Wildner, F.G. and Hauska, G. (1974) Arch. Biochem. Biophys. 164, 127—135
6 Kunert, K.J. and Böger, P. (1975) Z. Naturforsch. 30c, 190—200
7 Kunert, K.J., Böhme, H. and Böger, P. (1976) Biochim. Biophys. Acta 449, 541—553
8 Böhme, H., Kunert, K.J. and Böger, P. (1978) Biochim. Biophys. Acta 501, 275—285
9 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370—379
10 Bohner, H. and Böger, P. (1978) FEBS Lett. 85, 337—339
11 Wood, P.M. (1978) Eur. J. Biochem. 87, 9—19
12 Yakushiji, E. (1972) Methods Enzymol. 23A, 327—334; Böger, P. and H. Böhme (1980) in Handbook of Biosolar Resources (Mitsui, A. and Black, C.C., eds.), CRC Press, W.-Palm Beach, in the press
13 Maurer, H.R. (1971) Disc Electrophoresis, 2nd ed. Walter de Gruyter, Berlin
14 Powls, R.J., Wong, J. and Bishop, N.I. (1969) Biochim. Biophys. Acta 180, 490—499
15 Bendall, D.S., Davenport, H.E. and Hill, R. (1971) Methods Enzymol. 23A, 327—344
16 Appleby, C.A. (1969) Biochim. Biophys. Acta 172, 88—105
17 Vernon, L.P. and Kamen, M.D. (1954) J. Biol. Chem. 211, 643—662
18 Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570—572
19 Laemmli, U.K. (1970) Nature (Lond.) 227, 680—685
20 Dunker, A.K. and Rueckert, R.R. (1969) J. Biol. Chem. 244, 5074—5080
21 Radola, B.J. (1973) Biochim. Biophys. Acta 295, 412—428
22 Böhme, H. and Cramer, W.A. (1973) Biochim. Biophys. Acta 325, 275—283
23 Andrews, P. (1964) Biochem. J. 91, 222—233
24 Chervenka, C.H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, p.49
25 Spackman, O.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206

26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Bradford, M.M. (1976) Anal. Biochem. 72, 248—254
28 Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502—514
29 Garewal, H.S. and Wassermann, A.R. (1972) Biochim. Biophys. Acta 275, 437—441
30 Good, N.E., Winget, G.D., Winter, W., Conolly, T.N., Izawa, S. and Singh, R.M.M. (1966) Biochemistry 5, 467—477
31 Dickerson, R.E. and Timkovich, R. (1975) The Enzymes (P.D. Boyer, ed.), Vol. 11, pp. 397—547, Academic Press, New York, NY
32 Ben-Hayyim, G. and Schejter, A. (1974) Eur. J. Biochem. 46, 569—573
33 Teale, F.W.J. (1959) Biochim. Biophys. Acta 35, 543
34 Singh, J. and Wasserman, A.R. (1971) J. Biol. Chem. 246, 3532—3541
35 Lach, H.J., Ruppel, H.G. and Böger, P. (1973) Z. Pflanzenphysiol. 70, 432—451
36 Davenport, H.E. (1972) Proc. 2nd Int. Congr. Photosynthesis (Forti, G. et al., eds.), pp. 1593—1601, Junk, The Hague
37 Schmidt, G.H., Radunz, A. and Menke, W. (1977) Z. Naturforsch. 32c, 271—280
38 Hare, D.L. Stimpson, D.I. and Cann, J.R. (1978) Arch. Biochem. Biophys. 187, 274—275
39 Pettrigrew, G.W. (1974) Biochem. J. 139, 449—459
40 Laycock, M.W. (1972) Can. J. Biochem. 50, 1311—1325