Biochimica et Biophysica Acta, 592 (1980) 528—535

BBA 47915

PURIFICATION AND CHARACTERIZATION OF CYTOCHROME *f*-556.5 FROM THE BLUE-GREEN ALGA *SPIRULINA PLATENSIS*

HERBERT BÖHME, BIRGIT PELZER and PETER BÖGER

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.)

(Received March 18th, 1980)

Key words: Cytochrome f-556.5; Blue-green alga; Photosynthesis; (Spirulina platensis)

Summary

The membrane-bound cytochrome *f*-556.5 from the blue-green alga *Spirulina platensis* was purified to apparent homogeneity. Most of its properties are comparable to cytochrome *f* isolated from higher plants and green algae. It is clearly distinguishable from soluble cytochrome *c*-554, also present in *Spirulina*, which probably replaces the function of plastocyanin in photosynthetic electron transport.

1. The reduced form of cytochrome *f* exhibits an asymmetrical α-band with a maximum at 556.5 nm, and a pronounced shoulder at 550 nm. The β-, γ and δ-bands coincide with those described for *Scenedesmus* cytochrome *f*-553, with maxima at 524 (532), 422, 331 and a protein peak at 276 nm. The maximum of ferricytochrome *f* is at 410.5 nm; there is no indication of a weak 695 nm band, described for soluble *c*-type cytochromes. The purest preparations had a δ/protein-peak ratio of 0.8; the γ/α ratio was 7.3. Formation of a pyridine hemochromogen with a maximum at 550 nm indicated a *c*-type cytochrome. The molar extinction coefficient at 556.5 nm is 30 200, the differential extinction coefficient 21 500.

2. The molecular weight determined by gel filtration or SDS-polyacrylamide gel electrophoresis is 33 000 and 34 000, respectively.

3. The redox properties differ from those described for other cytochromes *f* isolated from green algae and higher plants: the midpoint redox potential is significantly more negative (+318 mV, pH 7.0) and from pH 6 to 10 no pH dependence is observed.

4. The isoelectric point was determined at pH 3.95, which is more acidic as compared to other cytochromes *f*.

5. Comparison of the amino acid composition indicated a distant relationship to higher plant cytochrome *f* and a closer relationship to cytochrome *f* from green algae.

Introduction

Cytochrome *f*, a membrane-bound component of photosynthetic electron transport was first described by Hill and Scarisbrick in higher plants [1] and purified since then from several plants [2—8] and recently, from green algae [9,10]. Its biochemical and biophysical properties distinguish it clearly from so-called soluble, plastidic cytochromes *c*, also present in eukaryotic algae, which, however, replace the function of plastocyanin [10—15]. There is, on the other hand, little information about the function and properties of cytochrome *f* in prokaryotic algae. The present paper describes for the first time comprehensively the isolation, purification and properties of cytochrome *f*-556.5 from *Spirulina*. The bearing of its unusual spectroscopic properties on proposed schemes of photosynthetic electron transport of blue-green algae will be discussed.

Methods

The blue-green alga *Spirulina platensis* (Nordts.) Com., strain Leonhard/Wouters, was obtained in large quantities from Prof. C.J. Soeder, Institut für Biotechnologie at the Kernforschungsanlage Jülich. 200 g of wet algal cell paste was washed and suspended in 200 ml of 50 mM phosphate buffer, pH 7.2, and subjected to three freeze-thawing cycles. The broken cells were homogenized in 500 ml of the phosphate buffer, mixed in a Waring Blendor at full speed for 30 s, and centrifuged for 15 min at 50 000 × *g*. The blue-green pellet was used for isolation of cytochrome *f*, whilst the supernatant contained cytochrome *c*-554. Its properties have been already described [16]. The pellet was then subjected to two washes with phosphate buffer and one wash with phosphate buffer (50 mM) containing 0.4 NaCl to remove soluble proteins. Chlorophyll was determined according to Arnon [17], and adjusted to 0.4 mg/ml. This suspension could be stored in the frozen state at −25°C. The extraction of cytochrome *f*-556.5 by organic solvents followed exactly the procedure described for Scenedesmus [10]. The yield after this first step was approx. 1.5 nmol cytochrome *f*/mg chlorophyll with an absorbance ratio (A_{331nm}/A_{276nm}) of 0.3. Further purification was achieved by repeated gel chromatography (Sephacryl-G 200) which could be combined with hydroxyapatite chromatography (Bio-Gel HTP). The Sephacryl-G 200 columns (90 × 2.5 cm) were equilibrated with 50 mM Tris-HCl (pH 8.0)/0.4 M NaCl, the hydroxyapatite column (1 × 5 cm) with 20 mM phosphate buffer, pH 7.2. The cytochrome was eluted by 45 mM phosphate buffer and could be recovered completely.

Polyacrylamide electrophoresis (15% gel [18]) yielded one red band measured densitometrically at 422—450 nm, dual-wavelength mode (Shimadzu UV 300, gel scan accessory). 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 (cf. Fig. 4). A partial purification of cytochrome *f* from *Anabaena flos-aquae* has been recently described, but no further data were given [19].

All other physical and chemical measurements have been described in a previous paper [10].

The following standard proteins were used for molecular weight determination by gel filtration (Sephadex-G 75, Pharmacia Calibration kit): ribonuclease (molecular weight of 13 000), chymotrypsinogen (25 000), ovalbumin (43 000), bovine serum albumin (67 000). For calibration of the SDS-polyacrylamide gels (15%) the following standard proteins were used (Sigma, Boehringer): horse heart cytochrome *c* (12 500), lysozyme (14 300), myoglobin (17 800), trypsin inhibitor (soybean, 21 500), chymotrypsinogen A (25 000), trypsin inhibitor (hen egg, 28 000), pepsin (34 700) ovalbumin (45 000).

Room- and low-temperature spectra were run on an Aminco DW 2 spectrophotometer or a Shimadzu UV 300. Fourth derivative spectra [20] were recorded by a single-beam spectrophotometer on line with a small computer (Nicolet, Mod. 1170) constructed by Dr. W. Schmidt of this faculty; half-bandwidth and $\Delta\lambda$ was 1 nm. Wavelengths were calibrated with a krypton spectral lamp (Oriel).

Results and Discussion

Absorption spectra The absorption spectrum of the reduced and oxidized form of cytochrome *f*-556.5 at room temperature is shown in Fig. 1. The reduced cytochrome exhibits the typical three absorption bands in the visible at 556.5 (α), 524 (β), and 422 (γ) nm, and two bands in the ultraviolet region of the spectrum, at 331 (δ) and 276 nm (protein). There are shoulders at 550, 532, 514, 508, 400, 365 and 283 nm. A spectral characteristic distinctive to other cytochromes *f* is the pronounced shoulder at 550 nm, resulting from a red-shifted α-peak at 556.5 nm. An α-peak at 556—557 nm has also been observed with partially purified *f*-type cytochrome from *Anacystis nidulans* [11], *Nostoc muscorum* [21], and *Anabaena variabilis* (Böhme, H., unpublished results). The γ/α absorbance ratio is about 7.3; a δ/protein-peak ratio of 0.8 indicates an essentially pure protein. The oxidized form can be obtained by treatment with excess (50-fold) of ferricyanide and removal of excess oxidant

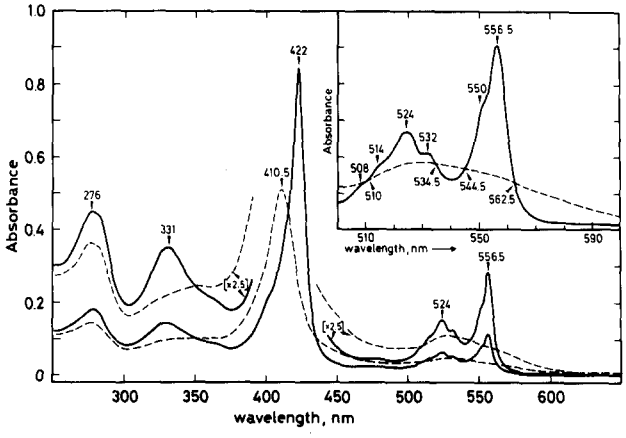

Fig. 1. Absorption spectrum of plastidic membrane-bound cytochrome *f*-556.5 from *Spirulina platensis*. Reduced (———), oxidized (- - - - -) forms; upper trace is a 2.5-fold scale expansion. Insert shows the α/β region enlarged; isosbestic points are indicated.

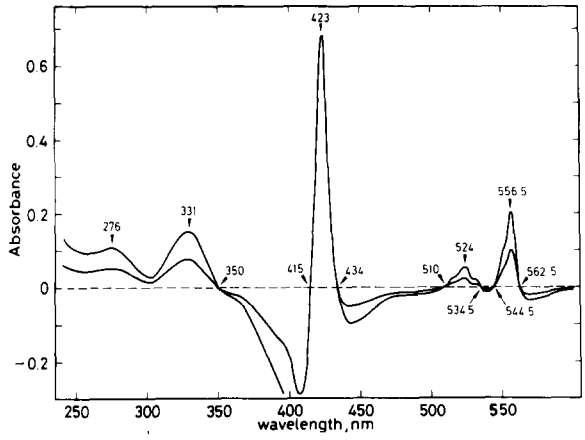

Fig. 2. Difference absorption spectrum (reduced minus oxidized) of cytochrome *f*-556.5. The sample was oxidized by ferricyanide and excess oxidant removed by gel filtration, recorded, then reduced by borohydride and recorded again; a 2-fold scale expansion was used to demonstrate isosbestic points.

by gel filtration (Sephadex G 25, coarse, equilibrated with 20 mM phosphate buffer, pH 7.2 and 80 mM NaCl). The maximum of the oxidized form is at 410.5 nm, whereas the α/β-region and the 331 nm band is replaced by a more diffuse spectrum. A 695 nm band ascribed to a methionine residue as sixth ligand of the heme iron in soluble *c*-type cytochrome is absent in the ferricytochrome *f* spectrum [22,23]. The difference spectrum (Fig. 2) reduced minus oxidized, shows isosbestic points at 562.5, 544.5, 534.5, 510, 434, 415 and 350 nm; the maximum of the γ-peak shifts to 423 nm. The reduced minus oxidized low-temperature spectrum (77 K) exhibits a fine structure comparable to other *f*-cytochromes with bands at 555, 549, 544, 538, 531, 525.5, 523, 520, 515.5, 513 and 507.5 nm (Fig. 3A). A similar resolution of bands of

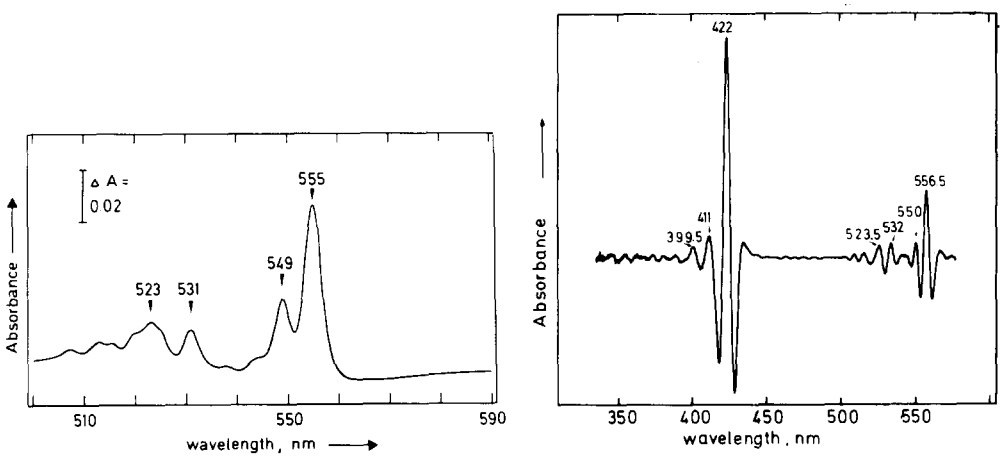

Fig. 3. A. Low-temperature (77 K) difference absorption spectrum of cytochrome *f*-556.5; half-bandwidth 0.7 nm. B. Fourth-derivative spectrum of reduced cytochrome *f*-556.5, half-bandwidth and $\Delta\lambda$ = 1 nm.

reduced cytochrome *c*-556.5 is obtained by a fourth-derivative spectrum at room temperature: 556.5, 550, 540, 532, 523.5, 514, 507.5, 422, 411 and 399.5 nm (Fig. 3B). Due to the better resolution, higher derivative and low-temperature spectra have been used to analyze complex mixtures of absorbance bands in biological material [20], e.g., cytochromes in thylakoid membranes.

The α-band of the pyridine hemochromogen is at 550 nm, indicative of a *c*-type cytochrome. The extinction coefficients for the α- and β-band were $\epsilon_{556.5} = 30.2$, and $\epsilon_{524} = 16$ ($mM^{-1} \cdot cm^{-1}$), respectively; the differential extinction coefficient was $\Delta\epsilon_\alpha = 21.5$ ($mM^{-1} \cdot cm^{-1}$).

Molecular weight. Gel filtration (Sephadex G-75) of cytochrome *f*-556.5 revealed a single symmetrical peak with an estimated molecular weight at 33 200 (average of three different runs). Polyacrylamide electrophoresis (15% gel) in the absence of SDS gave a single band moving close to the Bromophenol Blue marker front. This indicates a highly charged protein, and is generally characteristic of soluble plastidic *c*-type cytochromes with a molecular weight close to 10 000 (Fig. 4). In the presence of SDS and dithiothreitol a molecular weight of 34 000 was obtained (average of five different runs, with the protein molecular weight markers described in Methods). A molecular weight of 27 000—33 000 is generally indicative of monomeric cytochrome *f* [6—10].

Isoelectric focusing of cytochrome *f*-556.5 in thin-layer gels (Sephadex G-75, superfine), containing 2% ampholine (pH 3—5) gave a single band with a isoelectric point at 3.95 pH. This value is about 1—1.5 pH units more acidic than that reported for cytochrome *f* from *Sinapis arvensis* [7] and *Scenedesmus acutus* [10].

The midpoint redox potential (E_m) was determined by plotting the absorbance change (556.6—562.5 nm, dual wavelength mode) of the α-band versus the

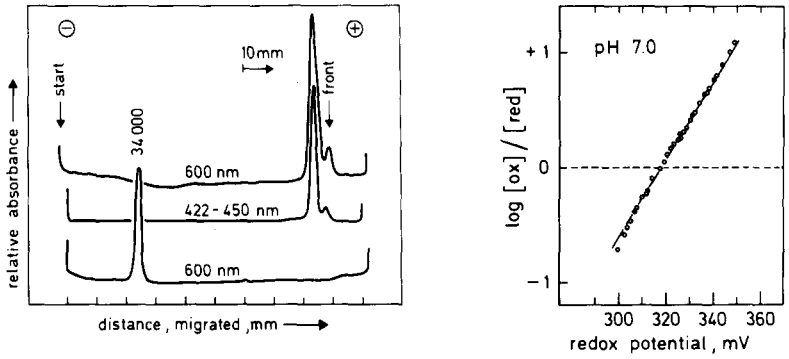

Fig. 4. Densitometric recordings of cytochrome *f*-556.5 bands after polyacrylamide electrophoresis in the absence (upper two traces) and presence of sodium dodecyl sulfate and dithiothreitol (lower trace). The middle trace shows a dual wavelength scan at 422—450 nm, which records the cytochrome only; after staining with Coomassie Brilliant blue no additional band becomes visible (upper trace). The lower trace shows the same cytochrome sample after denaturation with sodium dodecyl sulfate; the apparent molecular weight is 34 000.

Fig. 5. Redox titration curve of cytochrome *f*-556.5 at pH 7.0 and 20°C. The buffer was 0.2 M morpholinopropane sulfonic acid/NaOH, pH 7.0; wavelength pair used was 556.5—562.5 nm; •, oxidation by ferricyanide; ○, reduction by sodium ascorbate.

TABLE I

AMINO ACID COMPOSITION OF PLASTIDIC, MEMBRANE-BOUND CYTOCHROME *f*-556.5 (*SPIRULINA*) AND CYTOCHROME *f*-553 (*SCENEDESMUS*) AS COMPARED TO CYTOCHROMES *f* OF HIGHER PLANTS

Amino acid residue residue	*Spirulina platensis* cyt *f*-556.5	*Scenedesmus acutus* cyt *f*-553	*Raphanus sativus* cyt *f*-554 (Ref. 8)	*Sinapis arvensis* cyt *f*-554 (Ref. 7)
Asx	29	37	40	27
Thr	20	11	12	8
Ser	14	12	12	11
Glx	41	34	35	30
Pro	30	30	27	24
Gly	33	28	29	24
Ala	25	18	19	22
Val	47	32	20	16
Met	1	3	2	1
Ile	18	18	18	17
Leu	18	18	22	19
Tyr	12	11	11	8
Phe	9	10	10	7
Lys	11	24	22	19
His	3	2	3	4
Trp	—	—	1	2
Arg	8	7	13	7
Cys	2	2	3	2
Number of residues	321	297	299	248
Calculated molecular weight *	34 443	32 740	33 366	27 340

* Includes the heme prosthetic group.

redox potential of the solution measured simultaneously with a platinum-calomel combination electrode. An E_m value of +318 ± 4 mV at pH 7.0 was obtained, which is considerably more negative than those reported for other cytochromes *f* (Fig. 5) [6,7,10]. The generally observed pH-dependency (−60 mV/pH unit) at alkaline pH [3,10] is not observed with cytochrome *f* from *Spirulina*. We determined values of 312, 310, 304 and 300 (±4) mV at pH 6.0, 8.0, 9.0 and 10.0, respectively.

The amino acid analysis of *Spirulina* cytochrome *f*-556.5 is shown in Table I and compared to cytochrome *f* from other sources. (Also included is the amino acid analysis of *Scenedesmus* cytochrome *f*, which has not been published so far.) The degree of similarity of *f*-cytochromes was determined by statistical comparison of their amino acid composition [24]. The sum of the squared differences ($S\Delta Q$) was taken as estimate of protein relatedness (Table II). It was shown previously that unrelated proteins differ by more than 100 $S\Delta Q$ units [24]. The data of Table II indicate that cytochrome *f* and cytochrome *c* from the same organism (*Spirulina* or *Scenedesmus*, respectively) are totally unrelated, as expected. For *f*-type cytochromes from various sources a sequence of relationship can be found in the following order: blue-green algae → green algae → higher plants. Among cytochromes *f* from higher plants $S\Delta Q$ values of 35 and below indicated a very close similarity [7].

TABLE II

COMPARISON OF THE AMINO ACID COMPOSITION BY $S\Delta Q$ VALUES OF *f*- AND *c*-TYPE CYTOCHROMES ACCORDING TO REF. 24

Cytochrome	*Scenedesmus* cyt *f*-553	*Raphanus* cyt *f*-554 Ref. 8	*Spirulina* cyt *c*-554 Ref. 16	*Scenedesmus* cyt *c*-553 Ref. 10
Spirulina cyt *f*-556.5	59.0	133.7	276.9	288.4
Scenedesmus cyt *f*-553	—	42.1	263.0	218.0
Spirulina cyt *c*-554	—	—	—	56.2

Comparison of the polarity of (membrane-bound) cytochrome *f* and (soluble) cytochrome *c*, both isolated from *Spirulina*, by summing up the mole fractions of polar amino acids [25], yielded values of 39.2 and 41.8%, respectively. Although cytochrome *c* appears to be more polar than cytochrome *f*, both proteins are not particularly hydrophobic as a whole. With membrane proteins, which require detergents of organic solvents for extraction, values below 40% were generally obtained [25].

The data presented clearly show common features between isolated cytochromes *f* from higher plants, green and blue-green algae, concerning biochemical and biophysical properties. The red-shifted α-band of cytochrome *f* from blue green algae at 556—557 nm, however, necessitates a reinterpretation of light-induced absorbance changes measured with thylakoid membrane fragments. Data on redox reactions of a component absorbing at 557—558 nm and at 549—550 nm as reported [26,27] can be clearly assigned now to cytochrome *f* alone rather than to cytochrome *b*-559 and cytochrome *c*-549. The interpretation of light-induced redox reactions might be further complicated by the presence of soluble cytochrome *c*-554 which, however, replaces the function of plastocyanin in several blue-green algae (e.g. *N. muscorum*, *S. platensis* [16, 21]).

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft through its Sonderforschungsbereich 138. Our thanks are expressed to Margarete Stengel (laboratory of Professor C.J. Soeder) for growing the algal material. We also wish to thank Dr. I. Rasched and Mrs. M. Nickel for performing the amino acid analysis and excellent cooperation. The fourth-derivative spectra were measured by Dr. W. Schmidt of this faculty, to whom we also wish to express our thanks.

References

1 Hill, R. and Scarisbrick, R. (1951) New Phytol. 50, 98—111
2 Davenport, H.E. and Hill, R. (1952) Proc. R. Soc. Lond. Ser. B Biol. Sci. 139, 327—345
3 Bendall, D.S., Davenport, H.E. and Hill, R. (1971) Methods Enzymol. 23A, 327—344
4 Singh, I. and Wasserman, A.R. (1971) J. Biol. Chem. 246, 3532—3541
5 Nelson, N. and Racker, E. (1972) J. Biol. Chem. 247, 3848—3853
6 Matsuzaki, E., Kamimura, Y., Yamasaki, T. and Yakushiji, E. (1975) Plant Cell Physiol. 16, 237—246

7 Gray, I.C. (1978) Eur. J. Biochem. 82, 133—141
8 Tanaka, K., Takahashi, M. and Asada, K. (1978) J. Biol. Chem. 253, 7397—7403
9 Kamimura, Y. and Matsuzaki, E. (1978) Plant Cell Physiol. 19, 1175—1183
10 Böhme, H., Brütsch, S., Weithmann, G. and Böger, P. (1980) Biochim. Biophys. Acta 590, 248—260
11 Wood, P.M. (1977) Eur. K. Biochem. 72, 605—612
12 Böhme, H., Kunert, K.J. and Böger, P. (1978) Biochim. Biophys. Acta 501, 275—285
13 Wood, P.M. (1978) Eur. J. Biochem. 87, 9—19
14 Bohner, H. and Böger, P. (1978) FEBS Lett. 85, 337—339
15 Sandmann, G. and Böger, P. (1980) Plant Sci. Lett. 17, 417—428
16 Yamanaka, T., Fukumori, Y. and Wada, K. (1978) Plant Cell Physiol. 19, 117—126
17 Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wada, K. (1974) 357, 231—245
18 Maurer, H.R. (1971) Disc Electrophoresis, 2nd edn., Walter de Gruyter, Berlin
19 Kwok Ki Ho, Ulrich, E.L., Krogmann, D.W. and Gomez-Lojero, C. (1979) Biochim. Biophys. Acta 545, 236—248
20 Butler, W.L. and Hopkins, D.W. (1970) Photochem. Photobiol. 12, 439—450
21 Almon, H. and Böhme, H. (1980) Biochim. Biophys. Acta, in the press
22 Dickerson, R.E. and Timkovich, R. (1975) The Enzymes (Boyer, P.D., ed.), Vol. 11, pp. 397—547, Academic Press, New York
23 Böger, P. and Böhme, H. (1980) CRC Handbook of Biosolar Resources (Mitsui, A. and Black, C.D., eds.), Vol. 1, in the press
24 Marchalonis, I.I. and Weltman, I.K. (1971) Comp. Biochem. Physiol. 38B, 609—625
25 Capaldi, R.A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 930—932
26 Aparicio, P.J., Ando, K. and Arnon, D.I. (1974) Biochim. Biophys. Acta 357, 246—251
27 Tsujimoto, H.Y., McSwain, B.D., Hiyama, T. and Arnon, D.I. (1976) Biochim. Biophys. Acta 423, 303—312