Purification and Characterization of Cytochrome c_6 from the Unicellular Green Alga *Scenedesmus obliquus*

Röbbe Wünschiers[a], Thomas Zinn[a], Dietmar Linder[b] and Rüdiger Schulz[a]

[a] Fachbereich Biologie/Botanik, Philipps-Universität, Karl-von-Frisch-Str., D-35032 Marburg, Germany
[b] Biochemisches Institut, Justus-Liebig-Universität, Friedrichstr. 24, D-35392 Giessen, Germany

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Purification of a soluble cytochrome c_6 from the unicellular green alga *Scenedesmus obliquus* by a simple and rapid method is described. The purification procedure includes ammonium sulfate precipitation and non-denaturating PAGE. The N-terminal sequence of the first 20 amino acids was determined and shows 85% similarity and 75% identity to the sequence of cytochrome c_6 from the green alga *Monoraphidium braunii*. The ferrocytochrome shows typical UV/VIS absorption peaks at 552.9, 521.9 and 415.7 nm. The apparent molecular mass was estimated to be 12 kDa by SDS-PAGE. EPR-spectroscopy at 20K shows resonances indicative for two distinct low-spin heme forms.

Introduction

Cytochromes are well known heme proteins catalyzing one-electron transfer reactions. In cytochromes *c* the *c*-type heme is covalently bound to the protein through thioether linkages to cysteine residues. During redox-reactions the central iron changes between the Fe(II)- and Fe(III)-oxidation status.

In the photosynthetic electron transport chain of higher plants plastocyanin mediates the electron transfer between the cytochrome b_6f complex and photosystem I. In some eukaryotic algae and cyanobacteria, however, the copper containing plastocyanin is substituted by cytochrome c_6 (Davis *et al.*, 1980; Hervás *et al.*, 1992). While in some cyanobacteria cytochrome c_6 is the only electron mediator between the cytochrome b_6f complex and photosystem I, in other species and in many algae plastocyanin and cytochrome c_6 are interchangeable (Wood, 1978; Hervás *et al.*, 1995). Depending on the availability of copper, either plastocyanin (*pet*E gene) or cytochrome c_6 (*pet*J gene) is synthesized (Ho and Krogmann, 1984; Sandmann, 1986). In most organisms the regulation is controlled at the transcriptional level (Bovy *et al.*, 1992). In the green algae *Chlamydomonas reinhardtii* it was shown, however, that the synthesis of plastocyanin is controlled post-translationally, whereas cytochrome c_6 synthesis is regulated transcriptionally (Hill and Merchant, 1992).

In general cytochrome c_6 (formerly named soluble cytochrome *f* or cytochrome c_{553}) is a soluble protein located in the thylakoid lumen (Medina *et al.*, 1997). At present, the amino acid sequences of 15 prokaryotic and 11 eukaryotic cytochromes c_6 are known and the crystal structures of cytochrome c_6 from *Chlamydomonas reinhardtii* (Kerfeld *et al.*, 1995) and *Monoraphidium braunii* (Frazão *et al.*, 1995) are published at 1.9 Å- and 1.2 Å-resolution, respectively. They contain a single heme group which is covalently bound to two cysteine residues with the characteristic motif Cys-X-Y-Cys-His (Moore and Pettigrew, 1990; Campos *et al.*, 1993; Kerfeld *et al.*, 1995). The central iron atom is usually in the low-spin state. The protein consists of 83–89 amino acids, has a molecular mass of about 10 kDa and a redox potential of about +350 mV (Cohn *et al.*, 1989; Moore and Pettigrew, 1990; Campos *et al.*, 1993). Thus it belongs to the group of high potential cytochromes.

During the first purification steps of the hydrogenase from *Scenedesmus obliquus* (Schnackenberg *et al.*, 1993) cytochrome c_6 was found in hydrogenase active fractions. Thus cytochrome c_6 might be structurally and/or functionally coupled

Reprint requests to Dr. Schulz.
Tel.: +49-6421-282484, Fax: +49-6421-282057.

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to the hydrogenase. To learn more about cytochrome c_6 from *Scenedesmus* the protein was purified and further investigated as described in this work.

Materials and Methods

Organism and growth conditions

Cells of the wild type of the green alga *Scenedesmus obliquus* strain D_3 (Gaffron, 1939) were grown as batch cultures heterotrophically in a controlled environment shaker at 30 °C in darkness for 3 days. Cells were inoculated in 2 liter Erlenmeyer flasks filled with 750 ml of a basal nitrate-medium (Bishop and Senger, 1971) supplemented with glucose (0.5% (w/v)) and yeast extract (0.1% (w/v)).

Isolation of cytochrome c_6

In a typical purification procedure 9 liter of *Scenedesmus obliquus* batch cultures were harvested by centrifugation at 1,500 x *g* at the end of their logarithmic growth phase. The cells (about 100 g wet weight) were washed twice with phosphate buffer (50 mM, pH 7.5) and adjusted to 500 µl/ml packed cell volume with the same buffer. The concentrated cells were mixed with about 250 g glass beads (0.7 mm diameter) and disrupted in a vibrogen cell mill (Bühler, Tübingen, Germany) for 10 min at 4 °C (Senger and Mell, 1976). The glass beads were filtered off. The filtrate (about 240 ml and 17 mg/ml protein) was centrifuged at 300,000 x *g* for 1 h at 4 °C. The supernatant containing the soluble proteins (about 200 ml and 10 mg/ml protein) was then adjusted to an ammonium sulfate concentration of 50% (w/v) saturation and centrifuged for 10 min at 48,000 x *g* and 4 °C. The pellet was discarded and the supernatant (about 220 ml and 2 mg/ml protein) adjusted to 90% (w/v) saturation in ammonium sulfate. After centrifugation (see above) the supernatant was discarded and the pellet resuspended in 220 ml buffer (about 1 mg/ml protein). To concentrate the obtained solution it was ultrafiltrated with an Amicon pressure dialysis cell (Amicon GmbH, Witte/Ruhr, Germany) fitted with a 10 kDa Omega membrane (Filtron GmbH, Karlstein, Germany). The concentrated solution (about 14 ml and 16 mg/ml protein) was then applied onto a non-denaturing preperative 15% polyacrylamide column 40 x 2 cm (125 ml) in size. Electrophoresis was carried out as described by Davis (1964). After electrophoresis the orange colored cytochrome band was cut out and eluted from the gel using a BioTrap electroelution chamber (Schleicher & Schüll, Dassel, Germany) at 200 V constant voltage. The obtained fraction (about 1 ml and 720 µg/ml protein) was homogenous in cytochrome c_6 as shown by SDS-PAGE according to Fling and Gregerson (1986) and subsequent silver staining (Blum *et al.*, 1987).

Quantitative determinations

Packed cell volume (PCV) was determined by centrifugation of aliquots of the cell suspension in hematocrit tubes for 5 min at 1,400 x *g*. Protein was quantified by the method of Bradford (1979), using bovine serum albumin as a standard. Protein molecular masses were determined by 15% SDS-PAGE using low molecular weight standards (Pharmacia, Freiburg, Germany) (Weber and Osborn, 1969). Proteins were visualized by Coomassie R-250 staining (Chua and Bennoun, 1975). The concentration of cytochrome c_6 was determined spectroscopically using the molar extinction coefficient of $\varepsilon_{552.5} = 25.27\ \text{mM}^{-1}\ \text{cm}^{-1}$ established for cytochrome c_6 from *Monoraphidium braunii* (Campos *et al.*, 1993).

Amino acid sequencing

Prior to sequencing proteins were separated by SDS-PAGE on 15% acrylamide gels (10 µg protein content per lane) and subsequently transferred onto Immobilon PVDF membranes (Millipore, Eschborn, Germany). After staining with Coomassie blue R-250 cytochrome c_6 was cut off the membrane and directly used for N-terminal sequencing. Automated Edman sequencing was performed with an Applied Biosystems (Foster City, CA, USA) pulsed-liquid-phase sequencer (model 471A).

UV/VIS absorption spectra

Spectra were recorded at room temperature using a Shimadzu MPS-2000 two beam spectrophotometer (Shimadzu-Europa, Duisburg, Germany). For redox experiments the samples con-

tained 200 µM cytochrome c_6 dissolved 1:200 in phosphate buffer (50 mM, pH 7.5). Oxidation was achieved by addition of ammonium persulfate, reduction by adding Na-dithionite.

EPR spectroscopy

EPR spectra were recorded with an ESP 300E spectrometer equipped with an x-band microwave bridge ER 041 MR and a rectangular cavity. Cooling of the sample was performed by using a continuous-flow helium cryostate ESR 900. All EPR equipment used was build by Bruker, Oxford, England. Oxidation of the sample was achieved by ammonium persulfate. After oxidation the samples were frozen in liquid nitrogen in an EPR-cuvette (707SQ, Spintech, Remshalden).

Chemicals

All chemicals were of analytical reagent grade and purchased from Merck (Darmstadt, Germany) or Sigma (München, Germany) if not mentioned.

Results and Discussion

Purification of cytochrome c_6

For purification of the soluble cytochrome c_6 from *Scenedesmus obliquus* we established a purification procedure based on two subsequent ammonium sulfate precipitations and non-denaturating PAGE. The cytochrome precipitated at 90% ammonium sulfate saturation as an orange colored solid in the second precipitation step. The pellet was redissolved and concentrated by ultrafiltration. Subsequently the cytochrome was separated by preparative non-denaturating PAGE (Fig. 1A). The intensively orange colored band at a R_f of 0.47 was cut out of the gel. Analysis of the electroeluted proteins by SDS-PAGE revealed a single band with an apparent molecular mass of about 12 kDa (Fig. 1B). Starting with 100 g (wet weight) of *Scenedesmus obliquus* cells about 720 µg of cytochrome c_6 could be isolated from the alga. The purified protein was apparently homogeneous and thus suitable for N-terminal sequencing and spectroscopic characterization.

N-terminal amino acid sequences

From the newly isolated cytochrome 20 amino acids of its N-terminus could be determined. An alignment with the known sequences of other cytochromes c_6 of eukaryotic organisms is shown (Fig. 2). At positions 15 and 18 no phenylthiohydantoin derivates could be identified. From the amino acid alignment with different cytochrome c_6 sequences cysteines are suggested for this positions (Fig. 2). Within the sequenced N-terminus the typical heme binding site motiv -CXXCH-

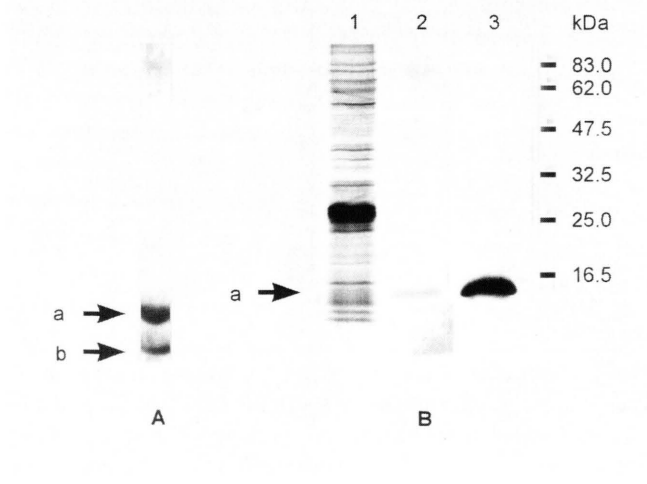

Fig. 1. **A)** Non-denaturating PAGE of the resuspended pellet of the 90%-saturation ammonium sulfate precipitate. The predominant colored bands are a) cytochrome c_6 and b) ferredoxin. **B)** SDS-PAGE of the resuspended pellet from the 90%-saturation ammonium sulfate precipitate (lane 1, Coomassie stained), the extracted cytochrome c_6 band from the non-denaturating PAGE (lane 2, Coomassie stained). In lane 3 the double quantity (10 µg) was administered and silver staining applied. a) marks the cytochrome c_6 bands.

Organism	1 20	%id/%si	Ref.
Euglena gracilis:	G-----GADV FADNCSTCHV	30/35	a
Euglena viridis:	S-----GAEV FGNNCSSCHV	35/40	b
Porphyra purpurea:	-ADLDNGEKV FSANCAACHA	65/65	c
Porphyra tenera:	-ADLDNGEKV FSANCAACHA	65/65	d
Monochrysis lutheri:	-GDIANGEQV FTGNCAACHS	55/70	e
Bumilleriopsis filiformis:	-ADIENGERI FTANCAACHA	60/65	f
Alaria esculenta:	-IDINNGENI FTANCSACHA	50/65	g
Petalonia fascia:	-VDINNGESV FTANCSACHA	50/65	h
Chlamydomonas reinhardtii:	-ADLALGAQV FNGNCAACHM	70/75	i
Monoraphidium braunii:	EADLALGKAV FDGNCAACHA	75/85	j
Bryopsis maxima:	GGDLEIGADV FTGNCAACHA	55/70	k
Scenedesmus obliquus:	SADLALGKQT FEANCAACHA	100/100	This work

Fig. 2. Alignment of the 20 N-terminal amino acids of all sequenced eukaryotic cytochromes c_6. Bold letters indicate similarity. Identical amino acids are connected by vertical lines. The heme binding cysteines and the heme iron axial ligating histidine are underlined. %id = percent identity, %si = percent similarity of the amino acids with respect to the sequence of *Scenedesmus obliquus*, as determined in this work. a: (Pettigrew, 1974), b: (Ambler *et al.*, 1991), c: (Reith and Munholland, 1995), d: (Ambler and Bartsch, 1975), e: (Laycock, 1972), f: (Ambler, R. P., unpublished results, cited by: Dickerson, 1980), g: (Laycock, 1975), h: (Sugimura *et al.*, 1981), i: (Merchant and Bogorad, 1987), j: (Campos *et al.*, 1993), k: (Okamoto *et al.*, 1987).

(Ambler, 1991) was found, suggesting that Cys15 and Cys18 establish thioether linkages to the heme. Hence, His19 is certainly one of the heme iron axial ligands. According to this arrangement it is suggested that the cytochrome c_6 from *Scenedesmus obliquus* belongs to the class I cytochromes *c* (Ambler, 1980). The derived sequence displays a very high similarity of 85% to the sequence of cytochrome c_6 from *Monoraphidium braunii* (Campos *et al.*, 1993).

UV/VIS spectroscopy of cytochrome c_6

The isolated, purified cytochrome was dissolved in phosphate buffer (50 mM, pH 7.5) and UV/VIS spectra were recorded. A typical absorption spectrum is shown (Fig. 3). In the as-purified status the absorption maxima were located at 552.9 nm (α), 521.9 nm (β) and 415.7 nm (γ or Soret band), 316.3 nm (σ) and 274.0 nm (protein). The ratio of the absorbances, Aγ/Aα, is 7.4. No changes of the absorption spectrum were observed on the addition of ascorbate or Na-dithionite. Therefore, it is obvious that the cytochrome was isolated in its reduced state (ferrocytochrome). Oxidation of the cytochrome (to ferricytochrome) by ammonium persulfate results in the replacement of the absorption maxima at 552.9 nm and 521.9 nm by a broad maximum at 526 nm, whereas the Soret band is shifted to 411.3 nm. The 316.3 nm-absorption maximum disappeared upon oxidation; instead a shoulder at 357 nm developed. Upon rereduction of the ferricytochrome by ascorbic acid the typical absorption spectrum of the ferrocytochrome reappeard, indicating that the redox potential is higher than 60 mV. Thus it can be con-

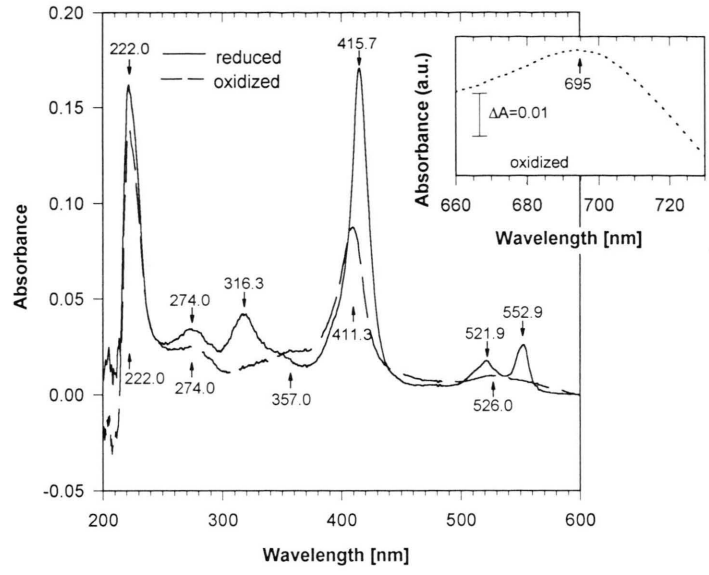

Fig. 3. UV/VIS spectrum of the purified cytochrome c_6 from *Scenedesmus obliquus*. The solid line shows the spectrum of the reduced state and the dashed line the cytochrome after oxidation with ammonium persulfate. The inset shows the 695 nm absorption peak of the oxidized protein at a concentration of 1 mM.

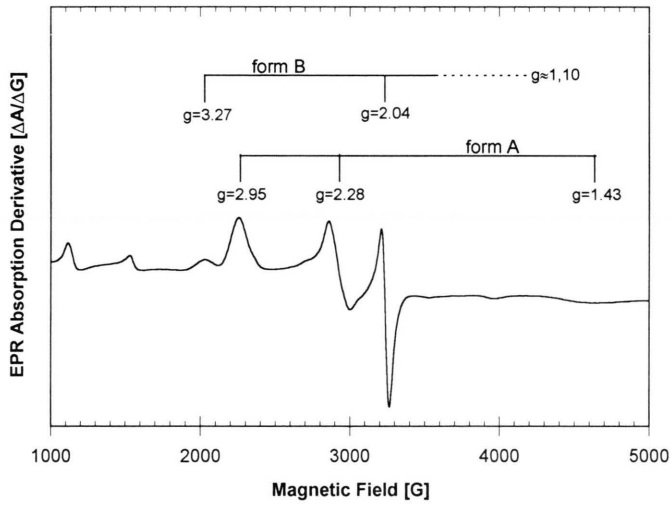

Fig. 4. EPR spectrum of the purified cytochrome c_6 from *Scenedesmus obliquus*. The spectrum shows resonances indicative for two distinct heme species: form A (bis-histidine) and form B (methionine-histidine), respectively. Measurements were performed at pH 5.5 and 20 K. The microwave power was 2.52 mW at 9.254 MHz microwave frequency, 100 kHz modulation frequency and 10 G modulation amplitude.

cluded that the cytochrome under investigation is of the high potential type.

At high concentrations (1 mM) the ferricytochrome displays a broad absorption peak at 695 nm (Fig. 3, inset). This feature is indicative of an axial heme iron coordination with the thioether group of a methionine (Schejta and George, 1964; Gupta and Koenig, 1971). These findings are in good agreement to previous spectroscopic measurements performed with purified cytochrome c_{553} from *Scenedesmus acutus* (= *S. obliquus* (Turp.), Kütz, strain 276.3a, Algal Culture Collection, Göttingen) by Böhme *et al.* (1980).

EPR spectroscopy

A typical X-band EPR spectrum of ferricytochrome c_6 at 20 K is shown (Fig. 4). The spectrum was measured at pH 5.5 which is near to the pH value of the chloroplast lumen. The spectrum exhibits the typical rhombic features of a low-spin heme (Fig. 4). However, it also shows resonances indicative for two distinct heme species, A and B. Species A reveals three well resolved g values at $g_x = 1.43$, $g_y = 2.28$ and $g_z = 2.95$. For species B only $g_y = 2.04$ and $g_z = 3.27$ could be identified. g_x was calculated to be ≈ 1.10 by assuming that

$\Sigma g_i^2 \approx 16$ (Taylor, 1977). The observed resonance at $g = 5.74$ is probably due to some high-spin heme and the feature at $g = 4.23$ can most probably be attributed to exogenous Fe^{2+}. Both signals were found in all samples, but with slightly varying intensities. This effect might be due to the sample preparation.

Using the TAYLOR formalism (Taylor, 1977) the crystal field parameters, tetragonal distortion Δ and rhombic distortion V, were calculated for both, species A and B (Table I). The parameters calculated for A suggest a bis-histidine heme iron axial ligation, whereas the crystal field parameters of species B are within a range typical for a methionine-histidine ligation. A similar situation could be observed for cytochrome c_6 of *Monoraphidium braunii* (Campos *et al.*, 1993) and that of *Anabaena* PCC 7119 (Medina *et al.*, 1997). However, by ^{1}H-NMR-measurements no evidence for a bis-histidine heme iron axial ligation was reported in both publications. Campos *et al.* (1993) concluded from their experiments that either the structural differences between the two species are not detectable by ^{1}H-NMR at room temperature or are only observable at the low temperature used in EPR-spectroscopy (20 K). From the obtained UV/VIS and EPR spectroscopic results we suggest a predominating methionine-histidine ligation, at least at room temperarure.

Table I. g Values (g_x, g_y, g_z) and crystal field parameters (planar distortion V/λ, axial distortion Δ/λ, rhombicity V/Δ) of the oxidized *Scenedesmus obliquus* cytochrome c_6. Both data sets are presented for species A (bis-histidine) and B (methionine-histidine). Crystal field parameters were calculated using the TAYLOR-formalism (Taylor, 1977). (1) was calculated by assuming $\Sigma g_i^2 \approx 16$ (Taylor, 1977).

Species	g_x	g_y	g_z	V/λ	Δ/λ	V/Δ
A	1.43	2.28	2.95	1.77	2.86	0.62
B	1.10(1)	2.04	3.27	1.13	3.01	0.38

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