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Cytochrome c_6 isolated from the marine diatom *Thalassiosira weissflogii*

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Abstract

A soluble monoheme *c*-type cytochrome c_6 was isolated from the marine diatom *Thalassiosira weissflogii*. The isolated protein shows an apparent molecular weight of 13 kDa and an isoelectric point of 3.6 in the ferric form and 3.8 in the ferrous form. The visible spectrum of the reduced cytochrome c_6 is typical of a *c*-type heme protein, with maxima at 273, 416 (γ-peak) and 553 nm (α-peak). The cytochrome c_6 isolated from *T. weissflogii* contains phosphoserine in its sequence. No plastocyanin was detected in the soluble extracts and no cross-reactivity was found with antibodies raised against cytochrome c_6 or plastocyanin from *Chlorella fusca* and *Anabaena* PCC 7119. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Thalassiosira weissflogii*; Chrysophyta; Diatom; Cytochrome c_6

1. Introduction

Cytochromes c_6 (formerly c_{553}) are soluble heme-containing proteins of 83–89 amino acids with a molecular weight of ca. 10 kDa and a redox potential of ca. +350 mV. They are class I *c*-type cytochromes with low-spin heme iron, with the heme covalently bound to the polypeptide. This cytochrome acts as electron carrier between the cytochrome b_6f complex and the photosystem I and is interchangeable with plastocyanin. The amino acid sequences of cytochrome c_6 from many cyanobacteria and green algae are known (Kerfeld, Anwa, Interrante, Merchant, & Yeates, 1995) and the crystal structure of the *Monoraphidium braunii* cytochrome has been resolved to 1.2 Å (Frazao et al., 1995). The isoelectric point of cytochrome c_6 can be basic, neutral or acidic in cyanobacteria. All green algae cytochromes c_6 described so far have acidic isoelectric points, as do plastocyanins from higher plants.

In some cyanobacteria, cytochrome c_6 is the only electron donor to PSI. In other organisms such as Chlorophyceace, it occurs in conjunction with plastocyanin and is progressively synthesised under copper deprivation (Sandmann, Reck, Kessler, & Böger, 1983). In higher plants only plastocyanin is present.

The *petJ* gene coding for cytochrome c_6 is nuclear in plants and green algae. Although no *petJ* sequence information is available for diatoms, the genes for cytochrome c_6 and plastocyanin are not present on the choloroplast genome sequence of the marine diatom *Odontella sinensis* contained in Genebank (Kowallik, Stoebe, Schaffran, Kroth-Pantic, & Freier, 1995). In plants and green algae, the protein is synthesised in the cytoplasm in the form of a preapocytochrome, which contains a transit peptide for further translocation into the chloroplasts and thylakoid lumen (Merchant & Bogorad, 1986; Bovy, de Vrieze, Borrias, & Weisbeek, 1992).

Sandmann et al., 1983 used difference spectroscopy to determine the presence of cytochrome c_6 and plastocyanin in a number of eukaryotic algae. All species of Crysophyceae, Xanthophyceae and Rhodophyceae examined, including the marine diatoms *Skeletonema* and *Phaeodactylum*, showed only cytochrome c_6. No plastocyanin was detected, even at high copper concentrations. As no cytochromes c_6 from diatoms have been isolated and characterised, we have undertaken the purification of cytochrome c_6 from *Thalassiossira*

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weisfologii that is described here. This study contributes to our broader knowledge of both the cytochromes and the evolutionary links between different photosynthetic organisms.

2. Experimental

2.1. Organism

The marine centric diatom *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle (clone ACTIN) was used for this study.

2.2. Growth of phytoplankton

Large volume (20 l) cultures were grown in 0.2 μm filtered Vineyard Sound (MA, USA) sea water (31‰) enriched with ESNW nutrients according to Harrison, Waters, and Taylor (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for Na_2glyceroPO_4 and selenium (as H_2SeO_3) was added to a final concentration of 10 nM. Trace metal additions were made according to Brand, Sunda, and Guillard (1983). Additions of iron and EDTA were 100 nM and 1 μM, respectively, for iron-limited cultures and 20 and 100 μM for iron-replete cultures. Sea water was sterile-filtered (0.2 μm) into autoclaved glass carboys then enriched with sterile nutrients. Macro nutrient (nitrate, phosphate and silicate) stocks were sterilised by autoclaving while iron, trace metal, selenium, EDTA and vitamin stocks were sterile-filtered (0.2 μm). The cultures were bubbled with sterile air and maintained at 20°C on a 14:10 h light:dark cycle at an irradiance of ca. 175 μE m^{-2} s^{-1} as measured with a photometer (Biospherical Instruments model QSP-100). Cells were harvested by centrifugation.

2.3. Cytochrome c_6 purification

20 g of cells (iron-replete and iron deplete) of *Thalassiosira weissflogii* were used for the isolation of the protein. These cells were resuspended in 40 ml of 0.1 M sodium phosphate, 0.1 M NaCl, 1 mM EDTA, pH 7.0 with 13 mM beta-mercaptoethanol and 1 μg/ml each pepstatin, leupeptin and aprotinin.

The cells were disrupted with ice-cold zirconium beads (500 μm) in the proportion 1:1, using a bead-beater (Bio-Spec Products, Bartlesville, OK) for two 1-min cycles in an ice and water bath with a 1-min cooling period in between.

Unbroken cells and debris were removed by centrifugation at $27000 \times g$ for 30 min (4°C). The resulting supernatant was precipitated with 60% ammonium sulphate (the pH was maintained at 7) with stirring overnight in the cold room. After 30 min centrifugation at $20000 \times g$, the supernatant was loaded onto a DEAE-cellulose column (1.5×30 cm) that had been equilibrated with 20 mM sodium phosphate buffer pH 7 plus 60% ammonium sulphate. Cytochrome c_6 was eluted in a reverse gradient of 60 to 0% ammonium sulphate in 20 mM sodium phosphate buffer pH 7. The fractions containing cytochrome (easily recognised by the characteristic absorption peak at 420 nm) were pooled and dialysed against 20 mM sodium phosphate buffer pH 7.

Further purification of these pooled fractions was obtained by HPLC, using a semipreparative anionic Hydropore-5-SAX column (Varian Associates, Walnut Creek, CA) using a linear gradient from 0.1 to 0.7 M NaCl in 20 mM phosphate buffer pH 7.0. Detection was performed with a Hewlett-Packard model 1050 diode array detector (Hewlett-Packard Co., Andover, MA). Cytochrome peaks were concentrated and dialysed in 50 mM Tris–HCl pH 8 using Centricon tubes (Amicon). This sample was further purified by FPLC (Pharmacia) using a MonoQ 5 column. This last step of purification was performed in a linear gradient using 50 mM Tris–HCl pH 8 as buffer A and the same buffer plus 1 M NaCl as buffer B.

2.4. Protein quantification

An extinction coefficient at 553 nm of 24.8 mM^{-1} cm^{-1} was assumed (Inda et al., 1997) and used for the quantification of pure preparations.

2.5. Phosphoserine determination

The presence of phosphoserine was assessed using mouse monoclonal antiphosphoserine from Sigma Bio Sciences.

2.6. Isoelectric focusing and SDS-PAGE

Electrophoresis and p*I* determination of the purified protein were performed in a Phast System from Pharmacia, with the products and directions given by the manufacturer.

2.7. Ultraviolet-visible electronic spectra

Spectra were recorded using a Kontron Uvikon 860 spectrophotometer.

2.8. Circular dichroism

CD spectra were obtained using a Jasco 710 spectropolarimeter at room temperature. The protein concentration was 1.43 μM for the oxidised form and 0.56 μM for the reduced form. Samples were prepared in 1 mM sodium phosphate buffer pH 7.

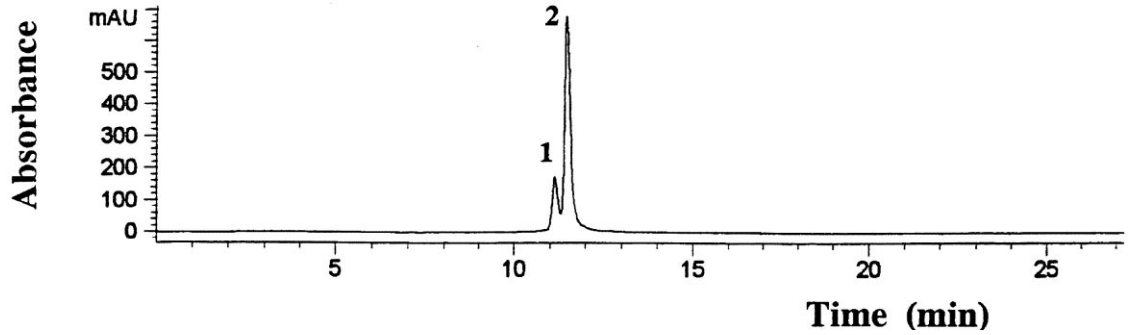

Fig. 1. HPLC elution profile of the cytochrome c_6 from *T. weissflogii*. Peak 1 corresponds to the oxidised form and peak 2 is the reduced form.

2.9. Antibodies

Cross-reactivity was studied by the Ouchterlony technique (Ouchterlony, 1949), using rabbit antibodies raised against *Chlorella fusca* and *Anabaena* PCC 7119 cytochrome c_6. Antibodies raised against plastocyanin from *Chlorella fusca* (8) (Inda et al., 1997) were also tested.

3. Results and discussion

Cultures of the marine diatom *Thalassiosira weissflogii* were used to isolate cytochrome c_6. We detected cytochrome c_6 in cells grown with replete iron (20 μM) and also in cells grown with limited iron (0.1 μM). The yield of cytochrome was 3 times less in the case of iron depletion. During ion-exchange HPLC column two cytochrome c_6 peaks were observed, corresponding to the oxidised (peak 1) and reduced (peak 2) forms, the majority being reduced protein. (Fig. 1).

The purified protein exhibited two bands in SDS-PAGE with apparent molecular weights of 13 (major) and 37 kDa (very minor), identified as monomeric and oligomeric cytochrome, respectively, using gel filtration in a Superose 12 HR column from Pharmacia. Oligomerisation was detected only in concentrated samples and has been reported in cytochrome c_6 from different sources (Kerfeld et al., 1995; Inda et al., 1997). Its functional significance has been supported by kinetic data (Carter et al., 1985), but is not yet clear. The isoelectric points were slightly different for the reduced (3.8) and oxidised (3.6) forms, as expected from the behaviour during anion-exchange chromatography. This diatom cytochrome c_6 is a very acidic cytochrome c_6, as in green and red algae, and as is plastocyanin in higher plants.

The visible-ultraviolet absorption spectra of cytochrome c_6 from *T. weissflogii* in the reduced and oxidised form are shown in Fig. 2. Ferrocytochrome c_6 showed maxima at 553 (α-peak), 522 (β-peak), 416 (γ-peak), 318 (δ-peak) and 273 nm (corresponding to the polypeptide chain). The ferrocytochrome exhibited a ratio of A_{553}/A_{273} of 0.91. Shoulders at 392, 354 and 291 nm were also present. The oxidised spectrum showed maxima at 361 nm, 409 (shift of the soret band) and 526 nm (instead of α and β peaks) and a small broad band at 690 nm. The 690 nm band indicates the presence of methionine as one of the axial ligands of the heme-iron (Moore & Pettigrew, 1990). A summary of properties of *T. weissflogii* cytochrome c_6 is shown in Table 1.

Immunodot tests using a phosphoserine antibody show the presence of phosphoserine in the cytochrome c_6 that we isolated from *T. weissflogii*. The significance of the observed phosphorylation process requires several experiments to allow any serious hypothesis, but is important to note that signal transduction via light-dependent redox control of reversible phosphorylation is a very common mechanism for controlling events related to light energy utilisation (Vener, Van Kan, Gal, Anderson, & Ohad, 1995).

Circular dicroism spectra of the oxidised and reduced states are shown in Fig. 3. The far UV-CD

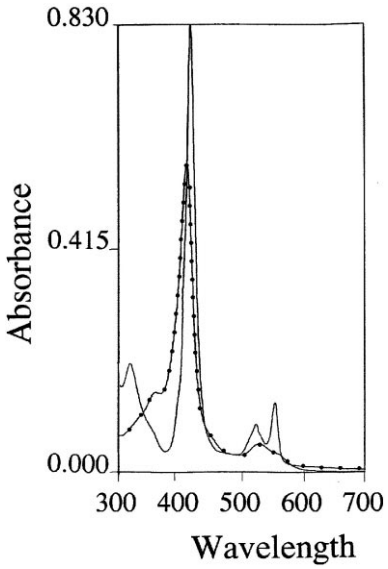

Fig. 2. UV/visible absorption spectra of cytochrome c_6 from *T. weissflogii* in its native auto reduced state and after oxidation (•) with $[Fe(CN)_6]^{3-}$.

Table 1
Summary of determined characteristics of *Thalassiosira weissflogii* cytochrome c_6

Apparent molecular weight, SDS-PAGE	13 kDa
pI	
Oxidised form	3.6
Reduced form	3.8
A_{553nm}/A_{273nm}	0.91
Absorbance maxima (nm)	
Oxidised form	reduced form
270	273
352	318
409	416
526	522
690	

spectra showed, both in the oxidised and the reduced state, two predominant bands at 222 and 208 nm, indicating a large proportion of secondary structure in the α-helix, as observed in cytochromes from other sources (Inda et al., 1997) as can be expected from the three-dimensional structure of the protein (Frazao et al., 1995). The aromatic region showed differences between the two states (not shown), indicating slight structural alterations of the aromatic residues upon reduction. CD spectra in the visible region (not shown) showed the expected changes in the heme group upon reduction.

Plastocyanin (reduced or oxidised) was not detected in the crude extracts and HPLC fractions from cells grown either in iron-replete and iron-deplete conditions. Copper additions to the culture media were 10 nM, an amount sufficient for regular synthesis of plastocyanin in other organisms. When iron was limited, the amount of cytochrome c_6 was 3 times lower than in iron replete conditions, but plastocyanin was still not present. Sandmann et al., 1983 did not find plastocyanin in two other diatoms and other non-green algae. They proposed that in the course of evolution, cytochrome c_6 dominated over plastocyanin in the older species, whereas cytochrome c_6 was the only one found in the oldest oxygenic photosynthetic organisms, namely some cyanobacteria (Sandmann, 1986).

T. weissflogii cytochrome c_6 was not crossreactive with *Chlorella* and *Anabaena* anticytochromes c_6 (data not shown) or *Chlorella* plastocyanin antibodies. The absence of recognition of green algal and cyanobacterial cytochrome c_6 antibodies is consistent with the antigenic differences expected from the phylogenetic distance between the respective groups.

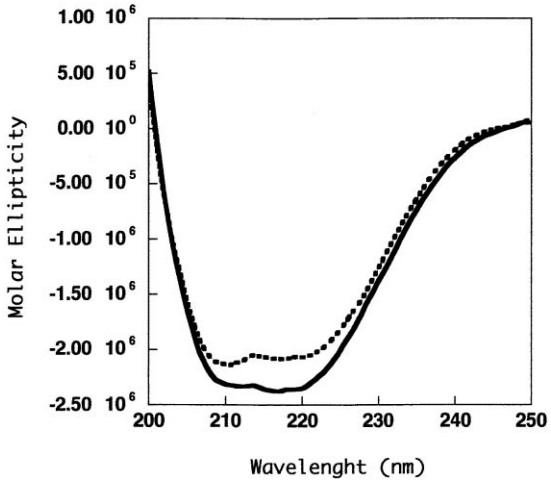

Fig. 3. UV-Circular dichroism spectra of cytochrome c_6 in the oxidised (· · ·) and reduced (———) states. The protein was in 1 mM sodium phosphate pH 7.0. Molar ellipticity, [θ], corresponds with degrees $cm^2\ dmol^{-1}$.

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