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Extinction coefficients and midpoint potentials of cytochrome c_6 from the cyanobacteria *Arthrospira maxima*, *Microcystis aeruginosa*, and *Synechocystis* 6803

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Abstract

Cytochrome c_6 is a soluble heme protein that serves as a photosynthetic electron transport component in cyanobacteria and algae, carrying electrons from the cytochrome *bf* complex to photosystem I. The rapid accumulation of cytochrome c_6 sequence data from a wide range of species, combined with significant advances in determining high resolution three-dimensional structures, provides a powerful database for investigating the relationship between structure and function. The fact that the gene encoding cytochrome c_6 can be readily modified in a number of species adds to the usefulness of cytochrome c_6 as a tool for comparative analysis. Efforts to relate cytochrome c_6 sequence information to structure, and structural information to function depend on knowledge of the physical and thermodynamic properties of the cytochrome from different species. To this end we have determined the optical extinction coefficient, the oxidation/reduction midpoint potential, and the pH dependence of the midpoint potential of cytochrome c_6 isolated from three cyanobacteria, *Arthrospira maxima*, *Microcystis aeruginosa*, and *Synechocystis* 6803.

Keywords: Photosynthesis; Cytochrome; Optical spectroscopy; Extinction coefficient; Redox potentiometry

1. Introduction

Energy transducing membranes, including the electron transport system in photosynthetic bacteria and algae, and the respiratory electron transport system in mitochondria, depend on small *c*-type cytochromes to carry electrons between large integral protein complexes [1]. These cytochromes link the membrane bound redox components by diffusing in the water phase surrounding the membrane [2]. In the photosynthetic apparatus of cyanobacteria and algae this role is fulfilled by cytochrome c_6[1], which operates between the cytochrome *bf* complex and photosystem I. In many species of cyanobacteria

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[1] In earlier literature cytochrome c_6 was often referred to as cytochrome *c*553.

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and algae a small copper protein can replace cytochrome c_6 (reviewed in [1]). It is not known why these organisms maintain two different proteins to serve what appear to be identical functions. In most species expression of genes for both cytochrome c_6 and plastocyanin is controlled by the copper content of the growth medium [3–7]. For cells grown without copper cytochrome c_6 is expressed, whereas if copper is available in the growth medium plastocyanin is formed. In contrast, plant chloroplasts depend solely on plastocyanin to carry electrons from the cytochrome *bf* complex to photosystem I, whereas mitochondria depend on a *c*-type cytochrome that is similar to cytochrome c_6 to link the cytochrome bc_1 complex and cytochrome oxidase.

Cytochrome c_6 genes have been cloned and sequenced from over 20 species [1] and high resolution three-dimensional structures have been determined for cytochrome c_6 from two cyanobacterial and from two algal species [1,8–10]. The sequence and protein structural data, and the fact that some of these organisms can be readily transformed (e.g., *Synechocystis* 6803 and *Chlamydomonas reinhardtii*) make cytochrome c_6 an excellent system for mutational studies directed toward investigating the relationship between structure and function. For example, how protein structure determines absorption spectra and the energetics of heme redox reactions can be investigated by directed mutagenesis and comparative analysis of cytochromes c_6 from different species [8,11,12]. Directed mutagenesis is also used to investigate the role of cytochrome c_6 in the electron transfer reactions linking the cytochrome *bf* complex and photosystem I (e.g. [6,13–15]). To facilitate these and other investigations, which depend on knowledge of the absorption spectrum, oscillator strength, and thermodynamic properties of the cytochrome heme, we isolated cytochrome c_6 from three species of cyanobacteria and determined absorption spectra, extinction coefficients, and oxidation reduction midpoint potentials[2].

[2] Spectra and physical data for photosynthetic cytochromes can be found and downloaded from our website: <http://www.life.uiuc.edu/whitmarsh/cytochrome>.

2. Materials and methods

2.1. Cell culture and enzyme purification

Arthrospira maxima was grown and cytochrome c_6 was purified as described in Gomez Lojero and Krogmann [16]. *Microcystis aeruginosa* was collected from a natural bloom and the cytochrome was purified as describe in Cohn et al. [17]. *Synechocystis* sp. PCC 6803 was grown under white light fluorescent lamps at approx. 200 μE m^{-2} s^{-1} in BG11 liquid medium prepared without copper and cytochrome was purified as described in Zhang et al. [5].

2.2. Determination of extinction coefficients

The extinction coefficient of cytochrome c_6 was determined by comparing the reduced minus oxidized absorption spectrum of cytochrome c_6 to that of the pyridine hemochrome as described elsewhere [18]. The extinction coefficient for the reduced minus oxidized pyridine hemochrome was taken from Berry and Trumpower [19]. Determination of the extinction coefficient at different protein concentrations and different pyridine concentrations revealed no significant differences. Spectroscopic measurements were done using a DW2 SLM/Aminco spectrophotometer modified by OLIS (Athens, GA) or a Hewlett-Packard HP8452A diode-array spectrophotometer. The data shown in Fig. 1 were gathered using the DW2 spectrophotometer. The wavelength was calibrated using a Hg emission line to within ± 0.2 nm.

2.3. Potentiometric redox titrations

Potentiometric redox titrations were done using a Radiometer P101 platinum electrode in combination with a Radiometer K401 calomel reference electrode as described elsewhere [18]. The ambient redox potential was calibrated either by a saturated solution of quinhydrone (Clark) or by a known ratio of ferricyanide/ferrocyanide [20]. The redox state of cytochrome c_6 was determined by its α-band reduced minus oxidized absorption spectrum. Absorbance measurements were done using either a DW2 SLM/Aminco spectrophotometer modified by OLIS, or an RSM1000 rapid scanning spectrophotometer (OLIS). Cytochrome c_6 was suspended in 40 mM KCl and

either 50 mM succinate (pH 4–5), MES (pH 6), KH_2PO_4 (pH 6.5–7.5), tricine (pH 8), or glycine (pH 9–10). Potassium ferricyanide and sodium dithionite were used as oxidant and reductant, respectively, and 2,3,5,6-tetramethyl-*p*-phenylenediamine ($E_{m,7}$ = 260 mV), or hydroquinone ($E_{m,7}$ = 280 mV) were added as additional mediators. Titrations done in the reductive or oxidative direction gave identical results. During spectral measurements the ambient redox potential was stable within 1 mV. Midpoint potentials were determined by fitting the Nernst equation to the spectral data using software from either Kaleidagraph or Microsoft Excel.

3. Results and discussion

3.1. Extinction coefficients

The reduced minus oxidized extinction coefficients for cytochrome c_6 from *A. maxima*, *M. aeruginosa* and *Synechocystis* 6803 over the wavelength range from 515 to 585 nm are shown in Fig. 1. The α-band peak of cytochrome c_6 is in a narrow range, between 552.2 and 552.6 nm. All of the β-band peaks are near 522 nm. Table 1 gives the extinction coefficients for the α-band peak of reduced cytochrome c_6 (reference wavelength of 580 nm), and of the reduced minus oxidized α-band peak minus the absorption minimum near 537 nm. To our knowledge, only one other cytochrome c_6 extinction coefficient has been published [21]. The value of 24.4 mM^{-1} cm^{-1} for the reduced cytochrome from *Synechocystis* 6803 reported here is the same as the value given by Diaz et al. [21] within the experimental error.

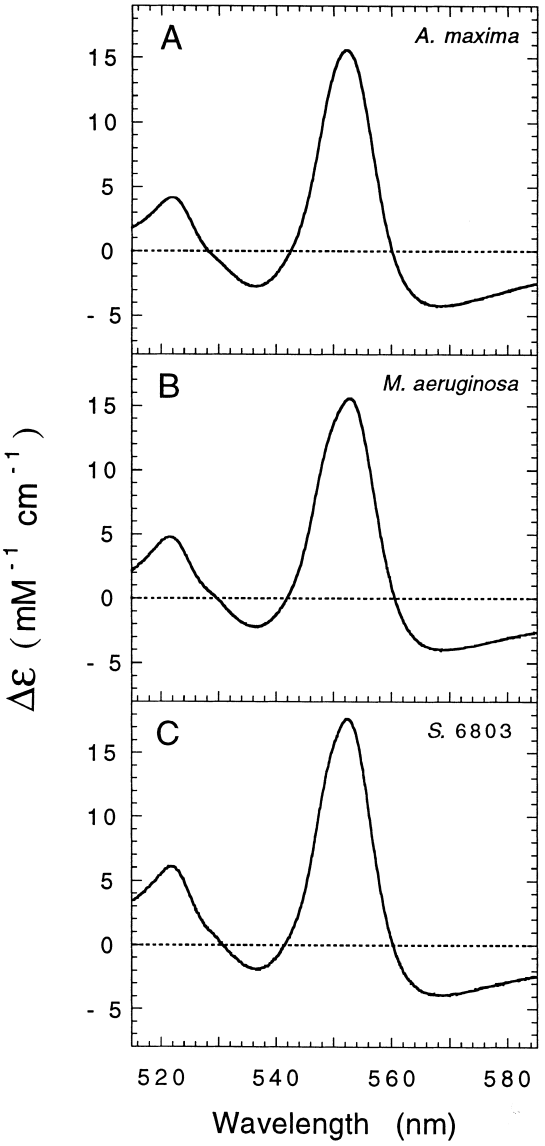

Fig. 1. Reduced minus oxidized extinction coefficient of cytochrome c_6 from the cyanobacteria *A. maxima* (A), *M. aeruginosa* (B), and *Synechocystis* 6803 (C). Cytochrome c_6 was oxidized in the presence of 0.25 mM potassium ferricyanide and subsequently reduced by addition of sodium dithionite. The procedure for determining the extinction coefficient is described in the text.

3.2. Midpoint potentials

Equilibrium redox titrations of cytochrome c_6 from *A. maxima*, *M. aeruginosa* and *Synechocystis* 6803 are shown in Fig. 2. Fitting the Nernst equation to the data reveals that each cytochrome behaves as a homogeneous redox component undergoing a one-electron redox reaction. For each of the three cytochromes the oxidation-reduction midpoint potential at pH 7.0 is given in Table 2. The midpoint potentials shown here are 20–30 mV lower than those determined at pH 7 for cytochrome c_6 from the red alga *Porphyridium cruentum* [22] and from the cyanobacterium *Nostoc* sp. PCC 8009 [23]. Diaz et al. [8] reported a midpoint potential of 324 mV at pH 7 for cytochrome c_6 from *Synechocystis* 6803, which is, within experimental error, the same as that reported here (Table 2). A midpoint potential of 336 mV at pH 7 has been reported for cytochrome c_6 from

Table 1
Comparison of cytochrome c_6 extinction coefficients from three cyanobacteria determined using the pyridine hemochrome assay

Cytochrome c_6 (source)	ε (mM^{-1} cm^{-1})	Δε (mM^{-1} cm^{-1})
A. maxima	ε (552.2–580 nm) = 22.9	Δε (552.2–536.5 nm) = 18.4
M. aeruginosa	ε (552.6–580 nm) = 22.3	Δε (552.6–536.3 nm) = 17.8
Synechocystis PCC 6803	ε (552.5–580 nm) = 24.4	Δε (552.5–537.0 nm) = 19.5

For the reduced cytochrome the extinction coefficients (ε) were calculated from the α-band peak using a reference wavelength of 580 nm. For the reduced minus oxidized cytochromes the extinction coefficients (Δε) were calculated from the α-band peak to the absorption minimum occurring between 536 and 537 nm. The extinction coefficients for each value are the average of three or more determinations. The standard deviation was 0.5 or less.

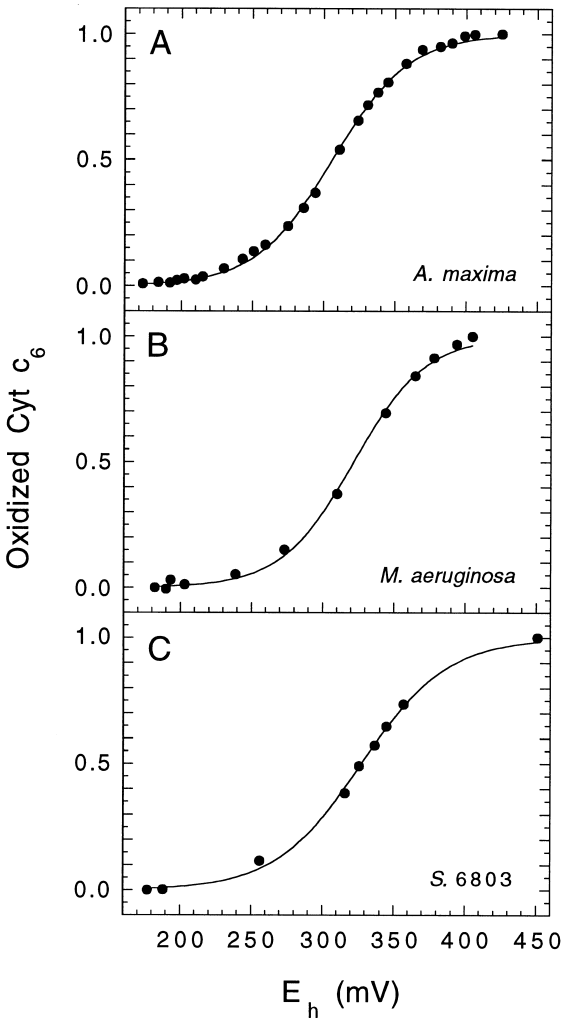

Fig. 2. Potentiometric titration of the redox midpoint potential of cytochrome c_6 from *A. maxima* (A), *M. aeruginosa* (B), and *Synechocystis* 6803 (C). The smooth curve was drawn using the Nernst equation giving the best fit to the data. The pH of the medium was 8 in A, 7 in B, and 6.25 in C. Further experimental details are given in the text. The midpoint potential and the number of electrons were selected as free parameters.

S. maxima [24]. Cytochrome c_6 from the green algae *Chlamydomonas reinhardtii* ($E_{m,7}$ = 370 mV [25]) and *Monoraphidium braunii* ($E_{m,7}$ = 358 mV [26]) have significantly higher midpoint potentials than those for the three cyanobacteria reported here (Table 2). Campos et al. note that cytochrome c_6 from *M. braunii* exhibits an unusual heme axial coordination [26], but conclude that distortion is not directly correlated with the higher midpoint potential. In common with cytochrome *f*, cytochrome c_6 has a midpoint potential significantly higher than most other soluble *c* cytochromes. Cramer and coworkers [27] determined the three-dimensional structure of cytochrome *f* and have suggested that its high midpoint potential may be due to the fact that the heme is shielded from the aqueous phase.

The dependence of the equilibrium midpoint potential of cytochrome c_6 on pH is shown in Fig. 3. There is little change in the midpoint potential from pH 4 to 8, indicating that the reduction and oxidation of cytochrome c_6 in this pH range do not involve protons. Above pH 8 each of the cytochromes shows a significant decrease in midpoint potential, indicating a p*K* on the oxidized form between pH 8 and 9, which is a common feature in *c*-type cytochromes (e.g. [8,28–30]). The p*K* observed above

Table 2
Comparison of the oxidation/reduction midpoint potentials of cytochrome c_6 at pH 7 from three cyanobacteria

Cytochrome c_6 (source)	$E_{m,7}$ (mV)
A. maxima	314
M. aeruginosa	321
Synechocystis PCC 6803	320

For *A. maxima* and *M. aeruginosa* the values are the average of three or more measurements. For *Synechocystis* the value is taken from Fig. 3. The standard deviation was 10 mV or less.

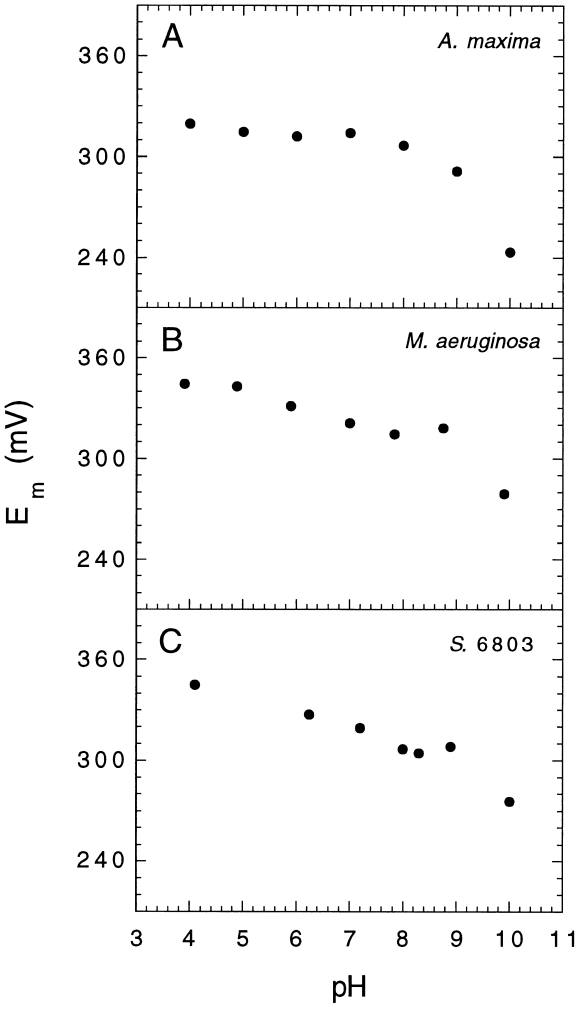

Fig. 3. The pH dependence of the redox midpoint potential of cytochrome c_6 from *A. maxima* (A), *M. aeruginosa* (B), and *Synechocystis* 6803 (C).

pH 8 is likely due to one of the heme propionates (e.g. [8]). It is noteworthy that in contrast to the heme in cytochrome c_6, the midpoint potential of free heme is pH-dependent over the pH range of 4–8. Because cytochrome c_6 operates in the water phase of the thylakoid vesicle, where the pH ranges from 5 to 8, the structure of cytochrome may be designed, in part, to remove the pH dependence of the midpoint potential of the heme. Like cytochrome c_6, the midpoint potentials of cytochrome *f* and P700 are pH-independent over this range. As a consequence, the free energy difference driving electron transfer between cytochrome c_6 and cytochrome *f* or P700 is pH-independent during electron transport, which can lower the pH by 2–3 units in the light.

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