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A comparative structural and functional analysis of cytochrome c_M, cytochrome c_6 and plastocyanin from the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract Cytochrome c_M is a new *c*-class photosynthetic haem protein whose physiological role is still unknown. It has been proposed previously that cytochrome c_M can replace cytochrome c_6 and plastocyanin in transferring electrons between the two membrane complexes cytochrome b_6–f and photosystem I in organisms growing under stress conditions. The experimental evidence herein provided allows us to discard such a hypothesis. We report a procedure to overexpress cytochrome c_M from the cyanobacterium *Synechocystis* sp. PCC 6803 in *Escherichia coli* cells in mg quantities. This has allowed us to perform a comparative laser flash-induced kinetic analysis of photosystem I reduction by the three metalloproteins from *Synechocystis*. The bimolecular rate constant for the overall reaction is up to 100 times lower with cytochrome c_M than with cytochrome c_6 or plastocyanin. In addition, the redox potential value and surface electrostatic potential distribution of cytochrome c_M are quite different from those of cytochrome c_6 and plastocyanin. These findings strongly indicate that cytochrome c_M cannot be recognised by and interact with the same redox partners as the other two metalloproteins.

Key words: Cytochrome c_M; Cytochrome c_6; Plastocyanin; Photosystem I

1. Introduction

A new *c*-class cytochrome (Cyt), the so-called Cyt c_M, has recently been found in photosynthetic organisms [1]. It is a small soluble protein, with a molecular mass of ca. 8 kDa, a slightly acidic isoelectric point (p*I*) of ca. 6, and a midpoint redox potential value of +150 mV at pH 7 [2], but its physiological function is still unknown. In the cyanobacterium *Synechocystis* sp. PCC 6803, the deletion of the Cyt c_M produces no discernible phenotypes when the cells are grown under standard conditions. In fact, Cyt c_M appears to be synthesised only under stress conditions (low temperature and high light intensity), when the synthesis of the two soluble metalloproteins Cyt c_6 and plastocyanin (Pc) is repressed [3].

Cyt c_6 and Pc are very well characterised redox carriers, which are located inside the thylakoidal lumen and transfer electrons between the membrane complexes Cyt b_6–f and photosystem I (PSI). These two proteins possess similar physicochemical and surface structural properties, which make them capable of replacing each other [4,5]. In fact, their relative synthesis is regulated by copper availability in such a way that the cells produce either Cyt c_6 or Pc in the absence or presence of copper, respectively [6].

It has been reported that a deletion mutant of *Synechocystis* lacking the *petJ* gene encoding Cyt c_6 is able to grow at near wild-type (WT) rates in copper free media, that is, when the synthesis of Pc is repressed [7]. Therefore, it has been suggested that there should be a third electron carrier transferring electrons from Cyt b_6–f to PSI, and that Cyt c_M could be a plausible candidate to play such a role. Cyt c_M could thus replace the other two metalloproteins in cells growing under stress conditions [3,8]. However, up to now no further evidence has been provided to support such a hypothesis.

The structure and function of Cyt c_6 and Pc have been intensively studied in a wide variety of organisms (see [5] and [9], for reviews), but little is known about Cyt c_M. In this work, we report a procedure to overexpress *Synechocystis* Cyt c_M in *Escherichia coli* that yields the recombinant haem protein in mg quantities. This has allowed us to carry out a comparative structural and functional analysis between Cyt c_M and Cyt c_6 and Pc from the same organism. In the light of our results, we can conclude that Cyt c_M is unable to replace either Cyt c_6 or Pc in donating electrons to PSI.

2. Materials and methods

2.1. DNA techniques

The direct primer CM1 (CCC CCA CTA GGA TAT GGA TTA TG) and the reverse one CM2 (CCA AAC CAA TCT GGG CTT ATC), designed from the known gene sequence of *Synechocystis* sp. PCC 6803 Cyt c_M [1], were used to amplify the regions containing the Cyt c_M open reading frame (ORF) from the genomic DNA of *Synechocystis* by the polymerase chain reaction (PCR). The resulting PCR products were cloned using the pGEM-T cloning kit (Promega), and pBluescriptII SK(+) (Stratagene) was used as expression vector. *E. coli* DH5α (Bethesda Research Laboratories) was used for cloning and plasmid construction. Other molecular biology protocols used were

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Abbreviations: Cyt, cytochrome; k_{bim}, second-order rate constant for PSI reduction; k_{inf}, bimolecular rate constant extrapolated at infinite ionic strength; k_{obs}, pseudo-first-order rate constant; LB, Luria–Bertani; Pc, plastocyanin; PCR, polymerase chain reaction; p*I*, isoelectric point; PSI, photosystem I

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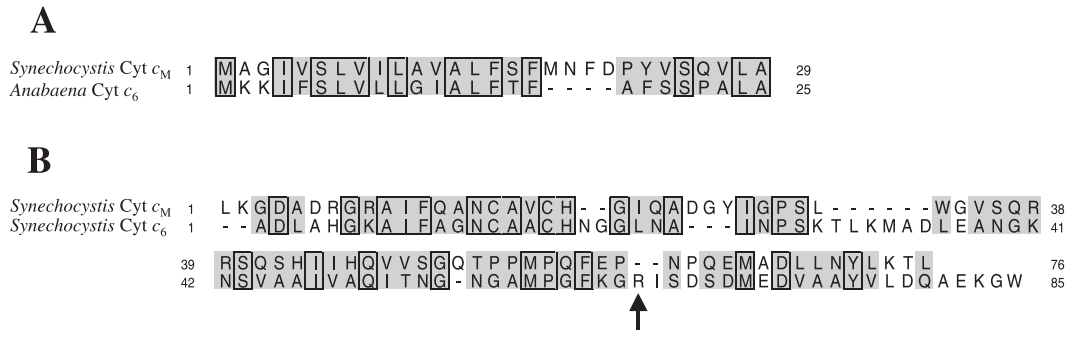

Fig. 1. Alignment of the amino acid sequences of the transit peptides from *Anabaena* Cyt c_6 and *Synechocystis* Cyt c_M (A), and codifying regions of Cyt c_M and Cyt c_6 from *Synechocystis* (B). Homologous regions are depicted in grey, whereas identical regions are boxed. The arrow points to the only arginyl residue of Cyt c_6.

standard. *E. coli* MC1061 cells [10] co-transformed with the plasmids pESCM (see Section 3) and pEC86 [11], which encode the *E. coli* genes required for Cyt *c* maturation [12], were used for expression of the cloned gene.

2.2. Biochemical procedures

Cells were grown in 3 l of standard Luria–Bertani (LB) medium [13] supplemented with 100 μg/ml ampicillin, 10 μg/ml chloramphenicol and 0.5 mM $FeCl_3$. Cyt c_M was extracted and purified from the periplasmic fraction as previously described for *Synechocystis* Cyt c_6 [14], with minor modifications. Protein concentration was determined spectrophotometrically using the absorption coefficients described by Cho et al. [2]. Purity of the resulting protein fractions was determined using an absorbance ratio A_{551}/A_{275} of ca. 1.1 for pure Cyt c_M. Cyt c_6 and Pc were purified from *Synechocystis* cells as described elsewhere [14,15]. *Synechocystis* PSI particles were obtained by β-dodecyl maltoside solubilisation as previously described [16]. Molecular mass, isoelectric point and redox potential of recombinant Cyt c_M were determined as reported in [17].

2.3. Kinetic analyses

Laser flash-induced kinetics of PSI reduction by Cyt c_M, Cyt c_6 and Pc were monitored by following the absorbance changes at 820 nm, and the kinetic analyses of the resulting oscilloscope traces were carried out according to the reaction mechanisms previously proposed [15,18].

2.4. Structural modelling

The structures of Cyt c_M and Cyt c_6 from *Synechocystis* were modelled using the SYBYL program (Tripos Inc.) in an SGI RC10000 workstation as described previously [14,19], for which the three-dimensional (3D) structure of Cyt c_6 from the cyanobacterium *Synechococcus elongatus* [20] was used as a template. The 3D structure of *Synechocystis* Pc used was that reported by Romero et al. [21]. The surface electrostatic potential distribution of Cyt c_M, Cyt c_6, and Pc were modelled using the Swiss-Pdb Viewer program as described in [22].

3. Results and discussion

Synechocystis Cyt c_M has been expressed previously in *E. coli* cells [2], but the resulting recombinant protein contained a methionine residue added to the predicted N-terminus and six histidine residues at the C-terminal end due to malfunction of the hypothetical transit peptide of Cyt c_M in *E. coli*. To investigate the possible functional role of Cyt c_M it is desirable to have a recombinant protein identical to the WT molecule, with no altered structural regions that could lead to misinterpretations of experimental results. Our first objective was thus to improve the experimental procedure to transform *E. coli* cells and overexpress Cyt c_M without structural modifications. Taking into account the close similarity between the hypothetical transit peptide of *Synechocystis* Cyt c_M and the transit peptide of Cyt c_6 from the cyanobacterium *Anabaena* sp. PCC 7119 (Fig. 1), the latter having a processing site (ALA) downstream from the N-terminal end that works properly in *E. coli* [17], we constructed a chimeric gene comprising the transit peptide of *Anabaena* Cyt c_6 and the region codifying for mature Cyt c_M from *Synechocystis*.

The chimeric gene has been constructed in a single PCR step, with the *pet*J gene from *Anabaena* and the ORF from *Synechocystis* Cyt c_M as templates. Four primers have been used: the direct primer previously utilised for the cloning of *pet*J gene from *Anabaena* [17], the reverse primer described in this work for amplifying the Cyt c_M ORF, and two primers (TCA GTA GCC CTG CTC TGG CTC TCA AAG GAG ATG CGG ATA G as the direct primer, and CTA TCC GCA TCT CCT TTG AGA GCC AGA GCA GGG CTA CTG A as the reverse one) that anneal to the same sequence on opposite strands, designed with half of its sequence complementing the transit peptide from *Anabaena* Cyt c_6 and the other half complementing the N-terminal end of mature Cyt c_M. The chimeric gene was cloned and expressed in *E. coli* cells, from which Cyt c_M was purified as described in Section 2. 12 mg of Cyt c_M were extracted from the periplasmic fraction of 3-L *E. coli* cell cultures, and 4 mg of pure Cyt c_M, with an

Table 1
Physicochemical parameters and rate constants of PSI reduction by *Synechocystis* Cyt c_6, Pc and Cyt c_M

Protein	Molecular mass (kDa)		p*I*	$E_{m,7}$ (mV)	k_{bim} (M^{-1} s^{-1}) pH 7.5	k_{bim} (M^{-1} s^{-1}) pH 5.5	k_{inf} (M^{-1} s^{-1})[a] pH 7.5
	SDS–PAGE	Gene sequence					
Cyt c_6	8.1	8.7	5.6	+324	8.8×10^6	14.4×10^6	1.3×10^7
Pc	9.5	10.4	5.5	+360	8.5×10^6	9.9×10^6	1.0×10^7
Cyt c_M	7.9	8.3	5.6	+150	0.1×10^6	0.1×10^6	0.1×10^6[b]

[a] k_{inf} is the k_{bim} extrapolated to infinite ionic strength.
[b] k_{bim} is independent of ionic strength.

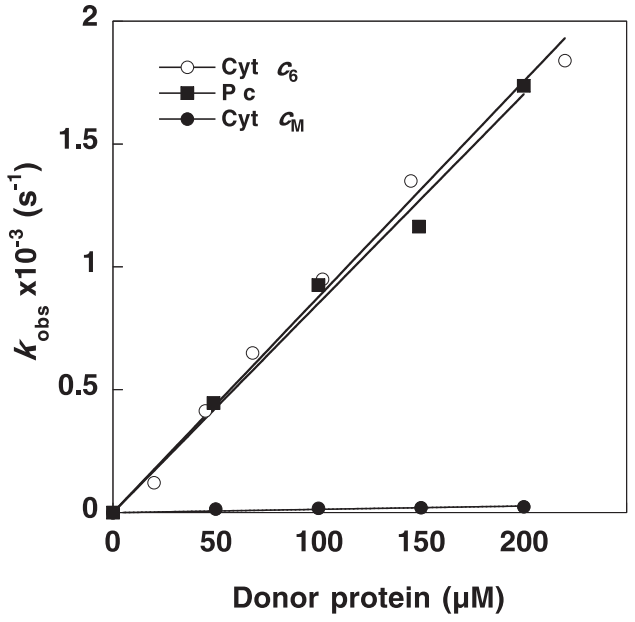

Fig. 2. Dependence upon donor protein concentration of the k_{obs} for *Synechocystis* PSI reduction by Cyt c_6, Pc and Cyt c_M. Temperature was 25°C and pH was 7.5.

A_{551}/A_{275} ratio for the reduced protein of 1.1, were finally obtained.

As shown in Table 1, the molecular mass of Cyt c_M deduced from the gene sequence (8.3 kDa) and that calculated by SDS–PAGE (7.9 kDa) is similar to those reported for *Synechocystis* Cyt c_6 and Pc [7,23]. The midpoint redox potential value of Cyt c_M determined in this work (+150 mV, at pH 7) is very similar to that previously described for the modified Cyt c_M [2], but significantly lower than that reported for Cyt c_6 and Pc (+350 mV, at pH 7) [23,24].

The cellular localisation of Cyt c_M is a key point to bear in mind when assessing its physiological role. In this context, the close similarity between the transit peptides of *Synechocystis* Cyt c_M and *Anabaena* Cyt c_6 (see Fig. 1A) suggests that Cyt c_M could, in fact, be located inside the thylakoidal lumen, as are Cyt c_6 and Pc. In addition, the amino acid sequence of Cyt c_M contains the VLA motif (see Fig. 1A), which is the same as that found at the site at which the transit peptide is cleaved from the 18-kDa polypeptide of the oxygen-evolving complex of spinach [1,25]; these findings suggest that the product of the Cyt c_M gene is processed after translocation to the lumen or to the periplasmic space, and that the mature protein is hydrophilic [1].

To check whether Cyt c_M could replace Cyt c_6 and Pc in their physiological role (see Section 1), we have performed a comparative laser flash-induced kinetic analysis of PSI reduction by Cyt c_M, Cyt c_6 and Pc. As shown in Fig. 2, the observed rate constant (k_{obs}) of the overall reaction is much lower with Cyt c_M than with Cyt c_6 or Pc. With all the three proteins, the values for k_{obs} depend linearly on protein concentration, indicating that the kinetics of PSI reduction by Cyt c_M follows a simple oriented collisional reaction mechanism, as do the two others [15]. The values for the second-order rate constant (k_{bim}), which are inferred from such a linear plot of k_{obs} versus protein concentration, are two orders of magnitude lower for Cyt c_M than for the other two donors of PSI, both at pH 7.5 and at a more physiological pH of 5.5 (Table 1); it should be noted that the k_{bim} values for the three proteins, in particular for Cyt c_M, do not significantly change with pH.

The ionic strength dependence of the reaction rates provides further evidence for the lack of reactivity of Cyt c_M towards PSI: for both Cyt c_6 and Pc, the rate constants increase as with salt concentration [14,26], but are independent of ionic strength with Cyt c_M (Table 1). We have observed previously that the interaction of PSI with both Cyt c_6 and Pc is mainly repulsive in *Synechocystis*, thus explaining why the rate constant increases with ionic strength and the overall reaction rather follows a collisional kinetic model [15]. The global charge of the two reaction partners is thus critical in assessing their respective affinities and interactions. The isoelectric point of recombinant Cyt c_M herein determined is 5.6 (see Table 1), a value close to the ones previously reported for the modified Cyt c_M [2], as well as for Cyt c_6 and Pc [23,24]. From an electrostatic point of view, Cyt c_M could thus behave similarly to the other two metalloproteins. However, not only the global charge, which defines the long-range movements, but also the surface potential distribution, which controls the short-range interactions, is critical for right orientation and complex formation. It is important to realise that Cyt c_6 and Pc exhibit two functionally equivalent surface areas: site 1, or the so-called 'north' hydrophobic pole, and site 2, or the electrostatically charged 'east' face. Site 1 harbours the haem group in Cyt c_6 and His-87, which is a solvent-exposed copper ligand, in Pc. Whereas site 2 is responsible for driving the electrostatic interaction process and making close contact with PSI, site 1 is mainly involved in the electron transfer itself. In order to look for the reasons why Cyt c_M reacts so slowly with PSI as compared with Pc and Cyt c_6, the surface electrostatic potential distribution of the three metalloproteins was analysed. In Fig. 3, the three molecules are shown in a similar orientation, thereby making evident that Cyt c_6 and Pc exhibit a similar distribution for their respective surface electrostatic potentials, whilst Cyt c_M shows a completely different pattern with a negatively charged area surrounding the solvent-exposed part of the haem group. In fact, Cyt c_M does not show surface areas equivalent to sites 1 and 2 of Cyt c_6 and Pc (Fig. 3).

In addition, Cyt c_6 and Pc contain one critical arginyl residue each (Arg-64 in Cyt c_6 and Arg-88 in Pc), similarly located at the molecular surface between sites 1 and 2. When such arginine is substituted by glutamate, neither of the two proteins is capable of reducing PSI [22]. Even though Cyt c_M contains three arginine residues in its sequence, none of them is located in a position equivalent to that of the loop containing the arginine in Cyt c_6 and Pc, and, in fact, Cyt c_M exhibits a deletion in the region corresponding to Arg-64 in Cyt c_6 (see Fig. 1).

From a thermodynamic point of view, it is difficult to see how Cyt c_M, with a midpoint redox potential of +150 mV, could be reduced by Cyt *f*, with a redox potential of +320 mV [27]. All these findings at the structural and functional level reveal that Cyt c_M is not a plausible candidate to act as a third electron carrier between Cyt b_6-*f* and PSI so as to replace Cyt c_6 and Pc. It could nevertheless play a functional role as a protective agent against photo-induced and/or oxidative stress in cells growing under stress conditions.

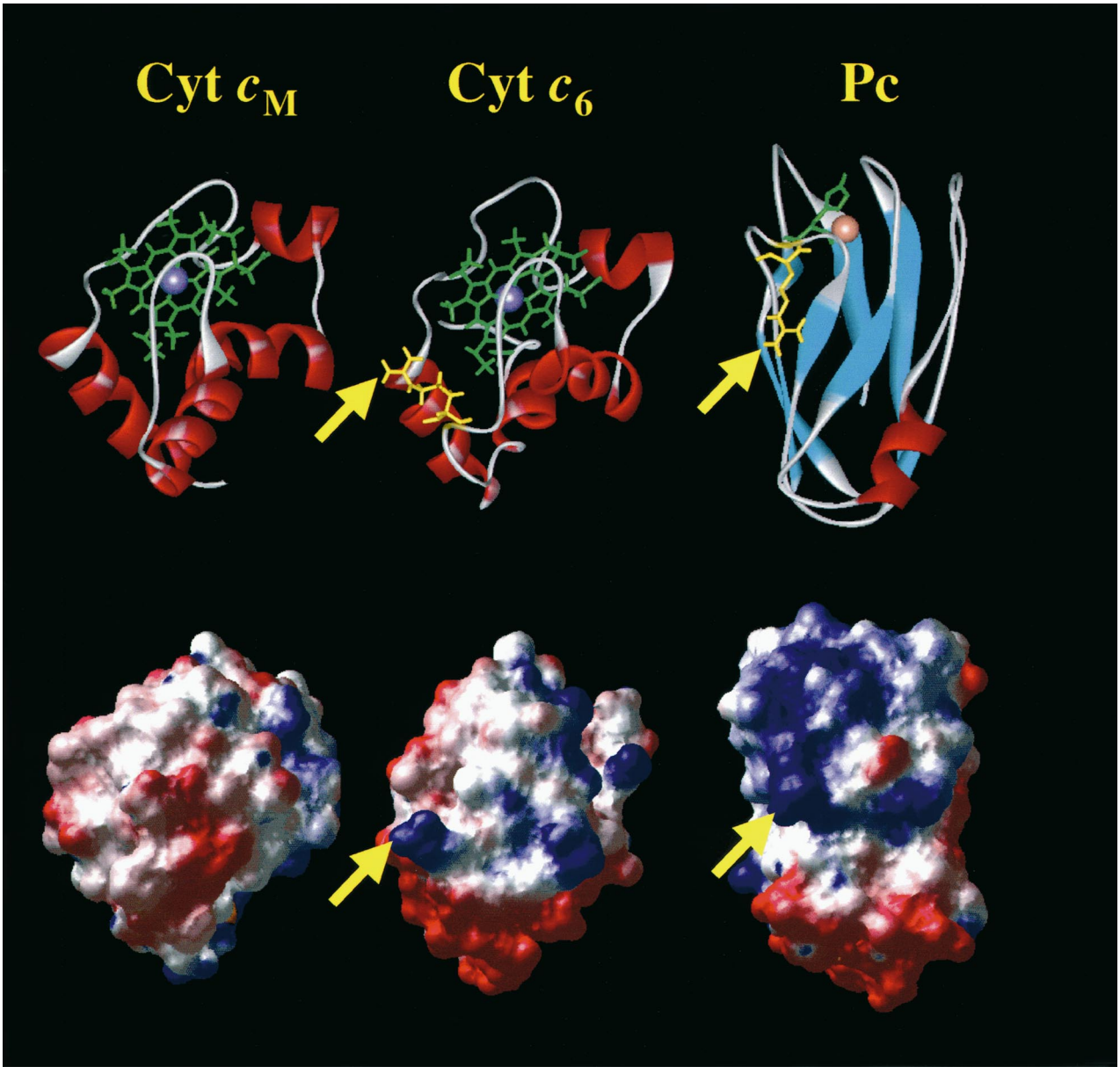

Fig. 3. Upper panel: Structural models of Cyt c_M, Cyt c_6, and Pc from *Synechocystis*. The haem group in Cyt c_M and Cyt c_6 and His-87 in Pc are depicted in green, whereas the only arginyl residues in Cyt c_6 and Pc are in yellow. Lower panel: Surface electrostatic potential distribution of Cyt c_M, Cyt c_6, and Pc. Simulations were performed assuming an ionic strength of 40 mM at pH 7.0. Negatively and positively charged regions are depicted in red and blue, respectively. The three molecules are similarly oriented in the upper and lower panels. Cyt c_6 and Pc are placed with their respective 'east' faces (site 2) in front and the north hydrophobic poles (site 1) at the top, whereas Cyt c_M is placed with its haem group at the same orientation as that of Cyt c_6. The arrows point to the guanidium group of the only arginyl residue in Cyt c_6 and Pc.

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