

Communication

Cytochrome c_M Is Probably a Membrane Protein Similar to the C Subunit of the Bacterial Nitric Oxide Reductase

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Abstract: Cytochrome c_M was first described in 1994 and its sequence has been found in the genome of manifold cyanobacterial species ever since. Numerous studies have been carried out with the purpose of determining its function, but none of them has given place to conclusive results so far. Many of these studies are based on the assumption that cytochrome c_M is a soluble protein located in the thylakoid lumen of cyanobacteria. In this work, we have reevaluated the sequence of cytochrome c_M , with our results showing that its most probable 3D structure is strongly similar to that of the C subunit of the bacterial nitric oxide reductase. The potential presence of an α -helix tail, which could locate this protein in the thylakoid membrane, further supports this hypothesis, thus providing a new, unexpected role for this redox protein.

Keywords: cyanobacteria; cytochrome c_M ; NorC; nitric oxide reductase



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1. Introduction

Electron transport chains play a fundamental role in the metabolism of all living beings. They consist of a series of redox reactions, spatially separated, in which electrons are transferred from a donor molecule to an acceptor one. In phototrophic eukaryotes, the respiratory and photosynthetic electron transport chains are spatially separated into specialized organelles (mitochondria and chloroplasts). However, in cyanobacteria, both electron transport chains are located in the thylakoid membranes and share some components, such as plastoquinone (PQ), plastocyanin (Pc) or cytochrome (Cyt) c_6 and the Cyt b_6-f complex.

In cyanobacteria, two c -type cytochromes involved in the electron transport chains have been described: Cyt c_6 and Cyt c_{550} . While Cyt c_6 is a soluble electron carrier involved both in photosynthesis and in respiration, Cyt c_{550} is structurally bound to photosystem (PS) II and is not directly involved in electron transport [1]. One further c -type cytochrome has been described in cyanobacteria: the so-called Cyt c_M [2]. The gene encoding for Cyt c_M is conserved in nearly every sequenced cyanobacterium [3,4], and it has been consistently studied in the unicellular organism *Synechocystis* sp. PCC 6803 [5,6]. In *Synechocystis*, the annotated gene contains two methionine codons (Figure 1), of which the distal one was proposed to be the actual start codon as it was preceded by a GA-rich motif, i.e., a probable ribosome binding site [2,6]. The sequence further comprised a hydrophobic amino-terminal region that was assumed to constitute either a membrane anchor or a signal peptide. However, due to its close similarity to the signal peptide of Cyt c_6 from *Anabaena* sp. PCC 7119, the latter was regarded as the most probable option [6].

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ATG GCC CCT GTA ATC GAA AAA AGT CCA ACT GTT GCC ACG GTA AAT < 45
  M  A  P  V  I  E  K  S  P  T  V  A  T  V  N

GCT TCC CCC ACT GGG ATA TGG ATT ATG GCA GGC ATT GTT TCC CTG < 90
  A  S  P  T  G  I  W  I  M  A  G  I  V  S  L

GTG ATA TTG GCA GTG GCT TTG TTT AGT TTT ATG AAC TTT GAC CCC < 135
  V  I  L  A  V  A  L  F  S  F  M  N  F  D  P
                                ▼
TAT GTA AGC CAG GTA CTC GCT CTC AAA GGA GAT GCG GAT AGG GGT < 180
  Y  V  S  Q  V  L  A  L  K  G  D  A  D  R  G
                                ▲
CGG GCC ATT TTT CAA GCT AAC TGT GCA GTT TGC CAT GGC ATC CAA < 225
  R  A  I  F  Q  A  N  C  A  V  C  H  G  I  Q

GCT GAC GGT TAC ATT GGC CCC AGT CTT TGG GGG GTA TCC CAA CGG < 270
  A  D  G  Y  I  G  P  S  L  W  G  V  S  Q  R

CGG TCC CAG AGC CAC ATC ATT CAT CAG GTG GTG AGT GGT CAA ACT < 315
  R  S  Q  S  H  I  I  H  Q  V  V  S  G  Q  T

CCT CCC ATG CCC CAG TTT GAA CCC AAT CCC CAG GAG ATG GCG GAT < 360
  P  P  M  P  Q  F  E  P  N  P  Q  E  M  A  D

TTA TTG AAT TAC CTC AAA ACC CTC TGA < 387
  L  L  N  Y  L  K  T  L  *

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Figure 1. Cyt c_M protein and gene sequences from *Synechocystis* sp. PCC 6803. The two potential translation initiation sites are underlined. The black triangle separates the hydrophobic region (orange) from the hydrophilic one (blue) and marks the point at which the signal peptide was presumed to finish, i.e., right after the VLA motif (boxed). The characteristic CXXCH heme binding motif (boxed) can also be observed.

Cho et al. [5] were the first to successfully detect the protein in cyanobacteria through Western blot analysis. Nevertheless, their results were not clear enough to conclude whether the protein featured the N-terminal hydrophobic domain in vivo or not, since the molecular weight of the protein detected by Western blotting did not match either of the two possible options [6]. As for the protein location, even though Bernroitner et al. [3] claimed to have determined that the thylakoid and plasma membranes are the exact places where Cyt c_M is located in the cell, these results have later been questioned [7] given the high likelihood that cross-contamination between membranes occurred. Ever since, no other author has declared to have successfully established the cellular region where the protein is placed, probably due to its extremely scarce expression levels under normal conditions [8].

Based on the hypothesis that the actual Cyt c_M protein sequence starts with the second methionine and that it contains a signal peptide to the thylakoid lumen—which would be subsequently released thereby generating a soluble globular protein—several authors have tried to determine its physiological function. As an alleged electron carrier similar to Cyt c_6 , speculations about the biological role of Cyt c_M have been mainly linked it to a hypothetical role in respiratory and photosynthetic electron transport chains. The fact that deletion mutants were able to carry out both processes without any significant phenotype [2] dismissed the possibility of Cyt c_M being a main component of any of these systems. However, there was a chance that Cyt c_M participated in respiration or photosynthesis as a secondary element, i.e., as a backup piece that is only expressed under certain conditions to substitute or complement a major constituent. Interestingly, the expression levels of Cyt c_M sharply increase in stress conditions such as low temperature

and high-intensity light whereas those of the two main electron carriers, plastocyanin (Pc) and Cyt c_6 , decrease [9]. Despite some further evidence indicating a “spare component” role for Cyt c_M in photosynthesis [10,11], we ruled this out by showing that the surface electrostatic potential of Cyt c_M is too different from those of Pc and Cyt c_6 and by proving that the kinetic interaction constant between Cyt c_M and PS I, to which Pc and Cyt c_6 donate electrons, is negligible [6]. Moreover, the low redox potential of Cyt c_M (+150 mV) makes it thermodynamically unlikely that it can be reduced by Cyt f (with a redox potential of +320 mV), as is the case with Pc and Cyt c_6 [6,12]. Consequently, only respiration remained as a real possibility. Initially, it was suggested that Cyt c_M could reduce respiratory terminal oxidases (COX) since it was kinetically plausible [3] and it was impossible to create a Δ cytM/PSI double mutant [10]. However, the hypothesis of Cyt c_M working as an electron donor in respiration was dismissed once it was observed that Δ cytM mutants have in fact a higher respiration rate under dark heterotrophic conditions [13], with the lack of COX not affecting this whatsoever [8].

In conclusion, none of the hypotheses formulated to this day regarding the physiological role or the cellular location of Cyt c_M has proved solid, and this matter remains altogether an open debate. Since most attempts to determine any of these have been based on the assumption that it is a small soluble protein located in the lumen of the thylakoid, we have reevaluated the sequence and structure of Cyt c_M in the present work. Our results suggest that, contrarily to what was previously thought, Cyt c_M could be a membrane protein with a structure similar to that of the C subunit of bacterial nitric oxide reductase.

2. Materials and Methods

For the sequence collection, we searched for protein sequences corresponding to the cytM gene appearing in either of the two main repositories of annotated cyanobacterial genes: CYORF [14] (<http://cyano.genome.jp/>, accessed on 11 May 2021) and CyanoBase [15] (<http://genome.microbedb.jp/cyanobase/>, accessed on 11 May 2021).

Secondary and 3D protein structure prediction was made with the tools Phyre² [16] (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>, accessed on 11 May 2021), I-TASSER [17] and AlphaFold [18].

The free software UCSF Chimera [19] (<https://www.cgl.ucsf.edu/chimera/>, version 1.15, accessed on 11 May 2021) was used to visualize protein 3D structures and to determine hydrophobicity and electrostatic potential, as well as for distance measurements and structure matching. For the electrostatic potential, the “Coulombic Surface Coloring” option was chosen. For comparison with NorC, we used the structure of nitric oxide reductase of *Pseudomonas aeruginosa* [20], which we obtained from the Protein Data Bank (<https://www.rcsb.org/>; PDB entry: 3O0R, accessed on 14 May 2021).

3. Results and Discussion

With the purpose of determining whether the N terminal part of the protein encodes for a signal peptide or a transmembrane helix, we retrieved all the protein sequences corresponding to the cytM gene that appear in either of the two main repositories of annotated cyanobacterial genes: CYORF and CyanoBase. A total of 18 sequences was obtained (Table 1), and they were then subjected to a structural prediction through Phyre², which uses the homology recognition (threading) approach.

The first striking result was that 15 out of 18 sequences produced almost identical structures with a long, protruding α -helix (or “tail”) corresponding to the initial section of the protein (Figure 2A). This was the precise same region whose nature—signal peptide or transmembrane domain—has not been clearly established to this day. In fact, the VLA motif, repeatedly alleged as the cleavage site of the signal peptide, is located exactly at the junction of the tail with the “body” of the protein in the newly proposed structure (Figure 2B). The length of this helix motif of 5.1 nm (the average membrane being between 5 and 10 nm wide; Figure 2C) and the remarkably hydrophobic nature of this section (Figure 2D) agree with a membrane anchoring function.

Table 1. Cyt c_M protein sequences retrieved from CyanoBase and/or CYORF.

Organism	Strain	Phyre ² Structure Matching	DB of Retrieval
<i>Gloeobacter violaceus</i>	PCC7421	Yes	CyanoBase
<i>Prochlorococcus marinus</i>	MIT9211	Yes	CyanoBase
<i>Synechococcus elongatus</i>	PCC6301	No *	CyanoBase & CYORF
<i>Synechococcus</i> sp.	WH7803	No *	CyanoBase & CYORF
<i>Synechocystis</i> sp.	PCC6803	Yes	CyanoBase & CYORF
<i>Nostoc</i> sp.	PCC7120	Yes	CyanoBase & CYORF
<i>Prochlorococcus marinus</i>	NATL1A	Yes	CyanoBase & CYORF
<i>Prochlorococcus marinus</i> subsp. <i>pastoris</i>	CCMP1986	Yes	CyanoBase & CYORF
<i>Synechococcus elongatus</i>	PCC7942	No **	CyanoBase
<i>Prochlorococcus marinus</i>	MIT9303	Yes	CyanoBase & CYORF
<i>Synechococcus</i> sp.	RCC307	Yes	CyanoBase & CYORF
<i>Synechococcus</i> sp.	WH8102	Yes	CyanoBase & CYORF
<i>Acaryochloris marina</i>	MBIC11017	Yes	CyanoBase & CYORF
<i>Cyanothece</i> sp.	ATCC51142	Yes	CyanoBase & CYORF
<i>Synechococcus</i> sp.	PCC7002	Yes	CyanoBase & CYORF
<i>Thermosynechococcus elongatus</i>	BP-1	Yes	CyanoBase & CYORF
<i>Microcystis aeruginosa</i>	NIES-843	Yes	CYORF
<i>Prochlorococcus marinus</i>	MIT9313	Yes	CYORF

The “Phyre²” column states whether the structure predicted for that specific sequence by the tool Phyre² matched most of the structures predicted for the other sequences or was different from the rest. * First model proposed by Phyre² did not match the rest, but the second one (the next most probable according to the software’s algorithm) did. ** None of the models proposed for this sequence by Phyre² matched the rest.

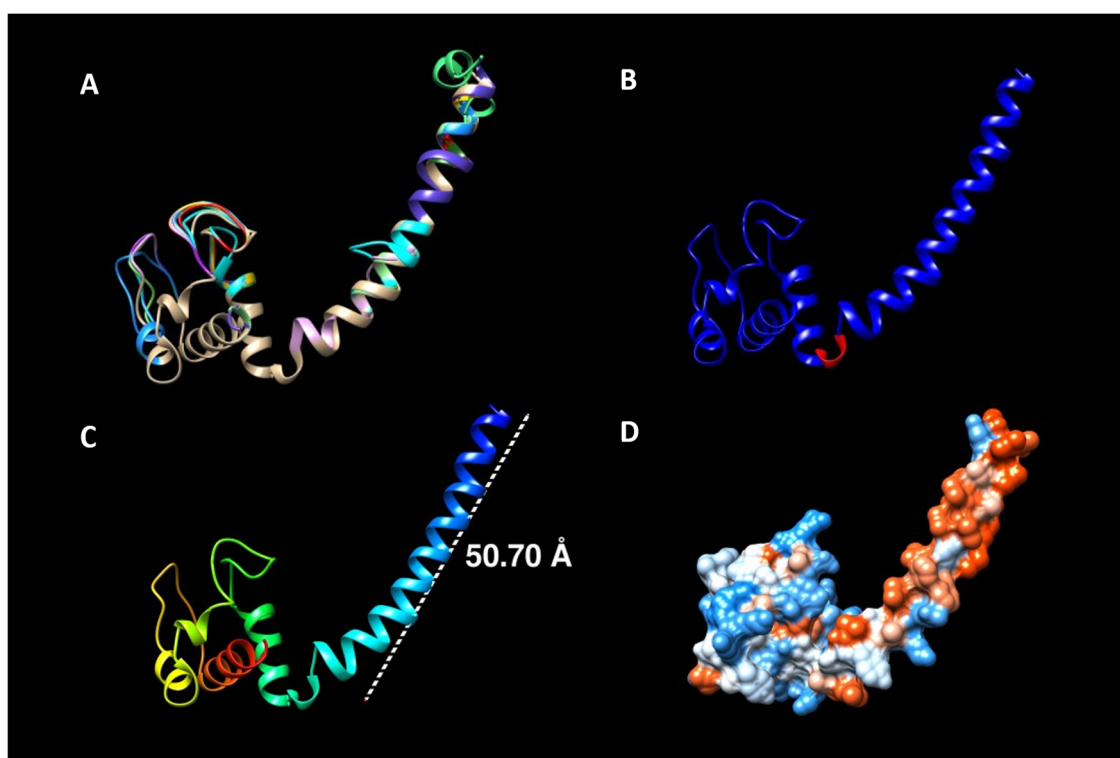


Figure 2. Visualization of the predicted 3D structures for Cyt c_M . (A) shows the superposition of all the 15 predicted structures that coincided, each one displayed in the ribbon representation and bearing a different color. Among all the sequences that gave rise to the same structure, that of *Nostoc* sp. PCC 7120 was used as consensus model for (B–D), since its prediction had both the sequence with the highest coverage (92%) and the structure with the highest confidence index (99.8%). (B) displays in red the place where the VLA motif would be located, whereas (C) shows the length of the tail in a rainbow-colored model. (D) represents the hydrophobicity surface of the model with an orange-hydrophobic/blue-hydrophilic palette. Note the considerably more hydrophobic nature of the tail.

At this point, it is important to bring up the fact that the signal peptides of Cyt c_6 and Cyt c_6 -like proteins from the cyanobacterium *Anabaena* sp. PCC 7119, having a processing site (AXA) downstream from the N-terminal end, are recognized by *E. coli* which results in transport of the proteins to periplasmic space, a subcellular compartment equivalent to thylakoid lumen, where the signal peptide is correctly processed [21–23]. However, the hydrophobic tail of the *Synechocystis* Cyt c_M is not recognized by *E. coli* as a signal peptide. For this reason, it has only been possible to express the soluble region of *Synechocystis* Cyt c_M in *E. coli* by substituting its hydrophobic tail for the signal peptide of *Anabaena* Cyt c_6 [6].

The template protein used by Phyre² for calculating those 15 structures happened to be the *c* subunit of nitric oxide reductase (NorC) from *Pseudomonas aeruginosa*. Thus, the similarity between the returned models and the original template were compared. Surprisingly, the overlap between both proteins was clear and almost total (Figure 3A). To confirm this, a second, independent prediction for the same 15 sequences was performed using I-TASSER, a different structure prediction software that incorporates the ab initio approach in its algorithm. In this new test, the structures obtained were, once again, almost identical to that of NorC (Figure 3B). We obtained similar results once more using AlphaFold, a novel deep learning algorithm that incorporates physical and biological knowledge about protein structure and that leverages multi-sequence alignments [18].

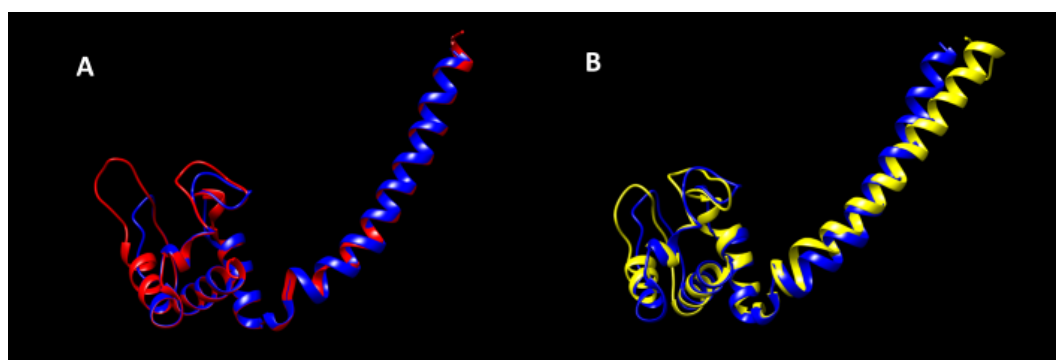


Figure 3. Comparative of the predicted structures of Cyt c_M and NorC. (A) shows the predicted structure for Cyt c_M by Phyre² (blue) vs. NorC (red) while (B) shows the same prediction (blue) vs. the one by I-TASSER (yellow). In both panels, the structure predicted by Phyre² is the one for Cyt c_M of *Nostoc* sp. PCC 7120, as in Figure 2.

Crucially, NorC is a subunit of nitric oxide reductase that has never been found in cyanobacteria, while the core subunit NorB is present in several cyanobacterial species [24] and its expression decreases in Δ cytM mutants [7]. Furthermore, Cyt c_M sharply increases its expression in oxidative stress conditions [9], a condition in which nitric oxide reductase is highly active [25].

In order to determine similarities in charges between Cyt c_M and NorC, we subsequently evaluated their surface electrostatic potential (Figure 4).

Also in this aspect, we found similarities between Cyt c_M and NorC that might be indicative of a similar function, with Cyt c_M acting as a functional part of a nitric oxide reductase and therefore being involved in the oxidative stress response. These included a positively charged tip at the end of the tail, a negatively charged region at the body closest to the tail, another positively charged region on the opposite side of the body and yet one more negatively charged pocket on the bottom. On the other hand, there are some clear differences too, such as a more extensive positive zone in the case of NorC, but the resemblances between both structures here are again undeniable. Moreover, the midpoint redox potential of Cyt c_M and NorC, a fundamental parameter to establish the function of redox proteins, are relatively close with +150 mV and +183 mV, respectively [6,26]. This is interestingly opposed to the major divergences found previously between the surface

electrostatic potential and midpoint redox potential of Cyt c_M and the ones of Cyt c_6 and Pc [6].

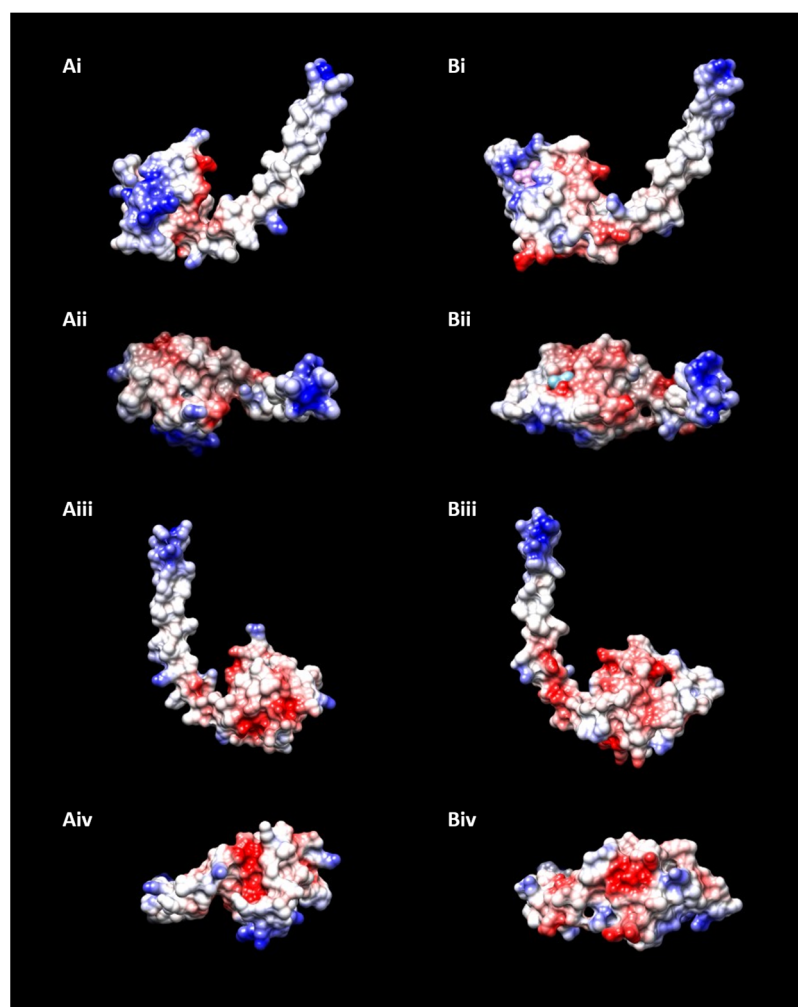


Figure 4. Predicted surface electrostatic potential of Cyt c_M and NorC. Cyt c_M (the same as in Figures 2 and 3) is represented in (A), whereas NorC (the same as in Figure 3) is represented in (B). The displayed POVs are front (i), top (ii), back (iii) and bottom (iv). The color code is as customary, with a blue-positive/red-negative/white-neutral palette.

4. Conclusions

Here we show simple yet solid evidence that Cyt c_M most probably has a transmembrane domain, and that it might well be a protein very similar to—or simply the equivalent of—the C subunit of nitric oxide reductase in cyanobacteria. If this last was the case, it would explain why it has been so difficult to assign any clear function to the protein so far, since all previous studies have focused on the soluble part of it when in fact it could hardly carry out any task without the rest of the protein it belongs to.

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