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In vivo photosystem I reduction in thermophilic and mesophilic cyanobacteria: The thermal resistance of the process is limited by factors other than the unfolding of the partners

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Abstract

Photosystem I reduction by plastocyanin and cytochrome c_6 in cyanobacteria has been extensively studied in vitro, but much less information is provided on this process inside the cell. Here, we report an analysis of the electron transfer from both plastocyanin and cytochrome c_6 to photosystem I in intact cells of several cyanobacterial species, including a comparative study of the temperature effect in mesophilic and thermophilic organisms. Our data show that cytochrome c_6 reduces photosystem I by following a reaction mechanism involving complex formation, whereas the copper–protein follows a simpler collisional mechanism. These results contrast with previous kinetic studies in vitro. The effect of temperature on photosystem I reduction leads us to conclude that the thermal resistance of this process is determined by factors other than the proper stability of the protein partners.

Keywords: Cyanobacteria; Cytochrome c_6; Laser flash spectroscopy; *Nostoc*; Photosynthesis; *Phormidium*; Plastocyanin; *Synechocystis*; Thermostability

Cyanobacteria are the only prokaryotic organisms performing oxygenic photosynthesis, with plastocyanin (Pc) and cytochrome c_6 (Cyt) acting as alternative electron donors to photosystem I (PSI) in the photosynthetic electron transfer pathway [1,2]. The way by which PSI, a membrane embedded complex, is reduced by the two small soluble metalloproteins Pc and Cyt has been extensively studied in vitro from a functional, structural, and mechanistic point of view [3–5]. From all these studies, it arises that Pc and Cyt are two very different proteins sharing a number of structural properties that allow both of them to reduce PSI with similar efficiency [6].

PSI reduction by Pc and Cyt, isolated from different sources, follows a hierarchy of kinetic models with a significant increase in efficiency: either an oriented collisional mechanism (type I), a mechanism involving complex formation (type II), or complex formation with rearrangement of the partners to properly orient the redox centres and allow an efficient, fast electron transfer (type III) [6]. Although the process of in vivo PSI reduction in eukaryotic organisms has been widely analysed (see [5], for a review), the information on cyanobacteria is rather scarce [7,8].

Previous studies have emphasized the high thermal stability of Pc from the thermophilic cyanobacterium *Phormidium laminosum* (T_M of ca. 80 °C, [9]) as compared to Pc from mesophilic cyanobacteria (T_M ranging from ca. 60 to 70 °C, [10]). However, the way by which the higher thermostability of proteins is related with the thermal resistance of organisms remains unclear. Indeed, *c*-type cytochromes—including photosynthetic Cyt—are well known to maintain their native folded structure at temperatures well above the thermal range of cell survival (see [11]).

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Here, we report an in vivo analysis of PSI reduction by Pc and Cyt in whole cells of different meso- and thermophilic cyanobacterial species. The efficiency of the reaction mechanism within a wide range of temperatures is investigated, its physiological meaning being discussed and compared with the in vitro data reported previously.

Materials and methods

Synechocystis sp. PCC 6803 and *Nostoc* sp. PCC 7119 were grown photoautotrophically in liquid BG-11 medium at 30 °C as previously described [8], either in the presence or absence of copper. *Phormidium laminosum* was cultured under the same conditions but at 45 °C [12]. When necessary, copper was added at 1 μM, whereas copper depleted medium was supplemented with 300 μM bathocuproinedisulfonic acid (BCSA) as a chelating agent to eliminate any traces of copper [8,13]. Cell cultures with or without copper were pre-adapted to the new conditions by repeated incubation cycles, and cell growth was monitored by spectrophotometrically measuring the chlorophyll content [14].

Isolation of proteins and immunoblotting experiments were carried out as previously described [8,15–17]. Proteins isolated from each cyanobacterial strain were immunodetected with polyclonal antibodies raised against Pc or Cyt from each organism, except in the case of *Nostoc* Pc, for which antibodies against *Synechocystis* Pc were successfully used. Antibodies raised against *Phormidium* Pc and Cyt were kindly provided by Dr. Beatrix Schlarb-Ridley (University of Cambridge, UK). All other antibodies were obtained by inoculating purified proteins into rabbits at the Centro de Producción y Experimentación Animal (Sevilla).

Cyanobacterial cells from each strain were harvested by centrifugation at different growing phases to test in vivo reduction of PSI by laser flash absorption spectroscopy, as previously described [8]. For the analysis of the temperature dependence of PSI reduction kinetics, measurements were performed at different temperatures ranging from 25 to 90 °C, and the values for the observed rate constant (k_{obs}) were normalized to those measured at 30 °C to obtain percentages of relative rates. Sigmoidal fitting of data allowed us to estimate the temperature value at which k_{obs} reached 50% of the initial value (T_{50}). The extent of photoactive PSI was determined by measuring the amplitude of the initial absorbance increase at 820 nm after the laser flash.

Results and discussion

Expression of Pc and Cyt

As previously reported [18–20], cyanobacteria having genes coding for both Pc and Cyt alternatively synthesize each donor depending on copper availability in the medium. In Fig. 1, this regulation is shown for *Synechocystis*, *Nostoc*, and *Phormidium*. Western blot analysis using specific antibodies demonstrated that every strain expressed Pc when copper was added to the medium, whereas Cyt was the donor protein synthesized in the absence of the metal. To ensure the removal of copper traces, the copper quelator BCSA was added to the culture medium [8,13]. In *Synechocystis* and *Nostoc*, the expression control was complete, showing undetectable levels of Cyt in the presence of copper and of Pc in the absence of the metal. However, *Phormidium* cells

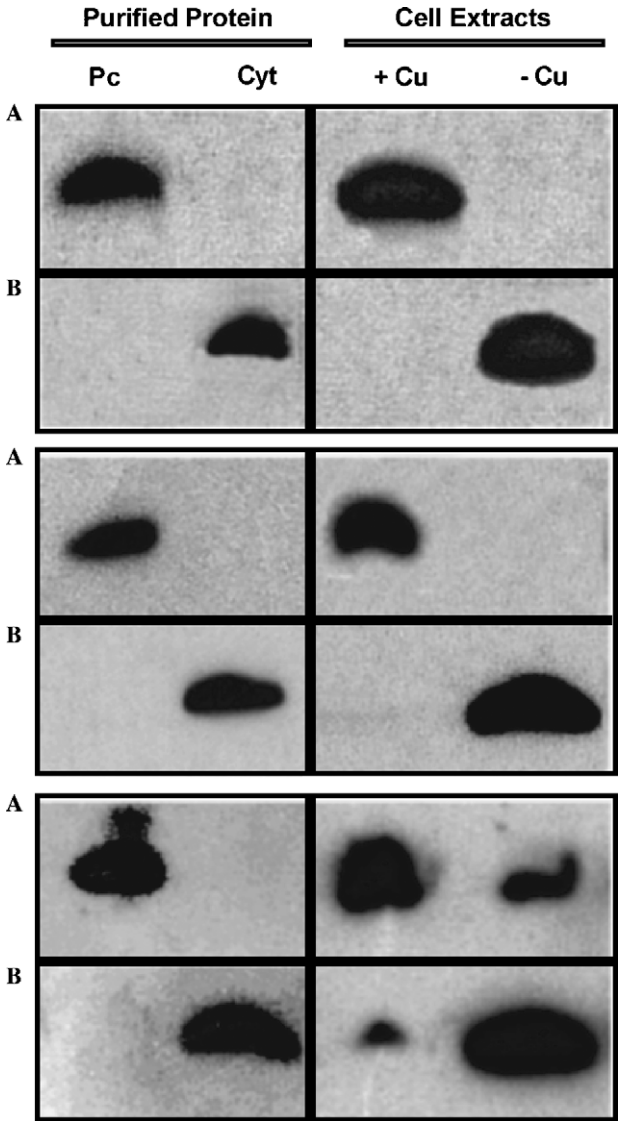

Fig. 1. Immunodetection of Pc and Cyt in wild-type cell cultures of different cyanobacteria grown in the presence and absence of copper. Western blots corresponding to *Synechocystis* (upper), *Nostoc* (middle), and *Phormidium* (lower) are shown. Cell extracts with 100 μg of total protein from each specie were hybridized with specific polyclonal antibodies raised against Pc (A) or Cyt (B) from each cyanobacterium, except in the case of *Nostoc* Pc for which antibodies against *Synechocystis* Pc were used. 0.1 μg of purified Pc and Cyt from each cyanobacterium was used as controls.

showed low basal levels of both proteins in their respective inhibitory conditions (Fig. 1). Whatever the donor was, there were no differences in the photoautotrophic growth in the presence or absence of copper in the medium in all the three tested species (not shown), making evident that Pc and Cyt have a similar efficiency in the global metabolism of cyanobacteria.

In vivo PSI reduction by Pc and Cyt

To test the ability of Pc and Cyt to reduce PSI in vivo in different cyanobacteria, we have performed laser flash

absorption spectroscopy measurements in whole cells cultured both in the presence or absence of copper, assigning the kinetic traces to Pc–PSI and Cyt–PSI interactions, respectively (see Fig. 1 and [8]). Fig. 2 shows that the kinetic traces corresponding to PSI reduction by Pc in the three cyanobacterial cells can be well fitted to a monophasic process, whereas the Cyt–PSI interaction follows in all cases biphasic kinetics, with a first fast component in the microsecond range. These results agree with the kinetic behaviour previously reported for in vivo PSI reduction by both donors in *Synechocystis* [8] and by Cyt in *Synechococcus* [7]. The fast phase in PSI reduction has been typically assigned to the formation of a donor–PSI complex prior to laser excitation. According to the so-called type II or III kinetic models, the transient complex is formed before electron transfer [21], as occurs in eukaryotic organisms both in vivo and in vitro conditions [22–24]. Thus, as a general rule in cyanobacteria, it seems that Cyt interacts with PSI in vivo by following a more complex and efficient mechanism than Pc does.

The results reported here for the in vivo reduction of PSI contrast with the kinetic behaviour previously described for the isolated proteins. Thus, in *Synechocystis* and *Phormidium*, monophasic kinetics corresponding to a single collisional mechanism (type I) have been reported for both Pc–PSI and Cyt–PSI interactions [10,25], whereas in *Nostoc* the same simple mechanism takes place for PSI reduction by Pc but the more sophisticated process found in eukaryotes occurs in the Cyt–PSI interaction (see [17,21]). The disagreement between the in vitro and in vivo results can be explained taking into account that the donor–PSI complex formation is a very precise process that can be altered by subtle changes coming from the different donor–PSI environments existing both in vitro and in vivo, namely the presence or absence of the thylacoidal membrane and detergent molecules. Besides, there can be differences in the protein–protein complex configurations in vivo and in vitro, allowing Cyt being docked in vivo in a more optimal arrangement regarding complex formation and electron transfer to PSI. Differences between in vivo and in vitro kinetic behaviour in other photosynthetic redox proteins have been previously reported [24,26,27].

Table 1 summarizes the values obtained for the observed rate constants corresponding to the monophasic kinetics (k_M) for Pc–PSI interaction and the fast (k_F) and slow (k_S) phases—as well as the percentage of the kinetic amplitude corresponding to the fast phase—in the Cyt–PSI interaction. Monoexponential kinetics with Pc and the slower component with Cyt showed k_M and k_S values of the same order of magnitude in the three cyanobacteria (Table 1). On the other hand, the fast phase for the Cyt–PSI interaction also showed values

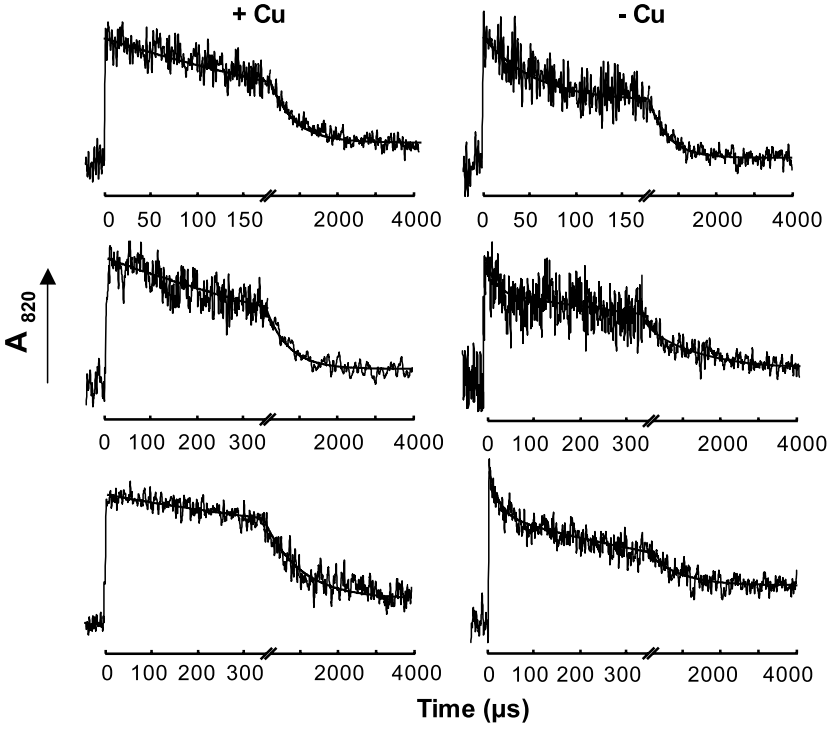

Fig. 2. Kinetic traces showing in vivo PSI reduction in *Synechocystis* (upper), *Nostoc* (middle), and *Phormidium* (lower). The cells were grown in the presence (left) or absence (right) of copper. The reaction cuvette contained an amount of cells equivalent to a total chlorophyll content of 150–300 μg mL^{-1}. Absorbance changes were recorded at 820 nm. Kinetic traces were fitted to either mono or biexponential functions.

Table 1
Observed rate constants for the in vivo PSI reduction in different cyanobacteria

Cyanobacterium	Plastocyanin	Cytochrome c_6	
	k_M[a] $\times 10^{-3}$ (s^{-1})	k_F[b] $\times 10^{-4}$ (s^{-1})	k_S[c] $\times 10^{-3}$ (s^{-1})
Synechocystis	2.7 ± 0.2	4.5 ± 0.6 (37%)	2.0 ± 0.2
Phormidium	1.8 ± 0.6	2.1 ± 0.5 (27%)	1.9 ± 0.6
Nostoc	3.2 ± 0.5	5.6 ± 0.6 (26%)	1.2 ± 0.7

[a] Observed rate constant for monophasic kinetics.
[b] Observed rate constant for the fast phase of biphasic kinetics; percentages of amplitude for the fast phase are shown in parentheses.
[c] Observed rate constant for the slow phase of biphasic kinetics.

of the same order in all cases, although *Nostoc* presents the faster rate. In every case, the percentage of total amplitudes corresponding to the fast phase was ca. 25–35%, no differences being observed throughout the culture growth. Although there are no significant differences among organisms, those with faster Pc–PSI reaction have also a faster fast phase for the Cyt–PSI interaction. In agreement with previous reports in vitro (reviewed in [4]), *Nostoc*—whose donors possess positive electrostatic areas—is the organism with the most efficient systems both in vitro and in vivo. From the k_M and k_S values and the reported kinetic constants for in vitro experiments (see Table 2 of [10]), an estimated value of ca. 200 μM for the effective concentration of donor protein at the surroundings of PSI can be determined.

Dependence of the kinetic parameters for the donor–PSI interaction with temperature

Up to now, the main focus of the effect of temperature on the donor–PSI system has been the analysis of the thermal stability of the individual donor proteins, as well as the study of the thermal resistance of PSI reduction in vitro [9,10,28–30]. However, no information has been provided on the latter process in vivo. Here, we have selected two cyanobacterial species, the mesophilic *Synechocystis* and the thermophilic *Phormidium*, to test in a comparative way the thermal resistance of the PSI reduction process by Pc and Cyt in vivo. Fig. 3 shows the temperature dependence of the relative rates for PSI reduction under conditions of Pc (upper panel) or Cyt (middle panel) expression, both in *Synechocystis* and *Phormidium*. The values of k_M and k_S were normalized taking those at 30 °C as 100%. From the fittings of data in Fig. 3, the values for the midpoint temperature (T_{50}) in relative rates of PSI reduction could be determined (Table 2). Higher T_{50} values are obtained in *Phormidium* as compared with *Synechocystis*, with both Pc and Cyt, as expected from the thermophilic nature of the former organism.

Interestingly, the T_{50} values of PSI reduction are quite below those obtained for the melting temperature (T_M) reported for the individual purified soluble proteins [9–11]. In addition, Fig. 3 (lower panel) and Table 2 also show that in both *Phormidium* and *Synechocystis*, the T_{50} value for PSI photoactivity is ca. 20 °C higher than the values obtained for both Pc–PSI and Cyt–PSI relative rates, no differences being observed in PSI photoactivity in the presence or absence of copper (not shown). These results suggest that, even in vivo, PSI maintains an apparently unaffected activity at temperatures that impair its

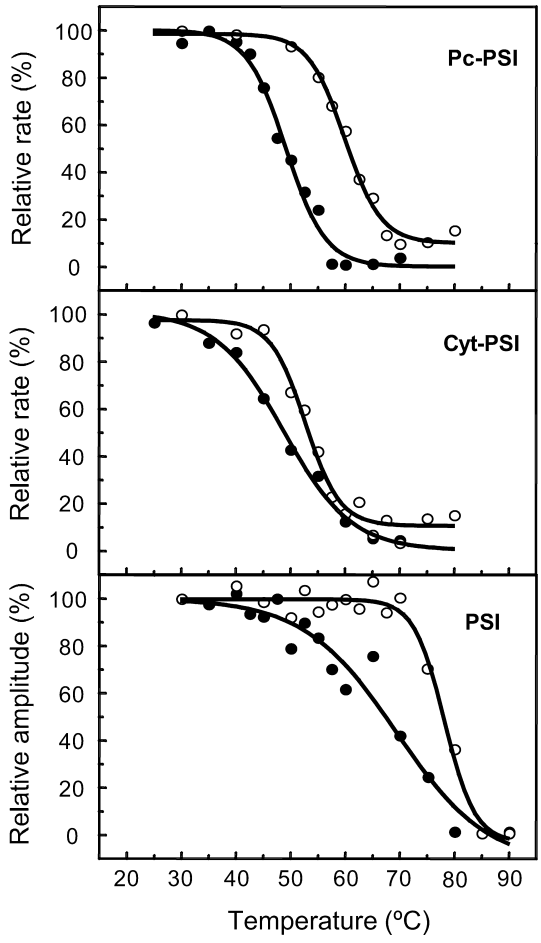

Fig. 3. Dependence on temperature of k_{obs} for the in vivo reduction of PSI by Pc (upper) and Cyt (middle), and of PSI photoactivity (lower). Values were normalized with respect to those obtained at 30 °C. Continuous lines represent the sigmoidal fitting of experimental data.

Table 2
Values for midpoint temperature (T_{50}) of in vivo Pc–PSI and Cyt–PSI interaction kinetic efficiency, and of photoactive PSI in *Synechocystis* and *Phormidium*

Cyanobacterium	T_{50} (°C)		
	Pc–PSI	Cyt–PSI	PSI
Synechocystis	49.2 ± 0.6	48.8 ± 0.8	69.4 ± 3.5
Phormidium	60.0 ± 0.4	54.1 ± 0.7	78.2 ± 0.7

physiological interaction with both Pc and Cyt, and also that the molecular interaction between the two donors and PSI is affected before the unfolding of the proteins takes place.

It is also interesting to note that the T_{50} value for the Pc–PSI system is higher than that for the Cyt–PSI system in *Phormidium*. This difference could arise from the different forces involved in the process. Thus, the Pc–PSI interaction has been reported to implicate a higher entropic and lower enthalpic component in the overall reaction as compared with the Cyt–PSI system [10]. Consequently, it can be concluded that in *Phormidium*, hydrophobic interactions are prevalent on electrostatic forces when Pc is used as electron donor instead of Cyt [10,31], the hydrophobic interactions being less affected by temperature changes than the electrostatic ones.

Concluding remarks

Based on the in vitro analysis of the different donor–PSI systems, it has been previously proposed that Cyt is the ancestral donor to PSI and was first "discovered" by Nature when cyanobacteria started to perform oxygenic photosynthesis [6]. So, in cyanobacteria, the interaction with PSI was first optimized with Cyt, whereas in these organisms Pc follows a simpler kinetic model. Later on in the evolution, in primitive eukaryotes, namely green algae, Pc became even more efficient than Cyt [6]. Our results support this evolutionary theory of the electron donation to PSI, Cyt showing a more efficient and complex mechanism than Pc in ancestral organisms like cyanobacteria.

The higher thermal stability of purified electron donors as compared with the overall kinetic rate of PSI reduction reveals that, in vivo, this process is limited by the weakening of the protein–protein interactions rather than by the unfolding of partners. The ability of proteins from thermophilic species to keep the native conformation at temperatures beyond their own physiological thermal range can be explained as a resistance mechanism to maintain the structural integrity of components without harmful disorganization at shocks of extreme temperatures. This thermal resistance seems to be particularly evident in membrane protein complexes, like PSI.

Acknowledgments

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