

Cytochrome *c*-549 – an endogenous cofactor of cyclic photophosphorylation in the cyanobacterium *Anacystis nidulans*?

Peter F. Kienzl and Günter A. Peschek

Biophysical Chemistry Group, Institute of Physical Chemistry, University of Vienna, Währingerstraße 42, A-1090 Vienna, Austria

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The cyanobacterium *Anacystis nidulans* synthesizes fairly large amounts of low potential cytochrome *c*-549 ($E'_0 = -260$ mV) during late logarithmic and stationary growth phases. We show here that isolated cytochrome *c*-549 catalyzed high rates of cyclic photophosphorylation by membrane preparations of *A. nidulans* when added in about 50% reduced state; fully oxidized or reduced cytochrome *c*-549 was far less efficient. A similarly decisive role of proper redox poising was documented with ferredoxin as the cofactor of cyclic photophosphorylation. Inhibitor studies indicated that plastoquinone and cytochrome *f* are involved in cyclic electron flow catalyzed by cytochrome *c*-549 and ferredoxin.

Low potential <i>c</i> -type cytochrome	Cytochrome <i>c</i> -549	Cyclic photophosphorylation	Ferredoxin
Redox poising	<i>Anacystis nidulans</i>	<i>Cyanobacteria</i>	

1. INTRODUCTION

Cyanobacteria (blue-green algae) are a major group of phototrophic prokaryotes uniquely capable of oxygenic, plant-type photosynthesis. Their photosynthetic mechanisms differ only little from those of chloroplasts [1,2]. Accordingly, chlorophyll-containing membranes and lyophilized cells prepared from, e.g., *Anacystis nidulans*, have been shown to catalyze both non-cyclic and cyclic photophosphorylation [3–5]. Ferredoxin, a low potential iron–sulfur redox protein ($E'_0 = -420$ mV) is believed to be the endogenous elec-

tron mediator of cyclic electron transport in photosystem I [5,6].

A low potential *c*-type cytochrome, cyt. *c*-549 ($E'_0 = -260$ mV), has been isolated, besides two other soluble *c*-type cytochromes (*c*-554 and *c*-552), from the cyanobacterium *A. nidulans* [7–9]. However, thus far the physiological functions of these cytochromes have received only scant attention. Cytochrome *c*-554 was recently shown to catalyze electron transfer from cyt. *f* to P700 in *Anacystis* membranes [10] thus replacing plastocyanin which is not synthesized by *A. nidulans*. Neither cyt. *c*-554 nor *c*-549 appear to be effective electron donors to the membrane-bound cyanobacterial cyt. *c* oxidase [11]. Attempts to re-isolate cyt. *c*-552 [8] were unsuccessful [11] but its possible identity with a periplasmic *c*-type cytochrome in *A. nidulans* was recently inferred from spectral measurements [12].

Cytochrome *c*-549 is almost undetectable in *A. nidulans* during early and mid logarithmic growth. Yet, in late logarithmic and stationary cells its in-

Abbreviations: cyt, cytochrome; chl, chlorophyll; Fd, ferredoxin; PMS, phenazine methosulfate; DSPD, disalicylidene-1,3-diaminopropane; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

tracellular concentration may reach the level of Fd (i.e., about $50 \mu\text{M}$; unpublished). This fact, together with its fairly negative redox potential prompted us to re-investigate the possible role of cyt. *c*-549 as an electron mediator for cyclic photophosphorylation in membrane preparations of *A. nidulans*.

2. MATERIALS AND METHODS

Axenic cultures of *A. nidulans* (*Synechococcus* sp., strain 1402-1, Göttingen) were grown at 38°C in an illuminated turbidostat as in [11]. Cells were maintained in the late logarithmic growth phase. For the isolation of Fd and cyt. *c*-549 harvested and washed cells were lyophilized and processed according to [8]. Solutions of cyt. *c*-549 and Fd (in phosphate buffer) were concentrated by lyophilization and dialyzed against 50 mM Tris-Hepes buffer (pH 7.6). Concentrations were adjusted by use of the following extinction coefficients ($\text{mM}^{-1} \cdot \text{cm}^{-1}$): Fd (oxidized; 423 nm), 7.8 [13] and cyt. *c*-549 (reduced; 549 nm), 20 [9].

Cytochrome *c*-549 and Fd were reduced with excess solid dithionite, followed by dialysis against N_2 -flushed Tris-Hepes buffer (pH 7.6). Since both reduced Fd and cyt. *c*-549 are easily autoxidized by atmospheric oxygen this was carefully excluded from all solutions containing reduced forms of either redox protein. Samples containing defined proportions of the reduced form of cyt. *c*-549 or Fd were obtained by anaerobic titration, in spectrophotometer cuvettes, with 10 mM sodium dithionite solution while continuously monitoring the height of the peaks at 549 and 423 nm, respectively.

Isolated membranes of *A. nidulans* were prepared by French press treatment (700 kg/cm^2) of lysozyme-pretreated cells [14], resuspended in 0.35 M mannitol containing 50 mM Tris-Hepes buffer plus 2 mM MgCl_2 (pH 7.6), and sedimented at $144000 \times g$ (4°C , 1 h). The washed membranes gave rates of 'endogenous' cyclic photophosphorylation of $<3\text{--}5 \text{ nmol ATP/h} \cdot \text{mg chl}$.

Cyclic photophosphorylation was measured on 3 ml anaerobic membrane suspensions (0.35 M mannitol, 50 mM Tris-Hepes buffer, 5 mM MgCl_2 , $10 \mu\text{M}$ DCMU; final pH 7.6; 10–25 $\mu\text{g chl/ml}$) in temperature-controlled plexiglass cuvet-

tes ($5 \times 5 \times 0.3 \text{ cm}$) at 25°C and $1.5 \mu\text{E/cm}^2 \cdot \text{min}$ of 707 nm light which was provided by a 1000 W Oriel Xenon lamp and passed through a Schott interference filter (half-band width 12 nm); 2 mM $\text{K}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7.6; containing about $10^6 \text{ cpm } ^{32}\text{P/ml}$) and 3 mM ADP were present as phosphorylation substrates. All experiments were performed under O_2 -free N_2 . Reactions were started by switching on the light (however, cf. table 1) and terminated with trichloroacetic acid. ATP was determined by liquid scintillation counting after extraction of unreacted phosphate [15].

3. RESULTS

Fig.1 shows that samples of cyt. *c*-549 or Fd containing different percentages of the reduced form displayed markedly different activities in catalyzing cyclic photophosphorylation. Highest rates were observed with about 50% of the redox proteins initially present in the reduced state. Under these conditions the overall redox state of the cofactors did not change measurably during the reactions when monitored spectrophotometrically (not shown). Thus the cofactors may be about 50% reduced also under steady-state conditions; in vivo measurements would be needed, however, to

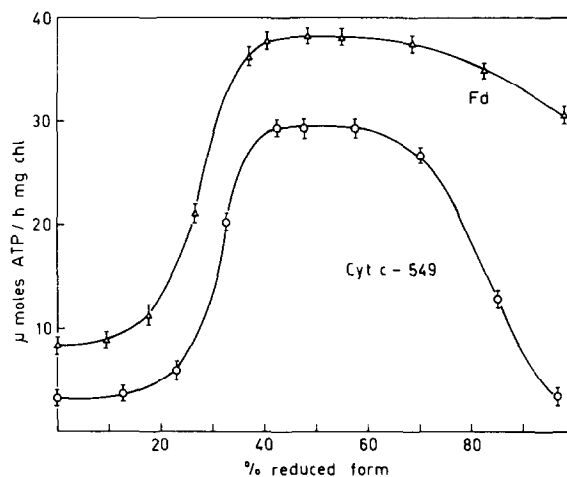


Fig.1. Rate of ATP formation by illuminated anaerobic membrane preparations of *A. nidulans* supplemented with Fd or cyt. *c*-549 containing different percentages of the reduced forms. Total concentration of each redox protein was $70 \mu\text{M}$.

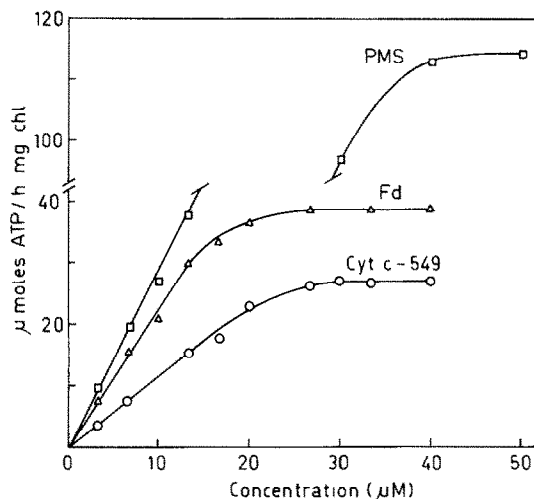


Fig. 2. Dependence of cyclic photophosphorylation on the concentration of the reduced form of added cofactors. Fd and cyt. *c*-549 were present in 50% reduced states. PMS was added together with 3 mM sodium ascorbate.

substantiate this conclusion. From a comparison of fig. 1 and 2 it is evident that the oxidized form of cyt. *c*-549 and Fd had little influence on the rate of ATP formation except at very low concentrations of the reduced form. Under optimum conditions Fd was only 30% more efficient than cyt. *c*-549 (fig. 1). Results with PMS, an electron mediator commonly used to obtain high rates of cyclic photophosphorylation by chloroplast preparations, are included in fig. 2, 3. Reaction rates with each of the cofactors were linear up to at least 8 min after the start of illumination (fig. 3).

Table 1 shows the effect of inhibitors on cyclic photophosphorylation mediated by Fd, cyt. *c*-549 and PMS. Clearly, the pathway of PMS-mediated cyclic electron flow is distinct from that with Fd or cyt. *c*-549 as none of the usual electron transfer inhibitors affected PMS-catalyzed cyclic photophosphorylation (cf. [16]). On the other hand, both cyt. *c*-549 and Fd appear to transfer electrons from the primary acceptor of photosystem I to plastoquinone since DBMIB was strongly inhibitory. EDAC, believed to block the reduction of cyt. *f* [17], likewise inhibited both types of reaction. Yet, clearcut distinction between Fd and cyt. *c*-549 is offered by the strikingly different effects of DSPD and CO on photophosphorylation mediated by either redox

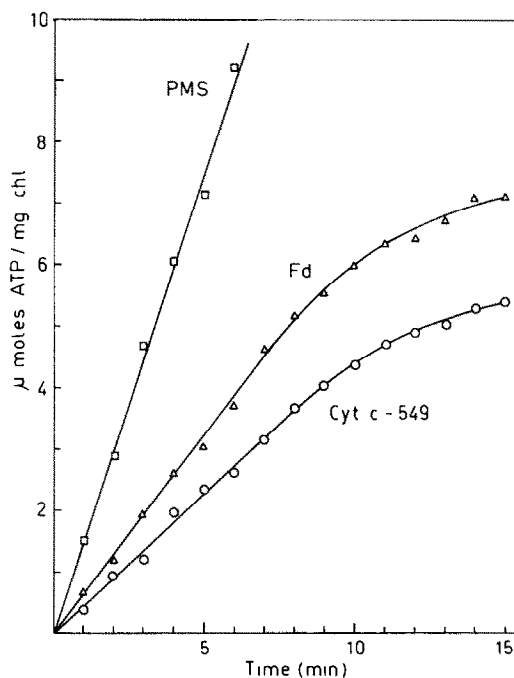


Fig. 3. Time course of cyclic photophosphorylation. Fd and cyt. *c*-549 were present in 50% reduced states (70 μM total). PMS (50 μM) was added together with 3 mM sodium ascorbate.

protein: DSPD, a compound known to interfere with Fd-supported photosystem I reactions [18], specifically inhibited Fd-mediated cyclic photophosphorylation while carbon monoxide specifically inhibited the cyt. *c*-549-mediated pathway (table 1); isolated cyt. *c*-549, like many other low potential *c*-type cytochromes, forms a complex with CO [7-9]. At the same time the effect of CO appears to leave little doubt that cyt. *c*-549 indeed can support cyclic photophosphorylation by the *Anacystis* membranes.

The uncoupler atebriol abolished ATP formation with all electron mediators employed, and the same was true of DCCD which blocks the membrane-bound ATPase. We did not test whether DCCD also inhibited electron flow, proper, as was recently shown with isolated chloroplasts [19]. Yet, the concentration of DCCD used in our experiments was rather low (cf. [19]) and it also inhibited the PMS-catalyzed phosphorylation (table 1). Thus we conclude that it acted primarily on the membrane-bound ATPase in our experiments.

Table 1

Effect of inhibitors on the rate of cyclic photophosphorylation catalyzed by Fd, cyt. *c*-549 and PMS in membrane preparations of *A. nidulans*

Inhibitor	Cofactor		
	Fd	Cyt. <i>c</i> -549	PMS
None	100	100	100
DCMU (50 μ M)	100	100	100
DBMIB (5 μ M)	5	12	110
DSPD (0.5 mM)	9	93	97
CO (100%) ^a	100	8	100
EDAC (0.5 mM)	14	19	96
Atebrin (0.1 mM)	<1	<1	<1
DCCD (10 μ M)	7	5	8

^a Membrane suspensions flushed with pure CO instead of N₂

Inhibitors were added to dark anaerobic membranes 5 min before adding the cofactors. After 5 min the light was switched on and pre-illumination was continued for another 5 min. Reactions were then started by addition of the phosphorylation substrates and terminated after 5–7 min. Rates are expressed as % of controls. Samples contained 10–25 μ g chl/ml. Fd and cyt. *c*-549 were added as anaerobic solutions reduced about 50% with dithionite (see section 2). Final concentrations (total) of Fd, cyt. *c*-549 and PMS were 70, 70 and 50 μ M, respectively. PMS was added together with 3 mM sodium ascorbate: 100% = 38.8 (Fd), 27.1 (cyt. *c*-549) and 114.5 (PMS) nmol ATP \cdot h⁻¹ \cdot mg chl⁻¹

4. DISCUSSION

Low potential *c*-type cytochromes capable of forming complexes with carbon monoxide were isolated from various sources, among them algae and cyanobacteria [20]. Little is known about their physiological functions in vivo. For example, cyt. *c*-548 from *Nostoc* MAC could not replace NAD(P)⁺ as an electron acceptor in the glucose oxidase reaction but was rapidly reduced by NAD(P)H in soluble cell-free extracts [21]. The function of cyt. *c*-549 as a terminal oxidase in *A. nidulans* is unlikely in view of, e.g., lack of inhibition by cyanide [7–9]; it may contribute, however, to the low rates of cyanide resistant respiration in this cyanobacterium [22].

These results demonstrate that cyt. *c*-549 can act

as an efficient electron mediator for cyclic photosynthetic electron transport coupled to phosphorylation in particle preparations of *A. nidulans*. Its negative redox potential and its fairly high abundance in late logarithmic to stationary phase cells let it appear plausible that cyt. *c*-549, besides Fd, functions as an endogenous cofactor of cyclic photophosphorylation under certain conditions in vivo. However, fully oxidized cyt. *c*-549 was a poor catalyst of cyclic photophosphorylation; this was also observed, though to a lesser extent, with Fd. Our findings thus confirm the pivotal importance of proper redox poising of the cyclic electron flow system: With chloroplast preparations partly reduced Fd permitted rates of cyclic photophosphorylation 15-fold higher than the rates catalyzed by (initially) fully oxidized Fd [23]. Owing to the fact that isolated cyt. *c*-549 ($E'_0 = -260$ mV), similar to Fd, inevitably is fully oxidized in solutions equilibrated with air its role as a cofactor of cyclic photophosphorylation might have escaped previous investigators.

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