# ON THE PATHWAY OF ELECTRON TRANSPORT IN CYCLIC PHOTOPHOSPHORYLATION

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## 1. Introduction

Cyclic photophosphorylation is defined as ATP synthesis from ADP and orthophosphate coupled to electron transport from the light-generated primary reductant of photosystem I of photosynthesis to the oxidizing side of PS I. Experimentally, it can be distinguished from any other type of photosynthetic phosphorylation on the basis of two criteria: (a) cyclic phosphorylation is not affected by specific inhibitors of photosystem II, such as CMU, DCMU and orthophenanthroline. (b) It requires activation by light of only PS I.

The pathway of electron flow in cyclic phosphorylation is still largely unknown. When tested in isolated chloroplasts prepared according to conventional methods, cyclic phosphorylation shows an absolute requirement for added electron transport cofactors [1]. Most effective are non-physiological compounds such as PMS and pyocyanine [1]. More recently ferredoxin was found to catalyze cyclic photophosphorylation, but the concentrations necessary to saturate the system were 20 to 100 times higher than those required to saturate the non-clycic photoreduction of NADP by the same chloroplast preparations [2].

Only by the use of "class 1" chloroplast preparations was it possible to demonstrate cyclic phosphorylation in the absence of added electron carriers [3]. The chloroplasts prepared in this way retain a considerable capacity for the complete photosynthetic process [4] and can reduce NADP in the light at substantial rates without any ferrodoxin added, with a stoichiometry of 2 ATPs made per NADPH produced [5]. It was previously shown that the chloroplast flavoprotein ferrodoxin-NADP reductase, NADPH-cytochrome *f* reductase [6, 7] is an essential catalyst of cyclic photophosphorylation [3].

In the present investigation we demonstrate that the cyclic electron flow can be diverted from the reducing side of PS I to $O_2$ by means of a low-potential, autoxidizable dye such as methylviologen ($E'_0 = -440$ mV) resulting in a complete inhibition of cyclic phosphorylation. Phosphorylation can be restored to rates considerably higher than the initial ones by feeding in electrons at the level of the flavoprotein by an NADPH-generating system. It is also shown that dibromothymoquinone, a potent inhibitor of plastoquinone-dependent reactions [8–10], does not affect cyclic phosphorylation at concentrations which drastically inhibit NADP photoreduction. It can therefore be concluded that cyclic phosphorylation requires the following pathway of elec-

*Abbreviations*:

| | |
|---|---|
| CMU | : 3-(4-chlorophenyl)-1,1-dimethylurea |
| DCMU | : 3-(3,4-dichlorophenyl)-1,1 dimethylurea |
| PS I | : photosystem I |
| PS II | : photosystem II |
| PMS | : phenazinemethosulphate |
| Fd | : ferredoxin |
| Fp | : flavoprotein |
| PC | : plastocyanin |
| MV | : methylviologen |
| DBMIB | : dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) |
| HEPES | : *N*-2 hydroxyethylpiperazine *N'*-2-ethane sulphonic acid |
| Chl | : Chlorophyll |

Table 1
Cyclic phosphorylation and phosphorylation coupled to flavoprotein oxidation by photosystem I.

| Additions | "Endogenous" system | PMS system |
|---|---|---|
| None | 20.0 | 556 |
| Methylviologen 1 mM | 2.3 | – |
| NADP 1 mM; $Gl_6$-P 5 mM $Gl_6$-P dh 100 units | 2.6 | 528 |
| Same plus methyl viologen 1 mM | 41.0 | – |

Conditions: HEPES buffer 0.06 M; pH 8.0; ADP 1 mM; $MgCl_2$ 4 mM; phosphate 2.5 mM containing 100,000 cpm of $^{32}P$; CMU 0.04 mM; chloroplasts containing 10 μg chlorophyll. In the PMS system, phenazinemethosulfate was added at the concentration of 0.02 mM. Dark controls were run with all additions indicated and no ATP synthesis occurred in the absence of illumination. Temperature: 22°. Illumination: saturating white light. All figures indicate specific activities, in μmoles of ATP·mg $Chl^{-1}\cdot hr^{-1}$.

tron transport: PS I → X → Fd → Fp → (cyt. *f*, PC) → PS I. Furthermore, the span Fp → (cyt. *f*, PC) → PS I → X → MV → $O_2$ is coupled to phosphorylation.

## 2. Methods

Chloroplasts were prepared from spinach leaves according to Heldt and Sauer [11]. Photophosphorylation was measured according to Avron [12], NADP reduction as previously described [5] and chlorophyll after Arnon [13]. Dibromothymoquinone was obtained through the courtesy of Dr. A. Trebst.

All other reagents were analytical grade commercial products. Glucose-6-phosphate dehydrogenase was purchased from Boehringer, Mannheim, Germany and dialyzed against buffer before use.

## 3. Results and discussion

### 3.1. *Pathway of cyclic electron transport*

Cyclic photophosphorylation in the presence of 0.04 mM CMU ( to inhibit completely any electron flow from PS II) was found to produce ATP at rates

Table 2
Effect of antimycin A on cyclic photophosphorylation.

| Additions | ATP formed (μmoles·mg $Chl^{-1}\cdot hr^{-1}$) |
|---|---|
| None | 13.5 |
| Antimycin A 0.02 mM | 6.2 |
| NADP 1 mM; $Gl_6$-P 5 mM; $Gl_6$-P dh 100 units and methylviologen 1 mM | 55.0 |
| Same, plus antimycin A | 48.5 |

Conditions: as in table 1 – Chlorophyll: 48 μg.

ranging from 15 to 50 μmole per mg chlorophyll per hr in a large number of experiments over a period of 3 years. The structural integrity of the chloroplast membrane is not required *during* the light reaction, but only during the isolation of the particles [14]. Table 1 shows two different and opposite conditions for virtually complete inhibition of cyclic phosphorylation. The first one is established by addition of methylviologen, a low-potential autoxidizable dye, which is a very efficient electron acceptor for PS I. Upon addition of methylviologen the electrons are withdrawn from the cycle and diverted to $O_2$. The second inhibitory condition is established by addition of NADPH and a NADPH generating system ($Gl_6$-P and $Gl_6$-P dehydrogenase). This system is capable of keeping the flavoprotein in the fully reduced state [15]. Since no electron acceptor is available to PS I, phosphorylation is inhibited (table 1). This condition does not affect PMS catalyzed cyclic phosphorylation because PMS cycle bypasses the flavoprotein (table 1). When methylviologen is added together with the NADPH generating system, phosphorylation is restored to a rate 2 to 4 times faster than the endogenous one (tables 1 and 2). These observations indicate that the cyclic electron flow has been "opened" and changed into an open chain as follows: NADPH → Fp → (cyt. *f*, plastocyanin?) → PS I → X → MV → $O_2$. Both pathways include a phosphorylating step. The rate of phosphorylation is higher in the non-endogenous cycle due to the fast reoxidation of methylviologen by $O_2$. Direct evidence for the participation of cytochrome *f* is not available. However, the well-

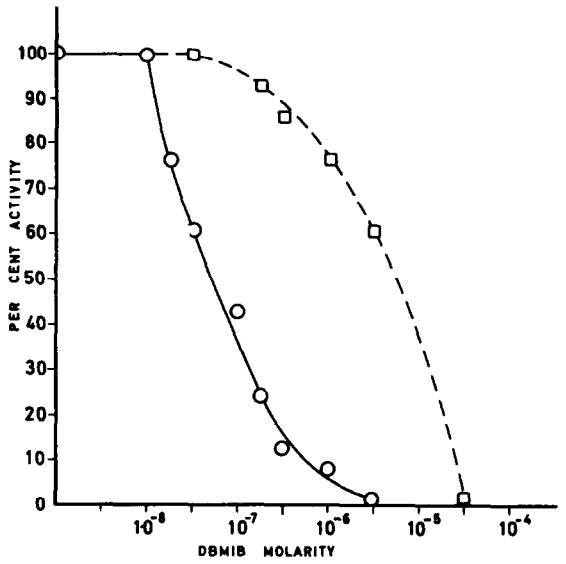


Fig. 1. Effect of DBMIB on cyclic phosphorylation and on NADP photoreduction. Conditions: cyclic phosphorylation, as in table 1. NADP reduction: HEPES buffer 0.06 M, pH 8.0; NADP 0.5 mM; Fd 0.003 mM; $NH_4Cl$ 1 mM; Chl. 33 μg o—o: NADP reduction; □—□ cyclic phosphorylation. Control specific activities were 180 and 27.7 for NADP reduction and cyclic phosphorylation, respectively.

known cytochrome *f* reductase activity of the flavoprotein [6, 7, 15], and the fact that steady-state kinetics of that reaction indicate the formation of a ternary complex NADPH–Fp–cyt.*f* [7] are suggestive evidence for the participation of this cytochrome. Furthermore, Racker and coworkers have demonstrated the participation of cytochrome *f* in PMS-catalyzed cyclic phosphorylation by immunological techniques [16]. Their system, a sonicated chloroplast preparation, does not catalyze phosphorylation without addition of electron carriers. Cytochrome *f* is not available to the antibody in our type of preparation [16]. Evidence that *b*-type cytochromes are not involved in the reconstituted, open chain pathway is provided by the lack of inhibition of this pathway by antimycin A (table 2). It can be seen that in the pathway NADPH → PS I → $O_2$ coupled phosphorylation is insensitive to antimycin A. The endogenous cycle is inhibited, though at a rather high concentration of the inhibitor. This last observation can be interpreted as suggestive evidence for the operation in spinach chloroplasts of the cyto-

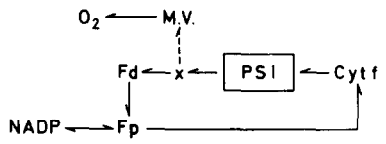


Fig. 2. Scheme of electron transport in cyclic phosphorylation.

chrome $b_{563}$ alternative pathway proposed by Hiyama et al. to explain their spectroscopic observations in *Chlamydomonas reinhardii* [17].

Fig. 1 shows that cyclic phosphorylation is relatively insensitive to dibromothymoquinone, a competitive inhibitor of plastoquinone reactions [8, 9, 10]. For comparison, it illustrates the high sensitivity of non-cyclic NADP reduction to this inhibitor. These observations are interpreted to indicate that the flavoprotein-catalysed cyclic electron flow does not include plastoquinone, or any carrier of potential lower than that of the quinone.

## 4. Conclusion

The investigations reported here support the following conclusions:

(a) cyclic photophosphorylation, dependent on PS I and catalyzed by the chloroplast flavoprotein [3] depends probably on the cytochrome *f* reductase activity of this enzyme. Plastoquinone is not involved. This conclusion is reached on the basis of the studies on the sensitivity to inhibitors (table 2, fig. 1).

(b) Cyclic electron flow can be interrupted in one of two ways: (i) at the electronegative side of PS I by the addition of an autoxidizable electron acceptor as methylviologen. This prevents the backflow of electrons towards the cytochrome *f*-side of PS I and therefore phosphorylation; or (ii) at the flavoprotein level, by keeping this enzyme reduced by NADPH and an NADPH generating system. In this case, electron flow around PS I is prevented by the lack of an electron acceptor at the reducing side.

(c) The simultaneous addition of methylviologen and an NADPH generating system restores phosphorylation at rates considerably higher than the endogenous cycle, due to the fast turnover of methylviologen. In these conditions the cyclic electron transport is changed into a PS I dependent chain from

NADPH to $O_2$. The lack of sensitivity of the phosphorylation coupled to this electron pathway to antimycin A indicates that *b*-type cytochromes are not involved.

This open chain photophosphorylation dependent on PS I seems to us a useful tool for further investigation of cyclic phosphorylation.

Fig. 2 summarizes the above conclusions.

## Acknowledgement

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