THE IMPORTANCE OF REDUCING CONDITIONS FOR THE INHIBITORY ACTION OF DBMIB$^+$, ANTIMYCIN A AND EDAC$^+$ ON CYCLIC PHOTOPHOSPHORYLATION

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Received 2 May 1977

1. Introduction

Cyclic photophosphorylation can be studied using intact mesophyll chloroplasts of the C_4 plant *Digitaria sanguinalis* by following pyruvate-dependent $^{14}CO_2$ fixation [1,2]. It has been experimentally verified that 2 molecules of ATP are required to fix 1 molecule of CO_2 in C_4 mesophyll preparations [3]. Other sources of ATP can be prevented by running the reactions under N_2 (to block pseudocyclic electron flow [4]) and by keeping the concentration of chlorophyll low to avoid accumulation of oxalacetate (to prevent noncyclic electron flow [1]). One of our interests has been to determine which electron transport components are involved in cyclic electron flow. We have attempted to do this by the use of several specific inhibitors: antimycin A which is thought to block the oxidation of cytochrome b_{563} [5], DBMIB which prevents the oxidation of plastohydroquinone [6], EDAC which probably blocks the reduction of cytochrome *f* [7] and DSPD which blocks photosystem I at the site of ferredoxin reduction [8].

Abbreviations: C_4 Plant, plant having the C_4-dicarboxylic acid pathway of photosynthesis; C_3 plant, plant having only the Calvin pathway of photosynthesis; 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; DSPD, disalicylidenepropanediamine; Chl, chlorophyll

It was previously reported [1,2] that in white light, cyclic photophosphorylation was sensitive to DBMIB, antimycin A and EDAC, indicating a role for cytochrome b_6, plastoquinone and cytochrome *f* in cyclic electron flow. It was also reported that in white light + DCMU, or in far-red light, the cyclic system was markedly less sensitive to these inhibitors. A proposed explanation was that 2 pathways for cyclic electron flow are possible and that electron transport from photosystem II regulates which pathway operates [2].

In this report, we show that in the presence of low levels of ascorbate, cyclic phosphorylation (in white light + DCMU or in far-red light) is completely sensitive to the inhibitors antimycin A, DBMIB and EDAC. This suggests that there is only one pathway for cyclic electron flow (through plastoquinone) and that the effectiveness of certain inhibitors requires a relatively reduced electron transport chain.

2. Materials and methods

2.1. *Mesophyll protoplast and protoplast extract preparation*

Mesophyll protoplasts were isolated from 2–4 week-old leaves of *Digitaria sanguinalis* as previously described [9] except they were purified from cellular debris by centrifugation (400 × *g*, 6 min) over a layer of Sigma Type P–F Ficoll similar to the method of

Larkin [10]. Protoplast extracts (suspension of intact chloroplasts containing cytoplasmic components) were prepared as previously discussed [11].

2.2. $^{14}CO_2$ fixation by mesophyll protoplast extracts

All reactions were performed in sealed ampules under N_2 at 35°C. The basic reaction mixture (150 μl) contained 0.3 sorbitol, 2 mM $MgCl_2$, 1 mM K_2HPO_4, 50 mM Tricine–KOH (pH 7.8), 3 mM pyruvate (potassium salt) and 3 mM $NaH^{14}CO_3$ (10 μCi/μmol). Reactions were initiated by the addition of chloroplasts (15–25 μg Chl/ml). Samples were taken at intervals up to 8 min and radioactivity fixed into acid-stable products determined as previously described [11]. Under these conditions, $^{14}CO_2$ fixation is linear with time and largely dependent upon cyclic photophosphorylation [1,2].

Where used, white light was provided by a General Electric Lucalox sodium discharge lamp (400 W) giving an irradiance of 1.5×10^5 ergs.cm^{-2}.s^{-1} at the reaction cuvette, as measured with a YSI Model 65 radiometer. Far-red light was provided by passing white light from a Sylvania Direct Light incandescent bulb (150 W) through two 2 mm Schottgen filters, giving 6.6×10^4 ergs.cm^{-2}.cm^{-1} at the reaction cuvette.

2.3. Chlorophyll determination

Chlorophyll was determined by the method of Wintermans and De Mots [12].

3. Results and discussion

The effect of ascorbate on pyruvate-dependent $^{14}CO_2$ fixation is shown in fig.1. Without added inhibitors (fig.1, control) ascorbate stimulated the rate of $^{14}CO_2$ fixation; this stimulation varied from day to day and in some cases ascorbate alone had no effect. Without added ascorbate, DCMU stimulated the rate of CO_2 fixation. A stimulation of cyclic photophosphorylation by DCMU has been previously reported for intact C_3 [13] and C_4 mesophyll chloroplasts [1,2] as well as with isolated thylakoids [5,14]. In the presence of DCMU, ascorbate had no effect. $^{14}CO_2$ Fixation (without ascorbate) was inhibited 90% by 6.7 μM DBMIB (fig.1, solid triangle) and this inhibition, as previously reported [1,2] was reversed

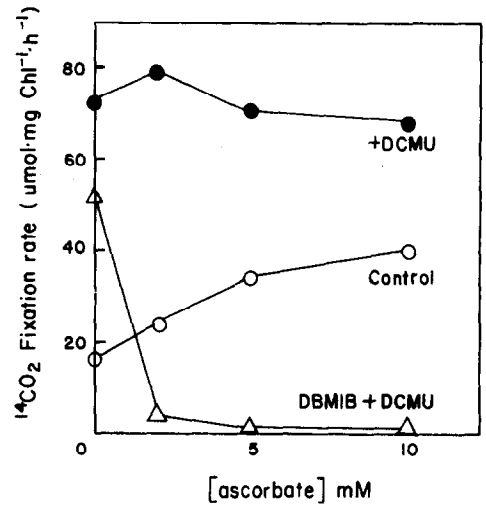

Fig.1. Effect of ascorbate on $^{14}CO_2$ fixation in white light under N_2 with mesophyll preparations of *Digitaria sanguinalis*. For details see Materials and methods section. Additions were as follows: DCMU 0.4 μM and DBMIB 6.7 μM.

by DCMU. As shown, however, $^{14}CO_2$ fixation in the presence of DBMIB + DCMU was completely inhibited by the addition of millimolar levels of ascorbate.

The ability of ascorbate to overcome the reversal of DBMIB inhibition caused by DCMU may be related to the fact that ascorbate is a strong reducing agent and is capable of donating electrons to photosystem II [15] and photosystem I [16]. In white light, in the absence of DCMU, the electron transport chain is in a reduced state and DBMIB is an effective inhibitor of the oxidation of plastohydroquinone. In the presence of DCMU in white light, the electron transport chain is more oxidized and DBMIB is no longer an effective inhibitor. The addition of ascorbate, however, keeps the electron transport chain reduced even in the presence of DCMU and hence DBMIB becomes an effective inhibitor.

It was previously reported that DCMU could overcome the inhibition of cyclic photophosphorylation (as measured by $^{14}CO_2$ fixation) by antimycin A and EDAC, as well as DBMIB, while inhibition by DSPD was insensitive to DCMU [2]. The effect of ascorbate on the inhibition of cyclic photophosphorylation by these inhibitors is shown in table 1. Consistent with a previous report [2], $^{14}CO_2$ fixation dependent on cyclic photophosphorylation was strongly inhibited

Table 1
Effect of ascorbate on the inhibition of cyclic photophosphorylation by certain electron transport inhibitors in white light[a]

Inhibitor	Additions		
	Control	+DCMU	DCMU + ascorbate
	(μmol.mg Chl^{-1}.h^{-1})[c]		
None	46	82	59
Antimycin A	5.4	29	3.6
DBMIB	8.1	36	4.1
EDAC[b]	3.9	45	3.8
DSPD	7.2	8.9	8.9

[a] Conditions for CO_2 fixation by mesophyll preparation of *Digitaria sanguinalis* was dependent on cyclic electron flow as described in Materials and methods. Concentrations were: DCMU 0.4 μM, ascorbate 10 mM antimycin A and DBMIB 6.7 μM, EDAC 0.3 mM and DSPD 0.3 mM

[b] In experiments with EDAC, the DCMU (where used) was added after a 3 min preincubation in the light

[c] All rates were calculated on the basis of cpm fixed from 3–8 min (linear phase)

(85–92%) by antimycin A, DBMIB, EDAC and DSPD. This can be taken to indicate that cytochrome b_{563}, plastoquinone, cytochrome *f* and ferredoxin are obligatory intermediates in the pathway of cyclic electron flow (see introduction). Low levels of DCMU (0.4 μM) stimulated the control rate of $^{14}CO_2$ fixation and reversed the inhibition of $^{14}CO_2$ fixation by antimycin A, DBMIB and EDAC, while inhibition by DSPD was unaffected. In all cases, 10 mM ascorbate reversed the effect of DCMU (table 1). The simplest explanation is that certain inhibitors (DBMIB, antimycin A and EDAC) require reducing conditions for effectiveness and that ascorbate provides this condition even in the presence of DCMU. In a recent study with intact spinach (C_3) chloroplasts, it was reported that ascorbate penetrates the chloroplast envelope slowly but nonetheless does interact with the electron transport chain [17]. The ability of ascorbate to provide reducing conditions was used previously by Gromet-Elhanan and Avron [18] to show that the uncoupling activity of desaspidin was dependent on the oxidation–reduction state of the system. As only low levels of ascorbate were required for maximal effect with intact C_4 mesophyll chloroplasts in the present study (fig.1) it may appear that the C_4 envelope is more permeable to ascorbate. However, the stimulation of $^{14}CO_2$ fixation by C_3 mesophyll chloroplasts by ascorbate is also maximal at relatively low levels [19].

In a previous communication we also reported that pyruvate-dependent $^{14}CO_2$ fixation in far-red light (cyclic photophosphorylation) was largely insensitive to DBMIB, antimycin A and EDAC; i.e., inhibitors that were ineffective in white light + DCMU [2]. As shown in table 2, inhibition by antimycin A and DBMIB in far-red was increased in the presence of ascorbate. This result is consistent with the proposed explanation for the effect of ascorbate. In far-red light, the electron transport chain between photosystem I and II will be relatively more oxidized than in white light and the inhibitors antimycin A and DBMIB are ineffective. Ascorbate causes the chain to become more reduced and antimycin A and DBMIB become effective inhibitors.

In far-red light, DCMU was very inhibitory to cyclic photophosphorylation (table 2). This has been observed previously with intact chloroplasts [2] and also with thylakoids [14] and has been explained on the basis that a low rate of electron transport from photosystem II is required for maximal rates of cyclic electron flow. The fact that ascorbate largely reverses the inhibition by DCMU is consistent with this proposal and the assumption that ascorbate acts by donating electrons to the transport chain.

Oxalacetate was also a potent inhibitor of cyclic photophosphorylation in far-red light (table 2, see

Table 2
Effect of ascorbate on $^{14}CO_2$ fixation dependent on cyclic photophosphorylation in far-red light with mesophyll preparations of *D. sanguinalis*[a]

Inhibitor[b]	Additions	Additions
	Control	Ascorbate + 10 mM
	(μmol.mg Chl^{-1}.h^{-1})	
None	26	29
Antimycin A	18	6
DBMIB	27	8
DCMU	8	32
Oxalacetate	5	8

[a] Conditions as described in the Materials and methods section

[b] Concentrations as given in the legend of table 1 and oxalacetate 0.5 mM

ref. [2]) and inhibition by oxalacetate could not be reversed by ascorbate. Inhibition by oxalacetate was explained as a loss of electrons from a closed cyclic system by reduction of oxalacetate to malate [2]. However, since the potential for oxalacetate reduction (i.e., loss of electrons from the cyclic system) is quite high, but the potential for electron donation from ascorbate is limited (without an intermediate donor such as dichloroindophenol), the inhibition could not be reversed.

If ascorbate increased the effectiveness (i.e., reduces the K_i) of an inhibitor by providing a reduced environment, then the concentration of inhibitor required to give a fixed level of inhibition of electron flow would be expected to be reduced by ascorbate. This was observed and data for DBMIB are given in fig.2. As shown, DBMIB was inhibitory to $^{14}CO_2$ fixation dependent upon cyclic photophosphorylation in white light in the absence of ascorbate, but inhibition was more pronounced in the presence of ascorbate (fig.3). In contrast, inhibition by DSPD was unaffected by ascorbate. This may indicate that action of DSPD is insensitive to the oxidation–reduction state of

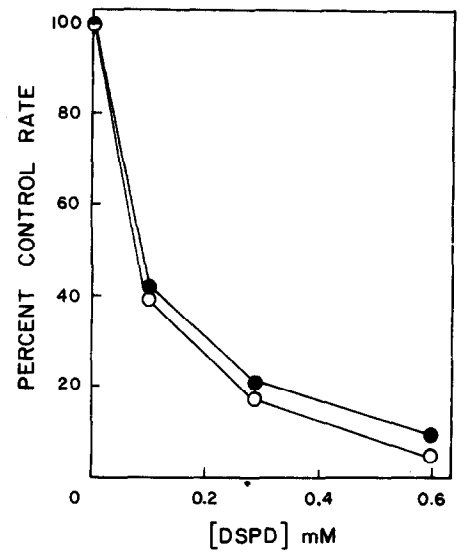

Fig.3. Effect of DSPD on the rate of $^{14}CO_2$ fixation with mesophyll preparations of *D. sanguinalis* in white light under N_2 in the presence or absence of 10 mM ascorbate. The control rates were 45 and 56 μmol.mg $Chl^{-1}.h^{-1}$ in the presence and absence of ascorbate, respectively. For details, see Materials and methods section.

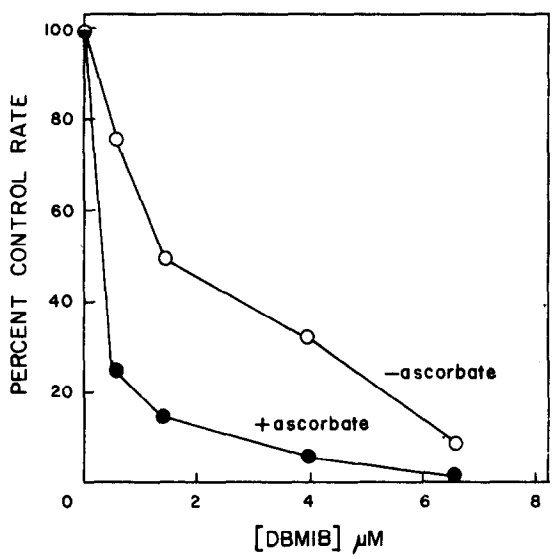

Fig.2. Effect of DBMIB on the rate of $^{14}CO_2$ fixation with mesophyll preparations of *D. sanguinalis* in white light under N_2 in the presence or absence of 10 mM ascorbate. The control rates were 41 and 58 μmol.mg $Chl^{-1}.h^{-1}$ in the presence and absence of ascorbate, respectively. For details, see Materials and methods section.

ferredoxin (apparent site of action of DSPD) or that the oxidation–reduction level of ferredoxin is not altered significantly by addition of DCMU or ascorbate in white light.

4. Concluding remarks

It was previously reported that DCMU could overcome the inhibition of cyclic photophosphorylation by DBMIB, antimycin A and EDAC [1,2]. A possible explanation given was that 2 pathways of cyclic electron flow exist and that the flow of electrons from photosystem II regulates which pathway operates. The DCMU reversal is further documented in this communication but a new interpretation is presented. On the basis that low levels of ascorbate restore inhibition of cyclic electron flow by DBMIB, antimycin A and EDAC (fig.1, tables 1 and 2) it is suggested that these inhibitors are most effective when the electron transport chain is under reducing conditions.
This condition is obtained in white light, white light + DCMU + ascorbate, or in far-red light + ascorbate.

When the electron transport chain would be expected to be relatively more oxidized (e.g., white light + DCMU or far-red light) the inhibitors were less effective. Furthermore, certain inhibitors (DSPD (table 1, fig.3) and KCN, see ref. [2]) appear to be insensitive to the redox level of the electron transport chain.

It thus appears that the data can all be explained within the framework of 1 pathway for cyclic electron flow in intact C_4 mesophyll chloroplasts.

Acknowledgements

The research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by the National Science Foundation Grant BMS-74-09611. The authors are grateful to M. Avron for discussions which lead us to this study.

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