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OXYGEN REQUIREMENT OF PHOTOSYNTHETIC CO_2 ASSIMILATION *

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Summary

In the absence of electron acceptors and of oxygen a proton gradient was supported across thylakoid membranes of intact spinach chloroplasts by far-red illumination. It was decreased by red light. Inhibition by red light indicates effective control of cyclic electron flow by Photosystem II. Inhibition was released by oxygen which supported a large proton gradient. Oxygen appeared to act as electron acceptor simultaneously preventing over-reduction of electron carriers of the cyclic electron transport pathway. It thus has an important regulatory function in electron transport. Under anaerobic conditions, the inhibition of electron transport caused by red illumination could also be released and a large proton gradient could be established by oxaloacetate, nitrite and 3-phosphoglycerate, but not by bicarbonate. In the absence of oxygen, ATP levels remained low in chloroplasts illuminated with red light even when bicarbonate was present. They increased when electron acceptors were added which could release the over-reduction of the electron transport chain. Inhibition of electron transport in the presence of bicarbonate was relieved and CO_2-fixation was initiated by oxygen concentrations as low as about 10 μM. Once CO_2 fixation was initiated, very low oxygen levels were sufficient to sustain it. The results support the assumption that pseudocyclic electron transport is necessary to poise the electron transport chain so that a proper balance of linear and cyclic electron transport is established to supply ATP for CO_2 reduction.

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* Dedicated to Professor W. Simonis on the occasion of his 70th birthday.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCMU, dichlorophenyldimethylurea.

Introduction

CO_2 reduction in the Calvin cycle requires ATP and NADPH in a ratio of 1.5 [1]. It is still unclear whether sufficient ATP can be formed during linear electron flow to $NADP^+$ to satisfy the ATP requirement of photosynthesis. Reported values of the number of ATP molecules synthesized during the transport of 2 electrons from water to a suitable acceptor such as $NADP^+$ range from 0.9 to 2.0 [2—4]. Whenever the ratio of ATP/NADPH is lower than 1.5, auxiliary reactions such as cyclic or pseudocyclic phosphorylation (Mehler reaction [5]) or a combination of both are necessary to supply ATP, which is not synthesized during linear electron flow from water to $NADP^+$. In previous publications we have presented evidence that in intact chloroplasts oxygen is reduced by the electron transport chain during CO_2-reduction [6]. However oxygen reduction is slow in spinach chloroplasts and presumably cannot provide much ATP [7]. In this report we present evidence that a main role of pseudocyclic electron transport is that of a safety valve reaction: By preventing overreduction of electron carriers of the cyclic pathway it permits cyclic electron flow to occur whenever ATP is needed. As has first been proposed by Arnon [8], it poises electron carriers of the cyclic electron pathway.

Materials and Methods

Intact chloroplasts were isolated from greenhouse-grown spinach (*Spinacia oleracea* L. var. Monatol) as described previously [6,7,9]). Chlorophyll was determined according to Arnon [10]. Intactness of the chloroplasts was determined by the ferricyanide method [11]. Our preparations contained no less than 83 and up to 93% intact chloroplasts. CO_2-dependent oxygen evolution was measured in a Clark-type electrode. Rates were between 101 and 161 μmol O_2/mg chlorophyll per h. The aerobic assay medium contained 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediamine tetraacetic acid, 10 mM NaCl, 0.5 mM KH_2PO_4 and 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes). Catalase was also added (1500 enzyme units/ml). The pH was adjusted to 7.6. Electron acceptors were $KHCO_3$, 3-phosphoglycerate, oxaloacetate or KNO_2 (all 2 mM). Chloroplast suspensions were made and kept anaerobic by adding 10 mM glucose and glucose oxidase (usually about 7 units/ml). H_2O_2 formed during glucose oxidation and by the Mehler reaction was decomposed by catalase. Transient oxygenation of anaerobic chloroplast suspension was produced by injecting known amounts of H_2O_2. The quenching of 9-aminoacridine fluorescence (concentration: 5 μm) was measured by a photomultiplier. Aerobic and anaerobic CO_2 fixation in the presence of $NaH^{14}CO_3$ was measured by removing aliquots from an illuminated chloroplast suspension, drying them on planchets after the reaction was terminated by acetic acid, and by counting the planchets in a windowless methane flow counter. Light intensities were determined with a radiometer model 65 A (Yellow Springs Instrument Co., Ohio). Short wavelength red light having a half band width from 633 to 673 nm was produced by passing a beam of white light through the following filter combination: Calflex C (Balzers, Liechtenstein), RG 630 (2 mm; Schott, Mainz), K-65 (Balzers, Liechtenstein).

The filter combination of the far red light was: Calfflex C, RG 695 (3 mm; Schott, Mainz), interference filter 720 nm (Baird Atomic Co.); halfbandwidth from 710 to 729 nm. For the radioactive experiments light passed only through an RG 630 cutoff filter.

Results

During coupled electron transport protons are pumped from the chloroplast stroma into the intrathylakoid space. Formation of the proton gradient, which is thought to be involved in ATP synthesis [12], can be monitored in intact chloroplasts by measuring the quenching of 9-aminoacridine fluorescence. 9-Aminoacridine penetrates into the chloroplasts in the neutral form and is protonated and trapped in the intrathylakoid compartment, when the latter is acidified on illumination [13]. The trapped 9-aminoacridine is nonfluorescent. The usefulness of 9-aminoacridine as a probe for ΔpH and its limitations have been discussed previously [14—16].

Fig. 1 shows the extent of 9-aminoacridine fluorescence quenching by intact chloroplasts under red or far-red illumination in the presence of different oxygen concentrations. In the absence of electron acceptors and oxygen, red light did not cause significant 9-aminoacridine fluorescence quenching, because linear electron flow was not possible and cyclic electron flow was inhibited by overreduction of electron carriers. It should be noted that the glucose/glucose oxidase/catalase system used for establishing anaerobic conditions in

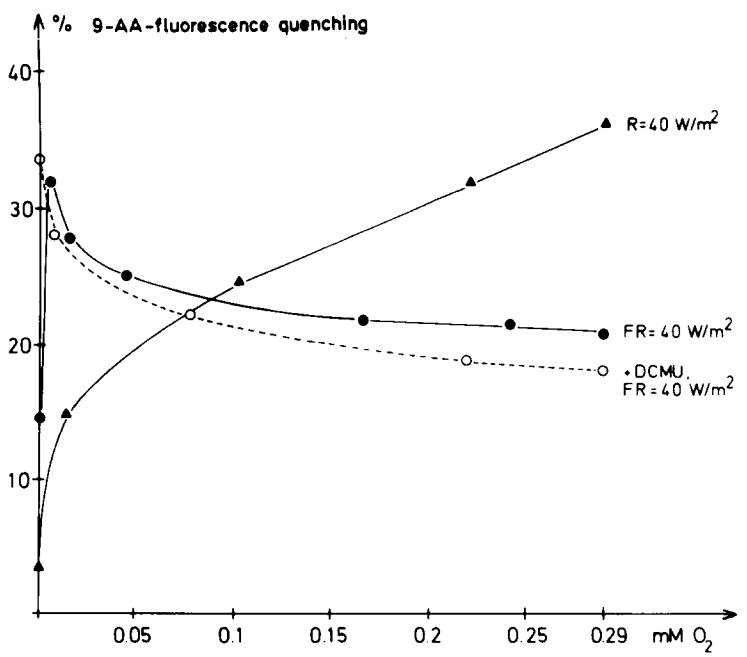

Fig. 1. Quenching of 9-aminoacridine (9-AA) fluorescence by intact chloroplasts illuminated with 40 W · m^{-2} red (R) or far red (FR) light, concentration of DCMU $5 \cdot 10^{-8}$ M. Chloroplast concentration: 23 $\mu g \cdot ml^{-1}$.

the chloroplast suspension does not give rise to ATP consumption which might affect the state of 9-aminoacridine fluorescence quenching. Though glucose enters the chloroplasts [17], it is not phosphorylated there [18]. In contrast to red light, far-red illumination produced a significant proton gradient under anaerobic conditions, showing that cyclic electron flow can operate under these conditions. With increasing oxygen concentrations 9-aminoacridine fluorescence quenching caused by red light increased dramatically. This is interpreted to reflect the release of over-reduction of the electron carriers by oxygen [19]. Under far-red illumination 9-aminoacridine fluorescence quenching reached its maximum at a very low oxygen concentration (7 μM or about 0.5% in the gas phase). Complete anaerobiosis or increased oxygen concentrations both caused partial inhibition of cyclic electron flow as indicated by the extent of fluorescence quenching. Only in the presence of a very low concentration of dichlorophenyl dimethylurea (DCMU, $5 \cdot 10^{-8}$ M) which interferes with linear electron transport from Photosystem II far-red-induced fluorescence quenching reached its maximum in the complete absence of oxygen (Fig. 1). Higher concentrations of DCMU decreased the proton gradient drastically, lower concentrations had little effect (results not shown). The response to DCMU indicates that even far-red light can sufficiently excite Photosystem II to cause some over-reduction of electron carriers and thereby decrease cyclic electron flow. Under the light intensity of the experiment, over-reduction was released either by DCMU or by 7 μM oxygen. Higher oxygen concentrations drained electrons from the cyclic pathway thereby inhibiting cyclic electron flow.

Fig. 2 shows the effect of the addition of nitrite, 3-phosphoglycerate, bicarbonate and oxygen on the quenching of 9-aminoacridine fluorescence by intact chloroplasts illuminated with red light under carefully maintained anaerobic conditions. When nitrite which does not consume ATP during reduction was added, fluorescence quenching increased dramatically indicating that inhibition of electron transport was relieved. Oxygen produced no further effect. The addition of 3-phosphoglycerate also relieved inhibition of electron transport and fluorescence quenching increased. Oxygen had only a minor effect. The reduction of 3-phosphoglycerate requires ATP and NADPH at a ratio of 1. When bicarbonate was added, fluorescence quenching increased only transiently in many experiments. Inhibition of electron transport was usually only relieved after oxygen was added, and there was a large fluorescence decrease on addition of oxygen. Fluorescence quenching remained significant even after the added oxygen was largely removed. However, a second illumination after a period of darkening under anaerobiosis was unable to produce much fluorescence quenching, and electron transport remained inhibited. The reduction of CO_2 requires ATP and NADPH at a ratio of 1.5.

Fig. 3 shows that oxygen can at very low concentrations initiate electron transport to CO_2 as indicated by fluorescence quenching, and that it needs to be present only for short times. After the main part of the oxygen had been removed by an enzymic oxygen trap the low oxygen concentration maintained by photosynthesizing chloroplasts was sufficient to sustain photosynthesis. Darkening, however, made the system unable to restart without the aid of another oxygen injection.

In Fig. 4 $^{14}CO_2$ fixation of intact chloroplasts is shown under aerobic and

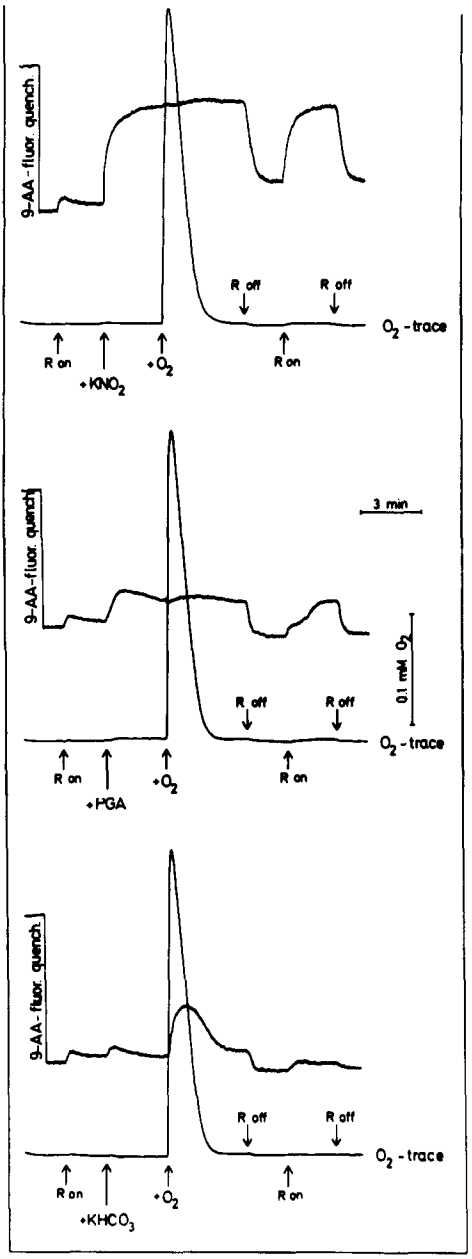

Fig. 2. Quenching of 9-aminoacridine (9-AA) fluorescence by intact chloroplasts illuminated with 40 W · m^{-2} red light (R) as affected by different substrates (nitrite, 3-phosphoglycerate and bicarbonate, each 2 mM) and oxygen. The sample was anaerobic before illumination. The oxygen trace indicates the time course of oxygenation and of removal of oxygen by a glucose oxidase/catalase trap. Concentration of chlorophyll 24 $\mu g \cdot ml^{-1}$.

anaerobic conditions. The results are consistent with the results of the 9-aminoacridine fluorescence experiments described above (Figs. 2 and 3). Under anaerobic conditions $^{14}CO_2$ fixation was inhibited. Inhibition was released by an oxygen pulse. After most of the added oxygen was removed by an enzymic

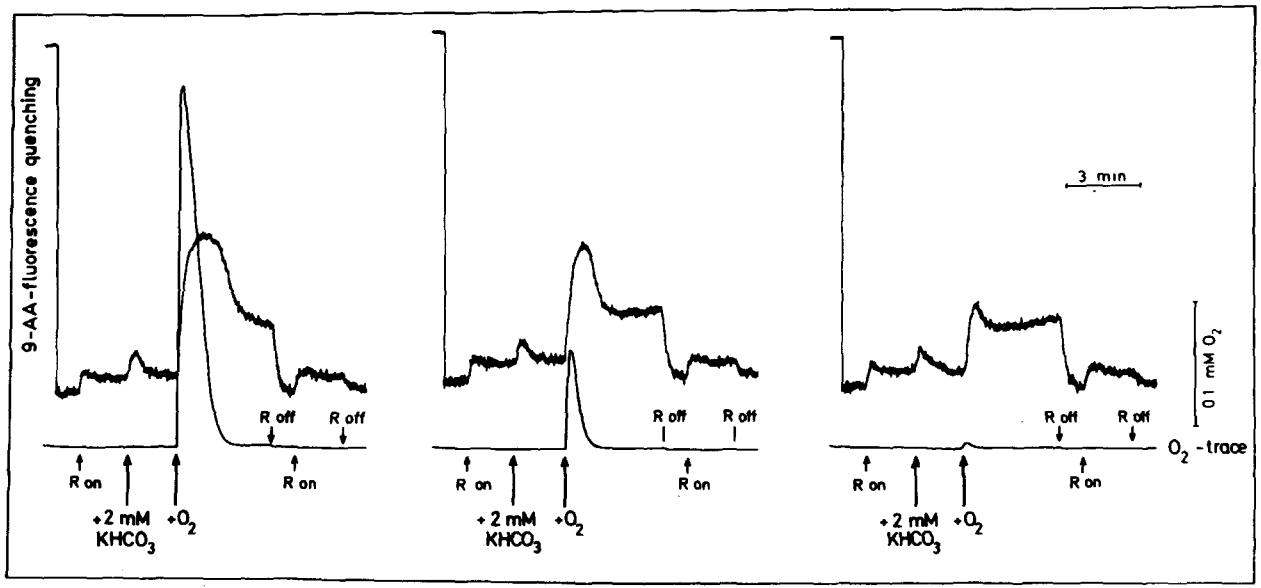

Fig. 3. Quenching of 9-aminoacridine (9-AA) fluorescence by intact chloroplasts illuminated with 100 W · m^{-2} red light (R) as affected by the addition of bicarbonate (2 mM) and of different concentrations of oxygen. Lower trace shows oxygen concentration in the sample which before illumination was anaerobic. Chlorophyll concentration: 25 $\mu g \cdot ml^{-1}$.

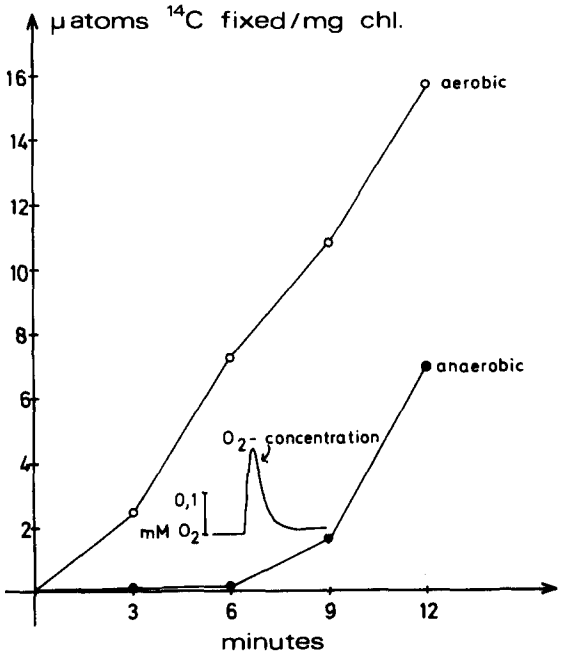

Fig. 4. Fixation of $H^{14}CO_3$ by intact chloroplasts under illumination with red light (RG 630 cutoff filter, intensity 110 $W \cdot m^{-2}$) under aerobic and anaerobic conditions. After 6 min illumination, H_2O_2 was injected into the anaerobic sample which produced the transient oxygenation shown in the insert.

trap, continuous photosynthesis maintained a stationary oxygen concentration of 8 μM in the sample. This low oxygen concentration was sufficient to maintain a rate of CO_2 fixation which was similar to that observed in the aerobic sample. It appears that lack of ATP is responsible for inhibition of CO_2 reduction under anaerobic conditions (Table I). Chloroplasts illuminated under anaerobic conditions in the absence of substrate or in the presence of bicarbonate contained less ATP than darkened chloroplasts. Addition of nitrite or admission of oxygen increased ATP levels considerably. Chloroplasts photoreducing CO_2 contained less ATP after a transient oxygen pulse than chloroplasts photoreducing CO_2 aerobically but much more ATP than chloroplasts kept strictly free of oxygen (not shown).

TABLE I

Adenylate levels (nmol/mg chlorophyll) and ATP/ADP ratios in intact chloroplasts in the dark and after 4 min illumination with red light (110 $W \cdot m^{-2}$) with (+) or without (—) oxygen (0.29 mM) or substrates (2 mM) present.

	Substrates	Oxygen	ATP	ADP	ATP/ADP
Dark	—	—	10.5	25.2	0.42
Light	—	—	8.7	21.8	0.40
Light	HCO_3^-	—	8.1	23.9	0.35
Light	HCO_3^-	+	36.7	15.8	2.43
Light	NO_2^-	—	39.6	13.5	2.93

Interestingly, inhibition of photosynthesis in the absence of oxygen was not always observed. In some experiments, intact chloroplasts showed nearly the same $^{14}CO_2$-fixation rate under anaerobic and aerobic conditions and no starting by oxygen was required. The significance of these deviations from normal behaviour will be considered below.

Discussion

Our data show that oxygen which is a rather inefficient electron acceptor in spinach chloroplasts [7] is nevertheless capable of shifting the redox state of carriers of the photosynthetic electron transport chain toward oxidation and thereby relieves inhibition by cyclic electron flow by over-reduction which is caused under anaerobic conditions by illumination with red light. In contrast, cyclic electron flow supported by far-red light is decreased by increased oxygen concentrations (Fig. 1) presumably by excessive oxidation of electron carriers. Obviously, cyclic electron transport is significant in intact chloroplasts only when the carriers of the cyclic pathway are optimally poised [19,20]. A role of oxygen in poising intact chloroplasts has been considered before [7,21,22]. Nitrite and oxaloacetate which require for reduction only electrons, but no ATP, and 3-phosphoglycerate which needs for reduction ATP and NADPH at a ratio of 1 also relieved the over-reduction caused by red light in anaerobic chloroplasts (Fig. 2). In contrast, CO_2 usually did not start electron transport. Bioenergetically, the reduction of CO_2 differs from that of 3-phosphoglycerate only in its higher ATP requirement. We attribute the failure of CO_2 to relieve over-reduction to this difference. If linear electron transport provides ATP and NADPH at a ratio below 1.5 and cyclic electron flow cannot occur, CO_2 cannot be reduced because of ATP deficiency. Indeed, anaerobic chloroplasts illuminated with red light were shown to be ATP deficient (Table I). There was sufficient ATP to start reduction of phosphoglycerate, but not enough to start reduction of CO_2 (Fig. 2). Oxygen initiated electron transport to CO_2 by permitting ATP synthesis to occur. It accomplished this by serving both as electron acceptor and by releasing inhibition of cyclic electron flow. As very low oxygen concentrations were effective, ATP synthesis during pseudocyclic electron transport to oxygen appeared to be less important than regulation of the redox state of electron carriers (poising) for cyclic electron flow. The starter role of oxygen for CO_2 reduction by anaerobic chloroplasts shows that oxygen is essential for photosynthesis if the coupling ratio (ATP/$2e$ ratio) of the electron transport chain is below 1.5. In the absence of oxygen, NADPH accumulates, and this inhibits cyclic electron even when CO_2 is present.

It has been mentioned that inhibition of photosynthesis by anaerobiosis was not invariably observed. Three explanations are possible. (1) Over-reduction does not always proceed to the point where cyclic photophosphorylation is effectively blocked. (2) Some endogenous electron acceptors such as oxaloacetate were still present in the inhibited chloroplast preparations and replaced oxygen as a starter of CO_2 reduction. (3) The ATP/$2e$ ratio of the electron transport chain of chloroplasts in the uninhibited preparations was above 1.5 and sufficient ATP for CO_2 reduction could be produced by electron transport

to NADP alone. There are reports of maximal ATP/2*e* ratios of 2 in the literature [2,3], although most data presently available favor maximal ATP/2*e* ratios of 1.33 [23]. Our experience leads us to favour explanation 1.

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