ROLE OF OXYGEN IN FERREDOXIN-CATALYZED CYCLIC PHOTOPHOSPHORYLATIONS

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1. Introduction

The enzymatic reactions responsible for the photosynthetic conversion of CO_2 to glucose or its equivalent require an excess of ATP over NADPH. Specifically, for one mole of CO_2 the required molar ratio of ATP to NADPH is 1.5 for C_3 plants [1] and 2.5 for C_4 plants [2,3]. From early studies of the light reactions of chloroplasts it appeared that these two components of assimilatory power might be generated by two distincts and competitive reactions: ATP by cyclic photophosphorylation [4,5] and NADPH by photolysis of water [6].

This concept [7] was abandoned with the finding of noncyclic photophosphorylation, in which ATP and NADPH are formed in an equimolar ratio (P/e_2 1), not competitively but concomitantly and in a mutually enhancing manner [8]. Cyclic photophosphorylation now came to be regarded as a second source of ATP in chloroplasts, one that supplements the insufficient ATP generated for CO_2 assimilation by noncyclic photophosphorylation and also serves as a source of ATP for processes other than CO_2 assimilation, for example, protein synthesis [8,9]. The assignment of this physiological role to cyclic photophosphorylation was strengthened by evidence that this process was catalyzed by ferredoxin, a native protein component of chloroplasts [10–13].

Some investigators sought to account for the extra ATP needed for CO_2 assimilation by contending that the P/e_2 ratio of noncyclic photophosphorylation may equal 2 and that, therefore, no additional source of ATP is needed [14–20]. A P/e_2 ratio of 2 would still fail to provide enough ATP for CO_2 assimilation by C_4 plants. Moreover, the derivation of such a ratio rests on assumptions and experimental approaches that are open to question (see Discussion).

Recent work has thrown new light on the nature of ferredoxin-catalyzed cyclic photophosphorylation and its contribution to the apparent stoichiometry of noncyclic photophosphorylation [21–23]. Under aerobic conditions, very low concentrations of ferredoxin (10 μM), the same as those required for $NADP^+$ reduction and an order of magnitude smaller than those used previously [13], catalyzed cyclic photophosphorylation with a high quantum efficiency, unmatched by other cofactors [22,23]. Furthermore, the aerobic, ferredoxin-catalyzed cyclic photophosphorylation was sensitive to inhibition not only by antimycin A [11] but also by dibromothymoquinone [21], an antagonist of plastoquinone [24] that serves as the natural energy-conservation site for cyclic photophosphorylation in chloroplasts [25].

In the presence of $NADP^+$, ferredoxin catalyzed simultaneously cyclic and noncyclic photophosphorylation, yielding, in the case of chloroplasts from young spinach (a C_3 plant) leaves, a P/e_2 ratio of about 1.5 (measured as ATP/O and ATP/NADPH ratios, c.f. [21,22]. The extra ATP was attributed to a concurrent cyclic photophosphorylation on the basis of inhibition by antimycin A, which, within a concentration range that did not inhibit noncyclic photophosphorylation, inhibited cyclic photophosphorylation and restored the intrinsic ATP/e_2 ratio of 1.0 to noncyclic photophosphorylation [21,22].

When operating by itself, without a concurrent noncyclic photophosphorylation, ferredoxin-catalyzed cyclic photophosphorylation was unexpectedly found

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to be regulated by NADPH [21,22]. This effect was related to a back reaction of NADPH (via ferredoxin) with the primary electron acceptor of photosystem II and a resultant improvement in redox poising [21–23]. The importance of poising for the functioning of cyclic photophosphorylation in chloroplasts has long been recognized, but its mechanism was obscure [26–28].

The experiments now reported were undertaken to test the validity of these observations under experimental conditions that would rule out complications that might result from reactions with oxygen. Specifically, evidence was obtained that:

(i) The ferredoxin-catalyzed phosphorylation under aerobic conditions is of the cyclic type and not of the pseudocyclic type [29,30] in which ATP formation is coupled to the reoxidation of reduced ferredoxin by molecular oxygen [13,31] and the formation of hydrogen peroxide [32].

(ii) The observed stimulatory effect of NADPH on ATP formation is not the result of noncyclic photophosphorylation that might be triggered by a reoxidation of NADPH by oxygen, followed by photoreduction of $NADP^+$, but reflects an enhancement of cyclic photophosphorylation brought about by a restriction of the electron flow from water [21,22].

2. Methods

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea* var. High Pack) grown in a greenhouse in a nutrient solution culture [33] and freshly harvested before each experiment. The preparation used consisted of 'broken' chloroplasts prepared as described [22] which contained the thylakoid membranes depleted of soluble chloroplast components. The broken chloroplasts were used within 20 min of preparation.

Oxygen evolution or uptake was measured polarographically in a Rank oxygen electrode as described by Delieu and Walker [34] and modified by Hiyama et al. [35].

Monochromatic illumination (554 nm) was provided by a light beam from a 250 W air-cooled tungsten–halogen lamp (Type ENH, General Electric Co.) passed through an interference filter (Baird Atomic Co.) with a 20 nm half-band width. The incident light intensity was measured with a radiation meter (Radiometer model 65, Yellow Springs Instruments Co.) that was calibrated against a National Bureau of Standards (Washington, DC) radiation standard.

The ATP formed was measured by the increase in labelled organic phosphate, determined by the method of Hagihara and Lardy [36]. Ferredoxin was isolated from spinach leaves [37]. $NADP^+$, NADPH, ADP, and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorophyll was determined as described [33].

3. Results

The reduction of oxygen to hydrogen peroxide by illuminated chloroplasts [38] is ordinarily a sluggish reaction which can be accelerated by the addition of a catalyst that is rapidly photoreduced by chloroplasts and reoxidized by molecular oxygen [39]. When the photoreduction of oxygen was coupled with ATP formation the result was a pseudocyclic photophosphorylation, i.e., a photophosphorylation superficially similar to cyclic photophosphorylation in that the only net product of the reaction was ATP [29,30].

Pseudocyclic photophosphorylation is, in reality, a variant of noncyclic photophosphorylation in which oxygen (1 O_2) consumed by the formation of 1 H_2O_2 is balanced by 1/2 O_2 generated photochemically by chloroplasts plus 1/2 O_2 released by the decomposition of 1 H_2O_2 by catalase [29,30], an enzyme normally present in many chloroplast preparations. However, catalase can also act as peroxidase that catalyzes the oxidation of ethanol to acetyladehyde by hydrogen peroxide [40]. Thus, the addition of both ethanol and catalase insures the presence of a 'trap' that removes hydrogen peroxide before its dissociation into oxygen and water and permits the identification of pseudocyclic photophosphorylation by a net uptake of 1/2 O_2.

Because chloroplasts photoreduce ferredoxin with water and ferredoxin so reduced is (in the absence of $NADP^+$) reoxidized by molecular oxygen [13,31] in a reaction that forms hydrogen peroxide [32] and induces pseudocyclic photophosphorylation [13], rigorous evidence was sought to establish that our aerobic, ferredoxin-catalyzed cyclic photophosphoryl-

ation was not, in fact, pseudocyclic photophosphorylation.

Accordingly, oxygen uptake and ferredoxin-catalyzed cyclic photophosphorylation were measured in the presence of the catalase–ethanol peroxide trap and in the presence of DCMU, also useful in identifying pseudocyclic photophosphorylation and in distinguishing it from the cyclic type. DCMU, as a strong inhibitor of photoproduction of oxygen, is thereby a strong inhibitor of pseudocyclic photophosphorylation [13]. By contrast, DCMU enhances ATP formation by ferredoxin-catalyzed cyclic photophosphorylation because, by decreasing the electron pressure from water, it acts as an artificial poising agent for the cyclic system [21–23].

Figure 1 (left) shows that, in the presence of a peroxide trap, there was indeed an oxygen uptake, suggestive of a pseudocyclic photophosphorylation. However, the oxygen uptake was small (ca. 0.25 μmol) and bore no relation to the ATP formed (2.57 μmol). Moreover, DCMU, far from inhibiting ATP formation, as would be expected in the case of pseudocyclic photophosphorylation, greatly increased ATP formation, as would be expected from its favorable action as a poising agent for cyclic photophosphorylation (fig.1, right) [21,22].

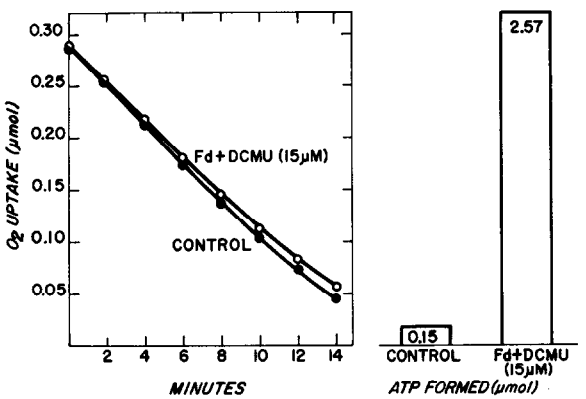

Fig.1. ATP formation and concurrent oxygen uptake by ferredoxin-catalyzed cyclic photophosphorylation in the presence and absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Left, time-course of oxygen uptake; right, ATP formed in 14 min. The reaction mixture (1.0 ml) contained broken spinach chloroplasts (see Methods) (equivalent to 332 μg chlorophyll), catalase (750 units), and the following (mM): Tricine–KOH buffer (pH 8.2), 200; $MgCl_2$, 5; ADP, 5; $K_2H^{32}PO_4$, 5; spinach ferredoxin, 0.01; and ethanol, 0.175. DCMU (0.015 mM) was added where indicated. Temperature, 20°C; incident monochromatic (554 nm) illumination, 2×10^4 ergs·cm^{-2}·s^{-1}; gas phase, air.

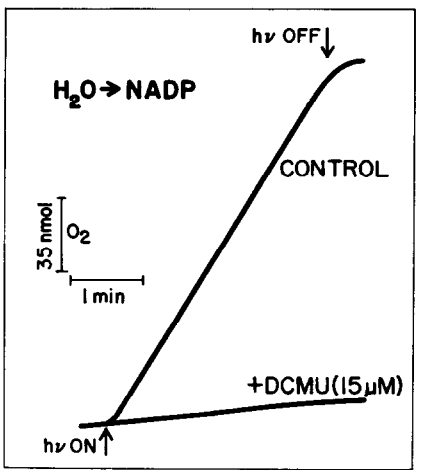

Fig.2. Photoproduction of oxygen as a measure of the extent of inhibition of noncyclic electron flow by the DCMU concentration used to poise cyclic photophosphorylation in fig.1. The reaction mixture (made anaerobic at the start by flushing with N_2) was as in fig.1 except that catalase and ethanol were omitted and $NADP^+$ (2.5 mM) was added. Temperature, 20°C; incident monochromatic (550 nm) illumination, 1×10^4 ergs·cm^{-2}·s^{-1}.

The concentration of DCMU used in fig.1 was large enough in relation to that of chlorophyll to give a marked decrease in electron flow from water, as measured separately by oxygen evolution (fig.2). We conclude, therefore, that the ferredoxin-catalyzed photophosphorylation under aerobic conditions (fig.1) was of the cyclic and not of the pseudocyclic type.

The nature of the stimulatory effect of NADPH on ferredoxin-catalyzed photophosphorylation was examined by experiments represented by fig.3. We previously concluded that the action of NADPH was similar to that of DCMU in improving poising of the cyclic system [21,22]. However, a remote possibility had to be ruled out that the added NADPH was being oxidized to $NADP^+$ by some unknown oxidase system. The $NADP^+$ that might be formed in this manner could then serve as the electron acceptor for noncyclic photophosphorylation and account for the increase in the total ATP formed.

The operation of such a concurrent $NADP^+$-linked

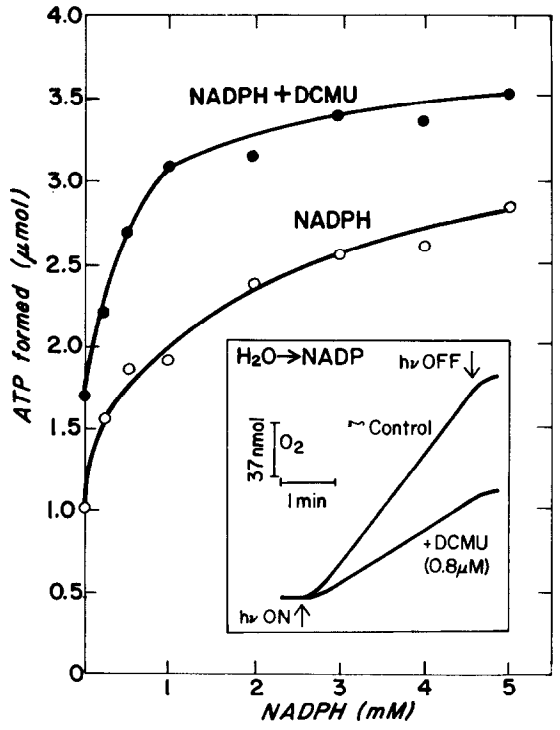

Fig.3. Stimulation by NADPH of ferredoxin-catalyzed cyclic photophosphorylation in presence and absence of DCMU. The reaction mixture was as in fig.1 except that catalase and ethanol were omitted, the concentration of DCMU was lower (0.0008 mM), and NADPH was added as indicated. The reaction was run in glass cuvettes (optical path, 2 mm) under aerobic conditions as described in [21]. Temperature, 15–18°C; illumination as in fig.1. Inset: Photoproduction of oxygen as a measure of the extent of inhibition of noncyclic electron flow by the DCMU concentration used above. Experimental conditions as in fig.2.

noncyclic photophosphorylation system should be inhibited by DCMU but, as fig.3 shows, the addition of both NADPH and DCMU actually enhanced the stimulatory effect of NADPH on ATP formation. The enhancement appeared to be the result of better poising of the cyclic system by NADPH plus DCMU than by NADPH alone [22]. The concentration of DCMU used, although low, was sufficient, when its effect on oxygen evolution was measured separately (see inset, fig.3), to give a significant decrease of electron pressure from water and thereby improve poising. To recapitulate, the data in fig.3 are consistent with other evidence [21–23] that points to NADPH acting (via ferredoxin) as a poising agent for aerobic, ferredoxin-catalyzed cyclic photophosphorylation.

4. Discussion

Three different sources of extra ATP have been proposed to account for the excess of ATP over NADPH needed for photosynthetic CO_2 assimilation: high P/e_2 ratios of noncyclic photophosphorylation [14–20], pseudocyclic photophosphorylation [41–44], or cyclic photophosphorylation [8,9,13,45].

The view that noncyclic photophosphorylation has a P/e_2 ratio close to 2 (indicative of two sites of phosphorylation) and therefore is able to provide all of the extra ATP needed for CO_2 assimilation by C_3 plants (though not by C_4 plants) rests in part on direct measurements and in part on assumptions and experimental approaches [14–20] that bear examination. The direct measurements are in conflict with recent studies in which ATP/NADPH ratios were found to be close to one [41,42]. When higher ATP/NADPH ratios were obtained, the excess ATP was traced to the aerobic cyclic photophosphorylation that operated concurrently with noncyclic photophosphorylation [21–23].

Computations of high P/e_2 ratios for noncyclic photophosphorylation are sometimes based on a subtraction of the basal electron flow under non-phosphorylating conditions from the overall electron flow under phosphorylating conditions [15]. The validity of this procedure has been questioned [46–48]. Furthermore, much of the argument in support of a P/e_2 ratio of 2 (and two sites of ATP formation in noncyclic photophosphorylation) is based on the use of different classes of artificial electron acceptors [17].

The remarkable reactivity of isolated chloroplast preparations enables them to interact with artificial electron acceptors and produce artifacts that often obscure and distort the true characteristics of physiological photophosphorylations. For example, cyclic photophosphorylation catalyzed by phenazine methosulfate has a very high light saturation requirement and is insensitive to inhibition by antimycin A and dibromothymoquinone [24] – three features that are

exact opposites of the characteristics of cyclic photophosphorylation by chloroplasts when catalyzed by its native catalyst, ferredoxin. In short, however useful artificial acceptors and donors may be for studies of photosynthetic electron transport, results obtained with them provide no compelling evidence for corresponding physiological features in vivo.

Noncyclic photophosphorylation aside, the remaining sources for the extra ATP needed for CO_2 assimilation are pseudocyclic and cyclic photophosphorylation. Each is catalyzed by ferredoxin but the optimal concentration of ferredoxin for pseudocyclic photophosphorylation is about an order of magnitude higher than that for noncyclic photophosphorylation (see fig.6, [49]) or the aerobic cyclic photophosphorylation [21]. This fact, combined with evidence presented in this and related studies [21–23] leads us to the conclusion that the source of the extra ATP needed for CO_2 assimilation is the aerobic, ferredoxin-catalyzed cyclic photophosphorylation. Other evidence favoring this conclusion comes from experiments on CO_2 assimilation that are reviewed elsewhere [50].

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