REGULATORY ELECTRON TRANSPORT PATHWAYS IN CYCLIC PHOTOPHOSPHORYLATION

Reduction of C-550 and cytochrome b_6 by ferredoxin in the dark

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1. Introduction

Recent work [1–4] has strengthened and extended in several important respects the concept that ferredoxin is the physiological catalyst of cyclic photophosphorylation in chloroplasts and that this type of phosphorylation is a source of ATP needed for CO_2 assimilation, protein synthesis and other energy-dependent reactions [5–10]. As reviewed in [2,11], noncyclic photophosphorylation alone cannot fully meet the ATP requirements of CO_2 assimilation because of the marked excess of ATP over NADPH that is needed: C_4 plants require 5 ATP and C_3 plants require 3 ATP per 2 NADPH used in the assimilation of 1 CO_2 to the level of sugar phosphate (cf. [12]).

The recent findings pertaining to cyclic photophosphorylation in chloroplasts may be summarized as follows:

(i) Cyclic photophosphorylation by chloroplasts proceeds optimally in the presence of air and is catalyzed by low concentrations of ferredoxin (10 μM), the same as those required for $NADP^+$ reduction [1,2];

(ii) Pseudocyclic photophosphorylation, which depends on electron transport from photoreduced ferredoxin to oxygen [7], is of minor importance as a source of ATP in chloroplasts [1,4];

(iii) In the presence of $NADP^+$, the ferredoxin-dependent cyclic and noncyclic photophosphorylations operate concurrently and generate an excess of ATP over NADPH with a high quantum efficiency. Thus, in chloroplasts from a C_3 plant (spinach), the concurrent operation of cyclic and noncyclic photophosphorylation yielded 3 ATP and 2 NADPH with a nearly theoretical total requirement of ~12 quanta [2,3].

(iv) In addition to its long-recognized sensitivity to inhibition by antimycin [13–16], ferredoxin-dependent cyclic photophosphorylation is sensitive to inhibition by low concentrations of DBMIB [1,17], the antagonist of plastoquinone [17]; this observation is consistent with the view that plastoquinone serves as an energy-conservation site in endogenous cyclic photophosphorylation ([18] cf. [19]).

(v) Ferredoxin-dependent cyclic photophosphorylation is driven by photosystem I but is regulated by a redox poising system that involves, in addition to molecular oxygen, an attenuated electron flow from photosystem II [1,2]. Specifically, maximum rates of ferredoxin-catalyzed photophosphorylation were found to be favored by a partial but not a total inhibition of photooxidation of water by photosystem II. For example, under illumination favorable for the operation of photosystem II, cyclic photophosphorylation by isolated chloroplasts was stimulated by intermediate concentrations and inhibited by higher concentrations of DCMU [1,2].

Some of these findings seem to be already well

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DBMIB, dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone)

established because they were recently corroborated, singly or in combination, by other investigators who used different experimental approaches and measurements [20–26] Among the recent findings that warranted further study was a novel redox poising mechanism for cyclic photophosphorylation, one that may operate by restricting electron flow from water through an electron transport in the dark from NADPH to C-550 [1,2] C-550 is the spectrally identified photoreactive component of chloroplasts that provides a reliable measure of the redox status of the primary electron acceptor of photosystem II [27,28] When C-550 is fully reduced all of the reaction centers of photosystem II close [28]

We had observed [1,2] that, in the presence of ferredoxin, NADPH partially reduced C-550 and stimulated cyclic photophosphorylation The stimulatory effect of NADPH was similar to that of far-red monochromatic light or to that of intermediate concentrations of DCMU These findings led to a proposal [1,2] that electron flow from NADPH to C-550 may function as a physiological redox poising mechanism for cyclic photophosphorylation, a mechanism that comes into operation when noncyclic photophosphorylation stops and NADPH accumulates [1,2,11] What was obscure was the path of electrons in this novel electron flow from NADPH to C-550

This communication reports evidence that the dark electron transport from NADPH to C-550 proceeds via ferredoxin and cytochrome b_6 and is sensitive to inhibition by antimycin and DCMU but not by DBMIB

2 Methods

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea*, var High Pack) grown in a greenhouse in a nutrient solution culture [29] and freshly harvested before each experiment The preparation used consisted of osmotically disrupted chloroplasts prepared as in [1], which contained the thylakoid membranes depleted of soluble chloroplast compounds, including ferredoxin

Absorbance changes of C-550 and cytochromes were determined with an Aminco DW-2 spectrophotometer. Light-induced absorbance changes of C-550 were measured at a cryogenic temperature (77 K) after preincubation of chloroplasts at room temperature in the dark in appropriate reaction mixtures with or without NADPH, ferredoxin, or a particular inhibitor The spectrophotometer was operated in the dual-wavelength mode for measuring absorbance changes of C-550 and in the split-beam mode for measuring absorbance changes of cytochromes

Chlorophyll was determined [29], ferredoxin was isolated and purified from spinach [30] and a crude hydrogenase preparation was made from *Clostridium pasteurianum* [31] by procedures reported from this laboratory The hydrogenase, when stored at −20°C under hydrogen gas, was stable for several weeks

3 Results

That preincubation of chloroplasts with NADPH and ferredoxin reduces C-550 in the dark and thereby diminishes the extent of its total subsequent reduction in the light was established [1,2] by measurements of changes in the characteristic absorption spectrum of C-550 In these experiments, the extent of prior reduction of C-550 during the dark preincubation treatment was measured by subsequent light-induced ΔA_{546}, the absorption peak of C-550 at cryogenic temperatures [27] The smaller the light-induced decrease in A_{546}, the greater the extent of prereduction of C-550 in the dark

As shown in fig 1, the maximum photoreduction of C-550 was observed in the control (No Addtn) treatment in which chloroplasts were preincubated in the dark without either NADPH or ferredoxin By contrast, the smallest photoreduction of C-550, ~50% as great as that in the control, followed the dark preincubation of chloroplasts with both NADPH and ferredoxin, a clear indication that NADPH plus ferredoxin reduced C-550 prior to illumination Figure 1 also shows that ferredoxin alone without added NADPH did not reduce C-550 during the dark preincubation period the extent of C-550 reduction induced by light was the same as that in the control Likewise, preincubation with NADPH alone, without added ferredoxin, was also ineffective in reducing C-550 in the dark The slight dark reduction of C-550 by NADPH alone is attributed to a small amount of residual ferredoxin that was still adhering to the thylakoid membranes

The results in fig 1 demonstrate that C-550 was

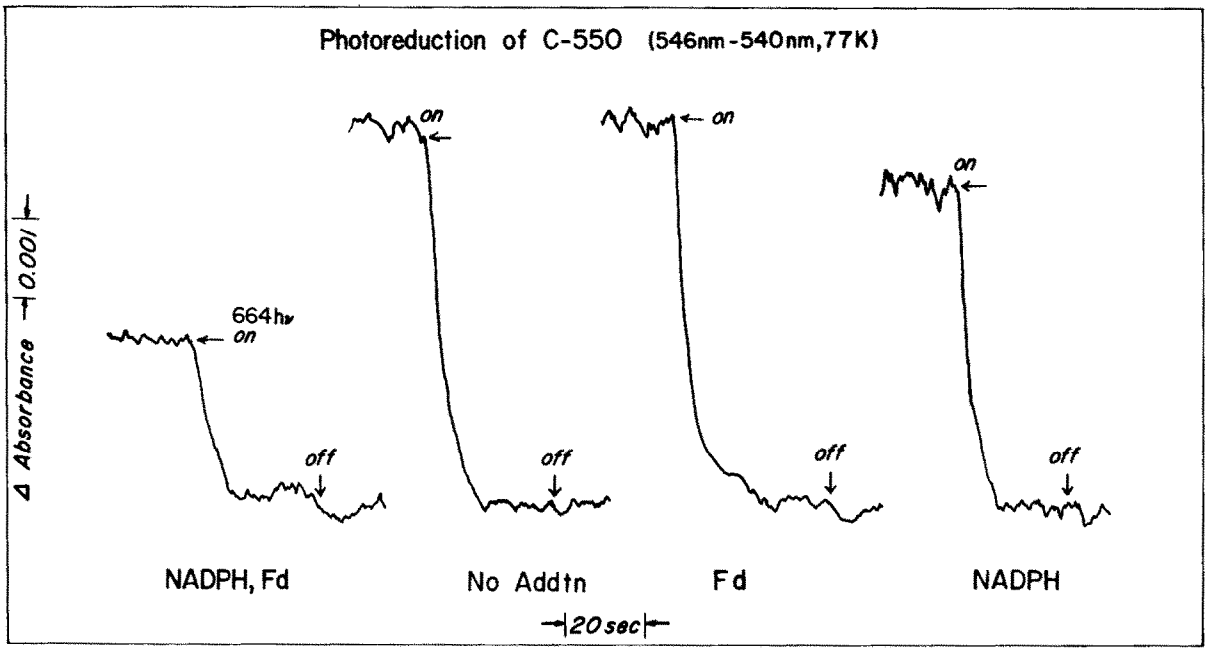

Fig.1. Effect of NADPH and ferredoxin on photoreduction of C-550 at 77 K. The basic reaction mixture (corresponding to 'No Addtn') contained spinach chloroplasts (500 μg) and (μmol): Tricine–KOH buffer (pH 8.35), 100; $MgCl_2$, 10. Spinach ferredoxin (0.02 μmol) and NADPH (5 μmol) were added where indicated. The final addition was that of 1.2 ml glycerol. A portion of the complete reaction mixture (final vol. 2.0 ml) was transferred to a special cuvette (3 mm lightpath), frozen, and illuminated at 77 K in a DW-2 Aminco spectrophotometer operated in the dual-wavelength mode (546–540 nm). The phototube was shielded from the monochromatic (664 nm) actinic illumination (intensity ~1 × 10^4 ergs.cm^{-2}.s^{-1}) by two Corning 4-96 filters.

reduced by NADPH in the dark only in the presence of ferredoxin as the electron carrier, i.e., NADPH was the source of reducing power but reduced ferredoxin was the effective reductant. Electron transfer from NADPH to ferredoxin, catalyzed by the membrane-bound ferredoxin-NADPH reductase of chloroplasts [32], provided a supply of reduced ferredoxin.

To elucidate the path of electron transport from reduced ferredoxin to C-550, chloroplasts were pre-incubated in the dark in the presence of inhibitors known to impede the forward (light-induced) electron transport to or from individual components of the electron transport chain of chloroplasts. Figure 2 shows that antimycin and DCMU increased appreciably the extent of C-550 reduction in the light after preincubation of the chloroplasts with reduced ferredoxin (i.e., NADPH plus ferredoxin) in the dark. Thus, antimycin and DCMU appeared to be effective inhibitors of electron transport in the dark from reduced ferredoxin to C-550. The inhibitory effect of antimycin suggested that the electron transport proceeded through cytochrome b_6, an electron carrier in the ferredoxin-catalyzed cyclic electron flow [13]. The reduction of cytochrome b_6 in the dark by reduced ferredoxin is supported by the spectrophotometric data presented below and is consistent with the immunological evidence [33] that an antibody to ferredoxin severely inhibits the photoreduction of cytochrome b_6.

The inhibitory effect of DCMU on the dark electron transport recalled earlier observations that, during the forward, light-induced electron flow from water, DCMU inhibited the oxidation but not the reduction of C-550 [27,34]. These observations suggested that the site of DCMU inhibition lay between C-550 and its nearest electron acceptor, probably plastoquinone. Accordingly, DCMU would be expected to inhibit not only electron transfer in the light between reduced C-550 and plastoquinone but also electron transfer

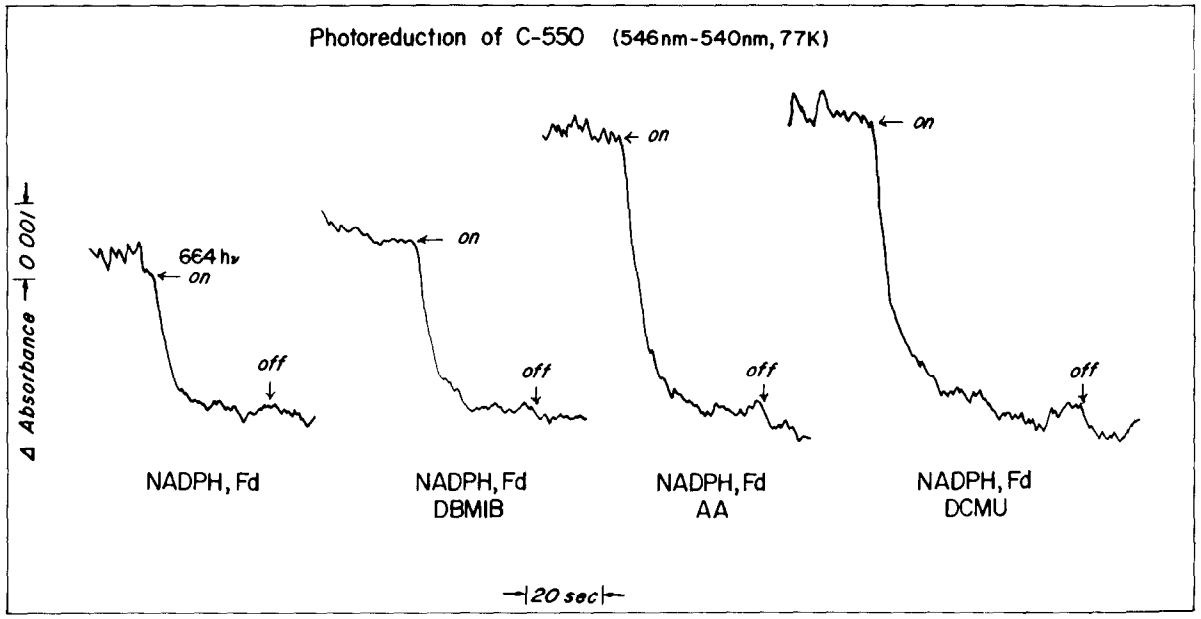

Fig 2 Effect of electron transport inhibitors on the reduction of C-550 by ferredoxin Experimental conditions as in fig 1 except that, where indicated, DBMIB (8 μM), antimycin A (18 μM), or DCMU (20 μM) was added prior to the addition of NADPH

in the dark between plastohydroquinone (or some other electron carrier that is reduced by ferredoxin via cytochrome b_6) and oxidized C-550

DBMIB, the antagonist of plastoquinone that inhibits the reoxidation of plastohydroquinone [17], was used to obtain evidence that the electron transport pathway from ferredoxin to C-550 involved plastoquinone in addition to cytochrome b_6 DBMIB inhibition was used [35,36] to implicate plastoquinone in the oxidation of photoreduced cytochrome b_6

As shown in fig 2, DBMIB did not inhibit the dark reduction of C-550 by ferredoxin This lack of inhibition might indicate that the site of DBMIB inhibition is not involved in electron transfer between cytochrome b_6 and C-550 but it might also be attributed to the special properties of DBMIB as an electron acceptor for photosystem II [37] and an oxidant of C-550 [38] Further work will therefore be needed to document the role of plastoquinone in electron transport from ferredoxin to C-550

Spectrophotometric evidence for the dark reduction of cytochrome b_6 by ferredoxin (kept reduced by NADPH) is presented in fig 3. The absorbance

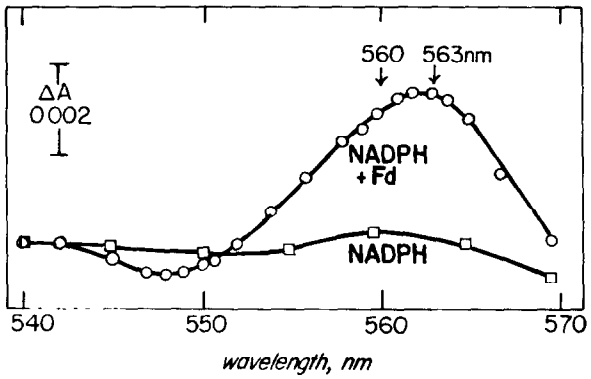

Fig 3 Absorbance changes in the 540–570 nm region produced by reduction of chloroplasts by NADPH and ferredoxin in the dark The reaction mixture (5 0 ml) contained chloroplasts (500 μg) in the following (μmol) Tricine–KOH buffer (pH 8 35), 500, NaCl, 100, and, where indicated, spinach ferredoxin, 0 05 After equilibration with N_2, 2 ml samples were each syringed into the sample cuvette (1 cm lightpath) (maintained under N_2) and the reference cuvette (open to air) The absorbance was recorded in an Aminco DW-2 spectrophotometer operated in the split-beam mode after the addition of NADPH (1 mM) to the sample cuvette and abscorbate (10 mM) to the reference cuvette Each point on the graph was corrected for baseline changes

changes in the α region showed a peak at 563 nm characteristic of cytochrome b_6 but the spectrum was not symmetrical. The increased absorbance around 560 nm is attributed to the α peak of the low-potential form of cytochrome b_{559} [39,40] which was also reduced by ferredoxin. Cytochrome *f* and the high-potential form of cytochrome b_{559}, normally present in the reduced state in isolated chloroplasts, were maintained in that state by the presence of ascorbate in the reaction mixtures. NADPH without ferredoxin (or ferredoxin alone) was ineffective as a reductant of cytochrome b_6 (fig.3).

Further evidence for the reduction of cytochrome b_6 by ferredoxin is given in fig.4. Here the stronger reducing power of H_2 gas plus hydrogenase replaced NADPH as the source of electrons for ferredoxin and resulted in a more complete reduction of cytochrome b_6, as reflected in the amplitude of the absorbance changes in the α region with a peak at 563 nm. The more complete reduction of cytochrome b_6 seemed to overshadow the concurrent reduction of the low-potential form of cytochrome b_{559} and gave a more symmetrical spectrum of reduced cytochrome b_5. H_2 alone, without ferredoxin, was ineffective (fig.4).

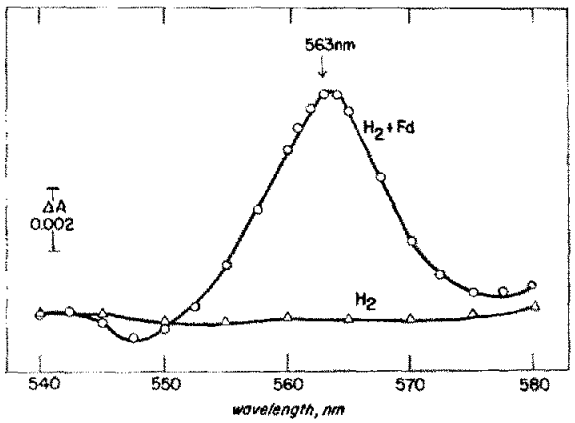

Fig.4. Absorbance changes in the 540–580 nm region produced by reduction of chloroplasts by H_2-hydrogenase in the presence and absence of ferredoxin. The reaction mixture (5.0 ml) contained chloroplasts (500 μg), 20 μl of crude hydrogenase (see section 2) and the following (μmol): Tricine KOH buffer (pH 8.35), 100; NaCl, 100; and, where indicated, spinach ferredoxin, 0.025. The reaction mixture was equilibrated with N_2 and added to the sample and reference cuvettes as in fig.3. Next, 5 mM ascorbate was added to the reference cuvette and the sample cuvette was equilibrated with H_2 gas. Each point on the graph was corrected for baseline changes.

4. Discussion

Previous investigations of reverse electron transport in chloroplasts, notably those in [41–44], have been concerned with an 'uphill' flow of electrons against the thermodynamic gradient, the required energy being supplied by ATP hydrolysis or by a proton gradient artificially induced by an acid–base transition. The source of electrons was a donor with a standard redox potential up to +300 mV and the terminal acceptor was Q, whose potential was estimated to be ~–35 mV [42]. Q, the hypothetical primary electron acceptor of photosystem II, is considered to be equivalent to C-550 [28].

The electron transport described here involves not a thermodynamically unfavorable, energy-dependent electron transfer from a more positive to a less positive potential but a thermodynamically favorable electron transfer from a strongly reducing chloroplast component (ferredoxin, $E'_0 = -420$ mV) [45,46], to C-550, a chloroplast component with a redox potential near 0 V. The direction of this dark electron flow is opposite to that of the familiar, forward, light-induced electron flow from C-550 to ferredoxin and $NADP^+$ in noncyclic photophosphorylation. The dark electron flow described here provides chloroplasts with a ferredoxin-dependent mechanism for poising cyclic photophosphorylation under conditions when NADPH accumulates, i.e., when cyclic photophosphorylation functions by itself without the concurrent operation of noncyclic photophosphorylation [1,2]. For this poising mechanism to be effective, electrons from ferredoxin need not actually reduce C-550 but only an adjacent electron carrier, like plastoquinone. Once such an adjacent carrier is reduced, the outflow of electrons from C-550 is blocked.

The dark electron flow from reduced ferredoxin to C-550 appears to involve cytochrome b_6 as one of the electron carriers and, like the light-induced cyclic flow, is inhibited by antimycin. The electron transport segment from reduced cytochrome b_6 to C-550 was found to be inhibited by DCMU.

Apart from the closing of reaction centers of photosystem II through the reduction of C-550 by NADPH-ferredoxin, attenuation of electron pressure from water and a resultant poising of cyclic photophosphorylation can also be experimentally accomplished:
(i) By the use of far-red illumination that is unfavor-

able for photosystem II or, under white light or short-wavelength illumination that is favorable for photosystem II, in one of two ways

(ii) By the use of selected concentrations of DCMU, i e , concentrations high enough to prevent over-reduction of cyclic electron carriers by photosystem II but not so high as to shut off photosystem II completely and bring about the over-oxidation of cyclic electron carriers, or

(iii) By having cyclic photophosphorylation function concurrently with $NADP^+$-linked noncyclic photophosphorylation and using $NADP^+$ to drain off electrons from water [1,2]

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