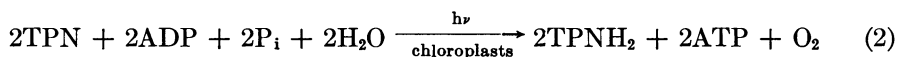# ROLE OF CHLOROPLAST FERREDOXIN IN THE ENERGY CONVERSION PROCESS OF PHOTOSYNTHESIS

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Chloroplast ferredoxin,[1-4] a water soluble iron protein with a redox potential[5] ($E_0' = -432$ mV, pH 7.55) close to that of the hydrogen electrode, is the most electronegative constituent isolated so far from the photosynthetic apparatus of green plants. Reduced ferredoxin has recently been identified[6] as the earliest, chemically defined reductant formed by chloroplasts at the expense of absorbed radiant energy. These properties are compatible with a role for ferredoxin as an electron carrier in cyclic (eq. 1) and noncyclic (eq. 2) photophosphorylation[7-10]—the two components of the photosynthetic energy conversion process that produces the assimilatory power (TPNH$_2$ and ATP[11]) to drive carbon assimilation by chloroplasts.[12-14]

$$ADP + P_i \xrightarrow[\text{chloroplasts}]{h\nu} ATP \qquad (1)$$

$$2TPN + 2ADP + 2P_i + 2H_2O \xrightarrow[\text{chloroplasts}]{h\nu} 2TPNH_2 + 2ATP + O_2 \qquad (2)$$

This article reports evidence for a role of ferredoxin in an endogenous type of cyclic photophosphorylation by chloroplasts (eq. 1), which proceeds anaerobically without added cofactors. A requirement for ferredoxin (then called "TPN-reducing factor"[1]) in noncyclic photophosphorylation (eq. 2) was already observed when this process was first discovered,[8] and this requirement has since been further documented.[9, 15-17] Evidence was also obtained several years ago[3] for a requirement of a "TPN-reducing factor" (i.e., ferredoxin) in what is now called pseudocyclic photophosphorylation[18] by chloroplasts, but the significance of these earlier observations for the mechanism of energy conversion in photosynthesis has only now been realized.

Methods.—"Broken" chloroplasts[28] (P$_{1s}$ or C$_{1s}$) were used in all experiments. The incandescent light used for illumination was filtered through a Jena G1 or G2 filter, which cut off light of wavelength shorter than 500 mμ. The ATP formed was estimated by the method of Hagihara and Lardy.[19] Other experimental procedures were the same as those used previously.[28]

Results and Discussion.—In the mechanisms currently envisaged in our laboratory,[10, 20] cyclic and noncyclic photophosphorylation share the same primary photochemical act which gives rise, within the chloroplasts, to an electron flow from an excited chlorophyll molecule to an endogenous, primary electron acceptor. If, as recent results have shown,[6] ferredoxin is the earliest primary electron acceptor that can now be isolated following the photochemical act, then it follows that photoreduction of ferredoxin should be a common feature of the electron flow pathways of either cyclic or noncyclic photophosphorylation. The two pathways would differ in the electron acceptors beyond ferredoxin. In the case of noncyclic photophosphorylation, the electrons from ferredoxin would be transferred to TPN,

whereas in the case of cyclic photophosphorylation they would "cycle" back to "electron-deficient" chlorophyll molecules[10, 20] via a chain of endogenous electron carriers.   (The transfer of electrons from reduced ferredoxin to TPN occurs only in the presence of a flavoprotein reductase which is contained in the chloroplasts.[21])

If this formulation of the role of ferredoxin is correct, then a transition from noncyclic to cyclic photophosphorylation should be regulated by the availability of TPN as the electron acceptor.   Figure 1 shows that this was experimentally observed.   The lowermost curve represents cyclic photophosphorylation in the absence of TPN.   The rate of ATP formation by cyclic photophosphorylation was lower than that by noncyclic photophosphorylation in the presence of TPN.   Noncyclic photophosphorylation continued at the higher rate as long as a supply of oxidized TPN was available (uppermost curve).   But when all of the available TPN was reduced (middle curve), the more rapid noncyclic photophosphorylation came to an end and the slower cyclic photophosphorylation took its place.   The rate of this "delayed" cyclic photophosphorylation was the same as the rate of the initial cyclic photophosphorylation in the absence of TPN (compare the slopes of the middle and lowermost curves in Fig. 1).   Where TPN was added (uppermost and middle curves of Fig. 1), its photoreduction was measured spectrophotometrically by absorption at 340 mμ.   The quantities of TPNH$_2$ found confirmed the stoichiometry of noncyclic photophosphorylation[8] (TPNH$_2$/ATP $= 1$).

The experiments represented by Figure 1 were carried out under aerobic conditions.   Forti and Jagendorf,[22] and Black et al.[23] have observed that under these conditions ferredoxin ("PPNR") stimulates an endogenous photophosphorylation which proceeds in the absence of added cofactors, but is dependent on, and consumes, molecular oxygen as the terminal electron acceptor.   Forti and Jagendorf[22] interpreted their data as ruling out the existence in chloroplasts of a truly endogenous cyclic photophosphorylation, i.e., a phosphorylation that would proceed without the participation of molecular oxygen as the terminal electron acceptor.   Evidence was needed, therefore, to show that ferredoxin could catalyze an endogenous cyclic photophosphorylation under anaerobic conditions, when oxygen was initially excluded and its photochemical production effectively prevented.

Evidence for an endogenous ferredoxin-catalyzed cyclic photophosphorylation, which proceeds anaerobically without the addition of other cofactors, was obtained after first establishing the experimental conditions under which this type of photophosphorylation can be observed.   These experimental conditions include a relatively low light intensity (illumination higher than 10,000 lux was inhibitory), an optimum pH about 8.2, and the use of p-chlorophenyldimethyl urea (CMU) as an inhibitor of oxygen evolution.   The significance of these experimental conditions will be discussed in a subsequent publication.

Table 1 shows the dependence of anaerobic cyclic photophosphorylation on the addition of ferredoxin.   In other experiments, it was found that under anaerobic conditions the ferredoxin requirement was much greater (up to tenfold) for cyclic photophosphorylation than for the noncyclic type.

As shown in Table 2 the anaerobic cyclic photophosphorylation catalyzed by ferredoxin was strongly dependent on the presence of CMU.   In the absence of CMU there was no significant photophosphorylation.   The addition of 10 μg of CMU in-

creased the rate of photophosphorylation about sevenfold, but 100 μg of CMU was inhibitory.
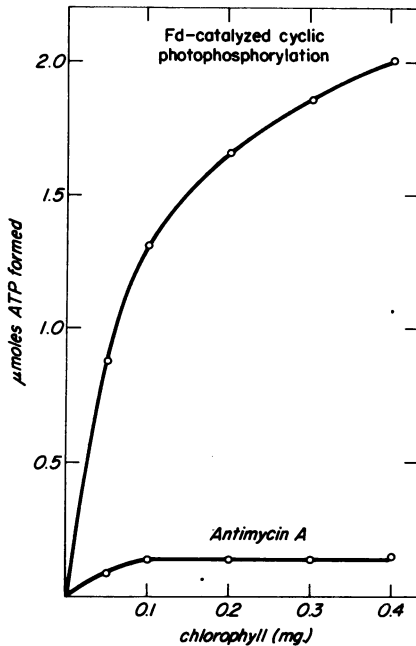

FIG. 1.—Transition from noncyclic to cyclic photophosphorylation, catalyzed by ferredoxin (Fd), as influenced by TPN availability.

The reaction mixture contained in a final volume of 4.5 ml, broken chloroplasts (C_{18}) containing 300 μg of chlorophyll, 1,500 μg of spinach ferredoxin and the following in μmoles: tris buffer pH 8.3, 150; $MgCl_2$, 7.5; ADP, 15; $K_2HP^{32}O_4$, 15; and TPN as indicated. The reaction was carried out in 1 cm optical path cuvettes under air at 15°C. Illumination was by incandescent light (10,000 lux) filtered through a Jena G2 filter. At the indicated times, 0.5 ml aliquots of the reaction mixture were withdrawn for ATP determination and 0.3 ml for TPNH$_2$ determination (see text). The ATP produced is plotted as μmoles ATP in 1.5 ml of reaction mixture.

FIG. 2.—Experimental conditions were as described in the legend for Table 1 except that 1,500 μg of spinach ferredoxin were used throughout and a variable amount of chlorophyll as indicated. 10 μg of antimycin A were added where indicated.

A notable feature of ferredoxin-catalyzed cyclic photophosphorylation was its sensitivity to antimycin A over a wide range of chlorophyll concentration (Fig. 2). Sensitivity to antimycin A has been observed in the endogenous anaerobic cyclic photophosphorylation by chromatophores of the purple bacteria, *Rhodospirillum rubrum*[24, 25] and *Chromatium*.[26] The inhibition of cyclic photophosphorylation in chromatophores does not occur in the presence of catalytic amounts of phenazine methosulfate,[24, 27] which evidently serves as a bypass around the site of antimycin A inhibition. Interestingly enough, the usual type of cyclic photophosphorylation in chloroplasts—measured in the presence of added cofactors and at relatively high chlorophyll concentrations—was also found to be resistant
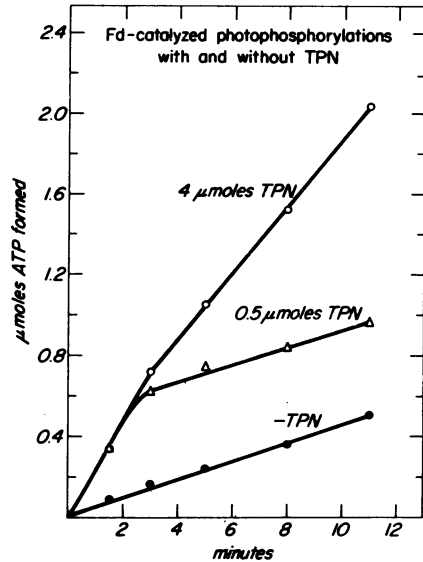
TABLE 1

EFFECT OF FERREDOXIN ON CYCLIC PHOTOPHOSPHORYLATION IN AN ATMOSPHERE OF ARGON

| Ferredoxin added (μg) | ATP formed | |
	cpm	μmoles
0	23	0.00
30	146	0.02
75	410	0.07
150	789	0.14
300	2,109	0.37
750	7,404	1.29
1,500	10,816	1.88

The reaction mixture contained, in a final volume of 1.5 ml: spinach ferredoxin as indicated, p-chlorophenyl-dimethyl urea (CMU), 2 μg; washed broken chloroplasts containing 100 μg of chlorophyll and the following in μmoles: tris buffer pH 8.0, 50; MgCl$_2$, 2.5; ADP, 5; K$_2$HP^{32}O$_4$, 5. The reaction was carried out in Warburg vessels in an atmosphere of argon at 15°C. Illumination was by incandescent light (10,000 lux) filtered through a Jena G2 filter.

TABLE 2 .

EFFECT OF P-CHLOROPHENYLDIMETHYL UREA (CMU) ON FERREDOXIN-CATALYZED CYCLIC PHOTOPHOSPHORYLATION IN AN ATMOSPHERE OF ARGON

CMU added (μg)	ATP formed (μmoles)
None	0.21
0.1	0.21
1.0	0.53
10	1.57
100	0.02

Experimental conditions were as described in the legend for Table 1 except that 1,500 μg of spinach ferredoxin were used throughout. CMU was added as indicated.

to antimycin A inhibition.[28] Since, in mitochondria,[29, 30] antimycin A inhibition is considered to be indicative of the participation of cytochrome b in electron transport, the sensitivity to antimycin A suggests a possible participation of the cytochrome b_6 component of chloroplasts[31, 32] in the ferredoxin-catalyzed cyclic photophosphorylation.

Concluding Remarks.—A previously puzzling feature of cyclic photophosphorylation in isolated chloroplasts, a feature which distinguished it from cyclic photophosphorylation in bacterial chromatophores, was a dependence on an added electron carrier such as vitamin K or phenazine methosulfate. A possible, though heretofore experimentally unsupported, explanation of this difference was that chloroplasts, but not chromatophores, automatically lost a soluble constituent in the process of isolation. The present findings point to chloroplast ferredoxin as being the water-soluble constituent of cyclic photophosphorylation which is, at least in part, lost from chloroplasts when they are removed from the cell. However, it is still premature to say what role bacterial ferredoxins[5] play in the mechanism of bacterial photophosphorylation. Differences between the two systems require further study. Thus, it has been found that the pyridine nucleotide-reducing system in chromatophores differs from that in chloroplasts. Chromatophores photoreduce DPN in preference to TPN and do so without the addition of a soluble cofactor such as ferredoxin.[26]

In chloroplasts, ferredoxin appears to be a branching point in the electron transport systems that result either in cyclic or in noncyclic photophosphorylation. Since TPN is required for noncyclic photophosphorylation but not for cyclic photophosphorylation,[33] it follows that when the photoreduced ferredoxin is reoxidized by TPN (with the aid of an appropriate flavoprotein enzyme[21]), noncyclic photophosphorylation would result. When oxidized TPN is unavailable

as an electron acceptor, the photoreduced ferredoxin would be reoxidized by a bound component of the grana, and cyclic photophosphorylation would result. It is thus possible to envisage that the availability of TPN as an electron acceptor would serve as a physiological regulator between cyclic and noncyclic photophosphorylation. TPN availability would, in turn, be regulated by the reoxidation of $TPNH_2$ in the course of reductive carbon assimilation (cf. ref. 8).

It is interesting to note that, although reduced ferredoxin is nonenzymatically oxidized by oxygen,[5] an appreciable leakage of electrons to O_2 (is always present around the chloroplasts *in vivo*) is prevented by the strong affinity of reduced ferredoxin, (a) for the TPN-reducing system, and (b) for the grana-bound electron carriers of cyclic photophosphorylation. We have observed, even under aerobic conditions, a cyclic photophosphorylation in isolated chloroplasts that was essentially independent of molecular oxygen as the terminal electron acceptor. A more detailed discussion of this point will be presented elsewhere.

* Aided by grants from the U.S. Public Health Service and the Office of Naval Research.

[1] Ferredoxin of chloroplasts is the same substance as the methemoglobin-reducing factor of Davenport, Hill, and Whatley,[2] the TPN-reducing factor of Arnon, Whatley, and Allen,[3] and the photosynthetic pyridine nucleotide reductase (PPNR) of San Pietro and Lang.[4] This change in nomenclature, discussed in more detail elsewhere,[5] is based on the recently discovered chemical and functional similarities of ferredoxins from bacteria and chloroplasts.

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[11] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, orthophosphate; TPN, $TPNH_2$, oxidized and reduced forms of triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.

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[33] Our recent experiments, with improved experimental methods, do not support our earlier conclusion[3] that TPN is also required for cyclic photophosphorylation.

THE DEFECTIVENESS OF ROUS SARCOMA VIRUS*

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The Bryan high-titer strain of Rous sarcoma virus (RSV) produces foci of transformed cells after infecting monolayers of chick embryo cells in tissue culture. The transformed cells take on the typical rounded appearance of cultivated Rous sarcoma cells and are no longer restricted to growth in a monolayer. When a stock of RSV is diluted beyond the end point for focus formation, another virus can readily be isolated which has been called Rous-associated virus (RAV).[1] RAV does not cause a noticeable morphological change in cells, but it induces resistance to RSV within a few days, and this resistance is the basis for the assay of RAV. RAV is indistinguishable from RSV in heat sensitivity, cellular site of maturation, growth rate, and immunological specificity.[1] It is known to differ from RSV only in its failure to produce either foci in tissue culture or sarcomas in the chicken. It does, however, produce leukosis in chickens, and can be considered a virus of the avian leukosis complex. In view of its close relationship to RSV and its presence in RSV stocks in higher titer than RSV itself, it seemed unlikely that RAV was a mere accidental contaminant of the RSV stock. A study was undertaken to clarify the relationship of the two viruses. Attempts were made to isolate a stock of RSV free of RAV by picking single foci of transformed cells. Although the transformed cells present in such foci multiplied indefinitely and maintained their distinctive morphology, they failed to produce either RSV or RAV. However, when RAV was added to such cells, they quickly produced large amounts of both RAV and RSV. We conclude from these observations that RSV is a defective virus which can only produce mature virus in the presence of a helper virus such as RAV.

Material and Methods.—RSV stock and assay: The high titer strain of RSV was used in the present studies. The stock was obtained from the medium of heavily infected monolayers of chick embryo cells. The medium was subjected to sonic vibration at 9 kc to disperse virus aggregates. The virus titer was assayed by focus formation on chick embryo cells.[2] The RSV stock contained 5×10^6 focus forming units (FFU) of RSV per ml and about 5×10^7 infectious units of RAV per ml (see below).

RAV stock and assay: RAV was isolated from a stock of RSV and was purified twice by limiting dilution *in vitro*. The stock used was obtained by disrupting infected chick embryo cells by sonic vibration. The virus was assayed by its interference with RSV infection as described