FACTORS INFLUENCING PHOTOSYNTHETIC ENHANCEMENT IN ISOLATED CHLOROPLASTS

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Summary: Photosynthetic enhancement of oxygen evolution (linked to CO_2 assimilation) in isolated chloroplasts was found to be governed by the supply of ATP. The addition of ATP (but not AMP) abolished enhancement that consistently occurred without added ATP. Enhancement in the $H_2O \rightarrow$ NADP reaction by chloroplasts was investigated in the light of one recent report that the phenomenon occurs when pure ferredoxin is replaced by a crude preparation (PPNR) and another report that the phenomenon is governed by Mg^{++} concentration. Fractionation of PPNR led to the isolation of a protein factor which when added to pure ferredoxin induced enhancement. However, the rate of NADP reduction with pure ferredoxin and without enhancement was greater than the maximum rate of NADP reduction with enhancement induced by either the protein factor of PPNR. The report that Mg^{++} concentration governs enhancement was not confirmed.

The term enhancement denotes the phenomenon that the rate of photosynthesis when measured under simultaneous illumination by red and far-red light is greater than the sum of the rates obtained with red and far-red light separately (1,2). Enhancement was first observed in intact cells engaged in complete photosynthesis but an understanding of the mechanism underlying this phenomenon has been sought from investigations of photosynthetic reactions in isolated chloroplasts (2).

In investigations with isolated chloroplasts, the most widely tested hypothesis was that enhancement indicated a collaboration, in series, of a long-wavelength (PSI) and a short-wavelength photosystem (PSII) in the light-induced electron flow from water to NADP. These experiments led to sharply conflicting results (2,3) and to a suggestion that the disagreements reflected ignorance of "definable conditions which will turn on or turn off enhancement" (2). Another proposed hypothesis (3) was that enhancement results from a joint contribution of ATP to CO_2 assimilation by two photosystems operating in parallel: cyclic photophosphorylation (PSI) and noncyclic photophosphorylation (PSII). In support of this hypothesis was an observed enhancement in isolated chloroplasts when engaged in CO_2 assimilation, measured as O_2 production, but not when engaged in the $H_2O \rightarrow$ NADP reaction (3).

The nature of enhancement has now been reinvestigated in isolated chloroplasts in the light of three recent reports. Sun and Sauer (4) reported that previous failures to observe enhancement in the $H_2O \rightarrow$ NADP reaction by

isolated chloroplasts resulted from too low a concentration of Mg^{++} (or Mn^{++}). Sane and Park (5) observed enhancement in the $H_2O \rightarrow$ NADP reaction by chloroplasts by replacing pure ferredoxin with a crude preparation (PPNR). Lastly, in an investigation concerned not with enhancement but with CO_2 assimilation by isolated chloroplasts, Schürmann et al. (6) obtained evidence for a role of cyclic photophosphorylation (or exogenous ATP) in shortening a lag period in CO_2 assimilation and stimulating the conversion of phosphoglycerate to carbohydrates.

In the present investigation, the controlling role of Mg^{++} on enhancement in the $H_2O \rightarrow$ NADP reaction, as proposed by Sun and Sauer (4), was not confirmed. An enhancement was induced in this reaction by replacing pure ferredoxin with PPNR, as observed by Sane and Park (5). We have now isolated from PPNR a protein factor which cannot by itself support NADP reduction but which produces enhancement when added as a supplement to pure ferredoxin. However, the enhancement that resulted from the addition of either PPNR or the protein factor gave maximum rates of NADP reduction that were consistently lower than the rates obtained without enhancement in the presence of pure ferredoxin. In other experiments, new evidence was obtained in support of the hypothesis that enhancement results from a joint contribution of ATP to CO_2 assimilation by cyclic and noncyclic photophosphorylation. The addition of ATP (but not AMP) abolished enhancement in isolated chloroplasts engaged in oxygen evolution linked to CO_2 assimilation.

METHODS

Broken spinach chloroplasts (C_{1S}) were prepared in NaCl by the method of Whatley and Arnon (7) and whole chloroplasts were made in sorbitol by the procedure of Kalberer et al. (8). Chlorophyll was determined as described by Arnon (9). NADP photoreduction by broken chloroplasts, oxygen evolution by whole chloroplasts, and enhancement were measured as described previously (3).

Purified spinach ferredoxin was prepared by the method of Tagawa and Arnon (10). Crude ferredoxin (PPNR) was prepared by a modification of the procedure of San Pietro and Lang (11).

A protein (fraction A) was obtained by passing the PPNR preparation over a DEAE-cellulose column (15 x 3 cm), equilibrated with water and by collecting the passed solution. The column was then eluted with 0.15 M Tris-HCl buffer, pH 7.3, containing 0.12 M NaCl. The eluate was combined with the passed solution and dialyzed overnight against 0.005 M Tris-HCl buffer, pH 8.0. The dialyzed solution was lyophilized, dissolved in 3 ml of water, and placed on a DEAE chromatography column (45 x 2.5 cm) equilibrated with 0.075 M Tris-HCl buffer, pH 7.3, containing 0.06 M NaCl. The column was

Table 1

EFFECT OF Mg^{++} CONCENTRATION ON ENHANCEMENT

($H_2O \rightarrow NADP$)

mM $MgCl_2$	ΔA_{340}/min x 10^3			Enhancement
	A	B	C	C / A+B
	708	650	(708+650)	
0	14.7	29.4	40.6	0.93
5	12.4	19.2	33.9	1.07
7.5	13.6	16.9	32.8	1.07
10	14.7	13.6	28.3	1.00
20	9.0	6.8	15.8	1.00

The reaction mixture (1.0 ml volume) contained broken chloroplasts equivalent to 100 µg chlorophyll and (in µmoles): K_2HPO_4, 2; ADP, 2; NADP, 2; ferredoxin, 0.01; Tricine [N-Tris-(hydroxymethyl)methyl glycine], 100; and $MgCl_2$ as indicated. Gas phase, air; temperature, 20°. Light intensity (ergs/cm^2 sec): 708 nm, 1.5 x 10^4; 650 nm, 4 x 10^2.

eluted with the same buffer solution and the eluate collected in 5-ml fractions. Fractions (42-53) that included the first protein peak (monitored by absorbance at 275 nm) were combined, dialyzed against 0.005 M Tris-HCl buffer, pH 8.0, lyophilized, and designated fraction A. Fraction A (but not a second protein peak in later fractions) had activity as described below. Prior to use, fraction A was suspended in water at a concentration that gave at 275 nm an absorbance of 1.0 (0.2-cm cuvette).

RESULTS AND DISCUSSION

Effect of Mg^{++} concentration.--Sun and Sauer (4) reported that Mg^{++} concentration (optimally, 7.5 mM) is the "principal controlling factor" of enhancement. This conclusion, which is in conflict with reports of enhancement in the presence of only 1 mM Mg^{++} (12) or even without any added Mg^{++} (13) and with reports of lack of enhancement in the presence of 10 mM Mg^{++} (5), is not supported by the experiments represented by Table 1. In these and other experiments [in which we used the experimental conditions of Sun and Sauer (4)] no enhancement was found in the photoreduction of NADP by water over a range of Mg^{++} concentration from 0 to 60 mM. Presence or absence of enhancement was determined by measuring the rate of NADP reduction in far-red light (A), in red light (B) and in simultaneous illumination by both beams (C). An enhancement effect is indicated when C/A+B > 1.

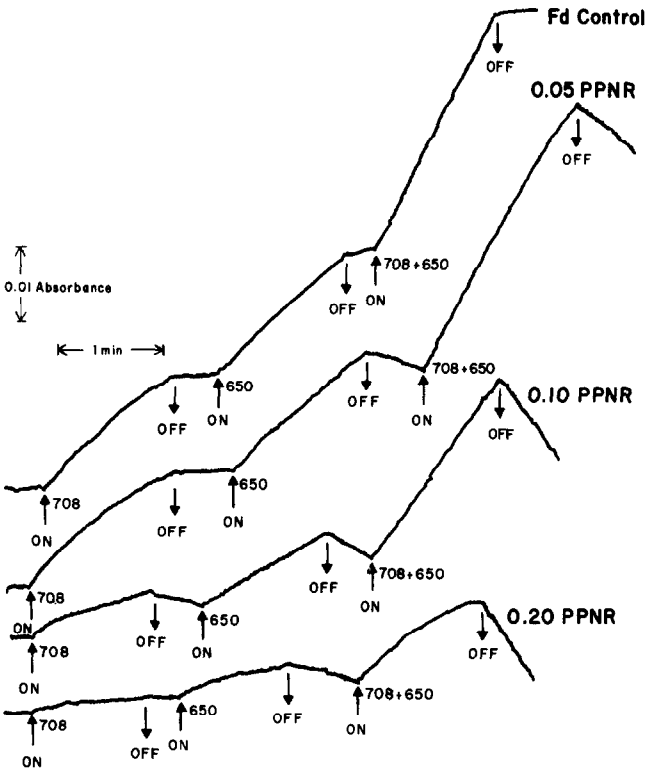

Fig. 1. Effect of PPNR on enhancement. Experimental conditions as in Table 1 except that, where indicated, increasing amounts of PPNR (ml) replaced pure ferredoxin and $MgCl_2$, 10 µmoles, was used throughout. Light intensity (ergs/cm^2 sec): 708 nm, 1.5 x 10^4; 650 nm, 4 x 10^2.

Effect of PPNR.--The effect of replacing pure ferredoxin by crude ferredoxin (PPNR) on the rate of the $H_2O \rightarrow$ NADP reaction in chloroplasts, at low intensities of red and far-red light, is shown in Fig. 1. Upon turning off the light, PPNR, unlike pure ferredoxin, was found to induce a back reaction, i.e., the reoxidation of $NADPH_2$. This dark back reaction was particularly pronounced at the higher concentrations of PPNR and, assuming that it also proceeded during the period of illumination, would influence the rates of NADP reduction observed at the high concentrations of PPNR. Such an effect of the back reaction would be pronounced at the low light intensities used in these experiments. In similar experiments made at saturating light intensities, the rate of NADP photoreduction did not decrease as much at the higher concentrations of PPNR even though high rates of the dark back reaction were observed.

In agreement with the findings of Sane and Park (5), the substitution of PPNR for pure ferredoxin gave an enhancement in the $H_2O \rightarrow$ NADP reaction by

Table 2

EFFECT OF PPNR AND FRACTION A ON ENHANCEMENT AND PHOTOCHEMICAL ACTIVITY OF CHLOROPLASTS IN THE PHOTOREDUCTION OF NADP BY WATER

	ΔA_{340}/min x 10^3					
	A	B	C	A+B	Enhancement	Activity Ratio $\frac{(A+B)_{PPNR}}{(A+B)_{Fd}}$
	708	650	(708+650)			
I. PPNR						
Ferredoxin	12.4	13.6	27.1	26.0	1.04	----
0.05 PPNR	10.2	12.4	27.1	22.6	1.20	0.87
0.10 PPNR	5.6	9.0	21.4	14.6	1.46	0.57
0.20 PPNR	1.1	3.4	9.0	4.5	2.00	0.17
II. Fraction A						
Ferredoxin (Fd)	9.0	11.3	20.3	20.3	1.00	----
Fd + fraction A	5.7	7.9	13.6	18.0	1.33	0.89

Experimental conditions in part I were the same as in Fig. 1 and in part II, the same as in Fig. 2.

chloroplasts (Table 2, part I). Enhancement was significant even when computed without correcting the rate of the forward light reaction for an assumed concurrent back dark reoxidation--a back reaction that could be measured only when the light was turned off. When such a correction is made--as was done by Sane and Park (5)--the calculated enhancement shown in Table 2 (part I) is appreciably higher.

An examination of the data in Table 2 (part I) reveals that the rate of NADP reduction in the presence of PPNR was less than that in the presence of ferredoxin under all conditions of illumination. However, in the presence of PPNR, the rate of NADP reduction under simultaneous illumination by the two light beams (C) was greater than the sum of the rates under illumination by separate light beams (A + B). Thus, PPNR, by a mechanism as yet unknown, brought about a computed enhancement ($C/A+B > 1$).

Pure ferredoxin gave maximal rates of NADP reduction under all illumination conditions and no enhancement resulted from simultaneous illumination by the two beams. The rate of NADP reduction with pure ferredoxin and without enhancement was consistently greater than the rate of NADP reduction with PPNR and with enhancement (Table 2, part I, last column).

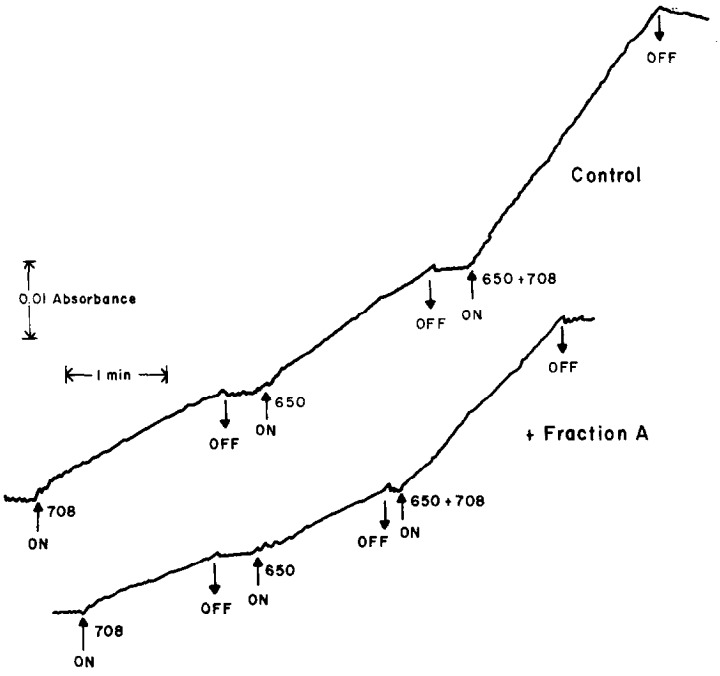

Fig. 2. Experimental conditions as in Fig. 1 except that fraction A (0.1 ml) replaced PPNR. Light intensity (ergs/cm^2 sec): 708 nm, 8 x 10^3; 650 nm, 7 x 10^2.

Effect of a protein factor.--A fractionation of the PPNR preparation yielded a protein factor (fraction A) which could not by itself support NADP reduction but which produced enhancement when added as a supplement to pure ferredoxin (Table 2, part II). The combination of ferredoxin and fraction A acted like PPNR in inhibiting the rate of NADP reduction and producing enhancement. When reduced 2,6-dichlorophenol indophenol replaced water as the electron donor for NADP reduction, PPNR or fraction A lowered the rate of electron transport but produced no enhancement. When ferricyanide was the electron acceptor, fraction A or PPNR produced neither a decrease in the rate of electron transport nor an enhancement effect. Fraction A was not replaceable by plastocyanin, ferredoxin-NADP reductase, or bovine serum albumin.

Unlike PPNR, fraction A did not give a reoxidation of $NADPH_2$ when the light was turned off (Fig. 2). It appears therefore that the PPNR component inducing inhibition of NADP reduction and enhancement can be separated from the factor(s) catalyzing the reoxidation of $NADPH_2$ in the dark. To recapitulate, with either PPNR or fraction A there was no true increment in maximal photochemical activity (implied in the photosynthetic enhancement concept),

Table 3

EFFECT OF ATP AND AMP ON ENHANCEMENT IN O_2 EVOLUTION LINKED TO CO_2 ASSIMILATION

	nmoles O_2/min			Enhancement
	A	B	C	$\frac{C}{A+B}$
	708	650	(708+650)	
Experiment I				
Control	8.2	16.5	33.0	1.34
+ATP	7.3	18.3	25.6	1.00
+AMP	8.2	18.3	34.8	1.31
Experiment II				
Control	3.6	18.3	40.2	1.83
+ATP	7.3	25.6	34.8	1.05
+AMP	5.5	18.3	44.0	1.85

The reaction mixture contained (per 1 ml) whole chloroplasts equivalent to 0.13 mg chlorophyll and (in μmoles): sorbitol, 350; HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) buffer, pH 7.6, 50; sodium pyrophosphate, 4; $KHCO_3$, 6.7; isoascorbic acid, 0.17; and ATP or AMP, 2. Gas phase, argon; temperature, 20°. Light intensity (ergs/cm^2 sec): 708 nm, 2 x 10^4; 650 nm, 1 x 10^4.

i.e., the rate of NADP reduction with pure ferredoxin and without enhancement was greater than the maximum rate of NADP reduction with enhancement induced by PPNR or fraction A. The mode of action of fraction A (or PPNR) in decreasing the rate of NADP reduction and producing enhancement will be further investigated.

Effect of ATP.--The conflicting reports on enhancement effects in electron transport by isolated chloroplasts are in sharp contrast with the consistent enhancement observed in oxygen evolution (linked to CO_2 assimilation) by whole cells (1,2) or intact chloroplasts (3). Further attention was therefore given to the possibility that enhancement results from the joint contribution by cyclic and noncyclic photophosphorylation of ATP for CO_2 assimilation. If this explanation is valid, enhancement should be influenced by exogenous ATP. Added ATP should replace that contributed by cyclic photophosphorylation, thereby eliminating the need for the cyclic process and abolishing enhancement. Table 3 shows that this was found to be the case. The addition of ATP (but not AMP) abolished enhancement in oxygen evolution by

chloroplasts engaged in CO_2 assimilation. The addition of ATP had no effect on the enhancement induced by PPNR or fraction A. We conclude, therefore, that enhancement consistently observed in chloroplasts and whole cells engaged in complete photosynthesis reflects the collaboration of cyclic and noncyclic photophosphorylation and is different from the "enhancement" associated with PPNR and the protein factor.

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