BBA 45856

THE EFFECTS OF PLASTOCYANIN ON PHOTOPHOSPHORYLATION

MARK M. ANDERSON AND RICHARD E. McCARTY
Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. (U.S.A.)
(Received May 19th, 1969)

SUMMARY

1. A simplified procedure for the large-scale isolation and purification of plastocyanin from spinach chloroplasts is reported. After electrophoresis on polyacrylamide gel, a pure protein was obtained as judged by analytical gel electrophoresis and by the fact that it contained no arginine.

2. Plastocyanin from spinach or cytochrome c_{552} from Euglena stimulated the photoreduction of $NADP^+$ in subchloroplast particles prepared by prolonged exposure of chloroplasts to sonic oscillation.

3. Photophosphorylation in the presence of pyocyanine or $NADP^+$ was enhanced by plastocyanin and cytochrome c_{552} whereas ferricyanide- or *N*-methylphenazonium methosulfate-dependent phosphorylation was not. Dichlorophenyldimethylurea abolished cyclic phosphorylation with pyocyanine and with *N*-methylphenazonium methosulfate in red light. This inhibitor had much less effect on phosphorylation in the presence of partially reduced pyocyanine or *N*-methylphenazonium methosulfate in white light.

4. Coupling factors 1 and 2 were required for optimal rates of pyocyanine- or $NADP^+$-dependent phosphorylation.

INTRODUCTION

Plastocyanin, a blue copper protein found in many photosynthetic organisms[1], has been shown to stimulate light-dependent $NADP^+$ reduction in chloroplasts which were exposed to sonic oscillation[2,3] or to detergents[4]. It also enhanced photooxidation of cytochrome *c* (refs. 5, 6) in detergent-treated chloroplasts. It has been reported that under similar conditions plastocyanin did not stimulate photophosphorylation[7]. However, GORMAN AND LEVINE[8] demonstrated that photophosphorylation in plastocyanin-deficient chloroplasts isolated from a mutant of *Chlamydomonas reinhardii* was less than 10% of that in chloroplasts from wild-type organisms.

It is the purpose of this communication to report a simplified procedure for the isolation and purification of plastocyanin from spinach chloroplasts and to show that phosphorylation in the presence of $NADP^+$ or pyocyanine in chloroplasts exposed

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, *N*-methylphenazonium methosulphate.

to sonic oscillation was considerably enhanced by plastocyanin as well as by two coupling factors for photophosphorylation. It is concluded that plastocyanin is not directly involved in cyclic electron flow and associated phosphorylation.

MATERIALS AND METHODS

Preparation of chloroplasts and subchloroplast particles

Chloroplasts were prepared from spinach as previously described[9]. Subchloroplast particles were prepared essentially as described by KATOH AND SAN PIETRO[2]. The chloroplasts, suspended in 50 ml of 0.05 M potassium phosphate buffer (pH 8.0), 0.4 M sucrose and 0.01 M NaCl, were exposed to sonic oscillation for a total of 4 min in a Branson 20-kcycles instrument at full output. The temperature during sonic treatment was kept below 8°. The mixture was centrifuged at 6000 × *g* for 10 min and the pellet was discarded. The supernatant fluid was centrifuged at 104000 × *g* for 60 min and the pellets resuspended in a volume of 0.4 M sucrose–0.01 M NaCl–0.02 M tris(hydroxymethyl)methylglycine (Tricine)–NaOH (pH 8.0) about one-half that of the chloroplast suspension which was exposed to sonic oscillation. After sedimenting the particles at 104000 × *g* for 1 h, the pellets were resuspended in a small volume of sucrose–NaCl–Tricine. The activities of these subchloroplast particles were stable for at least 1 month on storage at −80° under nitrogen. The plastocyanin content of these particles was less than 10% of that in chloroplasts.

Plastocyanin preparation

Spinach chloroplasts (3 mg of chlorophyll per ml) suspended in sucrose–NaCl–Tricine were precipitated with acetone as described by VAMBUTAS AND RACKER[10] except that the procedure was scaled up 15 fold. A crude extract was prepared from the acetone-precipitated chloroplasts and was fractionated with $(NH_4)_2SO_4$ as described[10]. The precipitate obtained between 20 and 45 % satn. of $(NH_4)_2SO_4$ was saved and the Coupling factor 1 (CF_1) it contained was purified by chromatography on DEAE-Sephadex[11]. All procedures described below were carried out at room temperature. The supernatant fluid of the $(NH_4)_2SO_4$ fraction was dialyzed overnight against 6 l of 10 mM potassium phosphate buffer (pH 7.0) with several changes of buffer. A small amount of ferricyanide was added to oxidize the plastocyanin and the entire solution was loaded on a 2.5 cm × 25 cm DEAE-cellulose[19] (Whatman, Type DE-23) column equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The column was eluted with a linear gradient of potassium phosphate buffer (pH 7.0) ranging from 10 to 400 mM. The total volume of the elution buffers was 2 l. Since the plastocyanin tended to become reduced on the column and since reduced plastocyanin migrated on the column slightly behind the oxidized form, the fractions were assayed for plastocyanin content spectrophotometrically in the presence of 0.33 mM $K_3Fe(CN)_6$. The fractions containing plastocyanin were combined and dialyzed overnight against 6 l of 30 mM potassium phosphate buffer (pH 7.0). The plastocyanin was concentrated by applying the dialyzed solution to a 3 cm × 3 cm column of DEAE-cellulose which was equilibrated with 30 mM potassium phosphate buffer (pH 7.0). The column was washed with 50 ml of the equilibrating buffer and the plastocyanin was eluted with 1 M potassium phosphate buffer (pH 8.0). The trailing fractions of plastocyanin which eluted from this column could be combined, dialyzed and concentrated as above if

maximal yields of plastocyanin were desired. The concentrated plastocyanin solution (15 ml) was passed through a 2 cm × 36 cm column of Sephadex G-75 equilibrated with 20 mM Tricine–NaOH (pH 8.0) and the peak fractions were combined and stored at −20°.

Preparation of other proteins

Ferredoxin was isolated from spinach chloroplasts by the procedure of SAN PIETRO AND LANG[12] and was partially purified, after the acetone step, by chromatography on DEAE-cellulose. Ferredoxin was eluted from the column with 1 M Tris–HCl (pH 8.0). The ferredoxin was about 50 % pure and was free of diaphorase activity. Ferredoxin–TPN^+ reductase was eluted from the DEAE-cellulose column with 0.2 M Tris–HCl (pH 8.0) and was purified by chromatography on DEAE-cellulose[13]. This preparation was over 75 % pure and was free of ferredoxin and plastocyanin. Coupling factor-2 (CF_2) was isolated either from chloroplasts or from acetone-precipitated chloroplasts[10] by the procedure of LIVNE AND RACKER[14]. Cytochrome c_{552} from Euglena chloroplasts was the generous gift of Dr. J. S. Kahn and was about 75 % pure. The blue copper protein from *Pseudomonas aeruginosa* was kindly supplied by Dr. D. C. Wharton. Bovine serum albumin (Fraction V) was defatted by the procedure of CHEN[15].

Analytical procedures

Soluble proteins were determined either by a direct spectrophotometric method[16] or by a colorimetric procedure[17]. Chlorophyll was estimated by the method of ARNON[18]. Plastocyanin was assayed spectrophotometrically in the presence of ferricyanide using the extinction coefficient reported by KATOH *et al.*[19]. Ferricyanide reduction was determined by the method of JAGENDORF AND SMITH[20]. Photophosphorylation with $^{32}P_i$ was assayed as described previously[9]. $NADP^+$ reduction was assayed in a reaction mixture which contained, in a volume of 1.0 ml, 50 mM Tricine–NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.25 mM $NADP^+$, 20 μg ferredoxin, 0.7 μg ferredoxin–$NADP^+$ reductase, 2 mM potassium phosphate buffer (pH 8.0), 3 mM ADP, 2.5 mM GSSG and 0.5 μg glutathione reductase[21] (in 20 mM Tricine–NaOH (pH 8) containing 2 % bovine serum albumin) and subchloroplast particles equivalent to 50 μg of chlorophyll. After illumination with white light ($2 \cdot 10^6$ ergs/cm^2 per sec after passage of the light through 10 cm of H_2O) for 2–5 min under nitrogen, the reaction was terminated by the addition of 0.01 ml 2 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The mixtures were then passed through 0.45-μ Millipore filters to remove the subchloroplast particles. The reduced glutathione formed in the light was taken as a measure of NADPH formation and was determined by the method of ELLMAN[22]. A dark control was run for each determination to obviate interference by colored proteins. The rates of $NADP^+$ reduction agreed well with those obtained by direct spectrophotometric assays of NADPH. The method for $NADP^+$ reduction determination reported here offers several advantages over the direct spectrophotometric determination of NADPH. Since 2 moles of GSH are formed per mole of NADPH and since the extinction coefficient of the dinitrophenolate ion is about twice that of NADPH, the assay is 4 times more sensitive than the direct method. Also, this method obviates problems with NADPH reutilization by side reactions.

Biochemicals

Tricine and 2-(*N*-morpholino)ethane sulfonic acid were purchased from General Biochemicals. Biochemicals and glutathione reductase were obtained from Sigma. DCMU was obtained from K and K Chemicals and was twice recrystallized.

RESULTS

Isolation and purification of plastocyanin

Acetone-precipitated chloroplasts are a convenient source of plastocyanin and several other chloroplast proteins. The extraction of plastocyanin from acetone-precipitated chloroplasts was, apparently, quantitative. KATOH *et al.*[19] reported that the ratio of plastocyanin to chlorophyll on a molar basis was 1:600. The yield of plastocyanin in our procedure varied from 1 mole per about 500 moles of chlorophyll to 1 mole per 450 moles of chlorophyll. The plastocyanin in the crude extracts was readily purified at room temperature by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and Sephadex G-75. The recovery of plastocyanin ranged from 40 to 50% (Table I). This recovery can be considerably increased by saving the trailing fractions from the DEAE-cellulose and Sephadex columns and concentrating them. The purified plastocyanin in the reduced form in 0.1 M potassium phosphate buffer (pH 7) gave a single boundary when analyzed in the analytical ultracentrifuge. The ratio of $A_{280\ nm}$ to $A_{597\ nm}$ of the purified preparation was 1.5:1.7. These ratios are somewhat higher than those reported by KATOH *et al.*[19]. Since the copper content of the purified protein was found to be 2 moles per 21000 g of protein in agreement with the results of KATOH *et al.* for homogeneous plastocyanin, the high ratio of $A_{597\ nm}$ to $A_{280\ nm}$ is probably not due to the presence of large amounts of contaminating protein. It is apparent, then, that the ratio of $A_{597\ nm}$ to $A_{280\ nm}$ is not a good criterion for purity of plastocyanin. This conclusion is borne out by the observations of KATOH *et al.* that $A_{597\ nm}$ of highly purified plastocyanin solutions decreased on storage. Similarily, we have found that repeated freezing and thawing of plastocyanin solutions (in 20 mM Tricine–NaOH, pH 8) considerably reduced $A_{597\ nm}$.

TABLE I

PURIFICATION OF PLASTOCYANIN

Fraction	*Volume (ml)*	*Protein (mg/ml)*	*Plastocyanin (μM)*	$\frac{A_{280\ nm}}{A_{597\ nm}}$	*Recovery (%)*
Dialyzed supernatant, after $(NH_4)_2SO_4$ fractionation	1760	1.17	3.7	32.6	—
Dialyzed combined fractions from DEAE-cellulose column	400	0.72	10.3	7.2	63.4
Combined fractions from Sephadex G-75 column	58	0.87	47.7	1.7	43.2

Plastocyanin may be further purified by preparative polyacrylamide gel electrophoresis[23]. The protein distribution in the fractions which eluted from the gel column is given in Fig. 1. Only two significant peaks were observed, a small peak comprising about 5% of the protein and a major peak which was identified as plastocyanin.

On analysis of the plastocyanin thus isolated by polyacrylamide gel electrophoresis[24] with a 10 % gel concentration and a Tris–glycine buffer system (which also contained 1 mM GSH) a single band was found. Furthermore, the purified plastocyanin contained no detectable arginine, which was found to be absent in plastocyanin by KATOH *et al.*[19]. These two criteria indicate that the purified plastocyanin was a single protein species.

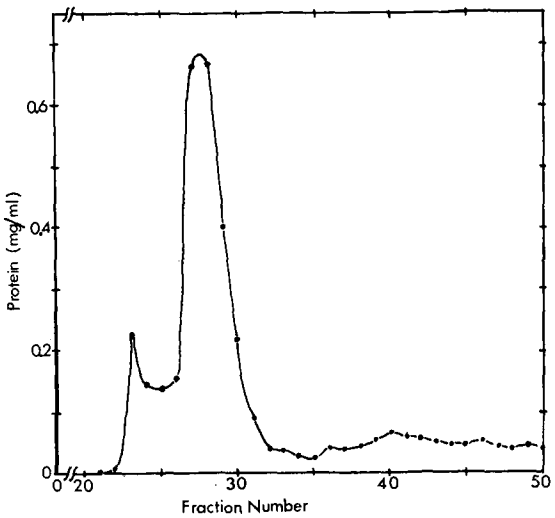

Fig. 1. Preparative polyacrylamide gel electrophoresis of plastocyanin. A preparative polyacrylamide gel electrophoresis column, similar to that designed by JOVIN *et al.*[23] was made by Dr. C. E. Furlong. A 1.5 cm × 2 cm resolving gel (12 % acrylamide, 0.4 % methylene bisacrylamide) was polymerized by the method of ORNSTEIN AND DAVIES[24]. A stacking gel (about 1.5 cm × 2 cm) containing 5 % acrylamide and 1.25 % methylene bisacrylamide was polymerized in the light in the presence of FMN[24]. The upper chamber buffer was 0.052 M Tris–glycine (pH 8.9) and contained 1 mM GSH. The lower chamber and elution buffer was 0.1 M Tris–HCl (pH 8.0). A concentrated solution of plastocyanin (about 10 mg/1.4 ml) was dialyzed for 3 h against 100 ml of a solution which contained 3.2 ml 1 M H_3PO_4 and 0.71 g Tris base per l. To the dialyzed solution, GSH was added to a final concentration of 1 mM and the solution was applied to the gel column. The electrodes were connected and the current adjusted to 10 mA at 100–150 V. The flow of elution buffer through the elution chamber was established at 0.25–0.5 ml/min and 1.5-ml fractions were collected. The entire operation was carried out at room temperature. The plastocyanin eluted from the gel column in about 2 h. $A_{280\ nm}/A_{597\ nm}$ of the eluted plastocyanin was 2.1.

Effect of plastocyanin and other redox proteins on NADP⁺ reduction

KATOH AND SAN PIETRO[2,3] showed that plastocyanin enhanced $NADP^+$ reduction with water as the electron donor in deficient subchloroplast particles. Whereas $NADP^+$ reduction was not observed at pH 6.5 in the absence of added plastocyanin, considerable $NADP^+$ reduction was detected at pH 8.0 (Table II). In some subchloroplast particle preparations, the rate of $NADP^+$ reduction in the absence of plastocyanin was as high as 10 μmoles per h per mg of chlorophyll and was stimulated only 50 % by plastocyanin. Yet, even in this preparation plastocyanin was absolutely required for $NADP^+$ reduction at pH 6.5. The observation that $NADP^+$ reduction can occur at pH 8 in the absence of plastocyanin indicates either that the removal of plastocyanin was not complete or that, at this pH, there is a partial bypass of the plastocyanin site.

TABLE II

EFFECT OF PLASTOCYANIN AND OTHER REDOX PROTEINS ON $NADP^+$ REDUCTION

$NADP^+$ reduction in plastocyanin-deficient subchloroplast particles was assayed as described in MATERIALS AND METHODS. For measurements at pH 6.5, 50 mM (*N*-morpholino) ethane sulfonate–NaOH was used. The plastocyanin and other proteins were added directly to the reaction mixture. No $NADP^+$ reduction was observed in the absence of ferredoxin.

pH	*Additions*	*$NADP^+$ reduction (μmoles NADPH formed per h per mg chlorophyll)*
Expt. 1		
8.0	None	1.8
	8.3 μM plastocyanin	11.4
6.5	None	0
	8.3 μM plastocyanin	12.5
Expt. 2		
8.0	None	3.9
	2.9 μM plastocyanin	23.2
	5.8 μM plastocyanin	29.9
	2 μM cytochrome c_{552}	11.6
	4 μM cytochrome c_{552}	14.5
	40 μg Pseudomonas blue protein	5.8
Expt. 3		
8.0	None	9.8
	6 μM plastocyanin	57.5
	8 μM cytochrome c_{552}	36.4
	Plastocyanin + cytochrome c_{552}	60.1

ELSTNER *et al.*[25] observed that cytochrome c_{552} from Euglena chloroplasts stimulated $NADP^+$ reduction with ascorbate–dichlorophenolindophenol as the electron donor in plastocyanin-deficient chloroplast preparations. As may be seen in Table II, cytochrome c_{552} at low concentrations was somewhat less effective than plastocyanin in enhancing $NADP^+$ reduction. At high concentrations, however, the rate of $NADP^+$ reduction in the presence of cytochrome c_{552} approached that observed in the presence of plastocyanin. In the presence of saturating amounts of plastocyanin, cytochrome c_{552} had no effect on $NADP^+$ reduction. A blue copper protein from *Pseudomonas aeruginosa*[26] had little effect on $NADP^+$ reduction, but in most experiments, a slight stimulation was observed. Similar enhancements of $NADP^+$ reduction were observed when ascorbate–dichlorophenolindophenol rather than water was used as the electron donor. However, neither plastocyanin nor cytochrome c_{552} had any effect on $NADP^+$ reduction in chloroplasts or in subchloroplast particles prepared by exposure of chloroplasts to 45 sec of sonic oscillation[27]. Only a very slight (less than 10%) stimulation of ferricyanide reduction in the plastocyanin-deficient subchloroplast particles was observed in agreement with the results of KATOH AND SAN PIETRO[3].

Stimulation of photophosphorylation by plastocyanin and cytochrome c_{552}

Plastocyanin and cytochrome c_{552} stimulated pyocyanine-dependent phosphorylation in deficient subchloroplast particles (Fig. 2). Cytochrome c_{552} was less effective

than plastocyanin, and the blue copper protein from Pseudomonas (5 μM) stimulated phosphorylation by only about 10 %. Although the rates of phosphorylation varied among preparations of deficient subchloroplast particles, pyocyanine-dependent phosphorylation in all preparations was stimulated at least 2-fold by plastocyanin. The very low rate of phosphorylation which occurred in the absence of pyocyanine was not enhanced by plastocyanin at the concentrations used in these experiments. The plastocyanin which was purified by polyacrylamide gel electrophoresis also stimulated pyocyanine-dependent phosphorylation. However, the cruder preparation was about 15 % more effective than the highly purified one indicating that another factor may be present in the cruder preparation. No stimulation of phosphorylation by plastocyanin in the presence of pyocyanine in chloroplasts was observed.

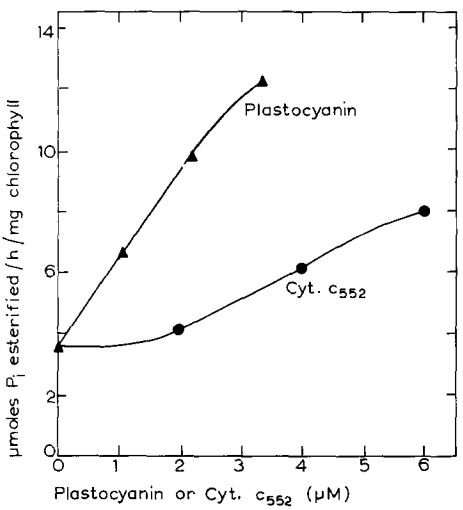

Fig. 2. Effect of plastocyanin and cytochrome c_{552} on pyocyanine-dependent phosphorylation in subchloroplast particles. Subchloroplast particles equivalent to 50 μg of chlorophyll were used. The plastocyanin and cytochrome c_{552} were added directly to the reaction mixture. The illumination time was 5 min and the gas phase was nitrogen.

Phosphorylation coupled to ferricyanide reduction was not enhanced by plastocyanin, even in a preparation of deficient subchloroplast particles in which pyocyanine-dependent phosphorylation was stimulated 4-fold (Table III). Since many workers use phenazine methosulphate (PMS), rather than pyocyanine, to support cyclic phosphorylation, it was of interest to test the effect of plastocyanin on PMS-dependent phosphorylation.

It was surprising to find that plastocyanin had no effect on PMS-dependent phosphorylation. Furthermore, the rate of pyocyanine-dependent phosphorylation in the deficient subchloroplast particles was only 10 % of that of PMS-dependent phosphorylation. Pyocyanine-catalyzed photophosphorylation in the presence or absence of plastocyanin was abolished by 20 μM DCMU, whereas PMS-dependent phosphorylation in white light was inhibited by only 15 % (Table IV). In red light, however, both PMS- and pyocyanine-dependent phosphorylation were fully sensitive to DCMU. Pyocyanine, which had been partially reduced by dithiothreitol, was much more

TABLE III

EFFECT OF PLASTOCYANIN ON PHOTOPHOSPHORYLATION WITH VARIOUS COFACTORS

Prior to assay of photophosphorylation, the deficient subchloroplast particles were diluted to final chlorophyll concentration of 0.5 mg/ml in a solution which contained 0.6 mg/ml bovine serum albumin and 20 mM Tricine–NaOH (pH 8). Aliquots of this mixture (0.1 ml) were incubated in a final volume of 0.5 ml with the indicated amounts of plastocyanin and 15 μmoles $MgCl_2$. After 10 min at room temperature, the remaining components of the photophosphorylation reaction mixtures were added in a volume of 0.5 ml and the tubes gassed with nitrogen and illuminated with $2.5 \cdot 10^6$ ergs/cm^2 per sec of white light. The pyocyanine and PMS concentrations were 50 μM. Little conversion of PMS to pyocyanine occurred under these conditions.

Cofactor	*Plastocyanin addition* (μM)	*Ferricyanide reduction* (μequiv/h *per mg chlorophyll*)	*Photophosphorylation* (μmoles P_i *esterified per h per mg chlorophyll*)
Expt. 1			
Ferricyanide	—	78.5	1.4
	3.3	85.0	1.3
Pyocyanine	—	—	3.7
	3.3	—	12.3
Expt. 2			
PMS	—	—	21.6
	10	—	20.4
Pyocyanine	—	—	1.7
	10	—	3.8

TABLE IV

EFFECT OF DCMU ON CYCLIC PHOSPHORYLATION

In Expt. No. 1, subchloroplast particles equivalent to 50 μg of chlorophyll were used. The illumination time was 5 min and the gas phase was nitrogen. Red light (> 620 nm) was provided by the use of a Corning glass filter (No. 2403). The plastocyanin concentration was 2.5 μM. In Expt. No. 2, pyocyanine was partially reduced with dithiothreitol. To 1.9 ml of a 0.5 mM solution of pyocyanine, 0.05 ml 0.25 M dithiothreitol and 0.05 ml 1 M Tris–HCl (pH 8.0) were added and the solution was gassed with argon. After 30 min at room temperature 0.1-ml aliquots of this solution were used as a source of reduced pyocyanine. The reactions were run in Thunberg tubes as follows: The pyocyanine was added to the side arm of the tube to which the otherwise complete reaction mixture had been added. The tube was immediately evacuated and then flushed with argon. The evacuation and flushing were repeated 3 times to assure anaerobiosis. Finally, the pyocyanine solution was tipped in and the tubes illuminated for 2 min with white light.

Expt. No.	*Mediator*	*Additions*	*Phosphorylation* (μmoles P_i *esterified per h per mg chlorophyll*)	
			White light	*Red light*
1	Pyocyanine	—	10.1	8.5
		DCMU (20 μM)	0	0
		Plastocyanin	28.0	22.9
		Plastocyanin + DCMU	0	0
	PMS	—	66.0	55.5
		DCMU (20 μM)	55.6	1.6
2	Pyocyanine	—	21.7	—
		DCMU (10 μM)	1.0	—
	Reduced pyocyanine	—	74.7	—
		DCMU (10 μM)	58.2	—

effective as a mediator of cyclic phosphorylation than oxidized pyocyanine. Furthermore, 10 μM DCMU inhibited phosphorylation in the presence of partially reduced pyocyanine by only 22 % and plastocyanin had no effect, as was the case with phosphorylation with PMS in white light.

Effects of coupling factors and plastocyanin on photophosphorylation

The rates of photophosphorylation in the plastocyanin-deficient subchloroplast particles were very low, even in the presence of plastocyanin. It was possible, then, that the subchloroplast particles were deficient in coupling factors as well as plastocyanin. Two coupling factors for photophosphorylation have been isolated. One factor, CF_1, is a latent Ca^{2+}-dependent ATPase[10] which is required for optimal rates of phosphorylation in deficient chloroplast preparations[10,28]. The other factor, CF_2, was extracted by treatment of chloroplasts with dilute solutions of NH_4OH (ref. 14) and was shown to enhance phosphorylation in the presence of CF_1 in subchloroplast particles which were treated with trypsin. It can be seen from Table V that CF_2 was also required for optimal rates of pyocyanine-dependent phosphorylation in subchloroplast particles prepared by the prolonged sonic treatment. CF_1 alone had little effect on phosphorylation, but CF_1 and CF_2 together enhanced phosphorylation 5-fold. Plastocyanin stimulated phosphorylation by 3-fold in the presence or absence of the coupling factors. This result indicates that our plastocyanin preparations were probably not contaminated with these coupling factors. Similar results were obtained with cytochrome c_{552}.

TABLE V

EFFECT OF CF_1 AND CF_2 AND PLASTOCYANIN ON PYOCYANINE-DEPENDENT PHOSPHORYLATION IN SONICATED CHLOROPLASTS

Aliquots (0.1 ml) of a subchloroplast particle mixture, prepared as described in the legend to Table III were placed in tubes immersed in an ice bath. To the appropriate tubes, CF_2 equivalent to 50 μg of protein was added. After 2 min CF_1 (40 μg of protein) and 3 nmoles of plastocyanin were added, where indicated. The volume in each tube was adjusted to 0.70 ml by the addition of water. Following the addition of 5 μmoles of $MgCl_2$, the tubes were removed from the ice bath. After 10 min at room temperature, the remaining components of the photophosphorylation assay mixture were added to bring the volume to 1.0 ml. The tubes were then illuminated for 5 min at room temperature. The gas phase was nitrogen.

Additions to subchloroplast particles	*Phosphorylation (μmoles P_i esterified per h per mg chlorophyll)*
None	2.8
CF_1	3.9
CF_2	10.5
$CF_1 + CF_2$	15.2
Plastocyanin	9.3
CF_1 + plastocyanin	12.6
CF_2 + plastocyanin	35.2
$CF_1 + CF_2$ + plastocyanin	43.2

Plastocyanin also markedly enhanced pyocyanine-dependent phosphorylation in trypsin-treated subchloroplast particles, indicating that plastocyanin is at least in part destroyed or dissociated by the trypsin treatment (Table VI). In these subchloro-

TABLE VI

EFFECT OF COUPLING FACTORS AND PLASTOCYANIN ON PYOCYANINE-DEPENDENT PHOSPHORYLATION IN TRYPSIN-TREATED SUBCHLOROPLAST PARTICLES

Subchloroplast particles, prepared by short exposure of chloroplasts to sonic oscillation[23] were treated with trypsin as described by LIVNE AND RACKER[14]. Aliquots of trypsin-treated subchloroplast particles (50 μg of chlorophyll) were suspended in 20 mM Tricine-NaOH–(pH 8) containing 0.66 mg/ml of bovine serum albumin, and were treated with CF_1 (30 μg), CF_2 (20 μg) and 4 nmoles plastocyanin as described in the legend to Table IV.

Additions to trypsin-treated particles	*Phosphorylation (μmoles P_i esterified per h per mg chlorophyll)*
None	5.1
CF_1	16.0
CF_2	32.4
CF_1 + CF_2	40.6
Plastocyanin	25.2
Plastocyanin + CF_1	31.8
Plastocyanin + CF_2	47.0
Plastocyanin + CF_1 + CF_2	41.2

plast particles, plastocyanin did not enhance phosphorylation when CF_1 and CF_2 were present. In addition, the plastocyanin stimulation of phosphorylation was less in the presence of either CF_1 or CF_2 alone than in the absence of coupling factors. Thus, it is possible that some other factor had been damaged by the trypsin treatment which may be rate limiting for phosphorylation in the presence of coupling factors.

Optimal rates of phosphorylation coupled to the reduction of $NADP^+$ in the sonicated chloroplasts were obtained only in the presence of plastocyanin, CF_1 and CF_2 (Table VII). In the absence of added coupling factors, plastocyanin stimulated

TABLE VII

EFFECT OF COUPLING FACTORS AND PLASTOCYANIN ON $NADP^+$ REDUCTION AND COUPLED PHOSPHORYLATION IN SONICATED CHLOROPLASTS

The incubation of the subchloroplast particles (50 μg of chlorophyll) with CF_1 (40 μg), CF_2 (50 μg) and plastocyanin (2.9 nmoles) was performed as described in the legend to Table IV. Duplicate incubations were carried out. To one tube of each set, a reaction mixture which did not contain $^{32}P_i$ was added and these tubes were used for the assay of $NADP^+$ reduction as described in MATERIALS AND METHODS. To the duplicate tubes of each set, a reaction mixture identical to that used for the $NADP^+$ reduction assays except that it contained $1.8 \cdot 10^6$ counts/min $^{32}P_i$, was added and $^{32}P_i$ esterification was assayed. The illumination time was 5 min and the gas phase was nitrogen.

Additions to subchloroplast particles	*$NADP^+$ reduction (μmoles/h per mg chlorophyll)*	*Phosphorylation (μmoles P_i esterified per h per mg chlorophyll)*	*P/2e*
None	10.2	0.39	0.038
CF_1	10.2	0.53	0.052
CF_2	10.4	1.10	0.105
CF_1 + CF_2	9.6	1.62	0.168
Plastocyanin	15.5	0.63	0.040
CF_1 + plastocyanin	14.5	1.26	0.088
CF_2 + plastocyanin	14.1	2.52	0.179
CF_1 + CF_2 + plastocyanin	14.5	2.96	0.204

photophosphorylation and $NADP^+$ reduction to a similar extent. In the presence of either CF_1 or CF_2, plastocyanin stimulated phosphorylation to a greater degree than $NADP^+$ reduction, resulting in an elevation of the P/2e ratio.

Lack of effect of plastocyanin on acid to base induced phosphorylation

JAGENDORF AND URIBE[29] showed that ATP synthesis takes place in darkness after a rapid increase of the pH of a chloroplast suspension from 4 to 8. If electron transport were required for ATP formation in this system, plastocyanin might be expected to enhance it. As shown in Table VIII, plastocyanin had no effect on the phosphorylation dependent upon the pH transition. However, pyocyanine-dependent phosphorylation, assayed in aliquots of the same incubation mixtures used for the acid to base induced ATP synthesis was enhanced by about 60 %. The stimulation of pyocyanine-dependent phosphorylation was low in this experiment since it was difficult to add enough plastocyanin to saturate the large amount of subchloroplast particles used. In control experiments, it was found that the pH transition had no effect on the stimulation of pyocyanine-dependent phosphorylation by plastocyanin, although it did lower the rates of phosphorylation by about 30–40 %.

TABLE VIII

LACK OF EFFECT OF PLASTOCYANIN ON ACID TO BASE INDUCED PHOSPHORYLATION

Subchloroplast particles (0.374 mg of chlorophyll) were incubated at room temperature in a final volume of 0.6 ml in a mixture which contained 3 mg bovine serum albumin, 8 μmoles Tricine–NaOH (pH 8.0), 5 μmoles $MgCl_2$ and 19 nmoles plastocyanin where indicated. After 10 min 0.05-ml aliquots were assayed for pyocyanine-dependent phosphorylation. The remaining portion of the mixture was assayed for acid to base induced phosphorylation as described[27]. All assays were performed in duplicate and the duplicates agreed within 5 %.

Plastocyanin added	*Phosphorylation*	
	Pyocyanine-dependent (μmoles $^{32}P_i$ esterified per h per mg chlorophyll)	*Acid to base induced (nmoles $^{32}P_i$ esterified per mg chlorophyll)*
–	9.6	10.1
+	15.1	9.0

DISCUSSION

The results presented in this paper show that plastocyanin-stimulated phosphorylation coupled to cyclic electron flow with pyocyanine and non-cyclic electron flow with $NADP^+$ in subchloroplast particles made deficient in plastocyanin by prolonged sonic treatment. This enhancement of photophosphorylation is probably not caused by proteins which contaminate the plastocyanin since homogeneous plastocyanin also stimulated. It is also apparent that plastocyanin is not the only protein resolved from the chloroplast membranes by the prolonged sonic treatment. Coupling factor-2, and to a lesser extent Coupling factor-1, considerably stimulated pyocyanine and $NADP^+$-dependent phosphorylation in the deficient subchloroplast particles. Since cytochrome c_{552} stimulated photophosphorylation and $NADP^+$ reduction in the deficient subchloroplast particles it is apparent that the specificity for plastocyanin is

not absolute. Since the chloroplasts used by FUJITA *et al.*[7] contained 70% of the plastocyanin of chloroplasts, the reported lack of stimulation of phosphorylation by plastocyanin is not too difficult to explain.

Ferricyanide reduction and associated phosphorylation were not stimulated by plastocyanin. Yet the rates of these reactions in plastocyanin-deficient chloroplasts from a mutant of Chlamydomonas were less than 10% of those in chloroplasts from wild-type organisms[8] suggesting that plastocyanin is required for ferricyanide reduction and phosphorylation. However, it is apparent that the characteristics of ferricyanide reduction in subchloroplast particles differ markedly from those in chloroplasts[2]. Thus, in subchloroplast particles, ferricyanide may readily accept electrons from the oxidation chain at a site before that of plastocyanin. In fact, C. T. LIEN AND T. T. BANNISTER (personal communication) have shown that dichlorophenolindophenol can be reduced at two sites in sonicated chloroplasts and that only one site is dependent on plastocyanin.

It would appear that plastocyanin is not directly involved in cyclic phosphorylation. The stimulation of pyocyanine-dependent phosphorylation by plastocyanin may be readily explained by the ability of plastocyanin to enhance the reduction of pyocyanine. It has been recognized that a mediator of cyclic electron flow must be partially reduced to give rapid rates of cyclic phosphorylation[30,31]. In chloroplasts, the reduction of pyocyanine from water can be accomplished by a Photosystem II reaction, since pyocyanine-dependent phosphorylation was inhibited by DCMU (ref. 32). In the sonicated chloroplasts, DCMU also abolished pyocyanine-dependent phosphorylation when oxidized pyocyanine was used. DCMU had little effect on phosphorylation in the presence of partially reduced pyocyanine and, therefore, it is probably that electrons from water can be used to reduce pyocyanine. Since pyocyanine would most likely be reduced at a site after that of plastocyanin, plastocyanin should stimulate pyocyanine reduction. PMS-dependent phosphorylation assayed in white light was not stimulated by plastocyanin and was insensitive to DCMU. In red light, however, DCMU abolished PMS-dependent phosphorylation. In white, but not red, light PMS is reduced nonenzymatically[30]. Thus, plastocyanin would not be required for the reduction of PMS in white light and therefore phosphorylation coupled to PMS-induced cyclic electron flow should be insensitive to DCMU, as was in fact observed. In red light, however, the reduction of PMS must be accomplished by the chloroplast electron transport chain using water as the electron donor and phosphorylation was therefore found to be sensitive to DCMU.

ARNON *et al.*[33] showed that PMS-dependent phosphorylation in the small particle produced by the action of digitonin on chloroplasts[34] (D-144) was independent of plastocyanin, even though the particles were deficient in plastocyanin. These results have been confirmed and extended in this laboratory*. Very high rates of PMS-dependent phosphorylation (300–600 μmoles P_i esterified per h per mg of chlorophyll) in the D-144 particles were found, whereas pyocyanine-dependent phosphorylation was less than 1% of PMS phosphorylation. If, however, reduced pyocyanine was used, the rates of phosphorylation approached those observed with PMS. Similarly, only a very low rate of PMS-dependent phosphorylation was observed in red light and phosphorylation under these conditions was markedly enhanced by

* G. HAUSKA AND R. E. MCCARTY, unpublished observations.

ascorbate, which reduced PMS. In no case was phosphorylation stimulated by plastocyanin in agreement with the fact that D-144 particles are incapable of System II-linked oxidation of water.

Finally, the lack of effect of plastocyanin on cyclic phosphorylation in plastocyanin deficient chloroplast fragments gives a clue to a possible site of energy conservation in photophosphorylation. Plastocyanin is thought to act in the chloroplast electron transport chain between cytochrome *f* and P_{700} (ref. 35). Thus, since cyclic phosphorylation does not require plastocyanin, it is possible to conclude that a phosphorylation site exists within the electron transport between the primary reductant of System I and P_{700}. It is possible, however, that the residual plastocyanin in the digitonin and sonic chloroplast fragments ($<5\%$) is enough to allow cyclic electron flow. In view of the high rates of cyclic phosphorylation with PMS which may be observed in the D-144 particle, this possibility is not too likely.

ACKNOWLEDGEMENTS

Thanks are due to Dr. C. E. Furlong for the loan of his preparative polyacrylamide gel column and for help in its use. Mrs. F. Farron performed the ultracentrifuge and amino acid analyses and Dr. D. C. Wharton kindly assayed the copper content of the plastocyanin. We are also in debt to Mrs. C. H. Coleman for her excellent technical assistance in some of these experiments. This work was supported by Grant GM-14409 from the National Institutes of General Medical Sciences. R. E. McCarty is a Career Development Awardee of the National Institutes of Health.

REFERENCES

1 S. Katoh, L. Suga, I. Shiratori and A. Takamya, *Arch. Biochem. Biophys.*, 94 (1961) 136.
2 S. Katoh and A. San Pietro, *The Biochemistry of Copper*, Academic Press, New York, 1966, p. 407.
3 S. Katoh and A. San Pietro, *J. Biol. Chem.*, 241 (1966) 3575.
4 L. P. Vernon, E. R. Shaw and B. Ke, *J. Biol. Chem.*, 241 (1966) 4101.
5 S. Katoh and A. Takamiya, *Plant Cell Physiol.*, 4 (1963) 335.
6 B. Kok, H. Rurainski and E. A. Harmon, *Plant Physiol.*, 38 (1963) 513.
7 R. Fujita, H. Sakurai, S. Katoh and A. Takamiya, *Plant Cell Physiol.*, 6 (1965) 689.
8 D. S. Gorman and R. P. Levine, *Proc. Natl. Acad. Sci. U.S.*, 54 (1966) 1665.
9 R. E. McCarty and E. Racker, *J. Biol. Chem.*, 242 (1967) 3435.
10 V. K. Vambutas and E. Racker, *J. Biol. Chem.*, 240 (1965) 2660.
11 A. Bennun and E. Racker, *J. Biol. Chem.*, 244 (1969) 1325.
12 A. San Pietro and H. M. Lang, *J. Biol. Chem.*, 231 (1958) 211.
13 M. Shin, K. Tagawa and D. I. Arnon, *Biochem. Z.*, 338 (1963) 84.
14 A. Livne and E. Racker, *Biochem. Biophys. Res. Commun.*, 32 (1968) 1045.
15 R. F. Chen, *J. Biol. Chem.*, 242 (1967) 173.
16 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1941) 384.
17 E. W. Sutherland, C. F. Cori, R. Haynes and N. S. Olsen, *J. Biol. Chem.*, 180 (1949) 825.
18 D. I. Arnon, *Plant Physiol.*, 24 (1949) 1.
19 S. Katoh, I. Shiratori and A. Takamiya, *J. Biochem. Tokyo*, 51 (1962) 32.
20 A. T. Jagendorf and M. Smith, *Plant Physiol.*, 37 (1962) 135.
21 D. D. Henley and E. E. Conn, *Arch. Biochem. Biophys.*, 46 (1953) 454.
22 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
23 T. Jovin, A. Chrambach and M. Naughton, *Anal. Biochem.*, 9 (1964) 351.
24 L. Ornstein and B. Davies, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
25 E. Elstner, E. Pistorius, P. Boger and A. Trebst, *Planta*, 79 (1968) 146.
26 T. Horio, *J. Biochem.*, 45 (1958) 267.
27 R. E. McCarty, *Biochem. Biophys. Res. Commun.*, 32 (1968) 37.
28 R. E. McCarty and E. Racker, *Brookhaven Symp. Biol.*, 19 (1966) 202.

29 A. T. JAGENDORF AND E. URIBE, *Proc. Natl. Acad. Sci. U.S.*, 54 (1966) 170.
30 A. TREBST AND H. ECK, *Z. Naturforsch.*, 166 (1961) 455.
31 J. S. C. WESSELS, *Proc. Roy. Soc. London, Ser. B*, 157 (1963) 345.
32 A. T. JAGENDORF AND M. MARGULIES, *Arch. Biochem. Biophys.*, 90 (1960) 184.
33 D. I. ARNON, H. Y. TSUJIMOTO, B. D. MCSWAIN AND R. K. CHAIN, *Comparative Biochemistry and Biophysics of Photosynthesis*, University of Tokyo Press, Tokyo, 1967, p. 113.
34 J. M. ANDERSON AND N. K. BOARDMAN, *Biochim. Biophys. Acta*, 112 (1966) 403.
35 G. HIND, *Biochim. Biophys. Acta*, 153 (1968) 235.