Biochimica et Biophysica Acta, 325 (1973) 255–265

BBA 46633

POLYLYSINE-ENHANCED EFFECTIVENESS OF PLASTOCYANIN IN PHOTOSYSTEM I

JERRY BRAND and ANTHONY SAN PIETRO

Department of Plant Sciences, Indiana University, Bloomington, Ind. 47401 (U.S.A.)

(Received May 21st, 1973)

SUMMARY

1. Chloroplast fragments from either *Chlamydomonas reinhardi* or spinach, which lack plastocyanin, or from *Euglena gracilis* depleted of cytochrome *c*552, require a large excess of exogenously added plastocyanin or cytochrome *c*552 to restore Photosystem I activity.

2. In the presence of a small amount of polylysine, Photosystem I activity of chloroplast fragments is stimulated greatly by plastocyanin or cytochrome *c*552, and the reaction is saturated at a lower concentration of these proteins. Higher concentrations of polylysine inhibit Photosystem I activity; the inhibition is not reversed by plastocyanin or cytochrome *c*552.

3. Salt protects chloroplast fragments from stimulation by polylysine *plus* plastocyanin or cytochrome *c*552, and also reverses this stimulation.

4. The data suggest that polylysine, at low concentration, enhances binding of plastocyanin or cytochrome *c*552 to chloroplast membranes, thereby increasing the effective concentration at their site of function. The total inhibition of Photosystem I activity, independent of the presence of plastocyanin or cytochrome *c*552, at higher polylysine concentrations is similar probably to that observed previously in chloroplasts which retain their plastocyanin.

INTRODUCTION

Many species of algae have a tough cell wall protecting the interior components of the cell. In these species rather severe methods are required to disrupt the cell wall and to isolate chloroplasts free of other cell components. Chloroplast fragments isolated from one such organism, *Chlamydomonas reinhardi*, are devoid of plastocyanin[1]. This blue copper protein which is widely distributed in photosynthetic organisms is membrane bound and functions as an electron carrier near Photosystem I[2,3]. Concomitant with the loss of plastocyanin, all Photosystem I activities are greatly diminished[2]. Restoration of these activities requires the addition of high concentrations of soluble plastocyanin. For example, the maximal rate of electron transfer from ascorbate/TMPD (*N,N,N,'N'*-tetramethyl-*p*-phenylene-

Abbreviations: TMPD, *N, N, N', N'*-tretramethyl-*p*-phenylenediamine dihydrochloride; Tricine, tris-(hydroxymethyl)methylglycine; DCMU, 3-(3',4'-dichlorophenyl)-1, 1-dimethylurea.

diamine dihydrochloride) to methyl viologen is observed only when plastocyanin is present in great excess over that originally occurring in the membranes.

Euglena species do not contain plastocyanin, its role apparently being filled by a cytochrome designated *c*552 (ref. 4). Cytochrome *c*552 will replace plastocyanin in Photosystem I reactions requiring exogenously provided plastocyanin. Again, a large excess of the cytochrome is required to saturate these reactions[5].

This paper focuses attention on the Photosystem I-mediated electron transfer from ascorbate/TMPD to methyl viologen in photosynthetic membranes devoid of plastocyanin. It is demonstrated that this reaction is saturated much more easily by plastocyanin or cytochrome *c*552 in the presence of polylysine. A comparison of this effect and that wherein polycations inhibit Photosystem I reactions in chloroplasts which retain their endogenous plastocyanin is presented.

METHODS

Chloroplast fragment preparation

C. reinhardi, wild type strain 137-c (+), was grown in Tris–acetate–phosphate medium as described by Gorman and Levine[6] in 500-ml flasks. The cells were harvested during log phase growth by centrifugation at $5000 \times g$ for 5 min, resuspended in 0.05 M phosphate buffer, pH 7.0, and sonicated at full power for 5 min with a Branson Sonifier. Large fragments and unbroken cells were removed by centrifugation at $4000 \times g$ for 5 min. The chloroplast fragment fraction was then collected by centrifugation at $40000 \times g$ for 20 min and resuspended in 0.05 M tris-(hydroxymethyl)methylglycine (Tricine) buffer, pH 8.0.

To prepare chloroplast fragments which retained their plastocyanin, cells were ground with sand in a mortar and pestle (5 g of sand per g wet weight of cells). The mixture remained moist, but contained no liquid above the sand. The mixture was vigorously ground for 1 min after which it was suspended in 0.05 M phosphate buffer, pH 7.0. This was filtered first through 4 layers of cheese cloth and then one layer of Miracloth. Whole cells and chloroplasts were removed by centrifugation at $4000 \times g$ for 5 min. Chloroplast fragments were collected by centrifugation at $12000 \times g$ for 10 min and resuspended in 0.05 M phosphate buffer, pH 7.0, for assays.

Euglena gracilis strain Z was grown in Hutner's acidic organotrophic medium[7] in 2-l batches continuously aerated with air passed through a potassium bicarbonate solution. The culture was grown under $5 \cdot 10^3$ ergs$\cdot$cm$^{-2}\cdot$s^{-1} illumination. Cells were collected by centrifugation at $4000 \times g$ for 5 min, resuspended in 0.02 M Tricine buffer, pH 4.5, and then sonicated for 15 min at full power with the Branson Sonifier. Chloroplast fragments which sedimented between $12000 \times g$ for 30 min and $40000 \times g$ for 2 h were collected and resuspended in 0.05 M Tricine buffer, pH 8.0, for assays.

Spinach chloroplasts, prepared by the method of Jagendorf and Avron[8], were resuspended in the chloroplast isolation medium and sonicated at full power for 5 min with the Branson Sonifier. The suspension was centrifuged at $4000 \times g$ for 5 min to remove cell debris and larger fragments. Chloroplast fragments essentially devoid of plastocyanin were collected at $40000 \times g$ for 30 min and resuspended in 0.05 M Tricine, pH 8.0, for assays.

It may be noted that a variety of sonication times, buffers, *etc.*, were used in the various chloroplast fragment preparations. This was so only because the preparations were also used in other experiments performed concurrently. Photosystem I-mediated electron transfer from ascorbate/TMPD to methyl viologen is extremely stable and relatively insensitive to the method of chloroplast fragment preparation.

All operations during and after disruption of the cells were performed near 0 °C. Chlorophyll concentration was determined spectrophotometrically[9].

Enzyme preparations

Plastocyanin was prepared as described previously[3].

Euglena cytochrome *c*552 was partially purified from *E. gracilis* strain Z cells grown as described above as follows: Cells were collected by centrifugation, resuspended in a minimum volume of 0.05 M phosphate buffer, pH 7.0, and kept frozen at −15 °C. When 1 l of frozen cells had been accumulated, they were thawed and sonicated in 120-ml batches with the Branson Sonifier at full power for 3 min. Cell fragments were removed by centrifugation at $40000 \times g$ for 30 min and the supernatant solution charged on a DE 11 column equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was washed with this same buffer; then the cytochrome *c*552 was eluted with 0.05 M phosphate buffer containing 0.25 M NaCl. The cytochrome fraction was dialyzed against 0.005 M Tris, pH 8.0, and thereafter placed on a DE 23 column equilibrated with the same buffer. The column was washed with 0.05 M Tris, pH 8.0. Cytochrome *c*552 was then eluted with a salt gradient of 0–0.3 M NaCl in 0.05 M Tris, pH 8.0. The cytochrome fraction was dialyzed against 0.05 M phosphate buffer, pH 7.0, and thereafter concentrated using a small DE 11 column. It was then dialyzed against 0.005 M phosphate, pH 7.0, diluted to a concentration of 60 μM, and individual aliquots stored in the freezer until needed. The visible spectrum of the cytochrome appeared identical to that reported previously although the absorbance in the ultraviolet region was several times higher than that reported for pure cytochrome *c*552 (ref. 10).

Photosystem I assay

Electron transfer from ascorbate/TMPD to methyl viologen was measured by the decrease in oxygen concentration in the reaction vessel as the photochemically reduced methyl viologen underwent autooxidazition by molecular oxygen. The assays were performed in a continuously stirred 1.3-ml reaction vessel equilibrated to 25 °C and illuminated with white light at an intensity of $1 \cdot 10^6$ ergs$\cdot$cm$^{-2}\cdot$s^{-1}. Concentrations of reagents used in individual assays are described in the respective legends.

Components were added in the following order: Polylysine, chloroplasts, other reagents, plastocyanin or cytochrome *c*552. This order of addition was important to insure polylysine binding to chloroplast membranes, as demonstrated previously[11].

Chemicals and reagents

Poly-L-lysine hydrobromide with an average molecular weight of $2.8 \cdot 10^5$ was obtained from Sigma Chemical Company. Methyl viologen was purchased from K and K Laboratories and TMPD from Eastman Organic Chemicals. 3-(3′,4′-

dichlorophenyl)-1,1-dimethylurea (DCMU) was kindly supplied by Dr Keelin T. Fry of E. I. DuPont. Other chemicals were reagent grade.

RESULTS

The green microalga *C. reinhardi* contains a single chloroplast occupying the greater portion of the interior of the cell. The chloroplast is very difficult to remove intact, but photosynthetic membranes can be isolated by sonic disruption followed by differential centrifugation[12]. These membrane fragments exhibit rather poor Photosystem I activity which is greatly stimulated by addition of exogenous plastocyanin. Interestingly, this reaction is difficult to saturate with respect to plastocyanin, as shown in Fig. 1 (circles). However, when a small amount of polylysine is present, a greatly enhanced effect is observed (Fig. 1, upper curve). Of more importance is the observation that plastocyanin saturates the reaction at a much lower concentration when polylysine is present. At a higher concentration of polylysine, plastocyanin becomes somewhat less effective (Fig. 1, lower curve).

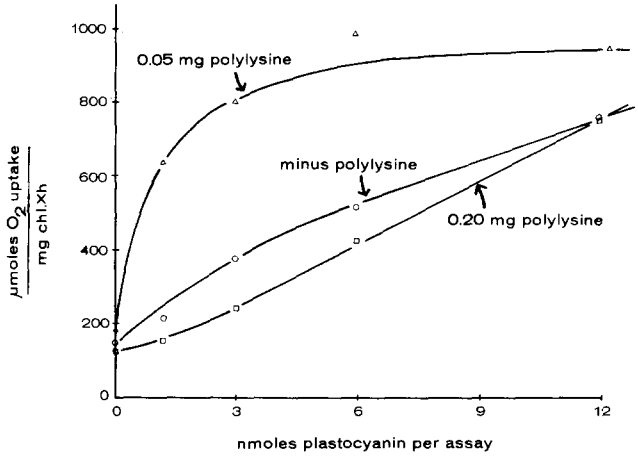

Fig. 1. Effect of plastocyanin on Photosystem I activity in sonicated *Chlamydomonas* chloroplast fragments. Electron transfer from ascorbate/TMPD to methyl viologen was measured as described in Methods. Each 1.3-ml assay contained the following, in μmoles: Tricine buffer, 50 (pH 8.0); sodium ascorbate, 1.5; TMPD, 0.1; DCMU, 0.01; methyl viologen, 0.1. Each assay also contained chloroplasts equivalent to 10.5 μg chlorophyll (Chl.) and the amount of plastocyanin indicated. Polylysine (0.05 or 0.20 mg) was present in each assay where indicated.

The interrelationship between plastocyanin and polylysine concentrations is shown in Fig. 2. *Chlamydomonas* fragments devoid of plastocyanin have little Photosystem I activity. This endogenous rate is further slightly decreased as polylysine is added to the assays (Fig. 2, lower curve). In the presence of 3 nmoles of plastocyanin the rate is increased somewhat in the absence of polylysine, but is increased much more in the presence of low concentrations of polylysine. However, as the concentration of polylysine is further increased, the reaction is inhibited

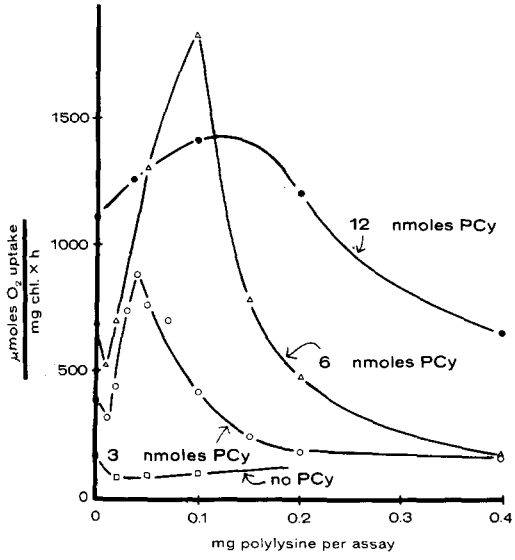

Fig. 2. Effect of polylysine on Photosystem I activity in sonicated *Chlamydomonas* chloroplast fragments. Assay conditions were as in Fig. 1. Each assay contained 15 μg chlorophyll (Chl.) and the amounts of plastocyanin (PCy) and polylysine indicated.

as noted earlier (Fig. 1). Similar results are obtained at higher concentrations of plastocyanin (Fig. 2, upper two curves).

It is clear from the data presented in Fig. 2 that there exists a relationship between the amount of plastocyanin added and the amount of polylysine required for maximal activity. For example, with 3 nmoles of plastocyanin, maximal activity is observed with about 0.05 mg of polylysine; with 6 nmoles of plastocyanin, 0.1 mg of polylysine is required. In addition, the actual observed maximal rate depends on the amount of plastocyanin present provided the amount of polylysine is quite low.

The results presented in Figs 1 and 2 suggest a dual and opposite role for polylysine. At low polylysine concentrations, it appears to enhance binding of plastocyanin to the photosynthetic membranes and thereby stimulate Photosystem I activity. In contrast, at higher polylysine concentrations, an inhibitory effect predominates, perhaps by preventing the functioning of plastocyanin, as described previously[13,14]. If this interpretation is correct, then membranes which retain their plastocyanin should not exhibit this stimulation by polylysine, but should be inhibited as has been described with spinach chloroplasts which retain their plastocyanin[11].

Chlamydomonas chloroplast fragments which retain their plastocyanin are rather difficult to prepare although a low yield can be obtained by hand grinding the cells with sand, followed by differential centrifugation. The effects of polylysine and plastocyanin on these fragments are shown in Fig. 3. These Photosystem I-competent fragments are active in the absence of polylysine (Fig. 3a, upper curve) and the activity is inhibited by 0.01 mg of polylysine (Fig. 3a, lower curve). Plastocyanin has very little effect on either activity; inhibited or uninhibited. The demon-

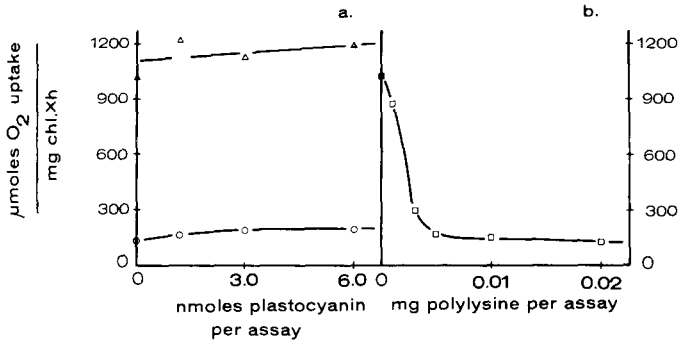

Fig. 3. Effect of plastocyanin and polylysine on Photosystem I activity in *Chlamydomonas* chloroplast fragments prepared by sand grinding. Assay conditions were as in Fig. 1. Each assay contained 12 μg chlorophyll (Chl.). Fig. 3a: (△) no polylysine; (○) *plus* 0.01 mg polylysine. No exogenous plastocyanin was present in the assays represented in Fig. 3b.

stration that polylysine inhibits these fragments in a manner analogous to that previously reported with spinach chloroplasts[11] is provided in Fig. 3b.

It was of interest to see if the stimulation was specific for *Chlamydomonas* and/or for plastocyanin. The flagellate *E. gracilis* permitted testing this question of specificity since it does not contain plastocyanin. The effect of polylysine on Photosystem I activity in *Euglena* chloroplast fragments in the presence of plastocyanin or *Euglena* cytochrome *c*552 is given in Table I. There is some stimulation by cytochrome *c*552 or plastocyanin alone but not as great as in the presence of polylysine. Even when the concentration of either plastocyanin or cytochrome *c*552

TABLE I

EFFECT OF POLYLYSINE ON PHOTOSYSTEM I ACTIVITY IN *EUGLENA* CHLOROPLAST FRAGMENTS

Assay conditions were as in Fig. 1. Protein additions refer to plastocyanin or cytochrome *c*552 as indicated. KCl was added as the first or last component of the assay mixture as indicated.

Additions to assay mixtures	*μmoles O_2 uptake per mg chlorophyll per h*	
Protein addition:	*Plastocyanin*	*Cytochrome c552*
No addition	145	145
3 nmoles protein	250	165
12 nmoles protein	650	310
3 nmoles protein *plus* 0.05 mg polylysine	500	170
3 nmoles protein *plus* 0.075 mg polylysine	850	580
3 nmoles protein *plus* 0.1 mg polylysine	525	865
3 nmoles protein *plus* 0.3 mg polylysine	155	180
3 nmoles protein *plus* 0.075 mg polylysine *plus* 300 μmoles KCl added first	200	160
3 nmoles protein *plus* 0.075 mg polylysine *plus* 300 μmoles KCl added last	210	215

was increased to 18 nmoles per assay (data not shown), there was significant stimulation but the system was not saturated with respect to either protein. As noted with *Chlamydomonas*, in the presence of a low plastocyanin or cytochrome *c*552 concentration small amounts of polylysine stimulate the reaction; higher concentrations of polylysine inhibit. Thus, plastocyanin and polylysine appear to function in *Euglena* exactly as in *Chlamydomonas*. Furthermore, the plastocyanin can be replaced by cytochrome *c*552 with the same results. Maximum stimulation was obtained with 0.05 mg of polylysine in the presence of plastocyanin and with 0.075 mg of polylysine in the presence of cytochrome *c*552 although the maximum rate was about the same for both. In addition, the stimulation by polylysine is prevented or reversed by potassium chloride (Table I, last two lines).

The results with algal chloroplast fragments suggest that higher plant chloroplast membranes devoid of endogenous plastocyanin might behave similarly. This view is substantiated by the data with spinach chloroplast membranes given in Fig. 4. Although plastocyanin stimulates the reaction, saturation is not achieved even with 12 nmoles of protein per assay. However, in the presence of 0.01 mg polylysine, maximal activity is achieved at a very low concentration of plastocyanin (about 1 nmole per assay).

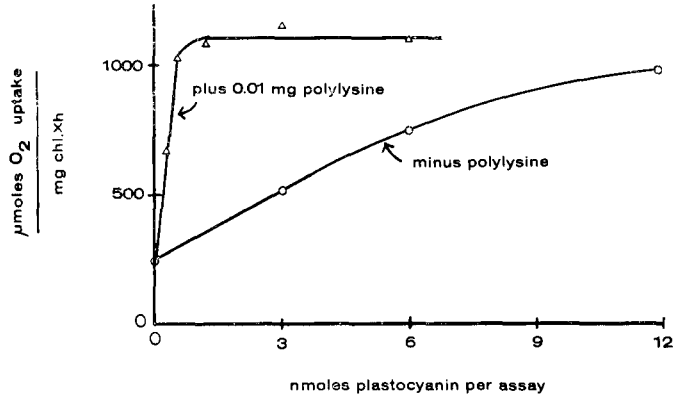

Fig. 4. Effect of plastocyanin on Photosystem I activity in sonicated spinach chloroplasts. Assay conditions were as in Fig. 1. Each assay contained 5.5 μg chlorophyll (Chl.); 0.01 mg polylysine per assay was present where indicated.

The experiment depicted in Fig. 5 demonstrates that in the presence of 3 nmoles of plastocyanin, low concentrations of polylysine stimulate Photosystem I activity whereas higher concentrations are inhibitory. Thus, spinach chloroplasts depleted of plastocyanin appear to respond to plastocyanin and polylysine in a manner analogous to that of algal chloroplast fragments.

In Fig. 6 is shown the effect of salt on Photosystem I activity in sonicated spinach chloroplasts. In the absence of polylysine or plastocyanin (squares) the activity is not significantly affected by KCl in the concentration range tested. This rate (about 400 μmoles of oxygen taken up per mg chlorophyll per h) is substantially lower than usually seen in electron transfer from ascorbate/TMPD to methyl viologen but is typical of rates observed after removal of endogenous plastocyanin.

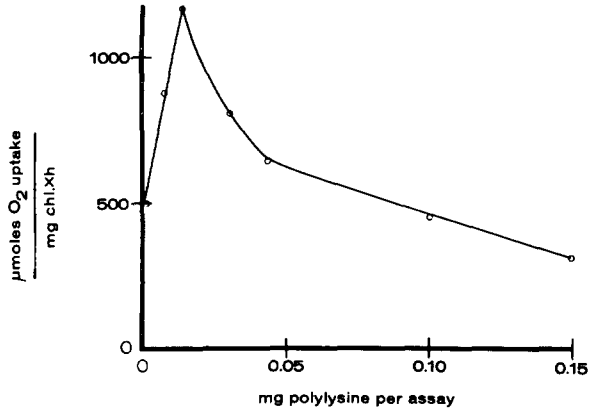

Fig. 5. Effect of polylysine on Photosystem I activity in sonicated spinach chloroplasts containing exogenous plastocyanin. Assay conditions were as in Fig. 1. Each assay contained 5.5 μg chlorophyll and 3 nmoles plastocyanin.

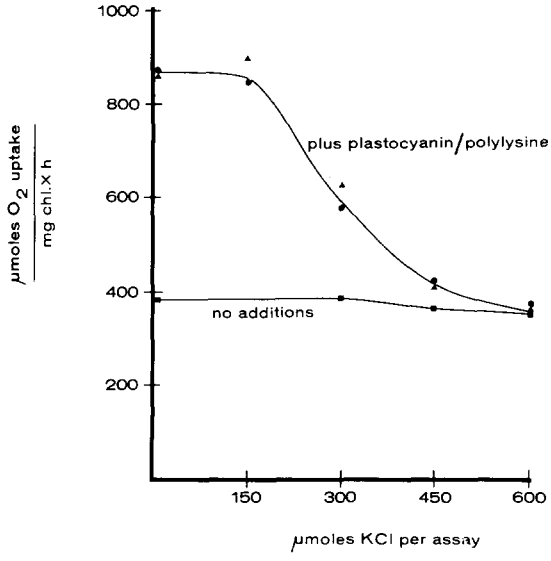

Fig. 6. Effect of salt on protecting and reversing the stimulation of Photosystem I by polylysine *plus* plastocyanin. Assay conditions were as in Fig. 1. Each assay contained 5.6 μg chlorophyll (Chl.) as sonicated spinach chloroplasts and the quantity of salt indicated. Assays represented by circles and triangles contained 3 nmoles plastocyanin *plus* 0.01 mg polylysine. In assays represented by circles KCl was added as the first component of the assay mixture; in those represented by triangles KCl was added last.

The upper curve of Fig. 6 (circles and triangles) represents assays performed in the presence of both polylysine and plastocyanin to afford maximum stimulation. When KCl is added prior to the addition of other components (triangles) stimulation is prevented or overcome. When KCl is added as the last component (circles) it reverses the stimulatory effect. Both prevention and reversal as defined here occur

at the same salt concentration. That is, the final result is the same whether KCl is added first or last. This is in sharp contrast to previous results obtained with fresh spinach chloroplasts in which the effect of polylysine was prevented by salt but could not be reversed[11].

DISCUSSION

Chloroplast fragments devoid of plastocyanin have deficient Photosystem I activity which can be reconstituted by providing plastocyanin exogenously. The maximum activity with NADP as electron acceptor is typically under 300 μmoles NADP reduced per mg chlorophyll per h and is achieved with about 0.6 μM plastocyanin in the assay mixture[15]. In contrast, when Photosystem I activity is measured as electron transfer from TMPD to methyl viologen, a much higher rate is obtained but the reaction is difficult to saturate with plastocyanin[16]. It appears therefore that the rate-limiting step in Photosystem I activity, at least with TMPD as donor, resides at a site beyond that where electrons are donated to methyl viologen. In this case, the rate-limiting step is the donation of electrons *via* plastocyanin to the photosystem.

Based on numerous experiments using electron transfer from ascorbate/TMPD to methyl viologen as a measure of Photosystem I activity, it is clear that the ratio of plastocyanin to chlorophyll is not the significant factor. Rather, the important parameter appears to be the absolute concentration of plastocyanin in the assay mixture. Using spinach chloroplast fragments prepared by sonication, maximal activity requires about 15 μM plastocyanin at high light intensities (Fig. 4).

Euglena cytochrome *c*552 can substitute for plastocyanin in Photosystem I reactions catalyzed by spinach as well as *Euglena* chloroplast fragments. In either case, saturation with respect to cytochrome *c*552 is difficult to achieve[5]. However, in the presence of a small amount of polylysine, maximal activity is achieved at much lower plastocyanin or cytochrome *c*552 concentrations (Figs 1 and 4; Table I). This lowered concentration is a function of the polylysine and chloroplast (as chlorophyll) concentration; it is not determined by a specific plastocyanin-to-chlorophyll ratio. Similarly, a maximal response of Photosystem I activity in spinach chloroplasts depleted of plastocyanin can be observed at less than 1 μM concentration of cytochrome *c*552 in the presence of polylysine.

Polylysine in the absence of plastocyanin or cytochrome *c*552 does not stimulate activity (Fig. 2). We interpret the effect of polylysine as permitting exogenously provided plastocyanin to act more efficiently in photosynthetic membranes depleted of endogenous plastocyanin. This effect could be explained by polylysine binding to both plastocyanin and the membranes and thereby increasing the effective plastocyanin concentration at its functional site. Previous results have demonstrated that polylysine effects Photosystem I reactions at or close to the site of function of plastocyanin[13,14]. The same interpretation would suffice when cytochrome *c*552 replaces plastocyanin.

Alternatively, polylysine might stimulate activity in these fragments simply by binding to the membranes, thereby changing their conformation such that plastocyanin is more efficiently utilized. In this vein, polylysine has been shown to effect ion movement and morphology of chloroplast membranes[17,18]. We favor

the former hypothesis, however, since polylysine was shown to bind soluble plastocyanin (Brand, J. unpublished).

The results presented here extend those reported previously wherein it was demonstrated that polylysine completely inhibits Photosystem I activity in chloroplasts which retain their plastocyanin[11]. Further, in those experiments the activity was not restored by subsequent addition of plastocyanin. The present results demonstrate that polylysine inhibits Photosystem I activity even in chloroplasts devoid of plastocyanin; however, a relatively large concentration is required when plastocyanin is present also. In this case, the mechanism of inhibition could still be similar to that operative when Photosystem I activity in chloroplasts not depleted in plastocyanin is inhibited. The concentration of polylysine required for inhibition differs in the two cases; with the depleted chloroplast fragments it is much higher.

One additional difference between chloroplast membranes containing and those depleted of plastocyanin may be noted in Fig. 6. Previous results indicated that chloroplasts which retained their plastocyanin were completely protected by salt from inhibition by polycations. However, once polycations were bound to the membranes, no amount of salt (or other reagent tested) was able to reverse the inhibition[11]. In the experiments with chloroplast membranes depleted of plastocyanin, KCl prevents (when added first) or reverses (when added last) the stimulation of Photosystem I activity by polylysine *plus* plastocyanin. We have also obtained similar results with $MgCl_2$ (data not shown). Since previous experiments demonstrated that polylysine was bound irreversibly to chloroplast membranes which retained their plastocyanin, the reversible reaction might be the binding of plastocyanin to polylysine. Confirmation of this view must await further experiments.

The observations reported here demonstrate the feasibility of studying reconstituted Photosystem I activity using a minimum of exogenous plastocyanin. The saturation kinetics and increased activity of plastocyanin in plastocyanin-depleted chloroplast fragments in the presence of polylysine suggest that the plastocyanin may be in a more physiological state than in the absence of polylysine. These results also suggest possibilities for the construction of functional artificial membranes.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Robert K. Togasaki for many helpful discussions and for providing the *Chlamydomonas* culture, and Ms Sydney Gray for valuable technical assistance. This investigation was facilitated by a research grant (GM 16314 to A.S.P.) from the National Institutes of Health, United States Public Health Service.

REFERENCES

1 Katoh, S., Suga I., Shiratori, I. and Takamiya, A., (1961) *Arch. Biochem. Biophys.* 94, 136–141
2 Levine, R. P. and Gorman, D. S., (1966) *Plant Physiol.* 41, 1293–1300
3 Siedow, J. N., Curtis, V. A. and San Pietro, A. (1973) *Arch. Biochem. Biophys.* in the press
4 Olson J. M. and Smillie, R. M. (1963) in *Photosynthetic Mechanisms of Green Plants* Publ. 1145, pp. 56–65, Natl. Acad. Sci. -Natl. Res. Council, Washington, D. C.

5 Elstner, E., Pistorius, E., Boger, P. and Trebst, A. (1968) *Planta* 79, 146–161
6 Gorman, D. S. and Levine, R. P. (1965) *Proc. Natl. Acad. Sci. U.S.* 54, 1665–1669
7 Schiff, J. A., Lyman, H. and Russell, G. K. (1971) in *Methods in Enzymology* (San Pietro, A., ed.) Vol. 23A, pp. 143–162, Academic Press, N. Y.
8 Jagendorf, A. T. and Avron, M. (1958) *J. Biol. Chem.* 231, 277–290
9 Arnon, D. I., (1949) *Plant Physiol.* 24, 1–15
10 Mitsui, A. (1971) in *Methods in Enzymology* (San Pietro, A., ed.) Vol. 23A, pp. 368–371, Academic Press, N. Y.
11 Brand, J., Baszynski, T. Crane, F. L. and Krogmann, D. W. (1972) *J. Biol. Chem.* 247, 2814–2819
12 Levine R. P. and Volkmann, D. (1961) *Biochem. Biophys. Res. Commun.* 7, 264–269
13 Brand, J., San Pietro, A. and Mayne, B. C. (1972) *Arch. Biochem. Biophys.* 152, 426–428
14 Ort, D. R., Izawa, S., Good, N. E. and Krogmann, D. W. (1973) *Biochim. Biophys. Acta*, in the press
15 Katoh, S. and San Pietro, A. (1966) in *The Biochemistry of Copper*, (Peisach, J,, Aisen, P. and Blumberg, W. E., eds) pp. 407–422, Academic Press, N.Y.
16 Baszynski, T., Brand, J. Krogmann, D. W. and Crane, F. L. (1971) *Biochim. Biophys. Acta* 234, 537–540
17 Dilley, R. A., (1968) *Biochemistry*, 7, 338–346
18 Berg, S., Cipollo, D., Armstrong, B. and Krogmann, D. W. (1973) *Biochim. Biophys. Acta*, in the press