EFFECTS OF THE PLASTOCYANIN ANTAGONISTS KCN AND POLY-L-LYSINE ON PARTIAL REACTIONS IN ISOLATED CHLOROPLASTS

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Received 12 February 1973

1. Introduction

A very large number of compounds are known to inhibit chloroplast electron transport at or near photosystem II. However, compounds which have been shown to block photosystem I are very few despite the great importance of such inhibitors in analyzing the pathway of photosynthetic electron transport and the sites of phosphorylation associated with it. Hauska et al. [1] demonstrated that an antibody to plastocyanin can be a useful inhibitor but only when applied to finely fragmented chloroplasts. Kimimura and Katoh [2] have recently reported that incubation of chloroplasts in $HgCl_2$ can inhibit electron flow at plastocyanin. However, prolonged exposure to $HgCl_2$ has a number of deleterious effects on the chloroplasts; moreover it strongly inhibits phosphorylation [3]. This leaves us only two specific photosystem I inhibitors which can be applied to unfragmented chloroplasts, i.e., polylysine (and certain other polycations) of Brand et al. [4] and KCN of Ouitrakul and Izawa [5]. Brand et al. [6] have located the site of polycation inhibition between cytochrome f and P_{700}. Izawa and associates (manuscript in preparation) have shown spectrophotometrically that KCN inhibits cytochrome f oxidation. Moreover KCN has been shown to react readily with isolated plastocyanin under the conditions required for effecting electron transport inhibition. Thus both polylysine and KCN appear to block electron transport at the level of plastocyanin involvement. However, the effect of polylysine on phosphorylating electron transport has not yet been explored. Furthermore, there are some discrepancies between the effects of polylysine reported by Brand et al. [7] and that of KCN reported by Ouitrakul and Izawa [5]. (For instance polylysine inhibited ferricyanide reduction only by 50% while KCN inhibited it almost completely).

The purpose of the present study was to compare these two inhibitions in well-coupled chloroplasts and under similar experimental conditions, to determine if the inhibitions were specific for photosystem I-requiring reactions as suggested by the plastocyanin involvement, and to determine the relation of the residual photosystem II reaction to phosphorylation. Using photosystem II and photosystem I partial reactions, we were able to show the photosystem I specificity of poly-L-lysine (M.W. 194,000) and to confirm the photosystem I specificity of the KCN treatment. Chloroplasts blocked with either inhibitor continue to phosphorylate during the reduction of lipophilic Class III acceptors. This electron transport which is entirely dependent on photosystem II is somewhat less efficient in phosphorylation when polylysine is used. Moreover, we found that exact conditions for polylysine inhibition were critical and therefore it was difficult to reproduce precisely various levels of polylysine inhibition.

2. Materials and methods

Chloroplasts were isolated from commercial spinach (*Spinacia oleracea* L.) as described elsewhere [8].

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The resulting stock suspension contained 800 μg chlorophyll/ml, 0.2 M sucrose, 0.005 M HEPES, and 2 mM $MgCl_2$. The procedure for measuring the reduction of the lipophilic oxidants, e.g. oxidized *p*-phenylenediamine (PD_{ox}), oxidized diaminodurene (DAD_{ox}), 2,5-dimethyl-*p*-benzoquinone (DMQ), is detailed in a paper by Saha et al. [8]. Photoreduction of dibromothymoquinone (DBMIB) was measured in a similar manner [9]. Electron flow from diaminodurene (DAD) to methylviologen (MV) was measured as described by Izawa et al. [10].

Cyanide-treated chloroplasts were prepared as described in a previous paper [5] by incubating chloroplasts for 90 min at 0° in a 30 mM solution of KCN buffered at pH 7.8. Control chloroplasts were suspended for the same time in a similar medium containing KOH instead of KCN.

In all experiments designed to test the effect of polycations on chloroplasts, poly-L-lysine of molecular weight 194,000 was used. The polylysine was dissolved in glass distilled water at 1 mg/ml. The appropriate amount of chloroplast suspension (see legend of table 1) was added to 0.4 to 0.6 ml of water at room temp. in the reaction cuvette prior to the addition of the polylysine. This room temp. water-shocking (see fig. 1) was essential to obtain inhibition of electron transport by polylysine. The addition of polylysine was followed immediately by addition of the sucrose and buffer, and then by the remainder of the reaction components. Control chloroplasts were water-shocked in a similar manner.

Photophosphorylation was measured using a modified version of the method of Avron [11].

3. Results and discussion

When Brand and his associates [4, 7] first studied the polycation inhibition of electron transport, they isolated the chloroplasts in media free of sucrose and salts. In contrast, we have found that the chloroplasts may be isolated in conventional isotonic, buffered media then suspended briefly in greatly diluted buffer just before addition of polylysine. Once the polylysine has been added the normal media can be restored. Thus the chloroplasts need be subjected to harmful hypotonic, salt-free conditions for only a few seconds. Therefore, our chloroplasts are more active, more tightly coupled and generally more intact than those employed by earlier investigators [4, 7]. Fig. 1 demonstrates the relationship between time of water shock and degree of inhibition of electron transport from water to ferricyanide. The time of water shock required to achieve 100% inhibition with 100 μg polylysine/ml varied with the different chloroplast preparations. To insure complete inhibition of electron flow through plastocyanin the chloroplasts were usually water shocked for 90 sec. Fig. 1 also shows that 50 μg polylysine/ml is sufficient to cause only 50% inhibition of the Hill reaction. This suggests that the polycation may be titrating negative charges on the site of inhibition and elsewhere and that 50 μg polylysine/ml does not supply enough positive charges to bind all available negative sites. Polylysine levels above 100 μg/ml resulted in excessive clumping of the chloroplasts and therefore made spectrophotometric assays very difficult.

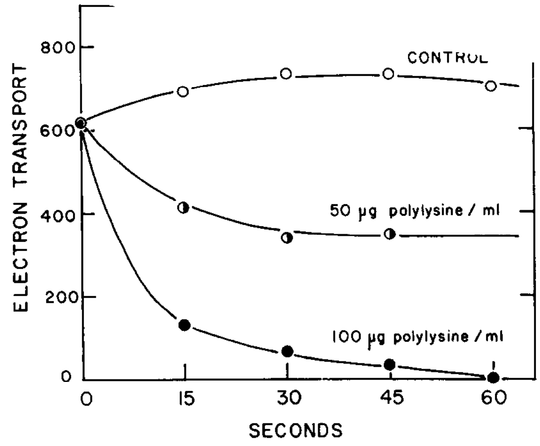

Fig. 1. Effect of water-shock-time on inhibition of electron transport from water to ferricyanide by polylysine. Rates are given in μequivalents/hr·mg chlorophyll. The reaction cuvette contained a 2 ml reaction mixture consisting of the following: 0.1 M sucrose, 50 mM tricine-NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.5 mM ADP, 5 mM P_i, 0.4 mM FeCy, 40 μg chlorophyll and polylysine as indicated. 0.5 ml of water at room temp. were placed in the cuvette. Then 0.05 ml of chloroplasts containing 40 μg chlorophyll from the stock suspension (see Methods) was added. After incubation for the indicated number of seconds the polylysine was added in 0.1 ml and this was followed immediately by sucrose and buffer and then by the other components of the reaction mixture.

Table 1 presents the data obtained from various partial reactions using KCN and polylysine-treated

Table 1
The effect of KCN or polylysine treatment on various pathways of electron transport (E.T.) and associated photophosphorylation (ATP) in isolated chloroplasts.

	KCN treatment*				Polylysine treatment*			
System	Condition	E.T.	ATP	(P/e_2)	Condition	E.T.	ATP	(P/e_2)
$H_2O \rightarrow$ FeCy	Control	401	253	(1.26)	Control	560	357	(1.24)
	KCN	35	5.2	(0.29)	Polylysine	20	0	
DAD → MV	Control	3250	580	(0.36)	Control	2750	440	(0.32)
	KCN	230	31	(0.27)	Polylysine	291	0	
$H_2O \rightarrow PD_{ox}$	Control	1070	310	(0.58)	Control	1390	429	(0.61)
	KCN	605	108	(0.36)	Polylysine	980	128	(0.26)
$H_2O \rightarrow DAD_{ox}$	Control	915	256	(0.57)	Control	1360	407	(0.59)
	KCN	415	70.5	(0.34)	Polylysine	830	112	(0.27)
$H_2O \rightarrow$ DMQ	Control	790	245	(0.62)	Control	1060	371	(0.70)
	KCN	292	44.2	(0.33)	Polylysine	590	62	(0.21)
$H_2O \rightarrow$ DBMIB**	Control	262	49.7	(0.38)	Control	272	41.5	(0.31)
	KCN	237	42.9	(0.36)	Polylysine	285	35.7	(0.25)

The reaction cuvette contained 2.0 ml of reaction mixture consisting of: 50 mM tricine-NaOH buffer (pH 8.0), 0.1 M sucrose, 2 mM $MgCl_2$, 1 mM $^{32}P_i$, 0.5 ADP, chloroplasts, the indicated electron acceptor, and the indicated electron donor system when used. The electron acceptor systems used were: 0.4 mM potassium ferricyanide (FeCy); 0.5 mM methylviologen (MV); 0.5 mM *p*-phenylenediamine plus 1.5 mM ferricyanide (PD_{ox}); 0.5 mM diaminodurene plus 1.5 mM ferricyanide (DAD_{ox}); 0.5 mM 2,5-dimethylquinone plus 0.4 mM ferricyanide (DMQ); 1×10^{-5} M dibromothymoquinone plus 0.4 mM ferricyanide (DBMIB). The electron donor system used was 1 mM ascorbate plus 0.5 mM diaminodurene (DAD). The amount of chloroplasts suspended in the 2.0 ml reaction mixture was equivalent to 40 µg chlorophyll for the systems $H_2O \rightarrow$ FeCy, DBMIB; 8 µg for DAD → MV; 20 µg for $H_2O \rightarrow PD_{ox}$, DMQ. Rates are expressed in µequivalents or µmoles ATP/hr·mg chlorophyll.

* For the procedures, see Methods.
** In reactions using DBMIB as the electron acceptor HEPES buffer at pH 7.5 was used.

chloroplasts. We were able to obtain nearly 100% inhibition of electron transport from water to ferricyanide by polylysine whereas previous investigators [7] saw only 50% inhibition of this system. This discrepancy is probably due to the "leaky" nature of those chloroplasts which had been exposed to prolonged osmotic shock. In leaky chloroplasts ferricyanide seems capable of accepting electrons not only at its normal hydrophilic photosystem I site, but also at a site close to photosystem II which is normally accessible only to lipophilic oxidants.

Saha et al. [8] have shown that lipophilic strong oxidants (e.g. oxidized *p*-phenylenediamine) are reduced very rapidly by illuminated chloroplasts and that the reaction is not stimulated by uncouplers or by the addition of ADP and P_i. Electron flow to such lipophilic oxidants is only about 50% as efficient in phosphorylation as is the flow of electrons to hydrophilic Hill acceptors such as ferricyanide. This suggests to us that lipophilic oxidants can accept electrons between two sites of phosphorylation. Table 1 shows that both KCN and polylysine prevent electron transport to Class I acceptors almost completely but have much less effect on electron transport to Class III acceptors. Oxidized *p*-phenylenediamine (PD_{ox}) is the best of the Class III acceptors, that is it has the smallest photosystem I-dependent component. By the same criterion dimethylquinone (DMQ) is the worst Class III acceptor tested. It is our contention that KCN and polylysine inhibit that portion of the electron flow through plastocyanin but hardly affects the transport of electrons from a donor close to photosystem II to Class III acceptors.

Dibromothymoquinone (DBMIB) is a unique Class III acceptor in that it acts not only as a lipophilic electron acceptor but also as an inhibitor of electron transport, probably by blocking at the site of plastoquinone involvement [12]. Therefore the reduction of this Class III acceptor has no photosystem I-dependent component and should not be effected by KCN or polylysine blocks. Table 1 shows this to be the case. These findings confirm the photosystem I specificity of polylysine inhibition and point out the similarity to KCN inhibition.

To complete the comparison of KCN and polylysine inhibitions it is necessary to consider phosphorylation. Dilley [13] has reported that polylysine is an uncoupler of phosphorylation at levels similar to those needed for inhibition of the photoreduction of P_{700}. We found that when polylysine was added to the 2 ml reaction mixture after the addition of 2 μmoles of $MgCl_2$ no phosphorylation occurred and electron transport from water to ferricyanide was stimulated. However, when the polycation was handled as described in Methods phosphorylation did occur when electrons flowed from water to a lipophilic oxidant. Table 1 shows that higher phosphorylation efficiencies were obtained when Class III acceptors were used with KCN-treated rather than with polylysine-treated chloroplasts. Furthermore, in some chloroplast preparations the polylysine treatment was found to uncouple even more severely than indicated in table 1. Since water shocking itself had little if any effect on phosphorylation efficiencies, we must conclude the partial uncoupling is due to the polycation.

On the basis of these experiments and similar experiments published elsewhere [4, 5, 7, 8] we conclude that polylysine and KCN treatments described provide convenient and specific inhibitors of photosystem I reactions, almost certainly by blocking the electron transport through plastocyanin.

Acknowledgements

We thank J.M. Gould for valuable consultation. This work was supported by a grant (GB 22657) from the National Science Foundation, USA.

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