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SILICOMOLYBDATE REDUCTION BY ISOLATED PEA CHLOROPLASTS

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Abstract—Conditions for the optimization of silicomolybdate reduction by isolated pea chloroplasts are described. Maximum rates of reduction are related to time of addition to the chloroplasts and the presence of an oxidizing cofactor, such as ferricyanide. Silicomolybdate or silicomolybdate plus ferricyanide reduction is only partially inhibited by a concentration of CMU which totally abolishes ferricyanide reduction. Evidence for a differing response of the two reduction sites to silicomolbydate is described.

INTRODUCTION

Girault and Galmiche [1] initiated the use of heteropoly anions in experiments with isolated chloroplasts. These authors reported that silicotungstate partially restored ferricyanide reduction in DCMU-treated chloroplasts. Subsequently Giaquinta *et al.* [2] introduced silicomolybdate which acted in a similar manner. Further studies by Barr *et al.* [3] demonstrated the reduction of silicomolybdate by isolated chloroplasts with either water or diphenylcarbazide as electron donor.

The reduction of silicomolybdate appears to occur at two sites: one at or before Q which continues to operate in the presence of electron transport inhibitors, and the second between Q and plastoquinone. Support for the second site was obtained by Giaquinta and Dilley [4] using the plastoquinone antagonist DBMIB. Addition of this compound to isolated chloroplasts did not affect electron transport to silicomolybdate in the presence of DCMU. Support for a silico reduction site at Q was made by Zilinskas and Govindjee [5] using fluorescence techniques with DCMU-inhibited chloroplasts. Additional evidence for a reduction site prior to the electron transport inhibitors site was provided by Robertson *et al.* [6] in experiments with chloroplasts isolated from leaves greened in the presence of CMU and sucrose. Oxygen evolution with ferricyanide as acceptor was absent, whereas with silicomolybdate the rate of oxygen evolution was similar to that of chloroplasts isolated from leaves greened in the absence of an electron transport inhibitor.

In this work with chloroplasts isolated from pea leaves, we have attempted to define more clearly the optimum conditions for silicomolybdate reduction in the presence of ferricyanide and an inhibitor of electron transport.

* *Abbreviations*: CMU = 3-(4'-chlorophenyl)-1, 1-dimethylurea; DBMIB = dibromothymoquinone; and DCMU = 3-(3', 4'-dichlorophenyl)-1, 1-dimethylurea.

RESULTS

Table 1 shows the effect of a variation in addition time of the electron acceptors on the final rate of oxygen evolution. If ferricyanide was added before, at, or during the illumination period, it had relatively little effect on the rate of evolution. With silicomolybdate on the other hand, addition before the time of illumination resulted in a 20% decrease in rate. In all subsequent experiments ferricyanide was added to the reaction mixture prior to illumination, and silicomolybdate at the point of illumination. The electron transport inhibitor used in these experiments was CMU. This is generally accepted to operate in the same manner as DCMU but with a different concentration threshold [7]. Figure 1 shows the effect of a range of CMU concentrations on on oxygen evolution with ferricyanide, silicomolybdate plus ferricyanide, and silicomolybdate alone. In this

Table 1. Effect of variation in time of addition of electron acceptor on rate of oxygen evolution. The reaction mixture contained in 3 ml: 0.03 M tricine–NaOH pH 8.0; 0.66 mM FeCy or 1 mg (0.182 mM) SiMo and chloroplasts equivalent to 70 μg/ml chlorophyll. Illumination was provided by a 300 W lamp giving 95 W/m² at the electrode. The temperature of the stirred reaction mixture was maintained at 20°

Time of addition of electron acceptor	μmol O_2 evolved/mg chlorophyll after 3 min illumination	
	FeCN	SiMo
0 in dark	0.628	0.498
1 min at illumination	0.628	0.599
2 „ „	0.612	0.601
3 „ „	0.594	0.589
4 „ „	0.590	0.579

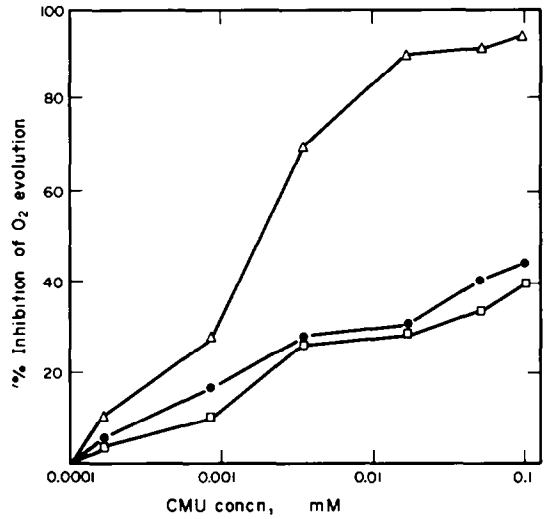

Fig. 1. Effect of CMU concentration on oxygen evolution with FeCy △; FeCy plus SiMo ●, and SiMo □. FeCy and CMU were added in the dark prior to illumination and SiMo upon illumination. Reaction conditions as described for Table 1.

experiment 1.8 μM CMU effected a 50% inhibition of oxygen evolution with ferricyanide as acceptor. This concentration of inhibitor suppressed oxygen evolution with silicomolybdate by only 24.5% and with silicomolybdate plus ferricyanide by 18.5%.

Figures 2a and b show the time course of oxygen evolution with the three electron acceptor systems used in Fig. 1. Figure 2b represents an experiment with the addition of 10 μM CMU, a concentration which totally abolished oxygen evolution with ferricyanide as oxidant. A conspicuous feature of these graphs is the progressive decline of oxygen evolution with silicomolybdate alone. The explanation is probably, as suggested by Giaquinta and Dilley [4], that when ferricyanide is present it acts as the final electron acceptor, thus eliciting a constant regeneration of oxidized silicomolybdate.

Figure 3 shows the effect of varying silicomolybdate concentrations on oxygen evolution. This graph also represents the effect of the addition of 0.66 μM ferricyanide and 10 μM CMU throughout the concentration range. With silicomolybdate alone there was a conspicuous difference in effect between an inhibited and a non-inhibited system. When the complete electron transport chain was in operation, and thus two sites for silicomolybdate reduction, an optimum rate was achieved at 0.5 mg. In contrast, in the presence of CMU and therefore only one reduction site, 1.0–1.5 mg of silicomolybdate was required for an optimum rate. However, the presence of ferricyanide lowered the optimum peak concentration to that of 0.5 mg. The addition of ferricyanide to the non-inhibited system resulted in a large increase in oxygen evolution at low levels of silicomolybdate (< 0.5 mg) but a constant rate was achieved between 0.5 and 1.5 mg.

DISCUSSION

In experiments with spinach and lettuce chloroplasts Zilinskas and Govindjee [5] reported that pre-illumination, before the addition of silicomolybdate, elicited a three-fold increase in rate of reduction. They suggested that illumination caused the repositioning of a structural component of the chloroplast so that Q would be located in a more exterior position, and hence donate electrons more efficiently to silicomolybdate. This effect was not demonstrated in pea chloroplasts where the optimum rate was achieved when silicomolybdate was added at the point of illumination (Table 1). This could indicate either a rapid photo-induced positional reorganization of the chloroplast membrane or alternatively, a chemical interaction between silicomolybdate and the chloroplast in the dark period, which reduced the subsequent photo-induced electron flow.

This investigation has confirmed that oxygen evolution in the presence of silicomolybdate is only partially inhibited by the electron transport inhibitor CMU. A concentration of CMU which totally abolished ferricyanide reduction, inhibited silicomolybdate reduction by less than 50%. To maintain this rate of reduction the level of oxidized silicomolybdate is critical. Whereas high concentrations > 50 μM limited reduction rates [5] possibly by acting as direct chemical quenchers of

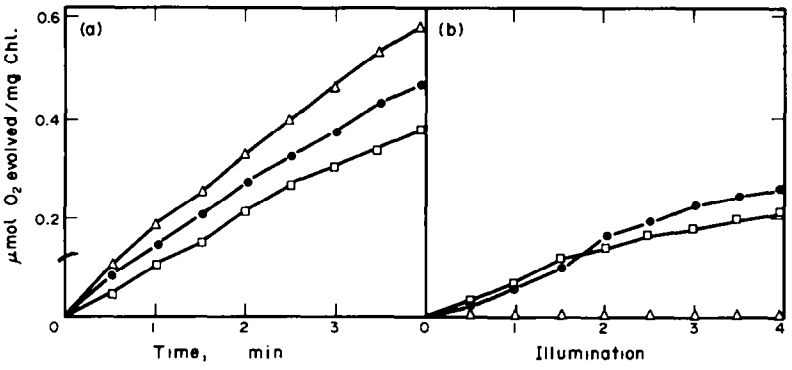

Fig. 2. Oxygen evolution as a function of time of illumination in the presence of FeCy △; FeCy plus SiMo ●, and SiMo □. 2A, no further additions; 2B plus 10 μM CMU. Reaction conditions as described for Table 1.

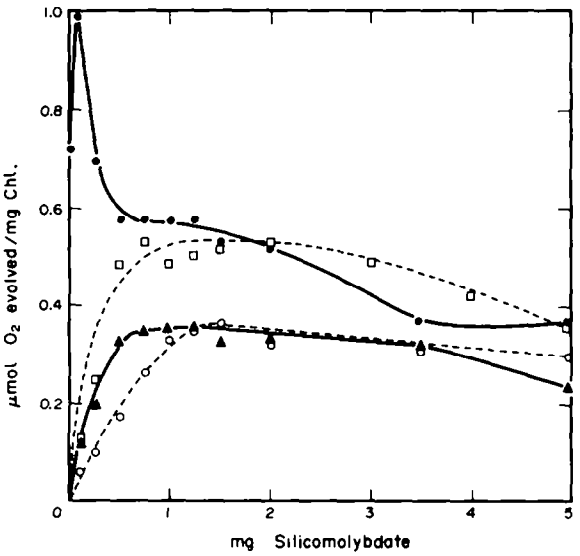

Fig. 3. Oxygen evolution after 3 m illumination as a function of SiMo concentration, 0.66 mM FeCy and 10 µ M CMU. ●—● FeCy and SiMo; □---□ SiMo; — FeCy, SiMo and CMU; ○---○ SiMo and CMU. Reaction conditions as described for Table 1.

fluoresence, the rate with an insufficient level progressively declined (Fig. 2). The addition of ferricyanide maintained sufficient silicomolybdate in the oxidized state, and thus the rate of oxygen evolution was not depressed. In this situation ferricyanide was not acting as a primary electron acceptor because a stimulation was achieved in the presence of CMU. In these experiments with pea chloroplasts we failed to find a silicomolybdate-induced inhibition of oxygen evolution in the presence of ferricyanide and an inhibitor of electron transport. The experiments of Zilinskas and Govindjee [5] showed a marked inhibition of oxygen evolution after 45 sec illumination under similar circumstances.

Although ferricyanide will mediate electron flow from silicomolybdate, it was evident that in a non-inhibited system and with a low concentration of silicomolybdate (0.1–0.2 mg) ferricyanide was acting as the primary electron acceptor (Fig. 3). As the concentration of silicomolybdate was increased to 0.5–1.5 mg the total inhibition of direct ferricyanide reduction appeared to be achieved. It would seem unlikely that this site for ferricyanide reduction is identical to the post Q site for silicomolybdate reduction, although Giaquinta and Dilley [4] obtained a small amount of ferricyanide reduction in the presence of the plastoquinone antagonist DBMIB, when oxygen evolution with silicomolybdate was unaffected.

Although there is good experimental evidence for two independent reduction sites for silicomolybdate, a difference was noted in relationship to silicomolybdate concentration. The optimum rate of oxygen evolution with electron flow from Q alone was dependent upon a higher concentration of silicomolybdate (1.5 mg) when compared with a situation in which both sites were operative (0.5 mg). It should be noted, however, that reduction at Q alone could be made more efficient by linking with ferricyanide. The higher concentration of silicomolybdate required for the pre-inhibitor site could be explained by the interaction of CMU with Q. It is generally agreed that the urea herbicides, which include CMU, function by inhibiting electron flow between Q and plastoquinone, but as yet the actual details of molecular involvement are unknown. A possibility is that the inhibitor interacts with a portion of the chlorophyll protein system of the chloroplast by means of a charge transfer complex, and this complex not only prevents electron flow between Q and plastoquinone, but also reduces the number of sites available for silicomolybdate reduction from Q. When ferricyanide was present, the limited silicomolybdate which linked with Q would be reoxidized, yielding a greater degree of oxygen evolution.

It is evident that the relationships between silicomolybdate and a particular chloroplast preparation are more complex than for most Hill oxidants such as ferricyanide and dichlorophenol-indophenol. As reduction may vary with plant species or isolation conditions, it would appear to be a wise precaution before embarking on an extensive series of experiments with silicomolybdate to ascertain the optimum time for addition to the experimental system and also to establish the optimum concentration of silicomolybdate required in the presence or absence of ferricyanide and/or electron transport inhibitors.

EXPERIMENTAL

Chloroplasts were isolated from leaves of 14–20 day old *Pisum sativum* cv. Meteor plants, grown under greenhouse conditions. The method was based on that of Izawa and Good [8]. Leaves were homogenized in an Ultra-Turrax homogenizor for 15 sec in a media containing 0.05 M tricine–NaOH buffer pH 7.5, 0.3 M NaCl and 0.003 M $MgCl_2$. After filtering through 4 layers of muslin and centrifugation at 8000 rpm for 1 min at 4°, chloroplasts were resuspended in media containing 0.005 M tricine–NaOH buffer pH 7.5, 0.1 M sucrose, 0.003 M $MgCl_2$ and 1 mg/ml bovine serum albumin. Oxygen evolution was measured in an electrode (Rank Bros.) linked to a Servoscribe chart recorder. Other details of experimental conditions are given in the figure legends. Chlorophyll content of chloroplast preparations was determined by the method of Arnon [9].

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