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INDUCTION OF A MEHLER REACTION IN CHLOROPLAST PREPARATIONS BY METHYL VIOLOGEN AND BY FERREDOXIN: EFFECTS ON PHOTOSYNTHESIS BY INTACT CHLOROPLASTS

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SUMMARY

CO_2-dependent oxygen evolution in illuminated spinach chloroplast preparations was found to be inhibited by addition of methyl viologen or ferredoxin. Under the conditions described, and in the absence of ribose-5-phosphate, the K_i for inhibition by methyl viologen was 0.4 μM and the K_i for inhibition by ferredoxin was 13 μM. The effect of osmotic shock on chloroplast oxygen uptake in the Mehler reaction implies that at the concentrations used neither methyl viologen nor ferredoxin freely penetrates the envelope of intact chloroplasts. The observed inhibition of CO_2-dependent oxygen evolution is therefore attributed in each case to a Mehler reaction in the broken chloroplasts of the preparations. Inhibition by methyl viologen (0.8 μM) and by ferredoxin (20 μM) could be reversed by ascorbate and by catalase. As reported [1] for flavin mononucleotide H_2O_2 is likely to be the chief inhibitory agent. The difference in mechanism of autoxidation between methyl viologen and ferredoxin is therefore less important in this system than is their common production of H_2O_2.

INTRODUCTION

H_2O_2 has been shown to be responsible for inhibition by flavin mononucleotide (FMN) of CO_2-fixation and CO_2-dependent oxygen evolution in isolated chloroplasts [1]. FMN resembles methyl viologen [2] in that the product of its autoxidation is superoxide ($O_2^{\cdot -}$), and H_2O_2 is produced as a result of subsequent dismutation of superoxide. Although the chloroplast envelope appears to be impermeable to FMN [1], there is evidence that it is permeable to

Abbreviation: FMN, flavin mononucleotide.

methyl viologen, since photosynthetic oxygen uptake which is mediated by methyl viologen has been reported to occur in intact chloroplasts [3]. If methyl viologen inhibits CO_2-fixation by competing with an electron acceptor within the intact chloroplast, then the sensitivity of the inhibition to added catalase and superoxide dismutase would be expected to differ from that of inhibition by FMN.

The mechanism of autoxidation of ferredoxin differs from that of methyl viologen and FMN [4], in that reduced ferredoxin may reduce both oxygen and superoxide. Dismutation of superoxide would then be expected to play no part in production of H_2O_2 by ferredoxin, and superoxide would exist only as a short-lived intermediate of the overall reaction. If superoxide is responsible for inhibition of CO_2-dependent oxygen evolution when a Mehler reaction is induced in chloroplast preparations, then ferredoxin would be expected to be less effective an inhibitor than methyl viologen or FMN, and there should be no reversal by superoxide dismutase of ferredoxin's inhibitory effect.

The inhibitory effects of methyl viologen were therefore compared with those of ferredoxin. As with FMN [1], inhibition should result from induction of a Mehler reaction and from any associated effects of $O_2^{\cdot -}$ or H_2O_2 on CO_2-dependent oxygen evolution.

MATERIALS AND METHODS

Chloroplasts were isolated from outdoor-grown spinach (*Spicacia oleracea* L.) in an isotonic sorbitol medium, and photosynthetic rates were measured in twin oxygen electrodes at 20°C. Experimental details are given in reference [1].

Bovine superoxide dismutase was purchased from the Microbiological Research Establishment, Porton, Wilts. NADP and bovine catalase (crystalline suspension) were purchased from Boehringer, Mannheim. Methyl viologen was purchased from Sigma, London. Ferredoxin from *Spirulina maxima* [5] was a gift from Dr. K.K. Rao and Professor D.O. Hall, King's College, London.

RESULTS

Table I shows rates of oxygen exchange obtained with different electron acceptors in shocked and in unshocked chloroplasts from a single preparation. Calculated percentages of intact chloroplasts were similar for oxygen evolution with either ferricyanide or NADP as electron acceptor. A lower value for the percentage of intact chloroplasts was obtained for oxygen uptake with ferredoxin, and even lower values for oxygen uptake with methyl viologen. In the latter case the apparent percentage of intact chloroplasts decreased with increasing methyl viologen concentration.

Fig. 1 shows the effect of increasing methyl viologen concentration on CO_2-dependent oxygen evolution. Figure 2 shows the corresponding effect of increasing ferredoxin concentration. In each case the inhibitory effect is seen to have been slightly offset by the presence of ribose-5-phosphate. This small effect

TABLE I

RATES OF PHOTOSYNTHETIC OXYGEN EXCHANGE BY OSMOTICALLY SHOCKED AND BY UNSHOCKED CHLOROPLASTS FROM A SINGLE PREPARATION.

Reaction conditions as for Fig. 1, but with DL-glyceraldehyde (10 mM) and NH_4Cl (5 mM) replacing $NaHCO_3$ and K_2HPO_4. Oxygen uptake was obtained with ferredoxin alone and with methyl viologen; NaN_3 (2 mM) was then present. Catalase (2×10^3 units) was present for oxygen evolution supported by ferredoxin with NADP.

Electron acceptor (final concentration)	Rate of O_2 evolution μmol (mg chl)$^{-1}$ h^{-1}		% of chloroplasts intact
	unshocked	shocked	
$K_3Fe(CN)_6$ (5 mM)	+169	+378	55
O_2 via ferredoxin (20 μM)	−45	−76	41
NADP (2 mM) via ferredoxin (10 μM)	+83	+169	51
O_2 via methyl viologen (20 μM)	−164	−272	40
O_2 via methyl viologen (100 μM)	−194	−293	34
O_2 via methyl viologen (500 μM)	−209	−293	29

of ribose-5-phosphate has been observed consistently in these experiments, and is particularly apparent at higher concentrations of methyl viologen or ferredoxin.

Table II shows the effect of methyl viologen at a concentration (0.8 μM) which gave over 90% inhibition of CO_2-dependent oxygen evolution in the absence of ribose-5-phosphate. Here again inhibition was seen to have been

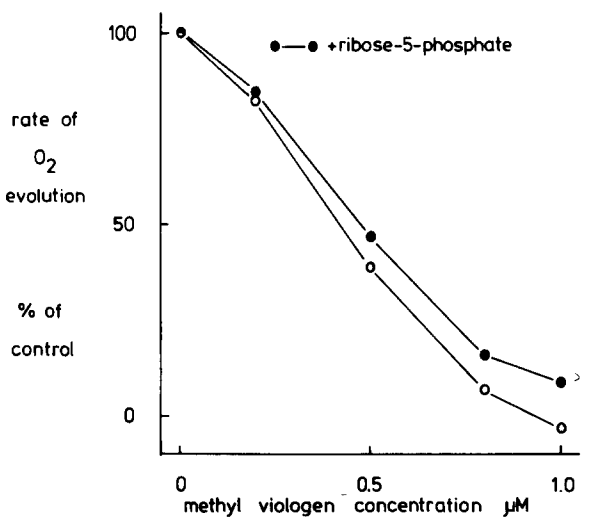

Fig. 1. Effect of increasing methyl viologen concentration on steady-state CO_2-dependent oxygen evolution by isolated chloroplasts, in the presence and absence of ribose-5-phosphate (1 mM). The reaction mixture (pH 7.6) also contained sorbitol (0.33 M), EDTA (2 mM), $MgCl_2$ (1 mM), HEPES (50 mM), K_2HPO_4 (0.5 mM), $NaHCO_3$ (10 mM) and chloroplasts (equivalent to 50 μg chlorophyll) in a final volume of 1.00 ml. 54% of chloroplasts were intact. The control rate of oxygen evolution was 37 μmol (mg chl)$^{-1}$ h^{-1}.

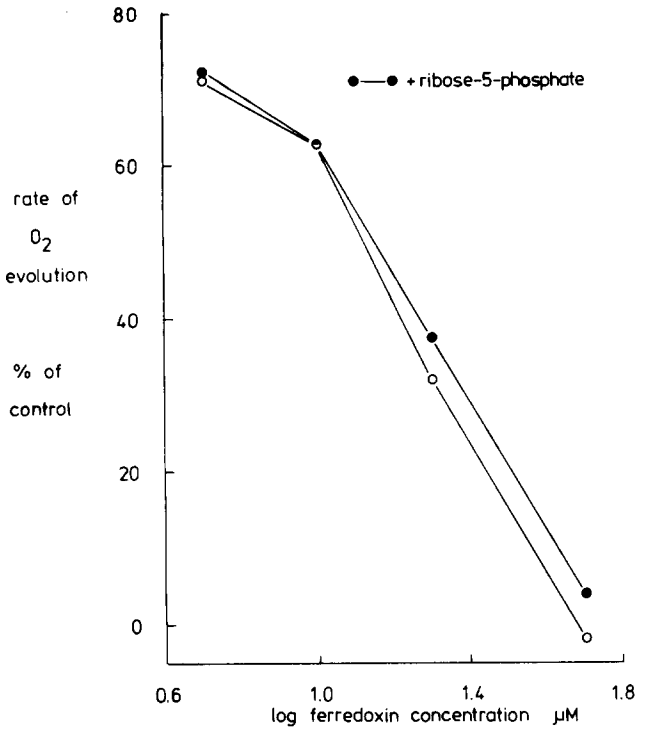

Fig. 2. Effect of increasing concentration of ferredoxin on steady-state CO_2-dependent oxygen evolution by isolated chloroplasts. Reaction conditions were as for the experiment of Fig. 1, with ferredoxin replacing methyl viologen. 52% of chloroplasts were intact. The control rate of oxygen evolution was 34 μmol (mg chl)$^{-1}$ h^{-1}.

TABLE II

STEADY-STATE RATES OF CO_2-DEPENDENT OXYGEN EVOLUTION BY ISOLATED CHLOROPLASTS:

Effects of methyl viologen (MV; 0.8 μM), ascorbate (2 mM) superoxide dismutase (SOD; 10^3 units) and catalase (2 × 10^3 units) in the presence and absence of ribose-5-phosphate (R5P; 1mM). Other conditions are as described for Fig. 1. 54% of chloroplasts were intact.

Addition	Rate of O_2 evolution μmol (mg chl)$^{-1}$ h^{-1}	
	without R5P	with R5P
None (control)	40	40
MV	3	6
MV, ascorbate	23	23
MV, ascorbate, SOD	29	28
MV, SOD	5	9
MV, catalase	31	32
MV, catalase, ascorbate, SOD	29	29

TABLE III

STEADY-STATE RATES OF CO_2 -DEPENDENT OXYGEN EVOLUTION BY ISOLATED CHLOROPLASTS:

Effects of *Spirulina* ferredoxin (Fd; 20 μM), ascorbate (2 mM), superoxide dismutase (SOD; 10^3 units) and catalase (2×10^3 units) in the presence and absence of ribose-5-phosphate (R5P; 1 mM). Other conditions as described for Fig. 1. 46% of chloroplasts were intact.

Addition	Rate of O_2 evolution μmol (mg chl)$^{-1}$ h^{-1}	
	without R5P	with R5P
None (control)	35	35
Fd	2	6
Fd, ascorbate	28	28
Fd, ascorbate, SOD	29	29
Fd, SOD	10	12
Fd, catalase	25	27
Fd, catalase, ascorbate, SOD	22	24

slightly greater in the absence of ribose-5-phosphate than in its presence. The presence of ascorbate reversed the effect of methyl viologen, and diminished the difference between the rates observed in the presence and in the absence of ribose-5-phosphate. Superoxide dismutase by itself had little effect on inhibition by methyl viologen. Catalase by itself had a protective effect slightly greater than that of ascorbate and superoxide dismutase together.

Table III records the results of an analogous experiment with ferredoxin (20 μM) as the inhibitor instead of methyl viologen. The results show an overall similarity to those of Table II, though in Table III superoxide dismutase is seen not to have added to ascorbate's effect in reversing the inhibition by ferredoxin. In contrast, superoxide dismutase on its own caused a reversal of inhibition by ferredoxin (Table III) which was larger than the corresponding reversal of inhibition by methyl viologen (Table II). Inhibition by ferredoxin (Table III) is also seen to have been reversed by catalase acting alone.

DISCUSSION

Robinson and Wiskich [3] have shown that uncoupled oxygen uptake by pea chloroplasts in the presence of methyl viologen (0.3 mM) is unaffected by sucrose concentration, despite the high initial percentage of intact chloroplasts in their preparations. They conclude that methyl viologen rapidly penetrates the chloroplast outer membrane. Table I shows that osmotic shock had some effect on oxygen uptake even with 0.5 mM methyl viologen. The chloroplast envelopes were therefore no more than partially permeable to methyl viologen even at relatively high concentrations. At lower methyl viologen concentrations the effect of osmotic shock was greater (Table I) though the persistence of

some penetration of the chloroplast by methyl viologen is indicated by the corresponding value for chloroplast intactness being lower than that obtained with ferricyanide-dependent oxygen evolution. In broken, washed chloroplasts methyl viologen competes with ferredoxin for electrons from photosystem I; with NADP present, oxygen uptake replaces oxygen evolution at methyl viologen concentrations greater than 1 μM (author's unpublished results).

Methyl viologen has a high affinity for oxygen and the initial product of its reaction with oxygen is $O_2^{\cdot -}$ [6]. Superoxide-mediated inhibition of CO_2-fixation and consequent production of glycolate has been shown to result from addition of methyl viologen (50 μM) to illuminated *Chromatium* suspensions [7]. Superoxide dismutase nevertheless had only a small effect in reversing inhibition by methyl viologen of CO_2-dependent oxygen evolution in the experiment of Table II. It is therefore likely that the low concentration of methyl viologen (0.8 μM) used in the experiment of Table II led to production of superoxide at a rate insufficient to overcome the protection offered by endogenous chloroplast superoxide dismutase [8]. Half-maximum oxygen uptake in broken chloroplasts is mediated by about 3 μM methyl viologen [4]. An initial and short-lived oxygen uptake of about 8 μmol (mg chl)$^{-1}$ h^{-1} was observed in the presence of methyl viologen and superoxide dismutase in the experiment of Table II (results not shown).

Substantial reversal by catalase of methyl viologen's inhibitory effect (Table II) indicates that H_2O_2 is the chief inhibitory agent. Though in general less toxic than superoxide, H_2O_2 in this case may have had the greater inhibitory effect because of the absence of catalase (other than as a contaminant) from intact chloroplasts [9]. The presence in chloroplasts of ascorbate peroxidase [10] makes reversal by ascorbate of methyl viologen's effect also consistent with inhibition having been caused primarily by production of H_2O_2. The further effect of superoxide dismutase may then be attributed to its suppression of ascorbate-stimulated oxygen uptake [11]. Superoxide dismutase-sensitive stimulation by ascorbate of a transient oxygen uptake which preceded CO_2-dependent oxygen evolution was seen in the oxygen-electrode traces from which the data of Table II are taken (results not shown). This Mehler reaction presumably continued even when net oxygen evolution had eventually appeared.

In contrast, oxygen uptake in the ferredoxin-mediated Mehler reaction is not stimulated by ascorbate and is inhibited by superoxide dismutase on its own [4]. Thus Table III records little further effect of superoxide dismutase when ascorbate was present, and superoxide dismutase by itself reversed inhibition by ferredoxin (Table III) to a greater extent than it reversed inhibition by methyl viologen (Table II). The protective effect of ascorbate in Table III is likely to be unrelated to ascorbate's reaction with superoxide, since this reaction does not occur during reduction of oxygen by ferredoxin [4]. Protection by ascorbate may instead be a result of its action as a substrate for a peroxidase [10], as previously mentioned, and the protective effect of catalase (Table III) again indicates that H_2O_2 is chiefly responsible for the initial inhibition. This view is

supported by the absence of a further effect of superoxide dismutase and ascorbate when catalase is present (Table II and III).

Thus, despite the fact that methyl viologen and ferredoxin differ in their mechanism of autoxidation [2], their most important feature in inhibition of CO_2-dependent oxygen evolution is their common production of H_2O_2. As with FMN [1], the tendency of ribose-5-phosphate to offset the inhibition implies either (i) that H_2O_2 in some way depletes chloroplasts of Calvin-cycle intermediates (as suggested by Kaiser [12]) and that ribose-5-phosphate can compensate for this depletion, or (ii) that the shortened pathway of CO_2-fixation from ribose-5-phosphate to triose phosphate excludes the step or steps at which inhibition by H_2O_2 otherwise occurs. It has been stated (H.W. Heldt; verbal report at the 4th International Congress on Photosynthesis, Reading, 1977) that the most H_2O_2-sensitive steps of the Calvin cycle are those catalysed by fructose bisphosphatase and by sedoheptulose bisphosphatase. This is consistent with and therefore adds plausibility to explanation (ii), though (i) cannot yet be entirely discounted.

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