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INDUCTION OF A MEHLER REACTION IN CHLOROPLAST PREPARATIONS BY FLAVIN MONONUCLEOTIDE: EFFECTS ON PHOTOSYNTHESIS BY INTACT CHLOROPLASTS

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SUMMARY

Addition of flavin mononucleotide (FMN) to spinach chloroplast preparations containing intact chloroplasts was found to result in inhibition of photosynthetic CO_2-dependent oxygen evolution. Since the inhibition could be reversed by catalase, it is attributed primarily to production of H_2O_2 in an FMN-mediated Mehler reaction which occurs in the broken chloroplasts of the preparations. Ascorbate and superoxide dismutase also reversed the inhibition though to a lesser extent. The inhibition was also offset by the presence of ribose-5-phosphate. The complete dependence of these effects on the presence of FMN is consistent with the view that intact chloroplasts do not themselves produce inhibitory concentrations of H_2O_2 under conditions optimal for photosynthetic CO_2-fixation.

INTRODUCTION

In the Mehler reaction isolated, illuminated chloroplasts use oxygen as a Hill oxidant, and H_2O_2 is thereby produced [1,2]. Mediators of the Mehler reaction, of which FMN is an example [3,4], pass electrons from photosystem I to oxygen by one of three possible routes [5]. In the case of FMN univalent reduction of oxygen occurs [6,7], and the superoxide anion ($O_2^{\cdot -}$) so formed may then dismutate to give H_2O_2 and O_2. In this respect FMN resembles methyl viologen [8,9]. The toxicity of $O_2^{\cdot -}$ makes it unlikely that reduction of oxygen by this mechanism occurs in vivo. Nevertheless, there is evidence (for a review see ref. 10) that oxygen is photoreduced in intact photosynthetic

Abbreviation: FMN, flavin mononucleotide.

systems such as whole algal cells. For isolated, intact chloroplasts a number of investigators have described results attributable to photosynthetic reduction of oxygen [11—13].

In the study described here low concentrations of FMN were employed in order to identify characteristic properties of chloroplast preparations in which reduction of oxygen can safely be assumed to accompany CO_2-fixation. Since it has been suggested that H_2O_2 is inhibitory to CO_2-fixation [12,13] the effect of catalase in the presence of FMN was considered to be of particular interest.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach *(Spinacia oleracea* L.) grown in the open in Oxford or purchased from F.A. Barker Ltd., Taplow, Bucks. In the latter case leaves were stored in polythene bags in a cold room and pre-illuminated for 30 min before use. Chloroplasts were isolated in a sorbitol medium as described elsewhere (Allen and Whatley, Plant Physiology, in press) but with ascorbate omitted from the homogenization and resuspending media. Chlorophyll was estimated by the method of Arnon [14].

Osmotically-shocked chloroplasts were obtained in the oxygen electrode by adding chloroplast suspension to approximately 10 vols. of H_2O. Addition of concentrated reaction medium 2 min later restored isotonic conditions and the final reaction volume of 1.00 ml.

Rates of oxygen exchange were measured in twin oxygen electrodes (Hansatech Ltd., King's Lynn, Norfolk) at 20°C with each electrode illuminated by a modified slide projector giving a saturating intensity of orange light.

Fixation of [^{14}C]bicarbonate (Radiochemical Centre, Amersham, Bucks.) into acid-stable products took place in the oxygen electrode vessel. Samples (5 μl) were withdrawn at intervals and placed on glass-fibre discs where they were acidified with 5N CH_3COOH (0.2 ml per sample) and then dried under an infra-red lamp. The discs were added to polythene scintillation vials containing 5 ml of scintillant (0.4% PPO and 0.01% POPOP in toluene) and the activity was measured in a Tracerlab liquid scintillation counter with a counting efficiency for ^{14}C of 93%.

Bovine superoxide dismutase was purchased from the Microbiological Research Establishment, Porton, Wilts., and was assayed by the method of McCord and Fridovich [15] using a Unicam SP8000 spectrophotometer. Xanthine oxidase and cytochrome *c* were purchased from Boehringer, Mannheim, as was bovine catalase (crystalline suspension). Catalase activity quoted is from the manufacturer's specification. Flavin mononucleotide, ribose-5-phosphate and sodium ascorbate were purchased from Sigma.

RESULTS

Table I presents the results of a standard measurement of the proportion of

TABLE I

RATES OF PHOTOSYNTHETIC OXYGEN EXCHANGE BY SHOCKED AND UNSHOCKED CHLOROPLAST PREPARATIONS

$K_3Fe(CN)_6$ (giving oxygen evolution) or FMN (giving oxygen uptake) as electron acceptors. Reaction conditions as for Fig. 1, but with DL-glyceraldehyde (10 mM) and NH_4Cl (5 mM) replacing $NaHCO_3$ and K_2HPO_4. With FMN NaN_3 (2 mM) was also present.

Expt.	Electron Acceptors	Rate of O_2 exchange, μmol (mg chl)$^{-1}$ h^{-1}		% of chloroplasts intact
		unshocked	shocked	
1.	$K_3Fe(CN)_6$ (5 mM)	141	357	60
	O_2 via FMN (20 μM)	94	243	61
2.	$K_3Fe(CN)_6$ (5 mM)	76	283	73
	O_2 via FMN (50 μM)	84	338	75

intact chloroplasts in each of two chloroplast preparations. In both cases osmotic shock increased the rate of oxygen uptake with FMN to the same extent as it increased the rate of oxygen evolution with ferricyanide, and so the 'intactness' value obtained did not depend on the electron acceptor used. This indicates that the envelope of intact chloroplasts is impermeable to FMN, and that an FMN-mediated Mehler reaction occurs only in broken chloroplasts even where the majority of chloroplasts present are intact.

FMN caused inhibition of CO_2-dependent oxygen evolution when present in illuminated suspensions containing intact chloroplasts. The dependence of this inhibition on FMN-concentration is shown in Fig. 1. The inhibitory effect of any given concentration of FMN was found to be slightly smaller in the presence of ribose-5-phosphate than in its absence. The FMN-concentration producing 50% inhibition of CO_2-dependent oxygen evolution (Fig. 1) was 1.8 μM in the presence of ribose-5-phosphate (1 mM).

This effect is also seen in the oxygen electrode traces reproduced in Fig. 2. For the chloroplast preparation used, and in the absence of FMN, ribose-5-phosphate had little effect on the linear rate of CO_2-dependent oxygen evolution which followed the usual lag-phase. This is shown in Fig. 2a. Fig. 2b shows the corresponding trace obtained in the presence of FMN (2 μM). When compared with the trace of Fig. 2a, the rate in the presence of ribose-5-phosphate has undergone 69% inhibition, and that in the absence of ribose-5-phosphate has undergone 80% inhibition. It is also seen (Fig. 2b) that in the presence of FMN the trace commenced with a short-lived consumption of oxygen.

Fig. 2c records that the presence of ascorbate (2 mM) increased the rate of the initial consumption of oxygen. Despite this effect, the final rate of oxygen evolution in the presence of ascorbate (Fig. 2c) was no lower than that observed in the absence of ascorbate (Fig. 2b). In the trace of Fig. 2d superoxide dis-

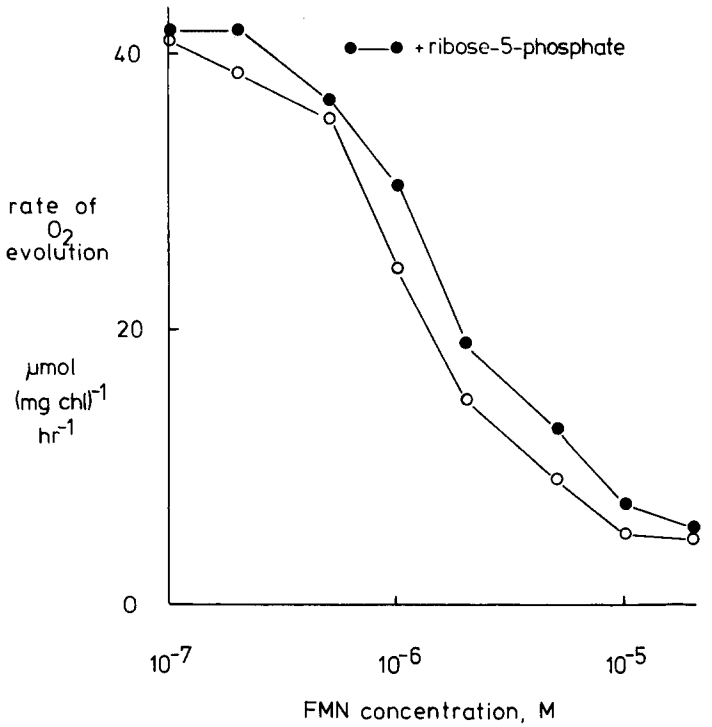

Fig. 1. Inhibition by FMN of CO_2-dependent oxygen evolution in isolated chloroplasts. The reaction mixture (at pH 7.6) contained sorbitol (0.33 M), EDTA (2 mM), $MgCl_2$ (1 mM), HEPES (50 mM), K_2HPO_4 (0.5 mM), $NaHCO_3$ (10 mM) and chloroplasts (equivalent to 50 μg chlorophyll) in a final volume of 1.00 ml. Ribose-5-phosphate (1 mM) was also present where indicated. 63% of chloroplasts were intact. The small stimulation by ribose-5-phosphate in the presence of FMN has been consistently observed in a number of other chloroplast preparations (see also Fig. 2).

mutase (10^3 units) was also present, and its effect is seen to have been to reverse the enhancement by ascorbate of the initial FMN-mediated oxygen uptake. In the presence of both ascorbate and superoxide dismutase (Fig. 2d) the inhibitory effect of FMN on steady-state oxygen evolution is to some extent relieved. Fig. 2e shows that superoxide dismutase on its own had a similar though smaller effect.

Catalase (2×10^3 units) was most effective in reversing inhibition of photosynthesis by FMN, as recorded in Fig. 2f. In the presence of catalase ribose-5-phosphate had little effect on the final rate of oxygen evolution even though FMN was still present. As shown in Fig. 2g the additional presence of ascorbate and superoxide dismutase did not add to the effect of catalase which on its own (Fig. 2f) provided most protection from inhibition of photosynthesis by FMN.

Fig. 3 shows that FMN also inhibited chloroplast CO_2-fixation and that the

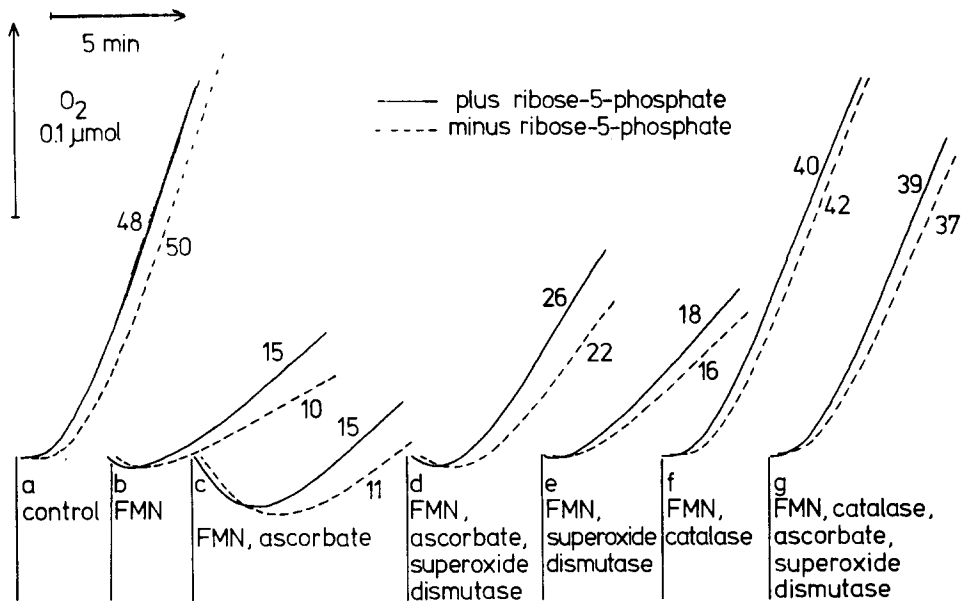

Fig. 2. Oxygen electrode traces showing CO_2-dependent oxygen evolution by isolated chloroplasts. FMN (2 µM), ascorbate (2 mM), superoxide dismutase (10^3 units) and catalase (2×10^3 units) were present where indicated. For the upper traces (continuous line) ribose-5-phosphate (1 mM) was present, for the lower traces (broken line) ribose-5-phosphate was absent. The upper and lower traces were obtained simultaneously though they are displayed with a horizontal separation equivalent to 20 s. Figures adjacent to linear parts of the traces are rates of oxygen evolution in µmol (mg chl)$^{-1}$ h^{-1}. Reaction conditions were as described for Fig. 1. 63% of chloroplasts were intact.

inhibition was reversed by catalase. Catalase by itself is seen to have had little effect on CO_2-fixation.

Table II shows the results obtained for CO_2-dependent oxygen evolution when ascorbate, superoxide dismutase and catalase are present but FMN is absent. The general lack of effect of the additions contrasts strongly with results such as those of Fig. 2. Of the four chloroplast preparations of Table II only one (No. 4) showed an appreciable response, whereby the rate of oxygen evolution was approximately doubled by addition of catalase. Such an effect of catalase in the absence of FMN has been found to be an exceptional occurrence, and is encountered only in chloroplast preparations which show photosynthetic activity of less than about 20 µmol O_2 (mg chl)$^{-1}$ h^{-1}. Although this is not apparent in Table II, it is usually associated also with a low percentage of intact chloroplasts.

DISCUSSION

The results in Table I suggest that the effect of FMN in the experiments

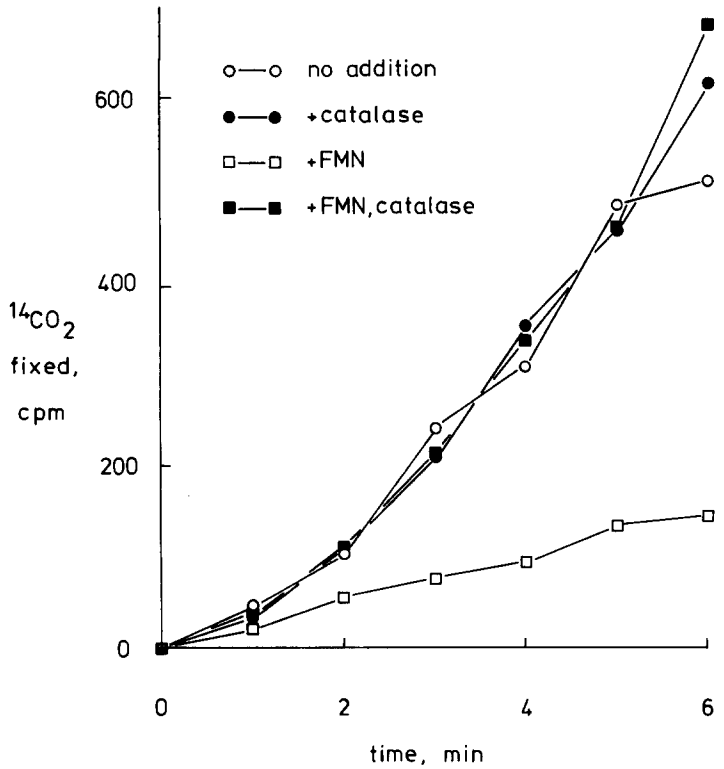

Fig. 3. Incorporation of ^{14}C into acid-stable products by isolated chloroplasts. FMN (2 μM) and catalase (2 × 10^3 units) were present where indicated. Other conditions are as for Fig. 1. 61% of chloroplasts were intact. Control rate of CO_2-dependent O_2 evolution was 26 μmol (mg chl)$^{-1}$ h^{-1}.

described here may be assumed to result from a Mehler reaction which takes place in broken chloroplasts incapable themselves of CO_2-fixation. Although the introduction of a Mehler reaction might be expected to diminish net oxygen evolution merely by superimposing a catalase-sensitive oxygen uptake on the overall reaction, the rate of oxygen uptake that is mediated by 2 μM FMN is unlikely to be greater than about 10 μmol (mg chl)$^{-1}$ h^{-1}, as seen at the beginning of the trace in Fig. 2b. The magnitude of this oxygen uptake is insufficient to account for the observed inhibition of CO_2-dependent oxygen evolution from 50 μmol (mg chl)$^{-1}$ h^{-1} (Fig. 2a) to 10 or 15 μmol (mg chl)$^{-1}$ h^{-1} (Fig. 2b). The possibility that FMN and catalase merely alter the overall stoichiometry of oxygen exchange in this system is also ruled out by the experiment of Fig. 3, which shows catalase-sensitive inhibition by FMN of CO_2-fixation itself.

Relief of inhibition by catalase indicates that H_2O_2 is the chief inhibitory agent, and that FMN is merely instrumental in its production. Inhibition of photosynthesis by H_2O_2 has previously been reported by Kaiser [12] and by Forti

TABLE II

EFFECTS OF ASCORBATE (2 mM), SUPEROXIDE DISMUTASE (10^3 UNITS) AND CATALASE (2 × 10^3 UNITS) ON CO_2-DEPENDENT OXYGEN EVOLUTION BY 4 SEPARATE CHLOROPLAST PREPARATIONS IN THE PRESENCE AND ABSENCE OF RIBOSE-5-PHOSPHATE (R5P; 1 mM).

Reaction conditions as for Fig. 1. FMN was absent throughout.

% intact chloroplasts		Rate of O_2 evolution μmol (mg chl)$^{-1}$ h^{-1}			
		no further addition	plus ascorbate	plus superoxide dismutase	plus catalase
53	+R5P	32	32	32	32
	−R5P	34	31	34	33
47	+R5P	37	37	44	38
	−R5P	39	34	40	36
65	+R5P	60	61	61	56
	−R5P	54	57	56	54
58	+R5P	12	17	15	29
	−R5p	8	12	10	22

and Gerola [13]. The smaller inhibitory effect of FMN in the presence of ribose-5-phosphate (Fig. 1) is consistent with the idea [12] that H_2O_2 inhibits CO_2-fixation by reacting with some intermediate of the reductive pentose phosphate pathway or by increasing the permeability of the chloroplast envelope to such an intermediate. In either case the chloroplast would then become depleted of substrates for CO_2-fixation. A possible alternative explanation is that the site of H_2O_2-inhibition does not lie on the shortened pathway of CO_2-fixation from ribose-5-phosphate to triose phosphate.

$O_2^{\cdot -}$ may also play some part in the inhibition. Ascorbate may enhance oxygen uptake in a Mehler reaction by virtue of its reduction of $O_2^{\cdot -}$ to H_2O_2 [8,9]. Fig. 2c shows that ascorbate does not decrease net oxygen evolution in the presence of FMN despite the fact that it increases the initial rate of oxygen comsumption. Ascorbate is also known to increase the rate of H_2O_2-production in the Mehler reaction [9]. If ascorbate is present together with superoxide dismutase (Fig. 2d) then the enhanced rate of oxygen uptake is no longer observed, the rate of H_2O_2-production may be assumed to be the same as if no ascorbate were present, and a reversal of FMN's inhibition of oxygen evolution is revealed. Superoxide dismutase in the absence of ascorbate has some protective effect of its own (Fig. 2e). This suggests that $O_2^{\cdot -}$ may resemble H_2O_2 in inhibiting CO_2-fixation, though the persistence of the effect of ribose-5-phosphate even in the presence of ascorbate and superoxide dismutase makes it possible that their mechanisms of inhibition differ. The fact that the rates of oxygen evolution with FMN and superoxide dismutase (Fig. 2e) are increased even more by the

further presence of ascorbate (Fig. 2d) argues for an additional protective effect of ascorbate which is unrelated to its reaction with $O_2^{\cdot -}$. Inhibition of photosynthesis by $O_2^{\cdot -}$ has also been suggested by Ziegler and Libera [16] who attributed a stimulation by superoxide dismutase of CO_2-fixation to the production of inhibitory $O_2^{\cdot -}$ by broken chloroplasts which were present in their preparations.

It is possible that inhibition of CO_2-fixation by $O_2^{\cdot -}$ or H_2O_2 results from diversion of newly-fixed carbon to glycolate. Glycolate synthesis caused by methyl viologen-mediated univalent reduction of oxygen in *Chromatium* has been reported [17]. It has also been suggested that a Mehler reaction provides H_2O_2 for glycolate synthesis in *Chlorella* [18]. Although in each case a transketolase reaction has been assumed to be involved, recent evidence [19] suggests that glycolate that is produced by intact chloroplasts originates instead from ribulose diphosphate.

In the present study production of H_2O_2 seemed to be the main cause of inhibition by FMN of chloroplast photosynthesis, as indicated by a substantial reversal of the inhibition by catalase. In contrast to the results of Fig. 2, Table II shows that ascorbate, superoxide dismutase and catalase usually have no effect on CO_2-dependent oxygen evolution where a mediator of the Mehler reaction has not been added. Moreover, Fig. 3 shows that under similar conditions catalase alone had no effect on fixation of $^{14}CO_2$. Ziegler and Libera [16] found an effect of superoxide dismutase on CO_2-fixation, though they attributed it to an endogenous Mehler reaction of broken chloroplasts just as the results of Fig. 2d and e are here attributed to an artificially-induced Mehler reaction. Catalase, however, has been reported to stimulate CO_2-fixation and associated oxygen evolution in the absence of a mediator of the Mehler reaction and in chloroplast preparations where a high proportion of chloroplasts are intact [11,12]. Thus the results of Table III may merely reflect a contaminant catalase activity which is high enough to prevent net production of H_2O_2 by intact chloroplasts themselves, but which is low enough to allow the additional burden of FMN-mediated production of H_2O_2 to inhibit photosynthesis appreciably.

Though chloroplast preparations of the type used here indeed contain a catalase activity of about 12 units $(mg\ chl)^{-1}$ (Allen, unpublished results) it is still possible that the simpler explanation of the non-effect of added catalase (Table II) is the correct one. This alternative view is that intact chloroplasts do not themselves release H_2O_2 under conditions optimal for CO_2-fixation. This is not necessarily to make the assumption that reduction of oxygen is absent from chloroplasts under physiological conditions. It does however require that the product of any such chloroplast oxygen reduction is H_2O rather than H_2O_2. Destruction of H_2O_2 within the chloroplast may be achieved by non-enzymic reduction of H_2O_2 by ascorbate [20] or by the action of glutathione peroxidase [21]. $O_2^{\cdot -}$ may be involved in photosynthetic oxygen reduction merely as a short-lived intermediate in reduction of oxygen by ferredoxin [22]. Any inactivation or overloading of these protective mechanisms must result in inhibition of photosynthetic CO_2-fixation.

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