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Polarographic Study of Oxaloacetate Reduction by Isolated Pea Chloroplasts

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ABSTRACT

Suspensions of pea chloroplasts, prepared by differential centrifugation, catalyzed oxaloacetate-dependent O_2 evolution (mean rate of 29 determinations 10.9 micromoles per milligram of chlorophyll per hour, SD 3.2) with the concomitant production of malate. At optimum concentrations of oxaloacetate, both reactions were light-dependent, inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and oxalate, and enhanced 2.5- to 4-fold by 10 millimolar NH_4Cl. At concentrations of oxaloacetate (<50 micromolar), 10 millimolar NH_4Cl was inhibitory. The ratio of O_2 evolved to malate produced was 0.39 to 0.58. The ratio of O_2 evolved to oxaloacetate supplied was commensurate with the theoretical value of 0.5.

Chloroplast suspensions contained both NAD- and NADP-malate dehydrogenase activities. It was concluded from oxalate inhibition studies and the promotion of oxaloacetate-dependent O_2 evolution by shocked chloroplasts by NADPH (but not NADH) that the reaction was mediated via the NADP enzyme.

Crude suspensions of isolated chloroplasts prepared by differential centrifugation exhibit OAA[1]-dependent O_2 evolution in the light (7–9, 18). This reaction is attributed to MDH activity coupled to the oxidation of a reductant produced by the light reactions (7, 9). However, with the exception of some important studies of the quantum requirements (6–8), few details are known about OAA-dependent O_2 evolution and the presumed associated synthesis of malate.

The light-coupled MDH activity of chloroplasts has been implicated as an essential component of the OAA/malate shuttle which represents one of several shuttles for the transfer of reducing equivalents from chloroplasts to the cytoplasm and, under certain conditions, *vice versa* (7, 14, 17, 23). Addition of OAA to illuminated intact chloroplasts should initiate a stoichiometric evolution of O_2 dependent on the light reactions, chloroplast-MDH, and pyridine nucleotides. According to the OAA/malate shuttle, addition of OAA to illuminated chloroplasts in the presence of a soluble extrachloroplast system to reoxidize malate exported from the chloroplasts, should stimulate continuing O_2 evolution due to recycling of OAA via chloroplast-MDH. This paper describes a study of OAA-dependent O_2 evolution (and the associated malate synthesis) by intact and osmotically shocked suspensions of crude washed chloroplasts under noncycling conditions.

In recent reviews of the OAA/malate shuttle (7, 14, 23) it is not clear whether NADH or NADPH serves to reduce OAA in the chloroplast. This uncertainty stems primarily from early reports that NAD-MDH (see Yamazaki and Tolbert [24] for references), but not NADP-MDH (9), was associated with chloroplasts prepared by differential centrifugation and that the activity of NAD-MDH associated with chloroplast suspensions prepared by this technique was sufficient to account for the coupled and uncoupled rates of OAA-dependent O_2 evolution (10). Density gradient centrifugation studies suggest that the NAD-MDH activity associated with crude suspensions of chloroplasts is associated with contaminating organelles (19, 24). The low activity of chloroplast NADP-MDH reported by Heber and Krause (9) can be attributed to activation *in vivo* by light and *in vitro* by various thiols (1, 4, 13, 21). A secondary aim of this study was to demonstrate that the OAA-dependent O_2 evolution catalyzed by crude chloroplast suspensions was mediated via NADP-MDH.

MATERIALS AND METHODS

Chemicals. LDH (in 7 M $(NH_4)_2SO_4$ and MDH (in glycerol) were obtained from Boehringer-Mannheim, West Germany. GSH reductase (in 3.6 M $(NH_4)_2SO_4$) was obtained from Sigma. All acidic substrates were adjusted to pH 7 with KOH. The concentration of solutions of OAA was ascertained immediately prior to use by reduction of NADH at 340 nm in 0.1 M Tris-HCl buffer (pH 7.6) in the presence of MDH.

Chloroplasts and O_2 evolution. Pea seedlings (*Pisum sativum* cv. Massey Gem) were raised in a growth cabinet as before (11) and harvested 13 or 14 days after imbibition. Chloroplasts were prepared by a modification of the method used previously (2) but which incorporated certain of the procedures of Nakatani and Barber (16). In summary, pea shoots (100 g) were blended in 250 ml of an icy slush of extracting medium (2) but with $MgCl_2$ at 0.2 mM. Chloroplasts were recovered by centrifugation at 2,500*g* for 30 s. A solution (50 ml) containing 0.33 M glucose, 0.1% (w/v) BSA, and 0.04 volumes of incubating medium (2) was added and gently swirled over the pellet. The washings were discarded and the pellet resuspended in 0.33 M sorbitol adjusted to pH 7.6 with Tris. After centrifuging at 2,500*g* for 20 s the supernatant solution was aspirated and the washed chloroplasts resuspended in 1 to 2 ml of incubating medium (2). Unless specified otherwise, these chloroplasts (referred to as crude washed chloroplasts) were used in all experiments. Purified chloroplasts were prepared by layering crude washed chloroplasts onto a discontinuous gradient of Ficoll 400 (30, 32, and 40%, w/v) in incubating medium and centrifuging at 5,000*g* for 7.5 min. Broken chloroplasts from the top of the gradient and purified chloroplasts from the 30/32% Ficoll interface were diluted 4-fold in incubating medium and, after centrifuging at 5,000*g* for 3 min, resuspended in incubating medium.

O_2 evolution by chloroplasts was measured polarographically at 25 C in a pair of O_2 electrodes supplied by Hansatech, Norfolk, England (model DW) and manufactured to the design of Delieu and Walker (5). Chloroplast intactness was measured by the ratio of the O_2 evolution rates of osmotically shocked and unshocked

[1] Abbreviations: OAA: oxaloacetate; NAD-MDH: NAD-specific malate dehydrogenase; NADP-MDH: NADP-specific malate dehydrogenase; LDH: lactate dehydrogenase; GSH: reduced glutathione; GSSG: oxidized glutathione; α-kG: α-ketoglutarate.

chloroplasts in the presence of 10 mM NH_4Cl using ferricyanide as electron acceptor (15). Chl was measured in ethanol as before (2).

Sonicated chloroplasts were prepared by treating intact chloroplasts, suspended in incubating medium, with a Rapidis 600 sonicator (Ultrasonics Ltd., Yorks, England) for 40 s. The soluble fraction (chloroplast extract) was recovered by centrifugation at 12,000*g* for 10 min.

Composition of Incubation Mixtures for O_2 Evolution by Illuminated Chloroplasts. Reactions were conducted in incubating medium containing 0.33 M sorbitol, 50 mM Hepes, 0.1% (w/v) BSA, 2 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ adjusted to pH 7.6 with KOH (2). Correct concentrations were maintained by addition of double strength incubating medium where necessary. Standard incubations were conducted in a volume of 0.5 ml containing 100 μg Chl/ml. DL-Glyceraldehyde (10 mM) was added to inhibit endogenous O_2 evolution (20). OAA-dependent O_2 evolution by intact chloroplasts was initiated with 0.4 to 0.8 mM OAA. The uncoupled rate was determined in the presence of 10 mM NH_4Cl.

(OAA plus NADPH)-dependent O_2 evolution was determined with osmotically shocked chloroplasts in the presence of 10 mM DL-glyceraldehyde. Intact chloroplast suspensions (1 volume) were diluted with approximately 2.5 volumes of water. NADPH (0.1 mM) was added to incubation mixtures (0.5 ml) containing shocked chloroplasts (220–300 μg Chl/ml). This effected the evolution of a small amount of O_2 which ceased within 30 s. (OAA plus NADPH)-dependent O_2 evolution was initiated by addition of OAA (0.8 mM).

Determination of Enzyme Activities in Chloroplast Extract. Unless specified otherwise, chloroplast extract was preincubated at 0 C for 40 min with 5 mM DTT and 5 mM $MgCl_2$ (13) prior to determining enzyme activities (treated extract). NAD-MDH and NADP-MDH were determined by monitoring OAA-dependent oxidation of NADH and NADPH at 340 nm at 25 C. Reaction mixtures (1 ml) contained 2 mM OAA, 0.15 mM NADH or 0.15 mM NADPH, 87.5 mM Tris-HCl (pH 7.6), and 10 μl treated extract (equivalent to approximately 8–10 μg Chl).

Two methods were used to determine transhydrogenase, and involved transhydrogenation from NADPH to NAD and NADH to NADP, respectively. For the first method, reaction mixtures (1 ml) contained 0.15 mM NADPH, 0.15 mM NAD, 2 mM pyruvate, 94 mM Tris-HCl (pH 7.6), and approximately 4 units LDH. For the second method reaction mixtures (1 ml) contained 0.15 mM NADH, 0.15 mM NADP, 0.3 mM GSSG, 90 mM Tris-HCl (pH 7.6), and approximately 5 units GSH reductase. For both methods, reactions were initiated with 10 μl chloroplast extract and monitored at 340 nm at 25 C and activity corrected for controls lacking chloroplast extract.

Determination of Malate. Samples (0.35 ml) from chloroplast incubation mixtures were treated with 0.117 ml 6% (w/v) $HClO_4$ and adjusted to pH 7 with KOH. Insoluble material was removed by centrifugation and malate in the supernatant solution was determined by the method of Hohorst (10).

RESULTS

Properties of Chloroplasts. The mean uncoupled rates of O_2 evolution for crude washed chloroplasts from cv. Massey Gem for ferricyanide and OAA were 205 (SD 30) and 37.2 (SD 8.6) μmol/mg Chl·h, respectively. The coupled rates for OAA and nitrite were 10.9 (SD 3.2) and 8.8 (SD 1.9) μmol/mg Chl·h, respectively, and the mean intactness 79% (SD 8). The rates are similar to those determined for cv. Massey Gem by the method used previously (11) but in general are 50 to 60% less than those determined for chloroplasts from cv. Feltham First (2, 3). However, the intactness was appreciably greater than that determined by the previous method for either cv.

Some Properties of OAA-dependent O_2 Evolution by Intact Chloroplasts. OAA-dependent O_2 evolution did not proceed in the dark and was inhibited (100%) by 1.7 μM DCMU. In the presence of 0.8 mM OAA the rate was constant for approximately 3 to 5 min and thereafter gradually decreased. The initial rate increased with the concentration of OAA up to approximately 50 μM (Fig. 1A); $V_{max}/2$ was estimated at approximately 15 μM. The rate was essentially independent of OAA concentration between 0.5 and 2.5 mM but higher concentrations were slightly inhibitory (*e.g.* 16% inhibition at 4.5 mM). When small amounts of OAA were supplied (up to 200 nmol/ml), O_2 evolution commenced in the usual way but ceased abruptly after a few minutes. Further additions of OAA reinitiated O_2 evolution (Fig. 2A) implying that the reaction had come to equilibrium after cessation of O_2 evolution. For the first addition of OAA, the ratio of O_2 evolved to OAA supplied approximated the theoretical value of 0.5 for the reduction of OAA to malate but both the ratio and the rate decreased with each successive addition of OAA (Fig. 2A). The abrupt cessation of O_2 evolution after each addition of OAA is in agreement with the high affinity of the reaction for OAA (Fig. 1A). Intact chloroplasts commenced O_2 evolution at the maximum rate within 5 s of supplying OAA.

Neither malate (10–40 mM) nor the transaminase inhibitor amino oxyacetate (5 mM) significantly affected the rate of OAA-dependent O_2 evolution; amino oxyacetate was also without significant effect on the O_2 to OAA ratio. However, oxalate inhibited O_2 evolution by intact chloroplasts (Fig. 3 C); the concentration required to effect 50% inhibition was approximately 4 to 5 mM. Chloroplasts did not catalyze O_2 evolution when OAA was replaced with α-kG or pyruvate (2 mM) nor did these compounds affect the rate of OAA-dependent O_2 evolution.

The effect of NH_4Cl on OAA-dependent O_2 evolution varied with the concentration of OAA (Fig. 1B) and NH_4Cl (Fig. 4). In the presence of 0.3 to 2 mM OAA, the rate increased 2.5- to 4-fold with NH_4Cl concentrations up to approximately 2 mM; higher concentrations (up to 20 mM) were not inhibitory. At lower

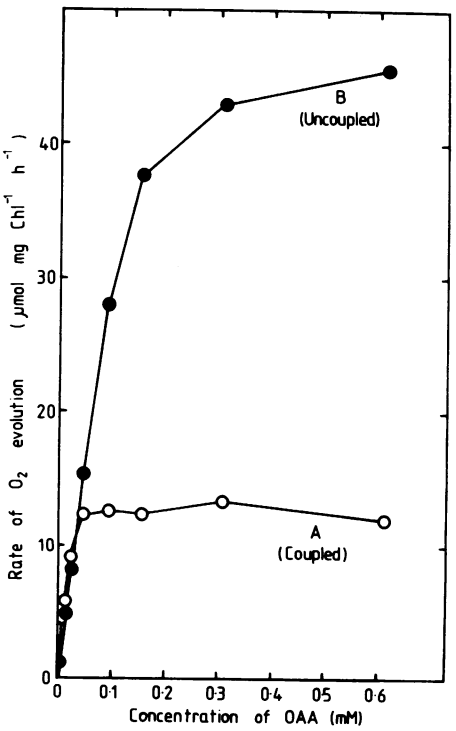

FIG. 1. Effect of concentration of OAA on the rate of O_2 evolution by illuminated intact chloroplasts in (A) the absence and (B) the presence of 10 mM NH_4Cl. Chloroplast intactness: 76%; Chl concentraton: 100 μg/ml.

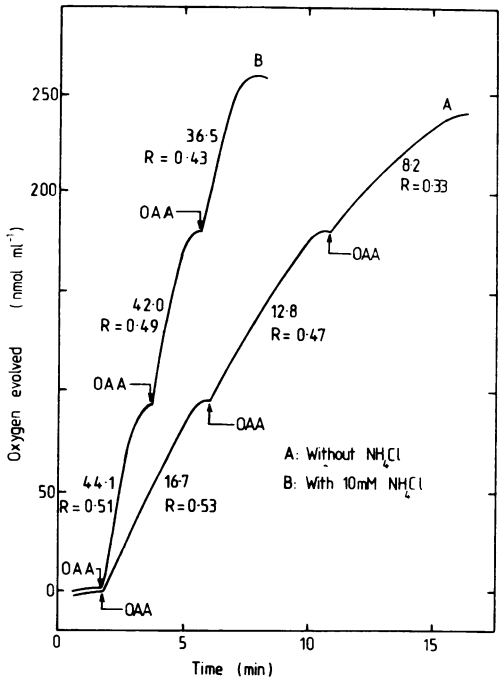

FIG. 2. Effect of limiting amounts of OAA on O_2 evolution by illuminated intact chloroplasts in (A) the absence and (B) the presence of 10 mM NH_4Cl. Reactions were timed from the moment of illumination and OAA (179 nmol/ml) was added as shown. Values beside the curves represent rates of O_2 evolution in μmol/mg Chl·h. Molar ratios of O_2 evolved to OAA supplied (R) for each successive addition of OAA are also shown. Chloroplast intactness: 75%; Chl concentration: 100 μg/ml.

concentrations of OAA, however, the enhancement effect was proportionately less and at concentrations less than 50 μM OAA, NH_4Cl (10 mM) was slightly inhibitory (Fig. 1). NH_4Cl (10 mM) did not significantly affect the O_2 to OAA ratio when OAA was supplied at 0.179 mM but the decrease in the ratio and rate of O_2 evolution for subsequent additions of OAA (Fig. 2B) was relatively much less than for reaction mixtures lacking NH_4Cl.

Light-dependent Synthesis of Malate from OAA by Intact Chloroplasts. OAA-dependent O_2 evolution was accompanied by the synthesis of malate. The ratio for O_2 evolved to malate synthesized by coupled chloroplasts was 0.39 to 0.58 (Table I). Malate synthesis was light-dependent, inhibited by 1.7 μM DCMU and 61 mM oxalate, and did not occur in the absence of OAA. NH_4Cl (10 mM) enhanced the rate of malate synthesis approximately 2-fold.

Enzyme Activities of Sonicated Chloroplasts. The activities of several enzymes associated with the soluble fraction prepared from sonicated chloroplasts were compared with the coupled and uncoupled rates of OAA-dependent O_2 evolution. The rates of NAD-MDH, after correcting for the appropriate stoichiometry, were sufficient to account for the uncoupled rates of OAA-dependent O_2 evolution (Table II). The NADP-MDH activity of untreated chloroplast extract was very low but when the extract was pretreated with 5 mM DTT and 5 mM $MgCl_2$ at 0 C for 40 min, enzyme activity was enhanced approximately 25- to 50-fold. The activity was sufficient to account for the coupled rate of O_2 evolution but in most experiments the activity did not fully account for the uncoupled rate (Table II). Transhydrogenase activity (NADH to NADP and NADPH to NAD) was not detected in treated chloroplast extracts using the glutathione reductase or LDH assays.

Both the NAD-MDH and NADP-MDH activities of treated chloroplast extract were inhibited by oxalate (Fig. 3, A and B); NADP-MDH (50% inhibition at approximately 4 to 5 mM) was more sensitive than NAD-MDH (50% inhibition at approximately 45 to 50 mM). NH_4Cl (10 mM) had no significant effect on the NAD-MDH and NADP-MDH activities of treated extract as determined by the spectrophotometric assay.

Properties of Purified Chloroplasts. The initial rate of OAA-

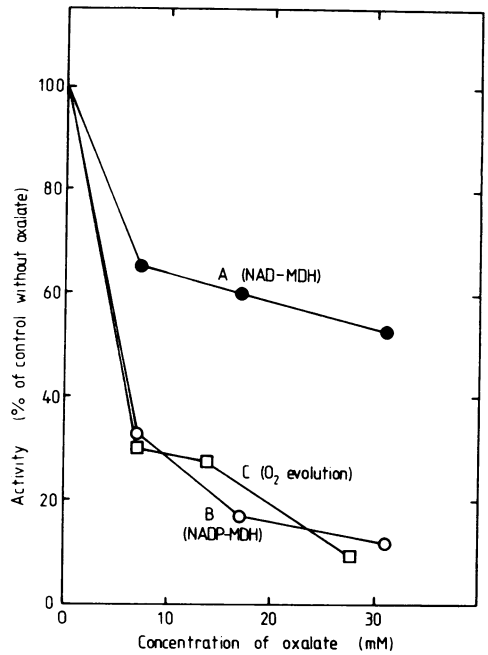

FIG. 3. Effect of concentration of oxalate on (A) NAD-MDH and (B) NADP-MDH activity of chloroplast extract and (C) uncoupled rate of OAA-dependent O_2 evolution by illuminated intact chloroplasts. Chloroplast extract (from 77% intact chloroplasts) was preincubated with 5 mM DTT and 5 mM $MgCl_2$ at 0 C for 40 min prior to determining the enzyme activities (A and B). The activities of NAD-MDH and NADP-MDH in the absence of oxalate were 209 and 52 μmol NAD(P)H oxidized/mg Chl·h, respectively. For O_2 evolution, chloroplasts (72% intact) were preincubated in the light at 25 C for 5 min in the presence of 10 mM NH_4Cl and oxalate before initiating the reaction with 0.8 mM OAA. The uncoupled rate of OAA-dependent O_2 evolution in the absence of oxalate was 19.0 μmol/mg Chl·h. All results are expressed as a percentage of the rates of appropriate controls lacking oxalate.

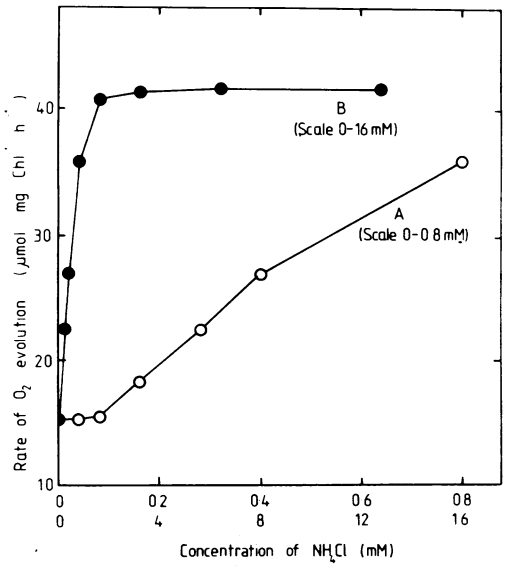

FIG. 4. Effect of concentration of NH_4Cl on OAA-dependent O_2 evolution by illuminated intact chloroplasts. Reaction mixtures contained 100 μg Chl/ml (chloroplast intactness 67%) and O_2 evolution was initiated with 0.8 mM OAA. A: scale 0 to 0.8 mM; B: scale 0 to 16 mM.

Table I. *OAA-dependent Malate Synthesis and O_2 Evolution by Illuminated Intact Chloroplasts*

Reaction mixtures contained 100 μg Chl/ml and were initiated with 0.8 to 0.96 mM OAA. Rates for O_2 evolution represent average values for the time periods specified.

Experiment No.	Chloroplast Intactness	Additions	Time Sampled	Malate Synthesized	O_2 Evolved	Ratio O_2 Evolved to Malate Synthesized
	%		min	μmol/mg Chl·h		
1	71	Nil	8	20.3	11.8	0.58
		NH_4Cl (10 mM)	4	43.0	24.5	0.57
		DCMU (3.3 μM)	9	2.3	0.9	(0.39)
		Oxalate (61 mM)	8	7.6	0	
		Dark	8	1.4	0	
2	88	Nil	16	10.2	4.5	0.44
		NH_4Cl (10 mM)	7.2	23.1	9.1	0.39
3	79	Nil	14	14.4	7.0	0.49
		Dark	14	1.1	0	

Table II. *NAD-MDH and NADP-MDH Activities Associated with Chloroplast Extracts in Relation to Coupled and Uncoupled Rates of OAA-dependent O_2 Evolution of Intact Chloroplasts*

Chloroplast extracts were prepared from sonicated chloroplasts and O_2 evolution was measured under standard conditions. Extracts were pretreated with 5 mM DTT and 5 mM $MgCl_2$ at 0 C for 40 min where indicated. Values in parentheses in columns 3 and 4 represent the rate of OAA reduction as calculated from the observed rate of O_2 evolution (theoretical stoichiometry 2.0).

Intact Chloroplasts				Chloroplast Extract		
Experiment No.	Intactness	O_2 evolution		Pretreatment	Enzyme activities	
		Without NH_4Cl	With 10 mM NH_4Cl		NAD-MDH	NADP-MDH
	%	μmol/mg Chl·h			μmol/mg Chl·h	
1	84	13.1 (26.2)	40.2 (80.4)	Nil	271	1
				DTT + $MgCl_2$	283	49
2	86	9.6 (19.2)	35.3 (70.6)	DTT + $MgCl_2$	283	105
3	77	9.2 (18.4)	38.9 (77.8)	Nil	217	0
				DTT + $MgCl_2$	209	46
4	69	14.7 (29.4)	41.2 (82.4)	Nil	270	2.3
				DTT + $MgCl_2$	272	60

dependent O_2 evolution varied from 0 to 17.7 μmol/mg Chl·h and, when active, decreased to zero after 5 to 7 min. NH_4Cl did not enhance OAA-dependent O_2 evolution. Relative to crude chloroplasts, purification enhanced the NADP-MDH to NAD-MDH ratio up to 4-fold but the activity of either enzyme was invariably sufficient to account for the observed OAA-dependent O_2 evolution. Broken chloroplasts, which also contained relatively high levels of NADP-MDH and NAD-MDH, did not catalyze OAA-dependent O_2 evolution.

(OAA plus NADPH)-dependent O_2 Evolution by Osmotically Shocked Chloroplasts. Partially shocked chloroplast suspensions were prepared by diluting crude chloroplast suspensions in approximately 2.5 volumes of water. This procedure, which decreased the intactness to 15 to 20%, was adopted to obtain a system which, in the absence of any additions, did not catalyze high rates of O_2 consumption in the light. Completely shocked or sonicated chloroplasts (0% intact) catalyzed high rates of O_2 consumption (up to 12 μmol/mg Chl·h). (OAA plus NADPH)-dependent O_2 evolution was not detected at less than 220 μg Chl/ml.

Partially shocked chloroplasts failed to catalyze OAA-dependent O_2 evolution but when NADPH (0.02–0.2 mM) was supplied O_2 evolution commenced immediately. The rate decreased during the first 30 s but then stabilized at 2.5 to 4.1 μmol/mg Chl·h for a period of 3 to 7 min before gradually decreasing to zero (Fig. 5A). NADH would not replace NADPH in this reaction (Fig. 5C).

In the absence of OAA, NADPH initiated O_2 evolution which ceased within 30 s (Fig. 5B). The amount of O_2 evolved varied with the amount of NADPH supplied (Table III, column 2). Subsequent addition of OAA effected an immediate resumption of O_2 evolution at rates of 1.9 to 5.4 μmol/mg Chl·h. These rates were sustained for 3 to 7 min, the time varying with the amount of NADPH supplied (Table III, column 4), before the rate declined. The initial rates of O_2 evolution were not highly dependent on the concentration of NADPH although 0.06 mM was optimal (Table III). Except at 0.2 mM NADPH, the amount of OAA-dependent O_2 evolution exceeded the amount of NADPH supplied (Table III, column 6), thus demonstrating that NADPH served a catalytic function. The results in Figure 5C and Table III demonstrate that the high but rapidly declining rate of O_2 evolution

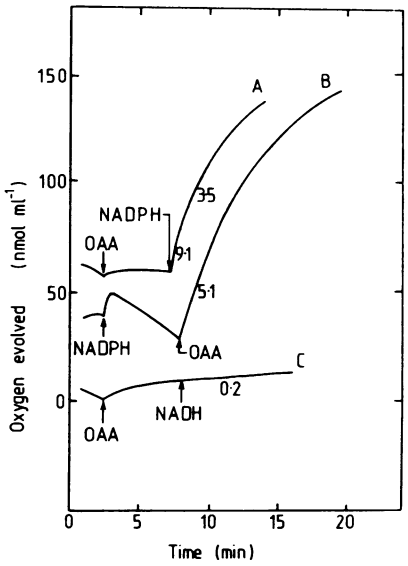

FIG. 5. Requirement for NADPH and OAA to effect O_2 evolution by partially shocked chloroplasts. Chloroplasts (80% intact) were shocked as described under "Materials and Methods" and incubated in the light in the presence of 10 mM DL-glyceraldehyde. Reaction mixtures (A–C) contained 233 μg Chl/ml and additions were made as shown; OAA was supplied at 0.8 mM and NADPH and NADH at 0.1 mM. Values beside the curves represent the rate of O_2 evolution in μmol/mg Chl·h.

Table III. *Effect of Concentration of NADPH on OAA-dependent O_2 Evolution by Partially Shocked Chloroplasts*

Chloroplasts (71% intact) were partially shocked as described under "Materials and Methods" and incubated with 10 mM DL-glyceraldehyde and NADPH at the concentrations shown. A small amount of O_2 was evolved during this period (column 2) but ceased after approximately 0.5 min. After a further 0.5 min, O_2 evolution was initiated by addition of 0.8 mM OAA. The initial rate of O_2 evolution (column 3) and the total amount of O_2 evolved at the time the reaction ceased (column 5) were recorded. All reactions were conducted in the light and contained 224 μg Chl/ml. A progress curve for a similar experiment is shown in Figure 5B.

Concentration of NADPH	O_2 Evolved Prior to Adding OAA	Initial Rate OAA-dependent O_2 Evolution	Time Initial O_2 Evolution Rate Sustained	Total OAA-dependent O_2 Evolution at Cessation of Reaction	Ratio OAA-dependent O_2 Evolution to NADPH Supplied
nmol/ml		μmol/mg Chl·h	min	nmol/ml	
0	0	0	0	0	
20	0	3.3	3	97	4.9
60	1.9	4.1	3	103	1.7
80	3.2	2.9	7	105	1.3
100	3.8	2.8	6	127	1.3
200	9.5	2.5	7	95	0.5

observed after addition of NADPH in Figure 5A was due to a short but rapid NADPH-dependent O_2 evolution (presumably due to an endogenous substrate [s]) in addition to the more sustained rate of OAA plus NADPH-dependent O_2 evolution. When OAA was supplied to partially shocked chloroplasts which had been preincubated in the light for various times, the rates of OAA-dependent O_2 evolution increased with the duration of the preincubation period (Table IV).

DISCUSSION

OAA-dependent O_2 evolution in the light was attributed to photosynthetically coupled NADP-MDH activity.

$$H_2O + NADP^+ \xrightarrow{hv} NADPH + H^+ + \tfrac{1}{2}O_2 \quad (I)$$

$$NADPH + H^+ + OAA \rightarrow NADP^+ + Malate \quad (II)$$

$$H_2O + OAA \xrightarrow{hv} \tfrac{1}{2}O_2 + Malate \quad (III)$$

The ratio of O_2 evolved to OAA consumed as determined by OAA-limiting experiments (Fig. 2A) and the ratio of O_2 evolved to malate produced (Table I) were in approximate agreement with the theoretical stoichiometry (reaction III). Like OAA-dependent O_2 evolution, the associated production of malate was light-dependent, inhibited by DCMU, and stimulated by 2 to 20 mM NH_4Cl (Table I). The value of 15 μM for $V_{max}/2$ for coupled OAA-dependent O_2 evolution is in approximate agreement with the K_m (apparent) for thiol-activated NADP-MDH from maize (25 μM) and spinach (30 μM) chloroplasts (13, 21). It is somewhat less than the value of 50 μM for the K_m (apparent) of the OAA translocator of spinach chloroplasts (7). This suggests that although the OAA translocator is operating at a smaller proportion of its maximum potential rate than NADP-MDH, it nevertheless does not affect the kinetics of the over-all reaction as judged by O_2 evolution. The coupled and uncoupled rates of OAA-dependent O_2 evolution (Table I) are commensurate with other values for pea chloroplasts (2, 18) but less than those reported for spinach (7, 8). The enhanced rate of O_2 and malate synthesis by 2 to 20 mM NH_4Cl at high concentrations of OAA (0.5–2.5 mM), implies that photophosphorylation is not required for the reaction *per se*.

Since purified chloroplasts are reported to contain NADP-MDH (21) but not NAD-MDH (19, 24), NADP-MDH is most probably the reductant in reaction II. Crude washed chloroplasts, which are effective in OAA-dependent O_2 evolution, contain presumably contaminating NAD-MDH (19, 24) at sufficient levels to account for the observed rates of OAA-dependent O_2 evolution whereas the NADP-MDH activity was not always sufficient (Table III). Rapid purification of chloroplasts on a discontinuous Ficoll gradient enhanced the ratio of NADP-MDH to NAD-MDH but purified chloroplasts did not consistently support OAA-dependent O_2 evolution and any activity could be accounted for by either enzyme. The relatively low levels of NAD-MDH associated with intact spinach chloroplasts purified by sucrose density gradient centrifugation, attributed to contamination (24), are also sufficient to account for the OAA-dependent O_2 evolution by crude pea chloroplasts. It was therefore necessary to seek other evidence to establish which enzyme was involved in OAA-dependent O_2 evolution in crude pea chloroplast preparations. Participation of NAD-MDH was made unlikely or ruled out by a series of observations. Addition of NADPH, but not of NADH, effected continuing OAA-dependent O_2 evolution by shocked chloroplasts (Table III and Fig. 5A). The kinetic data of oxalate inhibition of OAA-dependent O_2 evolution and NADP-MDH, but not NAD-MDH were similar (Fig. 3). The enhanced initial rates of OAA-dependent O_2 evolution by shocked chloroplasts with added NADPH following incubation in the light (Table IV) is consistent with the light activation of NADP-MDH of chloroplasts (1, 13). The absence of transhydrogenase activity in the chloroplasts demonstrates that transfer of a reducing equivalent from light-generated NADPH to NAD is not involved in OAA-dependent O_2 evolution. We presume that the discrepancy between the uncoupled rate of OAA-dependent O_2 evolution by intact chloroplasts and NADP-MDH of chloroplast extract (Table II) was due to incomplete extraction of the enzyme, incomplete activation of the enzyme by DTT and/or nonoptimum conditions for determining NADP-MDH activity.

As judged by spectrophotometric assay of chloroplast extracts prepared from chloroplasts following a period of illumination, complete activation of NADP-MDH is achieved in 60 s (1). We invariably observed commencement of OAA-dependent O_2 evolution at maximum rates within 1 to 5 s of supplying OAA to intact chloroplasts, irrespective of the period of preillumination. This method of monitoring NADP-MDH in intact chloroplasts, which does not require time to prepare extracts for estimating activity in secondary incubations, suggests that complete activation was achieved within 5 s. The relatively long preillumination periods required to effect OAA-dependent O_2 evolution at maximum rates of shocked chloroplasts suggest that the concentration of a membrane-bound or soluble factor(s) (1, 4, 12, 22) is rate-limiting in effecting light modulation of NADP-MDH in this system.

Table IV. *Effect of Preillumination of Partially Shocked Chloroplasts on OAA-dependent O_2 Evolution*

Chloroplasts were partially shocked as described under "Materials and Methods" and preincubated in the light for the times shown in the presence of 10 mM DL-glyceraldehyde and NADPH. O_2 evolution was initiated at the end of the preillumination period by addition of OAA. The chloroplast preparations prior to osmotic shock for experiments 1 and 2 were 72 and 84% intact, respectively. Reaction mixtures for both experiments contained 300 μg Chl/ml. For experiments 1 and 2 the concentrations of NADPH were 0.1 and 0.2 mM, respectively. For OAA, the corresponding values were 0.8 and 0.32 mM.

Experiment No.	Period of Preillumination	Initial Rate of OAA-dependent O_2 Evolution
	min	*μmol/mg Chl·h*
1	4	2.6
	7	3.5
	10	5.1
	13	5.4
2	4	1.9
	7	2.3
	12	3.2
	20	3.4

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