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THE REDOX STATE OF THE NADP SYSTEM IN ILLUMINATED CHLOROPLASTS

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Pyridine nucleotide levels were measured in intact spinach chloroplasts. The NADPH/NADP ratio was close to unity in darkened chloroplasts. On illumination, chloroplast NADP levels decreased rapidly. The decrease was more prominent at low than at high light intensities. In the presence of bicarbonate, NADP subsequently increased to reach a steady-state level. The kinetics of the increase were related in general, but not in detail, to the lag phase of photosynthesis. In the steady state, chloroplast NADP was sometimes, particularly during photosynthesis at high light intensities, less reduced in the light than in the dark. In the dark-light transition, phosphoglycerate reduction is driven by increases in the ratios NADPH/NADP and ATP/ADP. When photosynthesis accelerates after the initial lag phase, the NADPH/NADP ratio decreases and a high ratio of phosphoglycerate to triose phosphate becomes an important factor in driving carbon reduction. Under photosynthetic flux conditions, the redox state of the chloroplast NADP system appeared to be governed largely by the chloroplast ratio of phosphoglycerate to dihydroxyacetone phosphate and by the phosphorylation potential [ATP]/[ADP] [P_i]. The inhibitor of cyclic electron transport, antimycin A, increased reduction of the chloroplast NADP system. Even when reduction was almost complete in the presence of 5 μM antimycin A, photosynthesis was still significant at low light intensities. Electrons appeared to be effectively distributed between the cyclic electron-transport pathway and the noncyclic route to NADP at NADPH/NADP ratios as low as about 1. When bicarbonate was absent, the NADP system remained largely reduced in the light. The energy-transfer inhibitor, Dio-9, and uncouplers and agents which interfered with pH regulation of the Calvin cycle increased reduction of the NADP system while decreasing photosynthesis.

Introduction

Photosynthetic assimilation of CO_2 to sucrose or starch in plants using the Calvin cycle only (C_3 plants) requires ATP and NADPH at a ratio somewhat higher than 1.5. Isolated chloroplasts from spinach, a C_3 plant, demonstrate metabolic flexibility by being able to photoreduce not only CO_2, but also a number of other electron acceptors including glycerate [1]. Formation of phosphoglycerate and dihydroxyacetone phosphate from glycerate requires more than 2 mol of ATP/mol NADPH oxidized. In C_4 plants, somewhat more than 2.5 mol ATP/mol NADPH are needed for photosynthesis, and there are indications that in mesophyll cells of aspartate-forming C_4 species this ratio is still considerably higher [2].

Most data available on the coupling of electron flow to ATP synthesis indicate that reduction of NADP during oxidation of water provides insufficient ATP for carbon reduction. Additional ATP is believed to be synthesized by electron flow from water to oxygen (pseudocyclic electron transport) and by cyclic electron transport [3–8]. It has recently been shown that less oxygen is reduced and less H_2O_2 formed by intact chloroplasts when phosphoglycerate serves as electron acceptor instead of CO_2 [9]. Phosphoglycerate reduction requires less ATP than does CO_2 reduction. These findings and others pertaining

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Chl, chlorophyll.

to cyclic electron flow suggest that CO_2 reduction requires additional electron transport that is not needed for reduction of phosphoglycerate.

There is the question of how electron transport is regulated so as to satisfy the ATP requirement of photosynthesis with the least expenditure of light quanta. A mechanism has been proposed [3] which is based on the high affinity of the terminal component of the electron-transport chain for NADP. When the NADP pool of chloroplasts is depleted during illumination because NADPH cannot readily be oxidized for lack of ATP, electrons are supposed to be diverted to oxygen and into the cyclic pathway. Since in both cases phosphorylation accompanies electron transport, ATP becomes available for phosphorylation of carbon cycle intermediates thus permitting NADPH oxidation. Electrons are then diverted again to NADP. In this view, the redox state of NADP determines whether or not pseudocyclic and cyclic electron transport can occur.

It is not known within which range of redox states electrons are switched away from and back to NADP. Ferredoxin which is believed to distribute electrons to NADP, to oxygen and into the cyclic pathway has a redox potential 100 mV below that of NADP. At equilibrium between ferredoxin and NADP, ferredoxin is not much more than 1% reduced when NADP is 50% reduced.

In the present study, we have employed intact spinach chloroplasts which are capable of high rates of CO_2 assimilation to measure NADP levels and obtain information on the redox state of NADP during photosynthesis.

Material and Methods

Spinach was grown in a greenhouse under controlled light (12 h light and 12 h dark). Leaves were collected 2–3 h after the start of the light period. Intact chloroplasts were isolated from spinach leaves as described previously [10]. The isolated chloroplasts were suspended in a medium containing 330 mM sorbitol, 10 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM KH_2PO_4, 2 mM EDTA and 50 mM Hepes (pH 6.7). CO_2-dependent oxygen evolution was measured by a Clark-type oxygen electrode under illumination with red light (600–800 nm) at 20°C. The reaction mixture resembled the medium used for suspending the chloroplasts except that the pH was 7.6. It contained also 2 mM $NaHCO_3$ and 4 μg catalase/ml. The chlorophyll content of the samples was 35–50 μg/ml.

To determine levels of NADP and NAD, chloroplasts were illuminated in the same medium (2 ml) and the same vessel which were used to measure oxygen evolution. The reaction was terminated by adding $HClO_4$ to the chloroplasts during illumination or in the dark. The final $HClO_4$ concentration was 0.5 M. The mixture was kept at 20°C for 5 min to decompose NADPH and NADH and was then transferred to an ice bath. After centrifugation, triethanolamine (60 mM) was added to the supernatant, and the pH was adjusted to 7.6 by adding saturated K_2CO_3 solution. The neutralized solution was used to determine the amount of NADP and NAD by enzymic cycling [11]. The reaction mixture contained 33 mM Tris, 0.1 mM 2,6-dichlorophenolindophenol, 60 μM phenazine methosulfate, 2 mM EDTA and 10 μg glucose-6-phosphate dehydrogenase (per 3 ml) plus 3.3 mM glucose 6-phosphate for the determination of NADP, or 90 mM ethanol and 60 μg alcohol dehydrogenase (per 3 ml) for the determination of NAD (pH 7.5). Reactions were started by adding the enzymes. Dark levels of pyridine nucleotides were determined in extracts from chloroplasts which had been kept in the dark for 3–4 min at 20°C. The total amount of NADP plus NADPH was determined after oxidizing intrachloroplast NADPH by adding phenazine methosulfate (60 μM) to a chloroplast suspension in the dark. The levels of NADP and NADPH outside the chloroplasts were determined in the supernatant after removing chloroplasts by centrifugation. The amount of NADP and NAD was calculated by adding a known amount of the nucleotide to each reaction vessel. All data presented here were corrected for NADP and NADPH outside the chloroplasts. The percentage of extrachloroplast pyridine nucleotides varied in different experiments. Often, about 25% of measured NADP + NADPH was found outside the chloroplasts.

Results

Redox level of NADP in intact chloroplasts in the dark

Between 7 and 11 nmol NADP/mg Chl were found in darkened chloroplasts after extraction with $HClO_4$

which destroys NADPH. This corresponds to a stromal NADP concentration of about 0.4 mM. Attempts to measure NADPH after alkaline extraction which destroys NADP yielded variable and, in general, unreliable results. The reason for this is unknown. It had not been difficult to extract NADPH from freeze-dried chloroplasts by KOH [12]. Total pools of NADP plus NADPH were measured after adding phenazine methosulfate to a chloroplast suspension and extracting NADP subsequently with $HClO_4$. Phenazine methosulfate oxidizes NADPH to NADP nonenzymically. Total pools of NADPH plus NADP were between 14 and 24 nmol/mg Chl. The difference in the value before and after addition of phenazine methosulfate revealed the content of NADPH. The data showed that between 40 and 60% of the chloroplast NADP was reduced in the dark. This considerable reduction which is larger than that reported by Lendzian and Bassham [13] reflects the states of the chloroplast phosphoglycerate/glyceraldehyde phosphate system and of the glucose 6-phosphate/6-phosphogluconate system in the dark.

NADP levels during the lag phase of photosynthesis

Fig. 1 shows changes of the NADP level during 10 s illumination with low-intensity (14 W/m^2) and high-intensity (600 W/m^2) red light. The latter is sufficient to saturate photosynthetic oxygen evolution. Under low-intensity illumination, cyclic electron transfer has been suggested to be insignificant compared to that under high-intensity illumination [3]. After the red light was turned on, NADP was reduced rapidly and the level of NADP reached after 1 s illumination remained steady during the following 10 s at both light intensities. More NADP was reduced by the low-intensity than by the high-intensity illumination (about 85% vs. 75% of [NADP + NADPH]). To elucidate the reason why the extent of photoreduction was larger at the lower light intensity, we investigated the effect of antimycin A on the level of NADP. When added to chloroplasts which were illuminated at a high intensity, antimycin A (5 μM) decreased the level of NADP to that attained at low intensity (data not shown). The reagent did not affect greatly the level of NADP in chloroplasts illuminated at a low intensity. These results suggest that cyclic electron transfer is a factor in determining the redox level of NADP in the light, and that cyclic electron transfer does not contribute much to electron transport during the first seconds of illumination at a low intensity.

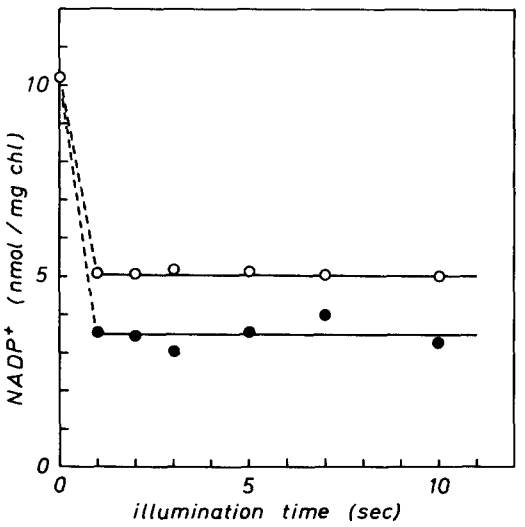

Fig. 1. The NADP content of intact chloroplasts during a 10 s illumination period. [NADP + NADPH] was 21.7 nmol/mg Chl. (○) 600 W/m^2 red light, (●) 14 W/m^2 red light.

The reduction state of NADP attained in the short period of illumination (Fig. 1) was retained for 10–60 s depending on the chloroplast preparation and light intensity (cf. Figs. 3, 6–8). Subsequently, the NADP level increased gradually (Fig. 2). This increase was already observed during the lag phase of photosynthesis. In the presence of antimycin A, the slow increase was suppressed, and the NADP level decreased gradually during illumination at 14 W/m^2 to reach almost full reduction of NADP. It should be noted that the high concentration of 5 μM antimycin A decreased photosynthesis by only one-third (Fig. 2).

Redox state of NADP during the induction phase and in the steady state of photosynthesis

Fig. 3 shows the time course of changes in chloroplast NADP during photosynthesis in the presence of 2 mM $NaHCO_3$ and in a subsequent dark period. On illumination with high-intensity red light (600 W/m^2), the NADP level in intact chloroplasts first decreased to reach 70–75% reduction of the NADP system as described for the first 10 s of illumination in Fig. 1. During the lag phase of photosynthesis, and also

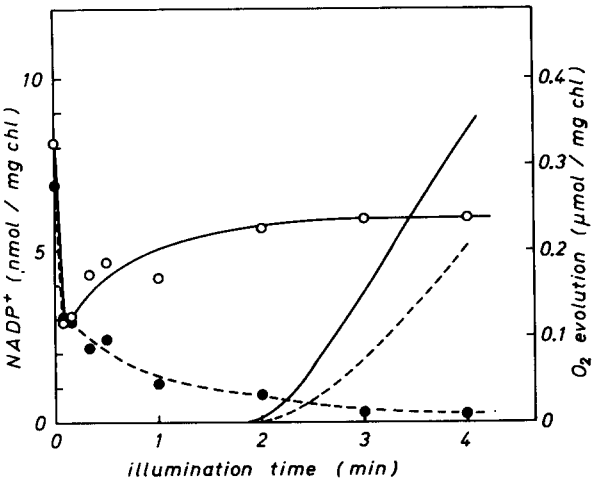

Fig. 2. Effect of antimycin A on the level of NADP in intact chloroplasts during illumination with low-intensity (14 W/m^2) red light. (○) No addition, (●) 5 μM antimycin A. Oxygen electrode traces of oxygen evolution in the presence (------) and absence (———) of antimycin A (5 μM) are also shown. 5 μM antimycin A inhibited photosynthesis by about one-third.

when photosynthesis accelerated, the NADP level increased and after 3 min illumination was higher than that before in the dark. The rate of photosynthesis in this experiment after 3 min illumination was 185 μmol CO_2 reduced/mg Chl per h. When the light was turned off, the NADP level first increased rapidly and then decreased slowly to the previous dark redox level.

Since it has been reported that NAD can be phosphorylated to NADP in the light [14–16], it appeared possible to attribute the kinetics of the chloroplast NADP level as shown particularly in Figs. 2 and 3 not only to changes in the redox state of NADP, but also to light-dependent changes in the size of the total pool of NADP plus NADPH. We therefore measured NAD in intact chloroplasts. The experiment of Fig. 4 reveals neither much reduction in the pool size of chloroplast NAD in the light nor an increase in the dark. It is known that only a small percentage of chloroplast NAD is reduced during illumination [12]. Measurements of chloroplast NADP levels after addition of phenazine methosulfate to oxidize NADPH (not shown) indicated that the total pool size of NADP plus NADPH remained essentially constant during illumination and in the dark. Therefore, light-dependent changes in the total pool size of the chloroplast NADP system (cf. Refs. 14–16) cannot be held responsible for the observed changes in chloroplast NADP levels during illumination (Figs. 2 and 3). Rather, the changes reflect changes in the redox state of NADP occurring during illumination. Since photosynthesis was light saturated in the experiment of Fig. 3, the observation was un-

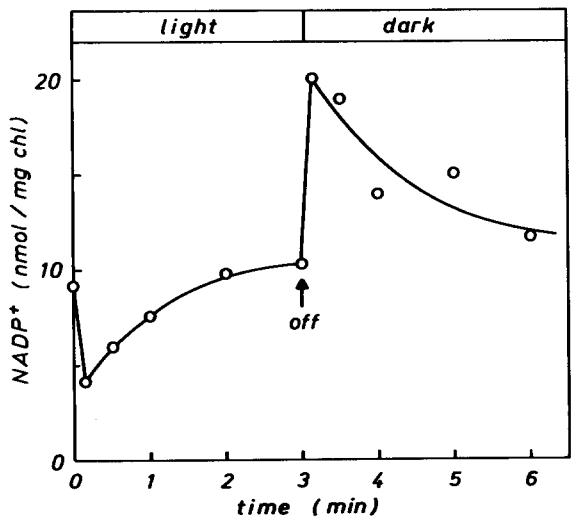

Fig. 3. Light/dark and dark/light transients in the level of NADP in intact chloroplasts. The intensity of red light was 600 W/m^2.

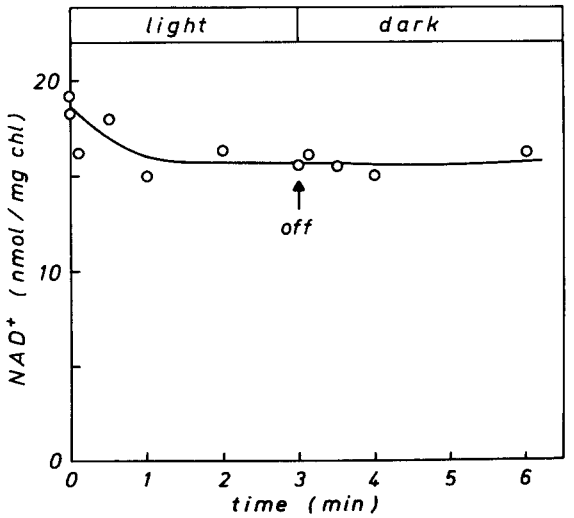

Fig. 4. The level of NAD in intact chloroplasts in the light and in the dark following a 3 min illumination period. The intensity of red light was 600 W/m^2.

expected that the redox state of NADP changed considerably during a 3 min illumination period. Under light saturation, photosynthesis is often believed to be limited by carbon cycle reactions, rather than the capacity of the electron-transport chain to provide NADPH. Photosynthesis of isolated chloroplasts is even more temperature dependent than photosynthesis of leaf cells. At low temperatures, rates of photosynthesis are very low and the lag in CO_2 fixation is considerably increased compared with photosynthesis at 20°C, presumably because enzymic reactions of the Calvin cycle are slowed down more than rates of transport of metabolites transferable by the phosphate translocator of the chloroplast envelope (cf. Ref. 2). It therefore appeared interesting to compare redox states of NADP in isolated chloroplasts photosynthesizing at two different temperatures. Fig. 5 shows that reoxidation of NADPH after the initial reduction of NADP was slower at 15°C than at 20°C. At 15°C, the level of NADP reached immediately after illumination was maintained for about 1 min, while at 20°C, it was maintained only for 30 s. It is particularly remarkable that the change from the reduced to the more oxidized state appeared to parallel the change in the rate of photosynthesis during the acceleration period of photosynthesis.

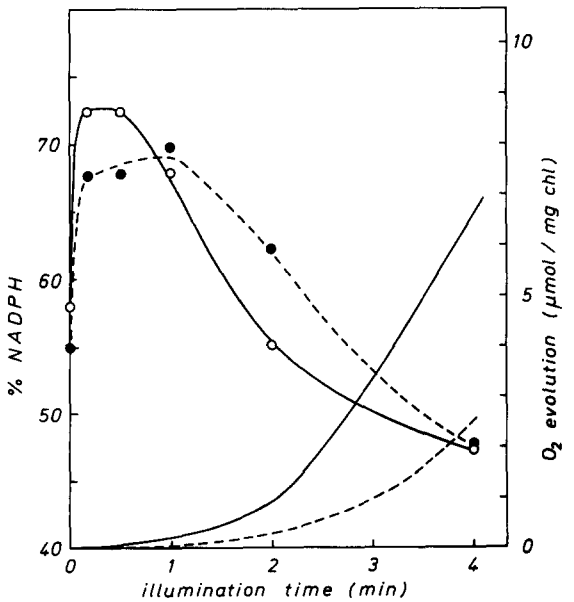

Fig. 5. The reduction state of NADP in intact chloroplasts and CO_2-dependent oxygen evolution by chloroplasts during illumination with 600 W/m^2 red light at 15°C (•------•) and 20°C (o———o). NADPH levels were calculated as the difference between [NADP + NADPH] and [NADP]. [NADP + NADPH] was 15.7 nmol/mg Chl.

Fig. 6 shows that the NADP level in chloroplasts remained low in the light when little CO_2 was available for reduction. Oxygen evolution during the first 4 min illumination reflects reduction of some endogenous CO_2. After 2 mM bicarbonate was added, oxygen evolution was resumed and the NADP level increased markedly to reach a steady state which was above the initial dark level. This was also observed in the experiments of Figs. 3 and 5, but not in the experiments reported by Lendzian and Bassham [17]. It shows that the NADP system of isolated chloroplasts is often more reduced in the dark than during CO_2 assimilation under high-intensity illumination. The small differences in the redox states of darkened and photosynthesizing chloroplasts do not lend support to the notion that the NADPH/NADP ratio plays a major role in regulating the activity of chloroplast enzymes [17].

Attempts were made to alter relative rates of electron transfer in the noncyclic and in the cyclic pathway by adding actimycin A, which inhibits cyclic electron transfer, and NH_4Cl, which dissipates the pH gradient across the thylakoids without affecting

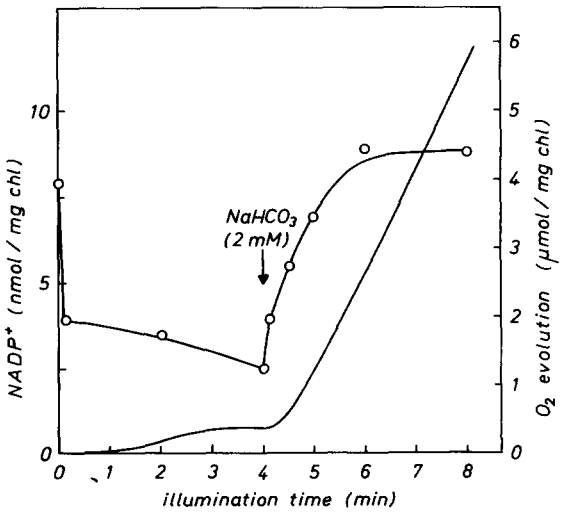

Fig. 6. Effect of $NaHCO_3$ on the level of NADP in illuminated chloroplasts. Chloroplasts were preilluminated with red light (600 W/m^2) without added bicarbonate for 4 min, and then 2 mM $NAHCO_3$ was added in the light.

photosynthesis at low concentrations [18,19]. NH_4Cl stimulates cyclic electron transport [20]. As shown in Fig. 7, antimycin A at 0.5 μM decreased the level of NADP by about 20% when added during steady-state photosynthesis under saturating illumination. Under these conditions, antimycin A was more inhibitory than at low light intensities. 0.5 μM antimycin A inhibited photosynthesis at light saturation by about 50% (cf. Fig. 2 for a smaller inhibition of photosynthesis by a high antimycin A concentration under low-intensity illumination). Higher antimycin A concentrations were more effective in shifting the redox state of NADP towards reduction than low concentrations. At the very low concentration of 0.3 mM, NH_4Cl added to chloroplasts during steady-state photosynthesis increased the NADP level without affecting photosynthetic oxygen evolution (Fig. 8). Higher concentrations (10 mM) were somewhat inhibitory and caused NADP reduction. When 45 mM sodium acetate, which decreases the stroma pH and thereby inhibits fructose bisphosphatase [21], was added in the presence of 0.3 mM NH_3Cl, the level of NADP decreased also indicating a more reduced state of the NADP system. Sodium acetate (45 mM)

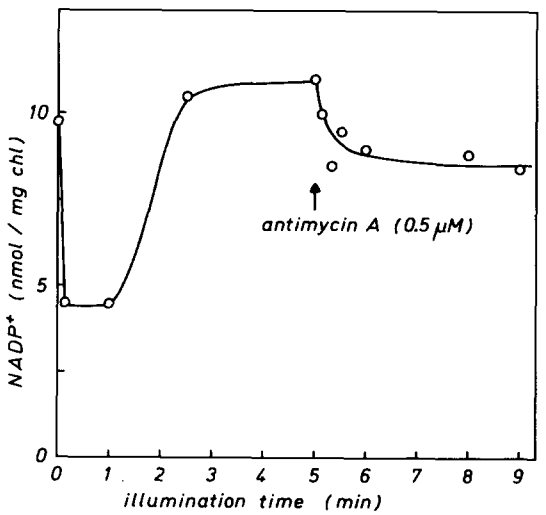

Fig. 7. Effect of antimycin A on the level of NADP in chloroplasts illuminated with high-intensity (600 W/m²) red light. 0.5 μM antimycin A inhibited CO_2-dependent oxygen evolution by about 50%. Higher concentrations of antimycin A were more inhibitory and also more effective in lowering the level of NADP than 0.5 μM. [NADP + NADPH] was 24 nmol/mg Chl.

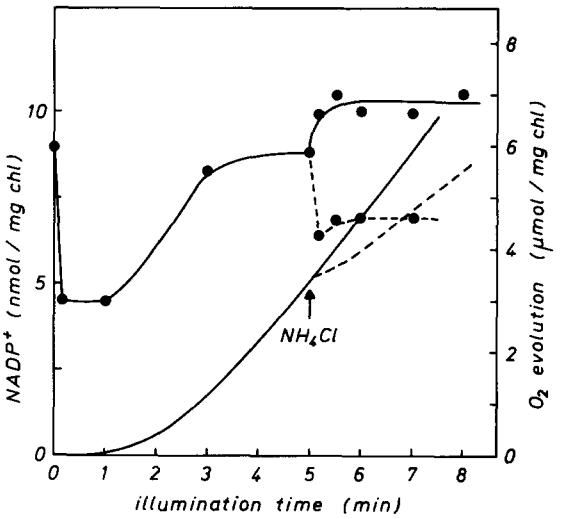

Fig. 8. Effect of NH_4Cl on the level of NADP in illuminated chloroplasts. The intensity of red light was 600 W/m². 0.3 mM (●———●) or 10 mM (●- - - - -●) NH_4Cl was added in the light. Oxygen electrode trace shows CO_2-dependent oxygen evolution: (———) 0.3 mM NH_4Cl, (- - - - -) 10 mM NH_4Cl. [NADP X NADPH] was 19.7 nmol/mg Chl.

inhibited CO_2-dependent oxygen evolution by about one-third.

An uncoupler of photophosphorylation from electron transfer, FCCP, or an energy-transfer inhibitor, Dio-9, also lowered levels of NADP while simultaneously inhibiting photosynthesis.

Discussion

In the light, levels of chloroplast NADPH and ATP are a function of the rate of production by electron-transfer reactions and associated phosphorylation and of the rate of consumption during substrate reduction. During photosynthesis both NADPH and ATP are consumed by the reduction of phosphoglycerate (PGA). In the dark, the reaction is very close to equilibrium;

$$\frac{[NADPH][H^+][ATP][PGA]}{[NADP][P_i][ADP][DHAP]} = K \qquad (1)$$

The equilibrium constant is calculated from the $\Delta G'_0$ values of Krebs and Kornberg [22] and Stryer [23] to be $7.25 \cdot 10^{-7}$. On illumination, production of

NADPH and ATP and the light-dependent alkalinization of the stroma [24] lead to displacement from equilibrium. As a result, phosphoglycerate is reduced to dihydroxyacetone phosphate (DHAP) [25]. The extent of displacement will be a function of light intensity and of the magnitude of carbon fluxes. As long as carbon fluxes are insignificant, i.e., during the lag phase of photosynthesis, displacement should be negligible and the reactants should assume a new balance which satisfies the equilibrium conditions. It can easily be seen from Eqn. 1 that a decrease in $[H^+]$ and in phosphoglycerate/dihydroxyacetone phosphate must be offset by an increase in NADPH/NADP and in the phosphorylation potential [ATP]/[ADP] $[P_i]$ to maintain equilibrium or a situation close to equilibrium. The relative contribution of both factors may be open to variation. In fact, Fig. 1 shows that at a low light intensity the NADP system is more reduced than at a high light intensity. This is a surprising finding in view of the fact that electron pressure within the elctron-transport chain which results from water oxidation must be greater at high than at low light intensities. Eqn. 1 suggests that the phosphorylation potential must be high when the ratio of NADPH to NADP is low and vice versa. Adenylate measurements which will be communicated in another contribution have verified this expectation. At a light intensity of 600 W/m^2 the phosphorylation potential was higher than at 14 W/m^2. Obviously, the energization of the thylakoid system by high light intensities which produces a high phosphorylation potential is also capable of decreasing the ratio of NADPH to NADP even when electron pressure within the electron-transport chain is high. The phenomenon of control of electron flow by the energization of the thylakoid system (photosynthetic control) has long been known [26]. In intact chloroplasts, it increases the quantum requirements of reactions such as oxaloacetate reduction during which no ATP is consumed [27]. Fig. 1 shows that it also affects the redox state of NADP during the lag phase of photosynthesis when electron fluxes are very small.

Fig. 2 shows that cyclic electron transport plays an important role in establishing the redox ratio of NADPH to NADP. After its initial reduction, NADP increased slowly during the lag phase of photosynthesis although electron consumption was negligible. This increase indicates a decrease in the ratio of NADPH to NADP. From Eqn. 1, this is expected to be brought about by an increased phosphorylation potential which, in turn, is supported by increased membrane energization. A low concentration of antimycin A was sufficient to prevent the secondary increase in NADP after its initial reduction. It is significant that antimycin A caused increased reduction of NADP already during the lag phase when consumption of ATP and electrons was slow compared with that during CO_2 reduction.

Antimycin A is known to inhibit cyclic electron flow and decrease membrane energization [5]. The data of Fig. 2 thus suggest that cyclic electron flow controls the redox state of NADP. On the other hand, earlier work had shown that the redox state of NADP controls cyclic electron flow [3]. When intact chloroplasts were illuminated with far-red light which preferentially excites Photosystem I, a proton gradient was formed which was decreased by adding oxaloacetate although this electron acceptor stimulates linear electron flow by oxidizing NADPH [3]. Thus, sensitive feedback systems distribute electrons between NADP and the acceptor of the cyclic electron-transport pathway. A low ratio of NADPH to NADP directs electrons to NADP, while an increased ratio diverts electrons into the cyclic pathway (Fig. 9). Oxygen plays an important regulatory role in this system by preventing excessive reduction of the electron-transport chain when NADP is largely reduced [28]. Electron transport to oxygen also provides ATP [8]. On the other hand, photosynthetic control which will be observed only at high light intensities, when the phosphorylation potential is high, restricts electron flow to NADP decreasing the NADPH/NADP ratio (Figs. 3 and 5). The physiological range of switching between cyclic phosphorylation and NADP reduction is surprisingly large. As indicated by the sensitivity of photosynthesis to antimycin A in the experiments of Figs. 2 and 7 and the redox ratios shown in Fig. 5, spillover of electrons into the cyclic pathway is considerable at an NADPH/NADP ratio as low as about 1. It has been mentioned that during the lag phase of photosynthesis, the reactants of Eqn. 1 cannot be far from equilibrium. However, under flux conditions, the reaction must be displaced from equilibrium as the free energy change of phosphoglycerate reduction must be negative. It has been shown previously that the phosphorylation

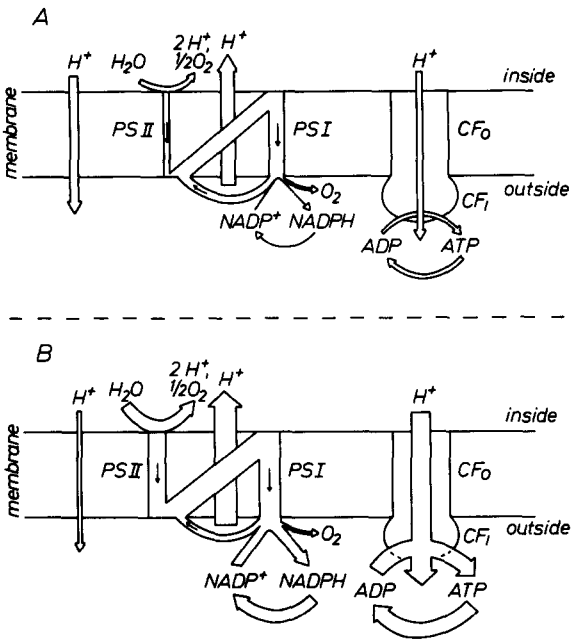

Fig. 9. Scheme of electron and proton fluxes during the lag phase and in the steady state of photosynthesis. (A) Lag phase: NADP reduction is restricted by the availability of NADP and electrons are diverted to oxygen [3,8,9] and into the cyclic pathway [3–5,20]. The proton gradient is large under high-intensity illumination and part of the electron flow is made possible by proton leakage across thylakoid membranes. Slow ATP turnover limits proton consumption during ATP synthesis. (B) Steady-state photosynthesis: At high light intensities, the proton gradient is also considerable and a low intrathylakoid pH controls the ratio of NADPH to NADP [26,31]. The NADP system may be less than 50% reduced and electrons are distributed between NADP, the cyclic pathway and oxygen. Proton leakage across thylakoid membranes is reduced and the main proportion of the proton flux serves to synthesize ATP. Arrows indicate fluxes of H^+ and electrons and turnover of ATP and NADP.

potential does not change much during photosynthesis of isolated chloroplasts [1]. Although ATP/ADP ratios slowly decrease while photosynthetic oxygen evolution accelerates [1], this decrease is largely compensated by a decrease in stromal P_i [29]. Figs. 2, 3 and 5–8 show that the NADPH/NADP ratio does not increase and actually decreases both during the lag phase of photosynthesis and when CO_2-dependent oxygen evolution accelerates. Its decrease must be overbalanced by an increase in another ratio, if the reaction system shown in Eqn. 1 is to change from quasi-equilibrium to a flux state. $[H^+]$ is known to remain largely constant above a light intensity of about 8 W/m^2 [21]. The only remaining ratio is that of phosphoglycerate/dihydroxyacetone phosphate which indeed has been observed to increase [19] after its initial light-dependent decrease [25]. Table I compares measured NADPH/NADP, ATP/ADP and phosphoglycerate/dihydroxyacetone phosphate ratios in the stroma of intact chloroplasts after different illumination times. While ATP/ADP ratios remain rather constant or even decrease during illumination [29] in a manner similar to that shown by the NADPH/NADP ratios, phosphoglycerate/dihydroxyacetone phosphate ratios increase dramatically. The data show that the increase in phosphoglycerate/dihydroxyacetone phosphate produces the deviation from equilibriuim which drives phosphoglycerate reduction during steady-state photosynthesis. The extent of displacement from equilibrium is indicated by the increase in the mass action ratio:

$$\frac{[NADPH][H^+][ATP][PGA]}{[NADP^+][ADP][P_i][DHAP]}$$

during illumination (Table I) and also by the sudden and almost complete oxidation of NADPH and the accompanying rise in NADP when the light is turned off (Fig. 3). Dark activation of glucose-6-phosphate dehydrogenase [17] and exchange of chloroplast phosphoglycerate for dihydroxyacetone phosphate across the chloroplast envelope then lead to a slow increase in the NADPH/NADP ratio until the original dark level of NADP is reestablished.

It should be noted that the mass action ratio calculated for the lag phase of photosynthesis, when carbon fluxes are still small (30 s illumination, Table I), does not differ very much from the equilibrium constant of the phosphoglycerate/dihydroxyacetone phosphate system ($7.25 \cdot 10^{-7}$, Eqn. 1). This is remarkable because the data of Table I are from different experiments and the measured metabolite concentrations do not necessarily represent thermodynamically relevant concentrations. Both themodynamically active free metabolites and metabolites bound to proteins are subject to extraction by $HClO_4$, and both are measured together.

The data of Fig. 5 show that considerable differ-

TABLE I

Calculated mass action ratios of phosphoglycerate reduction and measured ratio of NADPH to NADP, ATP to ADP and phosphoglycerate to dihydroxyacetone phosphate (PGA/DHAP) in the stroma of intact chloroplasts during illumination in the presence of bicarbonate. Metabolite ratios are from Fig. 5 and from experimental results published by Kobayashi et al. [1] and Lilley et al. [29].

	Illumination time (min)			
	0.5	1	2	4
NADPH/NADP	2.6	2.1	1.2	0.9
ATP/ADP	1.9	2	2.3	2.1
PGA/DHAP	1	2.2	10	12
$\frac{[NADPH][H^+][ATP][PGA]}{[NADP][ADP][P_i][DHAP]}$ [a] ($\times 10^7$)	50	90	270	230

ences in the rate of photosynthesis at 15 and 20°C did not affect the general pattern of NADP response to illumination. At both temperatures, the redox ratio established after 4 min illumination was comparable although the rate of photosynthesis at 20°C was twice that at 15°C. Only when CO_2 was unavailable during illumination did the NADPH/NADP ratio remain high in the light (Fig. 6). In the absence of photosynthetic flux, the ratio of phosphoglycerate/dihydroxyacetone phosphate was low [30] and reflected reaction equilibrium. The NADPH/NADP ratio decreased immediately after addition of bicarbonate as a consequence of the formation of a high phosphoglycerate/dihydroxyacetone phosphate ratio. This increase has not been observed in the work of Lendzian and Bassham [17]. A shift towards oxidation of the NADP system may be less pronounced or even absent when chloroplasts cannot maintain a high phosphorylation potential during photosynthesis (see Eqn. 1) or when the stromal phosphoglycerate/dihydroxyacetone phosphate ratio is not greatly increased during the transition from the lag phase to steady-state photosynthesis, i.e., at low light intensities. In fact, all conditions which either decreased the phosphorylation potential or the phosphoglycerate/dihydroxyacetone phosphate ratio caused an increase in the reduction of the NADP system. This can easily be understood in terms of Eqn. 1. The phosphorylation potential is expected to be decreased by inhibitors of cyclic electron flow such as antimycin A (Figs. 2 and 7), by energy-transfer inhibitors such as Dio-9 and by uncouplers such as FCCP or high concentrations of NH_4Cl (Fig. 8). The increase in NADP observed on adding a low concentration of NH_4Cl is interpreted to be caused by an increase in the stromal phosphoglycerate/dihydroxyacetone phosphate ratio. By slightly increasing the stroma pH, NH_4Cl can increase the activity of stromal fructose bisphosphatase which is redox and pH controlled and may limit flux in the Calvin cycle [30]. There is the question as to what extent the situation of intact isolated chloroplasts reflects conditions in a leaf system in vivo. In earlier work with leaves, freeze-stopping, freeze-drying and nonaqueous isolation procedures had been employed to isolated chloroplasts and measure the redox state of chloroplast pyridine nucleotides in vivo [12]. This work had shown the chloroplast NADPH/NADP ratio to increase rapidly on illumination and then to decline to values not far from that of the original dark redox state. It had not become clear whether the secondary change in the redox state was an artifact produced by NADPH oxidation during freeze-stopping of the leaves or whether it reflected physiological changes. The data of the present investigation show clearly that physiological events had been recorded. They permit understanding of light-dependent redox changes which previously had not been understood and give insight into the regulation of photosynthetic reduction. The present work also raises interesting questions regarding light activation of chloroplast enzymes and its control. An increase in the ratio of reduced to oxidized ferredoxin during illumination is thought to cause the reductive activation of key chloroplast enzymes [32]. Fructose bis-

phosphatase is known to be switched from practically zero activity to an activity which is capable of handling carbon fluxes [33]. The results of this work make one wonder how much the redox state of ferredoxin differs in vivo between dark and light. Recent work of Satoh and Katoh [34] suggests that electron transfer between ferredoxin and NADP is regulated.

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