Plant Physiol. (1983) 71, 568–573
0032-0889/83/71/0568/06/$00.50/0

Influence of Oxygen and Temperature on the Dark Inactivation of Pyruvate,Orthophosphate Dikinase and NADP-Malate Dehydrogenase in Maize[1]

Received for publication August 23, 1982 and in revised form October 24, 1982

HITOSHI NAKAMOTO AND GERALD E. EDWARDS
Botany Department, Washington State University, Pullman, Washington 99164

ABSTRACT

The influence of oxygen and temperature on the inactivation of pyruvate,Pi dikinase and NADP-malate dehydrogenase was studied in *Zea mays*. O_2 was required for inactivation of both pyruvate,Pi dikinase and NADP-malate dehydrogenase in the dark *in vivo*. The rate of inactivation under 2% O_2 was only slightly lower than that at 21% O_2. The *in vitro* inactivation of pyruvate,Pi dikinase, while dependent on adenine nucleotides (ADP + ATP), did not require O_2.

The postillumination inactivation of pyruvate,Pi dikinase in leaves was strongly dependent on temperature. As temperature was decreased in the dark, there was a lag period of increasing length (*e.g.* at 17°C there was a lag of about 25 minutes) before inactivation proceeded. Following the lag period, the rate of inactivation decreased with decreasing temperature. The half-time for dark inactivation was about 7 minutes at 32°C and 45 minutes at 17°C. The inactivation of pyruvate,Pi dikinase *in vitro* following extraction from illuminated leaves was also strongly dependent on temperature, but occurred without a lag period. In contrast, NADP-malate dehydrogenase was rapidly inactivated in leaves (half-time of approximately 3 minutes) during the postillumination period without a lag, and there was little effect of temperature between 10 and 32°C. The results are discussed in relation to known differences in the mechanism of activation/inactivation of the two enzymes.

In C_4 plants, two enzymes of the C_4 cycle are known to be light activated, NADP-malate dehydrogenase and pyruvate,Pi dikinase (4–6, 8, 12). Although both enzymes are located in the mesophyll chloroplast of C_4 plants there is considerable evidence from *in vitro* studies that the mechanisms of light/dark-mediated activation/inactivation of the enzymes are quite different.

Reducing conditions activate NADP-malate dehydrogenase (presumably via conversion of disulfide to sulfhydryl groups on the protein) while inactivation requires oxidizing conditions (6, 11, 13). Incubation of the inactive form of the enzyme with DTT plus thioredoxin leads to rapid activation. *In vivo* the apparent role of light is to generate a reductant through noncyclic electron flow which mediates activation of the enzyme, through either ferredoxin-thioredoxin reductase and/or a membrane-bound light effect mediator and ferralterin (1, 2).

Recently, Chapman and Hatch (3) and Nakamoto and Sugiyama (15) showed that pyruvate,Pi dikinase could undergo activation in the absence of reducing reagents (*e.g.* without DTT) suggesting that reduction/oxidation of the enzyme is not required in the activation/inactivation process even though activation is closely linked with the photosynthetic electron transport system (22). Adenine nucleotides and Pi appear to be key metabolites in controlling the activation/inactivation of the enzyme. Pi is required for activation while ATP + ADP is required for inactivation (3, 6–8, 15, 19). The following study was conducted with whole plants to evaluate whether differences in the mechanism of activation of the two enzymes may result in differences in response to change in environmental factors during inactivation *in vivo*.

MATERIALS AND METHODS

Plant Material and Reagents. *Zea mays* was grown in soil (five to six plants per pot) in a controlled environment with a day/night temperature regime of 27/22°C with a light/dark period of 14/10 h. Light was provided by a combination of fluorescent and incandescent lamps, giving a photosynthetic photon flux density of 700 $\mu E\ m^{-2}\ s^{-1}$. Plants were fertilized daily with nutrient solution as previously described (17). The most fully expanded leaves (fourth and fifth leaves from the bottom) were used from plants approximately 3 weeks of age. Biochemicals and reagent enzymes were obtained from Sigma Chemical Co.

Exposure of Leaves to Varying Levels of O_2 and N_2. Plants were removed from the growth chamber and transferred to a temperature-controlled room at 23 to 25°C. The plants were illuminated for 55 min by a General Electric 1000 w mercury vapor lamp giving a photosynthetic photon flux density of 1500 $\mu E\ m^{-2}\ s^{-1}$. One leaf blade was harvested (10–15 cm in length from the tip) to determine the initial light-activated state of the enzymes and stored as described later. Another leaf blade was quickly inserted in a Plexiglass chamber through the slit of a rubber stopper. The dimensions of the chamber were 16.4 (length) × 10.7 (width) × 8.3 cm (height) with two ports on opposing sides for passing through various gases. The desired gas was flowing through the chamber prior to inserting the leaf. The gas used was humidified by passing it through water. N_2 gas, 2% O_2-98% N_2, and compressed air were obtained commercially. Any CO_2 which may have contaminated the N_2 or 2% O_2-98% N_2 was removed by passing the gas through Ascarite II (Arthur H. Thomas Co.). CO_2 free air was also prepared by passing air through the Ascarite II. Gas was passed through the chamber (flow rate, 0.7 L min^{-1}) during each experiment until the leaf was harvested. After sealing the leaf in the chamber, illumination was continued for 2 to 3 min, giving a total illumination time of 60 min prior to placing the system in darkness. For the dark treatment, a green safe light was used which emitted less than 0.6 $\mu E\ m^{-2}\ s^{-1}$. A control experiment in complete darkness (N_2 *versus* air treatment) indicated there was no effect by the safe light on the state of activation. Through a

[1] This research was supported by a Herman Frasch Foundation Grant for Research in Agricultural Chemistry and National Science Foundation Grant PCM 82-04625.

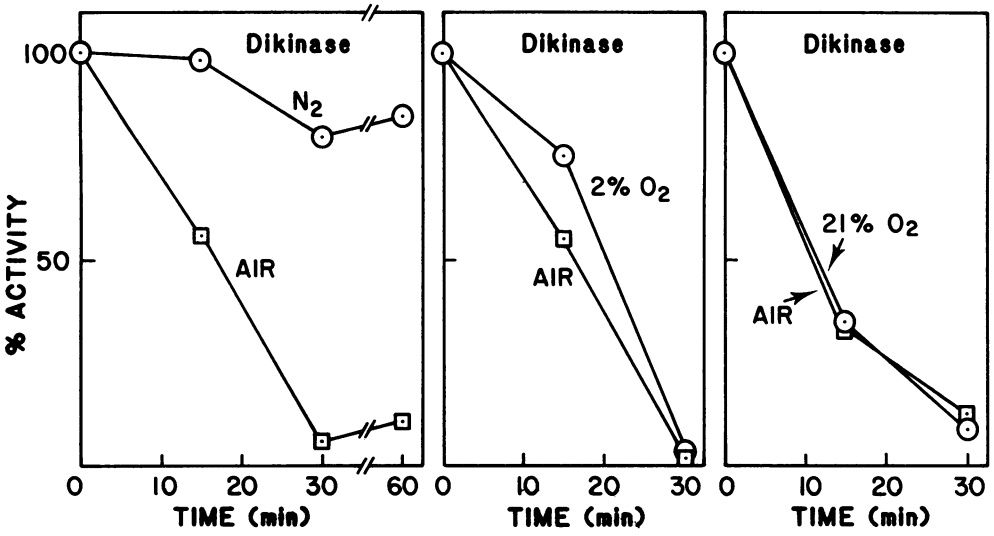


FIG. 1. Time course of the *in vivo* inactivation of pyruvate,Pi dikinase in the dark under different atmospheric conditions following illumination of a maize plant for 1 h at 25°C. The average initial activity at time zero was 152 μmol mg^{-1} Chl h^{-1}. O_2 treatments were 2% O_2-98% N_2, 21% O_2 (CO_2-free air), and air. The data are from a single experiment which is typical of data from similar treatments not shown.

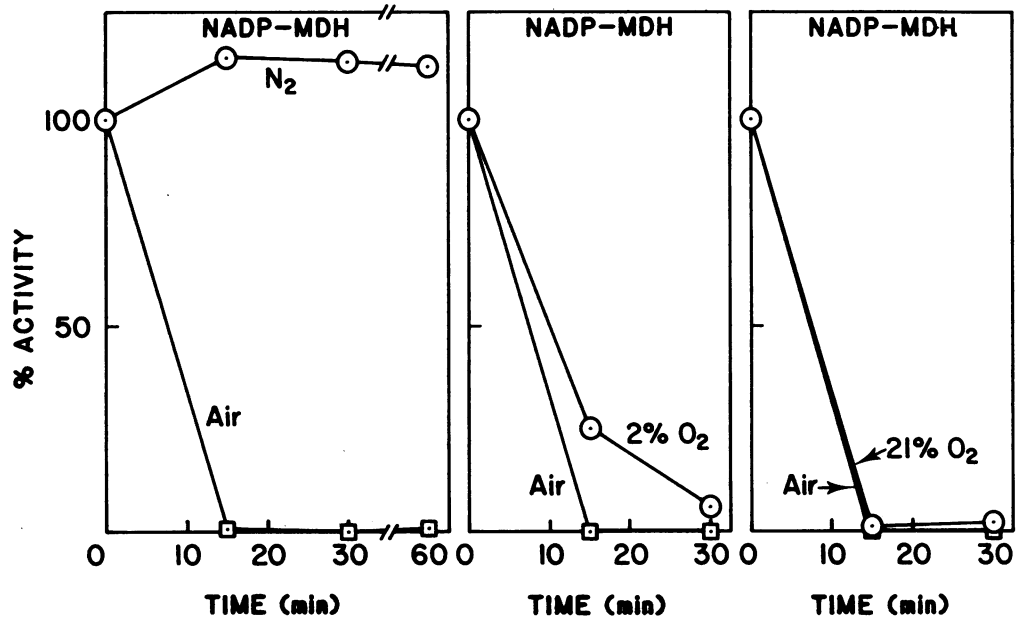


FIG. 2. Time course of the *in vivo* inactivation of NADP-malate dehydrogenase (MDH) in the dark under different atmospheric conditions following illumination of a maize plant for 1 h at 25°C. The average initial activity at time zero was 165 μmol mg^{-1} Chl h^{-1}. O_2 treatments were 2% O_2-98% N_2, 21% O_2 (CO_2-free air), and air. In Figures 1 and 2, at a given time, the same extract of maize was used to assay the two enzymes. The data are from a single experiment which is typical of data from similar treatments not shown.

number of treatments, the leaves were exposed to varying conditions prior to sampling and killing in liquid N_2. Thus, each enzyme assay was performed on a single leaf extract.

When leaves were taken from the chamber, the part of the leaf blade (about 1 g) which was under the gas treatment (*i.e.* N_2, 2% O_2-98% N_2, CO_2-free air) was cut and the midrib was removed. The leaf was immediately killed by immersion in liquid N_2 and then stored in liquid N_2 until extraction. The time from removal of the leaf from the chamber to killing in liquid N_2 was about 10 to 15 s (much shorter than the half-time for inactivation of the pyruvate,Pi dikinase and NADP-malate dehydrogenase) At the same time, the leaf blade of another plant in the same pot which was exposed to room air and darkness was sampled to compare with the treated leaf (Figs. 1 and 2). In one experiment, after exposure to N_2 gas in the dark, some leaves were further treated in room air prior to extraction (Fig. 3). The results of each experiment were obtained during a given day and leaves of similar age were sampled from several plants grown in the same pot.

In all *in vivo* experiments, sampled leaves were stored in liquid N_2. Comparisons of the leaves stored as described above in liquid N_2 with control experiments in which the enzymes were extracted without freezing the leaf showed that there was no significant difference in the extractable enzyme activities.

Incubation of Leaves at Various Temperatures during Dark Treatment. Before dark treatment, plants were illuminated as described previously for 1 h at 23 to 25°C. Following the illumination period, the plants were transferred to a room which was preset at the desired temperature. A leaf blade was immediately harvested for measurement of the initial activity (0 time) and other samples were taken at intervals during the dark; the midrib was removed, and the leaves were killed and stored under liquid N_2 as described above.

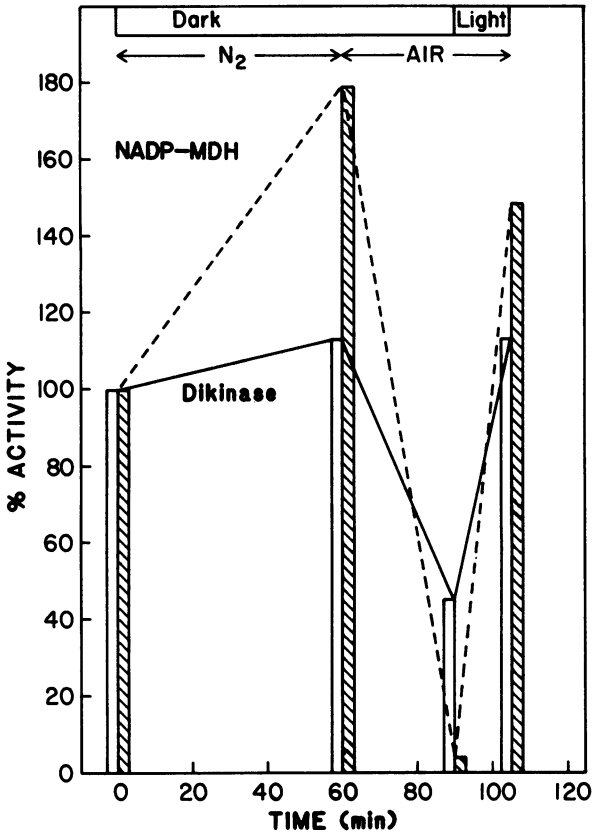


FIG. 3. Changes in the activity of NADP-malate dehydrogenase (MDH) and pyruvate,Pi dikinase under varying conditions following 1 h of illumination. Three leaves were treated under N_2 in the dark (one leaf per plant) as described in *Materials and Methods*. The leaves were sampled and extracted as follows: one leaf after 60-min N_2 treatment; one leaf after 60-min N_2 + 30-min air in the dark; and one leaf after 60-min N_2 + 30-min air in the dark + 15-min air in the light. The initial activities for NADP-malate dehydrogenase and pyruvate,Pi dikinase were 329 and 144 μmol mg^{-1} Chl h^{-1}, respectively. The data are from a single experiment.

Enzyme Extraction and Assay. The extraction procedure was carried out at room temperature using a chilled mortar and grinding medium. The grinding medium contained 4 volumes/g tissue of 0.1 M Tris-HCl (pH 7.5, 4°C), 10mM $MgCl_2$, 1 mM EDTA, 5 mM DTE[2] and 5% (w/w of leaf tissue) polyvinylpolypyrrolidone. The tissue was ground for 1.5 to 2 min and the homogenate was filtered through Miracloth. An aliquot of the filtrate was taken for Chl determination. For the assay of NADP-malate dehydrogenase, a 25-μl aliquot of the homogenate was added to the reaction mixture (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2 mM $NADPH_2$) and centrifuged at 14,000*g* for 1 min. The supernatant was assayed immediately at 25°C as previously described (9). The reaction was initiated by the addition of oxalacetate (0.5 mM) and the oxalacetate-dependent *A* decrease was measured at 340 nm.

For the assay of pyruvate,Pi dikinase, an aliquot of the same filtrate (400 μl) was rapidly desalted by passage through a column (0.8-cm diameter by 5-cm length) of Sephadex G-25 (superfine). The column was previously equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5, 25°C), 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTE, 0.2% (w/v) BSA, and 2.5 mM pyruvate. Desalting was accomplished by centrifuging the column at 1,400*g* for 3 min at room temperature after application of the sample (10). The eluate obtained with this method was not diluted and the yield was over 90% of the original sample. After Sephadex G-25 treatment, the eluate was centrifuged at 14,000*g* for 1 min and aliquots (25 μl) of the supernatant were immediately assayed. The enzyme was assayed in the direction of pyruvate to phosphoenolpyruvate through coupling with phosphoenolpyruvate carboxylase and NAD-malate dehydrogenase as previously described (5). The reaction was initiated by addition of ATP.

[2] Abbreviation: DTE, dithioerythritol.

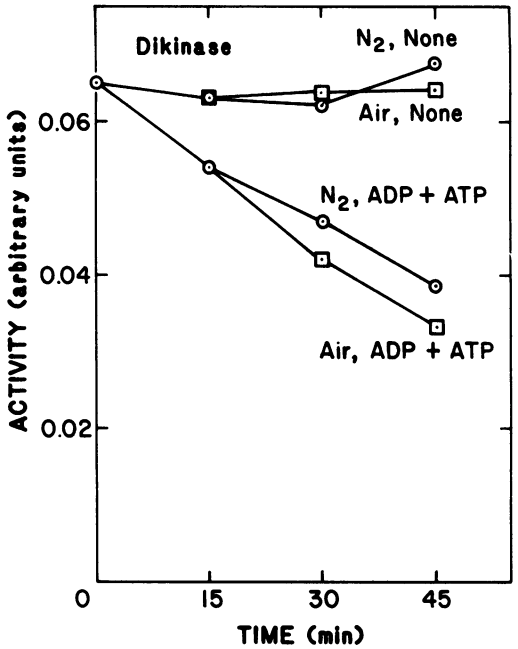


FIG. 4. ADP + ATP mediated *in vitro* inactivation of pyruvate,Pi dikinase in mesophyll chloroplast extracts of maize under N_2 or air. In the anaerobic treatments, the enzyme mixture was flushed with N_2 for 3 min in a small glass bottle (volume of 2.5 ml) before addition of ADP + ATP or water (in control). The bottle was covered with a rubber serum cap and two syringe needles were inserted, one serving as the inlet for the gas and the other for the outlet. The N_2 gas stream was humidified by passage through water in order to prevent sample evaporation. The enzyme samples were continuously flushed with N_2 except when samples were withdrawn with a microsyringe for enzyme assay. Samples were exposed to room air in the aerobic treatment. The temperature of incubation was 23°C and the initial enzyme activity was 0.42 unit/ml.

Chl Determination. Chl was determined by the method of Wintermans and De Mots (21) following extraction in 96% ethanol.

Preparation of Chloroplasts and Chloroplast Extracts. The procedure was basically the same as previously described by Sugiyama and Hatch (19). Plants were pre-illuminated to activate pyruvate,Pi dikinase. The midrib was either removed from pre-illuminated leaves in the light or under room light at 4°C to minimize dark inactivation of the enzyme. The tissue was blended for 8 s (4 s twice) at 60% of line voltage in a Polytron. Chloroplasts were isolated as previously described and stored frozen in liquid N_2. For experimentation, the chloroplast extracts were treated on Sephadex G-25. For each assay, 180 μl of the eluate was added to the incubation mixture, giving a total volume of 200 μl. This medium used for preincubation (under N_2 or air and at varying temperatures; see Figs. 4 and 6) contained 18 mM Tris-HCl, pH 8.3, 4.5 mM DTE, 9 mM $MgCl_2$, 0.18 mM EDTA, 1.8 mg ml^{-1} BSA, and in the presence or absence of 1mM ADP and 50 μM ATP. At various time intervals, samples of 25 μl were removed and placed in cuvettes containing the complete assay system for pyruvate,Pi dikinase. These were immediately mixed and activity was measured by following the *A* change at 340 nm and 25°C.

RESULTS

The Influence of O_2 on the Inactivation of Pyruvate,Pi Dikinase and NADP-Malate Dehydrogenase. O_2 was required for inacti-

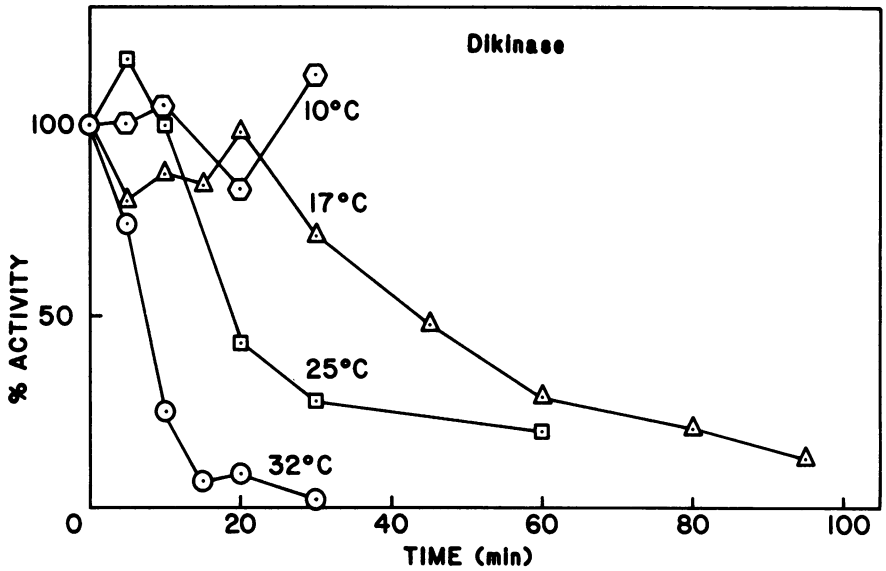


FIG. 5. Time course of *in vivo* inactivation of pyruvate,Pi dikinase in the dark at various temperatures following illumination of a maize plant for 1 h at 25°C. The average initial activity of pyruvate,Pi dikinase was 135 μmol mg^{-1} Chl h^{-1}. The data are from a single experiment.

vation of both pyruvate,Pi dikinase and NADP-malate dehydrogenase in maize leaves in the dark following light activation (Figs. 1 and 2). The rate of inactivation under 2% O_2 was only slightly lower than that at 21% CO_2 (without CO_2) or air (with CO_2). N_2 in the dark prevented inactivation of both enzymes. With NADP-malate dehydrogenase, there was a consistent although variable degree of increase in activity under N_2 in the dark following light activation (Figs. 2 and 3 and data not shown) which did not occur with pyruvate,Pi dikinase.

The prevention of inactivation of both enzymes in the dark by the treatment with N_2 is reversible (Fig. 3). Both enzymes were inactivated following transfer from the N_2 treatment in the dark to air. When the leaves were re-illuminated in the air, the enzymes were reactivated after 15 min to the original light-activated level.

The effect of N_2 *versus* air on the ATP + ADP-dependent inactivation of pyruvate,Pi dikinase *in vitro* was examined. The activated enzyme was obtained from chloroplasts isolated from pre-illuminated leaves. Inactivation occurred at similar rates under both air and N_2 when incubated with ATP + ADP (Fig. 4). NADP-malate dehydrogenase immediately lost activity following desalting of the chloroplast extracts, which would remove DTT (results not shown). It was previously shown that the activated state of partly purified NADP-malate dehydrogenase of maize is maintained under N_2 (13).

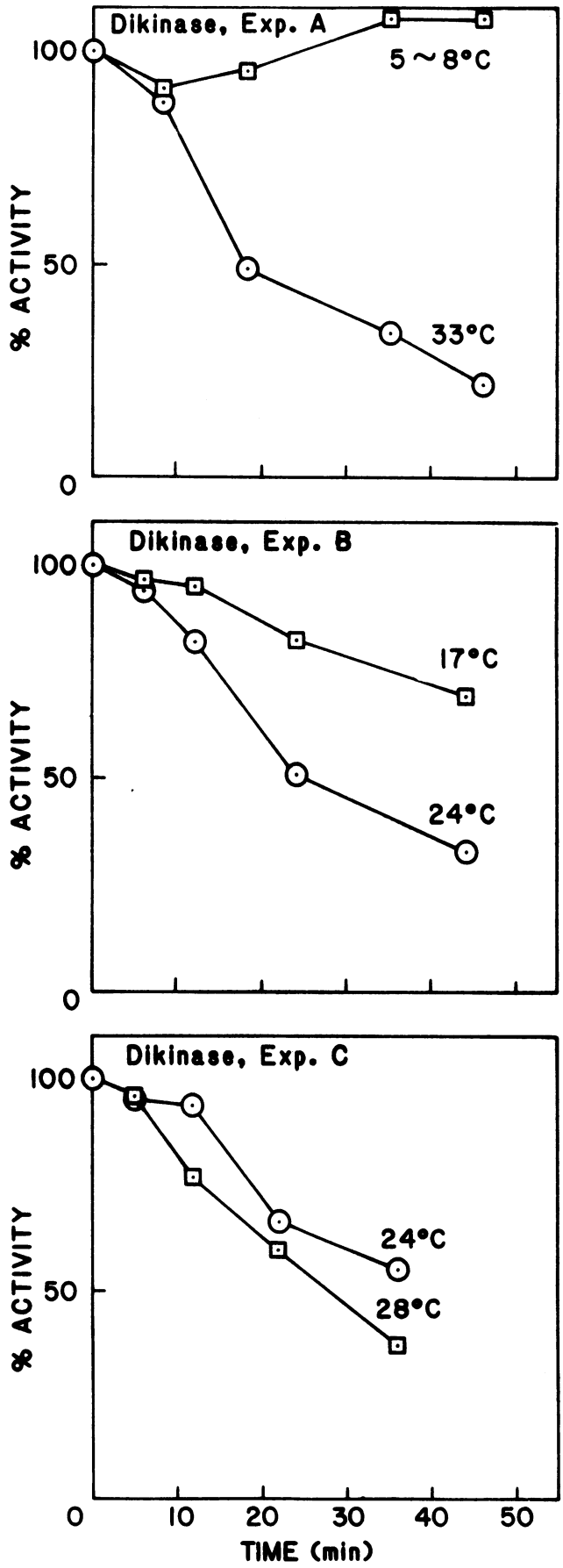
Influence of Temperature on the Inactivation of Pyruvate,Pi Dikinase and NADP-Malate Dehydrogenase. As the temperature was lowered during the postillumination period, there was an increasing lag period before inactivation of pyruvate,Pi dikinase proceeded in maize leaves (Fig. 5). The lag periods at 10, 17, and 25°C were more than 30, 25, and 10 min, respectively, while that at 32°C was less than 5 min. Following the lag period, the enzyme was inactivated and the rate of inactivation increased with increasing temperature. The half-time for dark inactivation was 7 min at 32°C, 20 min at 25°C, and 45 min at 17°C (Fig. 5). Therefore, *in vivo* inactivation of the enzyme proceeded in two phases, both showing a dependence on temperature. Inactivation of pyruvate,Pi dikinase *in vitro* was also strongly dependent on temperature, although there was no temperature-dependent lag period (Fig. 6). During the first 10 min of incubation, the ATP + ADP-dependent inactivation showed a slight lag, but this was independent of temperature. In the experiment of Figure 6, strict quantitative comparisons cannot be made between all temperatures inasmuch as results of Figure 6, A, B, and C, represent three different chloroplast preparations. In another experiment, the enzyme from one chloroplast preparation was incubated with ATP + ADP over a temperature range of 19 to 34°C for 30 min. An Arrhenius plot (log of the rate of inactivation *versus* $1/T$) of this data gave a linear function over the temperature range of the experiment with a Q_{10} of 2.0.

In contrast to the results with pyruvate,Pi dikinase, NADP-malate dehydrogenase was rapidly inactivated in leaves during the postillumination period (half-time of about 3 min; Fig. 7) and there was little effect of temperature on the rate of inactivation between 10 and 32°C. Also, there was no lag in the inactivation of NADP-malate dehydrogenase upon transfer from light to darkness.

DISCUSSION

The Influence of O_2 on the Inactivation of Pyruvate,Pi Dikinase and NADP-Malate Dehydrogenase. O_2 was required for the dark inactivation of NADP-malate dehydrogenase in maize leaves. Previous studies indicate that the mechanism of activation/inactivation of the isolated enzyme is through a reductive/oxidative process involving enzyme thiol/disulfide interconversion (11, 13). *In vivo* in the light, reductive power from photosynthetic electron transport is apparently used to activate the enzyme. In the dark, this source of reductant is unavailable and the enzyme may be oxidized via a a protein factor, possibly thioredoxin, with O_2 as the final electron acceptor (13, 16). Therefore, in the dark under anaerobic conditions, the enzyme may be maintained in the reduced, activated state. The fact that the activated state of the enzyme in the dark under N_2 tends to increase over that of the pre-illuminated state (Figs. 2 and 3) suggests that some reductant is available in the chloroplast to further activate the enzyme. Whether this increase in the level of activation in the dark is associated with an increased level of reduced pyridine nucleotides in the chloroplast under anaerobic conditions is uncertain. Leegood and Walker (14) have found that fructose-1,6-bisphosphatase can be activated in isolated chloroplasts in the dark under anaerobic conditions provided that triose-P is added to generate reductive power in the chloroplasts. Therefore, it appears that reduced pyridine nucleotides can serve as the electron donor to activate certain enzymes independently of light.

Surprisingly, O_2 is also required for inactivation of pyruvate,Pi dikinase in the dark in maize leaves. This cannot be readily interpreted mechanistically from current knowledge of the *in vitro*



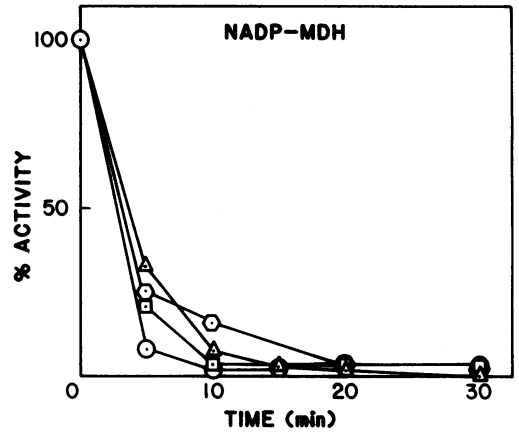


FIG. 7. Time course of *in vivo* inactivation of NADP-malate dehydrogenase in the dark at various temperatures following illumination of a maize plant for 1 h at 25°C. The average initial activity of NADP-malate dehydrogenase was 122 μmol mg^{-1} Chl h^{-1}, respectively. The experiment was with the same plant extracts as that of Fig. 5 and the data are from a single experiment. Temperatures designated by the symbols are 10°C (○), 17°C (△), 25°C (□), and 32°C (◎), respectively.

mechanism of inactivation. Recently, Chapman and Hatch (3) and Nakamoto and Sugiyama (15) showed that the enzyme can be activated in the absence of a reducing agent, and therefore suggested that reduction/oxidation of the enzyme is not required. The requirement for reducing agents in early studies with the enzyme may have been to stabilize the protein and/or the activating factor (see 3, 7, 15, 20). Inactivation (ATP + ADP-dependent) can occur in the presence of a high concentration of DTT (10 mM: Refs. 3 and 19) in contrast to NADP-malate dehydrogenase which can be activated by DTT and inactivated in the absence of DTT with soluble protein factor (13). In the present study, the ATP + ADP-dependent inactivation of pyruvate,Pi dikinase occurred under anaerobic and aerobic conditions (Fig. 4), which further suggests oxidation of the enzyme is not required for inactivation. *In vitro* studies indicate that the relative levels of ATP, ADP, AMP, PPi, and Pi are involved in controlling the state of activation/inactivation of pyruvate,Pi dikinase (3, 7, 15, 19, 20). Therefore, maintenance of activation in the dark under N_2 might be mediated through an effect on the levels of these metabolites in the chloroplasts. Alternatively, there may be additional factors involved in mediating inactivation of the enzyme *in vivo*, possibly involving oxidation/reduction of the enzyme or regulation of the activity of the inactivating factor protein. Chapman and Hatch (3) found that, at relatively high pH, reducing conditions were required to stabilize the enzyme and give maximum activation, which might be relevant *in vivo*. Under certain conditions in the chloroplast, reducing conditions could have a stabilizing effect and favor activation of the enzyme.

The Influence of Temperature on the Inactivation of Pyruvate,Pi Dikinase and NADP-Malate Dehydrogenase. The inactivation of pyruvate,Pi dikinase in leaves in the dark proceeded in two phases, both of which were influenced by temperature. First, there was a

FIG. 6. ADP + ATP mediated *in vivo* inactivation of pyruvate,Pi dikinase in mesophyll chloroplast extracts at various temperatures. Experiments A, B, and C were conducted with different chloroplast preparations. The percentage activity retained after ADP + ATP treatment is expressed as activity with ADP + ATP in the preincubation mixture/ activity without ADP + ATP in the preincubation mixture × 100. Occasionally, there was a slight increase in activity during the preincubation period in the absence of ADP + ATP, especially at the higher temperature. The average initial activity of the enzyme at time 0 was about 0.6 unit/ml of Sephadex G-25-treated chloroplast extract.

lag period which increased with decreasing temperature (phase I) and was followed by a temperature-sensitive decrease in activity of the enzyme (phase II). *In vitro* the time-dependent inactivation of pyruvate,Pi dikinase proceeded without a lag period (lacked phase I) and exhibited temperature sensitivity similar to that of phase II *in vivo*. Phase I may represent the time taken for change in levels of certain effectors (*e.g.* relative levels of adenine nucleotides) which are substrates in the inactivation process or control the activity of the inactivating protein factor.

The strong temperature dependence of inactivation of pyruvate,Pi dikinase between 17 and 32°C may be due to the influence of temperature on catalysis of inactivation by the unidentified protein-inactivating factor (assuming inactivation is a catalytic process as it is for activation; Refs. 19 and 20). Below this temperature, both the *in vivo* and *in vitro* temperature limitation could be due to a combined effect on the protein-inactivating factor and dissociation of the dikinase enzyme from an active to inactive form. Below about 12°C, the activated pyruvate,Pi dikinase dissociates and is converted from an active to inactive form. This results in a break in the Arrhenius plot around this temperature (18). Hatch (7) also found a break in the Arrhenius plot for the ATP + ADP-dependent inactivation of pyruvate,Pi dikinase at a temperature of 18°C, indicating a greater Q_{10} below this temperature.

The present study indicates differences in the temperature dependence of inactivation of pyruvate,Pi dikinase and NADP-malate dehydrogenase in maize leaves. It shows that O_2 has a role in the inactivating process with pyruvate,Pi dikinase as well as with NADP-malate dehydrogenase. More information about the mechanism of activation/inactivation is needed, particularly with pyruvate,Pi dikinase, in order to understand how the activation/inactivation *in vivo* is regulated by environmental factors.

Acknowledgments—The authors thank Dr. G. J. Williams, III for the use of his facilities for exposing plants to various environmental conditions.

LITERATURE CITED

1. ANDERSON LE, AH MOHAMED, AR ASHTON, R SCHEIBE, T BRENNAN, D BEN-BASSAT 1981 Light modulation: the light effect mediator (LEM) system. *In* G Akoyunoglou, ed, Photosynthesis IV. Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia, pp 293–302
2. BUCHANAN BB 1981 Photosynthetic enzyme regulation by the ferredoxin/thioredoxin and the ferralterin mechanisms. *In* G Akoyunoglou, ed, Photosynthesis IV. Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia pp 245–256
3. CHAPMAN KSR, MD HATCH 1981 Regulation of C_4 photosynthesis: mechanism of activation and inactivation of extracted pyruvate,inorganic phosphate dikinase in relation to dark/light regulation. Arch Biochem Biophys 210: 82–89
4. EDWARDS GE, SC HUBER 1981 The C_4 pathway. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants: a Comprehensive Treatise, vol 8. Academic Press, New York, pp 238–281
5. EDWARDS GE, M UJIHIRA, T SUGIYAMA 1980 Light and temperature dependence of the rate and degree of activation of pyruvate,Pi dikinase *in vivo* in maize. Photosynthesis Research 1: 199–207
6. HATCH MD 1978 Regulation of enzymes in C_4 photosynthesis. Curr Top Cell Regul 14: 1–27
7. HATCH MD 1981 Regulation of C_4 photosynthesis and the mechanism of light/dark modulation of pyruvate,Pi dikinase activity. *In* G Akoyunoglou, ed, Photosynthesis IV. Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia, pp 227–236
8. HATCH MD, CR SLACK 1969 Studies on the mechanism of activation and inactivation of pyruvate,Pi dikinase: a possible regulatory role for the enzyme in the C_4 dicarboxylic acid pathway of photosynthesis. Biochem J 112: 549–558
9. HATCH MD, CR SLACK 1969 NADP-specific malate dehydrogenase and glycerate kinase in leaves and evidence for their location in chloroplasts. Biochem Biophys Res Commun 34: 589–593
10. HELMERHORST E, GB STOKES 1980 Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. Anal Biochem 104: 130–135
11. JACQUOT JP, BB BUCHANAN, F MARTIN, J VIDAL 1981 Enzyme regulation in C_4 photosynthesis. Purification and properties of thioredoxin-linked NADP-malate dehydrogenase from corn leaves. Plant Physiol 68: 300–304
12. JOHNSON HS, MD HATCH 1970 Properties and regulation of leaf nicotinamide-adenine dinucleotide phosphate-malate dehydrogenase and malic enzyme in plants with the C_4-dicarboxylic acid pathway of photosynthesis. Biochem J 119: 273–280
13. KAGAWA T, MD HATCH 1977 Regulation of C_4 photosynthesis: characterization of a protein factor mediating the activation and inactivation of NADP-malate dehydrogenase. Arch Biochem Biophys 184: 290–297
14. LEEGOOD RC, DA WALKER 1981 Activation of fructose 1,6-bisphosphatase in darkened intact chloroplasts by NADPH. Arch Biochem Biophys 212: 644–650
15. NAKAMOTO H, T SUGIYAMA 1982 Partial characterization of the *in vitro* activation of inactive pyruvate,Pi dikinase from darkened maize leaves. Plant Physiol 69: 749–753
16. SCHEIBE R, LE ANDERSON 1981 Thioredoxin as dark effect mediator of chloroplast enzymes. *In* G Akoyunoglou, ed, Photosynthesis IV. Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia, pp 265–271
17. SCHMITT MR, GE EDWARDS 1981 Photosynthetic capacity and nitrogen use efficiency of maize, wheat, and rice: a comparison between C_3 and C_4 photosynthesis. J Exp Bot 32: 459–466
18. SHIRAHASHI K, S HAYAKAWA, T SUGIYAMA 1978 Cold lability of pyruvate,orthophosphate dikinase in the maize leaf. Plant Physiol 62: 826–830
19. SUGIYAMA T, MD HATCH 1981 Regulation of C_4 photosynthesis: inactivation of pyruvate,Pi dikinase in leaf and chloroplast extracts in relation to dark/light regulation *in vivo*. Plant Cell Physiol 72: 115–126
20. SUGIYAMA T 1981 Light modulator of photosynthetic enzyme activity: large modulator protein. *In* G Akoyunoglou, ed, Photosynthesis IV. Regulation of Carbon Metabolism. Balaban International Science Services, Philadelphia, pp 281–291
21. WINTERMANS JFGM, A DE MOTS 1965 Spectrophotometric characteristics of chlorophylls and their pheophytins in ethanol. Biochim Biophys Acta 109: 448–453
22. YAMAMOTO E, T SUGIYAMA, S MIYACHI 1974 Action spectrum for light activation of pyruvate phosphate dikinase in maize leaves. Plant Cell Physiol 15: 987–992