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Mechanism of reductive photoactivation of enzymes of C_4 pathway

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Abstract. Light, besides initiating primary photochemical processes, alters the redox state of soluble components in chloroplast. The present review attempts to cover the mechanism of reductive photoactivation of enzymes of photosynthetic carbon reduction cycle using key enzymes as examples. The reduced soluble components — ferredoxin, thioredoxin and NADPH, in turn, cause the reduction of disulphides to dithiols of chloroplastic enzymes. NADP-malate dehydrogenase is subject to activation by light through changes in NADPH/NADP. The key enzyme of C_4 photosynthesis-PEP carboxylase, though cytosolic, has been shown to be activated by disulphide/sulphhydryl interconversion by reductants generated in light through chloroplast electron transport flow. Pyruvate P_i dikinase activity is controlled by the adenylate energy charge. It remains unclear how light controls the activation of cytosolic enzymes.

Keywords. C_4 enzymes; ferredoxin-thioredoxin system; photoactivation; photosystem I.

1. Introduction

Light provides the necessary free energy for integrating the disorderly materials into more orderly forms through a process called 'photosynthesis' — the products of which sustain almost every form of life on earth. Light affects almost every phase of a plant's life and also has extensive control over the developmental blue prints — the genes (Moses and Chua 1988). A number of genes, particularly those of the photosynthetic enzymes and various parts of the photosynthetic apparatus are known to be under the regulatory control of light (Tobin and Silverthorne 1985). Sequences, flanking the genes upstream the TATA box, have also been identified which regulate the expression of genes through photomorphogenesis (Moses and Chua 1988).

Light also controls the post-translational activities of a number of photosynthetic enzymes which is essential, not only for the efficient operation of photosynthesis under fluctuating environmental conditions, but also for the overall economy of the cell. Exhaustive efforts have been made to understand the role and mechanisms by which light regulates the post-translational activities of photosynthetic enzymes of both C_3 and C_4 plants (see reviews, Buchanan 1980; O'Leary 1982; Edwards *et al* 1985; Woodrow and Berry 1988).

The present review deals with and updates the information on the mechanisms, particularly the stromal factors and nucleotide mediated photoactivation of enzymes of C_4 plants. The mesophyll cells of C_4 plants, through an additional pathway, are engaged in the uptake and concentration of CO_2. This is nature's way of suppressing the oxygenase activity of ribulose 1,5-bisphosphate carboxylase/ oxygenase (RUBISCO). The reductive pentose phosphate (RPP) pathway, by which CO_2 is converted to carbohydrate, is common in both C_3 and C_4 plants. No

attempt, in the present review, has been made to cover an equally important aspect of light mediated deactivation of key enzymes of oxidative pentose phosphate pathway.

2. Ferredoxin/thioredoxin system

Research on the role of light on the activation of enzymes began in the latter part of the sixties. Initially, experiments were done using either algal cells or leaf extracts but later on, isolated intact chloroplasts as well as purified (partly and homogeneously) enzymes were employed to study the phenomenon of photoactivation (Buchanan 1980). The increase in activity of various enzymes was thought to be due to *de novo* synthesis of proteins in light. However, experiments with inhibitors of protein synthesis clearly demonstrated that the effect of light was post-translational (Melandri *et al* 1970).

Three stromal protein factors *viz.,* ferredoxin (Fd), thioredoxin (thio), and ferredoxin/thioredoxin reductase (F/T R) together constitute a system—the ferredoxin/thioredoxin (Fd/thio) system which, of late, has become the best documented and widely accepted system explaining the photoactivation of fructose 1,6 -bisphosphatase (Fru-P2 ase) (Nishizawa and Buchanan 1981; Maheshwari *et al* 1988, 1990a), sedoheptulose 1,7-bisphosphatase (Sed-P2 ase) (Breazeale *et al* 1978), phosphoribulo kinase (PRK) (Avron and Gibbs 1974; Wolosiuk and Buchanan 1978), NADP-glyceraldehyde 3-phosphate dehydrogenase (NADP-GADP) (Buchanan *et al* 1978), NADP-malate dehydrogenase (NADP-MDH) (Hatch *et al* 1984; Edwards *et al* 1985) and to some extent of phosphoenolpyruvate carboxylase (PEPCase) (Gonzalez *et al* 1986; Maheshwari *et al* 1990b; Maheshwari and Bhardwaj 1991). Light induced electron transport reduces Fd on the reducing side of photosystem I (PS I). The reduced Fd, inturn, reduces thio, the reaction being catalysed by ferredoxin-thioredoxin reductase. The Fd/thio system has been shown to be a reversible system, i.e., it can catalyse both reduction and oxidation of enzymes in light and dark, respectively (Leegood and Walker 1980; Maheshwari *et al* 1990a,b) and therefore, availability of electrons to Fd, on the reducing side of PS I, is crucial for maintaining the enzymes in an activated state. There is evidence of light induced increase in the -SH content of chloroplast particularly, of the stromal proteins and it has been kinetically correlated with the reductive activation of photosynthetic enzymes (Slovacek and Vaughn 1982; Slovacek and Monahan 1983). Bhardwaj and coworkers (Maheshwari *et al* 1988, 1990a,b; Maheshwari and Bhardwaj 1991) have provided evidence that a thiol mediator is essential for the reductive photoactivation of photosynthetic enzymes.

RUBISCO — which occupies the 'driver's seat' in the RPP cycle, is shown to be activated by another enzyme system—the RUBISCO activase, independent of the Fd/thio system (Salvucci *et al* 1985; Portis *et al* 1986). Recently, Campbell and Ogren (1990a, b) showed that, in addition to RUBISCO activase, RUBISCO may require an effector, generated in light, for its activation. It has been shown to require effectors like ATP, NADPH and intermediates of RPP cycle for its activation (Chu and Bassham 1973; Hatch and Jenson 1979). Similarly, the ratio of NADPH to NADP was found to exert significant control over the activation state of NADP-MDH particularly, when the chloroplast redox state remains unaltered (Edwards *et al* 1985; Rebeille and Hatch 1986a,b).

3. NADP-malate dehydrogenase

Hatch and Slack (1969) for the first time showed the presence of a NADP specific malate dehydrogenase (MDH) in leaves of both C_3 and C_4 plants. It is exclusively located in the mesophyll chloroplasts where it catalyses the reduction of oxaloacetate (OAA) to malate (Hatch and Slack 1969). The light induced changes in the activity of NADP-MDH were detected almost at the same time in both C_3 and C_4 plants (Johnson and Hatch 1970; Johnson 1971). Inhibition of photoactivation of NADP-MDH by DCMU indicated the involvement of chloroplast electron transport chain (Hatch 1977). Incubation of NADP-MDH in air without DTT resulted in total loss of activity of enzyme (Johnson and Hatch 1970). Kagawa and Hatch (1977) observed that activation of NADP-MDH at pH 7·5 was absolutely dependent on a regulatory protein. However, the rate of activation at pH 9 was also increased by the regulatory protein. Thus, the activation of NADP-MDH was largely or solely dependent upon this factor at pH 8 or below but apparently only partially regulatory protein dependent at pH 9 (Kagawa and Hatch 1977). The increase in the rate of activation above pH 8 may be physiologically significant since stromal pH rises in light. Vidal *et al* (1978) observed a similar requirement of a protein factor for the DTT dependent activation of NADP-MDH from C_3 plants. This heat stable, trypsin sensitive protein was later identified as thio *m* (Buchanan 1980; Hutcheson *et al* 1981). Ashton and Hatch (1983a), by their inhibitor studies, have shown that only the active form of enzyme possesses -SH groups which are essential for maintaining the activation status of NADP-MDH. Thiol binding reagents such as Hg^{2+} and *p*-chloromercuribenzoate (pCMB) and Cd^{2+} and equimolar mixture of arsenite and 2,3-dimercaptoethanol-reagents which specifically binds to vicinal dithiol groups (Webb 1966), inhibited the light driven photoactivation of NADP-MDH. The inhibition, so produced, could be readily reversed by the addition of excess DTT indicating an easy access and more affinity of enzyme towards this reagent (Ashton and Hatch 1983a). However, the inhibition produced by alkylating agents such as iodoacetate, iodoacetamide and N-ethyl maleimide (NEM) was reported to be irreversible (Ashton and Hatch 1983a).

The pool of reduced thio, *in vivo,* was found to increase by more than 300% in light as compared to dark (Rebeille and Hatch 1986b). The pool of reduced pyridine nucleotide (NADPH) also recorded an increase from 20%, its value in dark, to more than 90% in light in less than 1 min (Rebeille and Hatch 1986b). The redox potentials (Eo'), at pH 7, for the dithiol-disulfide systems of thioredoxin and NADP-MDH were calculated to be about –0·30 and –0·33 V, respectively suggesting that a high activity of NADP-MDH, *in vivo,* can be achieved only when the ratio of reduced to oxidized thio is high (Rebeille and Hatch 1986a). NADPH (K_d = 250 μM) was found to totally reverse the NADP induced (K_d = 3 μM) inhibition of the reductive photoactivation of NADP-MDH (Ashton and Hatch 1983b). It was suggested that probably both NADP and NADPH compete for the same site on enzyme—the covalent binding site and the site has greater affinity for NADP than NADPH (Ashton and Hatch 1983b). Therefore, addition of substances like OAA and phosphoglyceraldehyde (PGA), which upon reduction *in vivo* yield NADP, reduced the level of activation of NADP-MDH (Leegood and Walker 1983). NADP inhibits the reductive photoactivation of NADP-MDH presumably by preventing the reduction of regulatory disulfide groups (Rebeille and Hatch 1986a,b).

Both, thio and NADP are in competition for the electrons from Fd on the reducing side of PS I. However, according to one estimate, the flow of electrons from Fd to NADP is in an order of magnitude higher than the corresponding flux from Fd to thio. However, the generation of NADPH *in vivo* depends upon the availability of NADP which in turn may be controlled by a number of other metabolic reactions involving either NADPH or NADP as coenzymes. Under conditions when the ratio of NADPH/NADP overrides the redox state of thio, the former may become the prime controller of the activity of NADP-MDH (Rebeille and Hatch 1986b). Similarly, *in vitro* also, if the concentration of reduced thio is kept constant, high activity of NADP-MDH can be obtained only at very high ratio of NADPH to NADP (Rebeille and Hatch 1986a).

NADP-malate dehydrogenase is believed to be a homotetramer and considerable controversy still exists with respect to the molecular weight of NADP-MDH in its various forms (Edwards *et al* 1985). The amino acid composition of the homogeneous preparation from the maize leaves also showed variations (Jacquot *et al* 1981; Jenkins *et al* 1986). The native enzyme contained 0·9 and 4·7 thiols per sub-unit in its inactive and active (DTT treated) forms, respectively as calculated by DTNB titrations (Jenkins *et al* 1986).

4. Pyruvate, phosphate dikinase

Initially, light/dark transitions of pyruvate, phosphate dikinase (PPDK) were thought to be mediated by disulphide/sulphhydryl interconversion with additional involvement of P_i and some nucleotides (Hatch and Slack 1969). The photoactivation was inhibited by sulphhydryl group modifying agents (Hatch and Slack 1969). Sugiyama (1974) isolated a high molecular weight protein factor, sensitive to heat and trypsin treatment, which may possibly be involved in the photoregulation of PPDK. Amino acid analysis of the purified PPDK revealed the presence of 34 -SH groups in the enzyme tetramer (Sugiyama and Shirahashi 1976). Later on, experiments with leaf extracts (Chapman and Hatch 1981), isolated chloroplasts (Sugiyama and Hatch 1981) and partially purified enzyme (Nakamoto and Sugiyama 1982) demonstrated that PPDK does not require thiols for its activation and in fact, undergoes a rapid ADP mediated inactivation even in the presence of high concentrations of DTT (10 mM).

A protein factor, isolated and purified from the mesophyll cells, was found to be involved in the ADP dependent inactivation (Sugiyama and Hatch 1981) and P_i dependent activation of PPDK (Burnell and Hatch 1983). ADP mediates inactivation by phosphorylating threonine residue on the catalytically active site of the enzyme (Burnell 1984; Burnell and Hatch 1984a; Ashton *et al* 1984). ATP phosphorylates another amino acid—histidine, in the near vicinity of the regulatory threonine residue, on the catalytically active site of the enzyme (Burnell and Hatch 1984a). The phosphorylation of histidine is a prerequisite for the ADP dependent inactivation of PPDK (Burnell and Hatch 1984b). No inhibition, atleast with ATP, would be observed if the activation reaction is carried out with monophosphorylated (histidine) PPDK (Burnell and Hatch 1984b). Phosphoenol-pyruvate (PEP), by favouring phosphorylation at histidine, promotes inhibition while pyruvate has an opposing effect (Burnell and Hatch 1984a,b).

The P_i dependent activation involves unusual (other than simple phosphatase

mediated hydrolysis) removal of phosphate from threonine (Burnell and Hatch 1983). The adenylate energy charge, *in vivo,* controls both the P_i dependent activation (Nakamoto and Edwards 1983) and catalysis of activated PPDK. The adenylate energy charge value in mesophyll cells was measured to be 0·60 in dark (Kobayashi *et al* 1979) and 0·95 in light (Stitt and Heldt 1985). Inhibition of cyclic and non-cyclic photophosphorylation inhibited the light activation of PPDK further proving that adenylate level, in mesophyll cells, is the critical factor controlling the activation of PPDK (Nakamoto and Edwards 1986; Nakamoto and Young 1990). In a recent development, pyruvate which has earlier been shown to promote activation (Burnell and Hatch 1984b; Burnell *et al* 1986) was reported to inhibit the activation (Nakamoto and Young 1990). They also contradicted the earlier reports that transport of pyruvate, across the chloroplast envelop, and its level increase in light (Flugge *et al* 1985; Ohnishi and Kanai 1987a,b). Instead, the level of pyruvate, in illuminated chloroplasts, was found to decrease rapidly may be as a consequence of the reaction catalysed by PPDK itself (Nakamoto and Young 1990).

5. Phosphoenolpyruvate carboxylase

The increase in activity of phosphoenolpyruvate carboxylase (PEPCase) in light was detected as early as in 1968 (Slack 1968) and since then has been reported from various C_4 species (Karabourniotis *et al* 1983). Depending upon the species, the photoactivation of PEPCase was apparent either as a decrease in K_m (PEP) (Karabourniotis *et al* 1983, 1985) or an overall increase in the extractable activity (Karabourniotis *et al* 1983) or a change in the sensitivity towards the effectors malate and G-6-P (Huber and Sugiyama 1986). Inhibition of activation of PEPCase in light by DCMU, MV, CCCP and DSPD suggest the involvement of photosynthetic electron transport chain in the photoactivation of PEPCase (Karabourniotis *et al* 1983; Samaras *et al* 1988; Maheshwari *et al* 1990a,b; Maheshwari and Bhardwaj 1991). Many workers have reported that thiol groups are essential for the activity of PEPCase (Stiborova and Leblova 1983; Iglesias and Andreo 1984; Gonzalez *et al* 1986; Maheshwari and Bhardwaj 1991). Four -SH groups per tetramer have been reported to be present at PEP binding site on enzyme probably in close proximity to the region which binds with methylene group of PEP (Gonzalez *et al* 1986). Kameshita *et al* (1979), with *E. coli,* and Stiborova and Leblova (1985) with maize leaf enzyme have reported similar results. Studies using specific site directed chemical modifications have revealed the presence of amino acids cysteine, histidine, arginine and lysine at the active site of PEPCase (Stiborova and Leblova 1985).

PEPCase is reported to be activated by thiol reagents and inhibited by thiol binding reagents (Raghvendra and Vallejos 1982; Iglesias and Andreo 1984; Maheshwari *et al* 1990b). G-6-P was found to protect, by shielding the essential –SH groups, against the inhibition particularly, by pCMB (Manetas and Gavalas 1982; Ignesias and Andreo 1984). G-6-P also acts as a positive modulator (Iglesias *et al* 1986; Wagner *et al* 1987) and stabilizer of enzyme in the assay medium (Angelopoulas *et al* 1988).

Though, PEPCase is now generally known to be a tetramer with four identical subunits (O'Leary 1982; Andreo *et al* 1987), reports of its presence in different oligomeric forms are also available in the literature (Stiborova and Leblova 1983;

Walker *et al* 1986; Wagner *et al* 1987). The active tetramer, upon incubation with pCMB and diethylpyrocarbonate which, modify the cysteine and histidine residues, respectively, not only lost the activity but also showed dissociation into dimers and monomers suggesting that both cysteine and histidine are essential for maintaining the integrity and hence the activity of enzyme (Walker *et al* 1986). Similar results have been obtained by altering the pH (Stiborova and Leblova 1985) and incubating the enzyme with NaCl (Wagner *et al* 1987). PEG was found to activate, specifically, the dark extracted enzyme (Huber and Sugiyama 1986). It should be pointed out here that PEG promotes enzyme and/or subunit interactions by a water exclusion mechanism (Miekka and Ingham 1978).

Phosphorylation of one or more serine residues in the day form of enzyme has been proposed to be responsible for the differential behaviour of PEPCase, extracted in light and dark, towards malate inhibition (Budde and Chollet 1986; Nimmo *et al* 1987). The concentration of malate in the mesophyll cells is believed to be very high (Hatch 1978; Leegood 1985) and an apparent increase in K_i of malate upon illumination, due to phosphorylation of protein, would make the enzyme less sensitive to feedback inhibition by malate or, in other words, make the enzyme more active in light (Budde and Chollet 1986; Nimmo *et al* 1987).

It has been proposed that the activation state of PEPCase, *in vivo,* is dependent upon the availability of PEP at the carboxylation site which, in turn, is regulated by the rates of synthesis and utilization of PEP (Samaras *et al* 1988). PEP *in vivo* is generated by the synchronized operation of NADP-MDH and PPDK—two chloroplastic photoactivated enzymes (Edwards *et al* 1985). Utilization of PEP needs bicarbonate, which on the other hand, if present in higher concentration, would diminish the availability of pyruvate by preventing the decarboxylation of malate in bundle sheath cells (Rathnam and Edwards 1977). Thus, light and internal CO_2 could be the main determinants of both synthesis and use of PEP and in turn, of the activation of PEPCase.

Temperature also affects the activity of PEPCase (Selinioti *et al* 1987; Samaras *et al* 1988). Due to higher activation energy of reaction catalysed by PEPCase, a higher temperature (>25°C) is favourable for the optimal activity of PEPCase (Selinioti *et al* 1987). The change in temperature *in vivo,* during light/dark transitions, may account for the increased activity of PEPCase in light (Selinioti *et al* 1987).

6. Conclusions

The presence of an additional pathway, the dicarboxylic acid pathway, in C_4 plants allow them to process higher light intensities and to sustain at low CO_2 concentrations and higher O_2 concentrations than their C_3 counterparts. Though, the response of various enzymes to both, the internal conditions of cell as well as external factors like light, have been studied in great detail, there still remain problems and loose ends which need to be solved and tightened up.

The photoactivation of PEPCase from the various species of C_4 plants has been consistently reported over the years, however, a clear and cohesive picture of mechanism of its activation is not yet available. There are evidences for and against the involvement of disulphide/sulphhydryl interconversions mediated by photo-synthetic electron transport chain, as in the case with most other enzymes of PCR

cycle, in the light-induced activation of PEP Case. For it to be conclusively right, efforts will have to be made to establish a link between light signal generated in chloroplast and cytoplasmic PEPCase. Similarly, single protein mediated activation and deactivation of PPDK is peculiar and unique and therefore, calls for further research. The influence of adenylate energy charge status of cell on the ATP/ADP ratio and, in turn, on PPDK as well as the effect of ratio of NADPH/NADP on the Fd/thio system mediated photoactivation of NADP-MDH, *in vivo,* will have to be studied in greater detail.

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