CHAPTER IV

DISCUSSION

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Light is perhaps the most important environmental factor affecting the plant metabolism. Its effects on the enzymes of photosynthetic as well as well as non-photosynthetic pathways are well documented (Zucker, 1972; Buchanan, 1980). Photoregulation of many of the enzymes of these pathways has been reported to be phytochrome mediated (Smith, 1975; Sharma, 1985). However, there are other photoregulated enzymes for which phytochrome mediation has not yet been demonstrated. Although the photoregulation of the enzyme glutamine synthetase has been well documented (Guiz et al.1979; Evstigneeva et al, 1981; Hirel et al, 1982; Nishimura et al, 1982; Canovas et al, 1986), its phytochrome mediation has not been demonstrated. Only very recently Tingey et al.(1988) have shown phytochrome regulation of GS at m-RNA level. Photoregulation of the enzyme NAD kinase has been postulated since 1960s as the levels of NADP were found to be higher than NAD in light than in dark (Oh-hama and Miyachi, 1959; Oh-hama et al, 1963; Ogren and Krogman, 1965). Muto et al.(1981) have shown a 30% increase in NAD kinase activity following light exposure of excised leaves or protoplasts from pea and wheat. Tezuka and Yamamoto (1972) have reported in vitro phytochrome control of NAD kinase in terminal buds of pea seedlings but Hopkins and Briggs (1973) could not confirm their results. Thus, no clear evidence for the involvement of phytochrome in its regulation in vivo is available.

In the present study, the activity of both GS and NAD kinase from pea terminal buds was found to be several fold higher in light than in dark grown seedlings and it increased with increasing period

of light illumination. The activity of both GS and NAD kinase also increased after exposure to short pulse of red light. The red light mediated increase for both the enzymes was reversed to dark level by immediate exposure to far-red light. The red-far- red reversibility thus clearly indicated the involvement of phytochrome in light mediated regulation of both GS and NAD kinase. Increasing the period of exposure beyond 5 min showed a decrease in phytochrome response. This may be because, increasing the period of red light exposure may alter the ratio of Pfr/P_{total} which may determine the biological action rather than Pr or Pfr alone (Smith, 1981; Schmidt and Mohr, 1982). A cessation of the red light mediated increase in enzyme activity is expected on increasing the period of dark incubation since Pfr disappears through a first order destruction process or reverts to Pr by dark reversion (Butler et al, 1963; Butler and Lane, 1964).

A number of phytochrome responses are found to be age dependent. Phytochrome control of lipoxygenase (Mohr and Oelze-Karow, 1976) and peroxidase (Sharma et al, 1979) are reported to be age dependent whereas that of nitrite reductase was independent of seedling age (Sopory et al, 1983). In the present study phytochrome regulation of both GS and NAD kinase was found to be independent of the age of the seedling between 5-8 day.

Escape period from reversibility by far-red light was found to be only of 2-3 min eventhough the increase in enzyme activities occurred many hours later. A short escape period in contrast to delayed appearance of enzyme activity suggests the existence of a rapid coupling reaction connecting

Pfr action to gene activation and increased enzyme activity. Similar short periods of Pfr action have been reported for many other phytochrome mediated responses, where the final photoresponse occurs hours or days after giving red light treatment. For example, in case of red light stimulated elongation of maize coleoptile sections, the photoresponse escapes from reversibility within 45 sec of red light treatment, pointing to a rapid action of phytochrome on this response, which manifests a few hours later (Warner et al, 1981).

Certain phytochrome mediated responses can also be induced by repeated short exposures to red light over a period of time. In case of light inhibition of cucumber hypocotyl elongation the response was found to be induced by repeated short exposures to red light (Hillman and Purves, 1966). Similarly, in mustard seedlings anthocyanin content was found to increase with increasing number of red light exposures given at intervals of 6 h darkness (Lange et al, 1971). In the present study also, the red light mediated response for both the enzymes was increased with increasing the number of red light exposures at intervals of 24 h darkness. This kind of response indicates that following each red irradiation there must be a fall in Pfr level by either dark reversion to Pr or by destruction followed by Pr synthesis. Repeated red light exposure is expected to maintain a higher level of Pfr leading to more pronounced signal initiation and consequently higher level of enzyme synthesis. The increase in activity of both the enzymes with repeated red light exposures was also completely reversed to the dark control level if each red light exposure was followed immediately by far-red light.

Studies on phytochrome regulation of various enzymes with protein-synthesis inhibitors have revealed that de novo synthesis of proteins can occur on phytochrome action (Sharma, 1985). In the present study, red light mediated increase in the activity of both GS and NAD kinase was inhibited by cycloheximide and not by chloramphenicol. Similar inhibition by cycloheximide and not by chloramphenicol has been reported for white light mediated increase in GS activity in rice leaves (Hirel et al, 1982). Inhibition of red light mediated increase in both the enzymes by actinomycin D suggested that the de novo synthesis of enzyme protein is controlled at transcriptional level. It also suggests the involvement of cytoplasmic protein synthesis machinery for the new synthesis of both GS and NAD kinase eventhough light mediated increase in both GS (Hirel et al, 1982) and NAD kinase (Muto et al, 1981) are known to occur in chloroplastic fraction. Most chloroplast proteins are encoded on nuclear genes and synthesized on cytoplasmic ribosomes (Ellis, 1983; Schmidt and Mishkind, 1986) which are then transported into the chloroplasts (Cline, 1986). The de novo synthesis of a number of other phytochrome regulated proteins has also been reported to be controlled at the level of transcription (Silverthorne and Tobin, 1984; Fourcroy, 1986; Sharma and Schopfer, 1987). The results of the present study on GS are supported by the recent studies of Tingey et al. (1988) who examined the effect of light on the steady state levels of transcripts for chloroplastic and cytosolic GS in pea leaves. The levels of GS_2 (chloroplastic GS) mRNA increased with red light treatment of etiolated pea plants and this effect was reversed by a subsequent pulse of far-red light. The level of mRNA for GS_1 (cytosolic GS) was not affected

by red and red-far-red light treatments. It was thus concluded that the light mediated induction of GS_2 mRNA expression was mediated through phytochrome.

NAD kinase has been reported to be dependent on Ca^{+2} and Ca^{+2} -dependent regulator protein, calmodulin (Muto and Miyachi, 1977; Anderson et al, 1980). However, Ca^{+2} -calmodulin dependency of GS, if any, has not yet been reported. In the present study it was found that only NAD kinase is Ca^{+2} -calmodulin dependent whereas GS is not. Since phytochrome regulation of NAD kinase was found to be controlled at the level of protein synthesis and calmodulin being a protein molecule, the possibility of phytochrome regulation of calmodulin synthesis cannot be ruled out. However, measurement of calmodulin levels in the terminal buds from different light treated seedlings showed that the calmodulin levels from the seedlings treated with either red, red-far-red or white light were not significantly different from the dark control level. Dieter (1986) has also shown that calmodulin levels in corn coleoptile remain unaltered after continuous far-red light irradiation. The phytochrome mediated increase in NAD kinase was thus not due to changes in the level of calmodulin but due to new synthesis of enzyme.

A short period of Pfr action (about 2-3 min) in contrast to delayed appearance of the two enzymes (8-12 h after light treatment) suggests the existence of a signal chain which connects Pfr action to final photoresponse. This signal chain may have one or more secondary messenger molecules to link Pfr action at genome level to increase the synthesis of enzymes. Although, the signal transduction pathway has not yet been identified, preliminary evidences are accumulating in favour

of polyphosphoinositide mediated signal transduction in plants, as in animals (Poovaiah and Reddy, 1987). This pathway employs a combination of second messenger that includes Ca^{+2} , inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Calcium has been proposed to act as second messenger in a number of phytochrome mediated responses (Roux et al, 1986). It is suggested that the photoactivation of phytochrome rapidly leads to an increase in Ca^{+2} concentration in certain subcellular compartments and this in turn activates calmodulin and calmodulin dependent enzymes in these compartments. Some of these enzymes could then catalyze the activities that would result in photomorphogenesis. A large number of experimental evidences have accumulated in favour of this hypothesis. For example, involvement of calcium has been shown in phytochrome mediated chloroplast rotation in Mougeotia (Dreyer and Weisenseel, 1979) and spore germination in Onoclea (Wayne and Hepler, 1984, 1985).

The red light mediated increase in Ca^{+2} cocentration may be caused by an increase in Ca^{+2} influx from the outside, a release from internal stores such as mitochondria, endoplasmic reticulum and vacuoles, a slowing down of outward Ca^{+2} -pump or a combination of these factors (Hepler and Wayne, 1985). Red light induces a small depolarization with a lag period of less than 1 sec (Weisenseel and Ruppert, 1977; Newman, 1981). This depolarization may be sufficient to open voltage dependent Ca^{+2} -channels and allow the ion to enter the cell. Red light has also been reported to inhibit Ca^{+2} -ATPase (Serlin et al, 1984) which in turn decreases outward Ca^{+2} pump. Existence of calcium channels (membrane bound proteins which control the entry of calcium to cytoplasm) in plants has been proposed on the basis of binding of

calcium channel blockers such as nitrendipin to pea shoot membrane (Hetherington and Trewavas, 1984), verapamil to zucchini and corn membranes (Andrejauskas et al, 1985; Drakeford and Trewavas, 1986) and nifidipine to membrane of carrot cells (Gillery and Ranjeva, 1986). However, so far, the nature of calcium channels in the plants have not been identified. In a few cases, effects of Ca^{+2} channel blockers such as verapamil and $LaCl_3$ have been demonstrated to cause specific inhibition of Ca^{+2}-dependent processes. Verapamil has been reported to inhibit cytokinin-stimulated bud formation in moss Funaria (Saunders and Hepler, 1983), to inhibit cytoplasmic streaming in the alga Micrasterias (Lehtonen, 1984) and to inhibit phototaxis in the alga Chlamydomonas (Nultsch et al, 1986). La^{+3}has been reported to inhibit red light mediated germination of Onoclea spores (Wayne and Hepler, 1985).

The results of the present study showed that spraying of seedlings with $CaCl_2$ in dark increased both GS and NAD kinase activity but in red light treated seedlings calcium did not show any additional response. This indicates that red light mediated increase may be linked to the release of calcium from organelles or membranes. Also pretreatment of seedlings either with a calcium chelator (EGTA) or with calcium channel blockers (verapamil, diltiazem, flunarizine and $LaCl_3$) abolished the red light mediated increase of both GS and NAD kinase without any significant change in enzyme levels in dark or red-far-red light treated seedlings. These studies thus confirm the role of calcium in phytochrome mediated regulation of the two enzymes.

5-Hydroxytryptamine (5-HT), an animal hormone, which stimulates the hydrolysis of PIP_2 has been shown to mimic red light mediated

Ca^{+2}uptake in maize protoplasts suggesting that phytochrome might stimulate the hydrolysis of PIP_2 whose product IP_3 somehow opens the Ca^{+2} channels in the membrane (Das and Sopory, 1985). 5-HT has also been shown to substitute for light in case of gravitropic curvature of corn roots and IP_3 level increases on either 5-HT or light treatment (Reddy et al, 1987). IP_3 has been demonstrated to release Ca^{+2} from microsomes isolated from various plants (Drobak and Ferguson, 1985; Reddy and Poovaiah, 1987; Schumaker and Sze, 1987). In the present study also, 5-HT was found to mimic the red light mediated increase in both the enzymes in dark and this effect was abolished by EGTA indicating the role of calcium in 5-HT action. Moreover, the effect of both calcium and 5-HT was abolished by cycloheximide suggesting that their effect was at the level of enzyme synthesis and not activation of the existing enzymes. Both $CaCl_2$ and 5-HT mimicked the red light effect only partially possibly due to the fact that red light can modulate Ca^{+2} concentration in the cell by controlling either influx and efflux from plasma membrane and/or release and uptake from internal organelles whereas externally supplied calcium or 5-HT may release Ca^{+2} from internal stores by IP_3 produced on hydrolysis of PIP_2 by calcium dependent phospholipase C. However, outward Ca^{+2}-pump may not be regulated on external supply of calcium or 5-HT. Thus, this increase in Ca^{+2} concentration may not be sufficient for pronounced signal initiation. Phytochrome mediated responses may thus be transduced via polyphosphoinositide system to mobilize calcium from internal stores, which in turn affects at genome level to increase the synthesis of enzymes.

Since both phytochrome and polyamines mediate plant growth and development primarily through their interaction with membranes,

it is likely that polyamines may play an important role in regulating phytochrome mediated responses. A possible relation between phytochrome and polyamine has also been suggested since phytochrome induced growth is accompanied by parallel changes in polyamine levels (Goren et al, 1982a). Studies on the effect of polyamines on phytochrome regulation of GS and NAD kinase showed that red light mediated increase in both the enzymes was abolished whereas in dark and red-far-red light treated seedlings enzyme levels remained unchanged However, guanidines, which are structurally related to polyamines, mimicked red light mediated response in dark and the reversibility by far-red light was found to be decreased. Thus both polyamines and guanidines have opposite effects on phytochrome mediated increase in GS and NAD kinase. A similar antagonistic and agonistic effect of polyamines and guanidines, respectively, has also been earlier demonstrated from this laboratory for phytochrome mediated regulation of peroxidase in maize scutellum slices (Rajbabu, 1985). Effect of polyamines and guanidines may occur at any one or more of the following steps linking phytochrome action to enzyme synthesis.

(i) Polyamines may directly effect the photoconversion of Pr to Pfr. Although the light induced conformational changes in the phytochrome molecule are not yet identified, Wong et al. (1986) have shown differential phosphorylation of Pr and Pfr form of phytochrome by polycation-dependent protein kinase associated with phytochrome molecule which may play an important role in phytochrome mediated responses in plants. Pr form of phytochrome is a preferred substrate for its autokinase activity and a specific N-terminal serine residue of the phytochrome is phosphorylated. Polyamines may stimulate this polycation-dependent autokinase activity causing increased

phosphorylation of Pr form. Polyamines have been shown to stimulate Ca^{+2} -dependent nuclear protein kinase in isolated pea nuclei (Datta et al, 1987). Another possibility is that the binding of polyamines to protein moiety of Pr form may not allow the conformational change to occur on red light treatment, as polyamines have been shown to bind to protein molecules (Folk, 1980; Apelbaum et al, 1988).

(ii) Recently, it has also been suggested that the phytochrome has highly reactive sulfhydryl residues located on the portion of the protein moiety which undergoes a conformational change on photo-conversion of Pr to Pfr (Smith and Cyr, 1988). There are also evidences to show that polyamines bind to -SH groups (Naik and Srivastava, 1981; Srivastava and Smith, 1982a) and if such a binding occurs with phytochrome it may not allow the conformational change to occur during light stimulated conversion of Pr to Pfr.

(iii) Although the precise nature of Pfr binding to membrane is not known, a number of evidences suggest that Pfr form of phytochrome causes its primary action at the membrane level. Polyamines, which are known to bind to membrane phospholipids (Chung et al, 1985), may interfere with Pfr binding to membranes.

(iv) Pfr, as a result of its binding to membranes, releases Ca^{+2} ions either directly or via polyphosphoinositide pathway. An indirect evidence for the involvement of polyphosphoinositide pathway in phytochrome regulation has been provided by the studies of 5-HT which stimulates hydrolysis of PIP_2 and mimics the red light mediated regulation of calcium uptake in maize protoplasts (Das

and Sopory, 1985). It was suggested that Pfr may stimulate the hydrolysis of PIP_2 whose products somehow open the Ca^{+2} channels in the membrane. The hydrolysis of PIP_2 is brought about by phospholipase C whose activity has been shown to be inhibited by polyamines in animal cells (Wojcikiewicz and Fain, 1988). Thougn there is no evidence yet to show that the enzyme in plants is inhibited by polyamines but its inhibition, if demonstrated, will result in the cessation of phytochrome mediated release of Ca^{+2} ions through polyphosphoinositide pathway. Results of the present study, where increase of both GS and NAD kinase by 5-HT in dark was abolished in the presence of spermine, may indirectly suggest that polyamines affect polyphosphoinositide metabolism which in turn inhibits calcium release.

(v) Polyamines may directly block calcium channels and thereby the release of calcium from membranes since polyamines have been found to block drug induced calcium release from sarcoplasmic reticulum by blocking calcium channels at very low concentration (Palade, 1987).

(vi) Apart from inhibition of calcium release from internal stores, polyamines may also affect at certain intermediate steps of signal transduction pathway such as phosphorylation of proteins by protein kinases. Reversible protein phosphorylation represents a fundamental and ubiquitous mechanism in the regulation of cellular activities (Cohen, 1985). Ca^{+2} -sensitive and phospholipid dependent protein kinase (protein kinase C) plays a crucial role in signal transduction process (Berridge, 1984; Nishizuka, 1986). Moruzzi et al. (1987)

have shown that in human erythrocyte vesicles micromolar concentration of polyamines greatly interfere with the protein kinase C activation process by inhibiting the formation of the active membrane-associated enzyme complex. The primary polyamine binding sites within membranes appear to be acidic phospholipids. Among them binding of spermine to phosphatidyl‿serine, phosphatiditylinositol and polyphosphoinositides (PIP, PIP_2) has been demonstrated (Chung et al, 1985; Tadolini and Varani, 1986; Meers et al, 1986). The mechanism proposed for protein kinase C activation by phosphatidyl‿serine, diacylglycerol and Ca^{+2} suggest that first the binding of protein kinase C occurs to a cluster formed by four phosphatidylserine molecules and Ca^{+2}. The succeeding binding of diacylglycerol to this complex causes dramatic decrease in Ca^{+2} requirement, possibly owing to a reorganization of the intra-complex bonds. The electrostatic interactions of spermine with phospholipids may decrease the pool of phosphatidyl‿serine involved in the formation of binding sites (Moruzzi et al, 1987). Spermine aligning parallel to the membrane may bind with its four charges three to four phosphatidylserine molecules (Tadolini et al, 1985; Chung et al, 1985) forming a cluster with no binding properties for the enzyme. Phosphatidylserine activation of protein kinase C has also been reported in plants (Elliott et al, 1988) and Ca^{+2} -dependent protein kinase of wheat germ was found to be inhibited by polyamines (Polya and Micucci, 1985).

(vii) Finally, polyamine may affect at the level of enzyme synthesis. However, when time course of spermine spraying was studied it

was found that treatment with spermine was essential prior to light treatment which suggests that it may be affecting one of the rapid steps of signal transduction and not at the level of enzyme synthesis. Polyamines can affect at the transcriptional level as synthesis of certain m-RNAs occurs very rapidly after red light treatment in pea terminal buds (Kaufman et al, 1986). However, this possibility is less likely since polyamines are generally associated with increased RNA synthesis (Bagni et al, 1971; Guilfoyle and Hanson, 1973).

In contrast to polyamines, guanidines mimicked the phytochrome mediated increase in GS and NAD kinase activity. The effect of guanidines was abolished by EGTA, cycloheximide as well as spermine suggesting that they may also affect at the membrane level to increase the PIP_2 hydrolysis and consequently the release of calcium from internal stores or they may directly open the calcium channels in the membrane. Guanidines may also inhibit the conversion of Pfr back to Pr on far-red light treatment since the reversibility by far-red light was decreased on treatment with these compounds.

Although, with the data presented in the thesis, it is not possible to give the precise mechanism by which polyamines and guanidines may control the phytochrome mediated responses but further studies on the possible explanations given above may provide the answer to the mechanism.

Since leguminous plants, especially pea seedlings, are known to contain a very active cell wall bound diamine oxidase (DAO) (Smith, 1985b; Federico and Angelini,1986) it raises the question whether the

effect of exogenously supplied di- and polyamines in the present study was due to the compounds themselves or due to their breakdown products resulting from DAO action. 1,3- Diaminopropane, one of the products of reaction on spermidine and spermine, was found to be equally effective. Recent studies have shown that DAO in lentil epicotyls is phytochrome mediated (Angelini et al, 1988). The enzyme is inhibited by phytochrome. If this is true for the pea enzyme also then it may be expected that di- and polyamines may enter the cells intact. This aspect, however, needs further study.

Also, the inhibitory control by polyamines on the phytochrome mediated processes in the present study was obtained when the seedlings were exogenously supplied with the compounds, whether the polyamines present in the cell will give a similar response is not yet known. In this connection, it may be of interest to note that phytochrome has been found to increase arginine decarboxylase (ADC) activity, a key enzyme involved in the biosynthesis of polyamines (Dai and Galston, 1981) and inhibit DAO activity (Angelini et al, 1988). These controls would lead to a build up of polyamine level in cells which in turn would result in an inhibitory control on phytochrome function. However, the significance of this control on the overall growth and development of plant remains to be elucidated.