CHAPTER V

SUMMARY

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Light, apart from providing energy for photosynthesis, also influences plant growth and development, collectively termed as photomorphogenesis and a photoreceptive substance, "phytochrome", involved in the process has been well identified. Phytochrome exists in two photointerconvertible forms, the red (Pr) form absorb light maximally at 660 nm and is converted to far-red (Pfr) form which absorbs maximally at 730 nm and is reverted to Pr form. Pfr is regarded as the physiologically active form. Phytochrome controls various aspects of plant metabolism including photosynthesis, photorespiration, fat and starch degradation, nitrate assimilation, nucleic acid synthesis and degradation as well as secondary product synthesis at the level of specific enzyme activities. A hypothesis where Pfr interacts directly with the genome and regulates the enzyme levels was proposed but certain rapid effects of phytochrome which occur within few seconds or minutes could not be explained on this basis. It was then postulated that the primary action of phytochrome is at the membrane level which would lead to secondary action at transcription, translation or enzyme activation or inactivation leading to developmental changes. The existance of a secondary messenger system has also been proposed to link the primary action of phytochrome at membrane level to secondary responses at genome level.

Calcium has been proposed to act as second messenger in certain phytochrome mediated responses. It was proposed that the photoactivation of phytochrome rapidly leads to an increase in Ca^{+2}

concentration which then acts as a chemical signal. The signal that initiates the response may not be Ca^{+2} ion itself but the complex between Ca^{+2} and calcium binding proteins such as calmodulin. The Ca^{+2} -calmodulin complex can act either directly on the effector system or indirectly on a regulatory system, usually a protein kinase, which through phosphorylation promotes or inhibits the activity of other enzymes. Recently, the role of polyphosphoinositides has also been emphasized in signal transduction in plants. It is believed that the breakdown products of phosphoinositides act as additional messengers to release calcium from intracellular stores. Studies with 5-hydroxytryptamine, which increases the hydrolysis of polyphosphoinositides in animal system, have shown that it can also mimic the effect of red light and thus indirectly suggesting a role of polyphosphoinositides in the transduction of phytochrome mediated responses.

Polyamines and guanidines having an ubiquitious distribution, have now been implicated as regulators of various developmental processes in higher plants. Many of the biological functions of these compounds can be attributed to their cationic nature and their electrostatic interactions with polyanionic components in the cell. Since polyamines and guanidines appear to function by controlling membrane properties and phytochrome responses are mediated through membranes, it was of interest to investigate their interactions, if any, on the phytochrome regulation of enzymes.

Photoregulation of number of enzymes has been shown to be mediated by phytochrome. However, there are other enzymes for which phytochrome mediation has not yet been demonstrated. Nitrate assimilation is known

to be regulated by light at the level of enzymes -nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT). Light regulation of NR and NiR is known to be mediated via phytochrome but its involvement in photoregulation of GS has not been demonstrated. Similarly, NAD kinase, which catalyzes the conversion of NAD to NADP, has been shown to be photoregulated and in one report phytochrome regulation of NAD kinase in vitro has also been demonstrated but it was not confirmed by others.

In the present study attempts were made to investigate the involvement of phytochrome in photoregulation of glutamine synthetase and NAD kinase and its interaction with polyamines and guanidines in pea terminal buds.

The activity of GS and NAD kinase was found to be more in light than in dark and it increased with increasing period of white light illumination. A maximum increase of 4 and 17 fold respectively was observed in light than in dark. Phytochrome regulation of the two enzymes was then studied in 5-day old dark grown seedlings by exposing them to red light for 5 min or to red followed immediately by far-red light for 5 min and then keeping them in dark. Both GS and NAD kinase increased from 8-12 hr after exposure to red light. A maximum increase of about 65 and 175% was obtained over the dark control level at 30 and 48 h for GS and NAD kinase respectively. The red light mediated increase for both the enzymes was reversed to dark level by immediate exposure to far-red light for 5 min. The red-far-red reversibility clearly indicated the involvement of phytochrome

in light mediated regulation of both GS and NAD kinase. A 5 min exposure of red and far-red light was found to elicit a maximal response. The time course of escape from reversibility by far-red light was evident from 30-45 sec and a complete escape occurred at about 2-3 min after red irradiation. The escape period of about 2-3 min indicates the time of Pfr action. Increasing the number of red light exposures from 1 to 4 at an interval of 24 h darkness increased GS activity from 0.7 to 2.5 fold and from 1.8 to 4.7 fold for NAD kinase over the dark control level. However, if red light on each day was followed immediately by 5 min of far-red light the enzyme levels were reverted to their dark control level.

Effect of various protein synthesis inhibitors was studied to show whether the increases in enzyme activities were due to de novo synthesis or due to activation of the preexisting enzyme. It was found that red light mediated increase in both the enzymes was due to de novo synthesis controlled at transcription level.

Since NAD kinase is known to be activated by calcium and calmodulin, it is possible that phytochrome may regulate this enzyme by altering the calmodulin levels. Therefore, calmodulin levels were measured in terminal buds from seedlings treated with different lights. To assay the calmodulin levels, NAD kinase free from calmodulin was isolated from light grown pea terminal buds by blue-sepharose and DEAE-sephadex chromatography with 55 fold purification and 95% yield. Tissue calmodulin content was estimated by comparing it with pure bovine brain calmodulin. Calmodulin content did not significantly differ in dark,

red, red-far-red or while light treated seedlings. Thus light mediated increase in NAD kinase was not due to change in the level of calmodulin but due to enzyme synthesis.

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) suggested the existance of 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. So far, only calcium has been proposed to act as secondary messenger in certain phytochrome mediated responses. To understand, the role of calcium in phytochrome regulation of the two enzymes, effect of calcium and its various antagonists was studied. Spraying of $CaCl_2$ to dark grown seedlings was found to partially mimic the effect of red light but spraying with EGTA, completely abolished the red light mediated increase of both the enzymes without any effect on dark control levels. Calcium channel blockers (Verapamil, Diltiazem, Flunarizine and $LaCl_3$) also abolished the red light mediated increase in enzyme activities. These studies confirmed that calcium plays an important role in phytochrome regulation of the two enzymes. Spraying of dark grown seedlings with 5-HT, which is known to mimic the red light effect, showed increase in both the enzyme activities and this effect was abolished by EGTA indicating that it acts via calcium release from membranes probably through IP_3 and/or DAG. The effect of both $CaCl_2$ and 5-HT was abolished by cycloheximide and the enzyme levels remained at dark control level, suggesting that the effect of calcium is at the level of enzyme synthesis and not activation of the enzymes.

These studies thus clearly indicate that the effect of phytochrome on GS and NAD kinase is mediated via calcium which may affect at genome level to increase the synthesis of the two enzymes.

Effect of polyamines and guanidines was studied on repeated red light mediated increase in enzyme activities. The seedlings were sprayed with the compounds an hour before red light treatment on day 5, 6 and 7. The red light mediated increase in both GS and NAD kinase was abolished by polyamines whereas in dark and red-far-red light treated seedlings the enzyme levels remained unchanged. However, spraying of seeedlings with guanidines increased GS and NAD kinase activity in dark by 65 and 100% respectively over the dark control values. Guanidines had no effect on red light mediated increase. However, reversal by far-red light was not complete in presence of guanidines. Thus polyamines have an inhibitory control while the guanidines appear to mimic the phytochrome response.

Since phytochrome mediated increase in case of both the enzymes was modulated by calcium, studies were carried out to investigate the interaction of polyamines and guanidines with calcium and 5-HT. When $CaCl_2$ and 5-HT were sprayed alongwith spermine, the effect of both calcium and 5-HT was abolished for both the enzymes. Effect of GAA was also abolished by spermine, EGTA or cycloheximide suggesting that GAA may affect at membrane level to increase the release of calcium from the internal stores, which in turn affects the synthesis of enzymes. Since spermine is able to abolish the effect of either phytochrome, 5-HT, calcium or guanidines, its effect may be due to inhibition of

calcium release from membranes. Also a combination of either phytochrome, calcium, 5-HT or GAA did not have any additive response suggesting that they increase enzyme levels by modulating free calcium concentration.

Kinetic properties of partially purified NAD kinase were also studied. Enzyme requires the presence of $MgCl_2$, $CaCl_2$, ATP, NAD and calmodulin for maximal activity. 12.5 nM concentration of $CaCl_2$ was sufficient to give optimal enzyme activity. Lineweaver and Burk plot for the substrates NAD and ATP gave a Km of 0.11 mM and 0.28mM respectively. Enzyme was found to be inhibited by a calcium chelator (EGTA) as well as by calmodulin antagonists (TFP, R_{24571} and W_7) which was reversible by $CaCl_2$ and calmodulin respectively. Polyamines and guanidines had no effect on the enzyme activity in vitro.

The present study, therefore, unequivocally for the first time demonstrates that the photoregulation of GS and NAD kinase is mediated by phytochrome. Phytochrome mediated increase in enzyme levels was due to the de novo synthesis, controlled at the level of transcription. Calcium was found to act as secondary messenger in the phytochrome regulation of the two enzymes. Indirect evidences suggest that IP_3 and DAG may also be involved in the release of calcium. Polyamines act antagonistic to phytochrome response whereas guanidines act agonistically and their effects are probably at membrane level affecting some of the initial steps of signal transduction pathway. Though, the precise mechanism of interaction of polyamines and guanidines with phytochrome is not known several possible explanations have been discussed which need further studies.