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Photocontrol of Enzyme Activation and Inactivation [and Discussion]

H. J. Newbury and J. W. Bradbeer

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Photocontrol of enzyme activation and inactivation

By H. J. Newbury
Department of Plant Biology, University of Birmingham, P.O. Box 363, *Birmingham B*15 2*TT, U.K.*

During recent years the question of whether phytochrome regulates certain plant enzyme activities by influencing the rates of enzyme synthesis or by post-translational activation mechanisms has been vigorously debated. There is now good evidence that phytochrome can regulate concentrations of specific messenger RNA populations in some situations. However, the capacity to exert control at a transcriptional level in some systems does not necessarily preclude the possibility that regulation could occur at a post-translational level elsewhere, or even within the same cells. Theoretical considerations apart, the evidence for activation of plant enzymes by phytochrome is not generally strong. Some of the enzymes whose activity is known to be modulated by phytochrome have also been shown to possess post-translational control systems or exist in inactive forms so that the molecular possibilities for such modulation do seem to occur. The lack of direct evidence for the control of such processes by phytochrome may well reflect the technical difficulties involved in this sort of investigation.

Introduction

The plant photoreceptor phytochrome is responsible for mediating a very large number of developmental responses to light (Smith 1975). Many of these responses are clearly apparent without the need for specialized measuring equipment because they result in morphological changes within the plant. At the other end of the spectrum lie a group of molecular responses that may be less obvious but are presumably of great importance during plant photomorphogenesis. A large amount of work has been carried out on a variety of enzymes since it has been found that their extractable activities are increased when etiolated seedlings are exposed to a pulse of red light or to continuous far-red light (Smith *et al.* 1976, 1977; Schopfer 1977). It is part of the dogma of developmental biology that such molecular events represent the means by which the genome exerts its control over development.

When considering the possible mechanisms by which phytochrome may regulate the activities of plant enzymes we normally pose the question 'synthesis or activation?' There is now strong evidence to support the 'synthesis' model; that is that this photoreceptor increases the activity *in vivo* of some enzymes by increasing their rates of production *de novo* and hence their concentrations within plant tissue. There is a tendency to ignore the role of degradative processes in such models even though the degradation rate is of equal importance as the synthetic rate in determining the accumulated level of an enzyme. This is largely because of the technical difficulties associated with the measurement of this process. The term 'activation' is used in this context as an indication that increased extractable activity can, alternatively, result from the post-translational modification of enzyme molecules (figure 1). Activation may mean relief from inhibition. However, the original question 'synthesis or activation?' is to some extent misleading because it implies that the two modes of control are mutually exclusive. There is no reason *a priori* why phytochrome could not regulate both the rate of synthesis and

the rate of post-translational activation of a specific enzyme within a particular tissue. Indeed one could argue that this would allow a finer modulation of enzyme activity. Hence the strong evidence that demonstrates phytochrome control of the synthesis of some enzymes and other proteins should not blind us to the possibility that this photoreceptor may also regulate enzyme

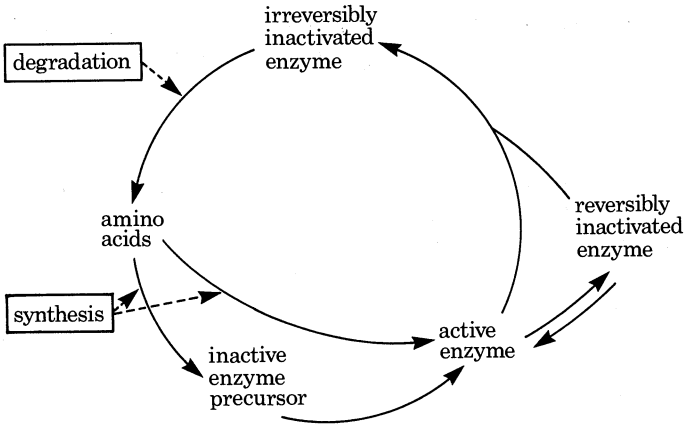

FIGURE 1. Scheme to show possible stages during enzyme turnover.

activities by post-translational mechanisms. However, if the evidence in favour of a role for phytochrome in the activation of plant enzymes is reviewed, the conclusion must be that it is at present very weak. The most obvious explanation for such a conclusion is that this method of control does not actually occur but there are other reasons that must also be considered.

TECHNICAL DIFFICULTIES IN THE DEMONSTRATION OF ACTIVATION

It is currently fashionable to investigate the controls of protein synthesis and gene expression so that perhaps the lack of evidence for phytochrome-regulated post-translational modifications partly reflects the neglect of this subject. There are good academic reasons to explain this neglect. If a research worker decides to investigate the possibility of phytochrome control of enzyme activation there seems to be no single experimental protocol that he can confidently adopt to obtain unequivocal results. Workers who wish to measure changes in the rate of synthesis of an enzyme can do so because they know quite a lot about the various stages of this process, i.e. the transcription of a particular gene followed by the translation of specific messenger RNA molecules. The more this system is investigated the more possible control points are discovered. However, a clear picture of the pathway that produces a specific protein is available and experiments can therefore be designed to measure rates of synthesis.

When, on the other hand, one wishes to test whether phytochrome regulates the post-translational modification of enzyme activities the relative ignorance of these processes is a severe handicap. Strong evidence for such control can only come from (1) an elucidation of the molecular mechanisms by which such enzyme modifications occur, and (2) a demonstration that the rate of modification is regulated by phytochrome. It is really the first of these that has caused problems because information derived from experiments involving eukaryotic organisms shows that there is a plethora of mechanisms by which activation or inactivation can occur.

Conformational changes within an enzyme can modify its activity, and these can occur, for example, as a result of the making and breaking of disulphide bridges. Such a system has been

demonstrated in higher plant chloroplasts where light generates reducing power, and transformations in the electron transport chain lead to the reduction of disulphide bonds in several photosynthetically important enzymes (Buchanan 1980). This leads to an activation of these enzymes. In this case phytochrome is not involved and the mechanism depends upon the trapping of light energy by chlorophyll. Enzyme activation has also been shown to occur by the removal of a small polypeptide by proteolytic cleavage (Neurath & Walsh 1976). This has been well documented for certain mammalian enzymes, especially serine proteases, although it is not clear if it occurs in the limited number of these enzymes identified in plants. Allosteric controls offer a further mechanism for the regulation of multimeric enzymes. Several of the enzymes under phytochrome control fall into this category including ascorbate oxidase and phenylalanine ammonia-lyase (PAL). In the latter case allosteric control has been shown to occur upon binding of certain phenolics (Boudet *et al.* 1971). Phytochrome control could therefore theoretically occur by modulation of the cellular concentrations of these compounds. In other cases active enzymes contain a metallic cation, and the presence or absence of this cofactor may represent a control point. Ascorbate oxidase contains copper, which can exist in several states. Both reversible and irreversible inhibitors have been shown to occur in plants, adding further possibilities to the list of post-translational control mechanisms (Wallace 1975). Because it is not possible to predict which of these types of modification may occur in the particular tissue under study it becomes extremely difficult to design satisfactory experiments to investigate enzyme activation.

Differential density labelling experiments

Some experiments have been designed specifically to distinguish between phytochrome regulation of enzyme synthesis and activation. Some of these involve the use of protein synthesis inhibitors and others use a combination of density labelling and CsCl gradient fractionation. Both methods have their critics and the latter has led to heated debate over the interpretation of results. A typical experimental protocol using differential density labelling as a measure of synthesis begins with seedlings growing in darkness in the presence of water. An extract is made from these seedlings; this is fractionated in a CsCl gradient, and the buoyant density of the band containing the enzyme under study is recorded. In parallel experiments seedlings are transferred to water containing a heavy isotope, commonly deuterium, and some seedlings are exposed to light and some maintained in darkness. After an incubation period extracts are made and again these are fractionated in CsCl gradients and the buoyant densities of the enzyme populations noted. The size of the density shift from the value for control enzyme (that was not exposed to density label) is said to be related to the rate of synthesis of enzyme. Thus if the labelled enzyme population extracted from seedlings exposed to light achieves a higher buoyant density than the labelled population extracted from etiolated seedlings then it is concluded that the enzyme is synthesized at a higher rate in light-treated seedlings. Refinements have been added to the technique but this is the rationale behind differential density labelling.

The technique has been used to compare density shifts of ascorbate oxidase extracted from mustard cotyledons raised in darkness or exposed to far-red light by two independent sets of workers (Attridge 1974; Acton *et al.* 1974). The groups broadly agreed that phytochrome controls the activity of this enzyme by regulating its rate of synthesis. However, after experiments with almost identical methods and tissues the same laboratories could not agree about the role of phytochrome in regulating the activity of PAL, with one group preferring the

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'synthesis' (Acton & Schopfer 1975) and the other the 'activation' model (Attridge *et al.* 1974). Interpretation of the results generated by this technique is a complex affair, and the controversy surrounding the PAL studies makes it very difficult for an independent reviewer to reach a clear conclusion on the basis of these results alone.

One weakness of the density labelling and CsCl gradient method as it has been used in the past is that density shifts of only the active forms of enzymes have been followed because gradient fractions have been assayed for catalytic activity. Any inactive form remains undetected although it may have great physiological significance. Critics of the technique state that results may be impossible to interpret unless it is known whether inactive precursor to the enzyme is either already present or is synthesized as a necessary stage in the production of active enzyme. Lamb & Rubery (1976) have said that differential (comparative) density labelling data of this kind cannot be properly interpreted without an understanding of the mechanism of enzyme 'turnover'. Some of these problems could be overcome if these techniques were to be combined with immunochemical detection of enzyme protein (see later) in gradient fractions rather than relying solely on assayable activity.

Density labelling experiments that employ deuterium oxide have also been criticised on technical grounds since it has been found that in one plant tissue deuterium leads to changes in some cellular membranes. Because the tonoplast is affected, vacuolar proteolytic enzymes are released into the cytoplasm and lead to a large increase in protein degradation rates (Cooke *et al.* 1979, 1980). If this effect is found to be more general then it will affect the interpretation of previous data obtained with mustard seedlings.

Phenylalanine ammonia-lyase in gherkin seedlings

Although differential density labelling experiments have been the subject of much controversy in some cases, the results seem only to lend themselves to one reasonable interpretation. This is true of the work carried out on the enzyme PAL in gherkin seedlings. If etiolated gherkin seedlings are exposed to continuous blue light a transient increase in extractable PAL activity occurs. However, after studies of density labelling *in vivo* it was found that active PAL was more heavily labelled if the seedlings were maintained in darkness than if they were illuminated by blue light (Attridge & Smith 1974); these results are most easily explained in terms of PAL activation. The rate of PAL synthesis seems to be lower after the illumination treatment than in darkness. It has been suggested that the higher PAL activity in the light leads to a build-up of reaction products, which inhibit enzyme synthesis (Johnson *et al.* 1975).

In this particular system there is also good evidence for a mechanism for the post-translation modification of PAL, leading to altered activity. A reversible inhibitor of this enzyme has been identified in gherkin hypocotyl extracts (Billett *et al.* 1978). It is non-dialysable, thermolabile, sensitive to proteolytic digestion, hydrophobic and has a molecular mass of less than 20 kDa. The inhibitor preparations will inhibit PAL from a number of plant sources and will also inhibit cinnamic acid 4-hydroxylase. Because this is the enzyme that catalyses the next reaction in the phenylpropanoid pathway and these two enzymes are often regulated coordinately in plants (Smith *et al.* 1967), the inhibitor seems to have a real physiological significance. It does not affect the activity of a wide range of other plant enzymes.

It may be that in this tissue photocontrol of PAL activity is achieved by regulation of the available concentration of inhibitor. To demonstrate this satisfactorily, additional evidence of

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the significance of the inhibitor *in vivo* is required. Protein synthesis inhibitor studies have already provided an indication that the PAL inhibitor must be continuously synthesized in gherkin seedlings to maintain its effect; treatment of etiolated seedlings with cycloheximide has been shown to lead to a massive increase in PAL activity (Attridge & Smith 1973). A more important feature missing from a proposed activation model in this system is an explanation of the way in which blue light might control the removal of inhibition. A proposal that this illumination may lower the rate of synthesis of the PAL inhibitor seems consistent with the limited experimental evidence but requires much more direct supporting evidence. The previous work on the regulation of PAL activity in gherkin seedlings has thrown up many interesting points and it is to be hoped that these will form the foundation of fresh work in this system.

Inactive enzymes

A proposal that the photocontrol of enzyme activity occurs, in some cases, by the post-translational regulation of the activity of enzyme molecules contains, by its very nature, a postulation that inactive or partly active forms of enzyme exist *in vivo*. Is there any direct evidence that they do occur? The bulk of the previous work in plant enzymology has quite naturally depended upon the catalytic activity of an enzyme for its detection, so that completely inactive forms of enzyme have remained completely undetectable. Immunochemical techniques do allow a recognition of these proteins when at least some antigenic sites are shared by active and inactive forms. Technically such investigations are time-consuming because of the necessity of raising, purifying and rigorously testing antiserum so that it reacts only with the protein under study.

Such experiments have been carried out on mustard ascorbate oxidase (Newbury & Smith 1981). Antiserum was raised against ascorbate oxidase from a *Cucurbita* species and was tested and purified by using this protein. Extensive tests showed that it cross-reacted with the enzyme from mustard cotyledons, and the simple technique of double-diffusion on agarose plates clearly demonstrated that the enzyme could be detected immunochemically in extracts from unimbibed seeds. These dry seed extracts exhibited no enzymic activity so that the only possible conclusion is that an inactive form of the enzyme does exist. No immunochemical differences could be distinguished between inactive enzyme extracted from seeds and an enzyme population with catalytic activity extracted from cotyledons exposed to light. To quantify the relative concentrations of ascorbate oxidase protein (active plus inactive forms) in extracts from mustard tissues a rocket immunoelectrophoresis method was used. When mustard seedlings were grown in darkness for 72 h the extractable activity of ascorbate oxidase in cotyledons reached a low but easily measurable level. If some seedlings were transferred to continuous far-red light at 36 h the cotyledon extracts showed markedly increased activity of the enzyme (figure 2*a*). When the same extracts were used for the immunochemical assay of ascorbate oxidase protein, the same concentrations were detected in both the etiolated and far-red light treated cotyledons and, indeed, in dry seeds (figure 2*b*). Unlike the results from density labelling experiments carried out on the same system (Attridge 1974; Acton *et al.* 1974) these data seem more consistent with photocontrol by activation of an inactive form.

However, this interpretation makes the assumption that the inactive form of the enzyme found in dry seeds is in fact a precursor to active enzyme *in vivo*; this may not necessarily be so. The inactive enzyme detected here could represent ascorbate oxidase that has previously exhibited

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activity during seed maturation but has subsequently been inactivated but not degraded. This concept of a time-lag between inactivation and degradation has previously been reported for chalcone synthase, which is detected in an inactivated form in parsley cells some time before it is degraded (Schroder & Schafer 1980). The accumulation of an altered form of β-fructofuranosidase that retained its immunochemical activity but lost its catalytic activity has been

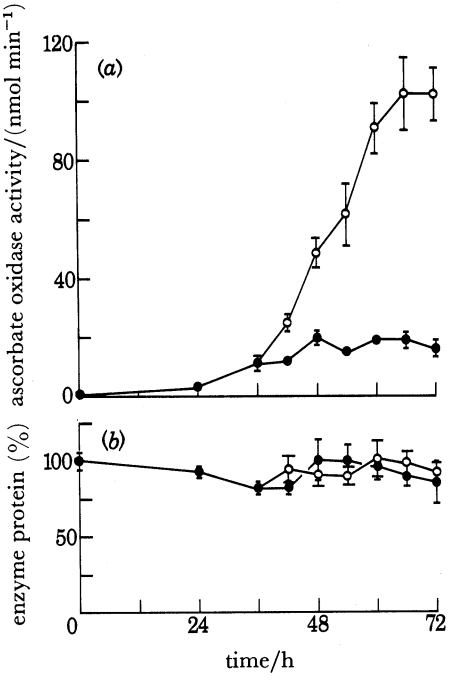

FIGURE 2. Levels of ascorbate oxidase activity and immunochemically detectable oxidase protein in mustard cotyledons. Mustard seedlings were grown in darkness (●) or transferred to far-red light after 36 h of darkness (○). Samples of cotyledon extract were desalted and assayed for ascorbate oxidase activity (*a*) or concentrated and subjected to rocket immunoelectrophoresis (*b*) to measure the amount of ascorbate oxidase protein, shown as the amount of enzyme protein per cotyledon pair as a percentage of the amount in the unimbibed seed. Standard errors are indicated.

reported in senescent tomato fruit (Nakagawa *et al.* 1980). Furthermore, ascorbate oxidase is reported to be an unusual enzyme in that it can be inactivated because of its catalytic action (Steinman & Dawson 1942). This 'reaction inactivation' is not caused by dehydroascorbate, the reaction product, but appears to be due to a highly reactive form of oxygen produced during the transformation. It seems possible that this process could be involved in the accumulation of immunochemically detectable inactive enzyme. At present the immunochemical data about this enzyme in mustard seedlings cannot be properly interpreted in the absence of information about the physiological significance of this inactive form. Once again a thorough understanding of the 'life-cycle' of an enzyme is lacking and work is in progress to clarify this situation.

THE SELECTIVE ADVANTAGE OF ENZYME ACTIVATION SYSTEMS

It is probably true to say that the cellular concentration of all plant enzymes is subject to regulation at the level of synthesis but that many enzymes have also evolved post-translational control systems. The discussion here is necessarily speculative and concentrates on the relative advantages of these two modes of control in various situations where a plant tissue changes some

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of its enzymic activities. One apparent advantage of an activation mechanism is the speed of formation of active enzyme. Activation may occur as a single step, whereas a dependence on *de novo* synthesis involves a time lag of several hours during which transcription and translation take place. In some developmental responses this may not matter because the plant tissue may have evolved an integrated and programmed control of gene expression that results in active enzyme becoming available at the exact time that it is required. It seems more likely that activation controls would be more effective in situations where the plant needs to respond quickly to an awaited environmental stimulus.

Seed germination is an interesting process in which to test these hypotheses. In this case either inhibition or an environmental dormancy-breaking signal triggers a large range of metabolic responses. There is evidence that some enzymes are present in seeds in inactive forms that are quickly activated early in germination (Shain & Mayer 1974). Germination and seedling establishment are critical stages in the life cycle of higher plants and it seems that the ability to mobilize certain enzymes rapidly by activation mechanisms represents a selective advantage. Other enzymes are synthesized *de novo* in germinating seeds; the most intensely studied is probably α-amylase, which is produced in barley aleurone cells after the release of gibberellic acid by the embryo (Filner & Varner 1967; Higgins *et al.* 1976). However, there is limited evidence for a third group of enzymes of which a cotton seed protease is an example. Here the messenger RNA for this enzyme is synthesized during seed maturation but is stored in the dry seed and only translated to yield the active enzyme during seed germination (Ihle & Dure 1969, 1972). In this system the delay caused by gene transcription during germination has been removed.

Attempts to relate these concepts to our understanding of the photocontrol of enzyme activities prove extremely difficult. In the germination system it is usually possible to relate changes in enzyme activity to physiological processes occurring within the seed. Many of the enzymes studied are hydrolytic in function and are involved in the degradation of seed storage reserves. In most of the studies involving phytochrome regulation of enzyme activity, seedlings have been transferred from darkness to red or far-red light; this has triggered an increase in the activity of some enzymes but it is usually very difficult to relate this in any causal way to changes that occur within the seedling. The strict catalytic functions of nitrate reductase, phenylalanine ammonia-lyase and ascorbate oxidase may be known but the developmental significance of their increases in activity during photomorphogenesis is not understood. Because of this, speculations on the relative advantages of 'activation' or 'synthesis' modes of control in these situations are not likely to be very profitable.

Conclusions

If we use the rigorous criteria outlined earlier that require (1) an understanding of the mechanism of post-translational modification, and (2) a demonstration that phytochrome, or the blue light receptor, controls the rate of these modifications, then there does not seem to be a single example of photocontrol (excluding chlorophyll-mediated mechanisms) of plant enzyme activation. However, this need not be because such mechanisms do not exist. Indeed there are some experimental systems that appear very worthy of further study.

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Discussion

J. W. Bradbeer (*Department of Plant Sciences, King's College London, U.K.*). Dr Newbury only gave a very small mention of the chloroplast carbon pathway enzymes known to be synthesized by dark-grown etiolated leaves and to have their synthesis promoted by phytochrome, although those carbon pathway enzymes that are subject to activation are activated by chloroplast metabolites and not by phytochrome. The state of knowledge with respect to the carbon pathway enzymes has been obtained from the application of a very wide range of techniques.

H. J. Newbury. I recognize that the group of enzymes to which Professor Bradbeer refers represent a well characterized example of light-induced post-translational activation in plants; my treatment of this area has been rather cursory not because this is not an interesting and important system for study but because the meeting has mainly been directed towards a discussion of photoreceptors not directly related to the photosynthetic process. Apart from being of general interest to students of plant molecular biology, research into the chloroplast carbon pathway enzymes has provided one of the few examples where the actual mechanism of post-translational modification has been properly elucidated.

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