Control by Phytochrome of the Level of Nicotinamide Nucleotides in the Cotyledons of the Mustard Seedling

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Long-term and short-term effects of phytochrome on the levels ("tissue contents") of NAD, NADH, NADP and NADPH were measured in the cotyledons of the mustard (Sinapis alba L.) seedling. It was found that long-term far-red light (which is considered to operate exclusively via active phytochrome, Pt), strongly increases the levels of NADP and NADPH, whereas this light treatment suppresses the levels of NAD and NADH below the levels present in the cotyledons of the dark grown seedling. The high levels of NADP and NADPH as well as the low levels of NADPH do not significantly respond. In the case of NAD and NADP, no significant changes could be induced by light pulses either in dark-grown seedlings or in seedlings pretreated with long-term far-red light. It is concluded that NADPH is not a direct result of NADP, nor from NAD.

It is further concluded that it is unlikely that nicotinamide nucleotides are links in any causal chain originating from Pt and leading to phenomena of photomorphogenesis. We favour the concept that the phytochrome-mediated changes caused by light pulses occur in the plastids.

Material und Methods

Standard techniques for photomorphogenic research with mustard seedlings were used. The seeds of Sinapis alba L. were purchased in 1969 from Asgrow Company (Hamburg, Germany). The

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** Abbreviation: Pt, far-red absorbing form of the phytochrome system.

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seedlings were grown at 25.0 ± 0.2 °C in the dark and experiments were started 36 h after sowing which is taken as time zero in the figures.

The standard far-red source 6 which maintains a low Pfr/Ptotal ratio in the seedlings 7,8 was used at an irradiance of 3.5 W·m⁻². The standard red source 6 which maintains a Pfr/Ptotal ratio of about 0.8 ⁷ was used at an irradiance of 675 mW·m⁻². Under continuous far-red light (onset 36 h after sowing) a steady state of the photosystem can be maintained in the mustard cotyledons and hypocotyl hook over at least 11 h ⁸. Continuous far-red light is considered to operate exclusively via phytochrome (Pfr) ¹⁻⁹. The time requirement for the shift from red light to far-red light and vice versa was approximately 1 min.

The use of the biological unit “pair of cotyledons” as a system of reference for the biochemical data was justified previously (cf. ¹). The major arguments in favour of the biological unit as a system of reference are the following: 1. During the experimental period (between 36 and 60 h after sowing) there is no significant increase of the DNA contents in the cotyledons ¹⁰. For this reason the biological unit “cotyledon” can be used as a system of reference instead of “cell” or “unit DDA”. There are enzymes, e.g. isocitrate lyase ¹¹, in the mustard cotyledons whose temporal development, i.e. increase and decline of extractable activity, is not influenced by phytochrome even if continuous far-red light is applied throughout the experimental period. The occurrence of enzymes whose rise and fall of extractable activity is phytochrome-independent shows that the phenomena of photomorphogenesis (photosresponses) are specific and that phytochrome control of development of an organ does not affect every aspect of metabolism and not even protein synthesis as a whole.

The data to be reported represent the nicotinamide nucleotide contents of whole cotyledons (“tissue contents”) without considering compartmentation and in situ binding of the nucleotides to protein. These disadvantages cannot be overcome at present since more sophisticated indirect measurements of the redox state of the NAD- and NADP-couples in rat liver cytoplasm and mitochondria ¹²,¹³ cannot readily be applied to plant tissue. Moreover the significance of these results and deductions is still under debate ¹⁴.

Extraction and determination of nicotinamide nucleotides: 40 seedlings were harvested under dim green safelight and 20 seedlings each were selected for extracting the oxidized or reduced nucleotides respectively. The organs (pairs of cotyledons, hypocotyls and roots) were isolated and frozen to liquid nitrogen temperatures in a precooled 9 ml teflon cell. The time needed for isolating the organs was about 15 min. The frozen organs were reduced to a powder together with a 9 mm tungsten carbide ball (pre-coloured) for 20 sec in a Micro-Dismembrator (Braun Melsungen A.G., Melsungen, West Germany). Extraction was accomplished by adding 3.5 ml 50% alcoholic 0.1 N HCl (oxidized nucleotides) or 3.5 ml 50% alcoholic 0.1 N KOH (reduced nucleotides) and immediately resuspending for 10 sec. The then liquified extracts were quantitatively transferred to centrifuge tubes and kept at 60 °C for 5 min. They were subsequently cooled to 0 °C. After centrifugation for 10 min at 39 100 × g and 0 °C, 2.5 ml of the supernatant was removed and 0.5 ml buffer (0.5 M triethanolamine, 0.4 M KH₂PO₄, 0.1 M K₂HPO₄·3 H₂O) added. The buffered extract of the oxidized nucleotides was adjusted to pH 7.2 – 7.4 with 6 N KOH; the extract of the reduced nucleotides was adjusted to pH 7.4 – 7.6 with 1 N HCl. After centrifugation for 10 min at 39 100 × g and 0 °C the clear supernatant was decanted and used in the assay.

Nucleotide determination

NAD (H⁺) and NADP (H⁺) were assayed after a modified method of Slater and Lawyer ¹⁵. The oxidized nucleotides allow the oxidation of alcohol with alcohol dehydrogenase (NAD) or the oxidation of glucose-6-phosphate with glucose-6-phosphate dehydrogenase (NADP). The nucleotides which are reduced during these reactions are reoxidized through phenazine-methosulfate while the hydrogen is accepted by 2,6-dichlorophenolindophenole. The reduction of 2,6-dichlorophenolindophenole was proportional to the concentration of NAD + NADH₂ or NADP + NADPH₂. The assays were carried out in semimicro cuvettes at 25 °C and the reactions followed with an Eppendorf photometer (Hamburg, West Germany) at 546 nm. The concentrations of the nucleotides were calculated from standards run at the same time. The reaction mixtures for NAD (H⁺) contained in a test volume of 1.06 ml: NADH₂ was assayed after a modified method of Slater and Lawyer ¹⁵. The oxidized nucleotides allow the oxidation of alcohol with alcohol dehydrogenase (NAD) or the oxidation of glucose-6-phosphate with glucose-6-phosphate dehydrogenase (NADP). The nucleotides which are reduced during these reactions are reoxidized through phenazine-methosulfate while the hydrogen is accepted by 2,6-dichlorophenolindophenole. The reduction of 2,6-dichlorophenolindophenole was proportional to the concentration of NAD + NADH₂ or NADP + NADPH₂. The assays were carried out in semimicro cuvettes at 25 °C and the reactions followed with an Eppendorf photometer (Hamburg, West Germany) at 546 nm. The concentrations of the nucleotides were calculated from standards run at the same time. The reaction mixtures for NAD (H⁺) contained in a test volume of 1.06 ml: 0.17 mmol phosphate buffer pH 7.4; 0.857 mmol ethanol; 0.185 µmol 2,6-dichlorophenolindophenole; 0.8 µmol phenazine-methosulfate; 60 µl alcohol dehydrogenase. For NAD 20 µl - 200 µl extract and for NADH₂ 50 µl - 200 µl extract were added and the difference in volume was adjusted by adding the appropriate amount of neutralized 50% alcoholic 0.1 N HCl or neutralized 50% alcoholic 0.1 N KOH. The reaction mixtures for NADP (H⁺) contained in a test volume of 1.02 ml: 0.07 mmol tris buffer pH 8.0; 0.185 µmol 2,6-dichlorophenolindophenole; 0.8 µmol phenazine-methosulfate; 2 µmol
glucose-6-phosphate; 1.4 U glucose-6-phosphate-dehydrogenase. The test mixture for NADP contained $2 \times 10^{-2}$ mmol neutralized 50% alcohol 0.1 N KOH. 50 µl – 200 µl extract were used for both NADP and NADPH₂ and the difference in volume was adjusted by adding the appropriate amount of neutralized 50% alcoholic 0.1 N HCl or neutralized 50% alcoholic 0.1 N KOH.

The following results were obtained in methodological experiments:

1. The kinetics of the test reaction are linear over 20 min. The velocity of the reaction is proportional to the nicotinamide nucleotide concentration up to 0.15 nmol/reaction mixture.

2. The recovery of standards added during the extraction was between 94% and 102% and proportional to the amount added.

3. Mixing experiments show that light treatment does not influence the assay of the nicotinamide nucleotides through formation of inhibitors or activators. Standards added to the reaction mixture are additive.

4. The nicotinamide nucleotide concentration is proportional to the number of seedlings or organs.

The average values given in the Figures are based on 8 – 12 independent experiment. Standard errors, as indicated by the bars, are in the range of 2 to 7 per cent.

**Results**

1. Long-term exposure of the seedlings to standard far-red light (onset of light at 36 h after sowing). Continuous standard far-red light which is considered to operate exclusively via phytochrome ($P_{fr}$)¹, ¹⁶ leads to the following effects on the level of nicotinamide nucleotides in the cotyledons:

   The levels of NADP and NADPH₂ are strongly increased as compared to those in the dark-grown seedling (Figs 1, 2); the increase of the levels of NAD and NADH₂ is retarded as compared to those in the dark-grown seedling (Figs 3, 4). If the far-red light is turned off at 12 h after the onset of light there is a rapid drop in the levels of NADP and NADPH₂ (Figs 1, 2). In the case of NADH₂ the level is increased after the light was turned off (Fig. 4) while in the case of NAD no significant change could be found (Fig. 3).

2. Short-term exposure of the seedlings to standard red and far-red light (5 min light pulses). Figs 2 and 5 show that a red light pulse causes a rapid but transient rise in the level of NADPH₂. This effect can always be obtained irrespective of the time of treatment. However, if the seedling was pretreated with long-term far-red light the peak rise is higher. Since the operational criteria for the involvement of phytochrome are clearly fulfilled (reversion of the inductive effect of a red light pulse by immediately following with a far-red light pulse)

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![Fig. 1. Kinetics of NADP contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h (following the sequence: 12 h far-red + 6 h dark).](image-url)
Fig. 2. Kinetics of NADPH\textsubscript{2} contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at 18 h following the sequence, 12 h far-red — 6 h dark.

Fig. 3. Kinetics of NAD contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h.

Fig. 4. Kinetics of NADH\textsubscript{2} contents in the mustard cotyledons in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h and following the sequence 12 h far-red — 6 h dark.
it can be concluded that the appearance of Pfr rapidly leads to a transient increase of the NADPH$_2$ level in the mustard cotyledons. At this point it must be remembered (cf. Methods) that for technical reasons there is a time lag of approximately 15 min between the end of the light treatment and the freezing of the isolated cotyledons to liquid nitrogen temperature. This time lag which cannot be shortened further precludes more precise statements about the actual rapidity of the Pfr-mediated response.

In the case of NADP (Fig. 1) a significant rise of the level following a red light pulse could only be observed if the seedlings were pretreated with long-term far-red light. Dark-grown seedlings do not respond significantly at either 36 h after sowing or at 54 h after sowing. In the case of NAD and NADH$_2$ (Figs 3 and 4) no significant changes could be induced by light pulses either in dark-grown seedlings or in seedlings pretreated with long-term far-red light.

3. Nicotinamide nucleotide levels in hypocotyl and radicle (taproot). The changes caused by long-term far-red light in the cotyledons can be observed in the other major organs of the mustard seedling only as a tendency. As an example Figs 6 and 7 show the amounts of NADPH$_2$ and NADH$_2$ in hypocotyl and radicle of the dark grown and far-red light grown mustard seedling. We notice that in the radicle (which has no potential to develop chloro-

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Fig. 5. Kinetics of NADPH$_2$ contents in the mustard cotyledons (cf. Fig. 2). Light pulses (5 min each) with red and/or far-red light were given at time zero and at 18 h in the dark.

Fig. 6. Kinetics of the NADPH$_2$ contents in the mustard hypocotyl and radicle in the dark and under continuous far-red light (onset of light at 36 h after sowing = time zero).
The present work shows that long-term far-red light which is considered to operate exclusively via phytochrome (Pfr) \(^1\) controls the levels of NAD, NADH, NADP and NADPH in the mustard seedling cotyledons. While the “mechanism” of this control is still obscure the data suggest that the increase of NADP and NADPH mediated by far-red light is related to the far-red mediated growth of the plastids \(^17\). We assume that the NADP and NADPH “induced” by far-red light is predominantly located in the growing plastids. The suppressive effect of far-red light on the level of NAD and NADH is possibly related to the fact observed previously \(^18\) that long-term far-red light transiently depresses the rate of respiration of the mustard seedling cotyledons.

Figs 1 and 2 document that the high level of NADP and NADPH is maintained by the far-red light. If the far-red light is turned off, the level decreases and nearly reaches the dark level within 8 h. In the case of NADH (Fig. 4) it is also obvious that the low level in far-red light is maintained by the light since the level tends to increase as soon as the light is turned off. It is concluded that the actual level of the nicotinamide nucleotides in the mustard cotyledons is determined throughout the experimental period by the rates of synthesis and destruction. The data are consistent with the view that far-red light increases the rate of synthesis in the case of NADP and NADPH whereas the light increases the rate of destruction in the case of NADH (and possibly of NAD). It is well known \(^{12-14}\) that the interpretation of the measured ratios of reduced and oxidized forms of the nicotinamide nucleotides remains a matter of debate even in the case of liver tissue in particular since the redox ratios of free nicotinamide nucleotides associated with various dehydrogenase reactions in the cytoplasm and in the mitochondria are different. For technical reasons in the present work only the “tissue contents” of the nicotinamide nucleotides could be determined. Therefore the notorious difficulties — the distinction between free and bound nucleotides and the uneven distribution within the cell (cytoplasm, mitochondria, plastids) — could not be overcome. Thus any attempt to interpret the measured ratios of the NAD- and NADP-couples at the present stage of our work would probably lead to an overinterpretation of the data. One may only notice that the ratios for the NADP-couple (of the order of 4 — 5) and for the NAD-couple (of the order of 1/10 — 1/20) differ greatly. This confirms the general observation, e.g. with liver tissue using the substrate couple method \(^{12,13}\), that the NADP-couple is much more reduced in situ than the NAD-couple.

Concerning the effects of light pulses we confirm previous reports that red light pulses will raise the in situ levels of NADP and NADPH. However, we question the interpretation given previously \(^3,4\) that the effect of a red light pulse is to be attributed to a phytochrome control of NAD kinase since no corresponding decreases of the NAD and/or NADH levels could be detected. At least in the case of NADH the decrease of the level would easily be detected if the rapid rise of the NADPH level was due to a rapid phosphorylation of NADH. While the interpretation of the previous investigators favouring a phytochrome activation of NAD kinases is probably not correct at least in case of the mustard seedling, it is difficult to suggest an alternative mechanism for the rapid rise of the NADP and NADPH levels upon the formation of Pfr. At present no substantiated suggestion for the underlying mechanism can be made.

The results of the present paper seem to exclude NAD and NADH as links in any causal chain be-
between Pfr and the final biochemical photoresponses such as end product accumulation, enzyme "induction" and enzyme "repression" (cf. 1). The fact that the NAD-couple does not significantly respond to light pulses makes it unlikely that it is a direct link (close to Pfr) in some causal chain between Pfr and the final photoresponses.

In the case of NADP/NADPH2 the fact is that both forms of the couple respond strongly and rapidly upon the formation of Pfr in the system by a red light pulse. The response is positive in both cases. This excludes the possibility that NADPH2 originates from NADP as suggested by in vitro data recently reported by Manabe and Furuya 4. We recall (Fig. 1) that the NADP level does not significantly respond if the red light pulse is given to a dark-grown seedling which has not received any light treatment before. It is only the NADPH2 level which responds to Pfr under all circumstances in principally the same manner. Thus one may consider only NADPH2 as a candidate for a link in the causal chain originating from Pfr. However, we must recall that the NADPH2 level changes with endogenous rhythms 20-22 and with variation in many environmental conditions (cf. 3). In addition by its very nature as a basic metabolite with rapid turnover NADPH2 would not be a likely candidate for a very specific 1 and very precise 23, 24 metabolic transformation of the Pfr signal eventually leading to photomorphogenetic phenomena. Therefore an alternative interpretation of the Pfr effect upon the NADPH2 level must be considered.

We suggest that the changes of the NADP-couple following red light pulse treatments reflect a control by phytochrome of some parts of the photosynthetic apparatus. Most of the constituents of the photosynthetic apparatus except chlorophyll are already present in the plastids of dark-grown or far-red light grown mustard seedlings (cf. 1). There are other data in print which possibly require a similar interpretation, e.g., the red far-red antagonistic effects observed by Michel and Thibaut 25 in kinetic studies on in vivo ATP synthesis in detached Zea mays leaves. The data suggest that photosynthetic phosphorylation is under the control of phytochrome.

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