Involvement of Thiol Groups in Blue-Light-Induced Phosphorylation of a Plasma Membrane-Associated Protein from Coleoptile Tips of *Zea mays* L.

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Light-induced phosphorylation of a 114 kDa protein in plasma membranes isolated from the tips of maize coleoptiles was investigated in the presence of several thiol reagents at the concentration of 1 mM. Dark phosphorylation of the protein was not affected but light-induced phosphorylation was inhibited 50% with iodoacetamide, 75% with N-ethylmaleimide and 93% with N-phenylmaleimide. Previous incubation of the inhibitors with mercaptoethanol abolished the inhibitory activity completely. N-phenyl-maleimide showed the same inhibition whether it was applied before or after irradiation of the sample. Involvement of thiol group(s) in processes after photoexcitation is discussed.

Introduction

Protein phosphorylation is involved in regulation of a great variety of cellular functions (see Krebs, 1994). A plasma membrane-associated protein that becomes phosphorylated on blue light exposure of etiolated tissues or membrane fractions isolated from them has been shown by physiological and genetic evidence to be involved in phototropism of many plants (review: Short and Briggs, 1994). In maize seedlings, this protein is concentrated in the coleoptile tip (Hager and Brich, 1993; Palmer *et al.,* 1993a, b), the region of the coleoptile with the highest sensitivity for phototropic stimuli (Sierp and Seybold, 1926; Lange, 1927). Hager *et al.* (1993) found that the degree of phosphorylation of this protein depended on the redox status of the membrane preparation. Plasma membranes when stored frozen or treated with hydrogen peroxide showed only a low level of light-dependent phosphorylation: higher levels of phosphorylation could be restored with reducing compounds such as NADH, NADPH, ascorbate, glutathione or dithiothreitol. The authors hypothesized that the transfer of excited electrons from the chromophore of the photoreceptor to the target should be facilitated by the reducing compounds. However, they did not discuss the nature of the presumed target. It is not known whether the protein contains prosthetic groups or cofactors. Apart from such moieties, cysteine residues are possible candidates for redox processes. We describe here inhibitor studies that showed involvement of thiol group(s) in light-dependent phosphorylation of this protein. The inhibitors must interfere with reactions occurring after photoexcitation, as they are equally effective when added before or after irradiation.

Materials and Methods

Seedlings of *Zea mays* L. (Northrup King hybrid pX9540) were grown for 5 d in total darkness at 23 to 25°C. Apical 1–5-mm sections of the coleoptile were harvested under dim red safelight and used for preparation of microsomal and plasma membrane as described by Palmer *et al.* (1993a). Aliquots of 200 µg plasma membrane protein were dissolved under dim red safelight in 100 µl 30 mM Tris-Mes (pH 7.5) containing 5 mM $MgCl_2$ and 0.5% Triton X-100. The aliquots were used for *in vitro* phosphorylation according to the protocol of Short *et al.* (1992); thiol reagents (final concentration 1 mM) were added either before or after irradiation with blue light as indicated in the Figures. The samples were incubated with 13.3 µCi [γ-^{32}P]ATP and 10 nmol of ATP for 2 min

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at 30°C. The reaction was stopped by boiling SDS-containing gel-loading buffer (Short *et al.*, 1992), and 50 μg protein of each sample were electrophoresed on 5 to 20% gradient polyacrylamide gels containing SDS (Gallagher *et al.*, 1988). The gels were dried and the phosphorylation of single bands was quantitated with a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA). Light-dependent phosphorylation was normalized on each lane by correcting to normalized values of a phosphorylated protein band that did not show any light-dependence of phosphorylation.

Iodoacetamide (IoA), N-ethylmaleinimide (NEM) and N-phenylmaleimide (NPM) were from Sigma, Munich. Stock solutions (10 mM) of IoA and NEM were prepared in water. NPM was dissolved in ethanol and then diluted with water (20 mM in 40% ethanol. The final concentration of ethanol in phosphorylation samples was 2%.

Results and Discussion

The plasma membrane preparation used in this study showed light-induced phosphorylation only in one protein band which migrates slightly faster than the 116 kDa marker (Fig. 1). We refer to this protein as a 114 kDa species in accordance with Palmer *et al.* (1993a, b). Hager and Brich (1993) and Hager *et al.* (1993) apparently studied the same protein although they designated it a 100 kDa protein. Although there might be differences between maize cultivars, this explanation seems unlikely given that Reymond *et al.* (1992) find values of 114 kDa for maize, wheat, sorghum, barley, and oat coleoptiles. As Hager and Brich used a steep gradient gel without a marker above 94 kDa, their estimate must be considered as only approximate. The protein can be detected in microsomal membranes before phase separation as well. Hence, all subsequent experiments were performed with microsomal membranes.

The possible involvement of thiol groups in phosphorylation was tested with several thiol reagents that were added to the membrane preparation at the beginning of the experiment (Fig. 2). Inhibition of the light-induced phosphorylation was indeed found; it increased in the following order: iodoacetamide (50% inhibition), N-ethylmaleimide (75% inhibition), N-phenylmaleimide (93% inhibition). The dark level of phosphorylation was unaffected, however. No significant difference in phosphorylation was found between irradiated

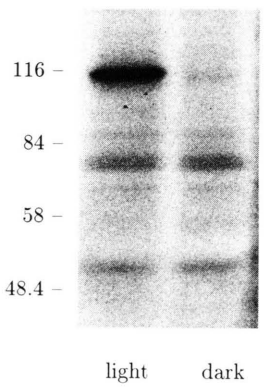

Fig. 1. *In-vitro* phosphorylation by [γ-^{32}P]ATP of a plasma membrane preparation from maize coleoptile tips. Blue light-induced phosphorylation is specific for the 114 kDa protein. The position of marker proteins is indicated at the left side.

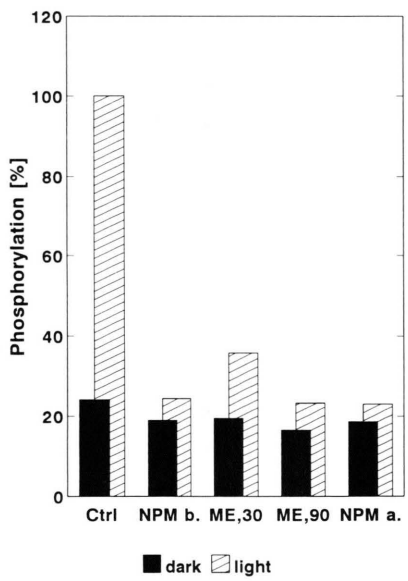

Fig. 2. Quantitation of phosphorylation of the 114 kDa membrane protein in the presence of several thiol reagents. Microsomal membranes were incubated for 1 min with the reagents (see below) before irradiation with 10^3 μmol m^{-2} of blue light and subsequent phosphorylation. Crtl = water control containing 2% ethanol; IoA, 1 mM iodoacetamide; NEM, 1 mM N-ethylmaleimide; ME/NEM, 1 mM N-ethylmaleimide preincubated for 5 min with 5 mM mercaptoethanol; NPM, N-phenylmaleimide; ME/NPM, 1 mM *n*-phenylmaleimide preincubated for 5 min with 5 mM mercaptoethanol. Mean values of 2–6 independent experiments.

and dark-control samples in the presence of N-phenylmaleimide. Phosphorylation in the presence of inhibitors never went significantly below the dark level of phosphorylation in the controls, indicating that light induction rather than the phosphorylation reaction itself was inhibited. Because the solubility of N-phenylmaleimide in water is poor, a stock solution of this inhibitor in 40% ethanol was prepared. The final concentration of ethanol (2%) in the reaction mixture resulted in about 20% inhibition of phosphorylation (not shown). We attribute this result to a general denaturing effect of the solvent. For valid comparisons between controls and inhibited samples, a 2% final concentration of ethanol was present in all samples. In order to demonstrate that inhibition by N-ethylmaleimide or N-phenylmaleimide was not itself a solvent effect of the inhibitors, we preincubated these reagents with an excess of mercaptoethanol before adding them to the membrane. No inhibition occurred in this case (Fig. 2). We conclude that the observed inhibition is indeed caused by a reaction with thiol groups of the protein.

The reaction of N-phenylmaleimide with the membrane is irreversible: when an excess of mercaptoethanol was added to the membrane 30–90 s later than N-phenylmaleimide, light-dependent phosphorylation could not be restored (Fig. 3). The inhibitor need not be present at the time of irradiation: The same inhibition of light induction was found whether N-phenylmaleimide was added before or immediately after (i.e. within 10 s) irradiation (Fig. 3). It should be noted that in these experiments as well, the dark level of phosphorylation was unaffected by the inhibitor.

In summary, a thiol group or thiol groups must participate in light induction of phosphorylation of the 114 kDa protein of maize. Since the inhibition increased with increasing hydrophobicity of the reagents, the sulfhydryl group(s) may well be buried in a hydrophobic region. However, since the more hydrophobic reagents lead to more bulky substituents at the sulfhydryl group, steric effects could also be the reason for differential inhibition. It had been demonstrated before that light activates a kinase and that the light signal for subsequent increased phosphorylation can be stored in the dark (Reymond *et al.*, 1992). Our experiments indicate that the sulfhydryl group that reacts with inhibitors might be an essential part of the stored light signal. The question whether a redox reaction or conformational changes (or both) are responsible for this light activation needs further investigation.

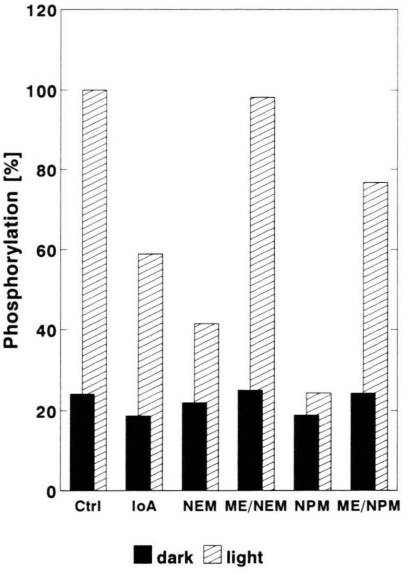

Fig. 3. Quantitation of phosphorylation of the 114 kDa membrane protein in the presence of N-phenylmaleimide (NPM) and mercaptoethanol (ME) added at different times with respect to each other and to irradiation. Crtl, water control containing 2% ethanol (data from Fig. 2); NPM b., N-phenylmaleimide added 1 min before irradiation (data from Fig. 2); ME,30, ME added 30 s after NPM; ME,90, ME added 90 s after NPM; NPM a., NPM added within 10 s after irradiation.

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Gallagher S., Short T. W., Ray P. M., Pratt L. H. and Briggs W. R. (1988), Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections. Proc. Natl. Acad. Sci. U.S.A. **85**, 8003–8007.

Hager A. and Brich M. (1993), Blue-light-induced phosphorylation of a plasma-membrane protein from phototropically sensitive tips of maize coleoptiles. Planta **189**, 567–576.

Hager A., Brich M. and Bazlen I. (1993), Redox dependence of the blue-light-induced phosphorylation of a 100-kDa protein on isolated plasma membranes from tips of coleoptiles. Planta **190**, 120–126.

Krebs E. G. (1994), The growth of research on protein phosphorylation. TIBS **19**, 439.

Lange S. (1927), Die Verteilung der Lichtempfindlichkeit in der Spitze der Haferkoleoptile. Jb. Wiss. Bot. **67**, 1–5.

Palmer J. M., Short T. W., Gallagher S. and Briggs W. R. (1993a), Blue light-induced phosphorylation of a plasma membrane-associated protein in *Zea mays* L. Plant Physiol. **102**, 1211–1218.

Palmer J. M., Short T. W. and Briggs W. R. (1993b), Correlation of blue light-induced phosphorylation to phototropism in *Zea mays* L. Plant Physiol. **102**, 1219–1225.

Reymond P., Short T. W. and Briggs W. R. (1992), Blue light activates a specific protein kinase in higher plants. Plant Physiol. **100**, 655–661.

Short T. S. and Briggs W. R. (1994), The transduction of blue light signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45**, 143–171.

Short T. S., Porst M. and Briggs W. R. (1992), A photoreceptor system regulating *in vivo* and *in vitro* phosphorylation of a pea plasma membrane protein. Photochem. Photobiol. **55**, 773–781.

Sierp H. and Seybold U. A. (1926), Untersuchungen über die Lichtempfindlichkeit der Spitze und des Stumpfes in der Koleoptile von *Avena sativa*. Jb. Wiss. Bot. **65**, 592–610.