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Phosphorylation of thylakoid proteins and synthetic peptide analogs

Differential sensitivity to inhibition by a plastoquinone antagonist

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Spinach thylakoids contain at least 8 proteins (8.3–58 kDa) whose phosphorylation is strongly inhibited by the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Reduction of DBMIB with ascorbate completely or partially relieves inhibition for all proteins other than the 27 kDa light-harvesting complex (LHC) II and a synthetic dodecapeptide (MRKSATTKKAVC). The peptide, an analog of the phosphorylation site of pea 27 kDa LHC II, is phosphorylated with the same site specificity, kinetics, redox control and sensitivity to DBMIB/ascorbate as the protein itself. The data indicate that synthetic peptides can be used to study the number, substrate specificity and redox regulation of thylakoid protein kinases.

Chloroplast; Protein kinase; DBMIB; Plastoquinone; Peptide synthesis

1. INTRODUCTION

Thylakoid protein phosphorylation is catalyzed by a protein kinase whose activation is dependent upon reduction of a component of the photosynthetic electron transport chain [1], probably PQ [2–4]. Prominent among the evidence implicating PQ are studies with electron transport inhibitors. The herbicide diuron, which blocks electron transport between PS II and PQ [5], prevents kinase activation [1], whereas $<1\ \mu$M DBMIB, which blocks electron transport between PQH_2 and the Rieske FeS protein of the cytochrome *bc* complex [6,7], does not prevent kinase activation [2]. However, at $>10\ \mu$M, DBMIB acts as a PS II electron acceptor and this is believed to be the reason why high concentrations of DBMIB inhibit thylakoid protein phosphorylation [2]. Ascorbate has been reported to maintain DBMIB in the reduced state and so abolish the secondary site of DBMIB action without affecting its ability to inhibit at the Rieske protein [8]. This result led us to expect that addition of ascorbate to thylakoids would prevent the DBMIB-induced inhibition of protein phosphorylation. However, as we now report, while ascorbate does restore thylakoid protein phosphorylation in the presence of DBMIB, the extent of recovery varies markedly among the phosphoproteins.

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Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; LHC, light-harvesting chlorophyll *a*/*b* complex; PQ, plastoquinone; PS, photosystem; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L. var. Hybrid 424) and peas (*Pisum sativum* L. var. Alaska) were grown as described [9].

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Intact spinach chloroplasts were isolated [9] and resuspended in hypotonic buffer (10 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$) for osmotic shock. Thylakoids were recovered at 10000 × *g* for 10 min and resuspended to 1 mg Chl/ml in buffer A1 (25 mM Tricine-NaOH, pH 8.0, 1 mM $MgCl_2$, 10 mM NaF), or buffer A10 (as for buffer A1 but with 10 mM $MgCl_2$).

Resuspended thylakoids were diluted to the indicated Chl concentration with buffer A1 or A10 and were incubated in an illuminated water bath (20°C, PAR = 200 $\mu E \cdot m^{-2} \cdot s^{-1}$) with [$\gamma$-^{32}P]ATP (200 μM, 200 μCi/μmol), supplemented where indicated with diuron, DBMIB and ascorbate. After 10–15 min, trichloroacetic acid was added to give a final concentration of 10% (v/v), the protein precipitates were collected by centrifugation at 10000 × *g* for 2 min, and the pellets were prepared for SDS-PAGE as described [10].

Peptides P1 and P2 were synthesized by the method of Merrifield [11]. The initial amino acid-resin and protected amino acids were obtained from Peninsula Laboratories. The following side chain protecting groups were used: tosyl for arginine, *O*-benzyl for threonine and serine, and benzyloxycarbonyl for lysine. All couplings were carried out using a 6-fold molar excess of *t*-butyloxycarbonyl-amino acid and were monitored by the ninhydrin reaction. The protected peptide resin was then dried and treated with HF/anisole for 1 h at 0°C. After extraction of the residual anisole with diethyl ether, the peptide was eluted from the resin with 5% aqueous acetic acid and purified by G-25 column chromatography prior to lyophilization and amino acid analysis [12].

P1 and P2 were labelled in vitro by incubation of pea and spinach thylakoids with [γ-^{32}P]ATP, separated from other phosphoproteins by SDS-PAGE and located by autoradiography. The bands of gel containing the peptides were fragmented by passage through a syringe (without needle) and stirred overnight at 4°C in a minimum volume of water. The peptides were recovered by lyophilization. Phosphoamino acid [13], protein [14] and Chl [15] analyses were as described.

3. RESULTS

When isolated spinach thylakoids are incubated in the light in the presence of [γ-^{32}P]ATP, at least eight proteins with molecular masses of 8.3–58 kDa become phosphorylated (fig.1). In the absence of ascorbate, both 10 μM diuron and 50 μM DBMIB completely block protein labelling, whereas, in the presence of 5 mM ascorbate, the inhibitory effect of DBMIB is largely abolished. Indeed, DBMIB reduced by ascorbate substantially reactivates protein phosphorylation that has been inhibited by diuron. We assume that DBMIB/ascorbate activates phosphorylation by reducing the PQ pool in two ways: (i) inhibition of PQH_2 oxidation [6] and (ii) by direct donation of electrons to the PQ pool. However, the activation of protein phosphorylation is not uniform. Although some proteins are heavily labelled, phosphorylation of other proteins remains significantly inhibited. Most conspicuous in this respect is the 27 kDa LHC II (90% inhibition). Other proteins showing significant inhibition (58–65%) are the 25 kDa LHC II and two uniden-

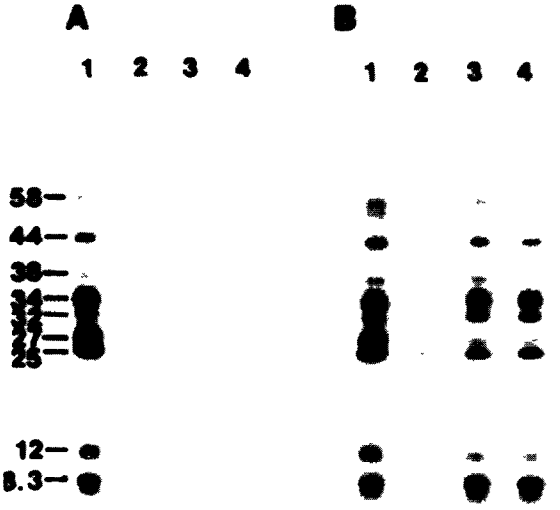

Fig.1. Effects of diuron, DBMIB and ascorbate on protein phosphorylation in spinach thylakoids. Thylakoids (0.25 mg Chl/ml) were incubated under kinase assay conditions (buffer A10) for 10 min and analyzed by SDS-PAGE. The gel was stained, dried and autoradiographed. A, without ascorbate. B, with ascorbate (5 mM). Lanes: 1, no additions; 2, with 10 μM diuron; 3, with 50 μM DBMIB; 4, with both diuron and DBMIB. Molecular masses in kDa.

tified proteins (12 and 58 kDa). Phosphorylation of the 8.3, 32, 34 and 44 kDa proteins is inhibited 0–16%.

Fig.2 shows phosphorylation of the 27 kDa LHC II and the 8.3 kDa protein as a function of DBMIB concentration in the presence of 5 mM ascorbate and 1 mg Chl/ml in buffer A10. This concentration of thylakoids corresponds to 20–25 μM PQ, if only those PQ molecules participating in electron transport are considered [16]. Phosphorylation of the 27 kDa protein is inhibited 50% by 2.5 μM DBMIB, whereas phosphorylation of the 8.3 kDa protein is not inhibited significantly by even 50 μM DBMIB.

How does DBMIB/ascorbate inhibit phosphorylation of the 27 kDa LHC II so selectively? Which factor is more important: the precise location/environment of the protein in the membrane, or the structure of its phosphorylation site? To investigate this question, we studied the ability of thylakoids to phosphorylate soluble peptide analogs of the phosphorylation site of the 27 kDa LHC II of pea [17]. From the sequence of two DNA clones [18,19], the N-terminus of the mature protein has been deduced to be (M)RKSATTK-KVASS..., where (i) methionine may be the first residue of the mature protein [20] or the last residue of the transit peptide of the precursor [19], and (ii) one or both of the threonines but none of the serines is phosphorylated [13,17]. We have synthesized P1, a peptide with structure MRKSATTK-

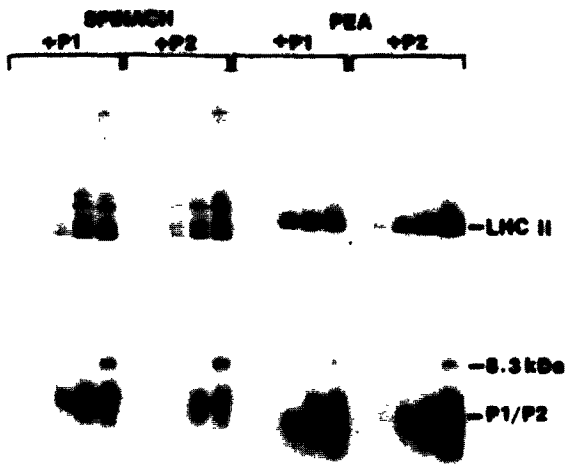

Fig.3. Phosphorylation of synthetic peptides by spinach and pea thylakoids. P1 and P2: 0.3 mg/ml of buffer A1. Five membrane concentrations were used; left to right: 0, 0.1, 0.2, 0.4 and 0.8 mg Chl/ml. After 15 min, the reaction mixtures were analyzed by SDS-PAGE. The wet, unstained gel was autoradiographed at −80°C.

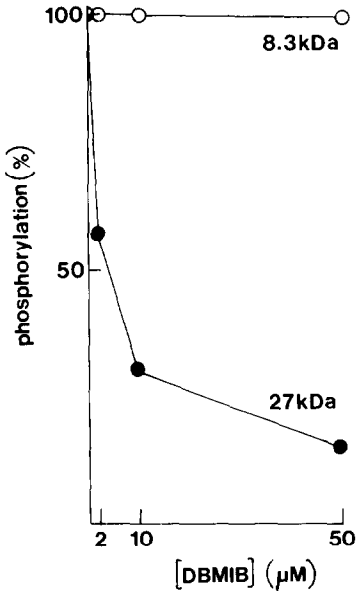

Fig.2. Sensitivity of the phosphorylation of the 27 kDa LHC II and the 8.3 kDa protein to DBMIB in the presence of 5 mM ascorbate.

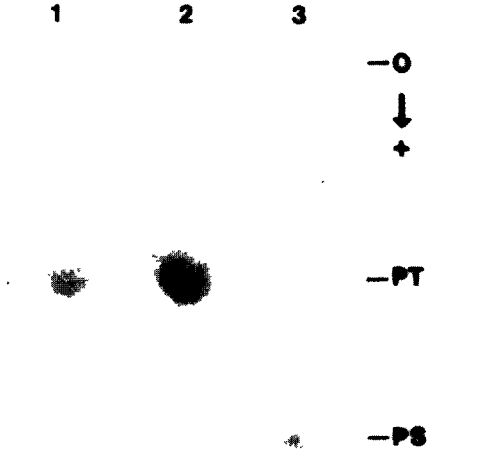

Fig.4. Phosphoamino acid analysis of P1 and P2. The synthetic peptides were: 1, P1 labelled by pea thylakoids; 2, P1 labelled by spinach thylakoids; 3, P2 labelled by pea thylakoids. PT and PS: phosphothreonine and phosphoserine standards.

KAVC, and P2, which has the same sequence as P1 except that the two threonines are replaced by serines.

Pea and spinach thylakoids phosphorylate P1 and P2 in addition to endogenous substrates (fig.3). In this experiment the $MgCl_2$ concentration during the kinase assay was 1 mM, optimal for the oligopeptide and near-optimal for LHC II but distinctly sub-optimal for the 32–58 kDa proteins (cf. fig.1). P1 is phosphorylated equally well by the two thylakoid preparations. The highest thylakoid concentration used corresponds to 0.8 mg Chl/ml. If we assume that LHC II accounts for about 50% of total Chl and that there are 6–13 Chl molecules per molecule of LHC II [17,21], the LHC II concentration is 35–75 μM. The concentration of P1 (0.3 mg/ml) corresponds to about 220 μM, i.e. 3–7-times greater than the LHC II concentration. Cerenkov counting of gel fragments indicates that about 5-times as much ^{32}P is incorporated into P1 as into LHC II. Thus, to a first approximation, exogenous P1 and endogenous LHC II are equally good thylakoid kinase substrates.

Like pea LHC II [13,17], P1 is phosphorylated on threonine (fig.4) but it is not yet clear whether one or both threonines are labelled. The fact that P1 is not phosphorylated on its serine indicates either that phosphorylation of P1 shows correct site specificity or that serine cannot be esterified by the kinase. Phosphorylation of P2 on serine (fig.4) argues strongly against the second alternative.

Fig.5 shows phosphorylation of thylakoids and P1 in the presence of diuron or DBMIB/ascorbate. Labelling of all eight endogenous phosphoproteins and P1 is sensitive to diuron but only the 27 kDa LHC II and P1 show extreme sensitivity to DBMIB/ascorbate. Phosphorylation of P1 is inhibited by 89%, close to the 90% inhibition seen above for the 27 kDa LHC II. We conclude that P1 is phosphorylated under redox control and that the amino acid sequence of P1 provides sufficient information to render its phosphorylation extremely sensitive to DBMIB/ascorbate. Attachment to thylakoids is unnecessary.

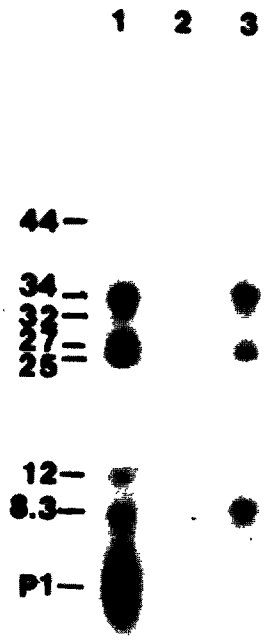

Fig.5 Inhibition of P1 phosphorylation by diuron and DBMIB in the presence of ascorbate (5 mM). Spinach thylakoids were incubated as in fig.1 but with addition of 0.3 mg P1/ml. After 10 min, proteins were analyzed by SDS-PAGE. The wet, unstained gel was autoradiographed at −80°C. Lanes: 1, no additions; 2, with 10 μM diuron; 3, with 50 μM DBMIB.

4. DISCUSSION

In principle, redox control of thylakoid protein phosphorylation could be due to an effect of $PQ(H_2)$ on either kinase activity itself or the conformation of protein substrates. A precedent for the latter phenomenon is provided by light-induced opsin phosphorylation in retinal rod cells [22] but the conformational mechanism seems unlikely in the case of thylakoids, since soluble exogenous substrates such as P1 (fig.5) and histones H1 and H2b [23] are subject to the same diuron-sensitive redox control as endogenous substrates.

How might DBMIB/ascorbate inhibit differentially the phosphorylation of thylakoid proteins? The answer depends crucially on how many distinct types of redox-controlled kinases and DBMIB-binding sites exist in thylakoids. The fact that phosphorylation of the 8.3 kDa protein and several other proteins is inhibited by diuron but not by DBMIB/ascorbate suggests that they are substrates of a PQ-controlled kinase. However, the sensitivity of the phosphorylation of P1 and 27 kDa LHC II to both diuron and DBMIB/ascor-

bate would indicate that binding of DBMIB to either the Rieske FeS protein or to the kinase itself selectively abolishes the ability of the latter to phosphorylate these substrates. Conversely, P1 and the 27 kDa LHC II could be substrates of a second kinase which is regulated by a redox component after PQ in the electron transport chain or which is directly inhibited by DBMIB. The possible existence of multiple kinases has been raised before [24–30], and in three cases [24,26,27], it was a difference in phosphorylation between LHC II and the 8.3 kDa protein which prompted such speculation. In any case, whether there is one kinase or two, our data raise as a distinct possibility the existence of a $PQ(H_2)$/DBMIB-binding site on the kinase molecules themselves.

Synthetic peptides will play a large role in distinguishing among these various hypotheses. It is clear that P1 is phosphorylated by pea and spinach thylakoids at the same site, at the same rate and with the same redox control and sensitivity to DBMIB/ascorbate as the 27 kDa LHC II. H. Michel in this laboratory has sequenced the phosphorylation site of the 8.3 kDa protein of spinach and is synthesizing an appropriate oligopeptide analog. We predict that this peptide will be phosphorylated by spinach thylakoids in a DBMIB/ascorbate-insensitive manner. Use of this peptide and P1 as kinase substrates should enable us to determine whether solubilization of thylakoids [23,29–31] releases a single kinase capable of phosphorylating both peptides or two kinases capable of discriminating between them.

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REFERENCES

[1] Bennett, J. (1979) FEBS Lett. 103, 42–344.
[2] Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature 291, 21–25.
[3] Horton, P., Allen, J.F., Black, M.T. and Bennett, J. (1981) FEBS Lett. 125, 193–196.
[4] Allen, J.F. and Horton, P. (1982) Biochim. Biophys. Acta 638, 290–295.
[5] Trebst, A. (1974) Annu. Rev. Plant Physiol. 25, 423–458.
[6] Bohme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352.
[7] Malkin, S. (1982) Biochemistry 21, 2945–2950.
[8] Aoki, M. and Katoh, S. (1983) Plant Cell Physiol. 24, 1379–1386.
[9] Bhalla, P. and Bennett, J. (1986) Arch. Biochem. Biophys., in press.
[10] Bennett, J. (1980) Eur. J. Biochem. 104, 85–89.
[11] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[12] Hirs, C.H.W. (1983) Methods Enzymol. 91, 3–8.
[13] Bennett, J. (1977) Nature 269, 344–346.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
[16] Amesz, J. (1973) Biochim. Biophys. Acta 301, 35–51.
[17] Mullet, J. (1983) J. Biol. Chem. 258, 9941–9948.
[18] Coruzzi, G., Broglie, R., Cashmore, A.R. and Chua, N.-H. (1983) J. Biol. Chem. 258, 1399–1402.
[19] Cashmore, A.R. (1984) Proc. Natl. Acad. Sci. USA 81, 2960–2964.
[20] Karlin-Neuman, G.A. and Tobin, E.M. (1986) EMBO J. 5, 9–13.
[21] Ryrie, I.J., Anderson, J.M. and Goodchild, D.J. (1980) Eur. J. Biochem. 107, 345–354.
[22] Thompson, P. and Findlay, J.B.C. (1984) Biochem. J. 220, 773–780.
[23] Clark, R.D., Hind, G. and Bennett, J. (1985) Molecular Biology of the Photosynthetic Apparatus (Steinback, K.E. et al. eds) pp.259–267, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[24] Millner, P.A., Widger, W.R., Abbott, M.S., Cramer, W.A. and Dilley, R.A. (1982) J. Biol. Chem. 257, 1736–1742.
[25] Markwell, J.P., Baker, N.R. and Thornber, J.P. (1983) Photobiochem. Photobiophys. 5, 201–207.
[26] Farchaus, J., Dilley, R.A. and Cramer, W.A. (1985) Biochim. Biophys. Acta 809, 17–26.
[27] Black, M.T., Foyer, C. and Horton, P. (1984) Biochim. Biophys. Acta 767, 557–562.
[28] Canaani, O. (1985) FEBS Lett. 188, 281–285.
[29] Lin, Z.-F., Lucero, H.A. and Racker, E. (1982) J. Biol. Chem. 257, 12153–12156.
[30] Lucero, H.A., Lin, Z.-F. and Racker, E. (1982) J. Biol. Chem. 257, 12157–12160.
[31] Coughlan, S.J. and Hind, G. (1986) J. Biol. Chem. 261, 11378–11385.