

SENSITIVITY OF THE PHOSPHORYLATION OF DIFFERENT PHOSPHOPROTEINS TO Q_o AND Q_i SITE INHIBITORS OF THE CYTOCHROME B/F COMPLEX IN CHLOROPLAST THYLAKOIDS

E. Liker¹, L. Cheng², G. Garab¹ and J. F. Allen²

¹Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, Szeged, P.O.Box 521, H-6701, Hungary and

²Department of Plant Cell Biology, University of Lund, Box 7007 S-220 07 Lund, Sweden

1. Introduction

Thylakoid membranes exhibit protein kinase activity which is activated by photochemical or chemical reduction of the plastoquinone pool (1,2). The major endogenous substrate for this activity is the light-harvesting chlorophyll a/b binding protein of photosystem II (LHCII), which is phosphorylated by the LHCII-kinase. Besides LHCII, several other thylakoid proteins are phosphorylated: D1/D2, CP43, the psbH gene product (9 kDa phosphoprotein) and several unidentified phosphoproteins.

The site of redox control of the activity(ies) of kinase(s) can be localized to the cytochrome b_6/f complex. Direct evidence that redox activation of the LHCII kinase requires participation of the cyt b_6/f complex was obtained from studies of a cyt b_6/f -deficient mutants of *Chlamydomonas* (3), maize (4) and *Lemna* (5). However, the mechanism of the activation of the LHCII kinase via the cyt b_6/f complex is unknown.

In thylakoid membranes the cyt b_6/f complex functions by transferring electrons from plastoquinol to plastocyanin, and protons are translocated across the membrane (6). There are two quinone binding sites in the cyt b_6/f complex, the Q_o and Q_i site, which are the sites of proton output and input, respectively. There are several reports on the effect of different inhibitors of the cyt b_6/f complex on the phosphorylation of thylakoid membranes (4,8). In most works the investigations of the inhibitory effects were confined to LHCII and the 9 kDa polypeptide.

We carried out a systematic investigation of the effect of Q_o site inhibitors (DBMIB, stigmatellin, bromanil) and Q_i site inhibitors (HQNO, MOA-stilbene) on the phosphorylation of the seven most intensely labelled phosphoproteins, LHCII, D1/D2, and the 56, 43, 20, 18 and 9 kDa proteins. The results show that all the Q_o site inhibitors have inhibitory effect on the thylakoid protein phosphorylation, whereas all the Q_i site inhibitors exhibit no inhibitory effect and some of them even slightly stimulate the phosphorylation. Our results indicate that the Q_o site or cyt b_6 or any component in the complex which locates between these two sites in the electrontransport chain is responsible for the redox control of the thylakoid protein phosphorylation.

2. Experimental Procedures

Pea thylakoid membranes were isolated according to Harrison et al. (9).

Protein kinase reactions were initiated by addition of ^{32}P ATP to a final concentration of 0.2 mM, specific activity of $100 \mu\text{Ci}/\mu\text{M}$ ATP in a reaction medium containing 50 mM Hepes, 5 mM MgCl_2 , 5 mM NaCl, 5 mM NH_4Cl , 0.1 M sorbitol and the inhibitors dissolved in 95 % ethanol (final ethanol concentration did not exceed 5 %). The protein kinase was activated by light. Phosphorylation reactions were terminated with ice cold 80 % acetone after 5 min. SDS-PAGE was performed on 12-25 % gradient gels according to Laemmli (10). Quantitation of the ^{32}P -labelled bands of SDS-gels were performed by phosphorimaging with a Fuji BAS2000 Bio-Imaging Analyzer. Background labelling was subtracted for each lane.

3. Results and Discussion

In accordance with earlier reports (cf. 2) we found that DBMIB at $3 \mu\text{M}$ almost fully inhibited the phosphorylation of LHCII, whereas the sensitivity of the 9 kDa phosphoprotein was significantly lower.

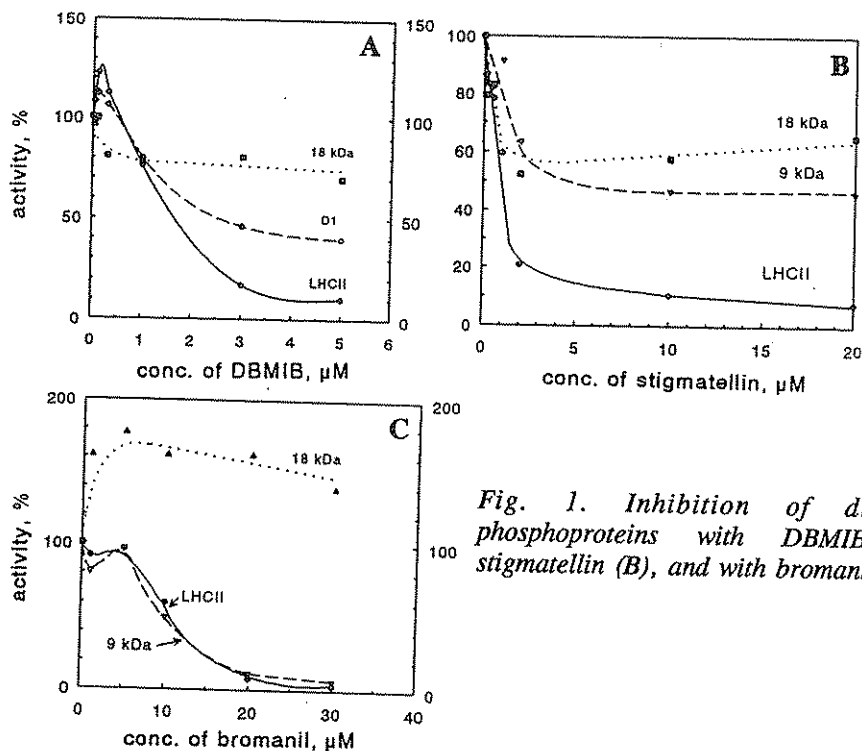


Fig. 1. Inhibition of different phosphoproteins with DBMIB (A), stigmatellin (B), and with bromanil (C).

Our data show that the inhibition of the 56 kDa protein by $3\mu\text{M}$ DBMIB closely resembles that of LHCII, whereas D1/D2, the 43 and 20 kDa proteins exhibit lower sensitivity. The 18 kDa protein appears to be largely insensitive to DBMIB. (The phosphorylation of this polypeptide was also insensitive to DCMU (data not shown).) Typical titration curves of different phosphoproteins, representatives of these three groups are shown in Fig. 1A.

Phosphorylation of LHCII and the 56 kDa protein could very effectively be inhibited with stigmatellin, which is a potent antibiotic inhibitor of the $\text{cyt } b_6/f$ complex at the Q_o site (11) (Fig. 1B). With both DBMIB and stigmatellin the I_{50} values for inhibition of phosphorylation were considerably higher than those for inhibition of the $\text{cyt } b_6/f$ complex where I_{50} values were around 50 nM (11,12). Similar shifts of the inhibition of the LHCII kinase was observed earlier with DCMU (cf. 2).

The phosphorylation of the second group of proteins (D1/D2, and the 43, 20 and 9 kDa proteins), which was less sensitive to inhibition by DBMIB than that of LHCII and the 56 kDa protein, was also less sensitive to inhibition with stigmatellin and the 18 kDa protein was the most insensitive polypeptide (data not shown).

Bromanil, which is a halogenated 1,4-benzoquinone and an efficient inhibitor at the Q_o site (12), inhibited the phosphorylation of all phosphoproteins except the 18 kDa protein, the phosphorylation of which was significantly stimulated (Fig. 1C). It must be noted, however, that at the concentration of half inhibitory effect, due to a covalent binding of bromanil to the thylakoids, the electron transport is inhibited irreversibly (13). This may explain why, with this inhibitor, no distinction could be made between the first and second group of phosphoproteins.

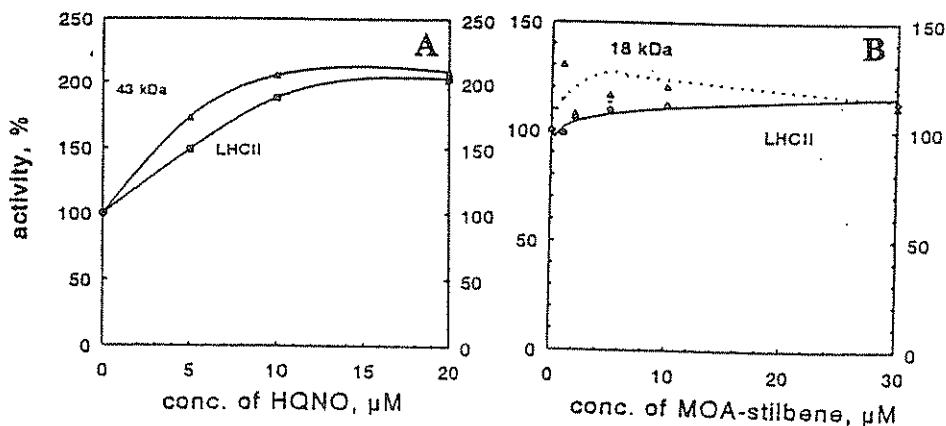


Fig. 2. Effect of HQNO (A) and MOA-stilbene (B) on phosphorylation of different phosphoproteins.

HQNO, which is a quinone analogue inhibiting at the Q_i site (14), enhanced significantly the phosphorylation of LHCII and other phosphoproteins of the thylakoid membrane (Fig. 2A). HQNO has been shown to raise the midpoint potentials of the cyt b563 molecules from -150 and -30 mV to -34 and +57 mV, respectively (15), and to significantly enhance the photoreduction of cyt b563 (13). Earlier, HQNO was reported not to affect phosphorylation (4). On the other hand NQNO, which inhibits at the same site (12), has been shown to enhance phosphorylation of LHCII by 40% (8).

MOA-stilbene, which is a recently introduced Q_i -site inhibitor (6), proved to be ineffective in the phosphorylation of thylakoid membrane proteins (Fig. 2B). In contrast with HQNO, this inhibitor does not alter the redox potentials of cyt b563, and only slightly enhances the reduction of these molecules (6).

In summary, our analysis of the effect of different inhibitors of the cyt b_6/f complex on the seven most intensely phosphorylated polypeptides, shows that the phosphorylation of the proteins, with the exception of the 18 kDa protein, can efficiently be inhibited by Q_o site inhibitors, whereas Q_i site inhibitors were either ineffective or enhance the phosphorylation. Thus, it is concluded that the redox control of the phosphorylation of the thylakoid membranes is found at the Q_o site itself, cyt b_6 or any other component between Q_o and Q_i sites of the cyt b_6/f complex.

Acknowledgements The authors acknowledge the financial support of Swedish Natural Science Research Council, OTKA III/2999 of Hungarian Research Fund and ESF Biophysics of Photosynthesis travel grant to E.L. We thank P.R. Rich for the gift of MOA-stilbene.

References

- 1 Allen, J.F. (1992) *Biochim. Biophys. Acta* 1098, 275-335
- 2 Bennett, J. (1991) *Annu. Rev. Plant Mol. Biol.* 42, 281-311
- 3 Lemaire, C., Girard-Bascou, J. and Wollman, F-A. (1987) in *Progress in Photosynthesis Research* (Biggins, J., ed.), Vol. IV., pp. 655-658, Martinus Dordrecht
- 4 Bennett, J., Shaw, E.K. and Michel, H. (1988) *Eur. J. Biochem.* 171, 95-100
- 5 Gal, A., Shahak, Y., Schuster, G. and Ohad, I. (1987) *FEBS Lett.* 221, 205-210
- 6 Rich, P.R., Magdwick, S.A., Brown, S., Jagow, G. and Brandt, U. (1992) *Photosynthesis Res.* 34, 465-477
- 8 Gal, A., Schuster, G. Frid, D., Canaani, O., Schweiger, H-G. and Ohad, I. (1988) *J. Biol. Chem.* 263, 7785-7791
- 9 Harrison, M.A. and Allen, J.F. (1991) *Biochim. Biophys. Acta* 1058, 289-296
- 10 Laemmli, U.K. (1970) *Nature* 227, 680-685
- 11 Oettmeier, W., Godde, D., Kunze, B. and Höfle, G. (1985) *Biochim. Biophys. Acta* 807, 216-219

- 12 Jones, R.W. and Whitmarsh, J (1988) *Biochim. Biophys. Acta* 933, 258-268
- 13 Oettmeier, W., Masson, K. and Dostatni, R. (1987) *Biochim. Biophys. Acta* 890, 260-269
- 14 Selak, M.A. and Whitmarsh, J. (1982) *FEBS Lett.* 150, 286-292
- 15 Clark, R.D. and Hind, G. (1983) *Proc. Natl. Acad. Sci.* 80, 6249-6253