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Regulation of thylakoid protein phosphorylation by the thiol redox state – the role of thioredoxin.

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Introduction

A number of thylakoid proteins belonging to photosystem II (PSII) are phosphorylated in a light dependent manner. Among these are the reaction center proteins of PSII, D1 and D2, as well as the major chlorophyll a/b light-harvesting antenna (LHCII) (Allen, 1992).

Thylakoid protein phosphorylation is believed to play an important role in the regulation of light energy distribution (state transitions), in long-term acclimation of the antenna size and in the control of D1 protein turnover (Allen 1992; Andersson and Aro 1997). Light activation of protein phosphorylation involves the reduction of plastoquinone (Allen, 1992) and, at least in the case of phosphorylation of LHCII, binding of plastoquinol at the quinol oxidising site in cytochrome bf (Vener et al. 1998).

Phosphorylation of LHCII in vivo has been shown to decrease at increasing light levels, in contrast to the phosphorylation of the PSII core proteins, which remains high also at elevated light levels (Rintamäki et al. 1997).

We have earlier shown that thylakoid protein phosphorylation in vitro is strongly affected by the thiol redox state (Carlberg et al. 1999). In spite of the fact that the phosphorylation is generally activated by reducing conditions, LHCII phosphorylation was shown to be inhibited by reduced dithiothreitol (DTT). This demonstrates the importance of a correct redox state of thiol groups, but also points to the possible existence of multiple levels of redox regulation for thylakoid protein phosphorylation. The PSII core phosphorylation, on the other hand, was much less sensitive and even increased in the presence of low concentrations of DTT. It was suggested that the reduced LHCII phosphorylation under high light conditions could be mediated by the thiol redox state, possibly via the thiol redox regulator thioredoxin (Rintamäki et al. 1997; Carlberg et al. 1999). In the present work we have investigated the influence of thioredoxin on thylakoid protein phosphorylation in vitro.

Materials and methods

Spinach thylakoid membranes where isolated by standard procedures and resuspended in 25 mM Tricine pH 8.0, 0.1 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl. Membranes were phosphorylated at 0.2 mg chl/ml in the presence of 10 mM NaF and 0.25 mM ATP, with or without [γ-^{32}P]ATP(0.02 mCi/mg chl) by illumination at 120 μmol photons $m^{-2}s^{-1}$ for 20 min unless otherwise indicated. In most experiments an ATP regenerating system consisting of 30

mM phosphocreatine and creatine phosphokinase (15 u/ml) was included. Spinach thioredoxin f was used at a concentration of 5 – 10 μM and was reduced by 0.1 mM DTT. Phosphorylation in the presence of 0.1 mM DTT alone was used as the control, to account for the inhibition produced by 0.1 mM DTT. The level of protein phosphorylation was analysed by SDS-PAGE, blotting onto PVDF membranes and visualised with phosphothreonine antibodies. Alternatively gels were dried and subjected to phosphorimaging/autoradiography.

Results

The presence of reduced thioredoxin had a marked negative effect on LHCII phosphorylation in vitro (Fig.1), in agreement with previous reports (Carlberg et al. 1999; Rintamäki et al. 2000). The inhibition appears to increase with time of phosphorylation (Fig.1) and was totally dependent upon light, as no inhibition was observed when protein phosphorylation was activated in the dark by the addition of NADPH and ferredoxin (not shown). However, light preincubation in the presence of reduced thioredoxin, before the initiation of protein phosphorylation by the addition of ATP, did not change the inhibitory pattern (not shown), indicating a requirement of ATP for the decrease in phosphorylation. Inhibition of LHCII phosphorylation by DTT does not display any of these characteristics. Also, in contrast to DTT, the effect of thioredoxin on protein phosphorylation in spinach thylakoids was found to be very similar for both LHCII and PSII core phosphorylation (not shown).

The CF1 ATPase is strongly activated by thioredoxin in a light dependent process (Mills et al.1980)) and a trivial explanation for the observed inhibition of protein phosphorylation, could be a decrease in available substrate due to ATP hydrolysis. Indeed, direct measurements of the ATP level in our system, during light incubation in the presence of reduced thioredoxin, clearly showed a rapid decrease in the concentration of ATP, reaching very low levels after 15 minutes. The presence of an ATP regenerating system significantly increased the level of protein phosphorylation (Fig.2). However, while the phosphorylation of D1 and D2, the reaction center subunits of PSII, was largely restored, the phosphorylation of LHCII was still

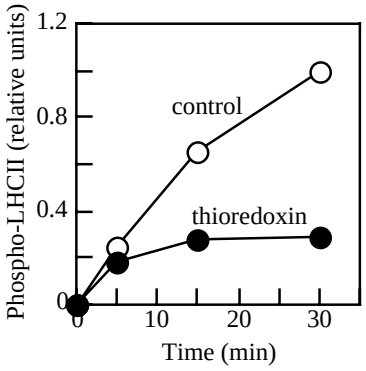

Fig.1 Time course for the phosphorylation of LHCII in the absence and presence of thioredoxin. Phosphorylation was analysed as incorporation of radioactive phosphate.

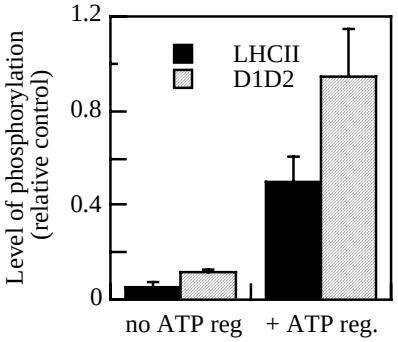

Fig.2. The effect of ATP regeneration on thylakoid protein phosphorylation in the presence of thioredoxin.
Phosphorylation was analysed with phosphothreonine antibodies.

inhibited, although to a much lower degree.

Addition of uncouplers partially counteracted the remaining inhibition of LHCII phosphorylation by thioredoxin. 2 μM nigericin increased the level of phosphorylation in the

presence of reduced thioredoxin by at least 30%. In some experiments nigericin abolished the inhibitory effect of thioredoxin. This indicates that the effect of thioredoxin could be related to the generation of a pH gradient across the thylakoid membrane. In the absence of an electron acceptor, the ΔpH of illuminated isolated thylakoid membranes will be very low. However, an activation of CF1 by added thioredoxin would create a ΔpH via reverse proton translocation driven by ATP hydrolysis. In agreement with this, the inhibition by thioredoxin is also decreased by the addition of ATPase inhibitors such as Fe-bathophenanthroline (Carlsson and Ernster 1980) or DCCD (not shown). In contrast to LHCII, the phosphorylation of D1/D2 in the presence of reduced thioredoxin was not significantly affected by nigericin.

It has previously been shown that thylakoid protein phosphorylation in intact chloroplasts was inversely correlated to the ΔpH (Oxborough et al.1987). Inhibition of phosphorylation was suggested to be correlated to the ΔpH-dependent formation of qE. Formation of qE is inhibited by antimycin A which was also shown to stimulate phosphorylation of LHCII (Oxborough et al 1987). In a number of our experiments, addition of antimycin A was found to increase the level of phosphorylation of LHCII in the presence of thioredoxin by a factor of around 2.

In marked contrast to the thioredoxin mediated inhibition of LHCII protein phosphorylation, the inhibition by 1 mM DTT was not affected by the addition of an ATP regenerating system, nigericin or antimycin A (Fig. 3). In Fig 3. it can also be seen that 1 mM DTT does not significantly affect the level of D1/D2 phosphorylation.

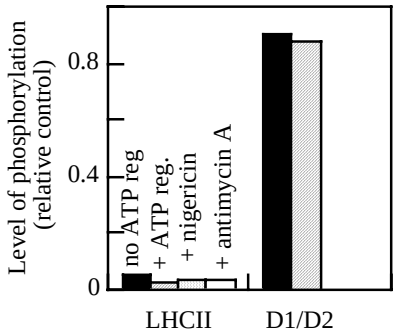

Fig 3. Protein phosphorylation in the presence of 1 mM DTT.
Phosphorylation was analysed with phosphothreonine antibodies. The concentration of nigericin or antimycin A was 2 μM

Discussion

In vitro incubation of spinach thylakoid membranes with thioredoxin, in the light and in the presence of ATP, inhibits thylakoid protein phosphorylation. The effect is more pronounced for LHCII phosphorylation than for D1/D2 phosphorylation, provided that an ATP regenerating system is present. In this respect, thioredoxin follows the pattern seen in vivo at high light levels as well as that displayed in vitro in the presence of DTT (Rintamäki et al.1997; Carlberg et al. 1999).

One major event taking place during the incubation with thioredoxin appears to be the activation of CF1. The active CF1 most likely generates a ΔpH across the membrane at the expense of added ATP. The observation that uncouplers counteract the thioredoxin induced inhibition of LHCII phosphorylation, indicates that the ΔpH is of importance for the decrease in LHCII phosphorylation. The DTT induced inhibition of LHCII phosphorylation, on the other hand, is not affected by uncouplers, indicating that a pH gradient is not important in this case. Incubation with 1 mM DTT under these conditions does give a significant rate of ATP hydrolysis (not shown), and presumably also a pH gradient. Major differences between DTT and thioredoxin, are size and membrane accessibility. Whereas the small DTT molecule probably will have easy access to its target in the membrane, thioredoxin might require a ΔpH induced conformational change in order for susceptible groups to become exposed. The effect

of antimycin A could suggest that this state is similar to qE, which has been proposed to involve the aggregation of LHCII (Horton et al 1996).

In conclusion, the effect of thioredoxin on LHCII phosphorylation in vitro, could either be due to the ΔpH alone or to a specific thioredoxin interaction dependent on the formation of a ΔpH. At present we cannot discriminate between these two possibilities. However, in support of the latter stands the marked sensitivity of LHCII phosphorylation to the thiol redox state.

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