The 9-kDa Phosphoprotein Involved in Photoinhibition

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Photosystem-II particles exhibit strong photoinhibition. Short-term illumination of photosystem-II particles with high-intensity light (5000 $\mu E/m^2 \times s$) leads to a typical change of the protein pattern on SDS-PAGE. Two proteins are mainly affected, namely the well-described 32-kDa herbicide-binding protein which probably is degraded [1] and, first published here, the 9-kDa phosphoprotein, whose function in the PS-II complex is still unknown. This protein is not degraded, but seems to be linked to other polypeptides of the PS-II complex. During light treatment new bands of 23, 41, 50 and 54 kDa appear in the protein pattern of SDS-PAGE.

A monospecific antiserum was produced against the 9-kDa phosphoprotein to investigate its fate. After light treatment the antibodies reacted with new proteins of higher molecular weights, most pronounced with a 23-kDa and a 41-kDa peptide.

Introduction

Exposure of thylakoids or photosystem(PS)-II particles (membranes) to high-light intensity results in a loss of electron-transport activity. This phenomenon is called photoinhibition (for review see ref. [2]). The PS-II region is essentially affected, the molecular mechanism of the damage is not clear and the target site of photoinhibition not yet known. In earlier publications photoinhibitory damage was correlated with the loss of the 32-kDa herbicide-binding protein (HBP or D-1 peptide) [1], the polypeptide carrying also the secondary quinone (Q_B) as electron acceptor. More recent studies have demonstrated that additionally the reaction center of PS II or a site close to it is a main target region for photoinhibition [3]. Another observation is a partial loss of chlorophyll fluorescence after high-intensity light treatment [4, 5], possibly indicating a mechanism of protection against photoinhibition. This has been interpreted as a transformation of part of the reaction center to a quencher of fluorescence [6]. Apparently, altered centers can still trap excitation energy, but convert it to heat instead to chemical energy. This type of damage may be beneficial to protect the antenna chlorophylls from photodestruction [6].

This paper presents data showing that the 9-kDa phosphoprotein is the first visually altered polypeptide in the PS-II complex by light stress and therefore possibly involved in the photoinhibition process.

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Materials and Methods

Thylakoid and PS-II particle preparation

Thylakoids were isolated from *Spinacia oleracea L. (strain Atlanta)* cultivated in the greenhouse. Twice washed young leaves were homogenized in 0.4 M NaCl, 2 mM $MgCl_2$, 0.2% bovine serum albumin (BSA) and 20 mM Tricine/NaOH [N-tris(hydroxymethyl)-methylglycine], pH 8.0, then filtered and centrifuged for 60 s at $300 \times g$. The supernatant was centrifuged for 10 min at $4{,}000 \times g$. The pellet was washed once with 0.15 M NaCl, 5 mM $MgCl_2$, 0.2% BSA and 20 mM Tricine/NaOH, pH 8.0. For thylakoid preparation the sediment was resuspended in 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM Tricine/NaOH, pH 7.8, and adjusted to 1 mg chlorophyll (Chl)/ml and stored on ice.

For preparation of PS-II particles the pellet was resuspended in 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM MES/NaOH [morpholinoethane sulfonic acid], pH 6.5 (called medium K-1), and adjusted to 2 mg Chl/ml. An aliquot of 0.5% Triton X-100 in K-1 was added giving a final Triton/Chl ratio of 25:1 (w/w). The mixture was stirred gently on ice for 30 min and then centrifuged for 20 min at $20{,}000 \times g$. The pellet was washed once with medium K-1 and adjusted to 1 mg Chl/ml. This suspension, containing the PS-II particles, was kept on ice or stored deep-frozen.

Protein phosphorylation

Thylakoids, equivalent to 1 mg Chl, were incubated in 5 ml of a medium consisting of 0.1 M sucrose, 10 mM NaCl, 10 mM NaF, 5 mM $MgCl_2$, 20 mM Tricine/NaOH, pH 7.8, 165 μM ATP including 8.3 MBq γ-[^{32}P]ATP (111 GBq/mmol, Amersham, Braunschweig, F.R.G.) for 7 min in red-light (Schott RG 610 nm cut-off filter) with low intensity of 100 μE/$m^2 \times s$ (measured with a quantum sensor LI-190 SB, made by LI-COR, Lincoln, Nebraska, U.S.A.). The ^{32}P-labeled thylakoids were then recovered by centrifugation at 10,000 × *g* for 10 min. The pellet was used either for SDS-PAGE or for photoinhibition treatment.

Photoinhibition (high-intensity light) treatment

Labeled thylakoids or PS-II particles were suspended in 0.3 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Tricine/NaOH, pH 8.0 (and 10 mM NaF in case of labeled thylakoids). Samples equivalent to 50 μg of Chl/ml were illuminated with white light from a quartz-halogen lamp at an intensity of 5000 μE/$m^2 \times s$ for different periods. After light treatment the thylakoids or PS-II particles were recovered by centrifugation at 10,000 × *g* for 10 min. The pellet was prepared for SDS-PAGE.

SDS-polyacrylamide gel electrophoresis (PAGE)

Samples were incubated with an approximately equal volume of solubilization buffer (4% (w/v) SDS, 2% DTT (w/v, 130 mM), 20% glycerol (v/v) and 80 mM Tris/HCl [tris-(hydroxymethyl)-aminomethane], pH 6.8) for 20 min at room temperature prior to electrophoresis on 17.5% polyacrylamide gels containing 4 M urea as described in [7]. ^{32}P-labeled proteins were detected in the gels by autoradiography. The dried gels were exposed for 2 days to Hyperfilm-MP sheets from Amersham, Braunschweig, F.R.G.

Preparation of the antiserum

Purification of the 9-kDa phosphoprotein was performed by cutting the stained protein from SDS slab gels. The slices from several gels were eluted by electrodialysis, pooled and thoroughly dialyzed against 10 mM sodium-phosphate buffer, pH 7.0. About 100 μg of the protein were emulsified with complete Freund's adjuvant and subcutaneously injected into the back of a rabbit. After 10 days, the rabbit was boostered with another 50 μg of the protein. After another 10 days the rabbit was blooded. The serum with the antibodies was obtained by simple centrifugation to remove all blood residue. Specificity of the antibody was assayed by immunoblotting.

Western blotting

After SDS-PAGE the gels were also used for Western blotting. Electrophoretic transfer of peptides to nitrocellulose (0.45 μm pore size, Schleicher und Schuell, Dassel/F.R.G.) was performed in 190 mM glycine, 25 mM Tris base and 20% (v/v) methanol at a pH of 8.4 for 2 h at a current of 0.4 A. The nitrocellulose was then removed and treated with 0.14 M NaCl, 10 mM sodium phosphate (PBS), pH 7.0, and 5% (v/v) newborn-calf serum followed by incubation at room temperature with the anti-9-kDa phosphoprotein antiserum diluted 1:5000. After incubation (12 h), the nitrocellulose was washed three times with PBS and incubated with a 1:5000 dilution of goat-anti-rabbit IgG labeled with peroxidase (Jackson, West Grove, Pennsylvania, U.S.A.) for 1 h and subsequently washed three times with PBS. Development of the color was performed in PBS with 0.018% (w/v) chloronaphthol and 0.006% (v/v) H_2O_2.

Miscellaneous

Comparisons of amino acid composition were made using the parameter $S\Delta n = \frac{1}{2} \times \Sigma(n_{iA} - n_{iB})^2$ of Cornish-Bowden [8], where n_i is the number of residues of an individual amino acid in protein A to be compared with protein B.

Results

The protein composition of PS-II particles is shown in Fig. 1, lane a. The arrows indicate the proteins which were diminished by a short high-light treatment. The upper one is the 32-kDa herbicide-binding protein whose role in photoinhibition is well described [2]. The lower one is the 9-kDa phosphoprotein whose light-dependent alteration is first described here.

Two parameters were used, to identify this protein as the 9-kDa phosphoprotein and to distinguish between cytochrome *b*-559, migrating close with the 9-kDa peptide. Firstly, thylakoids were phosphory-

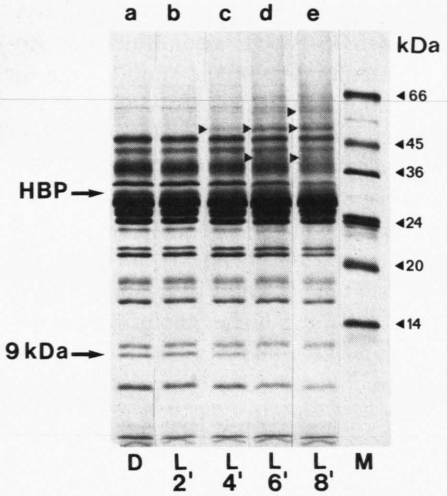

Fig. 1. SDS-PAGE of PS-II particles. Membranes were kept in the dark (D, lane a) or illuminated (L) with high-intensity light of 5000 $\mu E/m^2 \times s$ for different times (lane b to e). The gel shows the light-dependent decrease of both the herbicide-binding protein (HBP) and the 9-kDa phosphoprotein. The arrow-heads in lanes c–e point to the light-dependent increase of proteins at 41, 50 and 54 kDa. Molecular weight standards are given by the right lane (M).

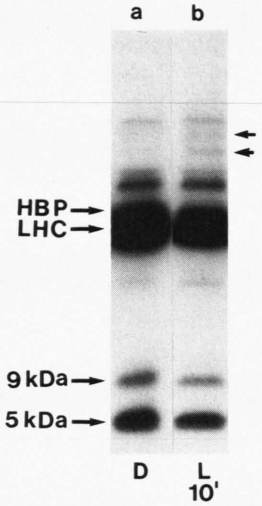

Fig. 2. Autoradiogram from a SDS-PAGE of thylakoids, phosphorylated by γ-[^{32}P]ATP. Thylakoids were phosphorylated (see Materials and Methods), washed and aliquots either kept in the dark (D, lane a) or illuminated (L) for 10 min with 5000 $\mu E/m^2 \times s$ (lane b). The strongly labeled proteins are a 5-kDa protein (probably the apoprotein of the cytochrome *b*-559), the 9-kDa phosphoprotein, proteins of the light-harvesting complex (LHC) and the herbicide-binding protein (HBP). After photoinhibition treatment some labeled proteins are diminished and new labeled proteins appear at 50 kDa and 54 kDa (indicated by the two arrows in lane b).

lated with γ-[^{32}P]ATP to label all proteins that were phosphorylated under the conditions described in Materials and Methods. As reported, the cytochrome *b*-559 will not be phosphorylated under these conditions [9]. Secondly, the amino acid composition of the 9-kDa protein was determined and compared with the known composition of both the 9-kDa phosphoprotein and the cytochrome *b*-559 taken from published data [10–13].

Lane a in Fig. 2 shows the autoradiogram of a typical SDS-PAGE from phosphorylated thylakoid proteins. The strongly labeled proteins are two proteins of the light-harvesting complex, the HBP, the 9-kDa protein and, noteworthy, a 5-kDa protein, which probably is an apoprotein of the cytochrome *b*-559 [14]. This protein was never reported before to become phosphorylated. The band at 9 kDa on the autoradiogram could be correlated with a protein, whose possible involvement in photoinhibition is described here.

To compare the 9-kDa protein in our gels with the known 9-kDa phosphoprotein and cytochrome *b*-559, the protein was purified as described in Materials and Methods for immunization and analyzed for amino-acid composition. Our data on amino-acid composition (not documented) yielded a probability higher than 95% for the phosphoprotein, using the method of Cornish-Bowden [8]. Data on both protein phosphorylation and amino-acid composition give strong evidence that the 9-kDa protein described in this paper is the 9-kDa phosphoprotein as reported previously [13].

The involvement of the 9-kDa phosphoprotein in the photoinhibition process is concluded from its decrease in the protein pattern of the PS-II particles after illumination with strong light. Fig. 1 (lane a–e) shows the time-dependent alteration of the protein pattern after a short high-light treatment. The loss of the phosphoprotein was more pronounced and somewhat faster than that of the HBP. The half-life time of the phosphoprotein during light stress, calculated from its loss on the SDS-PAGE, was approximately 5 min/±2 min, and about 7 min/±2 min for the HBP (values are means of 6 experiments). Electron-trans-

port activity, measured in the system 1,5-diphenylcarbazide (DPC) → 2,6-dichlorophenol indophenol (DCIP), was diminished by about 50% after a 5-min high-light treatment (not documented).

In PS II the 9-kDa phosphoprotein is the first visibly altered peptide under light stress. Unfortunately, in our SDS-PAGE system containing 4 M urea the HBP migrated closely with the LHC-proteins and could therefore hardly be visualized as a distinct band on the photograph of the gel. Determination of the loss of the HBP during light treatment was measured in gels with no urea present.

To follow the fate of the 9-kDa phosphoprotein, a polyclonal antiserum was produced by immunization with the purified protein. The antiserum was monospecific to this protein (Fig. 3, lane a). Neither the intact protein nor any degradation products could be observed by SDS-PAGE and immunoblotting techniques using the supernatant from particles which have been irradiated and subsequently pelleted (not documented). This indicates that neither the intact protein nor any degradation products greater than 2 kDa (this figure is determined by the resolution limit of the SDS-PAGE system) were released from the membrane.

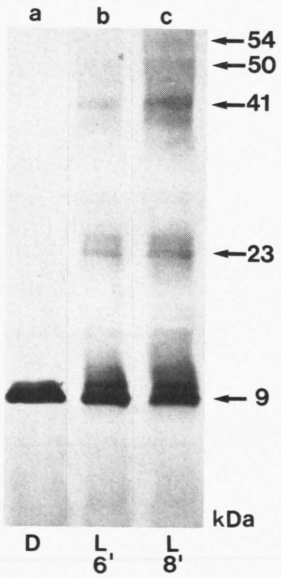

Fig. 3. Immunoblot of PS-II particles probed with antibodies against the 9-kDa phosphoprotein. PS-II particles were kept in the dark (D, lane a) or illuminated (L) with 5000 $\mu E/m^2 \times s$ for 6 min (lane b) and 8 min (lane c). After strong-light illumination the antibody reacted with new proteins at 23, 41, 50 and 54 kDa.

Analysis of the light-treated PS-II particles by immunoblotting and SDS-PAGE visualized new proteins. After a 6-min illumination the antibody against the 9-kDa phosphoprotein was reactive to new proteins of 23 kDa and 41 kDa (Fig. 3, lane b). After 8 min irradiation also proteins of 50 kDa and 54 kDa were recognized by the antibody, but with an unspecific diffuse background (Fig. 3, lane c). We conclude that these new proteins must contain the 9-kDa phosphoprotein or at least a part of it.

In the SDS-gel only the higher molecular weight peptides (41 kDa, 50 kDa and 54 kDa) could be directly seen as distinct bands (Fig. 1, arrows in lane c, d and e), because the smaller 23-kDa protein migrated together or very close to other bands of the PS-II particles.

The new proteins at 50 kDa and 54 kDa were also observed in an autoradiogram from a SDS-PAGE of light-treated γ-[^{32}P]ATP labeled thylakoids (Fig. 2, lane b), indicating that they contained at least one labeled, *i.e.* phosphorylated, polypeptide. As expected, also the ^{32}P-labeled 9-kDa phosphoprotein and the HBP were diminished during light stress (Fig. 2, lane b).

Photoinhibition phenomena occurred faster in PS-II particles. The degree of photoinhibition and loss of the 9-kDa phosphoprotein was much slower in thylakoids. Accordingly, the new bands described above could not be clearly seen with isolated thylakoids. In PS-II particles its exposed surface, corresponding to the lumenal surface of the grana thylakoids, becomes accessible to the medium [15]. In contrast to thylakoids the oxygen-evolving activity of PS-II particles is totally inhibited at pH 8.0 (data not shown). It was reported that inactivation of the oxygen-evolving complex (OEC) enhances photoinhibition [16]. Also a part of the OEC proteins are released in the medium during strong-light treatment (unpublished data).

Discussion

The most puzzling result of this study is the disappearance of the 9-kDa phosphoprotein and the formation of new proteins of higher molecular weight reactive with an antibody against the 9-kDa peptide. Marder *et al.* [17] recently reported on a 55-kDa protein in PS-II reaction center preparations from spinach reacting with antisera against the HBP (D-1 protein) and the D-2 protein, suggesting that

this new protein is composed of both, the D-1 and the D-2 protein. Also our antiserum against the D-1 protein [18] reacted in the 55-kDa region. The bands were slightly diffuse but appeared only after illumination of the membranes. Since the proteins are physically altered, possibly the recognition sites for the antibodies were modified or less accessible in the newly assembled polypeptides.

The mode of assembly of the described "surplus" proteins, formed during light stress, is not yet clear from our data. Both, the amino-acid composition of the 9-kDa phosphoprotein from spinach and the genetic determination of the amino-acid sequence of the 9-kDa phosphoprotein from wheat revealed no cysteine [10]. Accordingly, linkage to other peptides can not be the result of disulfide bridges mediated by cysteine. In the presence of 5 mM dithiothreitol (DTT) neither the 9-kDa phosphoprotein nor the HBP was diminished during high-light treatment for 10 min and the new peptide bands as mentioned above did not appear. Probably, this was caused by the inactivation of the PS-II activity by DTT. Electron-transport activity (DPC → DCIP) was totally inhibited by 5 mM DTT. Obviously, redox activity of the PS-II particles is a prerequisite for photoinhibition.

The stoichiometry of the newly-assembled 23-kDa and 41-kDa proteins, supposing a complete 9-kDa phosphoprotein as a component, suggests a linking to a 14-kDa and a 32-kDa peptide, respectively. It is of interest to assay their exact composition in future studies. The results may give an insight, whether the 9-kDa protein and the assembly of new proteins play a physiological role in PS II by adaptation to light stress.

Photoinhibition phenomena are accompanied by a partial loss of the variable fluorescence of Chl *a* [6], indicative of the formation of a fluorescence quencher during light stress. We speculate the 9-kDa phosphoprotein being functional as a quencher possibly together with other PS-II proteins.

Acknowledgement

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