Isolation of 32–35 kDa Thylakoid Proteins from *Chlamydomonas reinhardii*

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The protein composition of *Chlamydomonas reinhardii* thylakoids in the molecular weight range of 32–35 kDa was studied. The thylakoids were labelled with $^{32}P-P_i$ *in vivo* using P_i-starved cell cultures, solubilized with SDS and separated by polyacrylamide gradient gel electrophoresis. The following differentiation of proteins could be accomplished: two proteins were phosphorylated, which were also well stained with Coomassie blue, and one major protein was only detected by the silver staining procedure. The mobility of the latter protein is different in gels with urea, showing an apparently lower molecular weight.

In order to investigate whether a functional photosystem II is obligatory for protein phosphorylation, the phosphorylation of thylakoid proteins was studied with a photosystem II deficient mutant. The mutant, which had normal photosystem I activity, but lacked photosystem II activity, could synthesize ATP light dependently; its main labelled protein bands had a molecular weight of 32–35 kDa; it contained the light harvesting protein chlorophyll complex and an unknown protein at 22 kDa. The ^{32}P incorporation in photosystem II deficient cells was comparable to cells with functional photosystem II units.

Introduction

Our understanding of the structure and protein composition of the photosystem II complex is still rather unclear. The core of the reaction center in *Chlamydomonas reinhardii* is composed of three polypeptides with a molecular weight of 50, 47 and 3 kDa: Three peripheric proteins (35, 21 and 28 kDa) are so far identified as being loosely associated with the reaction centers [1]. The minimal number of polypeptides, which are involved in electron transfer, could be established by isolation and purification of the photosystem II complex [2], and by comparison of photosynthetic mutants with defects in this complex [3].

The state of photosystem II seems to be regulated by protein phosphorylation [4, 5] and by a photosensitive control mechanism [6]. The enzymes (protein kinase and dephosphorylase, protease), which are involved in these regulatory systems, are not identified. Either they are part of photosystem II, meaning their activities would have to be found within the complex, or additional protein components are reacting, which modulate the photosystem II activity.

The aim of this study was to investigate the phosphorylation of proteins in the molecular weight range of 32–35 kDa and to gather informations about the properties of these proteins. A further approach is the isolation of mutants with deficiencies in photosystem II to study their impact on the regulatory mechanisms, as discussed above.

Abbreviations: LHCP, light harvesting chlorophyll protein; Q_B, secondary quinone electron acceptor of photosystem II; SDS, sodium dodecyl sulfate. Herbicides: metribuzin, 4-amino-6-(*t*-butyl)-4-methylthio-1,2,4-triazine-5-one; atrazine, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNSJ, 2-iodo-4-nitro-6-isobutylphenol; ioxynil, 3,5-diiodo-4-hydroxybenzonitrile.

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Results and Discussion

Isolation of the 32–35 kDa membrane proteins

The proteins within the molecular range of 32–35 kDa of the thylakoid membranes of *Chlamydomonas reinhardii* cells were analyzed after phosphorylation by labeling with ^{32}P and by electrophoresis in two different systems. The properties of the proteins were compared and criteria for their differentiation were established.

Chlamydomonas reinhardii cells were grown in P_i-free medium for 2 days (P_i-starved cells), and

after the addition of $^{32}P_i$ illuminated for 20 min. The cells were quickly sonicated in 50 mM tricine NaOH buffer, pH 7.8, containing 100 mM NaF, and the thylakoid membranes were purified through a sucrose step gradient centrifugation (210000 × *g*, 1 h). The thylakoid membrane proteins were solubilized by SDS and separated by preparative gel electrophoresis according to the method of Chua [7]. The labeled band in the 32–35 kDa region was detected by autoradiography, excised, and the proteins transferred to an agarose slab gel. The proteins were recovered by centrifugation, dialyzed and concentrated. This concentrate was applied again for electrophoresis, but this time the separation occurred in presence of 4 M urea. This method proved to be a good procedure for separating the Q_B-protein from the ^{32}P labeled proteins. These proteins could be distinguished by differences in silver staining, but not with Coomassie blue, as already shown by Kyle *et al.* [8]. We could confirm the results that the Q_B-protein band is rather diffuse and poorly stainable with Coomassie blue (Table I). Since this band was not labeled with ^{32}P, we can conclude that this protein is not phosphorylated under our conditions. We assume that the properties of this protein do not change due to phosphorylation, *i.e.* the phosphorylated form has a different conformation, which can be stained by Coomassie blue. A significant dephosphorylation can be ruled out because results from preliminary experiments indicated that dephosphorylation of thylakoids did not occur in darkness and was suppressed by NaF.

The main ^{32}P-labeled protein had an apparent molecular weight of 34–35 kDa in the absence and presence of urea. In 4 M urea gels, however, another ^{32}P-labeled protein band with an apparent molecular weight of 37–38 kDa was observed. Further experiments on the amino-acid composition of these two proteins will answer the question of their identity.

Isolation, characterization and phosphorylation of photosystem-II deficient mutants

Mutants with deficient photosystem-II activity were isolated from wild-type cells of *Chlamydomonas reinhardii*. The cells were treated with 5-fluorodeoxyuridine and shortly exposed to UV irradiation. The plates were kept in the dark under heterotrophic conditions and the mutant clones were selected by chlorophyll fluorescence [9].

Table I. Properties of the 32–35-kDa proteins.

Protein	Apparent molecular weight [kDa]		Best staining procedure
	minus urea	plus urea	
Q_B-protein	35	32	silver
^{32}P-protein-I	35	35	Coomassie blue
^{32}P-protein-II	35	37	Coomassie blue

Table II. Comparison of wild-type cells (WT) and the mutant strain UV-77.

	WT	UV-77
Chlorophyll content (mg Chl/10^8 cells)		
light grown cells (1000 lux)	0.25	0.81
dark grown cells	0.12	0.17
Electron transport rates: intact cells		
$H_2O \rightarrow CO_2$ (μmol O_2/mg Chl × h) [a]	101.5	0
Electron transport rates: isolated thylakoids		
$H_2O \rightarrow$ DCPIP [b] (μmol DCPIP/mg Chl × h)	58	0
DPC → DCPIP [b] (μmol DCPIP/mg Chl × h)	85	0
DAD/Asc → methylviologen [c] (μmol O_2/mg Chl × h)	364	355

[a] The light dependent incorporation of ^{14}C was determined. The assay mixture contained cells equivalent to 0.1 mg chlorophyll, 10 mM Tris-sulfate buffer, pH 7.2, 10 mM $MgCl_2$ and [^{14}C]bicarbonate (1.5×10^7 Bq/mmol).

[b] The photosynthetic DCPIP reduction was followed spectrophotometrically at 600 nm. The assay mixture contained thylakoids equivalent to 10 μg chlorophyll (Chl), 10 mM tricine-NaOH buffer, pH 6.9, 5 mM $MgCl_2$, 20 mM KCl, 50 μM 2,4-dichlorophenol indophenol (DCPIP), 2.5 μM gramicidin D, plus or minus 0.5 mM diphenylcarbazide (DPC).

[c] The light dependent oxygen uptake was measured polarographically. The reaction medium contained thylakoids equivalent to 100 μg chlorophyll, 20 mM tricine-NaOH buffer, pH 7.4, 20 mM $MgCl_2$, 20 mM KCl, 0.1 mM methylviologen, 0.3 mM sodium azide, 2.5 μM gramicidin D, 0.5 mM diaminodurene (DAD), 50 μM DCMU and 3 mM sodium ascorbate (Asc).

The mutant strain UV-77 could grow only in a heterotrophic medium, in the dark or in light. The chlorophyll content varied considerably due to the growing conditions (Table II). The chlorophyll *a*/*b* ratio was similar for both samples with a value of 2, but the mutant UV-77 was not able to reduce CO_2. As shown in Table II, the isolated thylakoids had no photosystem II activity, but a similar photo-

system I activity as compared with the wild-type cells.

The lack of photosystem II activity could be correlated with the loss of proteins in this complex. Thylakoids were purified and solubilized by SDS and the proteins were separated by gradient polyacrylamide gel electrophoresis [7]. The following bands were greatly reduced or missing: 51, 46, 34, 23, and 18 kDa (data not shown).

It was of special interest to investigate whether phosphorylation of thylakoid proteins can still occur in this photosystem II deficient mutant. Therefore, P_i-starved cells were treated in light with $^{32}P-P_i$ and the purified thylakoids were isolated and solubilized. After the electrophoresis, the ^{32}P-labelled protein bands were identified by autoradiography and compared with wild-type thylakoids. Despite the deficiency of photosystem II, the 32–35 kDa band was labeled equally to the proteins of the wild-type, and the same observation was made by comparing the bands of the LHCP-complex. A significant difference was a heavily labeled band with a molecular weight of 22 kDa which was absent in the wild-type cells, but was the predominant band in the sample of the mutant UV-77 (data not shown). An analysis of the labeled protein(s) at the molecular weight range of 32–35 kDa is in progress, and the exact assignment of the labeling to a specific photosystem II protein can, therefore, not yet be accomplished.

The observation of the labeling of the LHCP-complex, despite a non-functional photosystem II, opens the question as to whether photosystem II is a part of the regulatory system in state transfers. It can be concluded that the phosphorylation of the LHCP-complex, and probably of the other 32–35 kDa protein as well, can occur without photosystem II activity, though it still depends on the reduction state of plastoquinone.

It is known that a special peculiarity of *Chlamydomonas reinhardii* thylakoids is that the plastoquinone pool can be reduced *via* NADH [10, 11]. Therefore, instead of the reduction of plastoquinone through photosystem II, NADH, which is provided by the oxidation of heterotrophic growth substrate (acetate), serves as the reductant for plastoquinone. A similar mutant was recently described, which also showed phosphorylation of thylakoid proteins despite the lack of photosystem II activity [12], whereas the mutant T_{44} grown at the nonpermissive temperature (absence of photosystem II) showed phosphorylation of the 32–35 kDa proteins, but not of the LHCP-complex [5].

Acknowledgements

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