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N-terminal sequence analysis of the 8 kDa protein in *Chlamydomonas reinhardii*

Localization of the phosphothreonine

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A phosphorylated 8 kDa protein of *Chlamydomonas reinhardii* thylakoids has been isolated and its N-terminal amino acid sequence determined by gas-phase sequencing. The sequence analysis of the 48 amino acid residues revealed that this protein is about 50% homologous to the psb H gene products of higher plants. In contrast to them, it contains an insert of seven amino acid residues (Ser-5 to Lys-11). The first threonine residue was phosphorylated as determined by ^{32}P detection during sequencing and also by analysis of the modified degradation products in the chemical reaction of the Edman degradation process. This latter method allows the identification of phosphorylated threonine residues without radiolabelling the protein.

Photosystem II; psb H protein; N-terminal sequence; Phosphothreonine; (*Chlamydomonas reinhardii*)

1. INTRODUCTION

The phosphorylation of thylakoid proteins is part of a regulatory system for ensuring the balance between the rates of photosystem I and photosystem II excitation (see for a review [1]). In *Chlamydomonas reinhardii* thylakoids two groups of phosphorylated proteins can be distinguished on the basis of their phosphorylation kinetics: proteins that are relatively rapidly phosphorylated are the LHC-II, a 33 kDa protein (not identical with the extrinsic protein of the OEC) and an 8 kDa protein, whereas several proteins of the photosystem II (psb A, psb C, psb D) are phosphorylated considerably slower.

The identity of the 8 kDa protein in *C. reinhardii* and whether it participates in the regulatory system mentioned above are unknown. It was suggested that the phosphorylated 8 kDa protein is the proteolipid moiety of CF_o [2], a small subunit of the cytochrome b_6f complex [3], cytochrome *b*-559 [4], a chlorophyll binding protein [5] or the psb H gene product [6,7].

The primary structure was used to characterize the 8 kDa protein and to localize the phosphorylated amino acid residue.

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Abbreviations: DTT, dithiothreitol; LHC-II, polypeptides of the light-harvesting chlorophyll *a/b* protein complex; Mops, 3-(*N*-morpholino)propanesulfonic acid; OEC, oxygen evolving complex; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. *Isolation of the 8 kDa phosphoprotein*

C. reinhardii cells were grown mixotrophically (5% CO_2 in air and 10 mM acetate) in light to the log growth phase. The cells were transferred to a phosphate free medium and depleted for 2 days. The cell culture was adjusted to 0.1 mg chlorophyll/ml, adapted to darkness (30 min), supplemented

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with [^{32}P]phosphate (37 MBq) and incubated at 23°C for 25 min in light (2.6×10^6 erg/cm^2 per s). The labelled cells were washed with 50 mM Tricine-NaOH buffer, pH 7.8, containing 100 mM NaF, and broken by sonication (7 times for 15 s, 100 W, Branson sonifier). The resulting thylakoids were washed with the same buffer, lyophilized and extracted by chloroform/methanol (1:2) in ice for 5 min. The thylakoid protein pellet was solubilized [9] and the proteins separated on preparative gels [10]. The phosphorylated protein bands were identified by autoradiography and by determining the apparent molecular mass.

The phosphorylated 8 kDa protein was electroeluted and electrodialysed [11]. The dialysate was directly used for reverse-phase chromatography (FPLC-ProRPC 5/10, Pharmacia). The binary gradient system consisted of (A) 0.1% TFA in water and (B) 0.08% TFA in CH_3CN. The protein elution was recorded by measuring the absorbance at 220 nm and the radioactivity was determined by liquid scintillation counting. The ^{32}P-labelled peak was pooled and concentrated to a small volume. The purity of the 8 kDa protein in this sample was analyzed by analytical gel electrophoresis and autoradiography.

2.2. *Preparation of the phosphorylated model peptide*

The model peptide (GRTGRRNSIHDIL) was synthesized with an Applied Biosystems peptide synthesizer. 600 mg of the resin-bound peptide were split off the resin with trifluoromethanesulfonic acid/TFA-thioanisole-ethanedithiol as described in the user's bulletin of Applied Biosystems according to [12,13]. 80 μmol of crude peptide were recovered after this procedure.

Aliquots were taken for peptide purification using a Vydac 218 TP 5415 C18 column.

The peptide was phosphorylated according to [14] with slight modifications: 80 nmol of the purified peptide were incubated in 1 nmol of a mixture containing 1 mM EGTA, 0.1 M KCl, 50 mM Mops, 1.5 mM $MgCl_2$, 1 mM ATP, plus some [γ-^{32}P]ATP, 7.5% glycerol and 300 mM catalytic subunit of the cAMP-dependent protein kinase for 18 h at 25°C.

The diphosphorylated peptide was purified by reverse-phase HPLC using a Vydac 218 TP 5415 C18 column and a linear gradient consisting of 0.1% TFA in water as solvent A and 0.08% TFA/84% acetonitrile as solvent B.

Aliquots of the purified peptide were taken for amino acid analysis and ^{32}P radioactivity determination to evaluate the phosphate content per mole of peptide.

Values found were between 1.7 and 1.9 mol phosphate per mol of peptide which means that the peptide contains two phosphate groups. Other aliquots were taken for amino acid sequence analysis.

2.3. *Amino acid analysis*

Samples were gas-phase hydrolysed with 6 M HCl at 150°C for 1 h. The analysis was performed by precolumn derivatization with phenylisothiocyanate and reverse-phase HPLC of the resulting PTC-amino acids [15,16].

2.4. *Sequence analysis*

The sequence analysis of the 8 kDa protein and the phosphothreonine containing model peptide was performed with an Applied Biosystems gas-phase sequencer equipped with an on-line PTH-analyzer [17].

3. RESULTS

3.1. *Isolation of the labelled 8 kDa protein*

The 8 kDa protein was isolated by two separation techniques: preparative gel electrophoresis and reverse-phase chromatography. This protein was purified to homogeneity with a yield of 20% (after gel electrophoretic separation of the thylakoid proteins) and the overall yield was equivalent to 0.8 μg protein/mg chlorophyll.

The short extraction of the pigments and phospholipids with $CHCl_3/CH_3OH$ (1:2) improved the autoradiographic identification of the labelled 8 kDa protein band without risking a loss of the hydrophobic protein. The major phosphorylated protein bands were recorded at 34, 33, 31, 29, 27, 8 and 4 kDa and the distribution of the ^{32}P label shows that approx. 11% of the radioactivity is present in the 8 kDa protein band. The protein was isolated from the excised gel band by electroelution and the subsequent electrodialysis step reduced significantly the SDS concentration in the sample.

The protein sample was subjected to FPLC–reverse-phase chromatography, and a binary gra-

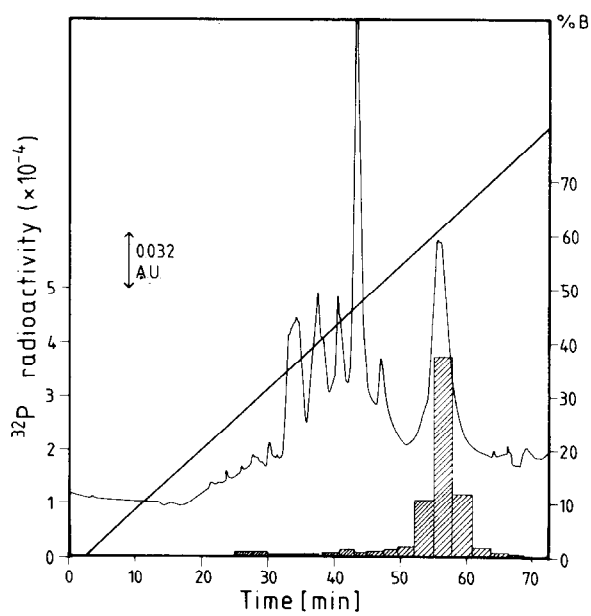

Fig.1. Preparative separation of the 8 kDa phosphoprotein by FPLC. The 8 kDa phosphoprotein was purified by reverse-phase FPLC chromatography. The binary gradient consisted of 0.1% TFA in water and 0.08% TFA in CH_3CN (1.16% per min, 0.5 ml/min); A_{220} and ^{32}P radioactivity were monitored.

dient system, TFA in water and TFA in acetonitrile, was applied. The elution diagram is presented in fig.1. Despite the fact that the 8 kDa protein band was excised rather sharply to avoid contaminations of other proteins, it was observed that at least five other proteins with similar R_f values comigrated with the 8 kDa protein. Only one of those proteins was labelled, and the most hydrophobic one eluted last from the column. The peak fraction was analyzed by gel electrophoresis and autoradiography and the results are presented in fig.2. This protein stains poorly with Coomassie blue, and the single radioactive band showed that the protein was isolated in intact form, since no other fragments were detected.

3.2. *Amino acid composition of the 8 kDa protein*

The amino acid composition of the *C. reinhardii* 8 kDa protein was determined and compared with the known composition of the wheat chloroplast psb H gene product [8]. The data given in table 1

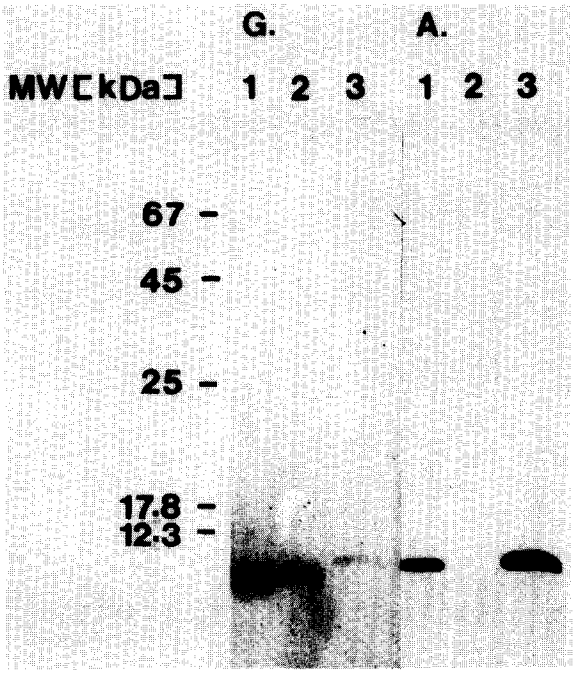

Fig.2. SDS-gel electrophoresis and autoradiography of the purified 8 kDa phosphoprotein. The integrity and purity of the 8 kDa protein were determined by electrophoresis, staining by Coomassie brilliant blue (G) and autoradiography (A). Lanes: 1, electroeluate; 2, unbound protein fraction of the FPLC; 3, ^{32}P-labelled peak fraction of FPLC (sample for sequence analysis).

Table 1

Amino acid analysis of the 8 kDa phosphoprotein in comparison to the wheat chloroplast psb H gene product

Amino acid	*C. reinhardii* 8 kDa protein[a] (mol/mol)	Wheat chloroplast psb H gene product (mol/mol)
Asx	3.6	5
Glx	3.9	4
Cyc	nd	0
Ser	8.7	7
Gly	8.7	7
Thr	7.4	6
Ala	6.4	6
His	0	0
Pro	7.0	5
Arg	1.8	2
Tyr	1.3	2
Val	7.2	4
Met	1.8	2
Ile	1.6	5
Leu	13.3	9
Phe	2.8	3
Lys	4.8	4
Trp	nd	1

[a] Values are not corrected for destruction or slow liberation of distinct amino acids

nd, not determined

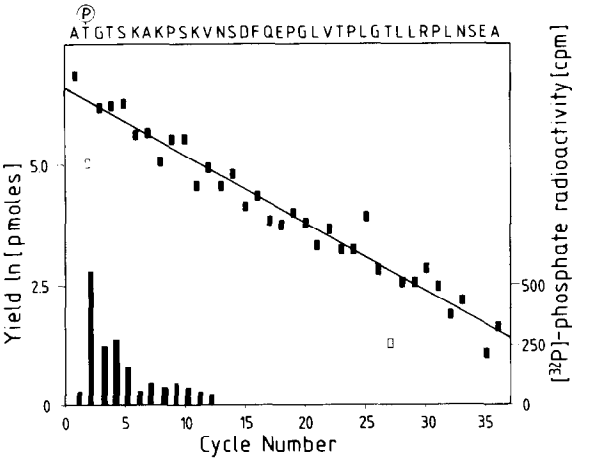

Fig.3. Sequence analysis of the 8 kDa phosphoprotein. About 1 nmol of the purified protein (72500 cpm) was applied onto the glassfiber disc of the gas-phase sequenator. Radioactivity (filled bars) was determined from each cycle and corrected for complete yield. The ln(yield) is plotted against the cycle number and the initial yield (850 pmol) and the repetitive yield (86.7%) were calculated from the residues drawn with filled signs.

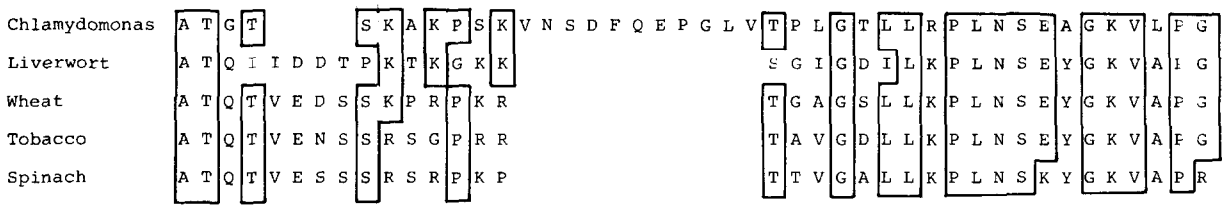

Fig.4. N-terminal amino acid sequence of the 8 kDa phosphoprotein from *C. reinhardii*, and alignment with respective psb H gene sequences from higher plant species (*Marchantia polymorpha* [18], *Nicotiana tabacum* [19], *Spinacea oleracea* [7] and *Triticum aestivum* [8]). The amino acid residues homologous to the *C. reinhardii* sequence are boxed.

show the similarity between both proteins with the high content of leucine and the absence of histidine.

3.3. *Sequence analysis of the phosphorylated 8 kDa protein*

The N-terminal amino acid sequence was determined (fig.3) and compared with the derived amino acid sequence of the psb H genes of *Marchantia polymorpha* [18], *Nicotiana tabacum* [19], spinach [7] and wheat [8] (fig.4).

The calculated initial yield was 850 pmol and the repetitive yield 86.7%, both calculated from the data in fig.3. The [^{32}P]phosphate liberated in each cycle was counted (filled bars in fig.3).

The threonine residue in position 2 was found to

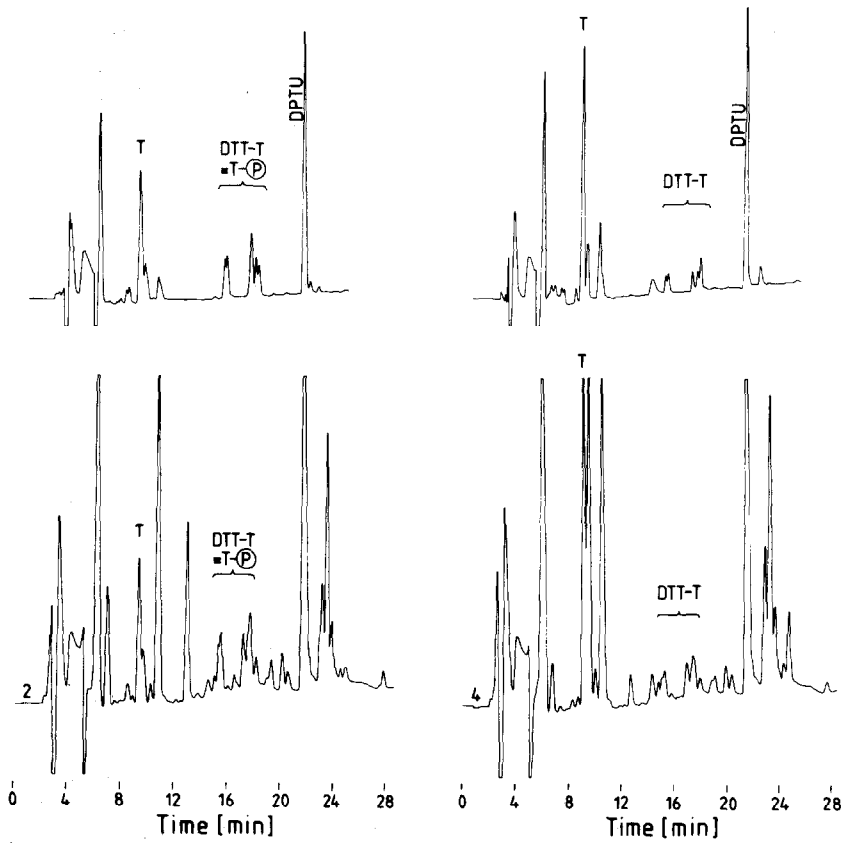

Fig.5. PTH-amino acid chromatograms of phosphorylated and non-phosphorylated threonines. The upper left chromatogram shows the result from the model peptide with a phosphothreonine in the third position, compared with the unphosphorylated peptide (right side). The lower two chromatograms show the results from degradation step 2 (phosphothreonine), left side, and step 4 (threonine), right side, of the 8 kDa phosphoprotein.

be phosphorylated. Although the ^{32}P released in this step represents only 0.8% of the initially applied radioactivity, the identification of the phosphothreonine in this position is unambiguous, since this amino acid behaves like the phosphothreonine residue in the model peptide (GRTGGRRNSIHDIL) as shown in fig.5. This assignment is also supported by the unmodified threonine in the fourth position of the 8 kDa protein which looks like the threonine in the chromatogram of the non-phosphorylated model peptide.

4. DISCUSSION

The sequence of the 8 kDa protein from a eucaryotic alga, like *C. reinhardii*, provides information about structural variability of the psb H gene products, since so far only sequences of higher plants are available [7,8,18,19,26]. The comparison with those reveals that the N-terminal part is similar: the first amino acid residue (M) is cleaved off and the NH_2-A-T(P)-X-T- sequence was found in all sources [6–8,18,19,26]. An SKXXP motive was also recognized as a conserved sequence at the N-terminus (fig.4). The alignment of all five sequences was possible by deletion and insertion of part of the *C. reinhardii* sequence, and thus a homologous stretch could be noted.

It is interesting to note that the subunits of photosystem II which are phosphorylated (psb A, psb C, psb D and psb H) have similar N-termini [20]. Despite the similarity of the phosphorylated sites there is a significant difference in the kinetics of phosphorylation. The $t(1/2)$ value for the 8 kDa protein is approx. 7 min, whereas the proteins of psb A, psb C and psb D are more slowly (longer than 20 min) phosphorylated (not shown).

The functional role of the protein phosphorylation is still unknown. Evidence has been presented which suggests that protein phosphorylation of photosystem II does attenuate the rate of electron flow between Q_A and Q_B [21]. The additional negative charge could lead to a stabilization of Q_A^- in light, and therefore decrease the rate of electron transfer in photosystem II [21,22], assuming that the 8 kDa protein is close to the accepting side of the reaction center of photosystem II. A recent hypothesis assigns the function of subunit H in reaction centers of photosynthetic eubacteria to the 8 kDa protein [23].

The method for the detection of the phosphorylated site was extended to the analysis of the PTH-DTT adducts of the modified amino acid deriving from phosphothreonine and threonine. The phosphate group of phosphothreonine is eliminated completely during the Edman degradation process, like the phosphate group of phosphoserine [24,25]. Therefore, the amount of DTT-addition products of the dehydroaminobutyric acid is increased compared with an unphosphorylated threonine. In the case of phosphothreonine some PTH-threonine is reconstituted due to the addition of water molecules. This reaction is different from that with phosphoserine which yields PTH-DTT-dehydroalanine exclusively.

There is a distinct difference between the shapes of the PTH chromatograms of a phosphothreonine and of its threonine. The PTH-threonine peak is lowered and in the case of phosphothreonine the DTT byproducts are increased.

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