Truncated recombinant light harvesting complex II proteins are substrates for a protein kinase associated with photosystem II core complexes

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Received 14 July 1998

Abstract Previous studies directed towards understanding phosphorylation of the chlorophyll *a/b* binding proteins comprising light harvesting complex II (LHC II) have concentrated on a single phosphorylation site located close to the N-terminus of the mature proteins. Here we show that a series of recombinant pea Lhcb1 proteins, each missing an N-terminal segment including this site, are nevertheless phosphorylated by a protein kinase associated with a photosystem II core complex preparation. An Lhcb1 protein missing the first 58 amino acid residues is not, however, phosphorylated. The results demonstrate that the LHC II proteins are phosphorylated at one or more sites, the implications of which are discussed.

Key words: Light harvesting protein; Protein kinase; Thylakoid membrane; Pea; Spinach

1. Introduction

The energy for transfer of electrons through the photosynthetic pathway originates from the absorption of light quanta by hundreds of chlorophyll molecules bound to proteins embedded in the thylakoid membrane. The dominant light harvesting complex in plants is associated with photosystem II (PS II) and is known as LHC II. It contains apoproteins (LHCPs) of 25–28 kDa, encoded by a family of nuclear genes called *Lhcb* (formerly *cab*) genes, which interact non-covalently with pigments including chlorophylls *a* and *b* and xanthophylls. Electron crystallography has revealed an ordered arrangement of pigments in LHC II trimers [1] which probably represents an evolutionary optimisation of antenna function for chloroplastic PS II. Stable complexes, similar in structure to LHC II monomers, can be reconstituted in vitro using purified endogenous proteins [2] or recombinant LHCPs and pigments [3,4].

Phosphorylation of LHC II proteins is believed to be one mechanism by which distribution of excitation energy between photosystems I and II is controlled. Activation of thylakoid protein kinase(s) correlates with the chemical reduction of specific electron carriers in the thylakoid membrane [5] and the resulting phosphorylation of LHC II is thought to initiate its detachment from PS II thereby decreasing excitation energy available for charge separation in the photosystem [6]. Although proteolytic digestion has shown a phosphorylation site close to the N-terminus [7] the position of the modified residue(s) within the amino acid sequence appears to vary according to the species. In a pea Lhcb1 Thr-5 is believed to be modified in preference to Thr-6 [8], where numbering of the amino acids begins at the arginine which is the first residue of the mature protein.

This sequence also contains a serine residue at position 3 which is not phosphorylated [8]. Analysis of *Lhcb* gene sequences from other species has revealed that neither threonine nor serine residues are conserved at positions 5 and 6 [8]. No consensus motif surrounds the phosphorylated residues in any species but there is a requirement for adjacent basic amino acids [9]. Isolation and identification of a single protein kinase catalysing LHC II phosphorylation has been a long-term research goal which has still to be achieved. Recent work has shown that a protein kinase activity copurifying with PS II (PS II-PK) catalyses phosphorylation of endogenous LHC II proteins on a timescale consistent with a function in intact thylakoids [10]. However, the involvement of more than one protein kinase in these modifications is indicated by the existence in the thylakoid membrane of distinct populations of LHC II with differing levels of phosphorylation [11,12].

Here we report the phosphorylation of a series of reconstituted, truncated recombinant LHC II proteins, based on the pea *Lhcb1*2* gene sequence. These modifications are catalysed by PS II core complexes containing PS II-PK derived from spinach. More than one residue along the length of the N-terminal domain of LHC II is therefore susceptible to phosphorylation.

2. Materials and methods

The pea *Lhcb1*2* clone (Fig. 1) was constructed stepwise by replacement of the 5′ proximal region of the reading frame in XLHCP-2 [3] with complementary synthetic oligonucleotides. A *Sph*I site was introduced at the translational start by exchange of the *Eco*RI-*Bam*HI fragment with the complementary oligonucleotides 5′-aattcattaaagaggagaaattaagcatgcgtag-3′ and 5′-gatcctacgcatgcttaatttctcctctttaatg-3′. The 5′ proximal region of the reading frame between the *Sph*I site and the second *Nco*I site, located at codons 15 and 16 of the mature protein, was then replaced by the complementary oligonucleotides 5′-cgtaaatctgctaccaccaagaaagtagcgagctctggaagcc-3′ and 5′-catgggcttccagagctcgctactttcttggtggtagcagatttacgcatg-3′. The N-terminal deletion

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; LHC II, light harvesting, chlorophyll *a/b* binding protein complex associated with photosystem II; Lhcb1, light harvesting protein of photosystem II; LHCP, light harvesting, chlorophyll *a/b* binding protein; OG, octyl glucopyranoside; PS II, photosystem II; PS II-PK, protein kinase associated with photosystem II; TCA, trichloroacetic acid; TX-100, Triton X-100

PII: S0014-5793(98)01046-1

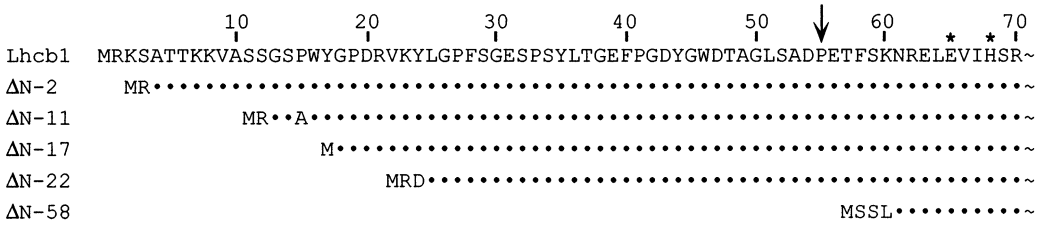

Fig. 1. N-terminal amino acid sequences of the pea Lhcb1*2 protein and derivatives. Numbering of amino acids begins one residue downstream from the leading methionine [22]. Dots indicate amino acid identity with Lhcb1. The start of the first transmembrane helix is marked by the arrow and asterisks identify residues implicated in chlorophyll binding [1].

mutants ΔN-2, ΔN-11, ΔN-17 and ΔN-22 have been described previously [13,14]. ΔN-58 was constructed by exonucleolytic digestion and re-ligation detailed by Paulsen and Hobe [15]. Full-length Lhcb1 and N-terminal deletion mutants thereof were over-expressed in *Escherichia coli*, extracted and reconstituted with pigments [16] with replacement of 1% (w/v) octyl glucopyranoside by 0.5% (w/v) CHAPS.

PS II core complexes were prepared from spinach as described in [10]. Phosphorylation of reconstituted LHC II protein (10 μg protein in 25 μl) by PS II core complexes (12 μg protein) was performed in the presence of 20 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 1 mM NaF, 1 mM DTT, 50 μM ATP and 0.5 μCi/μl [γ-^{32}P]ATP: 0.5% (w/v) CHAPS or 0.1% (v/v) TX-100 were also present where indicated. Reactions were terminated by addition of an equal volume of 20% (w/v) TCA: samples were prepared for electrophoresis as described in [17]. SDS-PAGE was carried out using 15% polyacrylamide gels (8.3×5.5 cm or 18×16 cm) and the buffer system of Laemmli [18]. Proteins were visualised by staining with Coomassie brilliant blue R250 and incorporated radioactivity was detected using a Molecular Dynamics PhosphorImager.

3. Results

3.1. Phosphorylation of expressed Lhcb1 protein after reconstitution

The rapid phosphorylation of endogenous spinach LHC II proteins by PS II core complexes isolated from the same species has been demonstrated [10]. A low concentration of detergent (0.003% TX-100) was included in this previous experimental system to inhibit protein aggregation during the incubation period. Reconstitution of Lhcb1 has typically been performed by detergent exchange from lithium dodecyl sulphate (LDS: 2%) to octyl glucopyranoside (OG: 1%) [16]. Such detergent conditions proved inhibitory to phosphorylation (data not shown), so a compromise was sought between conditions necessary to maintain solubility of reconstituted proteins and those required to maintain protein kinase activity. CHAPS was used in place of OG in the reconstitution procedure and its effect on subsequent protein phosphorylation was monitored in the presence and absence of TX-100.

Phosphorylation of the 43-kDa chlorophyll *a*-binding antenna protein of PS II (CP43) was apparent in complexes incubated in the presence of 0.1% (v/v) TX-100 and reconstitution buffer depleted in LDS by KCl precipitation [16] (Fig. 2, lane 1). The latter was included to determine the effects of components of the reconstitution buffer on protein kinase activity. Substitution of TX-100 by 0.5% (w/v) CHAPS diminished CP43 phosphorylation in PS II core complexes (Fig. 2, lane 2) but allowed incorporation of phosphate into Lhcb1, at the expense of modification of the former protein (Fig. 2, lane 3). Co-addition of TX-100 and CHAPS abolished phosphorylation of the CP43 protein (Fig. 2, lane 4). However, Lhcb1 became phosphorylated (Fig. 2, lane 5) to an extent similar to that observed in the presence of CHAPS alone (Fig. 2, lane 3).

3.2. Phosphorylation of truncated Lhcb1 proteins

The Lhcb1 protein and its truncated derivatives were reconstituted with 0.5% (w/v) CHAPS and phosphorylated by PS II-PK in the presence of the same detergent. The alterations in molecular weight resulting from the deletion of N-terminal amino acids of Lhcb1 are clearly resolved on the stained gel (Fig. 3A, lanes 2–7). Interestingly, all but the ΔN-58 derivative are phosphorylated by PS II-PK (Fig. 3B, lanes 2–7). No phosphate is incorporated into full-length Lhcb1 when it is incubated without PS II core complexes (Fig. 3B, lane 8) demonstrating that the protein kinase activity originates in the PS II preparation and is neither a contaminant carried over from the expression system nor an autocatalytic activity of the reconstituted Lhcb1 protein. It is apparent that the ΔN-2, ΔN-11, ΔN-17 and ΔN-22 proteins are more heavily phosphorylated than the full-length protein (Fig. 3B, lanes 3–7 and lane 2). In a vesicular preparation such an observation might be explained by the proteins adopting different preferred orientations, with only those whose N-terminal domains are exposed to the outside of the vesicles being accessible for phosphorylation. The reconstitution procedure followed here is, however, believed to produce micelles. In this case, the correlation between truncation of the N-terminal sequence and increased incorporation of radioactive phosphate would not be

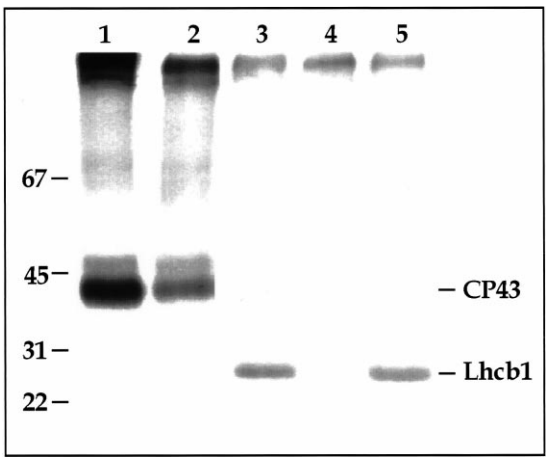

Fig. 2. Phosphorylation of reconstituted, recombinant Lhcb1 by PS II core complexes in the presence of various detergents. PhosphorImage of proteins after incubation with [γ-^{32}P]ATP for 21 min and electrophoresis on a 15% polyacrylamide gel. Lane 1, PS II core complexes + 0.1% (v/v) TX-100; lane 2, PS II core complexes + 0.5% (w/v) CHAPS; lane 3, PS II core complexes + 0.5% (w/v) CHAPS + reconstituted Lhcb1; lane 4, PS II core complexes + 0.5% (w/v) CHAPS + 0.1% (v/v) TX-100; lane 5, PS II core complexes + 0.5% (w/v) CHAPS + 0.1% (v/v) TX-100 + reconstituted Lhcb1. The positions of molecular weight standards and major phosphoproteins are indicated.

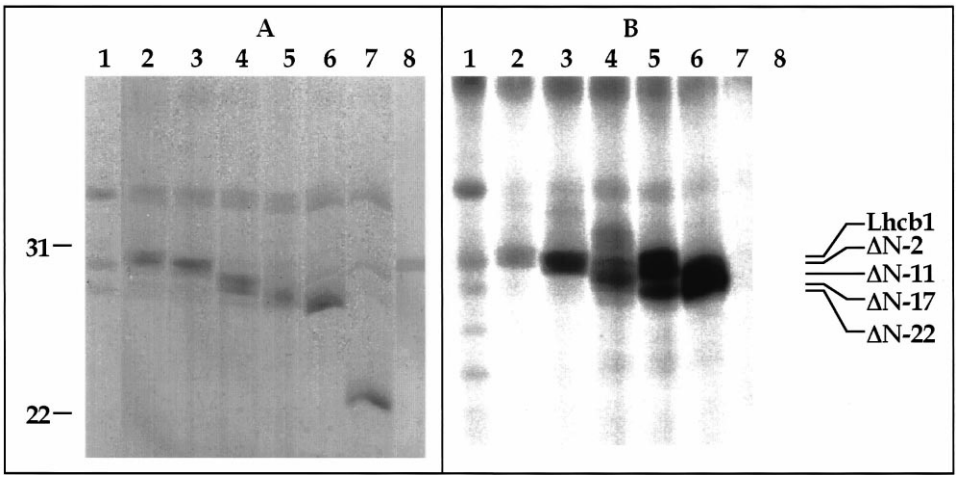

Fig. 3. Phosphorylation of reconstituted Lhcb1 protein and truncated derivatives by PS II core complexes. After incubation with [γ-^{32}P]ATP for 180 min and electrophoresis on a 15% polyacrylamide gel, proteins were visualised by staining with Coomassie blue (A) and PhosphorImaging (B). Lane 1, PS II core complexes; lane 2, PS II core complexes + Lhcb1; lane 3, PS II core complexes + ΔN-2; lane 4, PS II core complexes + ΔN-11; lane 5, PS II core complexes + ΔN-17; lane 6, PS II core complexes + ΔN-22; lane 7, PS II core complexes + ΔN-58; lane 8, Lhcb1. The positions of molecular weight standards and major phosphoproteins are indicated.

expected to result from different orientations of the proteins. Removal of the previously identified phosphorylation site at Thr-5 might instead expose additional residues for modification, but this does not explain why the ΔN-2 protein, which retains Thr-5, is more heavily phosphorylated than the full-length protein (Fig. 3B, lanes 2 and 3). Indeed access of PS II-PK to such additional phosphorylation sites might be expected to be hindered by increased shielding from the micelles as the N-terminal sequence becomes shorter. Such shielding does not appear to be significant here. Another physical property related to truncation of Lhcb1 is a reduction in the number of positively charged residues, Arg and Lys, in the N-terminal domain. The full-length protein contains six such residues within this domain, the ΔN-2 protein has five, the ΔN-11 protein has three, the ΔN-17 protein has two and the ΔN-22 has one. This segment is absent in the ΔN-58 protein. Negatively charged LDS molecules may remain associated with Arg and Lys residues after reconstitution, sterically or electrostatically hindering access of PS II-PK to phosphorylation sites: such an effect would be most obvious in the full-length and ΔN-2 proteins.

There is also evidence of multiple phosphorylation sites even within the truncated proteins lacking Thr-5. Two phosphoproteins are resolved in the ΔN-11 and ΔN-17 samples (Fig. 3B, lanes 4 and 5 respectively) and the diffuse nature of the signal from the phosphorylated ΔN-22 protein suggests that it, too, may originate from differentially modified proteins (Fig. 3B, lane 6). Phosphorylation-induced changes to protein migration, representing an apparent increase of up to 5 kDa in molecular weight with respect to unmodified proteins, have been described previously [19,20].

4. Discussion

The recognition of the full-length pea Lhcb1 protein as a substrate by spinach PS II-PK was not unexpected. The absence of a strict consensus motif in LHCPs from different species suggests that protein kinases operating in the thylakoid membrane have a fairly broad substrate specificity. Moreover synthetic peptides corresponding to LHCP N-terminal sequences with various permutations of the putative phosphorylation sites have been modified by spinach and pea thylakoids [8]. Longer incubation times were required to obtain detectable levels of incorporated phosphate in the recombinant Lhcb1 protein than was previously found to be necessary for the endogenous substrates [10]. Two factors may be responsible for this phenomenon: (i) the pea protein may be a less efficient substrate for spinach PS II-PK than is the endogenous LHC II; (ii) only a small population of Lhcb1 may be accessible for phosphorylation as a result of con-

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                                10        20        30        40        50        60        70
Lhcb1*2 Pea          RKSATTKKVASSGSPWYGPDRVKYLGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFSKNRELEVIHSR~
Lhcb1*1 Rice        RKTAAKPKPAASSG•P•••A••L•••••••••P••••••••••••••••••••••••••A••••••••••~
Lhcb1*1 S. pine     RKATGKKSVAASID•P••••••L•••••••••P••••••••••••••••••••••N•A••••••••••~
Lhcb2*1 Pea              RRTVKSAPE•I••••••P•••••••EQI•••••••••••••••••••••••••AR••••••••••~
Lhcb2*1 Rice             RRTVKSAPQ•I••••••P•••••••EQT•••••••••••••••••••••••••AR•••••L••••~
Lhcb2*1 B. pine          RRTVRSAPE•I••••••P•••••••EGT•••••••••••••••••••••••••A•••••••••C•~
Lhcb3*1 Pea                     GNDL••••••V•••••••AQT•••••••••••••••••••••••••A•A•••A•••••G•~
Lhcb3*1 Barley                  GNDL••••••V•••••••AQT•••••••••••••••••••••••••A•AR••A•••••G•~
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Fig. 4. Alignment of N-terminal amino acid sequences of Lhcb1, Lhcb2 and Lhcb3 from pea (dicotyledon), rice and barley (monocotyledons) and Scots pine and black pine (gymnosperms). All references are listed in [24] except pea Lhcb3*1 (EMBL X69215). Numbering of amino acids is for pea Lhcb1*2 and begins one residue downstream from the leading methionine [22]. Dots indicate amino acid identity and conserved potential phosphorylation sites are in bold.

straints imposed by reconstitution. The latter explanation is supported by the results obtained using the truncated Lhcb1 proteins rendering assessment of the significance of (i) difficult.

Successful reconstitution of the truncated Lhcb1 proteins [14,15] confirms that the excised regions of amino acid sequence are not important for protein folding and pigment binding: the highly cooperative nature of the pigment interactions renders this procedure very sensitive to small structural changes. Phosphorylation of four out of five of the truncated derivatives of Lhcb1 indicates that there are residues susceptible to this modification throughout the N-terminal domain. Previous workers have identified Thr-5 in pea Lhcb1 as the sole phosphorylation site [8]. Presumably this is the modified residue observed here in the full-length and ΔN-2 proteins. Prediction of putative phosphorylation sites in the other truncated proteins is not trivial as all contain threonine and serine residues (Fig. 1). Phosphoserine residues have been identified in thylakoid proteins by phosphoamino acid analysis [21] and mass spectrometry [22]. The conservation of potential phosphorylation sites in the first hydrophilic domain of Lhcb proteins from various species is highlighted in Fig. 4. Aside from two residues at positions 34 and 37 these sites are dispersed throughout the N-terminal region. The absence of phosphorylation in the ΔN-58 protein indicates that residues within the loop regions between the helices and the C-terminal domain are not susceptible to modification.

These results, although not based on in vivo protein sequences, may still be applicable to LHC II complexes within the thylakoid membrane. Nilsson and coworkers have proposed that phosphorylation close to the N-terminus of a pea LHCP induces formation of a short α-helix in that domain [23]. Such a structural change, mediated by a distinct protein kinase, might expose previously shielded hydroxyl groups within Lhcb1 for phosphorylation. The ΔN-11, ΔN-17 and ΔN-22 proteins (Fig. 1) could also be considered as modified Lhcb3 proteins rather than truncated Lhcb1 species (Fig. 4). In this context their phosphorylation suggests a mechanism by which modification of specific types of Lhcb proteins could promote a range of responses to different conditions.

The large-scale isolation of LHCPs through use of recombinant techniques is a promising route for structural analysis. Here we have demonstrated that proteins produced in this way are substrates for phosphorylation by a protein kinase associated with PS II. The results presented highlight the importance of unambiguous identification of phosphorylated residues within a protein by sequencing analysis prior to commencement of structural studies. Sites identified by other groups may have been obtained following phosphorylation under different experimental conditions, possibly catalysed by a different thylakoid enzyme.

Acknowledgements: We thank Jens Forsberg for useful discussions. H.D.-H. was supported by a DFG Post-Doctoral Research Fellowship and H.L.R. by an NFR Post-Doctoral Fellowship. NFR and FRN support as research grants to J.F.A. is also acknowledged.

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