A CK2 site is reversibly phosphorylated in the photosystem II subunit CP29

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Abstract Protein phosphorylation is a major mechanism in the regulation of protein function. In chloroplast thylakoids several photosystem II subunits, including the major antenna light-harvesting complex II and several core complex components, are reversibly phosphorylated depending on the redox state of the electron carriers. A previously unknown reversible phosphorylation event has recently been described on the CP29 subunit which leads to conformational changes and protection from cold stress (Bergantino, E., Dainese, P., Cerovic, Z. Sechi, S. and Bassi, R. (1995) *J. Biol Chem.* 270, 8474–8481). In this study, we have identified the phosphorylation site on the N-terminal, stroma-exposed domain, showing that it is located in a sequence not homologous to the other members of the *Lhc* family. The phosphorylated sequence is unique in chloroplast membranes since it meets the requirements for CK2 (casein kinase II) kinases. The possibility that this phosphorylation is involved in a signal transduction pathway is discussed.

Key words: Cold stress; Light-harvesting complex; Chlorophyll; Photosynthesis

1. Introduction

In the chloroplast, phosphorylation of membrane proteins is responsible for many of the physiological responses to changes in incident light and redox poise. The major substrate involved in this regulative process is the light-harvesting complex of photosystem II (LHCII). When PSII is overexcited with respect to PSI, the plastoquinone pool becomes reduced and thus activates a kinase bound to the cytochrome b6/f complex [1,2]. This process results in phosphorylation of the major trimeric LHCII complex in an N-terminal, stroma-exposed site [3] leading to the monomerisation of LHCII, disconnection from PSII RC [2,4] and migration of LHCII monomeric subpopulations from grana to stroma membranes [2,5,6] where they can transfer excitation energy to PSI [5] and thus oxidise the plastoquinone pool. This process can therefore be understood in terms of regulation of photosystem antenna size by the transfer of LHCII units away from the overexcited photosystem [2]. The underlying mechanism is a conformational change of the LHCII N-terminal portion which undocks this subunit from PSII and increases its affinity for PSI [2].

More recently, it was reported that photoinhibitory conditions induce reversible phosphorylation of CP29, another PSII subunit PSI [7]. Although there is strong homology between CP29 and LHCII and both proteins are phosphorylated in their stroma-exposed domains, CP29 phosphorylation does not induce changes in its distribution between chloroplast membrane domains. Moreover, the activity of the phosphorylating enzyme is affected differently by a number of electron transport inhibitors with respect to the case of LHCII [7]. CP29 phosphorylation induces a conformational change which affects the spectroscopic properties of the complex [8] and possibly provides protection from photoinhibition [7]. In order to elucidate the basis for the different effects of phosphorylation on these two highly homologous proteins, we have identified the site of phosphorylation in CP29. The results show that the phosphorylation site of CP29 differs in both the location within the protein structure and the sequence specificity with respect to the one in LHCII, thus supporting the view that the post-translational modifications are catalysed by distinct protein kinases. Moreover, the CK2-like structure of the CP29 site suggests that this phosphorylation event might be involved in the signal transduction pathway leading to the control of transcription of nuclear genes [9,10].

2. Materials and methods

2.1. In vivo labelling

Maize seedlings were grown in a growth chamber at 25/22°C day/night at a light intensity of 200 μE m^{-2} s^{-1} and 80% humidity. After 2 weeks, 14 seedlings were cut under water at the base of their epicotyl and quickly transferred into 0.5 ml of 40 mM HEPES/KOH, pH 7.5, in small glass vials (2 plants in each vial). ^{33}P-orthophosphoric acid (285 μCi; Amersham) was added to each vial, and seedlings were allowed to incorporate radioactivity for 3 h at 25°C in the light, with the evaporative demand increased by keeping the relative humidity (60%) of the growth chamber low. Buffer solution (HEPES–KOH, pH 7.5) was added to each vial as needed during this period. Following incorporation, seedlings were kept in the light for 6 h at 4°C in order to induce CP29 phosphorylation [7].

2.2. Preparation and fractionation of thylakoid membranes

Leaves were cut and cooled in ice. Thylakoids were rapidly isolated [11]. Thylakoid membranes were washed twice in 0.8 M Tris, 5 mM EDTA, then resuspended in water at 2 mg chlorophyll/ml and solubilized by adding an equal volume of 2% dodecylmaltoside in water. Samples were solubilised in ice for 5 min with occasional mixing, spun for 5 min at 10000×*g* and rapidly loaded onto a 0.1–1 M sucrose gradient, containing 10 mM HEPES, pH 7.6, and 0.06% DM. The gradient was then spun in a Beckman SW 60 rotor at 50000 rpm (336000×*g*) for 9 h at 4°C. Individual green bands were harvested

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Abbreviations: BBY, PSII membrane preparation; CK2, casein kinase 2; Chl, chlorophyll; CP, chlorophyll–protein; DM, dodecyl-maltoside; EDTA, ethylenediaminetetraacetic acid; ELFE, electroendoosmotic electrophoresis; HEPES, *N*-2-(hydroxy-ethyl)piperazine-*N'*-2-ethanesulfonic acid; IEF, isoelectrofocusing; LHCII, light-harvesting complex of PSII; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; rCP29, recombinant CP29 reconstituted from the apoprotein derivatives overproduced in bacteria; RC, reaction centre; SDS, sodium dodecyl sulphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol

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with a syringe. Green bands were analysed by SDS-PAGE using the Tris-sulphate buffer system [12]. Fractions containing CP34 were pooled and loaded into a flat-bed IEF [13]. Fractions were analysed by SDS-PAGE.

2.3. Isolation of overexpressed CP29 apoprotein from bacteria

CP29 was isolated from the SG13009 strain transformed with either of the two CP29 constructs following a protocol previously described [14]. The phosphorylated form of CP29 (CP34) was isolated as in Croce et al. 1996 [8].

2.4. Purification of recombinant or native CP29–CP34 complexes for proteolysis

In order to obtain a fully purified complex which did not contain any residual contamination by bacterial or thylakoidal proteins in a form useful for proteolysis, IEF purified protein was further fractionated by electroendoosmotic electrophoresis (ELFE) [15].

2.5. Proteolysis

For proteolysis the Tris–H_2SO_4 buffer resulting from ELFE purification was substituted by 50 mM NH_4HCO_3, 0.1% SDS. Endoproteinase glu-C (V8, Sigma) was added at an enzyme/substrate ratio of 1:10 and incubated for 2 h at 37°C. The reaction was stopped by adding 1% SDS and boiling for 1 min before rapid cooling in ice. The sample was concentrated by Centricon SRS (Amicon), then sample buffer was added before loading onto SDS-PAGE. Proteolytic fragments fractionated by SDS-PAGE were electroblotted to Immobilon-P membrane, stained with Coomassie Blue, destained and sequenced with an automated protein-sequenator (Applied Biosystem model 475A) [16–18].

2.6. Protein and pigment concentration

The concentration of the CP29 apoprotein purified from *Escherichia coli* inclusion bodies was determined spectrophotometrically at 280 nm using an E_{1cm} (mg/ml) of 1.58. Chlorophyll concentration was determined by the method of Porra et al. [19].

2.7. Electrophoresis

The buffer system of Shäger and von Jagow [20] was used. Acrylamide concentration was 18%.

2.8. ^{33}P detection

Radioactivity on SDS-PAGE gels was detected by electronic autoradiography using a Packard Instant-Imager instrument (Camberra-Packard).

3. Results

3.1. Peptide mapping of native and recombinant CP29

We have identified the phosphorylation site in CP29 by limited proteolysis after labelling with ^{33}P. Two major problems make this approach difficult in the case of CP29. Firstly, the phosphorylation site is probably located in the N-terminal part of the molecule and CP29 is N-terminally blocked [7], the identification of N-terminal fragments by sequence is impossible. Secondly, since CP29 is a pigment-binding membrane protein, the proteolytic treatment is much less effective and reproducible than in the case of soluble proteins.

The first problem was overcome by using overexpressed CP29 [14] which is not N-terminally blocked as shown by the sequence obtained (Table 1) which contains the putative N-terminal residue of CP29 preceded by three amino acids included due to the cloning strategy. The second problem was solved by subjecting the proteins (either isolated from thylakoids or from *E. coli* inclusion bodies) to an electroendoosmotic electroporesis (ELFE) step. This procedure yields the protein in 0.1% SDS solution and also extracts bound pigments, thus making the proteolytic sites more accessible. In these conditions, a reproducible proteolytic pattern was obtained using V8 protease, which was identical for the N-terminal blocked protein extracted from thylakoids and for the recombinant protein. A hypothetical model for CP29 structure is shown in Fig. 1A in which the V8 sites are indicated as well as the putative phosphorylation sites facing the stromal side of the membrane [7]. In Fig. 2 the V8 proteolytic pattern is shown while the N-terminal sequences of relevant proteolytic bands are shown in Table 1. Primary sequence analysis with the PROSITE package [21] identified four putative sites on the stroma-exposed N-terminal stretch and helix C–helix A loop (Fig. 1). Phosphorylation on one or more of these sites could be recognised by the ^{33}P-labelling pattern after V8 proteolysis. In fact site 1, 2, 3 or 4 phosphorylation would yield labelling on, respectively, bands e, b, b+c, or d.

The same procedure was applied to the CP29 extracted from thylakoid membranes and the same proteolytic pattern was obtained. However, the N-terminal containing fragment (a, e) could not be sequenced.

3.2. Isolation of the phospho-CP29 from ^{33}P-treated plants

Unlike other thylakoid phosphoproteins, CP29 can only be phosphorylated in vivo. To overcome this problem, we fed maize seedlings with ^{33}P-orthophosphate and then exposed the plants to photoinhibitory conditions. Following treatment for 6 h at 4°C and 300 μE m^2 s^{-1}, thylakoid membranes were isolated and fractionated by sucrose gradient ultracentrifugation after solubilisation with DM. Four green bands were obtained whose polypeptide composition and autoradiography are shown in Fig. 3. The upper two bands contained antenna proteins among which CP29 and LHCII were phosphorylated. The third green band contained PSII core complex including five ^{33}P labelled bands: *psbH*, D1, D2, CP43 and the D1+D2 heterodimer. An additional band at 27 kDa can be attributed to residual LHCII. The fourth green gradient band contained a rather pure PSI–LHCI complex which was not labelled. This is interesting in the light of recent reports of LHCI phosphoproteins [22]. The gradient fractions 1 and 2 were pooled and subjected to preparative IEF for the isolation of CP34 according to [8] (results not shown). The different phosphoproteins were well resolved by IEF: fractions 1–5 contained different LHCII subpopulations, fractions 7–10 contained CP29 in its phosphorylated and unphosphorylated forms and fractions 14–15 contained CP43. The *psbH* phosphoprotein was, instead, distributed over the entire pH range. In order to further purify phospho-CP29 and to obtain this protein in the pigmentless form suitable for proteolysis, IEF fractions 7–10 were pooled and subjected to ELFE, thus obtaining a pure phospho-CP29 preparation.

3.3. Proteolysis of phospho-CP29

The purified phospho-CP29 was treated with V8 protease,

Table 1
N-terminal amino acid sequences of Glu-C fragments obtained from native and recombinant CP29

Fragment	kDa	Sequenced residues	Sequence deduced from Lhcb4 cDNA
a	20	MRIRIF*	$—_1$RF
b	17	AGGIIGTRF	$_{77}$AGGIITRF
c	16.4	SSEVKSTPLQP	$_{87}$SSEVKSTPLQP
d	11	KILXN	$_{135}$KRLYP
e	7.68	MRIRF*	$—_1$RF

*This fragment could only be sequenced from the recombinant protein.

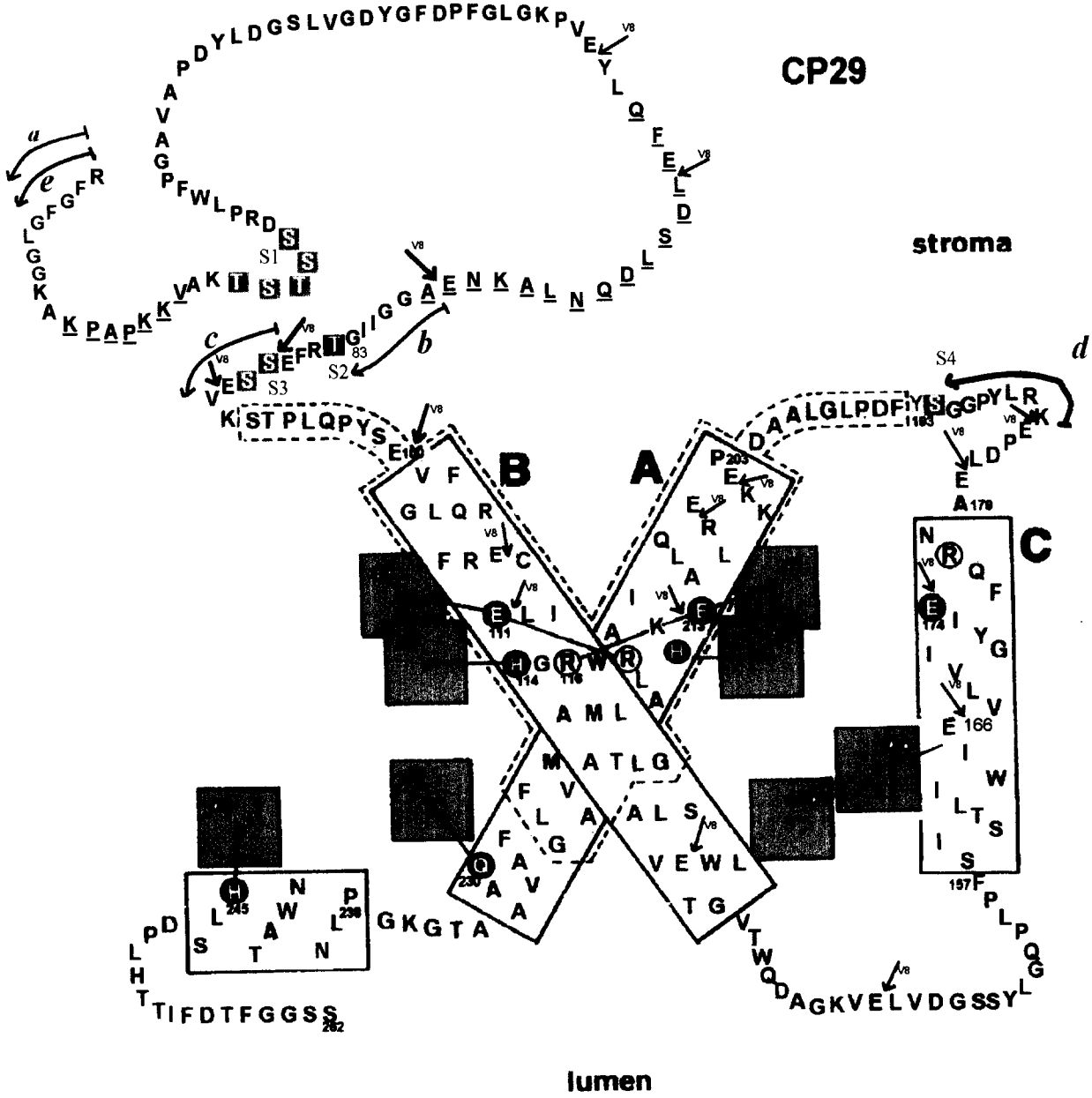

Fig. 1. Model of CP29 chlorophyll–protein obtained on the basis of cDNA deduced sequence [23] and on the LHCII structure [24]. S (1–4), putative phosphorylation site; a–e (arrow), sequenced residues of proteolytic fragments; V8↓, V8 protease (glu-C) putative proteolytic site.

fractionated by SDS-PAGE and the gel exposed to electronic autoradiography. The results (Fig. 4) show that only two bands were labelled by ^{33}P, namely bands a and b. In particular band e, containing the N-terminal fragment of CP29, was not labelled at all, thus implying that the putative site 1 was not phosphorylated. Also site 4 could be excluded on the basis of the lack of labelling of band d. The labelling was detected on band a, including all the N-terminal domain as well as helix B and helix C, and band b, whose N-terminal end was identified as Ala^{77}. Band c, starting with Ser^{87}, was not labelled, implying that the phosphorylated residue was located in the 77/87 fragment. The only possible site in this fragment is threonine-83.

3.4. Identification of phospholamino acids in LHCII and CP29

In an independent experiment, we have subjected phospho-LHCII and phospho-CP29 to complete hydrolysis and then separated the amino acids by high voltage electrophoresis. In the case of all of the LHCII subpopulations and of CP29, the only phospho-amino acid detected was phospho-threonine (not shown). This result confirms previous data on LHCII and supports the conclusion that site 2, namely threonine-83, is the phosphorylation site of CP29.

4. Discussion

The results of the peptide mapping of phospho-CP29 yield the unequivocal conclusion that threonine-83 is the residue that is reversibly phosphorylated in this chlorophyll a/b protein in response to photoinhibitory conditions. This conclusion comes from the previous finding that CP29 is phospho-

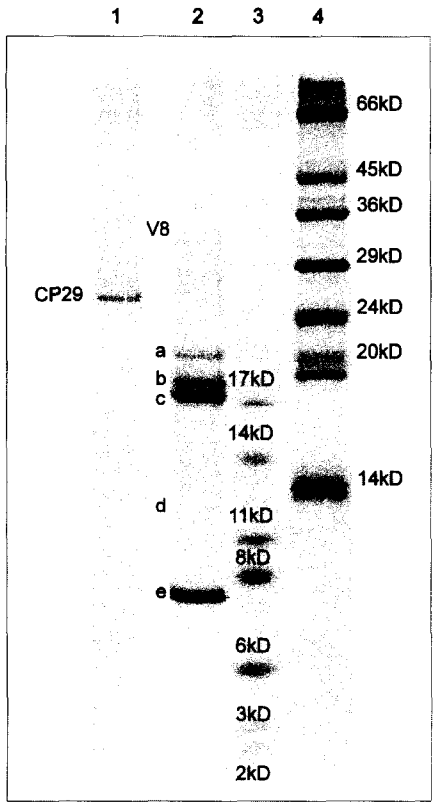

Fig. 2. Proteolytic mapping of recombinant CP29. SDS-PAGE: lane 1, recombinant CP29 (undigested, 5 μg protein); lane 2, as in 1 but after treatment with V8 protease (30 μg protein); lane 3, low MW standards; lane 4, high MW standards (other conditions as in Section 2).

rylated on the stromal side of the protein [7] and from the following experimental evidence. (a) Out of the four putative sites detected by sequence analysis (Fig. 1), sites 3 and 4 are ruled out since they have serine residues while phospho-amino acid analysis shows only the presence of phosphothreonine. Accordingly, the proteolytic fragments (bands c and d) containing these sequences are not labelled by ^{33}P. (b) Site 1 includes both threonine and serine but the proteolytic fragment encompassing this site (band e) is not labelled. (c) N-terminal sequencing shows that the fragments in bands b and c only differ by 10 amino acid residues and yet band b is labelled while band c is not. In the 77–87 fragment, threonine-83 is the only phosphorylable residue.

When compared to the results so far obtained with thylakoid phosphoproteins, this result confirms the trend that all chloroplast kinases are highly specific for threonine while phosphoserine and phosphotyrosine, which are often substrates for cytoplasmic kinases, have not been detected so far [1]. Nevertheless, all the thylakoid phosphoproteins so far identified have sites very close to their N-terminal, within 6 residues. This is quite different from the case of CP29 which is phosphorylated at residue 83. This raises the question of whether the physiological effect of this post-translational modification is similar to that of previously described thylakoid phosphorylations. The role of thylakoid membrane phosphorylation is far from being clearly identified; however, at least in the case of LHCII a clear relation has been established between the LHCII phosphorylation and the disconnections of LHCII units from PSII. The mechanism of this process is a phosphorylation-induced conformational change within the N-terminal, stroma-exposed domain of the molecule leading to changes in the molecular recognition properties of LHCII with respect to the surrounding PSII subunits. This has been proposed to consist into a charge compensation in the positively charged N-terminal region of LHCII yielding the formation of an α-helix which could be docked in the cavity between the ends of helices A and B, protruding into the stroma. This effect is probably confined to the N-terminal since no evidence has been so far reported for long-range conformational changes. This is clearly different from the case of CP29 since it was shown that phosphorylation does not affect the distribution of CP29 between grana and stroma membranes nor its connection to PSII core ([7]; Mauro et al., unpublished results) while a long-range conformational change has been reported leading to spectral change of tetrapyrrole pigments (mainly chlorophyll b) bound to the hydrophobic part of the molecule [8]. Since the two chlorophyll b molecules in CP29 are probably coordinated to residues lo-

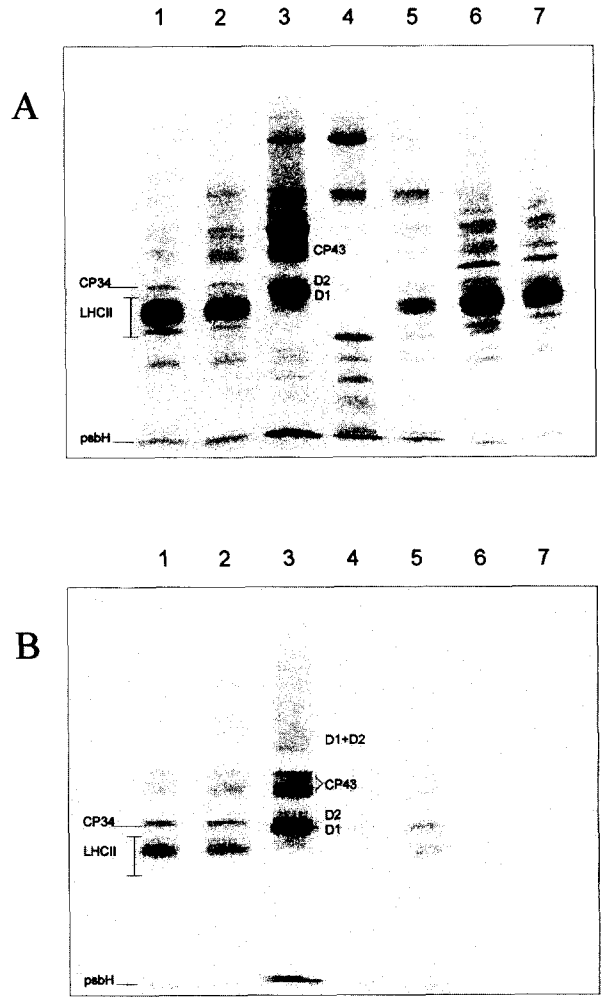

Fig. 3. Polypeptide composition of green bands obtained by sucrose gradient fractionation of ^{33}P-labelled thylakoids. Lane 1, sucrose band 1 containing monomeric complexes; lane 2, sucrose band 2 containing trimeric complexes; lane 3, sucrose band 3 containing PSII core complex; lane 4, sucrose band 4 containing PSI–LHCI complex; lane 5, ^{33}P-labelled thylakoids; lane 6, PSII membranes (cold-treated, unlabelled); lane 7, PSII membranes (unlabelled). A: Coomassie Blue-stained gel. B: Autoradiography; CP34: phosphorylated form of CP29.

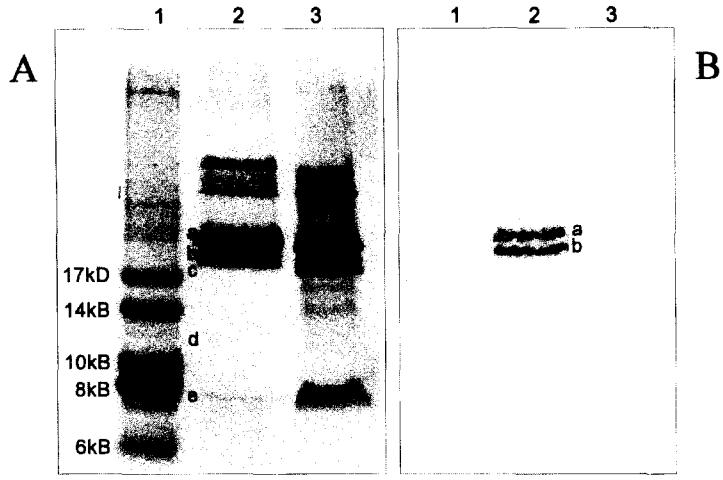

Fig. 4. Proteolytic mapping of in vivo ^{33}P-labelled CP34. A: Coomassie Blue stain of SDS-PAGE. B: Autoradiography. Lane 1, low MW standards; lane 2, CP34 ^{33}P-labelled after treatment with V8 protease (10 μg protein);lane 3, rCP29 (20 μg) after treatment with V8 protease. Numbers on the left refer to the MW of standards. a–e: Proteolytic fragments whose sequence is reported in Table 1.

cated near the C-terminus and the stromal end of helix C [23], the effect of phosphorylation could extend though the lumenal side of the protein. How this occurs can only be a matter of speculation since the structure of the N-terminal of LHCII was not resolved by the structural analysis [24] and moreover the N-terminal domain of CP29 is significantly divergent for both the primary sequence and length. Sequence analysis by secondary structure-prediction algorithms suggests the presence of two α-helices between residues 10–17 and 62–77. However, the proteolysis on site $_{64}$ Glu, yielding fragment e, casts doubts on the real presence of this second helix. On the basis of the proximity of the threonine-83 residue to the binding site of the lutein, likely to be in the region $_{48}$GFDPF [25] it can be hypothesised that a conformational change around threonine-83 can be propagated to a long distance by the lutein polyenic chain.

A primary sequence comparison between the different *Lhcb* proteins reveals that the IGTRFE site is conserved in barley and Arabidopsis sequences [26,27] with the only exception of a F>T substitution in position 85. This site is part of an insertion sequence which is only present in CP29 while it shows no homology with other *Lhc* proteins [28] consistently with the finding that the photoinhibition induced reversible phosphorylation is restricted to CP29. Another interesting feature of the CP29 phosphorylation site is that the surrounding sequence is completely different from those of the previously reported phosphorylation sites of chloroplast proteins as shown by the sequence of known thylakoid phosphoproteins reported in Table 2 [1]. It is interesting to notice that the CP29 phosphorylation site fits the requirement for CK2 type kinases found in animals due to the acidic residue in position +3 with respect to the phosphorylated residue [29,30]. This finding supports the previous suggestion that the CP29 kinase is different from LHCII kinase and from PSII core subunits kinases based on its sensitivity to different electron transport inhibitors [7]; moreover, CK2 kinases are involved in signal transduction pathways leading to cell differentiation and complement system [29,30]. Recent work by Allen et al. [10] and by Escoubas et al. [9] has shown that redox poise of the chloroplast controls the transcription of *Lhc* nuclear genes through a signal transduction pathway involving kinase and phosphatase activities. The same conditions producing repression or derepression of transcription, which can be mimicked by the use of the two herbicides DCMU and DBMIB, modifying the redox state of plastoquinone pool, respectively, causes activation or inactivation of the CP29 kinase [7]. This effect may be aimed to decrease the PQ over-reduction synergistically to the repression of *Lhc* gene transcription. An alternative view is that CP29 has a role in sensing both the trans-membrane pH gradient, as shown by the presence of a DCCD binding site [31] and of excess light through the plastoquinone redox state [7]. In this case CP29 phosphorylation could be the first step of the signal transduction pathway.

Table 2
Sequence of phosphorylation sites in chloroplast membrane proteins and a CK2 kinase

Protein	Organism	Sequence
Lhcb1	maize	AcRKT(P)AAKA.....
Lhcb2	spinach	AcRRT(P)AKSV.....
Lhcb4	maize	$_{77}$AGGIIT(P)RFESSE.....
D1	spinach	AcT(P)AILERR.....
D2	spinach	AcT(P)IAVGK.....
psbD	spinach	AcT(P)LFNGTL....
psbH	spinach	$_{1}$AT(P)QTVESSSR..
CK2		S/TXXE/D....

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