A single point mutation (E166Q) prevents dicyclohexylcarbodiimide binding to the photosystem II subunit CP29

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Abstract **Energy-dependent quenching of chlorophyll fluorescence (qE) reflects the action of a powerful mechanism of protection from photoinhibition in which the low pH in the chloroplast lumen induces dissipation of excess excitation energy. Dicyclohexylcarbodiimide (DCCD), a protein-modifying agent, is a powerful inhibitor of qE and has been shown to react with acidic residues, in a hydrophobic environment, involved in proton translocation. The CP29 subunit of photosystem II has been proposed to be the site of qE quenching and shown to bind DCCD. We have hypothesised, on the basis of the CP29 protein sequence and of the structure of light-harvesting complex II protein, that glutamic acid 166 is the DCCD binding site. In this study, we have produced recombinant proteins either with wild-type sequence or carrying a mutation on the 166 position. We show that the mutant protein does not bind DCCD. This identifies E166 as the site whose protonation may lead to a conformational change triggering qE.**

Key words: qE quenching; Light-harvesting complex; Chlorophyll; Photosynthesis

1. Introduction

Non-photochemical fluorescence quenching (qN), in the photosynthetic apparatus of higher plants, is originated by a set of physiological mechanisms. As a result, when light intensity exceeds the transport capacity of the electron transfer chain, excess excitation energy is channelled away from reaction centres. qN is caused by three processes which have been called qE (energy quenching), qT (state transition quenching) and qI (irreversible, or slowly reversible, quenching). qE is the major qN component in higher plants at physiological irradiances and is able to decrease by a factor of 2 the optimum quantum yield of photosystem II (PSII) when photosynthesis is saturated [1]. An absolute requirement for qE, with an apparent pK of 4.5–5, is the presence of a transmembrane pH gradient. This pK value could be shifted: in the presence of dibucaine [2] qE could be formed at reduced pH gradient while an increased pH gradient was required in the presence of antimycin A [3]. This indicates that an obligatory component of qE behaves like an enzyme whose activity is regulated through changes of 'K_m' [4].

Since PSII is a shallow trap [5], qE quenching could occur either in the PSII antenna or in the reaction centre (RC). Models for quenching in the RC suggest that low pH inactivates the donor side thus increasing the lifetime of P680+ which results either in a direct quenching or in non-radiative charge recombination with Q_A^- [6]. However, it was shown that qE function does not require an active PSII [7].

The alternative site of qE is the PSII antenna, which is composed of at least eight different chlorophyll binding proteins [8,9]. Horton and co-workers proposed the major LHCII complex as responsible for qE since LHCII aggregation in detergent solution induces quenching of fluorescence [10], which can be modulated by added xanthophylls [11], and scattering changes similar to those shown in leaves [12,13]. Alternatively, it has been proposed that the minor chlorophyll a/b proteins are the site of quenching on the basis of their high violaxanthin and zeaxanthin content [8,9]. Support for this second hypothesis was given by the finding that the qE inhibitor dicyclohexylcarbodiimide (DCCD) [14] binds to CP26 and CP29 but not to LHCII or CP24 [15]. In the present study, we have investigated CP29 as a target site for DCCD. Sequence analysis of this protein, in the light of the homologous protein LHCII structure [16], identifies the glutamic acid residue 166 as a putative binding site for DCCD since it is both in hydrophobic environment and lumen exposed. By using the method for in vitro reconstitution of overexpressed CP29 [17] we have produced recombinant (r) CP29 with the wild-type (WT) sequence or carrying the single point mutation E166Q. We show that the mutation prevents DCCD binding. This result supports the view that CP29 is the site of the qE quenching mechanism in PSII.

2. Materials and methods

2.1. DNA constructions

Plasmids were constructed using standard molecular cloning procedures [18]. Bacterial hosts were *Escherichia coli* strain TG1 [19] and strain SG13009 [20]. Mutant E166Q was obtained according to Yukenberg et al. [21]. The sequence was determined by the dideoxy method [22] by an automated apparatus (Applied Biosystems Model 377).

2.2. 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 [23,24]. The phosphorylated form of CP29 (CP34) was isolated as in Croce et al. [25].

2.3. Pigment isolation and reconstitution of CP29 pigment complexes

These procedures were performed as described in Giuffra et al. [17]. HPLC analysis was as in [9,17].

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Abbreviations: Chl, chlorophyll; DCCD, dicyclohexylcarbodiimide; DM, dodecyl-maltoside; HEPES, *N*-2-(hydroxy-ethyl)piperazine *N'*-2-ethanesulphonic acid; IPTG, isopropyl thiogalactoside; LDS, lithium dodecyl sulphate; LHCII, light-harvesting complex of PSII; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; rCP29, recombinant CP29 reconstituted from the apoprotein derivatives overproduced in bacteria; SDS, sodium dodecyl sulphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol

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2.4. Purification of reconstituted CP29

In order to obtain a fully purified complex which did not contain any residual contamination by bacterial proteins, the reconstituted CP29 was purified by preparative IEF [26] followed by ultracentrifugation in glycerol gradient (15–40% including 0.06% DM and 10 mM HEPES pH 7.6; run was for 12 h at 60 000 rpm in a SW60 Beckman rotor) in order to eliminate ampholytes.

2.5. Protein and pigment concentration

The concentration of the CP29 apoprotein purified from *E. coli* inclusion bodies was determined by the bicinchoninic acid assay [27]. For stoichiometric (pigments/protein ratio) determination, the protein concentration was determined by the ninhydrin method [28]. Chlorophyll concentration was determined by the method of Porra et al. [29].

2.6. Isolation of native CP29

CP29 was isolated from maize PSII membranes as previously described [8,26].

2.7. DCCD labelling

DCCD labeling was performed according to Jahns and Junge [30], using [^{14}C]DCCD (Amersham). Unlabelled DCCD was purchased from Sigma. Radioactivity was determined by using a Packard Instant Imager.

2.8. Electrophoresis

The buffer system of Shäger and von Jagow [31] was used. Acrylamide concentration was 14%.

2.9. Spectroscopy

Absorption spectra were recorded at room temperature using a Kontron DW2 spectrophotometer. Fluorescence emission and excitation spectra were measured at room temperature with a Jasco FP-777 spectrofluorimeter. Circular dichroism (CD) spectra were obtained at 8°C with a Jasco 600. All spectra were recorded in the presence of 0.06% DM.

3. Results

3.1. Reconstitution and pigment binding properties of WT rCP29 and E166Q mutant

The apoproteins purified from *E.coli* carrying either the construct pQBH22 or pQBH22E166Q coding for CP29 respectively with the WT sequence [32] or with the single point mutation E166Q were reconstituted as previously described [17] using a pigment mixture in which Chl a and Chl b were present in a 8:1 ratio.

The resulting holoprotein with WT sequence was identical to the native CP29, extracted from thylakoids, as detected by fluorescence emission, fluorescence excitation, circular dichroism, absorption spectroscopy and HPLC pigment analysis. Fig. 1A shows the absorption spectra of these proteins which had a Chl a/b ratio of 3.0.

The complex obtained with the E166Q apoprotein was very similar to the WT complex as judged from the absorption spectra; however, distinct differences could be observed in the mutant minus WT difference spectrum showing that the former was enriched in wavelengths around 670 nm in the Qy transition region (Fig. 1B). Peaks in the difference spectrum were also detected at 473 (+) and 495 nm (−) probably due to differences in carotenoid absorption. Table 1 shows the pigment composition of native, recombinant WT and the E166Q mutant as obtained by HPLC analysis. The carotenoid composition was very similar in the mutant and WT proteins, suggesting that differences in absorption could be due to electrochromic shift induced by the replacement of a negative charge by a positive charge in position 166 or to changes in pigment-protein interactions.

3.2. DCCD binding

The WT and the mutant recombinant proteins were incubated in the presence of [^{14}C]DCCD at pH 7.8 and the product of the reaction analysed by SDS-PAGE and autoradiography. As a reference, native CP29 and LHCII, purified from thylakoid membranes, were also analysed for DCCD binding. As shown in Fig. 2A, native CP29 as well as the recombinant protein with WT sequence were heavily labelled with [^{14}C]DCCD while the E166Q mutant and LHCII were not. CP29 apoprotein isolated from *E. coli* inclusion bodies but not reconstituted with pigments did not show DCCD binding.

CP29 can be reversibly phosphorylated in photoinhibitory conditions [33] inducing a conformational change yielding a higher apparent molecular mass in SDS-PAGE and a modification of the spectral properties of the complex [25]. When a mixture of the two forms was treated with DCCD, both were labelled with the same intensity thus suggesting that phosphorylation does not significantly modify DCCD binding (Fig. 2B).

4. Discussion

The finding that neither an active PSII nor an oxygen evolving complex is needed for qE quenching [7] has supported the view that the site of this dissipative mechanism is located in the PSII antenna system. This structure is composed of the major LHCII complex, binding more than 60% of PSII chlorophyll, and of three minor proteins called CP29, CP26 and CP24. The latter form a bridge between the PSII core complex and LHCII as suggested by biochemical studies [34] and confirmed by structural analysis [35].

The fluorescence decrease induced by low lumenal pH is thought to be a mechanism for diversion of excess energy from PSII in order to limit the decrease of lumenal pH before the donor side is damaged. OEC disruption leads to photoinhibition even in weak light through the stabilisation of P680+ and photo-oxidative damage [7,36]. According to this view, the qE mechanism involves a change to a dissipative state of the antenna system with a pK in the range of 5.0–5.5 [37].

The formation of zeaxanthin, although also enhanced at low lumenal pH, has a slower kinetic of both accumulation

Table 1
Pigment composition of chlorophyll proteins

Sample	Chl b	Lutein	Neoxanthin	Violaxanthin	β-Carotene
nCP29	34	19	6.1	11.3	1.0
rCP29WT	34.7	17.1	7.9	8.9	0
rCP29E166Q	33.0	18.0	7.4	7.9	0

Values are in moles per 100 moles of Chl a.

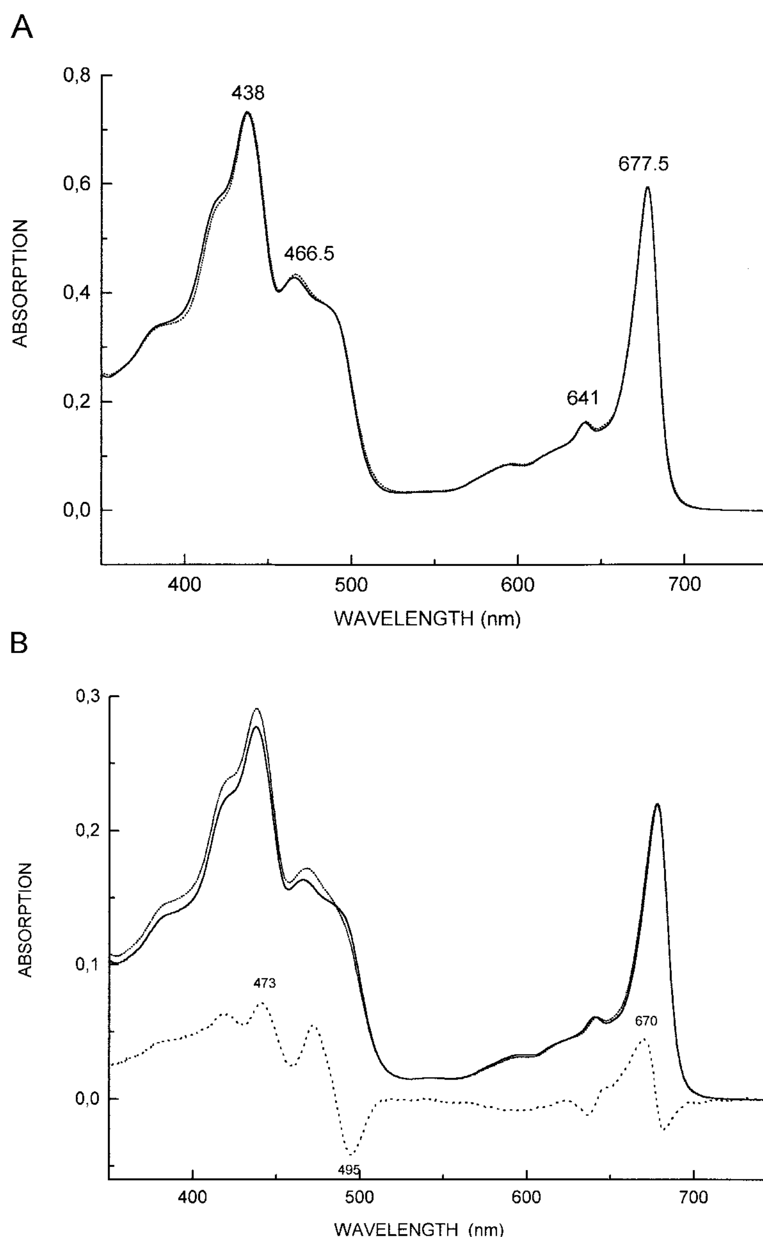

Fig. 1. A: Absorption spectra of native (solid line) and recombinant (dotted line) CP29 reconstituted with Chl a/b ratio of 8.0 during refolding. B: Absorption spectra of rCP29 with WT sequence (solid line) and with the E166Q mutation (dotted line). The lower line represents the difference spectrum (dashed line).

and decay than qE thus suggesting it might be a secondary process of further amplification of the quenching since the qE can be obtained in the absence of zeaxanthin [38,39].

Where is the primary process located and by which mechanism is it obtained? According to previous work of Jahns and Junge [30] and of Walters et al. [15], dicyclohexylcarbodiimide, a powerful and specific inhibitor of qE [14], binds to the two minor antenna proteins CP26 and CP29. The binding sites of CP26 have been recently identified as glutamic acid residues, on the lumenal exposed loop between helices B and C and on the C-terminus, by a careful chemical analysis [40]. These residues are not involved in pigment binding according to the homology with the LHCII protein [16]. The effect of their protonation with respect to changes in pigment-pigment and pigment-protein interactions may, however, involve long-range conformational changes.

These DCCD binding residues in CP26 are not conserved in the CP29 sequence. In this study, we have tested the hypothesis that, in CP29, DCCD binding is located in a different site. Analysis of PSII antenna protein sequences, searching for substitution which might affect ligation of pigments with respect to LHCII (Fig. 3), shows that a glutamic acid residue in a hydrophobic environment is present in CP29 (at position 166) and in CP26 but not in CP24 and LHCII while proline 82 of the LHCII sequence is substituted for a valine in CP29. In particular E166 is well buried into the hydrophobic domain of the protein, a condition for the shift of the pK from 4.2 to 5.2. Acidic residues in a hydrophobic environment have been

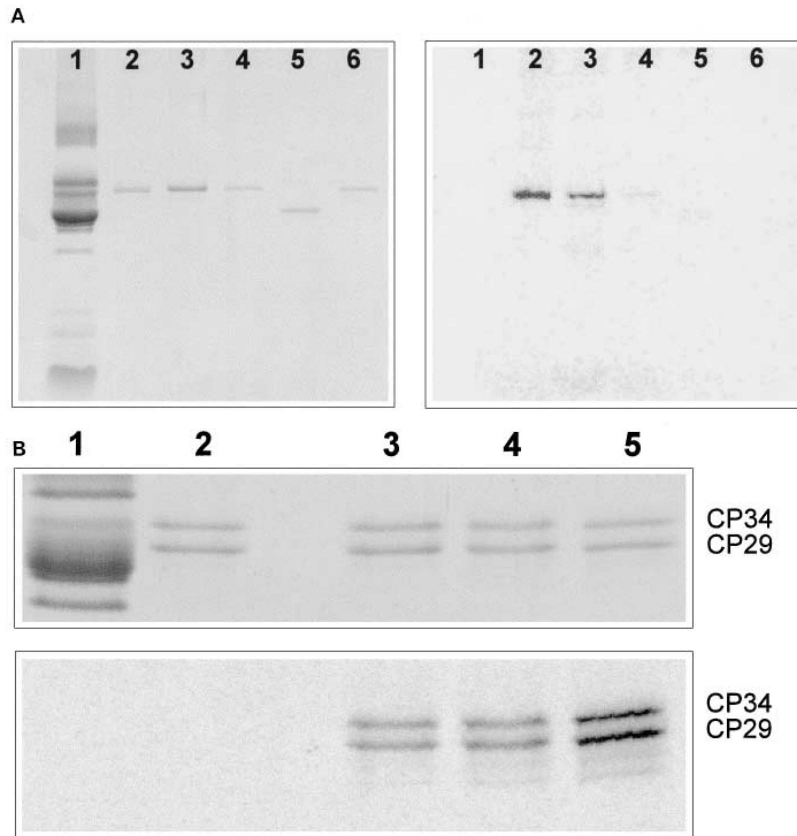

Fig. 2. A: DCCD binding to CP29. Left panel: electrophoresis; right panel: autoradiography of chlorophyll a/b proteins after treatment with [^{14}C]DCCD. Lane 1, maize PSII membranes, unlabelled; lane 2, nCP29; lane 3, rCP29WT; lane 4, rCP29 E166Q; lane 5, LHCII purified from maize thylakoids; lane 6, CP29 apoprotein treated with [^{14}C]DCCD without previous reconstitution with pigments. B: DCCD binding to the phosphorylated and unphosphorylated forms of CP29. Lane 1, thylakoids; lane 2, unlabelled CP29 and CP34 fraction; lanes 3–5, as in lane 2 but labelled with respectively 70, 140 and 210 μM [^{14}C]DCCD. CP34 is the phosphorylated form of CP29. Upper panel, SDS-PAGE; lower panel, autoradiography.

identified as DCCD binding sites in proton translocating proteins [41].

We have shown here that the single point mutation E166Q is able to abolish the DCCD binding to CP29 thus implying that this residue is involved in proton exchange. This is due to the particular environment present in CP29 and recreated by the folding of the recombinant apoprotein with pigments, as proven by the fact that the unfolded protein is unable to bind DCCD.

This proton binding site may be the primary site by which the thylakoid proton gradient initiates qE. There are several ways by which this protonation may lead to a quenching effect: (i) it may trigger a conformational change in the polypeptide that results in movement of chlorophylls towards each other or in bringing chlorophyll(s) closer to zeaxanthin [42]; (ii) it may alter the local electric field around the zeaxanthin or chlorophyll molecules so as to promote energy transfer [43]; (iii) if the protonable residue is a chlorophyll binding site, it may directly cause a different location of chlorophyll within the protein to give rise to quenching by proximity with Chl or carotenoids. As pointed out by Crofts and Yerkes [37], an efficient quencher can be obtained in chlorophyll solutions at concentrations similar to that present in thylakoids by the formation of dimers which introduce additional energy levels allowing thermal pathways for de-excitation [44]. In higher plant light harvesting complexes, chlorophyll molecules are held apart by coordination to a number of ligands provided by the LHC apoproteins [16]. It can be hypothesised that quenching in LHC proteins may derive from a change in the affinity of one or more Chl ligands for its protein coordination site. While the locations of DCCD sites in CP26 in hydrophilic loops on the lumenal surface may suggest a long-range conformational change (i, see above), the identification of E166 as DCCD site in CP29 may suggest that the mechanism proposed by Crofts and Yerkes [37] (iii, see above) is acting in CP29, provided that E166 is a Chl binding site. Sequence analysis (Fig. 3) shows that in the homologous protein LHCII, the glutamine residue in the corresponding position is binding chlorophyll [16]. However, this glutamine is substituted for by a glutamic acid residue in CP29. The changes in the absorption spectra of the E166Q mutant with respect to WT, while pigment composition, as determined by HPLC analysis, is very similar, indicate that both carotenoid and chlorophyll organisation may be affected by the mutation. Further spectroscopic characterisation of this mutant and the analysis of mutants carrying other residues in this position are needed in order to clarify this point. High light stress induces another modification in CP29 consisting in the reversible phosphorylation at the threonine residue 83 [33,45] which induces a long-range structural change [25]. It could well be conceived that in stress conditions the two effects (phosphorylation and protonation) interact with each other. The result

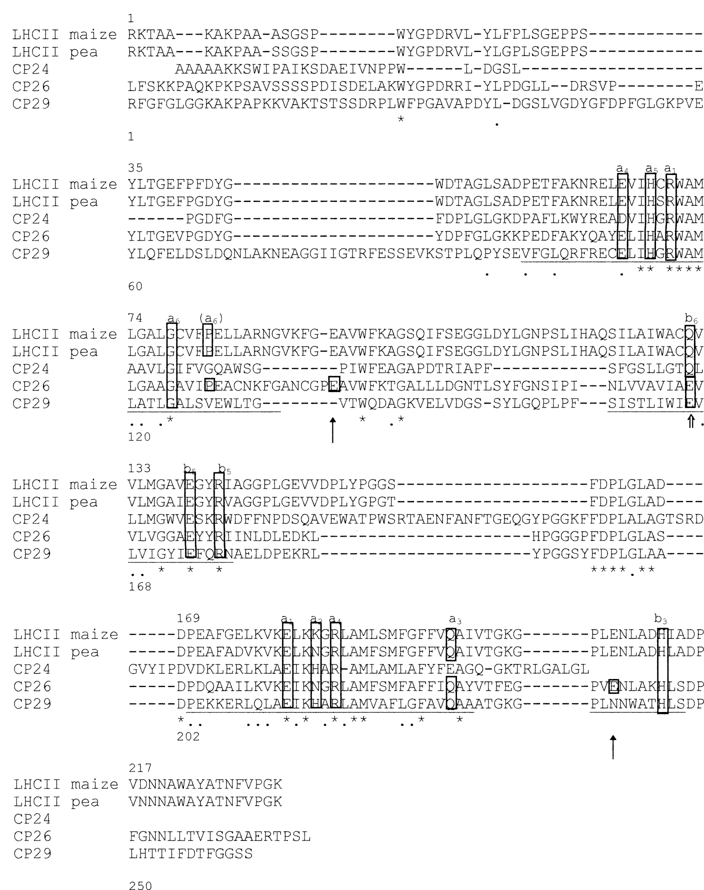

Fig. 3. Sequence comparison of Chl a/b proteins as deduced from cDNA sequences. Numbers in the upper left refer to sequence position in LHCII according to Kühlbrandt et al. [16]; numbers in the lower left refer to the sequence position in CP29 according to Bergantino and Bassi [32]. Thin arrows, DCCD binding sites in CP26 according to Walters and Horton [40]; arrowhead, DCCD binding site in CP29; underlined, helices; *, identical residues; •, conservative substitution.

that both the phosphorylated and unphosphorylated forms of CP29 could bind DCCD shows that the conformational change induced by phosphorylation [25] does not prevent the DCCD binding event. How the two mechanisms contribute to the modulation of CP29 function will be the subject of further research.

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