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The molecular mechanism of the control of excitation energy dissipation in chloroplast membranes

Inhibition of ΔpH-dependent quenching of chlorophyll fluorescence by dicyclohexylcarbodiimide

A.V. Ruban, R.G. Walters and P. Horton

Robert Hill Institute, Department of Molecular Biology and Biotechnology, PO Box 594, Firth Court, University of Sheffield, Sheffield, S10 2UH, UK

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Non-radiative dissipation of absorbed excitation energy in chloroplast membranes is induced in the presence of the trans-thylakoid proton motive force; this dissipation is measured as high energy state quenching of chlorophyll fluorescence, qE. It has been suggested that this results from a low pH-induced structural alteration in the light harvesting complex of photosystem II, LHCII [(1991) FEBS Letters 292, 1–4]. The effect of the carboxyl-modifying agent, dicyclohexylcarbodiimide (DCCD), on energy dissipation in chloroplast membranes has been investigated. At concentrations below that required to inhibit electron transport, DCCD caused a decrease in the steady state ΔpH, completely inhibited qE and also inhibited the low pH-dependent induction of qE. DCCD binding to polypeptides in the 22–28 kDa range correlated with inhibition of qE. It is suggested that DCCD reacts with amino acid residues in LHCII whose protonation is the primary event in the induction of energy dissipation. This LHCII domain may be identical to one forming a proton channel linking the site of PSII-dependent water oxidation to the thylakoid lumen [(1990) Eur. J. Biochem. 193, 731–736].

Photosynthesis; Thylakoid membrane; Light harvesting complex; Chlorophyll fluorescence; Proton channel

1. INTRODUCTION

In saturating light the photosynthetic apparatus of higher plants is protected from photoinhibition by the induction of increased non-radiative energy dissipation [1,2]. This protective mechanism is most commonly measured as the non-photochemical quenching of chlorophyll fluorescence, the major part of which is induced in response to the energisation of the thylakoid membrane, i.e. the formation of a ΔpH [3]. This quenching has therefore been called qE. It is the acidification of the thylakoid lumen upon illumination that is the primary trigger for qE formation; hence it is possible to induce qE in the dark by acidification of isolated thylakoid membranes [3,4].

Correspondence address: A.V. Ruban, Robert Hill Institute, Department of Molecular Biology and Biotechnology, PO Box 594, Firth Court, University of Sheffield, Sheffield, S10 2UH, UK.

Abbreviations: F_m, maximum level of chlorophyll fluorescence with photosystem II centres closed; F_o, minimum level of chlorophyll fluorescence with photosystem II centres open; LHC, light harvesting complex; LHCII, light harvesting complex of photosystem II; PSII, photosystem II; DCCD, dicyclohexylcarbodiimide; qE, non-photochemical quenching of chlorophyll fluorescence by the thylakoid pH gradient; q^9-aa, quenching of 9-aminoacridine fluorescence.

The mechanism by which lumen acidification leads to increased energy dissipation has not been established. One suggestion has been that, upon lumen acidification, electron donation to the PSII reaction centre is slowed down and a 'quenching' species is formed in the PSII reaction centre [5]. This suggestion is related to the hypothesis that qE is caused by a switch of PSII from an active to an inactive quenched state [6]. The fact that the thermal de-excitation corresponding to qE occurs in competition with photochemistry [7] suggests that it occurs by quenching in the antenna of PSII. It has been suggested that such quenching results from the presence of zeaxanthin, a carotenoid formed by light-dependent de-epoxidation of violaxanthin [8]. Recently, we have suggested an alternative model in which qE results from pH-dependent structural change in LHCII, the light harvesting complex of PSII [9]. This model for qE is based on the principle that the physical properties of the pigments bound to LHCII are modified following structural changes in the protein, initiated as a result of protonation of lumen-exposed amino acid residues; the modification of the pigment environment provides quenching centres in the LHCII. There are several lines of experimental evidence to support this hypothesis. Firstly, 77 K chlorophyll fluorescence spectra after induction of qE indicate preferential quenching of bands

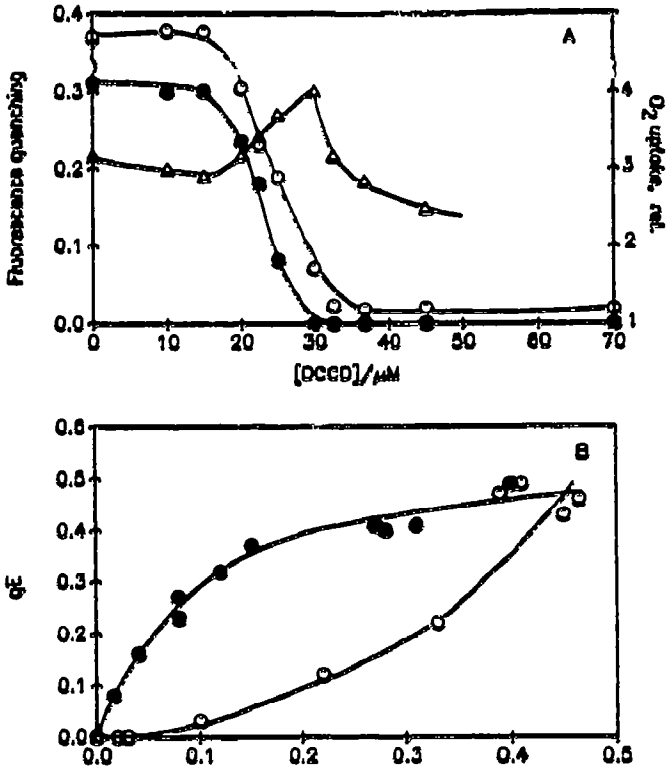

Fig. 1. (A) Effect of DCCD concentration on qE (●), ΔpH (q9-aa) (○) and rate of electron transport (△). Both qE and q9-aa are the amplitudes of fluorescence quenching expressed as a proportion of the maximum fluorescence levels obtained following relaxation upon adding 10 μM DCMU. The rate of O_2 uptake is given in relative units. (B) Relationship between ΔpH and qE obtained from titration with either DCCD (○) or nigericin (●).

at 680 and 700 nm, which arise from LHCII rather than the PSII reaction centre [10]. Secondly, antimycin A (an inhibitor of qE) also inhibits aggregation of LHCII in vitro [9]. Not only do these effects occur with the same submicromolar concentration dependency but the absorbance changes at 530 nm accompanying both qE and LHCII aggregation are inhibited by antimycin [11]. A further important feature of qE, the reversible modulation of its pH sensitivity by treatments which induce changes in zeaxanthin content [12], is also more readily explained by the LHCII model rather than by an alteration in the PSII reaction centre or by an obligatory involvement of zeaxanthin.

An important prediction of this model is that qE will depend on the protonation of lumen-facing glutamate and/or aspartate residues on the LHCII. The predicted secondary structure of LHCII [13], together with the images of LHCII derived from electron diffraction of 2D crystals [14] indicates that there are several such amino acid residues. Previous work has shown that covalent modification of thylakoid membrane carboxyl residues with dicyclohexylcarbodiimide (DCCD) inhibits H^+ release into the lumen following water splitting [15]. Interestingly, there was evidence that the reaction of DCCD with a small group of LHC polypeptides with molecular weights of 20–28 kDa was responsible for this effect ([16] W. Junge, personal communication). Also, covalent modification of isolated LHCII with DCCD has been described [17].

In this paper, we show that DCCD binding to these thylakoid polypeptides is correlated with inhibition of qE irrespective of whether it is generated by light-induced ΔpH or acidification in darkness. We suggest that protonation of the carboxyl group(s) in the LHCII complex is the primary event that leads to a structural change in LHCII and the induction of energy dissipation.

2. MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves which had been pre-illuminated to induce light activation of qE [12]. Simultaneous measurements of chlorophyll fluorescence using a Walz PAM fluorimeter, 9-aminoacridine fluorescence and oxygen concentration upon illumination of thylakoids were made as described previously [18]. Actinic light was provided at an intensity of 300 μmol/m²/s and the electron acceptor was methyl viologen (0.1 mM) and incubations continued for 5 min at 20°C. Induction of fluorescence quenching by acidification was carried out exactly as described in an earlier paper [4] using 20 mM ascorbate to suppress the effects of acidification of PSII redox reactions. Dicyclohexylcarbodiimide (either from Sigma or Aldrich, assayed to be 99% pure) was dissolved in ethanol and added such that volumes never exceeded 1% of the reaction volume. Labelling of membrane proteins by [^{14}C]DCCD (Amersham) during illumination of thylakoid suspensions was assayed in a manner similar to that described by Jahns and Junge [15]; thylakoids were pelletted by centrifugation, washed, re-pelletted, and either added direct to sample buffer or extracted with methanol/chloroform [19]. After SDS-PAGE according to Laemmli [20] using 15% acrylamide, DCCD-binding was assayed by densitometry of autoradiographs of the dried gel. Total binding (specific and non-specific) to thylakoid membranes was assayed by scintillation counting of aliquots of the reaction medium and the supernatant after centrifugation.

3. RESULTS

Fig. 1A shows the effect of a titration of qE, ΔpH and electron transport rate against the concentration of DCCD. The principle effect at low concentration is the inhibition of qE and ΔpH, and a stimulation of electron transport. These observations are indicative of an uncoupling effect of DCCD at 25–30 μM. At this DCCD concentration it has previously been observed that H^+ release from H_2O splitting is 'short-circuited'; i.e. the H^+ are not deposited in the lumen but instead go to the stromal side of the membrane where they are bound upon plastoquinone reduction [15]. At higher DCCD concentration (50 μM), inhibition of electron transport to methyl viologen was observed; again, this inhibitory effect at high concentration of DCCD is the same as reported by Jahns and Junge [15]. The decrease in ΔpH

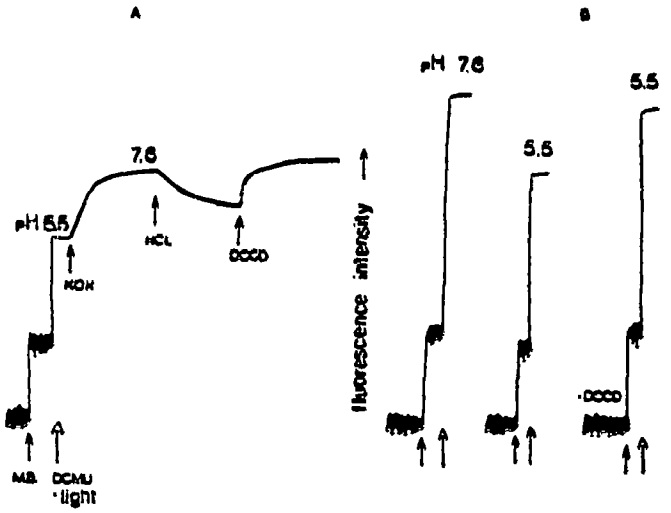

Fig. 2. Effect of DCCD on the chlorophyll fluorescence yield at pH 7.6 and 5.5. In A, successive additions of alkali and acid were used to adjust the pH. In B, DCCD was added prior to recording the fluorescence. DCCD concentration was 35 μM. Solid arrows indicate turning on the fluorescence measuring beam (M.B.) and open arrows, the turning on of the weak actinic light and DCMU addition.

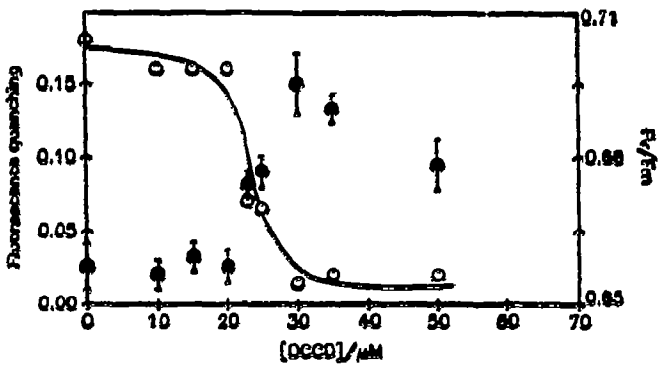

Fig. 3. Effect of DCCD concentration on the quenching of chlorophyll fluorescence on lowering the pH from pH 7.6 to pH 5.9. The experiment was performed as for Fig. 2B. Fluorescence quenching refers to the proportional change in F_m (○). Also shown (●) is the F_v/F_m ratio.

is consistent with this H^+ short circuit, although it would not be predicted that ΔpH would be almost completely eliminated. We find that DCCD markedly increases the decay of the ΔpH upon darkening. Thus the half-time for the relaxation of the quenching of 9-aminoacridine, following a saturating 1 s light pulse chosen to give equal amplitudes of quenching ± DCCD, changes from 12 s to 4 s in the presence of 30 μM DCCD. This increase in H^+ conductance was not observed by Jahn and Junge [15]. The decrease in qE could therefore be a predictable and trivial consequence of the elimination of the ΔpH in the presence of DCCD. However close inspection of the data in Fig. 1A shows that qE is more sensitive to DCCD than is the ΔpH. This is clearly illustrated in Fig. 1B where the relationship between qE and ΔpH is shown for titration with DCCD compared to the uncoupler, nigericin. As shown in earlier work [12,21], the curve obtained by uncoupler titration was roughly hyperbolic, with the first 60% decrease in ΔpH causing a negligible change in qE, and thereafter a steep decline in both parameters was observed. An exactly similar curve is obtained if the ΔpH is titrated by alteration in light intensity [12,21]. In contrast, the titration with DCCD gave an immediate decline in qE with only small changes in ΔpH and most of the qE was lost with only a 50% decline in ΔpH. This data shows that the inhibition of qE is not a consequence of the elimination of the ΔpH, but that DCCD is exerting a direct effect.

The possibility that DCCD is having a direct effect on qE was further tested by examining fluorescence quenching brought about by acidification of thylakoids in the absence of electron transport. This quenching is identical to the ΔpH-dependent qE provided that the effects of low pH inhibition of the electron donor side of PSII are avoided [4]. This was achieved in two ways: firstly, by adding ascorbate to the reaction mixture [4], and secondly, by using light-activated chloroplasts, pH-dependent quenching was measured without the requirement to use extreme acidification [4]. Fig. 2 shows the effect of adding DCCD to light-activated thylakoid membranes in the presence of ascorbate at pH 7.5 and 5.5. At pH 5.5, the variable fluorescence ($F_m - F_o$) value is approx. 30–40% below that at pH 7.5 (Fig. 2A). Addition of alkali to bring the pH back to 7.5 reverses most of the low pH-dependent quenching. The quenched state can then be restored by acidification to pH 5.5. Subsequent addition of DCCD to chloroplasts at pH 5.5 causes a reversal of quenching; addition of DCCD

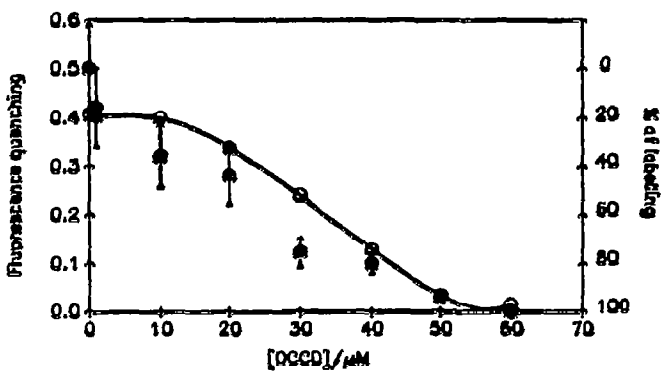

Fig. 4. Comparison between the inhibition of qE (○) and incorporation of ^{14}C DCCD into LHC polypeptides (●). The labelling of each of the 4 LHC polypeptides was expressed as a percentage of the maximum and the mean of all polypeptides calculated. The experiment was performed exactly as described for Fig. 1. For further details see text.

at pH 7.5 had negligible effect on fluorescence (not shown). A similar result was obtained if DCCD was added prior to acidification (Fig. 2B); in this experiment incubation at pH 5.5 lowered the variable fluorescence by 27% and the F_o level of fluorescence by 15% below that at pH 7.6. If DCCD was added prior to acidification there was negligible difference between the fluorescence levels at pH 5.5 and 7.6.

Titration of quenching of fluorescence by acidification against DCCD concentration shows that the half maximum effect occurs at approx. 22 μM DCCD (Fig. 3); this is very close to the concentration that inhibits ΔpH-dependent qE (Fig. 1A). Also shown in Fig. 3 is the effect of DCCD on the ratio F_v/F_m; low pH quenching lowers this ratio to approx. 0.66 from a control value of 0.7. As expected, DCCD inhibits this decline. It should be noted that at higher concentrations of DCCD, when electron transport is being inhibited (see Fig. 1A), there is also a decline in F_v/F_m. This data confirms that DCCD inhibits qE directly.

In previous work it has been suggested that DCCD has this effect on ΔpH by binding to five LHC polypeptides in the molecular weight range 20–28 kDa (20, 22, 24, 25 and 27 kDa) [15]. We have repeated this experiment and obtained very similar results: 4 polypeptides were labelled by [^{14}C]DCCD with apparent molecular weights of 22, 24, 26 and 29 kDa. These bands showed a broadly similar concentration dependency for labelling. The labelling of these polypeptides correlated well with the inhibition of qE (Fig. 4). For labelling and qE inhibition, a sigmoidal concentration dependency was observed, and the concentration for a half-maximum effect was 30 μM in both cases. It should be pointed out that measurement of total non-specific binding of DCCD to the thylakoid membrane did not show this sigmoidal behaviour (not shown), adding further support to the notion that, rather than the non-specific effect of incorporating a hydrophobic molecule into the thylakoid membrane, it is the covalent binding of DCCD to LHC polypeptides which is responsible for the inhibition of qE.

4. DISCUSSION

In this paper it has been shown that qE is inhibited by the carboxyl-modifying agent, DCCD. This inhibition is associated with, but not caused by, a decrease in ΔpH across the thylakoid membrane. The very different relationship between qE and ΔpH when the latter is changed by uncoupling with nigericin compared to that observed upon titration with DCCD points to a direct effect on qE. Similarly, the inhibitory effect of DCCD on qE generated by acidification of thylakoids cannot be explained by its possible action as an uncoupler. Previous work by Jahns and Junge has shown that DCCD binding, under identical conditions to those used here resulted in the inhibition of PSII-dependent H^+ release into the thylakoid lumen [14]. These workers have obtained evidence that this effect of DCCD is due to modification of amino acids on LHC polypeptides ([15], W. Junge, personal communication). Consistent with this, we have shown here that the binding of DCCD to these polypeptides correlates with the inhibition of qE. The main difference between our data and that of Jahn and Junge is that we observe uncoupling rather than short-circuiting of H^+ from the donor to the acceptor side of PSII. This difference may be due to differences in chloroplast preparation or plant species, and perhaps results from the differing extents of labelling of the 4 LHC polypeptides. It is possible that uncoupling is an additional separate effect of DCCD, but, alternatively, the blocking of qE may itself result in a decrease in the ability of the thylakoid to form a ΔpH.

The data suggest that there may be a link between the putative LHCII H^+ channel associated with H_2O oxidation and the control of energy dissipation. Protonation of amino acids within the channel may be the primary trigger for development of qE. When this channel is blocked by DCCD, qE is prevented. Results of previous experiments using the reagent, dibucaine, have also suggested that qE is promoted by localised H^+ domains around PSII [22]. There may be other contributing factors: binding of DCCD to isolated LHCII causes a release of bound Ca^{2+} [17] and it is possible that it is this that links the H^+ channel to qE. There have been suggestions before that qE involves changes in Ca^{2+} to the thylakoid membrane (E. Weis, personal communication).

The data described in this paper provide further evidence that qE is predominantly a process occurring in the LHCII complex and not in the reaction centre of PSII. There is no evidence that DCCD binds to any PSII polypeptide, either in our experiments or in those of Jahns and Junge [16]. Furthermore, it will perhaps be possible to identify the key glutamate or aspartate residues on LHCII that are involved in the primary event of qE. Further work is now in progress to achieve this important objective, with the possibility of elucidating the molecular mechanism of a process of major importance in the protection of plants against environmental stress.

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