The Relationship between the Binding of Dicyclohexylcarbodiimide and Quenching of Chlorophyll Fluorescence in the Light-Harvesting Proteins of Photosystem II†

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ABSTRACT: The relationship between the binding of dicyclohexylcarbodiimide (DCCD) to isolated light-harvesting proteins of photosystem II and the inhibition of chlorophyll fluorescence quenching by DCCD have been investigated. For a range of different complexes an approximately linear relationship was obtained between the efficiency of DCCD binding and the DCCD-dependent reversal of fluorescence quenching. The most efficient labeling was found for the minor light-harvesting complexes, CP29 and CP26. In the case of the former, five different preparations were compared including two reconstituted complexes in which a putative DCCD-binding site had been mutagenized. Again, an approximately linear relationship between DCCD binding and the extent of reversal of fluorescence quenching was found. However, the binding of DCCD was found to occur at least an order of magnitude faster than the change in fluorescence. The results are discussed in terms of the multiplicity of DCCD-binding sites and the influence of protein structure on both the binding of DCCD and the fluorescence quenching mechanism.

The light-harvesting system of photosystem II (LHCII)[1] consists of six different polypeptides, Lhcb1−Lhcb6, which are organized into four distinct pigment protein complexes, CP29, LHCIIb, CP26, and CP24, (also known as LHCIIa, LHCIIb, LHCIIc, and LHCIId, respectively) (*1*, *2*). These complexes bind Chl *a*, Chl *b*, lutein, neoxanthin, and violaxanthin, and their function is to absorb light and transfer energy to the PSII reaction center. The proportion of excitation energy which is transferred to the reaction center compared to that dissipated as heat is controlled by the induction of a nonphotochemical quenching mechanism. The increase in quenching, which occurs when light is in excess of electron transport capacity, is caused by the acidification of the thylakoid lumen as a result of light-dependent proton translocation (*3*), and the de-epoxidation of violaxanthin to zeaxanthin via the xanthophyll cycle (*4*). There is considerable evidence that nonphotochemical quenching occurs in LHCII, with CP29 and CP26 possibly having a key role (*5*−*7*). This suggests that these proteins have dynamic structural properties in which proton binding sites control the interactions between bound pigments, thereby determining the probability of energy dissipation (*8*). Direct evidence for light-dependent, reversible changes in the macroorganization of LHCII have been provided by CD of thylakoid membranes (*9*−*11*).

Isolated LHCII components show strong, reversible changes in nonphotochemical fluorescence quenching when the detergent concentration is altered around the critical micelle concentration (*12*−*14*). We have used this system to explore the dynamic behavior of LHCII and extensive spectroscopic investigation have been carried out (e.g., refs *15*−*17*). Fluorescence quenching was displayed by all LHCII components tested, although CP29 and CP26 showed larger amplitudes of quenching than LHCIIb (*18*). Direct evidence has been obtained that the quenching mechanism is associated with changes in protein−protein interaction, which was displayed in vitro as oligomerization or sometime large-scale aggregation (*12*, *19*). It has been found that both low pH and the xanthophyll cycle carotenoids control quenching (*12*, *18*, *20*). This observation, as well as spectroscopic analyses, showed that in vitro quenching possessed many similarities to nonphotochemical quenching in vivo (*8*).

DCCD binds to LHCII in isolated thylakoids (*21*, *22*) and causes an alteration in PSII proton transfer (*23*), an inhibition of light-dependent change in CD (*11*), and an inhibition of nonphotochemical quenching (*22*, *24*). DCCD binds to carboxy amino acid residues in hydrophobic domains of several membrane proteins, and DCCD binding generally indicates a biological function of these residues in proton binding and/or translocation. It was found that it is the minor LHCII components, CP29 and CP26, that bind DCCD (*22*), suggesting that the DCCD binding sites on these LHCII

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[1] Abbreviations: CD, circular dichroism; DCCD, dicyclohexylcarbodiimide; DM, *n*-dodecyl *β*-maltoside; IEF, isoelectric focusing; LHCII, light-harvesting complexes of photosystem II.

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proteins play a role in the quenching process. The specificity for DCCD binding to CP29 and CP26 as opposed to the more prevalent LHCIIb in vivo is preserved after the proteins have been isolated and purified (*22*). This observation allowed the identification of the active residues on CP26 as being two glutamate residues close to the lumen-facing surface (*25*). In an other approach, Lhcb4 polypeptide expressed in *E. coli* was used to reconstitute the CP29 complex, which bound DCCD. Using site-directed mutagenesis, it was shown that when glutamate residue E166 was replaced, DCCD binding was strongly reduced (*26*). This residue is in the hydrophobic interior of the protein and is suggested to be involved in Chl binding. Thus, DCCD may bind to different sites on these two proteins: acidic sites close to the lumen surface (as found for CP26), and putative Chl binding sites in the hydrophobic interior (as for CP29).

DCCD inhibits or reverses the in vitro fluorescence quenching in CP29 and CP26, but not LHCIIb (*18*, *25*). These data suggested that the residues bound by DCCD are involved in quenching in vitro, perhaps giving the first insights into how protein dynamics control LHCII function. It is therefore crucially important to more rigorously establish the relationship between DCCD binding and fluorescence quenching. In this paper, a quantitative analysis has been carried out regarding the efficiency of DCCD binding and inhibition of fluorescence quenching in a number of different preparations of LHCII, including mutagenized CP29. It was found that there was a strong correlation between DCCD binding and the effect on Chl fluorescence, implicating specific amino acid residues in the control of LHCII function. Further evidence for multiple DCCD binding sites was obtained.

MATERIALS AND METHODS

Preparation of PSII Antenna Complexes

The LHCII complexes, CP29, CP26, and LHCIIb, were prepared from dark-adapted spinach leaves by an isoelectric focusing procedure described previously (*27*, *28*). CP29 and CP47 complexes were also isolated using cation-exchange chromatography essentially as described by Henrysson et al. (*29*); for this tris-washed, NaSCN-treated PSII-membrane fragments (2 mg Chl/mL) were solubilized in 2% sulfobetaine 12, 2% digitonin at pH 6.0 in 10 mM MES/NaOH for 30 min at 4 °C. After centrifugation to remove unsolubilized material, the filtered sample was loaded onto a HPLC cation-exchange column (TSK 3SW, Tosohaas) preequilibrated with 0.1% sulfobetaine, 0.05% *n*-dodecyl β-maltoside (DM). Proteins were eluted with a 0−500 mM NaCl gradient, and the fraction at approximately 300 mM was collected, concentrated, and desalted prior to a second chromatography. The identity of the CP47 and CP29 fractions was confirmed by Western blotting using monoclonal antibodies directed against CP29 and polyclonal antibodies against CP47 and the high degree of purity by examination of silver-stained gels.

To prepare LHCIIb monomers, the LHCIIb sample was treated with phospholipase A_2 from bee venom (Sigma) for 48 h at room temperature in the presence of 20 mM $CaCl_2$ in a sterilized Eppendorf tube at a Chl concentration of 500 μM. Immediately after treatment, a sample was applied onto a seven-step exponential sucrose gradient from 0.15 to 0.87 M sucrose. The gradient buffer contained 20 mM HEPES and 200 μM DM. After 18 h of centrifugation at 200000*g*, the monomer band was located at approximately 0.25 M sucrose with a yield of 60%. The LHCIIb trimers were located at around 0.45 M sucrose.

CP29 Reconstitution and Mutagenesis

DNA Constructs. Plasmids were constructed using standard molecular cloning procedures (*30*). Bacterial hosts were *Escherichia coli* TG1 strain (*31*) and SG13009 strain (*32*). Site-directed mutants E166Q and E166V were obtained according to Geisselsoper et al. (*33*). The sequence was determined by the dideoxy method using an automated apparatus (Applied Biosystems Model 377).

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 (*34*).

Pigment Isolation and Reconstitution of CP29-Pigment Complexes. These procedures were performed as described in Giuffra et al. (*35*).

Purification of Reconstituted CP29. To obtain a fully purified complex which did not contain any residual contamination by bacterial proteins, the reconstituted CP29 was purified by ion-exchange chromatography (*35*) followed by ultracentrifugation (12 h at 60000 rpm in a SW60 Beckman rotor) using a glycerol gradient (15 to 40% including 0.06% DM and 10 mM HEPES, pH 7.6).

[^{14}C]DCCD Treatment and Analysis

[^{14}C]DCCD treatment was carried out at room temperature in 20 mM HEPES buffer at pH 8.0 for 15 min at a DCCD concentration of 250 μM and 2−3 μM Chl. Chloroform-methanol extraction procedure was used in the end of the treatment to remove unreacted DCCD and to precipitate the protein as described by Wessel and Flugge (*36*). After denaturing of the protein, the sample was subjected to SDS PAGE with 15% acrylamide. After staining with 0.5% Coomassie Blue, the destained gel was dried and scanned (HP Scanjet 4C) into a PC for digital analysis of the band densities using Optimas software. Autoradiography was performed using a high-performance β-max autoradiography film (Amersham). The exposure time was 1−2 weeks. The developed film was scanned and analyzed in a same way as stained gels. DCCD-binding efficiency was calculated as a ratio between the band area on the autoradiography film and the corresponding band on the gel.

Fluorescence Measurements

Chlorophyll fluorescence quenching was carried out as described in Ruban et al. (*12*). Briefly, complexes were diluted to a Chl concentration of 3 μM in 20 mM HEPES buffer at pH 8.0 to give a final DM concntration of 4 μM. and then the pH was lowered to 5.5 to further induce quenching. Fluorescence was measured using a Walz PAM 101 fluorimeter, and the DCCD effect was quantified as the difference between the fluorescence level established 5 min after addition of DCCD (F) minus the low pH-quenched fluorescence level (F') and divided by maximum fluorescence (F_{max}) achieved by diluting of sample in buffer containing

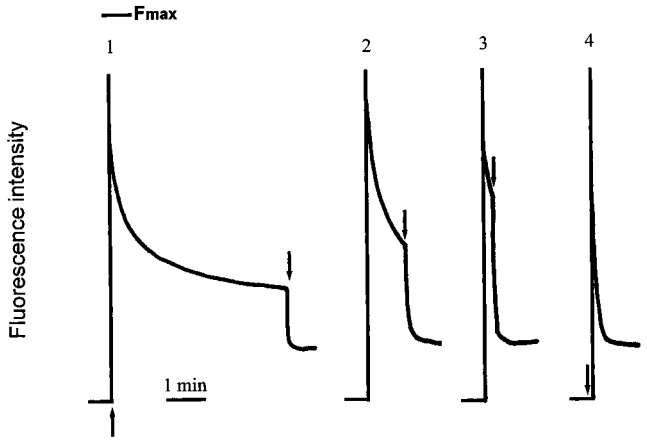

FIGURE 1: Fluorescence quenching in isolated LHCII. In trace 1, a sample of CP29 was diluted into buffer giving a final concentration of 4 μM DM (upward arrow) and incubated for 4 min before acidification to pH 5.5 (downward arrow). In traces 2 and 3, acidification was 1 min and 20 s after dilution. In traces 4, acidification took place before dilution. F_{max} is the fluorescence level of the complex in 200 μM DM. For further details see Materials and Methods.

200 μM DM. The DCCD concentration was 250 μM unless stated otherwise.

Absorption Measurements

Absorption spectra of LHCII were recorded using a Aminco DW2000 spectrophotometer operated in the split beam mode, with a scan rate of 5 nm/s and a slit width of 1 nm. Spectra were recorded for complexes diluted in buffer with or without DM, at pH 8.0 or pH 5.5, and for a low-pH sample incubated for 3 min with 250 μM DCCD. Difference spectra were calculated using DW2000 software.

RESULTS

In Figure 1 the general features of the quenching of Chl fluorescence in isolated LHCII are shown. Upon dilution into low detergent concentration there occurs a spontaneous quenching (trace 1). After several minutes the fluorescence approaches a minimum level; further incubation results in a continuation of slow quenching, but sample instability prevents a true end point being reached. However, acidification to pH 5.5 causes a rapid further quenching to the minimum level. If the sample is acidified earlier, this minimum level is reached more quickly (traces 2 and 3), so that if dilution is made at a starting pH of 5.5 the fluorescence is quenched maximally within 30 s (trace 4). These data indicate that the spontaneous quenching and the low-pH-induced quenching are not separate effects on LHCII, but a part of a continuum of quenched states of the complex arising from the same quenching mechanism. Clearly, acidification accelerates the rate of reaching the state of maximum quenching. Further support for the suggestion that the low-pH-induced quenching and the spontaneous quenching occur by a common mechanism comes from the similarity of the absorbance changes associated with them (Figure 2). The absorption difference spectra for both quenching stages have the same bands in the blue (435, 455, 485, and 510 nm) and red regions (661 and 685 nm).

Figure 3 shows the responses of a range of Chl binding proteins to quenching conditions. Stronger quenching was

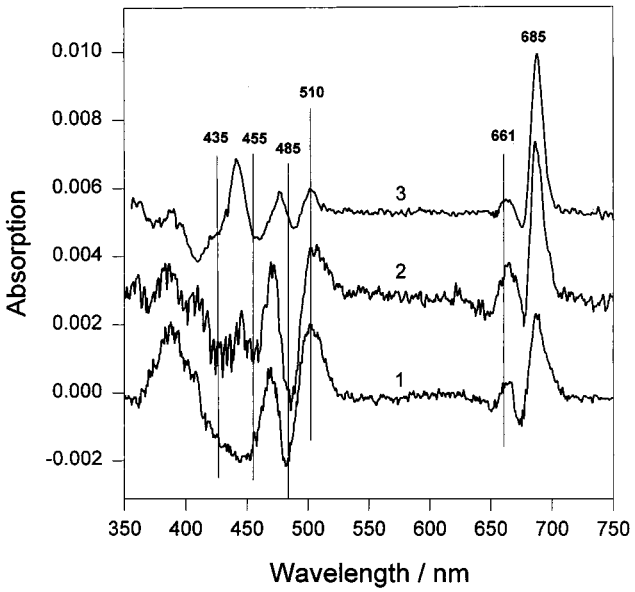

FIGURE 2: Absorbance changes associated with quenching of LHCII. Spectrum 1: difference spectrum dilution in buffer-*minus*-dilution in 200 μM DM as carried out in Figure 1, trace 1. Spectrum 2: difference spectrum pH 5.5-minus-pH 8.0, again as in Figure 1. Spectrum 3: difference spectrum before-minus-after addition of DCCD, as described in Figure 3. For further details see Materials and Methods.

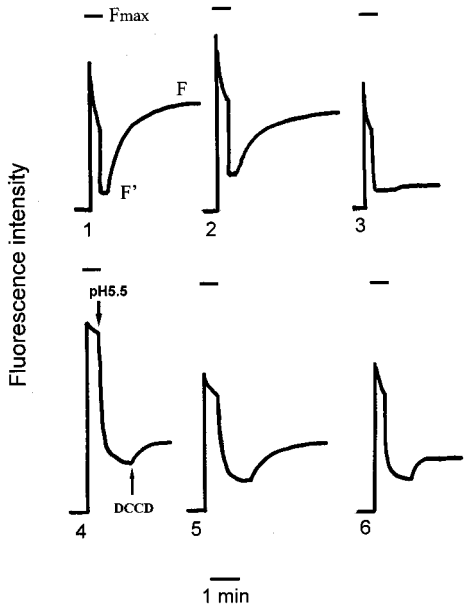

FIGURE 3: Reversibility by DCCD of chlorophyll fluorescence quenching in different pigment−protein complexes of PSII. (1), CP26; (2), CP29; (3), CP47; (4), LHCIIb from IEF; (5), LHCIIb monomer; (6), LHCIIb from the sucrose gradient. Horizontal bars indicate the level of maximum fluorescence for each complex in 200 μM DM, which was used in calculation of the extent of DCCD-induced fluorescence increase (see Materials and Methods).

shown by the minor LHCII components CP29 and CP26 compared to the major complex, LHCIIb. Also shown is the response of monomers and trimers of LHCIIb and the core complex CP47, which all show similar quenching behavior. This tendency for fluorescence quenching is probably shared by all Chl protein complexes.

Figure 3 also shows the effect of DCCD on each complex. For CP47 and LHCIIb (traces 3 and 4) almost no effect of DCCD was observed. However, for CP26 and CP29 (traces

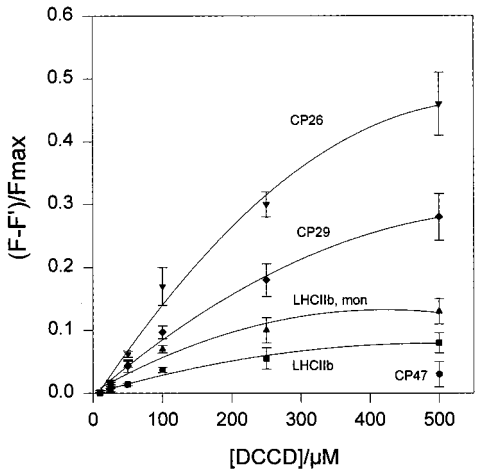

FIGURE 4: Concentration dependencies of DCCD-induced fluorescence recovery for five pigment−protein complexes of PSII. Data represent an average of 3 to 5 experiments. For experimental details see legend to Figure 3 and also Materials and Methods.

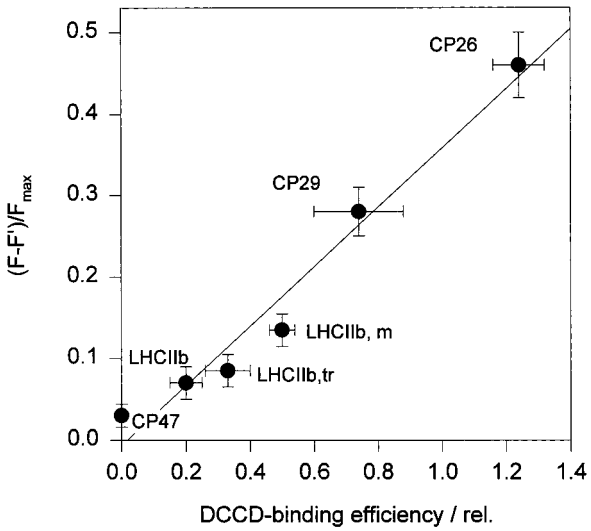

FIGURE 5: Plot of the extent of DCCD-induced fluorescence recovery vs DCCD-binding efficiency for different PSII antenna complexes. LHCIIb, m and LHCIIb, tr correspond to monomers and trimers of LHCIIb, respectively. Data represent average of 3 to 4 experiments. Data obtained as in Figure 3.

1 and 2) quenching was substantially reversed. In the case of CP26, addition of DCCD took the fluorescence level slightly above that seen before acidification, consistent with the view that all quenching arose from the same mechanism. The absorption difference spectrum for DCCD addition is the same as for the spontaneous and low-pH-induced quenching (Figure 2), indicating that DCCD causes reversal of the changes in LHCII that are responsible for quenching. In Figure 3 it is also shown that monomerization of LHCIIb leads to an increase in the effect of DCCD compared to trimers. For each complex the DCCD effect has a similar concentration dependency, approaching saturation above 500 μM (Figure 4).

It is important to point out that the effect of DCCD on the fluorescence yield of LHCII can be observed both as a reversal of quenching and as an inhibition of quenching. The fluorescence level reached if DCCD is added to CP29 in detergent micelles prior to dilution and acidification is approximately the same as that found when DCCD is added to the diluted quenched state (not shown).

To explore the relationship between the effect of DCCD on the fluorescence quenching and its covalent binding to the complexes it was necessary to establish an accurate method to quantify both processes. Fluorescence could be quantified by the normalization of the amount of DCCD-induced fluorescence increase to the maximum fluorescence. To quantify labeling the Coomassie-stained gel and the autoradiograph were both scanned and the image intensity was quantified. The method was validated by determining the binding efficiency of a CP29 complex determined following electrophoresis at a range of sample loading, from 0.2 to 5.0 μmol of Chl. A constant binding efficiency was found over a 10-fold difference in sample loading.

Figure 5 shows the efficiency of DCCD binding to the different complexes, and the extent of DCCD-induced Chl fluorescence increase. There was negligible binding of DCCD to CP47. The least effective LHCII substrate for DCCD was the LHCIIb fraction isolated by preparative IEF. Purification of IEF-prepared LHCIIb by sucrose gradient centrifugation, to give the LHCIIb trimer preparation, also led to a significant increase in labeling efficiency. This may result from removal of molecules weakly associated with the complex remaining from the IEF procedure, such as lipid or ampholines. Conversion of trimers into monomers increased the binding efficiency by a further 60%. However, CP26 and CP29 bound DCCD with the highest efficiency, respectively 4 times and 2.5 times stronger than LHCIIb trimers. It was found that there was an approximately linear correlation between the amount of labeling of each preparation by DCCD and the extent of the increase in fluorescence upon DCCD addition to the quenched complex. This applied to all complexes studied from CP47 (lowest) to CP26 (highest).

To further investigate the relationship between DCCD binding sites and the effect of DCCD on fluorescence quenching, we used reconstituted CP29 in which the putative DCCD binding site, E166, has been replaced with a valine (V-mutant) or glutamine (Q-mutant) (*26*). The calculated efficiency of DCCD binding was reduced in the mutant complexes by 50% compared to wild-type (Figure 6), although there was still significant binding under the conditions used in these experiments. Associated with this reduction in DCCD binding was a decrease in the amount of DCCD-induced fluorescence change. Also shown in Figure 6 is the response of two preparations of native CP29; the first, a complex prepared by the HPLC purification procedure, showed the same behavior as the reconstituted wild-type. In contrast, the native complex prepared by sucrose gradient purification of the IEF fraction showed double the binding efficiency of either the reconstituted or HPLC-purified complex. For all these CP29 complexes, an approximately linear relationship between DCCD binding and the change in Chl fluorescence quenching was found.

It was considered whether the fluorescence effect arose solely from a nonspecific effect of the insertion of a hydrophobic molecule into the LHCII structure that is nevertheless correlated to covalent binding. While the strength of the correlation shown in Figures 5 and 6 makes this unlikely, further evidence came from the total lack of effect of dicyclohexylurea on CP29 fluorescence (data not shown).

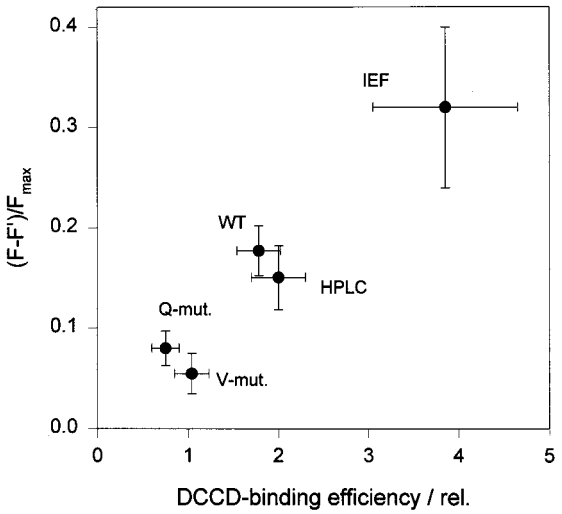

FIGURE 6: Plot of the extent of the DCCD-induced fluorescence recovery vs efficiency of DCCD binding for different CP29 preparations. IEF, HPLC, isoelectric focusing and cation exchange preps; WT, reconstituted wild-type CP29; Q-mutant V-mut, reconstituted CP29 with point mutations of E166 by glutamine and valine, respectively. Data represent the average of 3 experiments.

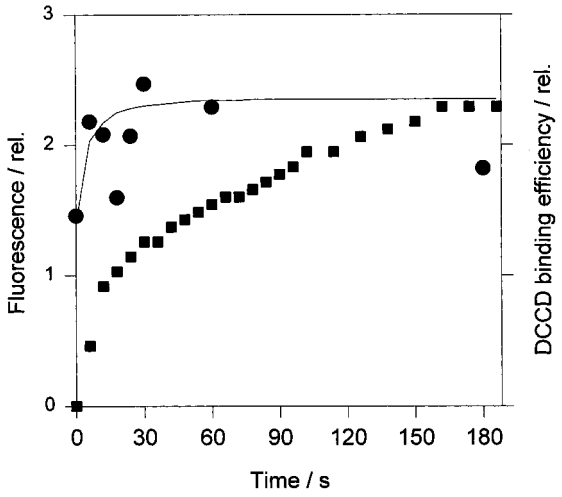

FIGURE 7: Kinetics of DCCD binding to CP29 and DCCD-induced fluorescence recovery. In the DCCD binding experiment, (circles) aliquots of sample were taken after given time intervals, mixed with ammonium acetate (final concentration 1.2 mM) to block the DCCD binding reaction and put into the denaturing mixture. Fluorescence (squares) was measured as described in Materials and Methods.

The mechanistic relationship between DCCD binding and its effect on fluorescence was explored by examining the kinetics of the two processes. It was found that whereas the DCCD-induced fluorescence rise is rather slow, taking approximately 3 min for completion, DCCD binding occurred within the fastest sampling time (6 s) (Figure 7). This suggested that the effect of DCCD on fluorescence was an effect secondary to its binding to the protein; i.e., binding to the protein induces a slower structural change that results in the elimination of quenching.

DISCUSSION

DCCD binds specifically and covalently to CP26 and CP29 in vitro and in vivo (*22*). In vivo this binding is associated with the inhibition of nonphotochemical quenching (*24*). In vitro it has been associated with an inhibition Chl fluorescence quenching induced by low pH and detergent dilution (*18*, *25*). We establish here that the covalent binding of DCCD to LHCII in vitro and its inhibitory effect on quenching of Chl fluorescence are directly related. Across a range of 10 different complexes, an approximately linear relationship between the efficiency of DCCD binding and the extent of inhibition of quenching was observed. This linear relationship indicates that the DCCD binding sites of each complex are equally effective in disrupting the quenching process, even though DCCD is binding to different amino acid residues (*25*, *26*). CP26 contains at least two binding sites (*25*), and both of these would therefore appear to be associated with inhibition of quenching. Similarly, for CP29 we have shown that residue E166 (*26*) has a functional role in the control of quenching.

For CP29, mutagenesis of E166 reduces labeling by DCCD. In these experiments a higher concentration of DCCD was used than in an earlier study (*26*), and significant labeling was found. The interpretation of these data is that there are two binding sites on the wild-type complex, perhaps with differing affinity for DCCD, one of which is the E166 residue replaced in the mutants. However, this extra site(s) seems to be equivalent to E166 in giving rise to an incremental inhibition of fluorescence.

There are clearly indirect influences which control DCCD binding. Thus, the sample of CP29 prepared by IEF has almost double the amount of labeling found in either a wild-type reconstituted complex or a native complex prepared by HPLC. Thus, features associated with LHCII structure and/or organization seem to influence DCCD binding. For example, in both the HPLC and reconstituted complex the lipid composition is lower than in the IEF fraction. Thus, the difference in DCCD binding between the HPLC and IEF preparations of CP29 may be due to the proportion of complexes that bind DCCD rather than the exposure of a different binding site. Thus an interpretation of the experiments with CP29 is that there is more than one DCCD binding site and that the efficiency of binding to these sites is determined by external factors relating to protein structure and environment. This may reflect differences in accessibility to DCCD. Similarly, for LHCIIb, DCCD binding can be enhanced by structural changes such as monomerization. The DCCD-binding residues identified for CP26 are present in LHCIIb and may become available during this treatment. DCCD-binding to LHCIIb monomers approaches 40% of that bound to CP26.

The observation that the rates of DCCD binding and fluorescence recovery are different suggests that DCCD binding has an effect on protein structure rather than a direct effect on the pigments involved in quenching. DCCD binding induces a slow change in conformation or organization of the complex that eliminates fluorescence quenching. This suggestion is consistent with the evidence that quenching in vitro results from oligomerization of these proteins (*19*). Evidence for a structural effect of DCCD comes from its inhibition of the light-dependent CD changes in thylakoids which arise from alteration in LHCII macroorganization (*11*).

While the effect of DCCD is specific for CP29 and CP26, fluorescence quenching was observed for all LHCII components and for CP47. Quenching is apparently triggered by exposure of the complex to a polar environment, and protonation accelerates the response of the protein to this

pressure perhaps by neutralizing negative charges. Proton binding sites, which may include the DCCD binding sites, are therefore key structural triggers controlling the interactions within and between LHCII components. The ability of DCCD to bind to and cause structural change in a complex is perhaps an expression of the conformational and photophysical flexibility of the complex. The dynamic range of quenching is larger for CP29 and CP26 than for LHCIIb.

It has been argued that the quenching of fluorescence inducible in isolated LHCII occurs by the same fundamental mechanism as nonphotochemical quenching in vivo (*8*). While this has yet to be proven, the similarity of spectral indicators and the analogous effects of DCCD in vitro and in vivo support this view. It is important to discuss the quantitative and qualitative differences between LHCII quenching in vitro and that which may occur in vivo. The dynamic range of LHCII fluorescence yield is clearly larger in vitro than in vivo. In vitro, there is more conformational freedom and the complex can readily collapse into a quenched state much deeper than in vivo where the surrounding environment (other complexes, lipids, grana structure) will restrict protein rearrangements and conformational transitions. For example, LHCII in vivo appears to exist in a three-dimensionally ordered chiral macrodomain (*10*, *11*). Similarly the fluorescence yield of unquenched state in vitro can approach that of free Chl (*37*), whereas in the thylakoid membrane LHCII never exists in this completely unquenched state. Hence, in vitro we observe much larger changes and a tendency toward irreversibility, for example, as protein interactions increase and large aggregates are formed. The in vivo states of the PSII antenna falls within the continuum of states of quenching observed in vitro.

In this work we have explored the dynamic features of the individual antenna complexes which are associated with PSII, and have shown how certain amino acid residues within these complexes play a key role in regulating function. It is important to point out that in vivo these complexes are arranged precisely and stoichiometrically in a PSII supercomplex (*38*, *39*). Therefore, elucidating the structural interaction between different complexes within the supercomplex is necessary in order to understand how these key residues in individual complexes control the light-harvesting system as a whole.

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